



THE COPPER METABOLISM OF WARM-BLOODED ANIMALS

by

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A thesis presented in fulfilment of the conditions
for the degree of Doctor of Science of the University
of Adelaide.

March, 1964.

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PREFACE

1. Background of the Investigations

From 1932 onward, Dr. H.W. Bennetts of the Western Australian Department of Agriculture, was investigating a disease of lambs designated "enzootic ataxia". Preliminary observations were made in 1936 (Bennetts and Chapman, 1937, Aust. Vet. J., 13, 138) which showed that the livers of affected animals contained much less copper than those of normal animals. In 1937 the candidate joined Dr. Bennetts to carry out the biochemical work of this investigation. It was shown unequivocally that the disease was associated with low levels of copper in the pastures grazed and in the tissues of affected animals, also that copper supplements prevented the onset of the disease. In 1939-1940 and again in 1944-1946, the candidate collaborated with Dr. Bennetts in the investigation of a disease of cattle ("falling disease") characterized by fibrosis of the heart muscle and by sudden death. Here again the disease was associated with very low levels of copper in pastures and in the tissues of affected animals. Both these diseases were almost entirely species specific, and at this early stage of investigations on copper deficiency it seemed likely that there was a

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marked difference in copper metabolism between species.

During 1940-1943 the candidate was stationed at the Animal Nutrition Laboratory, Adelaide, then a part of the Division of Animal Health and Nutrition of the Council for Scientific and Industrial Research. Much of this period was spent investigating the balance technique for measuring the loss of copper from sheep. This work showed that the copper in the liver of sheep was in a relatively stable combination, a fact which was of considerable importance in the comparative studies on the biochemistry of copper made later.

In all the above investigations, interest was centred on sheep and cows because of their economic importance, and it was not possible to make any studies on other species. As early as 1931, Cunningham (Biochem. J., 25, 1267) had shown that the levels of copper in the livers of sheep and cows were much higher than in some other species, but these observations were completely ignored and no attempts were made to assess their significance. From 1952 onward it was possible for the candidate to devote his time almost entirely to the investigation of the differences in the copper metabolism which occur between different species of warm-blooded animals. Toward the end of 1961, internal changes within C.S.I.R.O. made it

necessary to terminate these studies. Many important questions remain unanswered, but sufficient data have been accumulated to put forward a provisional classification of the types of copper metabolism in warm-blooded animals.

2. The Thesis

This thesis naturally falls into six Sections:-

- (1) A trilogy dealing with comparative aspects of the copper metabolism of warm-blooded animals.
- (2) A group of three published papers and some unpublished work on various aspects of normal copper metabolism.
- (3) A group of six papers relating to field studies on copper deficiency in stock.
- (4) Two papers relating to copper toxicity in humans and in sheep respectively.
- (5) An unpublished review of the data in Sections 1 - 4 and their relation to published papers of other workers.
- (6) A group of miscellaneous publications to indicate the experience of the writer in fields outside that of copper metabolism.

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Details of the proportion of work claimed as the candidate's own are given at the beginning of each Section, and acknowledgment of technical and other assistance is given at the end of each paper. The conception and planning of Section 1 was carried out by the candidate alone.

None of the published papers in this thesis have been submitted for a previous degree at this or any other University. Some of the unpublished data on clover oestrogens by Beck and Kowala (Paper 6:18) has been used by Mr. Kowala in a thesis for the degree of M.Sc. at the University of Western Australia.

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March 1964.

SECTION 1

SECTION 1



A COMPARATIVE STUDY OF THE COPPER METABOLISM OF WARM-
BLOODED ANIMALS

The initial purpose of these investigations was to obtain information on the reasons why the sheep is more susceptible to copper poisoning than other species. As the work progressed, it was realized that this starting point was only one phase of wider questions relating to the absorption, storage and excretion of copper by animals generally. The work described in this Section is confined to studies on warm-blooded animals and has the purpose of defining the patterns of storage and excretion of copper in such animals.

This Section contains three papers, of which the candidate was the sole author.

- 1:1 "The copper content of the liver and blood of some vertebrates." Aust. J. Zool., 1956, 4, 1.
- 1:2 "Observations on the copper metabolism of the domestic fowl and duck." Aust. J. Agric. Res., 1961, 12, 743.
- 1:3 "The copper metabolism of warm-blooded animals with special reference to the rabbit and the sheep." ibid., 1963, 14, 129.

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It had been thought that the liver was the main storage organ for copper in most, if not all, animals, both under physiological conditions and also under conditions of excess intake. For this reason the investigations reported in the first paper were designed to find out the concentration of copper in the livers of a wide range of species, living wherever possible under natural conditions. The results obtained were rather surprising. Most species showed a "normal" liver copper level of under 50 p.p.m. Cu (on dry matter), while a group of totally unrelated animals (ruminants, the duck, the frog and certain fish) showed very much higher levels. The significance of these differences was examined in the second and third papers.

In the second paper various aspects of the storage and excretion of copper were studied in the domestic fowl and duck. These species are characterized by low and high liver copper levels respectively. Both species could be fed the same diet, thus eliminating any effects from differing dietary components on the availability of copper.

The third paper describes similar experiments which were made with the rabbit and the sheep. These species were chosen because they show low and high liver

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copper levels respectively, and also because they were convenient animals for metabolic studies.

As pointed out in the Preface, these investigations had to be terminated earlier than had been expected. However, sufficient data has been accumulated to classify provisionally the patterns of storage and excretion in warm-blooded animals. This classification is given at the end of the third paper.

Considerable technical assistance has been received in the carrying out of the above investigations and such assistance is acknowledged in detail at the end of each paper. The original conception of the investigation and detailed planning were done by the candidate alone. All analytical work (except for the general analysis of feed stuffs in the second and third papers), was done either by the candidate or by assistants under his direct supervision.

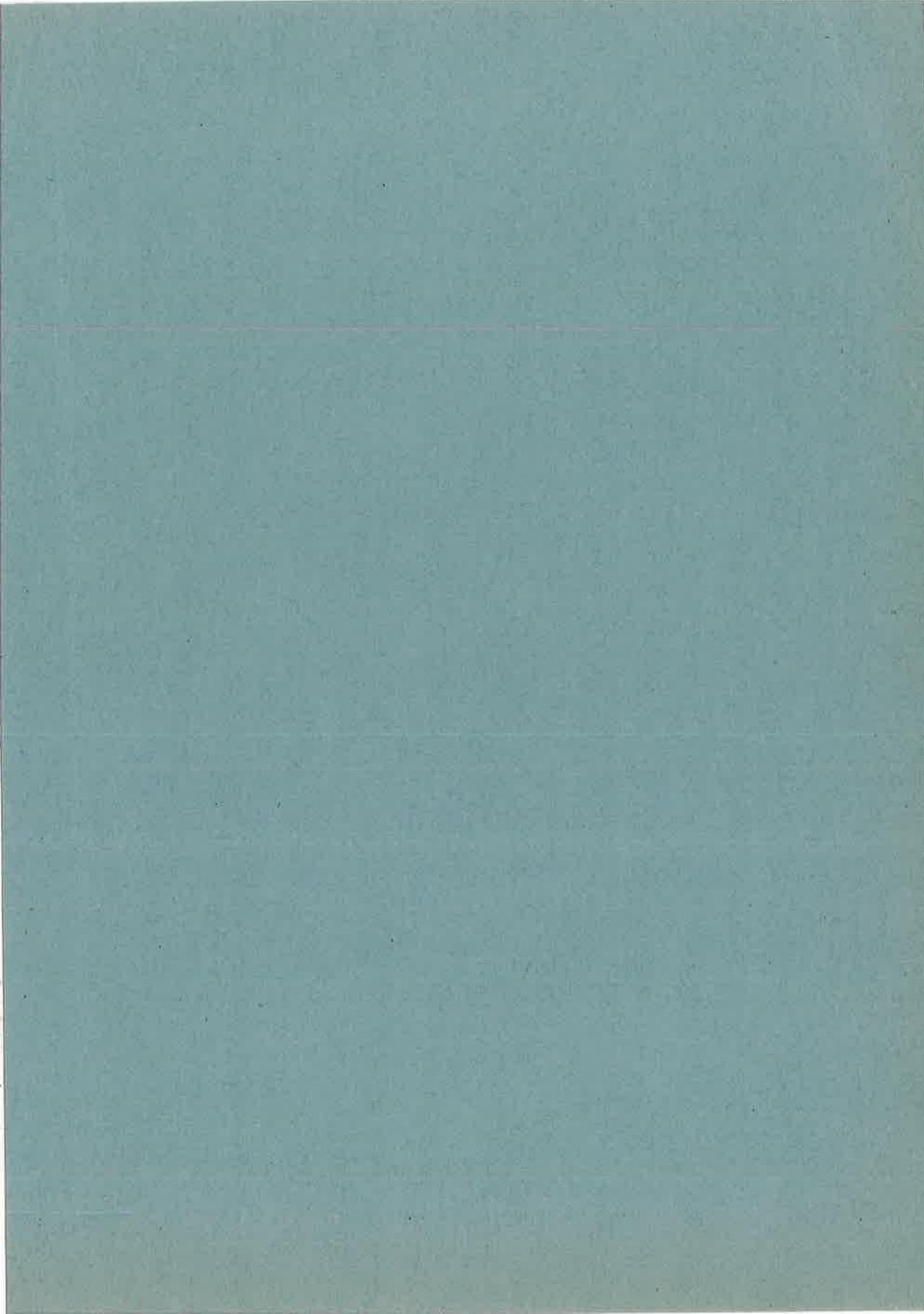
PAPER 1:1

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THE COPPER CONTENT OF THE LIVER AND BLOOD OF SOME
VERTEBRATES

By A. B. BECK

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Australia



THE COPPER CONTENT OF THE LIVER AND BLOOD OF SOME VERTEBRATES

By A. B. BECK*

(Manuscript received August 3, 1955)

Summary

Determinations have been made of the concentration of copper in the blood and liver from a wide range of vertebrate species.

The blood copper levels show trends which do not follow the phylogenetic relationships implied in current systems of classification. The highest levels are found in the pig (1.4 mg copper/l whole blood), and the lowest in the domestic fowl and turkey (0.23 mg/l). Marsupials show low values (0.3-0.4 mg/l), whereas in most other species the values lie between 0.5 and 1.0 mg/l. It is suggested that the usual range in an individual species represents the optimum for the physiological requirements of this species.

The concentration of copper in the liver of most species lies below 50 p.p.m. copper on a dry weight basis. High values are found in the ruminant, the duck, the frog, and in certain fish. From a consideration of the data presented, it seems probable that the high liver copper level characteristic of some species is due, not to a higher intake of copper or to a greater absorption, but to a lesser ability to restrict the storage of copper in the liver.

Although there is no suggestion of sex difference in liver copper levels of most species, a highly significant difference ($P < 0.001$) has been noted in the Australian salmon (*Arripis trutta* Bloch & Schneider).

I. INTRODUCTION

A recent review by Underwood (1953) has shown the paucity of information on the copper levels in the liver and blood of vertebrates except in a few species which have been studied in detail. Relatively little new information has appeared since the review by Elvehjem (1935) and, furthermore, much of the earlier work is of limited value because of doubtful analytical methods, particularly in the case of blood.

As early as 1931 Cunningham showed that the copper level in the liver of sheep and cows was much higher than in certain other species. This fact generally has been ignored and no attempt seems to have been made to assess its significance.

The present investigation has been carried out to extend Cunningham's observations by examination of the liver and blood of as wide a range of species as was available in Western Australia. It was designed to ascertain if systematic variations of copper levels occurred in the different classes and orders of vertebrates and to provide basic data necessary for a proper study of the comparative biochemistry of copper. It was also hoped that some light would be thrown on the anomalous copper status of the sheep and the cow.

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The present paper includes data from adult non-pregnant animals only, as numerous workers have shown that the pattern of copper metabolism is different in pregnant animals and in the embryo.

II. MATERIAL STUDIED

Samples of liver and blood from horses, pigs, and poultry were obtained from slaughter-house material. Blood was collected from the carotid artery. The values for sheep were obtained both from experimental animals, and from animals slaughtered for rations. Wild species were usually obtained by shooting, and where it was possible to obtain blood samples these were obtained by puncture of the heart or of adjacent arteries. The samples from "Ord River" are from country adjacent to the Kimberley Research Station, some 45 miles east of Wyndham. The specimens of the giant toad (*Bufo marinus* L.) were obtained by air freight from the canefields of Queensland.

The whale liver samples were obtained from dead animals on the flensing decks of the whaling companies operating in Queensland and Western Australia. In all cases the animals had been dead for at least 3 hr and the "blood" sample, obtained by cutting arteries in the snout, consisted of a variable mixture of whole blood and serum or plasma. Iron determinations were done on these samples to obtain some indication of the amount of red cells present.

Although there is no suggestion that normal seasonal variations in diet have any significant effect on levels of copper in blood or liver, the date of sampling is indicated for all wild species. Such dates have been omitted from domestic, commercial, or laboratory animals which exist on a more uniform diet.

The classifications of Simpson (1945) have been followed in the arrangement of results. In naming fish, the papers of Whitley (1940, 1948), Olsen (1953), and Thomson (1954) have been followed.

III. ANALYTICAL METHODS

Copper determinations were made by means of the diethyldithiocarbamate complex in amyl alcohol after destruction of organic matter by nitric, sulphuric, and perchloric acids (Eden and Green 1940).

Where haemoglobin levels are reported, they are calculated from iron content which was estimated by the dipyriddy method (Jackson 1938). It has been assumed that blood contains 1 per cent. non-haemoglobin iron and that haemoglobin contains 0.34 per cent. iron.

In the present investigation the results for liver copper values are subject to errors from two sources. The first is due to an uneven distribution of copper in the liver: duplicate samples from the same liver of whales, kangaroos, birds, crocodiles, and fish have given results which may differ by up to 20 per cent. although the difference is usually less.

This source of error particularly applies to the wallaby livers from Ord River where transport difficulties made it necessary to collect small samples (1-3 g dry weight); it also applies in the case of whales, where, of necessity, only a small portion of the liver was taken for analysis. In most cases, however, a fairly large portion of the liver was taken, dried, and subsampled. With smaller animals the whole liver was analysed.

The second source of error is due to the presence of variable amounts of fat in the liver. This acts as a diluent, and lowers the concentration of copper in the tissues. With mammals the percentage of fat is normally quite low, and no correction has been made for fat content. Only three mammalian livers (two cat and one leopard seal) were noted to be obviously fatty, and in these cases the results were recalculated to a basis of 5 per cent. fat. Bird livers normally show no obvious signs of fat although one batch of livers from domestic fowls was noted to be very fatty. The copper content of this batch was calculated to a basis of 15 per cent. fat which was the mean level in a random selection of "non-fatty" livers from fowls.

In the case of reptiles, amphibia, and fish, the percentage of fat in the liver is extremely variable, and may rise to high levels. In earlier samples such fatty livers were extracted with petroleum ether, and the determination of copper was made on the fat-free residue. Subsequently, in tests on very fatty frog and fish livers it was found that some copper (up to 10 per cent.) was dissolved by the petroleum ether. Although this error is of no great consequence, in all later samples from reptiles, amphibia, and fish, the dried liver was extracted with petroleum ether by standing and decantation until fat-free. The percentage of fat-free material was thus obtained, and both this material and the extracted fat were digested together for copper estimation. In the results for reptiles (see Appendix 1) where no comment is made, the samples were not obviously fatty, and analysis is on the dry liver without correction for fat content.

The blood was usually obtained with a stainless steel needle and an all-glass syringe. The brass butts of the needles were removed, and the needles were mounted in heavy rubber tubing. During the course of the investigation it was found that some stainless steel needles contained copper, and that appreciable amounts (up to 2 μg) could dissolve during the passage of blood through the needle. Subsequently all needles were tested by soaking for several hours in dilute ammonia solution; the addition of a solution of sodium diethyldithiocarbamate showed if copper was present. The blood samples from marsupials and the wild turkey, taken between Ord River and Port Hedland, were obtained with needles which were no longer available for testing when this source of error was discovered. However, as the results for these marsupials are the same as, or even lower than, results for similar species elsewhere it is fairly certain that no contamination has occurred.

TABLE 1
THE COPPER CONTENT OF THE LIVER AND BLOOD OF ADULT ANIMALS
Values from published papers. Liver values as p.p.m. copper on dry
weight basis; blood values as mg copper/l on whole blood

Species	Blood Copper (mg/l)	Liver Copper (p.p.m.)
Man		
Male	0.96 ± 0.13 ^(b)	25 ^(a)
	1.01 ± 0.02 ^(c)	24 ^(d)
Female	1.00 ± 0.11 ^(b)	—
	1.07 ± 0.02 ^(c)	—
Rat	0.99 ^(e)	12 — 18 ^(e)
	—	10 ^(a)
	—	11 ^(f)
	—	34 ± 2.9 ^(h)
Rabbit	0.69 — 0.86 ^(g)	9 ^(a)
	—	23 ± 3.6 ^(h)
Guinea pig	—	17 ^(a)
	—	23 ± 3.5 ^(h)
Badger	—	22 ^(a)
Pig	1.54 — 1.66 ⁽ⁱ⁾	41 ^(a)
	—	21 ^(d)
	—	15 — 20 ^(j)
Sheep	0.4 — 1.6 ^(k)	237 ^(a)
	—	190 — 446 ⁽ⁿ⁾
Cow	0.7 — 1.7 ^(k)	161 — 200 ^(l)
	1.3 — 1.5 ^(l)	77 ^(a)
	—	70 ^(d)
Horse	—	15 ^(a)
	—	21 ^(d)
Domestic fowl	—	12 ^(a)
	—	18 ^(d)
Fish		
Various species*	—	149 — 333 ^(m)
Herring	—	14 ^(a)
<i>Torpedo marmorata</i>	0.48 — 0.74 ^(o)	—
<i>Salmo trutta</i>	—	982 ± 147 ^(p)
		(range 165 — 1470)

* These values are on fat-free, dry weight basis.

(a) Cunningham (1931). (b) Lahey *et al.* (1953). (c) Sachs *et al.* (1943). (d) Elvehjem (1935). (e) Boyden, Potter, and Elvehjem (1938). (f) Lindow, Peterson, and Steenbock (1929). (g) Fontaine and Leloup (1946). (h) Lorenzen and Smith (1947). (i) Schultze, Elvehjem, and Hart (1936). (j) Harvey (1952). (k) Beck (1941). (l) Sahai and Kehar (1951). (m) Baldassi and Vignato (1942). (n) Albiston *et al.* (1940). (o) Leloup (1949). (p) Dewey, D. W., and Nicholls, A. G. (unpublished data 1955).

IV. RESULTS

The results for the copper content of liver and blood of the species examined are set out in Appendices 1 and 2 respectively. The figures are

generally presented as the mean, together with the standard deviation of the mean, and the range of the observations. Standard deviations are not given when the number of observations is less than four.

A selection of data from the literature is given for comparison in Table 1.

V. DISCUSSION

(a) *Blood Copper Levels*

In the few cases where it is possible to make comparisons with other workers, the agreement is fairly close. Because of difficulties of collection, and because many of the earlier samples were collected with defective needles, the number of observations is not as great as is desirable.

The very low haemoglobin levels of the coelacanth blood suggest that it may not have been an entirely normal sample.

There seem to be no figures published for the copper content of the whole blood of birds, but Warburg and Krebs (1927) and Locke, Rosbash, and Shinn (1934) report low serum levels. The statement by Underwood (1953) that "in birds there is a striking concentration of copper in the nucleated red blood cells" seems to have been made with little real evidence, but it may be noted that in three blood samples from domestic turkeys the mean copper level of the whole blood was 0.22 mg copper/l while the corresponding figure for plasma was 0.11.

In an earlier investigation (Beck 1941) blood copper values as low as 0.4 mg/l were found in so-called "normal" sheep. It is now considered that levels of about 0.8 mg/l represent the lower limit of normality. Values between 0.4 and 0.8 are occasionally found in apparently normal sheep but these appear to indicate either an incipient copper deficiency or the presence of some factors causing a derangement of copper metabolism.

The blood copper levels show some curious variations which do not parallel the phylogenetic relationships implied in current systems of classification. Among placental mammals, the pig shows uniformly high values (1.4-1.5 mg copper/l). The rat, sheep, cow, and man show intermediate levels (*c.* 1 mg/l), while relatively low levels occur in the guinea pig and the rabbit (0.5-0.7 mg/l). Insufficient figures are available to draw any conclusions about Carnivora. Large marsupials show low values (0.3-0.4 mg/l). Still lower values are obtained from the fowl and the domestic turkey (generally 0.2-0.3 mg/l). These samples were collected from healthy commercial birds, and the possibility of a deficient copper intake is extremely improbable. The duck and the Antarctic birds show slightly higher levels, while in the emu and the wild turkey (one value only) the blood copper is within the range of the higher mammals. The limited number of fish, reptile, and amphibian bloods generally show values within this range.

There is no suggestion from this or other investigations that, for animals on diets of normal copper content, the blood copper levels are determined by dietary copper. Thus, the groups showing the highest and lowest values (the pig and the fowl respectively) are fed commercially on very similar diets. It is probable, however, that all species will show lowered blood copper levels when fed on diets which are extremely low in copper.

In the species which have been studied in detail, there is considerable evidence to show that the concentration of copper in blood can be altered markedly by physiological and pathological conditions (see review by Cartwright 1950). Thus, in man, it has been shown by numerous workers that during pregnancy there is a large rise in maternal blood copper whereas the foetal blood copper is low. In the sheep, there is no change in maternal blood copper but high levels are observed in the new-born lamb (McDougall 1947). Infections of various kinds cause a rise of levels in man; similar rises have been observed by the author in a limited number of cases in sheep, pigs, and kangaroos. In some species, haemorrhage causes increased levels (Warburg and Krebs 1927), and severe exercise causes rises in the blood copper of sheep (Dick 1954b) and of man (Daum 1949). Lowered levels occur in the nephrotic syndrome and in Wilson's disease. Thyroid activity also influences blood copper levels (Fontaine and Leloup 1946, 1947; Daum 1951).

Although no explanation can be given for these variations, it seems likely that they have some physiological significance. The fact that blood copper levels can vary so readily may possibly provide the clue to the differences between species. As a tentative hypothesis it is suggested that the level of copper usually encountered in the blood of healthy members of any species is determined solely by the physiological requirements of that species.

(b) Liver Copper Levels

In this investigation the values found agree closely with the relevant data of other investigators except in the case of the guinea pig. No explanation can be given for this one particular difference.

The sex difference in the liver values of the Australian salmon (*Arripis trutta* Bloch & Schneider) was highly significant ($P < 0.001$) in both 1953 and 1954. In the closely related ruff (*A. georgianus* Cuv. & Val.) the number of male livers analysed was quite small but the results did not suggest any similar difference. In no other animal species was there any evidence of sex difference.

The values for the toad (*Bufo marinus*) show a very wide variation for which no satisfactory explanation can be given.

A consideration of the data in Appendix 1 shows that the liver copper of most species lies below 50 p.p.m., and that very much higher values are found only in totally unrelated species: the ruminant, the duck, the frog,

and certain species of fish. Intermediate levels have been found in the guinea pig, in some fish, and possibly among Carnivora. While no explanation for these differences can be made at present there are several points which may be stressed.

In a study of this type, most of the livers are from animals of unknown dietary history, but the results suggest that dietary copper is not an important factor in determining liver copper levels. It is usual for fowls and ducks from commercial flocks to be fed similar diets, and yet the mean liver copper of the former is low (15 p.p.m.) and the latter is high (153 p.p.m.). The whale shows low copper levels (21 p.p.m.) in spite of the fact that the diet consists entirely of Crustaceae which presumably contain fairly large amounts of copper derived from their respiratory pigment haemocyanin. In certain parts of the Wiluna area of Western Australia the natural herbage is high in copper (10-20 p.p.m. on dry material). Sheep grazing in these areas show high liver copper values (up to 2700 p.p.m.), yet kangaroos grazing on the same areas show liver values of only 13-17 p.p.m. Diets abnormally high or low in copper will cause corresponding changes in the concentration of copper in the liver of most, if not all, species; nevertheless, there is evidence from this and other work to show that for some species the copper intake may vary within fairly wide limits without a corresponding change in liver levels. Thus, kangaroos grazing on the copper-deficient coastal areas between Gingin and Yanchep show values which are no lower than those from normal areas or from the high-copper Wiluna area. Kangaroos are not restricted in their grazing, as are sheep, but some differences in liver copper levels would be expected if these were determined by the copper content of the herbage. The work of Cunningham (1931) with rats and domestic fowls also suggests that moderate increases in dietary copper will have little effect on the liver copper levels of these two species.

The sheep and the cow are different from most other mammalian species in that, within the normal range of diets, the concentration of copper in the liver varies with the intake. Dick (1954a) has shown, for crossbred sheep in pens, that the liver storage is directly proportional to the intake when the dietary copper lies between 4 and 18 mg daily. Under field conditions in Western Australia, there is generally a good correlation between liver copper levels and the copper levels of pastures in the growing period. Less information is available concerning the cow, but experience in the areas of copper deficiency has shown that the levels of copper in the liver follow the levels in the pasture (Bennetts *et al.* 1941, 1948). Under conditions of high copper intake, the cow appears to have a greater ability to regulate liver storage than the sheep. The work of Cunningham (1946) with cows showed that daily doses of copper sulphate, at the rate of 0.3-0.5 mg copper/lb live weight for 5-11 months, had no untoward effect on the animals and the liver copper was raised to only 570 p.p.m. In the experiments of Dick (1954a) a group of five sheep

received 33.6 mg copper daily (approx. 0.45 mg copper/lb live weight) for a period of 5½ months; two sheep died from copper poisoning after 5 months, and the mean liver copper of the remaining sheep was 2340 p.p.m. at the conclusion of the experiment.

The development of high liver copper levels on diets of normal copper content could be due either to increased absorption or decreased excretion. Although the sheep is characterized by high liver copper levels, there is little evidence to support the idea of excessive absorption in this species. In a large series of experiments Dick (1954a, 1954b) has shown that, on relatively high intakes of copper (10-30 mg per day), the liver storage is between 3 and 4 per cent. of the copper ingested. Urinary copper is extremely small, and unpublished figures obtained by the author show values below 10 µg/l. On the other hand, the rat maintains a low liver copper level in spite of a relatively high absorption of copper from the diet. Values obtained by Cunningham (1931) show that rats receiving a diet low in copper excrete some 26 per cent. of dietary copper in the urine. Lindow, Peterson, and Steenbock (1929) obtained similar results with rats on a normal diet.

It should be noted also that the rat is extremely tolerant to high doses of copper. Cunningham (1931) found that growth and reproduction was possible in rats fed 7.5 mg copper daily (approx. 500 p.p.m. copper in diet). By contrast, the sheep is very susceptible to increases of dietary copper, and if this is raised above about 20 mg daily (i.e. 20 p.p.m. copper in diet) excessive accumulation of copper occurs in the liver and copper poisoning may occur. The pig is another animal with a low liver copper level, and recent work has shown that normal growth and development will occur when diets containing 250 p.p.m. copper are fed (Barber, Braude, and Mitchell 1955a, 1955b; Bowler *et al.* 1955).

An explanation of the above observations may be offered in terms of differences in ability to restrict the storage of copper in the liver, due to differences in the avidity of the liver cells for copper or in the ability of the animals to excrete stored liver copper. Appropriate data to check this are not available for the rat, but Dick (1954b) has given pertinent values for another animal of low liver copper level, the rabbit. In his experiments, rabbits were given daily intravenous injections of 100 and 500 µg copper (as lactate) for 209 days. At the end of the period, the mean liver copper level of the control group was 7 p.p.m., of the group receiving 100 µg copper only 10 p.p.m., and of the highest copper group, 62 p.p.m.

Comparable experiments with the sheep do not seem to have been carried out, but the following balance experiment by the author is of interest. A sheep was given an intravenous injection of 20 mg copper; a fortnight later the dose was repeated. Four similar doses were then

given at weekly intervals, and the animal was slaughtered. Faeces and urine were collected throughout the experiment. Of the 120 mg of copper injected, 104 mg was retained in the body, and at least 70 per cent. of this was found in the liver.

A consideration of the data available for the sheep, rat, and rabbit suggests strongly that the development of the characteristically high liver copper level of the sheep is due to a lesser ability to restrict the storage of copper in the liver rather than to the absorption of excessive amounts. If this hypothesis applies to other species with high liver copper levels, it would be expected that the duck would be more susceptible to increases of dietary copper than the domestic fowl, and experiments are being carried out to investigate this point.

VI. ACKNOWLEDGMENTS

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APPENDIX 1

CONCENTRATION OF COPPER IN LIVERS

Values as p.p.m. on dry weight basis. S.D. not given when number of observations less than four

Species	Details	No. of Observations	Liver Copper (p.p.m.)	
			Mean and S.D.	Range
PRIMATES				
Orang-outang (<i>Pongo pygmaeus</i> Hoppius)	Zoological gardens, died from unknown causes, no obvious pathological changes	1	—	27
RODENTIA				
White rat (<i>Rattus norvegicus</i> Erxleben)	Laboratory animals	5	13.2 ± 0.5	12-15
Guinea pig (<i>Cavia porcellus</i> Pall.)	Laboratory animals	33	77.3 ± 5.6	29-205
LAGOMORPHA				
Wild rabbit (<i>Oryctolagus cuniculus</i> L.)	Beverley, Narrogin, Kojonup, and Borden; July-Aug. 1952	7	15.8 ± 0.6	14-19
Rabbit (<i>O. cuniculus</i>)	Laboratory animals	4	14 ± 3	9-20
CARNIVORA				
Cat (<i>Felis catus</i> L.)	Mongrels	6	49 ± 11	9-75
Dog (<i>Canis familiaris</i> L.)	Mongrels	3	80	22-154
Fox (<i>Vulpes vulpes</i> L.)	South Merredin, Mar. 1953	3	32	23-44
Elephant seal (<i>Macrorhinus proboscideus</i> Peron & Lesueur)	Heard I., Feb. 1954 and 1955. One male, nine females	10	66 ± 6	34-90
Leopard seal (<i>Hydrurga leptonyx</i> Blainville)	Heard I., Feb. 1955, males	2	95	84-105
Weddell seal (<i>Leptonychotes weddellii</i> Lesson)	Sandefjord Bay, Antarctica, Feb. 1955, females	3	38	24-46
PERISSODACTYLA				
Horse (<i>Equus caballus</i> L.)		6	14.8 ± 1.1	12-19
ARTIODACTYLA				
Pig (<i>Sus scrofa</i> L.)	Toodyay district	14	18.9 ± 0.9	15-25
Goat (<i>Capra hircus</i> L.)	Pt. Hedland district, Sept.-Nov. 1954	6	300 ± 62	157-590
Sheep (Merino) (<i>Ovis aries</i> L.)	(a) Beverley, dry grazing, Apr.-May 1952	6	329 ± 47	140-451
	(b) Beverley, green herbage, June-Nov. 1952	21	298 ± 28	123-584
	Mean of (a) and (b)	27	305 ± 24	123-584

APPENDIX 1 (*Continued*)

Species	Details	No. of Observa- tions	Liver Copper (p.p.m.)	
			Mean and S.D.	Range
CETACEA				
Humpback whale (<i>Megaptera nodosa</i> Bonnaterre)	(a) Carnarvon, W.A., June 1952	13	18.9 ± 1.2	15-30
	(b) Pt. Cloates, W.A., Aug. 1952	16	20.4 ± 1.3	12-32
	(c) Brisbane, Qld., Sept. 1954	8	25.6 ± 2.2	18-38
	Overall mean (a)-(c)	37	21.0 ± 0.9	12-38
MARSUPIALIA				
Grey kangaroo (<i>Macropus ocydromus</i> Gould)	(a) Darkan, June 1951	2	20	—
	(b) Margaret R. area, May-Oct. 1952	7	18.9 ± 0.8	15-21
	(c) Pingrup area, Sept. 1952	4	16.7 ± 0.6	15-18
	(d) Kojonup area, Nov. 1952	2	22	19-26
	(e) Gingin-Yanchep area, Dec. 1952 and Apr. 1954	2	22	20-24
	Overall mean (a)-(e)	17	19.3 ± 0.7	15-26
Red kangaroo (<i>M.</i> <i>rufus</i> Desmarest)	(a) Merredin, July 1949	1	—	9
	(b) Pt. Hedland district, June 1952	3	13	13-14
	(c) Albion Downs station, Wiluna, July 1953	6	15.5 ± 0.4	14-17
	(d) Lorna Glen station, Wiluna, July 1953	2	13.5	13-14
	(e) Cue, Mar. 1954	1	—	16
	Overall mean (a)-(e)	13	14.2 ± 0.6	13-17
Euro (<i>M. robustus</i> Gould)	(a) Pt. Hedland district, June 1952	6	13.0 ± 1.0	10-17
	(b) Albion Downs station, Wiluna, July 1953	3	15	12-17
	Overall mean (a) and (b)	9	13.6 ± 0.8	10-17
Wallaby (<i>M. agilis</i> Schwarz)	(a) Ord R., June 1952	10	16.3 ± 0.7	14-20
	(b) Gogo station, Fitzroy R., June 1952	3	15.3 ± 0.3	15-16
	(c) Ord R., Dec. 1952- Jan. 1953	9	16.6 ± 0.7	14-20
	(d) Ord R., June 1953	13	18.3 ± 0.6	15-21
	Overall mean (a)-(d)	35	17.0 ± 0.4	14-21
Brush wallaby (<i>M.</i> <i>irma</i> Jourdan)	Bindoon, July 1952	1	—	19
Tammar (<i>Thylogale</i> <i>eugenii</i> Desmarest)	Recherche Archipelago, Feb. 1954	2	18.5	15-22

APPENDIX 1 (Continued)

Species	Details	No. of Observa- tions	Liver Copper (p.p.m.)	
			Mean and S.D.	Range
Quokka (<i>Setonix brachyurus</i> Quoy & Gaimard)	(a) Laboratory animals	4	16.5 ± 0.9	14-18
	(b) Rottnest I., Dec. 1952	9	13.7 ± 0.8	9-17
	(c) Rottnest I., Nov. 1953	5	11.0 ± 0.5	10-13
	(d) Bald I., June 1954	2	9	8-10
	Overall mean (a)-(d)	20	13.1 ± 0.7	8-18
Rock wallaby (<i>Petrogale hacketti</i> Thomas)	Recherche Archipelago, Feb. 1954	1	—	23
Rat kangaroo (<i>Bettongia penicillata</i> Gray)	Narrogin-Williams district, Aug. 1954	2	27	21-33
AVES				
Domestic fowl (<i>Gallus gallus</i> L.)	(a) Australorp pullets, Mar. 1952	12	12.7 ± 0.5	9-15
	(b) Australorp pullets, July 1952	7	15.1 ± 0.7	13-18
	(c) Australorp adult birds*	10	15.1 ± 0.6	12-18
	(d) White leghorn cockerels	4	14.2 ± 0.5	13-15
	(e) White leghorn hens, 1 year culls	10	17.0 ± 1.6	14-31
	(f) White leghorn hens, 2 year	4	14.5 ± 0.6	13-16
	(g) Australorp × white leghorn hens	4	14.7 ± 0.5	14-16
	Overall mean (a)-(g)	51	14.8 ± 0.4	9-31
White muscovy duck (<i>Cairina moschata</i> L.)	(a) All female	12	164 ± 33	82-500
	(b) 7 female, 3 male	10	63 ± 7	37-102
	(c) All male	12	218 ± 39	88-555
	Overall mean (a)-(c)	34	153 ± 21	37-555
Turkey (<i>Meleagris gallopavo</i> L.)		6	13.5 ± 0.2	13-14
Crow (<i>Corvus ceciliae</i> Mathews)	Ord. R., June 1952	6	41 ± 5	25-56
Little corrella (<i>Kakatoë sanguinea</i> Gould)	Ord. R., June 1952	3	16	16-17
Galah (<i>K. roseicapilla</i> Vieillot)	Gogo station, Kimberleys, June 1952	3	17	15-19
Kite hawk (<i>Milvus migrans</i> Boddaert)	Ord R. and Pt. Hedland, June 1952	3	18	17-19
Wedgetail eagle (<i>Uroaëtus audax</i> Latham)	Ord R., June 1952	1	—	26
Brown hawk (<i>Falco berigora</i> Vigors & Horsfield)	Wiluna, July 1953	1	—	23

* Birds not laying, livers fatty, results calculated on basis of 15% fat.

APPENDIX 1 (Continued)

Species	Details	No. of Observa- tions	Liver Copper (p.p.m.)	
			Mean and S.D.	Range
Butcher bird (<i>Cracticus torquatus</i> Latham)	Perth, Sept. 1952	1	—	12
Kimberley kookaburra (<i>Dacelo leachi</i> Vigors & Horsfield)	Ord R., June 1952	1	—	26
Bee eater (<i>Merops ornatus</i> Latham)	Ord R., June 1952	2	—	15
Pied goose (<i>Anseranas semipalmata</i> Latham)	Ord R., June 1952	2	46	45-47
Wagtail (<i>Rhipidura leucophrys</i> Latham)	Perth, June 1951	1	—	29
Wild turkey (<i>Eupodotis australis</i> Gray)	Ord R., June 1952	2	31	29-33
Emu (<i>Dromaius novae- hollandiae</i> Latham)	Lorna Glen station, Wiluna, Sept. 1953	4	28 ± 4	17-37
Southern skua (<i>Stercorarius skua lonnbergi</i> Mathews)	Heard I., Mar. 1954	3	18	17-20
Storm petrel (<i>Oceanites oceanicus</i> Kuhl)	Heard I., Mar. 1954	1	—	21
Giant petrel (<i>Macronectes giganteus</i> Gmelin)	Heard I., Mar. 1954, young bird	1	—	30
Rockhopper penguin (<i>Eudyptes chrysocome</i> Forster)	Heard I., Mar. 1954, Feb. 1955	6	15 ± 0.6	13-17
Macaroni penguin (<i>E. chrysolophus</i> Brandt)	Heard I., Mar. 1954, Feb. 1955	3	15	13-16
Gentoo penguin (<i>Pygoscelis papua</i> Forster)	Heard I., Mar. 1954, Feb. 1955	5	13 ± 1.5	11-19
REPTILIA				
Freshwater tortoise (<i>Chelodina oblonga</i> Gray)	Perth, Oct. 1952	1	—	34
Crocodile (<i>Crocodylus johnsoni</i> Krefft)	Ord R., June-Nov. 1952	4	17.7 ± 2.6	11-23
Brown snake ("Linga")	Ord R., Nov. 1952	1	—	45
Brown snake (<i>Demansia nuchalis</i> Guenther)	Beverley, just emerged from hibernation, Oct. 1953	1	—	40
Blue-tongued lizard (not identified)	Ord R., Oct. 1952	1	—	19*

* Livers fatty, results on fat-free basis.

APPENDIX 1 (*Continued*)

Species	Details	No. of Observa- tions	Liver Copper (p.p.m.)	
			Mean and S.D.	Range
Bob-tail lizard (<i>Trachysaurus rugosus</i> Gray)	(a) Beverley, June 1952	1	—	12
	(b) Perth, Oct. 1952	1	—	10
	(c) Merredin, hibernat- ing, June 1953	1	—	11.5*
	(d) Perth, Feb. 1955	3	14*	11-16
	(e) Rottnest I., Feb. 1955	3	16*	11-20
	Overall mean (a)-(e)	9	14 ± 1	10-20
Skink (<i>Lygosoma</i> <i>trilineatum</i> Gray)	Cheyne Beach, Mar. 1953	1	—	10
Skink (<i>L. microtis</i> Gray)	Cheyne Beach, Mar. 1953	1	—	10
Skink (<i>Egernia</i> <i>napoleonis</i> Gray)	Cheyne Beach, Mar. 1953	1	—	10
AMPHIBIA-ANURA				
Frogs:				
<i>Lymnodynastes d.</i> <i>dorsalis</i> Gray	(a) Perth, Oct. 1952	1	—	865
	(b) Cheyne Beach, Mar. 1953	1	—	124
<i>Hyla aurea raniformis</i> Keferstein	Cheyne Beach, Mar. 1953	5	293 ± 58	172-454
Giant toad (<i>Bufo</i> <i>marinus</i> L.)	Qld., Mar. 1953 and Apr. 1954	14	468 ± 140*	10-1640
PISCES: ELASMOBRANCHII				
Blue whaler shark (<i>Carcharhinus mackieii</i> Phillips)	Rottnest I., Dec. 1952	1	—	27*
	(a) Cheyne Beach, Mar. 1953	1	—	50*
Grey nurse shark (<i>Carcharias arenarius</i> Ogilby)	(b) Denmark, W.A., Apr. 1954	1	—	35*
	Cheyne Beach, Mar. 1953	1	—	18*
Gummy shark (<i>Emissola antarctica</i> Günther)	Cheyne Beach, Mar. 1953	1	—	18*
School shark (<i>Galeorhinus australis</i> Macleay)	Rottnest I., Dec. 1953	2	13	11-15*
Stingray (<i>Urolophus</i> <i>mucosus</i> Whitley)	Cheyne Beach, Mar. 1953	1	—	10*
Southern shovelnose ray (<i>Aptychotrema</i> <i>vincentiana</i> Haacke)	Cheyne Beach, Mar. 1953	1	—	13
PISCES: TELEOSTII				
Mullet (not identified)	Ord R., June 1952	1	—	335

* Livers fatty, results on fat-free basis.

APPENDIX 1 (Continued)

Species	Details	No. of Observa- tions	Liver Copper (p.p.m.)	
			Mean and S.D.	Range
Bream (<i>Mylio butcheri</i> Munro)	Swan R., Sept. 1952- Mar. 1953	5	544 ± 202	108-1260
Cobbler (<i>Cnidoglanis macrocephalus</i> Cuv. & Val.)	(a) Swan R., Dec. 1952	3	10	9-11
	(b) Cheyne Beach, Mar. 1953	1	—	11*
Sand flathead (genus <i>Planiplora</i>)	Swan R., Jan. 1953	1	—	108
Australian salmon (<i>Arripis trutta</i> Bloch & Schneider)	Cheyne Beach, Mar. 1953 and Apr. 1954			
	(a) Male	18	15.0 ± 1.2*	10-30
	(b) Female	26	45.2 ± 3.9*	20-94
Ruff (<i>A. georgianus</i> Cuv. & Val.)	(a) Cheyne Beach, Mar. 1953, all female	5	62 ± 11*	26-88
	(b) Busselton, Jan. 1954, sexes not recorded	3	36 ± 8*	22-51
	(c) Denmark, W.A., Apr. 1954, 1 male, 5 females	6	59 ± 7*	44-80
	(d) Cheyne Beach, Apr. 1954, 1 male, 4 females	5	44 ± 4*	36-55
	Overall mean (a)-(d)	19	52 ± 4*	22-88
Skipjack (<i>Usacaranx georgianus</i> Cuv. & Val.)	(a) Cheyne Beach, Mar. 1953	1	—	22
	(b) Busselton, Jan. 1954	2	23*	16-31
Flathead (<i>Trudis bassensis westraliae</i> Whitley)	Cheyne Beach, Mar. 1953	1	—	10*
Sand whiting (<i>Sillago bostockii</i> Castelnau)	Busselton, Jan. 1954	3	24*	12-40
King George whiting (<i>Sillaginodes punctatus</i> Cuv. & Val.)	Busselton, Jan. 1954	1	—	44*
Trumpeter (<i>Helotes sexlineatus</i> Quoy & Gaimard)	Busselton, Jan. 1954	2	23*	22-23
Garfish (<i>Reporhamphus melanochir</i> Cuv. & Val.)	Busselton, Jan. 1954	2	15*	14-17
Flounder (order Heterosomata)	Busselton, Jan. 1954	1	—	29*
Rock cod (family Epinephelidae)	Busselton, Jan. 1954	1	—	13*

* Livers fatty, results on fat-free basis.

APPENDIX 1 (Continued)

Species	Details	No. of Observations	Liver Copper (p.p.m.)	
			Mean and S.D.	Range
Sea mullet (<i>Mugil cephalus</i> L.)	Cheyne Beach, Mar. 1954:			
	(a) Male	1	—	176*
	(b) Female	2	256*	117-395
Morwong (<i>Psilocranium nigricans</i> Richardson)	Cheyne Beach, Mar. 1954, female	1	—	59*
Coelacanth (<i>Latimeria chalumnae</i> Smith)	Madagascar:			
	(a) Adult male, Oct. 1954	1	—	36*
	(b) Adult female, Nov. 1954	1	—	47*

* Livers fatty, results on fat-free basis.

APPENDIX 2

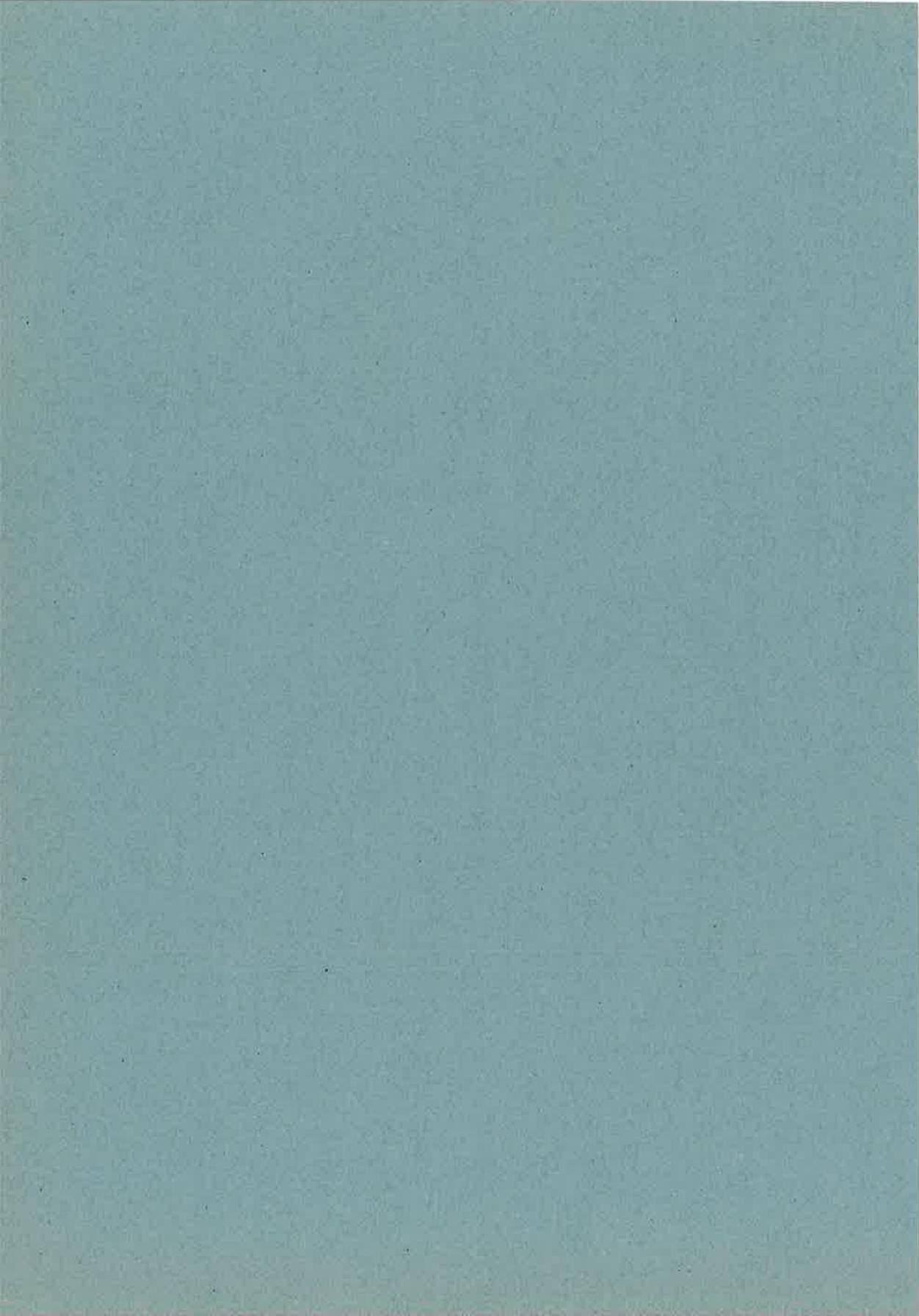
COPPER CONTENT OF BLOOD

Values expressed as mg copper/l of whole blood. Where no details are given, these are as in Appendix 1. Species names are the same as in Appendix 1

Species	Details	No. of Observations	Blood Copper (mg/l)	
			Mean and S.D.	Range
Rabbit	Laboratory animals, female	3	0.75	0.70-0.79
Guinea pig	(a) Males	15	0.49 ± 0.01	0.40-0.58
	(b) Females	10	0.55 ± 0.03	0.40-0.76
	Overall mean (a) and (b)	25	0.52 ± 0.02	0.40-0.76
Cat	—	4	0.99 ± 0.13	0.80-1.38
Dog	—	3	0.70	0.59-0.79
Elephant seal	—	10	1.19 ± 0.04	0.90-1.43
Leopard seal	—	2	0.80	0.78-0.82
Weddell seal	—	2	1.29	0.90-1.68
Pig	(a) Mt. Barker district	15	1.42 ± 0.05	1.20-1.82
	(b) Northam district	15	1.37 ± 0.05	1.10-1.60
	Overall mean (a) and (b)	30	1.40 ± 0.03	1.10-1.82
Sheep (Merino wethers)	Merredin			
	(a) June 1949	51	1.00 ± 0.02	0.76-1.35
	(b) Feb. 1950	69	0.98 ± 0.01	0.75-1.28
	(c) Apr. 1954	40	1.09 ± 0.02	0.81-1.32
	Overall mean (a)-(c)	160	1.02 ± 0.01	0.75-1.35
Horse	—	2	0.75	0.72-0.78
Whale	Carnarvon, June 1952. (Post mortem samples, mixture of plasma and blood: Haemoglobin 7.6-11.7 g/100 ml)	12	1.18 ± 0.03	1.04-1.35
Grey kangaroo	Various localities	4	0.41 ± 0.02	0.38-0.45
Red kangaroo	Various localities	8	0.35 ± 0.02	0.24-0.41

APPENDIX 2 (*Continued*)

Species	Details	No. of Observa- tions	Blood Copper (mg/l)	
			Mean and S.D.	Range
Euro	Pt. Hedland and Wiluna	6	0.35 ± 0.03	0.27-0.44
Wallaby	Ord R., June 1952	5	0.34 ± 0.02	0.27-0.39
Quokka	(a) Rottnest I., 1953	5	0.29 ± 0.04	0.16-0.42
	(b) Laboratory animals	2	0.43	0.33-0.52
	Overall mean (a) and (b)	7	0.33 ± 0.04	0.16-0.52
Domestic fowl	—	51	0.23 ± 0.01	0.11-0.47
Duck	—	38	0.35 ± 0.01	0.22-0.45
Turkey	—	14	0.23 ± 0.01	0.18-0.28
Emu	—	4	0.64 ± 0.03	0.55-0.71
Wild turkey	—	1	—	0.54
Giant petrel	—	1	—	0.30
Skua	—	2	0.39	0.35-0.42
Gentoo penguin	—	5	0.50 ± 0.03	0.43-0.58
Macaroni penguin	—	3	0.53	0.50-0.55
Rockhopper penguin	—	4	0.37 ± 0.02	0.32-0.43
Bobtail lizard	Perth and Rottnest I.	5	0.78 ± 0.01	0.75-0.82
Giant toad	—	8	0.46 ± 0.04	0.25-0.67
Australian salmon	Busselton, May 1954	8	0.58 ± 0.02	0.45-0.64
Ruff	Cheyne Beach and Denmark, W.A., 2 males, 9 females	11	0.71 ± 0.07	0.56-0.97
Sea mullet	—	3	0.53	0.47-0.66
Morwong	—	1	—	0.43
Coelacanth	Female (haemoglobin, 2.8 g/100 ml)	1	—	1.01



PAPER 1:2

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**OBSERVATIONS ON THE COPPER METABOLISM OF THE DOMESTIC
FOWL AND DUCK**

By A. B. BECK

OBSERVATIONS ON THE COPPER METABOLISM OF THE DOMESTIC FOWL AND DUCK*

By A. B. BECK†

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Summary

Experiments have been carried out to determine whether the concentration of copper in the liver of the domestic fowl and duck can be raised by a moderate increase of dietary copper. Groups of both species were fed for 12 weeks on the same basal diet, to which was added copper sulphate to increase the copper intake two- and fivefold. No significant increase in the liver copper concentration was noted in either species.

When copper was administered to both species by intravenous injection, it was rapidly excreted, mostly in the bile. In the fowl a significant amount was excreted through the caeca, but the experiments did not suggest that these organs were important in controlling copper storage.

Studies on the relationship between liver copper storage and age showed that there was a rapid increase in the duck after 3 weeks of age. No such changes were observed in the fowl.

I. INTRODUCTION

In an earlier publication (Beck 1956) it was found that the liver copper concentration of most species was less than 50 p.p.m. (dry matter basis). However, in a few unrelated groups of animals (ruminants, ducks, frogs, and certain fish) very much higher concentrations were regularly recorded.

The present paper describes observations made to obtain information on the storage and excretion of copper in the domestic fowl (*Gallus gallus* L.) and the Muscovy duck (*Cairina moschata* L.). These species were chosen because they are characterized by low and high liver copper levels respectively and also because they could be fed the same diet, any differential effect of other dietary factors on copper storage thus being eliminated.

II. CHEMICAL METHODS

Copper was determined by the method of Eden and Green (1940). Care was taken, particularly with faecal material, to avoid overheating the digestion flask above the level of the acid. Unless this precaution was observed, insoluble material was baked onto the glass and low recoveries of copper were obtained.

All liver copper levels in this paper are reported on a dry matter fat-free basis. Fat was determined by extraction with petroleum ether (b.p. 50–70°C). The copper

* This work was carried out at the Animal Health and Nutrition Laboratory and the Poultry Research Station, both of the Department of Agriculture of Western Australia.

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content of feeds and of all tissues is reported on the dry matter basis. With bile and blood the values are expressed as milligrams per litre.

Molybdenum was determined by the method of Piper and Beckwith (1948) and inorganic sulphate by a benzidine sulphate method developed by Dick and Bingley (personal communication). Iron was determined in blood after wet digestion by a modification of the method of Mayer and Bradshaw (1951). Levels of haemoglobin have been calculated on the basis that it contains 0.34% iron, and that 1.6% of the iron in blood is in an inactive form (Rostorfer 1949).

III. EFFECT OF INCREASING DIETARY COPPER ON THE LIVER COPPER LEVELS OF COCKERELS AND DUCKS

(a) *Experimental*

Groups of day-old White Leghorn cockerels and day-old Muscovy ducks of mixed sexes were purchased from commercial hatcheries. It was originally intended to use drakes for the feeding tests, but at this stage of the experiments we were unable to determine the sex of the day-old duck with any high degree of certainty. All birds were fed for 3 weeks on a standard poultry mash containing vitamins A and D. This mash had the following composition: protein ($N \times 6.25$), 18.8%; calcium, 1.50% Ca; phosphorus, 1.04% P; manganese, 53 p.p.m. Mn; molybdenum, 0.41 p.p.m. Mo; copper, 5.7 p.p.m. Cu; inorganic sulphate, 0.43% SO_4 .

During the preliminary feeding period there was a heavy mortality among the cockerels from some unidentified disease, but the remaining birds grew normally.

At the end of the preliminary 3 week period, six cockerels and six ducks were killed and blood, liver, and bile collected for the determination of pre-experimental copper values. The balance of each species was then randomized into three groups each of approximately 12 birds, which were housed in galvanized iron cages. The groups were fed on: (1) the basal diet described above; (2) the basal diet with copper sulphate added to give a total copper content of 12.5 p.p.m.; (3) the basal diet with copper sulphate added to give a total copper content of 27 p.p.m. The copper was added to the diet in the form of $CuSO_4 \cdot H_2O$, which was finely ground with starch as a diluent and then thoroughly mixed into the diet.

After 6 weeks' feeding, approximately half of the birds in each of the groups were killed and samples of blood, bile, and liver collected from each bird for copper determination. In addition copper determinations were made on kidneys, feathers, and bones from some of the birds.

The remainder of the birds were kept on their respective diets for another 6 weeks. At the end of this period the birds were killed and blood, liver, and bile samples were collected. The other tissues were omitted as no differences were noted in the samples collected after the first period.

(b) *Results*

The addition of copper to the diet had no effect on growth rate or on haemoglobin levels.

The results of the analyses of tissues are set out in Tables 1 and 2.

The increase of dietary copper by factors of 2.2 and 4.8 did not result in any significant increase in the concentration of copper in the liver of either cockerels or ducks at either period of sampling.

There was no significant difference in copper concentration due to sex among the experimental ducks. The large total amount of copper in one group of ducks (Table 2, 15 weeks, 12.5 p.p.m. diet) was due to the fact that the birds in this group were predominantly males, with greater body weight and correspondingly larger livers.

The bile copper values were extremely variable and no clear-cut pattern could be distinguished.

The results in Table 2 show that the liver copper levels of ducks at 3 weeks of age were significantly lower ($P < 0.001$) than those of the older birds. No differences due to age were noted with the cockerels. These observations were re-investigated in separate experiments described in Section VII.

IV. FATE OF COPPER IN COCKERELS AFTER INTRAVENOUS INJECTION

(a) *Experimental*

First cross Australorp \times White Leghorn cockerels were used for these experiments. The birds were 16–20 weeks of age and the body weight was generally between 2.0 and 2.3 kg. The birds were run in galvanized iron wire cages. When food consumption had become constant, the faeces were collected for 48 hr. The treated birds were injected with 1 mg copper (as sulphate) in 1 ml of isotonic saline solution through the wing vein. Faeces were collected for the following 12, 24, 48, or 96 hr period, after which the animals were slaughtered. Non-injected controls were run with each experiment. In all cases, samples of liver and blood were collected, also the contents of the duodenum, the small and large intestines, and the caeca; bile was collected whenever possible. In some of the first tests the walls of the duodenum, the small and large intestines, and the caeca were collected and also the gizzard contents, the kidneys, and urine samples (from ureters). No differences were noted in the concentration of copper in the urine or in the gizzard contents. The tissues of the injected birds contained slightly more copper but the differences were not considered sufficiently great to warrant collection in later tests.

One laying hen (together with an untreated control) was also injected and killed after 24 hr to see if the pattern of excretion was similar to that of the cockerels.

An attempt was made to locate the sites of copper storage in the liver by means of the rubanic acid staining methods of Uzzman (1956) and Howell (1959). Three cockerels were given two injections each of 1.5 mg copper at a 6 hr interval and were killed 12 hr after the first injection.

(b) *Results*

The results from the studies on the fate of copper given to cockerels by intravenous injection are given in Table 3.

The figures for the laying hens were very similar to those for the cockerels and have not been included.

TABLE 1
 COPPER CONTENT OF THE LIVER, BLOOD, AND BILE OF COCKERELS AT DIFFERENT LEVELS OF COPPER INTAKE
 Liver copper levels as parts per million on fat-free dry tissue. Figures given are means, with range of values in parenthesis

Age (weeks)	3	9	9	9	15	15	15
Cu in diet (p.p.m.) after 3 weeks	5.7	5.7	12.5	27	5.7	12.5	27
Number in group	6	5	4	6	5	6	5
Blood Cu (mg/l)	0.16*	0.19 (0.17-0.22)	0.19 (0.16-0.22)	0.17 (0.16-0.19)	0.21 (0.18-0.23)	0.22 (0.19-0.26)	0.19 (0.17-0.21)
Liver Cu (p.p.m.)	13.5† (12-17)	16.0 (13-20)	17.1 (15-19)	16.7 (16-19)	15.2 (14-16)	15.6 (14-17)	16.3 (14-18)
Liver Cu (μg)	14 (12-15)	79 (67-98)	68 (55-83)	74 (62-87)	127 (105-146)	114 (103-138)	116 (91-141)
Bile Cu (mg/l)	—	4.1*	4.7*	6.0*	3.5 (2.6-4.3)	4.3 (2.5-7.8)	3.6 (2.8-5.3)

* Bulk sample.

† Fat determinations not done on these samples—fat-free values calculated on assumed 5% fat basis.

TABLE 2
 COPPER CONTENT OF THE LIVER, BLOOD, AND BILE OF DUCKS AT DIFFERENT LEVELS OF COPPER INTAKE
 Liver copper levels as parts per million on fat-free dry tissue. Figures given are means, with range of values in parenthesis

Age (weeks)	3	9	9	9	15	15	15
Cu in diet (p.p.m.) after 3 weeks	5.7	5.7	12.5	27	5.7	12.5	27
Number in group	6	6	6	6	9	8	8
Blood Cu (mg/l)	0.27 (0.23-0.32)	0.28 (0.25-0.31)	0.29 (0.26-0.32)	0.27 (0.23-0.30)	0.34 (0.27-0.38)	0.29 (0.27-0.34)	0.32 (0.26-0.35)
Liver Cu (p.p.m.)	39† (26-73)	154 (121-191)	186 (136-275)	176 (120-250)	192 (75-273)	203 (94-288)	183 (111-251)
Liver Cu (μg)	87 (47-159)	1910 (1500-2330)	2230 (1360-3090)	1980 (1410-2700)	2150 (900-3160)	3670 (1920-5000)	2330 (1490-3420)
Bile Cu (mg/l)	4.5*	2.2 (1.4-3.1)	5.4 (3.2-8.7)	5.4 (2.2-9.0)	3.8 (1.9-5.4)	4.7 (3.2-7.2)	5.0 (2.7-6.7)

* Bulk sample.

† Fat determinations not done on these samples—fat-free values calculated on assumed 5% fat basis.

TABLE 3

COPPER LEVELS OF TISSUES AND VISCERAL CONTENTS OF COCKERELS AFTER INTRAVENOUS INJECTION OF COPPER

Copper injected, 1 mg Cu: liver values as parts per million copper on fat-free dry basis, other material on dry matter basis only; copper in food, 8.8-10.0, mean 9.4 p.p.m. Mean values and ranges given. A number in parenthesis indicates number of samples analysed when less than number of birds in group

Hours After Injection	No. of Birds	Blood (mg Cu/l)	Liver		Duodenal Contents (p.p.m. Cu)	Bile (mg Cu/l)	Small Intestine Contents (p.p.m. Cu)	Caecal Contents (p.p.m. Cu)	Large Intestine Contents (p.p.m. Cu)	Faeces (p.p.m. Cu)	
			(p.p.m. Cu)	(mg Cu)						Pre-Injection	Post-Injection
Controls	14	0.23 (13) 0.18-0.27	16.6 13.5-20.2	0.14 0.10-0.19	13.6 (13) 11.1-15.3	4 (9) 3-6	17.0 13.6-22.4	85 61-107	25 17-40	21.1 19.6-22.4	20.8 14.8-24.0
12	5	0.45 (4) 0.27-0.54	72 51-88	0.62 0.49-0.79	16.6 15.5-17.5	17 (3) 16-18	29.7 26.0-34.4	134 102-166	47 34-59	22.0 20.1-23.4	28.3 21.1-38.6
24	5	0.33 0.29-0.41	54 40-65	0.46 0.39-0.55	15.4 14.0-18.2	34 21-41	21.2 19.0-24.0	153 108-210	41 32-49	21.6 21.2-22.0	34.1 30.4-40.0
48	5	0.28 (4) 0.25-0.32	25 17-33	0.24 0.17-0.36	14.5 13.5-17.1	11 (4) 7-19	17.6 16.5-18.7	100 89-107	28 19-48	21.2 20.4-22.3	30.2 26.2-34.1
96	5	0.22 (4) 0.21-0.24	17.7 16.2-21.1	0.16 0.13-0.20	13.4 12.7-14.5	3 (1) —	16.9 16.4-17.2	75 62-83	23 20-27	21.5 20.6-22.5	27.6 (4) 26.6-28.5

The regression of copper levels on the logarithm of time (to base 2) was determined. In the case of blood, liver, duodenal, and large intestinal contents the regression was found to be linear. In the case of the contents of the small intestine it was necessary to include a quadratic term, and for caecal contents a cubic regression was used to give in all cases $P < 0.001$. The regression equations are as follows, y denoting the copper content and x the log (to base 2) of hours after injection;

Blood	$y = 0.682 - 0.238x$
Liver	$y = 140.4 - 64.0x$
Duodenal contents	$y = 20.29 - 3.47x$
Large intestinal contents	$y = 77.61 - 28.04x$
Small intestinal contents	$y = 90.60 - 79.78x + 21.52x^2$
Caecal contents	$y = -55.80 + 308.1x - 135.1x^2 + 16.567x^3$

Because of high variation within the copper levels of post-injection faeces it was not possible to obtain a regression curve. The maximum excretion seems to be between 12 and 24 hr after injection.

Insufficient data were available to examine the bile copper figures, but the maximum excretion of copper seems to be at about 24 hr after injection.

As a check on experimental technique, the fat-free dry weights of the livers were examined. No significant differences were noted between any of the injected groups or between injected groups and the controls. A similar examination of the copper levels of pre-injection faeces showed no significant differences between the treated groups or these and the controls.

The data on post-injection faeces did not prove suitable for making up balance sheets on copper storage. There was usually a decrease of food intake in the 24 hr after the injection, with a corresponding decrease in faecal output. The 12-hr faecal samples showed wide variations in copper content. This was because the collection period was approximately the same as the interval between caecal evacuations. The faecal samples thus contained either no, one, or two lots of the high-copper caecal material.

The livers of the birds injected for histological studies contained 191, 193, and 215 p.p.m. copper, but no stained copper was observed in any of the sections.

V. FATE OF COPPER IN DRAKES AFTER INTRAVENOUS INJECTION

Detailed studies were not made on drakes for two reasons: (1) the high and very variable concentration of copper in the liver would make it difficult to assess storage unless very large groups were used; (2) the semi-fluid nature of the excreta made it impossible to handle the faeces with the facilities available.

In the first experiment with drakes, six birds were given a total amount of 2.5 mg copper in three injections spaced at 2-day intervals; this amount of copper was sufficient approximately to double the original liver copper level if all were retained in the liver. The birds, with an equal number of controls, were killed 96 hr after the last injection. The mean liver copper concentration of the injected group was 282 p.p.m. (range 245-380 p.p.m.) and the corresponding figures for the controls were: 259 p.p.m. (168-321 p.p.m.).

In a second experiment three drakes were given two intravenous injections each of 1 mg copper at 9 a.m. and 4 p.m. The birds (with two controls) were killed 24 hr after the first injection, and tissues and the contents of the intestinal tract analysed as in the case of the cockerels.

The results for this experiment are set out in Table 4. No significant differences in liver copper levels due to treatment were noted in either experiment.

TABLE 4
COPPER CONTENT OF VISCERAL CONTENTS AND TISSUES OF DRAKES AFTER INTRAVENOUS INJECTION OF COPPER

2 mg Cu given and birds killed 24 hr after injection. Copper levels of livers as parts per million on dry fat-free tissue; other material as dry material only: copper in diet, 9.4 p.p.m.

	Blood (mg Cu/l)	Bile (mg Cu/l)	Liver		Small Intestine Contents (p.p.m. Cu)	Caecal Contents (p.p.m. Cu)	Large Intestine Contents (p.p.m. Cu)
			(p.p.m. Cu)	(mg Cu)			
Controls (2 birds)	0.35	3.2	194	4.60	21	39	16
	0.31	4.6	268	5.25	13	46	15
Injected (3 birds)	0.50	20	157	2.98	25	54	27
	0.49	25	393	6.24	18	101	18
	0.54	16	286	3.80	26	98	21

VI. STORAGE AND EXCRETION OF COPPER IN CAECECTOMIZED COCKERELS

(a) Experimental

The caecectomy was performed by the method of Sunde *et al.* (1950) and Sunde (personal communication, 1957). A fairly heavy mortality was experienced and the number of birds available for experiment was limited. The birds were allowed to remain for 4 weeks on a normal diet before being used for experiments. Two birds were injected as above with 1 mg copper and killed 48 hr after injection to determine the amount of copper remaining in the liver. Two non-injected birds were used as controls. In the second experiment small groups of caecectomized birds were fed for 12 weeks on a mash to which was added copper sulphate to give 40 p.p.m. copper. These were compared with entire birds on the same diet, and also with entire and caecectomized birds on the basal diet, which contained 7.6 p.p.m. copper.

(b) Results

The livers of the two birds which were injected with copper were found to contain 17.0 and 20.2 p.p.m. copper. The livers of the control birds contained 11.7 and 13.0 p.p.m. copper.

In the feeding tests the individual liver copper levels were as follows: basal diet, entire birds 16.6, 16.0 p.p.m., caecectomized bird 18.6 p.p.m.; high copper diet, entire birds 15.4, 15.1 p.p.m., caecectomized birds 14.5, 14.6 p.p.m.

VII. CHANGES WITH AGE OF LIVER COPPER STORAGE IN THE DRAKE AND THE COCKEREL

(a) *Experimental*

Day-old Muscovy drakes and White Leghorn \times Australorp cockerels were purchased from commercial hatcheries.

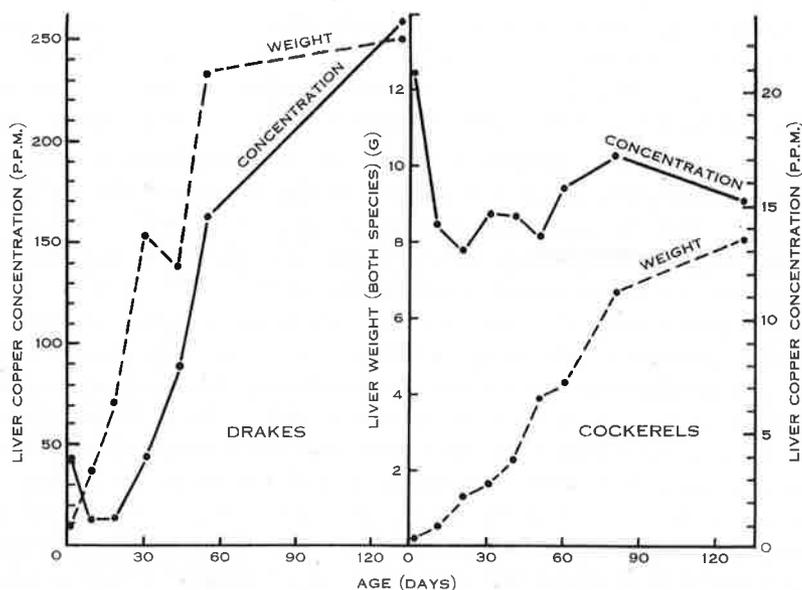


Fig. 1.—Liver copper concentrations and liver weights of drakes and cockerels. Both values based on fat-free dry weight.

The drakes were kept in wooden cages and fed poultry mash similar to that described in Section III(a). Groups of five birds were killed at approximately 10 day intervals until 8 weeks, and a final killing was made at 19 weeks.

The cockerels were kept in galvanized iron cages and fed a commercial poultry mash (7–8 p.p.m. copper). Groups of six birds were killed at 10 day intervals for 80 days and a final killing was made at 131 days.

Some lameness was noted in the group of cockerels killed at 61 days. This was apparently a mild form of staphylococcal arthritis. The infection was reflected in a smaller increase in both body and liver weight at this killing.

(b) *Results*

The data for liver copper concentration and weights of the livers (fat-free dry matter) are set out in Figure 1.

The decrease in liver weight in the duck between 31 and 44 days apparently represents some physiological change in the liver, as the mean body weight of the groups had increased from 656 to 1008 g in this period.

Although there was a decrease in the concentration of copper in the livers of both species during the first 10 days, the total amount of copper increased owing to the rapid growth of liver tissue.

VIII. GENERAL DISCUSSION

All experiments indicate that the high levels of copper in the liver of the duck are not due to an inability of this species to regulate storage. The failure of increased dietary copper to increase liver copper, the rapid elimination of injected copper, and the pattern of storage with age, all indicate that the development of high liver copper levels is under physiological control. Similar experiments indicate that the fowl has a considerable ability to prevent permanent storage of copper in the liver.

The fate of injected copper in cockerels can be clearly followed from the data in Table 3 and from the regression equations. Twelve hours after the administration of the copper a small but significant amount remained in the blood stream. A small amount had been secreted directly into the duodenum, the caeca, and the small and large intestines; some had already appeared in the faeces. Some 50% of the dose injected had been deposited in the liver, and excretion of copper through the bile had already commenced. By 24 hr the blood copper was still above normal and excretion of copper through the bile was at a maximum. The liver still contained an appreciable proportion of the amount injected (*c.* 30%). By 48 hr the levels in the intestinal contents had almost returned to normal but a small amount of injected copper still remained in the liver; this had disappeared by 96 hr. From the regression equation it was calculated that the maximum concentration in the caecal contents was approximately 160 p.p.m. copper at a time 18.5 hr after injection; also that 25–30% of the injected copper was excreted by these organs. If it is assumed that the volume of bile per unit of liver weight was the same as in the birds used by Schmidt and Ivy (1937), then approximately 50% of the injected copper had been excreted in the bile. The general pattern of excretion is similar to that observed by Mahoney *et al.* (1955) for dogs and pigs. The small but significant excretion into the duodenum was unexpected, but the work of Comar, Davis, and Singer (1948) suggested that such excretion may occur in cows.

Although some copper was almost certainly excreted through the caeca of normal cockerels, the experiments with caecectomized birds did not suggest that these organs played a very important part in the elimination of copper from the body. In drakes the caeca are very much smaller organs and their part in copper excretion is probably negligible.

The experiments do not give any clear picture concerning the absorption of copper. The absence of any marked increases in bile copper levels following an increase of dietary copper, suggests that the additional copper was not absorbed. If it is assumed that our cockerels secreted 15 ml bile daily, the bile copper indicates that, on a normal diet (10 p.p.m. copper), about 5% of the dietary copper is absorbed.

As some copper is almost certainly excreted through the caeca and some may be excreted directly into the intestines, the total absorption of copper is probably well in excess of the figure given above.

No explanation can be given for the striking increase of copper storage in the drake after 3 weeks of age, nor for the complete absence of such changes in the cockerel.

It is intended in a later paper to relate the data from the present investigation to similar studies on certain mammals.

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PAPER 1:3

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THE COPPER METABOLISM OF WARM-BLOODED ANIMALS WITH
SPECIAL REFERENCE TO THE RABBIT AND THE SHEEP

By A. B. BECK

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THE COPPER METABOLISM OF WARM-BLOODED ANIMALS WITH SPECIAL REFERENCE TO THE RABBIT AND THE SHEEP*

By A. B. BECK†

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Summary

Groups of rabbits were fed for 12 weeks on the same diet at two levels of copper intake (8.5 and 30 p.p.m. Cu). There was no real increase in the liver copper levels at the higher copper intake.

When rabbits were injected intravenously with 1 mg Cu, the excess copper was eliminated from the liver in 96 hr. Some copper was excreted through the bile and some appears to have been excreted directly into the caecum.

Experiments are described which show that, by contrast, the sheep very slowly lost excess copper from the liver. The rate of loss was the same whether the copper had been given orally or intravenously.

The patterns of copper storage and excretion in warm-blooded animals are outlined, and it is postulated that the unusual copper metabolism of the sheep is due to a limited capacity to excrete excess copper from the liver.

I. INTRODUCTION

The results of numerous investigations have indicated that the sheep has a pattern of copper metabolism which is different from that of other mammals. The concentration of copper in the liver is higher than in most species, and increases to toxic levels with moderate increases of dietary copper which have little or no effect on other species. Furthermore, in the sheep alone there is very little difference between the liver copper levels of the newly-born and adult animals.

A contribution to the understanding of the differences in copper metabolism between species of animals would be made by further information on the following aspects:

- (a) The physiology of copper absorption, the availability of copper in the normal diets of different species, and possible effects of microbial action on the chemical nature of dietary copper in the ruminant.
- (b) The forms of transport of copper in different species.
- (c) The nature of the chemical linkages of copper in liver, and in particular the differences which exist between species normally possessing high and low liver copper levels.
- (d) The factors involving the release of copper from the liver both for physiological processes and also after abnormal copper storage.

*This work was carried out at the Animal Health and Nutrition Laboratory and the Merredin Research Station, both of the Department of Agriculture of Western Australia. The balance studies on ewes were carried out in 1940-1943 at the Animal Nutrition Laboratory, Adelaide, then part of the Division of Animal Health and Production, C.S.I.R.

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(e) Quantitative aspects of the different channels of excretion, and information on the chemical nature of copper in bile.

(f) Factors causing altered copper metabolism in pregnancy and in late foetal life.

A comprehensive investigation of these points was begun in 1952, and data on storage and excretion of copper in some species has been published (Beck 1956, 1961*a*, 1961*b*). As it has become necessary to terminate these investigations, the present paper presents further information which has been obtained concerning copper metabolism in the rabbit and the sheep. These data demonstrate a marked difference in the excretory capacity of the sheep as compared with other species which have been studied.

On the basis of these and other published data, a provisional classification is given for the patterns of copper storage and excretion in warm-blooded animals.

II. CHEMICAL METHODS

These are as described previously (Beck 1956, 1961*a*).

III. EFFECT OF A MODERATE INCREASE IN DIETARY COPPER ON LIVER COPPER LEVELS IN THE RABBIT

Provided that the level of dietary copper is sufficiently high, it is probable that all species will store copper in the liver. Data on liver storage under such conditions is available for the domestic fowl (Mehring *et al.* 1960), the bovine (Cunningham 1946), the sheep (Dick 1954*a*), the pig (Lucas and Calder 1957), the rat (Boyden, Potter, and Elvehjem 1938) and the rabbit (Hall and Mackay 1931). However, little information is available on the effect of increases of dietary copper within physiological levels (*c.* 5–30 p.p.m. Cu), apart from observations on the sheep (Dick 1954*a*) and the domestic fowl and duck (Beck 1961*a*).

The results of feeding experiments by Eden (1940, 1941), injection studies by Dick (1954*b*), and histological studies by Cavallero (1958) strongly suggested that the rabbit would have an efficient mechanism for the excretion of excess copper. However, detailed experiments which actually confirmed this point had not been made.

(a) Experimental

Twenty male rabbits, approximately 8 weeks of age, were randomized into two groups. The animals of each group were kept together in cement pens and fed *ad lib*. The standard laboratory diet was used for the control group: this consisted of bran, pollard, crushed oats, and meatmeal, together with bone meal and vitamins A and D. The composition of the diet was as follows: protein ($N \times 6.25$), 16.8%; Ca, 0.74%; P, 0.69%; Cu, 8.5 p.p.m.; Mo, 0.67 p.p.m.; inorganic sulphate, 0.65% SO_4 (all values on dry matter). Copper sulphate was added to the diet of the second group by the method of Beck (1961*a*) to bring the copper content to 30 p.p.m. Cu (dry matter).

The animals were castrated at 16 weeks of age. All animals were slaughtered after a feeding period of 12 weeks. Samples of blood and liver were collected from all animals, and bile was collected from the gall bladder whenever possible.

(b) Results and Discussion

The addition of copper to the diet had no effect on growth rate or on haemoglobin levels.

The analysis of tissues is set out in Table 1.

Statistical examination showed that there was no significant difference in the mean bile copper level between the two groups. As only one blood sample was taken from each animal and as all animals were not killed on the same day, it is not possible to assess the significance of the difference in blood copper levels between the two groups.

TABLE 1

EFFECT OF COPPER INTAKE ON TISSUE COPPER LEVELS IN THE RABBIT

Values for liver as parts per million on fat-free dry matter basis. Mean value given with range of values in parenthesis. Copper in diet as parts per million on dry matter basis

Feeding Treatment	Dietary Copper (p.p.m.)	Blood Copper ($\mu\text{g/ml}$)	Bile Copper ($\mu\text{g/ml}$)	Liver Copper	
				(p.p.m.)	(mg)
Controls	8.5	0.74 (0.54-1.00)	1.4 (0.5-3.5)	13.9 (9.1-16.7)	0.25 (0.22-0.29)
Copper fed	30	0.89 (0.66-1.14)	1.7 (1.1-2.4)	16.1 (12.3-19.9)	0.29 (0.22-0.35)

The variability in liver weight was relatively high, but the means for the two groups did not differ significantly (mean fat-free dry weight of all livers, 18.3 g; coefficient of variation, 16.2%). In order to ascertain the differences in copper storage, an analysis of variance of the total liver copper was made. The differences were significant ($0.01 > P > 0.001$).

Although the increase of copper storage in the supplemented group was statistically significant, it was very small in absolute amounts. It is considered that the control animals may have been on a slightly suboptimal copper intake, and that the increased intake merely allowed the liver storage to reach "normal" levels.

IV. STUDIES ON THE LOSS OF COPPER FROM THE LIVER OF THE RABBIT AND SHEEP

*(a) Excretion of Copper in the Rabbit after Intravenous Injection**(i) Experimental*

Groups of castrated males (usually six animals per group), 16-20 weeks of age and about 2 kg in weight, were used for these experiments. During the experimental period they were housed in galvanized iron cages with 0.75 in. mesh stainless steel bottoms. The faeces were retained on an 0.25 in. mesh stainless steel screen, while the urine passed through to a polyethylene collector. To prevent dusting of the food

onto the urine, the diet was moistened before feeding. The diet used was that described in Section III, and copper sulphate was added to give 10.0 p.p.m. Cu (on dry matter).

Faecal refection was prevented by the use of $8\frac{1}{2}$ – $9\frac{1}{2}$ in. diameter plywood collars (centre holes, $1\frac{3}{4}$ –2 in. diameter). During the longer collection periods, it was necessary to remove the collars for about $\frac{1}{2}$ hr each day to allow animals to perform personal hygiene.

Animals were trained in the cages until food consumption was constant. Faeces were collected for 24 hr and the animals injected in the ear vein with 1 ml isotonic saline solution containing 1 mg Cu as sulphate. Animals were killed 12, 24, 48, and 96 hr after injection. At slaughter the following material was collected: blood, the liver, the contents of the caecum, and bile whenever possible. Contents of the small intestine were not collected, as there was, generally, very little food residue in this portion of the gut at the time of slaughter.

Faeces and urine were collected in the pre-injection period and in the post-injection periods for 12, 24, and 48 hr only.

As the volume of bile available in these experimental animals was rather small (usually 0.1–0.4 ml), an additional two rabbits were injected with 1 mg Cu and killed 24 hr after injection. Food was withheld from the animals 18 hr before slaughter in order to increase bile volume. Data from these two animals were not included with those from the experimental groups.

Also 24-hr faecal samples were collected in the metabolism cages from six non-injected rabbits with collars. The samples were separated into the soft caecal faeces and the hard normal faeces (Thacker and Brandt 1955) to ascertain the difference in copper content.

(ii) *Results and Discussion*

The results of the analysis of tissues and excreta are set out in Table 2.

The blood copper level of animals killed 12 hr after injection was not significantly higher than that of the control animals.

The total amount of copper in pre-experimental urine samples did not differ significantly from the amount excreted in the first and second 24 hr after injection. The mean value for the 32 samples was 7.4 μg Cu per 24 hr (range 1.3–16.4 μg).

As in the previous experiment, there was a considerable variation in liver weight, but there was no significant difference in the means of groups (mean fat-free dry weight of all livers, 20.1 g; coefficient of variation, 27.1%). Total amounts of copper were used for comparison of storage. About 70% of the injected copper was found in the liver 12 hr after the injection. This slowly decreased, and by 96 hr the amount was not significantly greater than in the controls.

A significant elevation ($P < 0.01$) of caecal copper level was noted 12 hr after injection. This probably reached a maximum between 24 and 48 hr and then slowly decreased. At 96 hr the concentration was still significantly higher ($P < 0.01$) than

in the controls. The fact that the 12-hr levels had increased markedly while bile copper levels were still relatively low suggests a direct secretion of copper into the caecum.

The samples of caecal faeces from the six non-injected rabbits always contained a greater concentration of copper than normal faeces, but the differences were not great. The mean copper content of moisture-free caecal faeces was 30 p.p.m. (range 26–37) and for normal faeces, 25 p.p.m. (range 24–27). The daily weights of caecal and normal faeces were very similar.

TABLE 2

EXCRETION OF COPPER IN THE RABBIT AFTER INTRAVENOUS INJECTION

Mean value given with range of values in parenthesis. Means generally are for six animals: when less, the number is indicated in bold face type and in parenthesis

Hours after Injection	Liver Copper (mg)	Bile Copper ($\mu\text{g}/\text{ml}$)	Caecal Contents (p.p.m. Cu)*	Faecal Copper (p.p.m.)*	
				Pre-Injection	Post-Injection
Control	0.25 (0.22–0.29)	1.9 (0.5–4)	31 (5) (27–34)	25.1 (5) (23.0–27.0)	—
12	0.97 (0.82–1.08)	8 (5) (3–14)	43 (35–50)	27.3 (26.5–28.6)	28.8 (26.7–31.8)
24	0.79 (5) (0.62–0.90)	22 (4) (7–57)	54 (5) (48–62)	28.3 (4) (27.3–28.7)	33.9 (4) (30.4–40.7)
48	0.47 (0.31–0.63)	19 (5) (7–30)	52 (42–65)	24.8 (5) (23.1–25.8)	36.4 (5) (34.3–40.3)
96	0.33 (0.26–0.44)	7 (5) (5–9)	41 (38–46)	—	—

*Dry matter basis.

The bile copper concentration showed large variations, but the maximum appeared to be reached between 24 and 48 hr after injection. The two rabbits killed 24 hr after injection, and fasted prior to slaughter, showed copper concentrations for bile and total amounts of liver copper in the same range as the experimental group (bile, 10 and 14 $\mu\text{g}/\text{ml}$; liver, 0.61 and 0.70 mg Cu).

The results of these experiments indicate that, as with the domestic fowl, the rabbit had a considerable capacity for excreting copper after intravenous injection. This excretion seemed to be mainly through the bile, with a possible small amount through the caecum, but the channels of excretion have not been defined as clearly as in the fowl.

(b) *Comparison of the Losses of Copper from the Liver of the Sheep after Oral and Intravenous Administration*

In seeking an explanation for the unusual storage of copper in the liver of the sheep, the writer considered the possibility that liver copper might be in a different chemical form from that in other species. It was postulated that this special form was synthesized in the rumen or gut from dietary copper, transported to the liver, and deposited there unchanged. The conversion of dietary cobalt to vitamin B₁₂ was taken as an analogy.

This hypothesis was examined by increasing the liver copper level of one group of sheep by oral administration to allow for the formation of the hypothetical compound. The liver copper level of a second group was increased by intravenous injection of copper, which thus eliminated any possible changes in the intestinal tract. If the rate of loss of copper from the two groups was different, it would strongly suggest that the copper supplements had been stored in different forms.

(i) *Experimental*

A liver sample was taken by the biopsy technique of Dick (1944) from 82 sheep at the Merredin Research Station in November 1957. A second liver sample was taken from all sheep in August 1958. Animals showing very high or low copper levels were excluded from the main experiment, and the 40 sheep which showed the least variation at the two samplings were chosen from the remainder. These sheep were paired in order of descending liver copper levels, and sheep from each pair randomized into two groups. Treatment was then allotted to one group at random. One group was allowed to run on good quality farm pasture. The other was run in a small paddock and fed a weighed amount of high quality wheaten hay daily. A solution containing copper sulphate was sprayed onto this hay. Alkyl sulphate and glycerine were added to the solution to allow better spreading on the hay and to prevent drying out of the solution with subsequent loss of copper. Cobalt at the equivalent of 1 mg Co per sheep per day was also added to the copper solution. The amount of copper used was equal to 39 mg Cu per sheep daily over the feeding period, and as the sheep consumed the hay completely each day, it is considered that this amount of copper was ingested almost quantitatively. It was necessary to restrict feed intake to keep body weights the same as in the group on pasture.

Liver biopsy samples were taken from the supplemented group after 13 weeks of feeding. The pairs from the pasture group were then injected with amounts of copper sulphate in isotonic saline, calculated to bring the liver copper levels to those of corresponding sheep in the supplemented group. As the amount of copper to be injected was quite large, it was given in divided doses over several days to avoid toxic effects. Three days after the last injection, biopsy samples were taken to determine actual copper levels.

Both groups were then run together and fed low-copper meadow hay from a copper-deficient area for 14 weeks and wheaten hay for a further 5 weeks. Final liver biopsy samples were taken at the end of feeding, in April 1959.

Two sheep showing a high liver copper level at both the November 1957 and August 1958 samplings, and three sheep showing a low liver copper level at the same dates, were run with the copper-fed group. It was considered that the persistent difference in liver copper levels might be an indication of a different type of copper metabolism.

In order to determine the amount of copper lost between the last two sampling dates it was necessary to calculate liver weights. This was done by determining the ratio of fat-free dry liver weight to body weight on small groups of sheep (spare animals from original group) which had been running with experimental animals and which were killed at the same times. The mean ratios were 0.00258 (range 0.00226–0.00289; six sheep) and 0.00274 (range 0.00242–0.00300; five sheep) respectively.

TABLE 3
ANALYSIS OF HAY USED IN STUDIES ON LOSS OF COPPER IN SHEEP
Values as means and standard error on dry matter basis

	No. of Samples	Copper (p.p.m.)	Molybdenum (p.p.m.)	Inorganic Sulphate (%)	Crude Protein (N × 6.25) (%)
Wheaten hay	4	3.8 ± 0.13	0.45 ± 0.08	0.24 ± 0.03	9.2 ± 0.43
Meadow hay	1	2.3	0.74	0.21	8.0

Analysis of the whole liver of four experimental animals gave copper values within 5% of those obtained from the biopsy sample.

The analyses of hay samples used in the experiment are set out in Table 3.

(ii) Results and Discussion

Liver copper levels and the calculated losses of copper are set out in Table 4.

Statistical examination of the data showed that there was no significant difference between the amounts of copper lost by the fed and injected groups, and that there was no significant correlation between amounts injected and amounts lost. Excess copper was eliminated very slowly from the liver of the sheep. This is in marked contrast to the rapid loss of approximately equal doses (in terms of body weight) from the liver of the fowl and the duck (Beck 1961*a*) and the rabbit. The results give no support for the hypothesis that the copper stored in sheep liver after oral administration is in a different form from that stored after intravenous injection.

The amounts of copper lost by the small groups of "high" and "low" liver copper sheep were not greatly different, and both lay within the range of values of the "medium" liver copper sheep of the main experiment.

Recently Gracey and Todd (1960) have published limited data showing a similar slow loss of copper from the livers of sheep which have received excess dietary copper.

(c) Studies on the Excretion of Copper in Sheep after Intravenous Injection

Earlier investigations had shown that balance studies were of limited value for providing information concerning the absorption and storage of copper in sheep. The errors due to sampling of food and faeces, analytical errors, and variations of faecal output are relatively high, and are usually greater than the daily gain or loss of copper under physiological conditions. The technique, however, has value in studying the loss of copper after the injection of milligram doses of copper.

TABLE 4
LOSS OF COPPER FROM THE LIVER OF THE SHEEP

Pre-experimental values as parts per million copper on dry matter basis, others on fat-free dry matter basis. Values for groups as mean with range of values in parenthesis

Experimental Group	Pre-Experimental Liver Copper (p.p.m.)		Dose of Copper Injected per Sheep (mg)	Post-Experimental Liver Copper (p.p.m.)		
	Nov. 1957	Aug. 1958		After Feeding or Injecting Dec. 1958	After Feeding Low-Cu Diet, Apr. 1959	Calculated Loss (mg)
Copper-fed sheep	108 (42-170)	94 (54-154)	—	607 (345-1150)	389* (65-830)	23·1† (4-47)
Copper-injected sheep	119 (37-219)	94 (50-160)	51 (25-102)	514 (270-800)	252 (75-510)	27·2† (11-40)
High-copper sheep (copper-fed)	286 234	310 294	— —	970 960	790 820	14·5 5·5
Low-copper sheep (copper-fed)	27 47 27	22 19 20	— — —	270 370 385	145 175 145	14·5 22·5 34·5

*Eighteen animals only; 20 at all other group samplings.

†Mean of difference between 18 pairs, 3·63 mg Cu : S.E. of mean difference, 3·69 mg.

During these earlier observations it was noted that the injection of up to 0·3 mg Cu/kg body weight produced little, if any, increase in urinary excretion of copper, but that a marked elevation occurred if the dose was above 0·5 mg/kg. With the larger doses, the maximum excretion was on the second day after the injection, and the levels of copper in the urine remained elevated for relatively long periods. As the blood copper had returned to normal in less than 24 hr, the results suggested kidney damage with a subsequent leakage of copper from the blood. No studies were made on this aspect, but recent investigations made on mice by Vogel (1960) suggest that necrosis of the proximal convoluted tubular epithelium

may have occurred. A similar pattern of urinary excretion has been found in mice by Gitlin, Hughes, and Janeway (1960).

(i) *Experimental*

Collection of faeces and urine from ewes was made in crates similar to those described by Marston (1935). The metal grating was replaced by a wooden grating and the separation of faeces and urine was done by a heavily tinned iron wire screen. With wethers, a similar type of crate was used but the faeces were collected in a canvas bag attached to the sheep by a leather harness. Urine was voided through a wooden grating onto a polyethylene collector.

TABLE 5
BALANCE STUDIES ON WETHERS AFTER INTRAVENOUS INJECTION OF COPPER
Values as milligrams copper

	Days of Collection	Copper Intake in Period			Copper Output in Period			Balance
		Feed	Injected	Total	Urine	Faeces	Total	
<i>Sheep 1:</i>								
Pre-injection	10	26.1	—	26.1	0.12	25.1	25.2	+ 0.9
Post-injection (1)	10	25.5	20	45.5	0.15	25.6	25.75	+ 19.75
(2)	10	26.3	—	26.3	0.10	27.1	27.2	— 0.9
Total	20	51.8	20	71.8	0.25	52.7	52.95	+ 18.85
<i>Sheep 2:</i>								
Pre-injection	10	26.6	—	26.6	0.11	24.5	24.6	+ 2.0
Post-injection (1)	10	26.3	20	46.3	0.16	25.1	25.3	+ 21.0
(2)	10	26.4	—	26.4	0.10	26.8	26.9	— 0.5
Total	20	52.7	20	72.7	0.26	51.9	52.2	+ 20.5

Merino sheep were used. The daily diet consisted of 600–700 g cereal chaff, 100 g lucerne chaff, 50 g gluten, and 5 g sodium chloride, together with 0.3–0.5 mg Co. Correction was made where necessary for copper in the drinking water.

Copper was injected into the jugular vein of the ewe in isotonic saline solution containing 5.0 mg Cu (as sulphate) per ml. With wethers a solution containing 1 mg/ml was used and the injection dose was divided to avoid toxic effects.

(ii) *Results and Discussion*

Table 5 gives the data on the intake and output of copper in two wethers each injected with a total amount of 20 mg Cu, given in three doses over a 24-hr period. A positive balance was shown by both sheep in the pre-injection period and this, at least with sheep 2, was greater than experimental errors. If the pre-experimental balances were maintained throughout the experiment, sheep 1 and 2 would have lost the equivalent of only 15 and 17% of the injected copper during the 20-day collection period.

The data for the excretion after multiple injections of copper into a ewe are set out in Table 6. The results again indicate that the sheep liver has the capacity to complex quite large amounts of copper in a relatively stable form. After the injection

TABLE 6

LOSS OF COPPER FROM A EWE RECEIVING MULTIPLE INJECTIONS OF COPPER SULPHATE

Values as milligrams copper. Second injection 13 days after first; subsequent injections at 7-day intervals

Period	Days of Collection	Intake in Feed	Injected	Total Copper Received	Copper Excreted			Copper Retained
					Faeces	Urine	Total	
Pre-injection	14	23.1	—	23.1	22.5	0.2	22.7	(0.4)
Injection (1)	13	20.8	20	40.8	22.4	1.0	23.4	17.4
(2)	7	10.7	20	30.7	10.8	0.9	11.7	19.0
(3)	7	11.4	20	31.4	16.2	0.6	16.8	14.6
(4)	7	12.8	20	32.8	13.4	0.9	14.3	18.5
(5)	7	12.5	20	32.5	17.1	0.5	17.6	14.9
Total	41	68.2	100	168.2	79.9	3.9	83.8	84.4

TABLE 7

COPPER CONTENT OF BILE

Mean values given with range of values in parenthesis

Species	Number of Animals	Bile Dry Matter (g/100 ml)	Bile Copper		Liver Copper (p.p.m.)*
			(μ g/ml)	(p.p.m.)*	
Sheep	21	11.3 (7.1-16.1)	0.21 (0.03-0.63)	2.0 (0.33-8.9)	214 (95-380)
Bovine	3	12.4 (9.3-17.1)	0.04 (0.03-0.05)	0.35 (0.23-0.54)	81 (56-116)
	9	8.4 (6.9-12.2)	0.06 (0.02-0.16)	0.76 (0.16-1.95)	—
Pig	8	12.9 (8.8-16.2)	0.56 (0.11-1.6)	4.3 (0.7-13.0)	—

*Dry matter basis.

of 100 mg Cu, only 11.7 mg was excreted in the faeces. These observations were made before the importance of small injection doses was appreciated, and interpretation of the urinary excretion is not possible.

(d) *Copper Content of Sheep Bile*

As it is certain that the bile is the main channel of copper excretion in most species, some preliminary observations were made on sheep bile. Some samples were also collected from cattle and pigs for comparison. Data on these samples are given in Table 7. The samples were from healthy abattoir animals.

A characteristic of these figures is the extreme variability in the concentration of copper. A similar variation has been noted in the fowl and duck (Beck 1961*a*) and the rabbit, but in the absence of data on bile volumes it is not possible to interpret these variations.

V. GENERAL DISCUSSION

In the experiments with the fowl and rabbit, and in the balance study with the two wethers, the amounts of copper injected were all very close to 0.5 mg Cu/kg body weight. In the large experiment with sheep the doses were greater but were still of the same order (mean 1.09; range 0.49–2.25 mg/kg). Although the time taken to eliminate these doses was very different in the sheep as compared with the rabbit and the fowl, the mean total daily loss of copper from each species was very similar (0.1–0.2 mg). This similarity is possibly fortuitous, but interpretation of results must be made with caution until data are available for other animals.

The difference of copper metabolism between the sheep and the other species studied is almost certainly related to the slower rate at which the concentration of excess copper is lowered in the liver of the sheep. It is postulated that this factor is responsible for the rapid increase in liver storage which occurs in the sheep under conditions of increased dietary intake. The limited excretory capacity by itself will not explain the high liver copper level of the sheep as a species, for the duck has both a high liver copper level and a high excretory capacity.

Sufficient data are now available to classify provisionally the patterns of copper storage and excretion in warm-blooded animals. When the intake of copper is within physiological levels (i.e. between about 5 and 30 p.p.m. in diet) there are apparently three or four patterns of storage and excretion among species.

(1) The first type is represented by the sheep, which on a normal diet has a high liver copper level (100–400 p.p.m. on dry matter). This level rises with relatively small increases of dietary copper (Dick 1954*a*). If the liver copper is raised either by feeding or by injection, the excess copper is eliminated very slowly from the liver. It is possible that other ruminants may be similar, but a comparison of the results of Cunningham (1946) and Dick (1954*a*) indicates that the cow has a greater capacity to control copper absorption or storage, or both.

(2) The second type is represented by the adult duck, which also has a high liver copper level (80–500 p.p.m. on dry matter). By contrast with the sheep, however, the duck very rapidly eliminates excess copper from the liver. It would appear that the young duck has a different pattern of copper metabolism (Beck 1961*a*).

(3) The third type of metabolism probably occurs in most species. Animals of these species are characterized by low liver copper levels (usually under 30 p.p.m. on dry matter) and by the fact that moderate increases of dietary copper cause no increase in liver levels. The present investigations have shown that the domestic fowl

and rabbit follow this pattern, and other data indicate that the pig (Lucas and Calder 1957), the mouse (Vogel 1960), and the rat (Boyden, Potter, and Elvehjem 1938), are similar.

Data for the pig (Lucas and Calder 1957) indicate that there is a threshold level of dietary copper (between 70 and 130 p.p.m.) above which liver copper levels do increase rapidly. Limited data in the literature suggests that the other species may behave in a similar manner, but that the threshold varies considerably with different animals.

(4) There may be another pattern of storage in the foetal liver of mammals (Underwood 1956; Pryor 1960; Beck 1961*b*), but this has not yet been studied in detail. As sheep alone seem to show little difference in late foetal and adult liver copper levels (McDougall 1947), it may be that the pattern of foetal storage persists into adult life in this species.

Recent observations on caeruloplasmins in man (Richterich 1961) may help to elucidate the differences in the above groups of animals. In the human foetus and in cases of Wilson's disease, the liver copper level is high and only one form of caeruloplasmin is present in the serum. In the normal adult the liver copper level is low, and two forms of caeruloplasmin exist in the serum. Similar studies should be made on species with normally high liver copper levels.

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SECTION 2

SECTION 2

OBSERVATIONS RELATING TO THE NORMAL METABOLISM OF COPPER IN MAMMALS

This Section comprises three published papers and some unpublished data on copper metabolism; all of these investigations were carried out by the candidate.

- 2:4 "Studies on the copper content of the milk of sheep and of cows." Aust. J. Exp. Biol. and Med. Sci., 1941, 19, 145.
- 2:5 "Changes in the levels of copper in milk during lactation" - unpublished.
- 2:6 "Studies on the blood copper of sheep and of cows." Aust. J. Exp. Biol. and Med. Sci., 1941, 19, 249.
- 2:7 "The liver copper levels of foetal and newly born marsupials and whales." Aust. J. Sci., 1961, 24, 245.

Papers 2:4 to 2:6 were the direct outcome of the studies on copper deficiency described in Section 3. These investigations were partly made to confirm some earlier observations of Bennetts and Chapman (Aust. Vet. J. 1937, 13, 138) and more particularly, to establish "normal" values of copper in milk and blood. Some of the earlier values in the literature were open to suspicion

Section 2

- 2 -

due to faulty analytical methods.

In Paper 2:6 it was pointed out that no changes of blood copper levels occurred in ewes just prior to lambing. This was in contrast to the situation in man, where the maternal blood rises sharply toward the end of gestation. These observations on ewes have been confirmed by subsequent investigations (Eden, A., *Biochem. J.*, 1941, 35, 813; and McDougall, E.I., *J. Agric. Sci.*, 1947, 37, 329). McDougall also found that the blood copper of the new-born lamb was considerably higher than in the mother. This, too, is in contrast to man, where the blood copper level is lowered in the new-born.

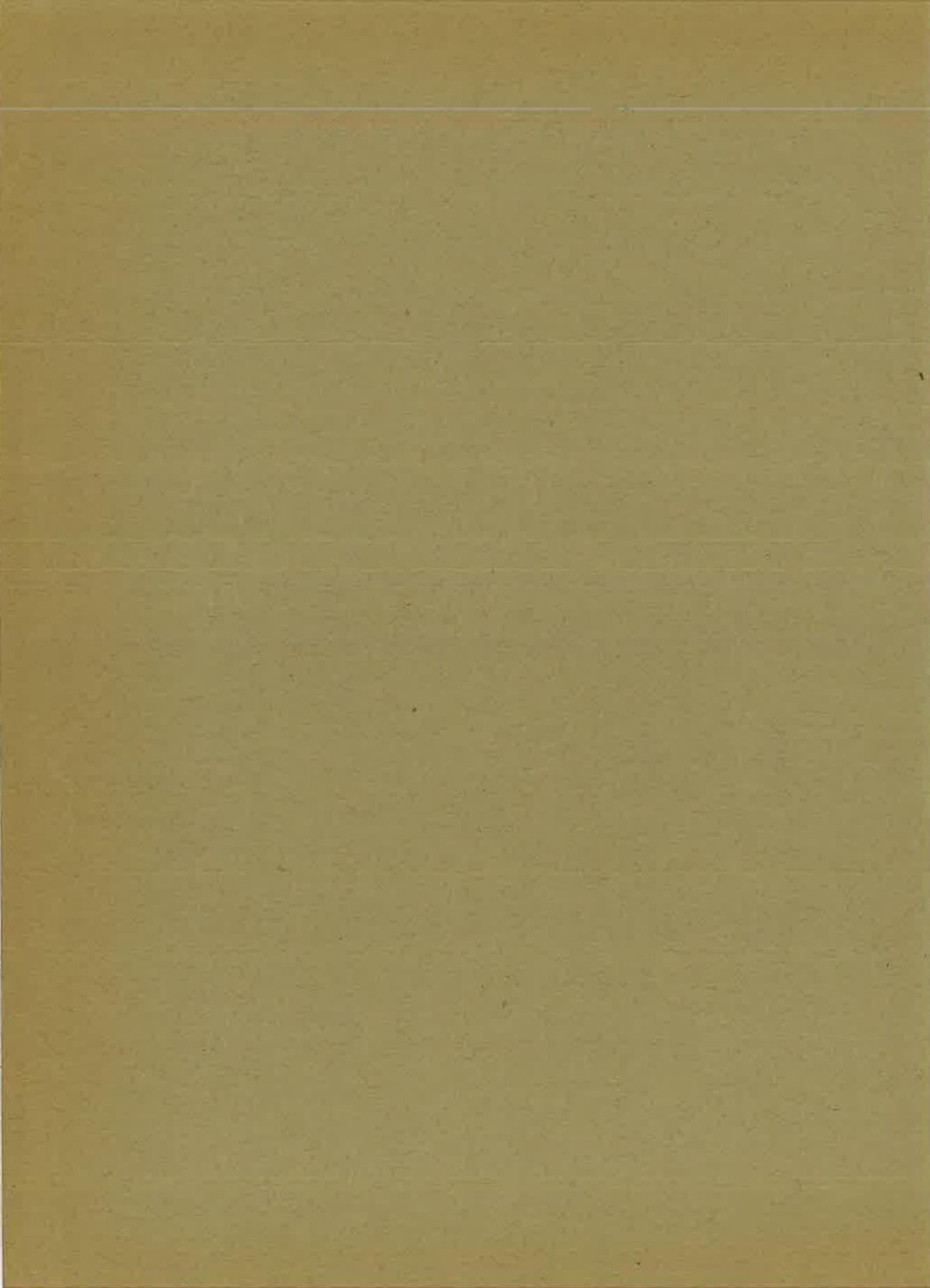
The Paper 2:7 is a short note giving some incidental information gathered during the initial studies on the establishment of the "normal" liver copper levels of different species (Paper 1:1). It provides further confirmation of observations of other workers that the copper metabolism of the foetus is different from that in the adult.

PAPER 2:4

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STUDIES ON THE COPPER CONTENT OF THE MILK OF
SHEEP AND OF COWS

by A. B. BECK



STUDIES ON THE COPPER CONTENT OF THE MILK OF SHEEP AND OF COWS

by A. B. BECK

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Observations made by Bennetts and Chapman (1937) suggest that lowered copper intake in sheep causes a very marked lowering of the milk copper. This is in contrast with the effect of a dietary deficiency of other inorganic constituents (Groenewald, 1935), which causes no such lowering of the concentration in the milk. Other observers had found that feeding an excess of copper salts to cows (Elvehjem, Steenbock and Hart, 1929) and to sheep (Thomas, 1937) caused no increase in milk copper.

The copper content of milk from normal ewes and cows as determined by various workers is shown in Table 1.

TABLE 1.

Authors.	Animals.	Milk copper range. mg. per litre.
Elvehjem <i>et al.</i> (1929)	Cows	0.123-0.184
Krauss and Washburn (1934)	"	0.14 -0.17
Grimmer (1934)	"	0.19 -0.34
Conn <i>et al.</i> (1935)	"	0.051-0.132
Sylvester and Lampitt (1935)	"	0.09 -0.17
Echava (1936)	"	0.31 -0.40
Miwa (1938)	"	0.22 -0.37
Grimmer (1934)	Ewes	0.27 -0.29
Thomas (1937)	"	0.46

Bennetts and Chapman (1937) found 0.12 and 0.19 p.p.m. respectively in the milk of two "normal" ewes which had run on copper deficient pastures for two months and had lambed about three weeks previously. In none of the results reproduced in Table 1 is any attention directed to a relation between the milk copper levels and the period of the lactation in which the samples were taken. The present paper is concerned with the presentation of further data concerning the variations of the copper content of the milk of normal sheep and cows and with the effect of lowered copper intake on the milk copper concentration.

Methods. The milk samples were taken directly into glass containers. The cows' milk samples were a composite of the foremilk from the four quarters and were usually taken in the afternoon. An estimation on the morning and afternoon foremilk of two cows disclosed no difference in copper concentration, nor were the strippings significantly higher than the foremilk.

A suitable quantity of the milk (50-100 ml.) was evaporated in a silica basin with 5 ml. of 20 p.c. magnesium nitrate (Cu free) per 50 ml. milk. After ashing at an incipient red heat, the ash was dissolved in hydrochloric acid and filtered on an acid-washed filter paper. The small amount of carbonaceous residue usually contained a few microgrammes of copper so the filter paper and residue were digested with 1 ml. H₂SO₄, 0.4 ml. 60 p.c. HClO₄ and sufficient HNO₃ to destroy organic matter and carbon. The mixture was evaporated to sulphuric fumes, cooled and diluted. Mixing this digest with the HCl extract precipitates calcium sulphate so the two solutions must be extracted separately. Both were neutralized with NH₃ and carefully brought to pH 3

with dilute HCl using brom-phenol-blue as indicator. The subsequent procedure was as described by Sylvester and Lampitt (1935); the dithizone solution used to extract the $H_2SO_4-HClO_4$ digest was also used for the original HCl solution. A blank was done on all reagents. Although somewhat lengthy, the method gives excellent results with duplicates and with recoveries of added copper.

The blood copper was estimated by the method of Tompsett (1934).

Results.

1. *The copper content of normal ewes' milk.* The first sheep tested were pure bred merinos depastured in the drier agricultural areas of Western Australia. The sheep were run on natural pasture which previously was found to have the following copper content: 10.6 p.p.m. (dry basis) on 24/7/39, 6.0 p.p.m. on 30/8/39 and 5.2 p.p.m. on 27/3/40 when the pasture was dry. Supplementary feeding with cereal hay was necessary after lambing in April until the end of June. Green feed was only available during July and August, but there was ample dry feed and both ewes and lambs remained in excellent condition throughout.

The first samples were taken ten to fifteen days after the ewes had lambed. The results of the analysis of the milk and blood at monthly intervals for a period of four months are given in Table 2.

TABLE 2.

Copper content of milk and blood of merino ewes.
(Both as mg. Cu per litre. Figures for blood in italics.)

GROUP 1: MERREDIN.

Ewe.	24/4/40.	22/5/40.	26/6/40.	31/7/40.	28/8/40.
B193	0.63 0.96	0.16 0.92	0.11 0.96	— 0.96	0.08 1.10
B243	0.24 0.89	0.14 0.90	0.08 1.13	0.06 1.13	0.11 1.29
B261	0.43 0.79	0.15 0.73	0.08 0.98	0.06 1.13	0.08 1.14
V70	0.64 0.78	0.16 0.83	0.09 0.88	0.04 0.93	0.10 0.92
V75	0.36 0.69	0.16 0.48	0.07 0.60	0.04 0.66	0.10 0.79
V79	0.41 1.18	0.19 0.90	0.16 1.05	0.07 1.12	0.12 1.44
Mean of milk copper	0.452	0.160	0.098	0.054	0.098
S.D.	±0.157	±0.017	±0.033	±0.013	±0.015

“B” sheep four-toothed. “V” sheep full-mouth.

The second group (Table 3) consisted of seven crossbred (merino-Border Leicester) ewes from a locality of slightly better rainfall and grazing conditions where the natural pastures had a copper content of 8 to 13 p.p.m. (dry basis). Ample green feed was available from the end of June onward. As in the case of the first group, the first samples were taken about a fortnight after lambing and subsequently at monthly intervals for three months. The last samples of milk were obtained with difficulty as the ewes were drying off. Blood copper determinations were also made and are included in Table 3.

TABLE 3.

Copper content of milk and blood of cross-bred ewes.
(Both as mg. Cu per litre. Figures for blood in italics.)

GROUP 2: BEVERLEY.

Ewe.	11/6/40.	11/7/40.	6/8/40.	3/9/40.
B4	0.53 0.87	0.48 1.05	0.14 1.06	0.22 0.84
B34	0.42 0.97	0.31 1.45	0.16 1.10	0.14 1.14
B55	0.44 0.96	0.36 1.38	0.16 1.17	0.21 1.05
B91	0.20 0.98	0.11 1.25	0.11 1.13	0.13 0.94
B112	0.48 0.75	0.18 0.96	0.14 1.46	0.19 0.94
B241	0.38 0.92	0.18 1.10	0.06 1.10	0.12 1.06
B270	0.39 0.94	0.25 0.96	0.12 1.11	0.17 1.09
Mean of milk copper	0.406	0.267	0.127	0.169
S.D.	±0.105	±0.126	±0.035	±0.040

B112, B241, B270, six-tooth; B4, B34, B55, full-mouth; B91, aged.

2. *The copper content of normal cows' milk.* The first group of cows (Table 4) were grazed on natural pasture, the copper content of which was 9 to 10 p.p.m. (dry basis) in the Spring months. These cows were pure bred Guernseys, mastitis-free and all details of their past history were available. During the months of January to June, the animals were on dry grazing and were receiving as a supplementary ration increasing quantities of mixed cereal silage (4.2 p.p.m. Cu dry basis) with some green lucerne and a crushed grain-concentrate mixture. From July, cereal chaff was substituted for the silage. Green feed was not available in the paddocks in any quantity until the beginning of August.

TABLE 4.

Copper content of cows' milk.

(Mg. per litre. Blood copper figures for four cows are in italics.)

GROUP 1: MURESK.

Cow No.	Weeks after calving at first test.	Weeks of test.							
		0	2	6	10	14	16	18	22
Sub group A									
100	44	0.04	0.05	*	—	—	—	—	—
130	40	0.05	0.03	0.05	0.05	—	0.04	—	—
70	37	0.03	0.02	0.05	*	—	—	—	—
151	36	0.03	0.03	0.05	*	—	—	—	—
86	1.5	0.38	0.18	0.10	0.06	0.08	—	—	0.07
		<i>0.92</i>	<i>0.96</i>	<i>0.86</i>	<i>1.02</i>	<i>0.85</i>	—	—	<i>1.08</i>
69	0.5	0.14	0.23	0.12	0.06†	0.11	—	0.07	—
Sub group B									
		0	1	2	3	4	5	8	12
108	8	0.06	—	—	—	0.07	—	0.06	—
167	4	0.13	—	—	—	0.11	—	0.05	—
188	4	0.10	—	—	—	0.06	—	0.11	0.07
199	1	0.12	0.17	0.27	—	0.19	0.18	0.10	0.09
		<i>1.01</i>	<i>1.02</i>	<i>1.06</i>	<i>1.01</i>	<i>1.40</i>	<i>0.93</i>	<i>0.98</i>	<i>1.10</i>
562W	1	0.06	0.09	0.06	0.07	0.06	—	0.06	—
		<i>0.94</i>	<i>0.93</i>	<i>0.85</i>	<i>1.00</i>	<i>1.25</i>	—	<i>0.90</i>	—
100	1	0.07	0.07	0.10	—	0.10	0.08	0.09	—
Sub group C									
		0	1	2	3	4	5	6	11
70	1.5	0.12	0.09	0.12	—	—	—	0.07	—
201	1.5	0.10	0.18	0.11	0.22	0.08	0.14	0.10	0.06
		<i>1.00</i>	<i>0.86</i>	<i>0.96</i>	<i>0.69</i> ‡	<i>0.82</i>	<i>0.77</i>	<i>0.90</i>	<i>1.03</i>
DF117	2	0.10	0.09	0.10	—	0.07	—	—	—
151	2	0.12	0.11	0.09	—	0.07	—	—	—
Sub group D									
		0	1	2	3	4	6	7	8
76	2	0.23	0.16	0.16	0.13	0.11	—	—	0.07
204	1.5	0.34	0.23	0.19	0.19	0.11	—	—	0.09
210	1	0.24	0.16	0.15	0.12	—	—	0.09	—
203	1	0.29	0.29	0.19	0.25	—	0.20	—	—

* Cows drying off, see below for next lactation. † Cow sick. ‡ Tuberculin injection on previous day.

Sub groups A-C: Summer calving.

A Cows 69 and 86 calved January, 1940, others March to May, 1939.

B Calved January to April, 1940.

C Calved May, 1940.

Sub group D: Winter calving, July, 1940.

Cows arranged in order of calving.

Blood copper was estimated simultaneously with most of the milk samples. As no systematic variation occurred, the blood copper figures are given (Table 4) for only four of the cows, selected as representing the four main types of variation which occur in the milk copper figures.

The second group of cows (Table 5) were grazed on irrigated pastures, the copper content of which was from 9 to 14 p.p.m. (dry basis). A blood copper determination was done on all cows on 5/9/40; most of the figures lay within the normal range (0.87 to 1.04 mg. per litre) but six of the cows (Nos. 14, 20, 28, 42, 46 and 66) showed rather higher values (1.19 to 1.66 mg. per litre).

TABLE 5.
Copper content of cows' milk.
(Mg. per litre.)

			GROUP 2: HARVEY IRRIGATION AREA.						
Cow No.	Breed.*	Weeks after calving at first test.	Weeks of test.						
			0	1	2	3	4	6	
Sub group A									
53	SXJ	3	0.05	0.04	—	—	0.05	0.04	
51	J	2.5	0.06	0.04	—	—	0.05	—	
36	J	2.5	0.10	0.05	—	—	—	0.05	
52	SXJ	2.5	0.09	0.05	—	—	—	—	
28	S	2	0.07	0.06	0.05	0.07	—	—	
50	S	1.5	0.06	0.05	0.04	0.04	—	—	
Sub group B									
68	SXJ	1.5	0.12	0.10	0.09	—	0.08	—	
66	GS	1	0.10	0.10	0.07	—	—	—	
20	S	0.5	0.06	0.06	0.08	—	—	—	
55	GS	0.5	0.13	0.11	0.16	0.11	0.14	0.12	
Sub group C									
29	S	1	0.07	0.10	0.09	0.05	—	—	
46	S	1	0.08	0.06	—	—	0.05	—	
14	S	0.5	0.10	0.06	0.05	—	—	—	
42	S	1.5	0.13	0.13†	—	—	—	—	
22	GF	0.5	0.20	0.21	—	—	—	—	

* S, Shorthorn; J, Jersey; F, Friesian (Holstein); X, cross; G, grade.

† Circumstances prevented the continuation of the experiment beyond this date.

Calving dates: Sub group A, early June; B, early July; C, August, 1940.
Cows arranged in order of calving.

3. *The effect of decreased copper intake on the milk copper levels of sheep and cows.* Milk samples were taken from a number of experimental ewes at Gingin, a copper-deficient area in Western Australia (see Bennetts and Chapman, 1937). Three groups were available: (1) Controls grazing on copper-deficient pastures, 2-3 p.p.m. (dry basis), (2) sheep having access to a salt lick containing 0.5 p.c. copper sulphate, and (3) sheep running on pasture which had been top dressed with 20 lb. copper sulphate per acre and contained 10-12 p.p.m. copper (dry basis). Group 3 may be regarded as normal. As some of the sheep of Group 2 showed low blood copper figures, it is probable that all the animals were not taking an adequate amount of lick. The blood copper figures are included in Table 6 to give some indication of the copper status of the animals.

TABLE 6.
Milk copper of Gingin sheep.
(Mg. per litre. Blood copper figures in italics.)

Group.	Sheep No.	9/8/39.		22/8/39.		19/9/39.	
1 (Cu-deficient pasture only)	133	0.03	0.15	0.04	—	0.13	0.59*
	234	0.02	0.07	0.02	0.07	0.02	0.10
	282	—	0.14	0.03	0.06	0.01	0.16
	289	0.01	0.11	0.02	0.09	0.01	0.17
2 (Cu-deficient pasture plus Cu-lick)	229	—	0.32	0.04	0.28	0.05	0.49
	283	—	—	0.07	0.67	0.10	1.04
	286	0.11	0.50	0.07	0.48	0.05	0.61
	295	0.15	0.84	0.11	0.74	0.10	1.04
3 (High Cu pasture)	249	—	0.99	0.11	1.00	0.10	0.89
	255	—	—	0.09	0.84	0.15	0.98
	267	—	—	0.09	0.44	0.11	0.86
	290	—	0.34	0.10	0.75	0.09	1.02

* See note in text.

Sheep 290 lambed 24/6/37; Nos. 229, 234, 249, 286 between 27/6/40 and 18/7/40; others in June.

The analysis of a number of milk samples from copper deficient cows is given in Table 7. These cows were from two "Falling Disease" areas (Bennetts and Hall, 1939) and were grazing on pastures containing between 1 and 2 p.p.m. copper (dry basis).

TABLE 7.
Milk copper levels of copper deficient cows.
(Mg. per litre.)

Calving date.	Group A.		Calving date.	Group B.	
	1/11/39.	13/12/39.		28/11/39.	12/12/39.
8/10/39	0.04	0.02	14/ 7/39	0.01	0.01
11/ 5/39	0.01	0.02	17/ 9/39	0.025	0.02
2/10/39	0.015	0.02	3/11/39	0.02	0.015
2/10/39	0.05	0.01	23/ 9/39	0.02	0.01

DISCUSSION.

Several facts emerge from the data for normal animals. In all cases, the concentration of copper in the blood is higher than in the milk. The mammary glands apparently have no mechanism for concentrating copper in the milk as occurs with some other inorganic constituents. Since the milk copper can vary enormously without any corresponding variation in the blood level, it is reasonable to assume that the transfer of copper from the blood to the milk is not one of passive diffusion but is subject to physiological control.

The fact that ewes and many cows give greatly increased milk copper in the early part of the lactation period, seems to have escaped the attention of other workers, and it is obvious that the stage of lactation is important when milk copper levels are considered. The Guernsey cows from Group 1 show this phenomenon more than the Shorthorns from Group 2, but the fact that sheep also show raised levels in the early lactation period suggests that it is far more fundamental than merely the effect of breed. The age of the cows apparently has no influence in deciding whether or not raised milk copper levels will occur. Although the limited data are insufficient to draw any conclusions concerning seasonal effects, it should be noted that there is a greater tendency for raised levels among cows calving in the winter months when the copper content of the pastures is higher.

The slight rise which occurs with almost all the normal sheep on the last sampling may be associated with the "drying off" of the ewes.

The values obtained for most of the copper deficient ewes confirms the original observation of Bennetts and Chapman (1937) that, under these circumstances, the milk copper may fall to very low levels. There is no obvious reason for the sudden rise of both blood and milk figures for ewe 133 on 19/9/39 (blood figures re-checked). The animal was slaughtered on 17/10/39 and its liver found to contain only 9.2 p.p.m. copper (dry basis) indicating a state of acute copper deficiency.

Unfortunately, no samples were taken from these copper deficient ewes shortly after lambing. However, the data of Bennetts and Chapman show that in such ewes the milk copper is slightly higher shortly after lambing, but still far below that of a normal sheep. The milk of three such ewes collected 1, 2 and 3 weeks after lambing contained 0.05, 0.03 and 0.05 p.p.m. respectively. This point has been confirmed by samples taken from an acutely copper-deficient ewe from another area, 0.09 mg. per litre being found two weeks after lambing.

It is apparent that under conditions of low copper intake, cows can produce milk with a copper content much below normal. Only two of the figures obtained lie within the normal range, and these are from cows within a few weeks of parturition.

SUMMARY.

An examination of the copper content of the milk of ewes and cows from normal and copper deficient areas of Western Australia has been made. It was found that:

The copper content of the milk of normal ewes fell progressively from values of 0.20 to 0.64 mg. copper per litre in early lactation to 0.04 to 0.16 mg. per litre several months later.

Similar falls were observed in the milk of some cows, but were not apparent in others. A considerable variation in the values was obtained, but the majority of the figures lay between 0.05 and 0.20 mg. copper per litre.

There was no correlation between blood copper levels and the milk copper variations.

Sheep and cows grazing on copper deficient pastures (1 to 3 p.p.m. copper dry basis) show reduced levels of copper in the milk. Values down to 0.01 to 0.02 mg. copper per litre were obtained in most cases.

Acknowledgments. The writer wishes to acknowledge his indebtedness to the Western Australian Department of Agriculture for providing laboratory facilities, and to officers of that Department for assistance in collecting the milk samples.

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CHANGES IN THE LEVELS OF COPPER IN MILK DURING
LACTATION

by

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Unpublished Data

The studies on copper in milk of ewes and cows were extended to humans and to mares. These data have been referred to by Underwood ("Trace Elements in Human and Animal Nutrition", Academic Press, 1962, second edition, page 66), but full details have never been published.

The following levels of copper were obtained in human milk samples collected during the period 1942 to 1945.

<u>Subject</u>	<u>Copper content of human milk</u>							
	Values as mg Cu per litre							
	<u>Weeks of Lactation</u>							
	<u>1</u>	<u>2</u>	<u>4</u>	<u>8</u>	<u>12</u>	<u>16</u>	<u>20</u>	<u>+24</u>
A (second lactation)	0.89	0.67	0.55	0.48	0.39	0.27	0.27	0.19
A (third lactation)	0.62	-	0.42	0.38	0.28	0.27	0.24	0.15
B	-	0.68	0.44	0.50	0.30	0.40	0.38	-
C	0.68	0.57	0.44	0.42	-	-	-	-
D	0.47	-	0.46	0.41	0.41	-	-	0.23

All the above samples showed a downward trend in

copper levels during lactation, although in subject D this trend is very small.

After these studies were done, an earlier paper by Lesné, Zizine, and Briskas (Rev. Path. Comp. 1936, 36, 1369) was noted. Using rather unsatisfactory chemical methods these workers had observed similar trends in copper levels during lactation. Their values tend to be considerably higher than those obtained by the writer.

Similar studies were made on mare's milk. Some samples showed similar trends, but in general the changes were smaller.

Copper content of mare's milk
Values as mg Cu per litre

<u>Subject</u>	<u>Weeks of Lactation</u>			
	<u>1</u>	<u>2</u>	<u>4</u>	<u>8</u>
Mare				
A	0.47	-	0.19	0.21
B	0.63	-	0.19	0.15
C	0.38	-	0.23	0.21
D	0.31	0.14	0.14	0.14*
E	0.23	0.17	0.12	-
F	0.36	0.12	0.14	0.20*
G	0.17	0.13	0.20*	0.15*
H	0.33	0.14	0.16	0.14*

*These determinations were done on small samples with probable higher errors due both to sampling and to chemical methods.

The occurrence of changes of copper levels in cow's milk during lactation (Paper 2:4) seems to have passed unnoticed until they were discovered again by Koppejan and Mulder (Proc. 13th Intern. Dairy Congress, The Hague, 1953, 3: 1400. Cited by King and Dunkley in following reference). More recent studies have been made by King and Dunkley (J. Dairy Sci., 1959, 42: 420) and King and Williams *ibid*, 1963, 46: 11) in relation to the spontaneous development of oxidized flavour in milk.

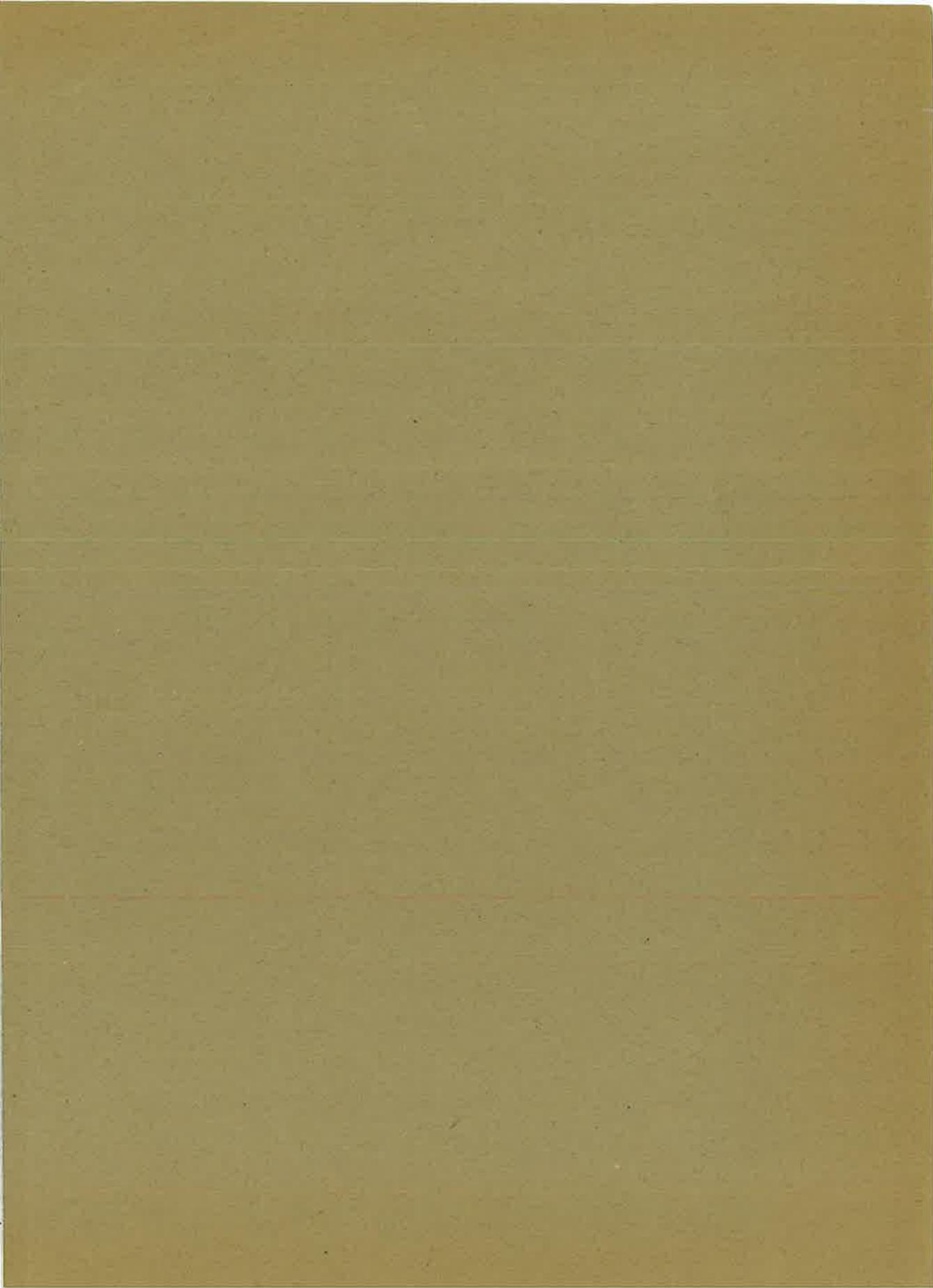
In Paper 2:4 it was suggested that the transfer of copper from the blood to the milk was not one of passive diffusion, but was subject to physiological control. An interesting confirmation of this hypothesis is found in recent observations by Barker (Ph.D. Thesis, University of Western Australia, 1960, p. 95). Studies on the marsupial Setonyx brachyurus have shown that in this species the copper content of the milk is higher than that of the blood.

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PAPER 2:6

STUDIES ON THE BLOOD COPPER OF SHEEP AND OF COWS

by A. B. BECK



STUDIES ON THE BLOOD COPPER OF SHEEP AND OF COWS

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(From the Department of Agriculture, Perth, Western Australia, and the Council for Scientific and Industrial Research).

(Accepted for publication 16th August, 1941.)

Published data for the copper content of the blood of normal sheep show values extending over a wide range.

TABLE 1.

Observer.	Range (mg. Cu per litre).
Tompsett (1934)	1.56-1.80
Bennetts and Chapman (1937)	0.64-0.87
Eden (1939)	0.16-1.64
Albiston <i>et al.</i> (1940)	0.8-2.0
Innes and Shearer (1940)	0.47-2.81
Eden (1941)	0.13-2.10

Tompsett (1934) gives values of 1.80 to 2.23 mg. Cu per litre for ox blood, while Sjollem (1938) states that the normal figure for cattle is about 1.0 mg. per litre. Apart from the recent observations of Eden (1941) there seem to be no figures available concerning the effect of pregnancy on the blood copper level of sheep and cows, but it has been shown by Tompsett and Anderson (1935) that there is a rise in the copper content of the maternal blood of humans during the last few months of pregnancy.

The object of the present investigation was to obtain the range of blood copper values in normal sheep and cows under Western Australian conditions. The corresponding figures for haemoglobin were also obtained as existing data show a wide variation (see Underwood *et al.* (1939)). Collateral studies were also carried out on animals under conditions of naturally-occurring copper deficiency to provide information on the relation between low blood copper and haemoglobin levels.

EXPERIMENTAL.

Chemical methods. The blood copper of normal animals was estimated by the trichloroacetic acid method of Tompsett (1934) using 20 ml. of blood and 50 ml. of the acid filtrate for analysis. Tests showed that no correction was necessary for the volume of the protein precipitate (*cf.* Thivolle and Laugier, 1938). Acid washed filter papers were used and the readings were made in an ordinary colorimeter with micro-cups.

With bloods of low copper content this method was used for routine analysis, a known amount of copper being added to bring the colour to a suitable intensity. In spite of obvious objections this procedure gives fairly accurate results (probably within ± 0.05 mg. Cu per litre). In the study of the relation between blood copper and haematopoiesis more precise values were necessary and the copper content was estimated by dry ashing 30-40 ml. of blood with magnesium nitrate (Van Niekerk, 1937) and extracting with dithizone as in the method of Sylvester and Lampitt (1935). With bloods of low copper content the accuracy of this method is probably ± 10 p.c.

Haemoglobin was estimated by the acid haematin method using a Newcomer disc which had been standardized against oxygen capacity (1 p.c. Hb = 1.34 vols. p.c. O₂).

Experimental animals. Observations were made on four groups of merino sheep grazing on natural pastures at Merredin in the drier portion of the Western Australian wheat belt: (1) 11 two-toothed wethers brought from Beverley just prior to the experiment, (2) 10 pregnant four-toothed ewes, (3) 4 ewes originally in group 2 which did not lamb, (4) 12 pregnant full-mouth ewes. The copper content of the natural pastures was found to fall from 10 p.p.m. Cu (dry basis) when young to 5-6 p.p.m. when mature. Green feed was only available to the above sheep on the last two samplings.

The results given in Table 3 were from mature Border Leicester-merino cross-bred ewes grazing at Beverley, Western Australia, where the pastures contain 7 to 13 p.p.m. Cu (dry basis)

during the growing period. This area appears to have a slightly higher copper status than Merredin. Green feed was available after the first sampling.

The Muresk cows (Table 5) were pure-bred Guernseys and the figures presented were a series of irregular determinations made on 17 cows over a period of 6 months (January to July, 1940). Samples were taken up to about two months before calving, and then from about one week after calving.

The Harvey cows (Table 5) were mainly Shorthorns and Jerseys. All samples were taken on 5th September, 1940. These animals were grazing on pastures which contained 9 to 14 p.p.m. (dry basis); these values are slightly higher than those usually encountered in Western Australia. The Perth animals were stall fed. Further details of the Muresk and Harvey cows have been described elsewhere (Beck, 1941).

The copper-deficient sheep (Table 6) were from Gingin (Bennetts and Beck, 1941) and the "Falling Disease" areas (Bennetts *et al.*, 1941). The deficient cows (Table 7) were all from the last areas. Pastures in these localities contain 1 to 3 p.p.m. Cu (dry basis). The figures given in Tables 7 and 8 are representative of a larger collection of data and have been selected as being typical of the blood changes which occur under conditions of naturally-occurring copper deficiency. It will be noted that in many cases there is a rise in blood copper in the late spring. This occurs generally in these areas, but the exact cause is unknown.

Results.

The data for normal cows and sheep are given in Tables 2, 3 and 5, and for deficient animals in Tables 6 and 7. Blood copper levels for sheep just before and after parturition are given in Table 4.

DISCUSSION.

The data for normal sheep in Western Australia confirm the findings of other workers that the blood copper levels for sheep under conditions of normal copper intake (5-10 mg. Cu per day) can vary within wide limits (0.4-1.6 mg. Cu per litre) without any effect on the health of the sheep. Most of the figures, however, lie between 0.6 and 1.2 mg. per litre. There is no apparent reason for the definite seasonal variation in the figures for the Merredin wethers, a variation which is quite absent in the ewes. The younger age of the wethers and the fact that these sheep previously came from an area of slightly higher copper status may be contributory factors, but no evidence is available to suggest that sex alone may produce such a difference.

The observation by Eden (1941) that the blood copper of individual sheep can vary very widely is amply confirmed by the results for the sheep of Table 2. The differences between the highest and lowest values for individual sheep were distributed as follows:

Range of differences. mg. Cu per litre.	Per cent. of wethers. Group 1.	Per cent. of all ewes. Groups 2, 3, 4.
0.22-0.39	18	46
0.40-0.59	73	35
0.60-0.78	9	19

There was, however, a definite tendency for sheep with higher or lower values to maintain their respective positions throughout the test.

Blood samples were taken from four of the sheep at 10 a.m. and 4 p.m. on three of the monthly tests. The values obtained in the morning were generally very slightly higher than those of the afternoon, but the difference was not significant.

There was no significant difference between the blood copper levels of the pregnant and non-pregnant ewes (Table 2). The values in Table 4 show that samples taken just after parturition show normal values. Samples taken one to four days before lambing generally show the same values as those obtained after lambing. Eden (1941) observed no rise in blood copper levels during the last few months of pregnancy.

The haemoglobin figures are generally slightly higher than those reported by Underwood *et al.* (1939) for sheep in another part of the Western Australian wheat belt.

TABLE 2.

Blood copper and haemoglobin levels of Merredin merino sheep.
(Blood Cu as mg. per litre; Hb as gm. per 100 ml.)

	3/11/39.	13/12/39.	17/1/40.	28/2/40.	27/3/40.	24/4/40.	22/5/40.	26/5/40.	31/7/40.	28/8/40.
Group 1. Two-tooth wethers (eleven animals).										
Blood Cu: Mean	1.03	0.95	0.78	0.88	0.73	0.75	0.66	0.66	0.76	0.88
Highest	1.23	1.06	0.96	1.03	0.87	1.16	0.88	0.90	1.03	1.34
Lowest	0.84	0.86	0.60	0.70	0.54	0.48	0.55	0.43	0.48	0.68
Haemoglobin: Mean	11.1	13.4	11.7	10.7	11.4	12.1	13.3	12.3	12.3	12.3
Blood Cu. General mean = 0.81, S.D. \pm 0.17. Range, 0.43-1.34. Difference for significance between monthly means = 0.11 (P = 0.01).										
Haemoglobin. General mean = 12.1, S.D. \pm 1.37. Range, 9.3-16.1.										

Group 2. Four-tooth ewes. Pregnant (ten animals).										
Blood Cu: Mean	0.96	1.05	0.93	1.14	0.86*	0.89*	0.86	1.00	0.97	1.09
Highest	1.09	1.15	1.03	1.52	0.99	1.11	0.98	1.33	1.21	1.29
Lowest	0.79	0.86	0.84	0.93	0.74	0.64	0.73	0.80	0.76	0.92
Haemoglobin: Mean	12.7	13.9	13.4	11.3	11.9	12.4	13.4	11.8	11.3	11.7
* Lambing between these two dates.										
Blood Cu. General mean = 0.97, S.D. \pm 0.15. Range = 0.64-1.52. Difference for significance between monthly means = 0.13 (P = 0.01).										
Haemoglobin. General mean = 12.4, S.D. \pm 1.27. Range = 10.1-15.6.										

Group 3. Four-tooth ewes. Non-pregnant (four animals).										
Blood Cu: Mean	0.97	1.18	0.95	1.11	0.87	0.93	—	0.99	0.95	1.00
Highest	1.09	1.53	1.04	1.48	0.93	0.96	—	1.05	1.05	1.16
Lowest	0.84	1.02	0.89	0.94	0.79	0.87	—	0.93	0.82	0.77
Haemoglobin: Mean	13.4	14.9	13.7	12.3	12.9	13.7	—	14.1	13.2	12.8
Blood Cu. General mean = 0.99, S.D. \pm 0.16. Range = 0.77-1.53. No significant difference between means.										
Haemoglobin. General mean = 13.4, S.D. \pm 1.20. Range = 11.5-17.0.										

Group 4. Full-mouth ewes. Pregnant (twelve animals).										
Blood Cu: Mean	—	0.99	0.94	1.02	0.82*	0.98*	0.82	0.92	0.94	1.11
Highest	—	1.40	1.27	1.61	1.17	1.18	1.00	1.10	1.16	1.44
Lowest	—	0.59	0.64	0.83	0.51	0.69	0.48	0.60	0.64	0.77
Haemoglobin: Mean	—	15.6	14.3	12.1	12.4	12.7	13.6	13.1	13.0	11.8
* Lambing between these two dates.										
Blood Cu. General mean = 0.95, S.D. \pm 0.19. Range = 0.48-1.61. Difference for significance between means of months = 0.12 (P = 0.01).										
Haemoglobin. General mean = 13.2, S.D. \pm 1.81. Range = 9.9-17.4.										

Blood copper of all Merredin sheep. Mean = 0.92, S.D. \pm 0.19. Range = 0.43-1.61.
Haemoglobin of all Merredin sheep. Mean = 12.6, S.D. \pm 1.57. Range = 9.3-17.4.

TABLE 3.

Blood copper and haemoglobin of seven cross-bred ewes.
(Blood Cu as mg. Cu per litre, Hb as gm. per 100 ml.)

Blood Cu: Mean	0.91	1.16	1.16	1.01	0.97
Range	0.98	1.45	1.46	1.14	1.17
	0.75	0.96	1.06	0.84	0.84
Haemoglobin: Mean	12.0	12.1	10.9	13.5	12.9

Samples taken 11/6/40 to 1/10/40 at four-weekly intervals. First sample taken two weeks after lambing.
Blood copper. General mean 1.04, S.D. \pm 0.16. Range = 0.75-1.46. Difference for significance between monthly means = 0.18 (P = 0.01).
Haemoglobin. General mean = 12.3, S.D. \pm 1.28. Range = 10.0-14.2.

TABLE 4.

Blood copper of normal sheep near to parturition.

(mg. Cu per litre).

(Approximate hours of test before (-) or after (+) lambing given in brackets.)

Sheep No.	27/3/40.	10/4/40.	24/4/40.
B. 131	0.99	0.86 (+ 72)	0.80
B. 243	0.94	1.07 (+ 24)	0.89
V. 72	0.77	0.79 (+ 24)	0.92
V. 80	1.17	—	1.07 (+ 12)
B. 267	0.74 (- 24)	0.87	0.64
B. 244	0.74 (- 48)	0.94	0.95
V. 75	0.51	1.01 (- 96)	0.69
V. 77	0.81 (- 96)	0.92	1.18

TABLE 5.

Blood copper and haemoglobin levels of normal cows.

Locality		Muresk	Harvey	Perth
No. of observations		79	15	2
Blood Cu (mg. per litre):	Mean	0.97 ± 0.16	1.14 ± 0.27	0.98
	Range	0.69 — 1.47	0.82 — 1.66	0.97, 1.00
Haemoglobin (gm. per 100 ml.):	Mean	11.7 ± 1.11	14.9 ± 1.22	11.2
	Range	8.6 — 15.0	11.6 — 16.8	11.8, 10.6

TABLE 6.

Blood copper and haemoglobin levels of copper-deficient sheep.

Top figures, blood copper (mg. per litre): those by trichloroacetic acid method in italics, others by dry ashing.

Lower figures, haemoglobin (gm. per 100 ml.).

Sheep No.	Period of test.	Breed and sex.†								
1	April–Nov., 1939	X E	<i>0.43</i>	<i>0.20</i>	0.19*	0.07*	0.07	0.10	0.12	0.17
			11.5	9.9	8.1	6.3	6.5	5.4	9.7	10.5
2	April–Nov., 1939	X E	<i>0.42</i>	<i>0.26</i>	<i>0.18</i>	<i>0.09*</i>	<0.05*	<i>0.14</i>	0.15	0.28
			12.5	12.1	11.3	12.7	11.1	10.5	10.9	12.5
3	April–Nov., 1939	X E	<i>0.50</i>	<i>0.12*</i>	0.13*	0.14	0.06	<i>0.16</i>	0.15	0.23
			12.2	10.5	10.4	8.0	9.9	9.3	12.3	12.3
4	April–Nov., 1939	X E	<i>0.43</i>	<i>0.23*</i>	0.19*	0.11	0.09	<i>0.17</i>	0.31	0.31
			13.2	9.6	11.2	10.6	8.5	9.9	11.5	12.6
5	March–Nov., 1939	M E	<i>0.27</i>	<i>0.34</i>	<i>0.18</i>	<i>0.10</i>	<i>0.07*</i>	<0.05*	0.09	<i>0.09</i>
			14.6	15.1	14.4	13.2	10.5	6.1	4.6	3.7
6	March, 1939–Aug., 1940	M E	<i>0.92</i>	<i>1.08</i>	<i>0.64</i>	<i>0.36</i>	<i>0.22</i>	<i>0.21*</i>	<0.05*	<i>0.14</i>
			13.2	11.7	13.7	13.2	13.4	7.9	9.1	7.6
			<i>0.19</i>	<i>0.41</i>	+	<i>0.33</i>	<i>0.27</i>	0.14*	0.14*	0.25
7	May, 1939–Nov., 1940	M W	9.0	9.8	+	12.2	12.6	11.0	9.4	6.5
			<i>0.31</i>	<i>0.18</i>	<i>0.05</i>	<i>0.22</i>	<i>0.11</i>	<i>0.16</i>	<i>0.16</i>	<i>0.14</i>
			14.2	13.9	13.2	13.6	12.7	10.5	12.7	14.1
			+	<i>0.22</i>	0.17	0.14	0.16	0.19	—	
			+	13.7	11.4	13.4	11.9	8.3	8.4	11.0

Haemoglobin levels below 9.0 are regarded as evidence of anaemia, but only levels below 7.0 are considered to indicate severe anaemia.

* Samples generally taken at monthly intervals.

† Sheep lambled between these two dates.

+ Summer period (December to April) during which no samples were collected.

† E, ewe; W, wether; M, merino; X, Border Leicester-merino cross.

TABLE 7.

Blood copper and haemoglobin levels of copper-deficient cows.

Top figures, blood copper (mg. per litre) : those by trichloroacetic acid method in italics, others by dry ashing.

Lower figures, haemoglobin (gm. per 100 ml.).

Cow No. Period of test.

1	May, 1939-	<i>0.35</i>	<i>0.23</i>	<i>0.15</i>	<i>0.16</i>	<i>0.12</i> *	<i>0.17</i> *	0.11	<i>0.08</i>
	Sept., 1940	10.2	11.8	11.0	12.6	12.0	9.0	8.9	11.9
		+	<i>0.33</i>	<i>0.42</i>	0.36	0.36	<i>0.25</i>	0.18	—
		+	11.4	10.8	10.2	10.7	11.0	5.2	6.5
2	May, 1939-	<i>0.37</i>	<i>0.21</i>	<i>0.19</i>	<i>0.19</i>	<i>0.29</i>	0.12	0.14	<i>0.05</i>
	Sept., 1940	9.8	11.2	12.2	12.8	11.5	10.1	11.0	13.6
		+	<i>0.11</i> *	0.44	0.36	0.17	0.29	0.12	—
		+	10.6	10.2	10.0	9.9	8.6	5.4	7.1
3	May, 1939-	<i>0.20</i>	<i>0.21</i> *	<i>0.50</i> *	<i>0.39</i>	<i>0.29</i>	0.18	0.22	<i>0.15</i>
	Sept., 1940	11.0	11.9	10.8	11.1	10.0	9.5	10.5	11.7
		+	—	<i>0.42</i>	<i>0.30</i>	<i>0.41</i>	<i>0.26</i>	0.16	—
		+	10.4	10.2	9.9	9.8	10.5	4.7	8.3
4	May, 1939-	<i>0.25</i>	<i>0.38</i>	<i>0.20</i>	<i>0.17</i>	<i>0.09</i>	0.17	0.09	<i><0.05</i> *
	Sept., 1940	10.4	11.8	12.7	12.6	11.3	9.2	10.8	12.8
		+	—*	<i>0.37</i>	<i>0.16</i>	0.24	0.20	0.09	—
		+	—	9.2	8.9	9.9	8.8	4.7	7.2
5	May-December, 1939	<i>0.17</i>	<i>0.15</i>	<i>0.15</i>	<i>0.11</i>	<i>0.07</i>	0.11	0.16*	0.09#
		9.0	9.2	9.4	11.0	9.5	7.7	9.7	10.3

Haemoglobin levels below 9.0 are regarded as evidence of anaemia, but only levels below 7.0 are considered to indicate severe anaemia.

Samples generally taken at monthly intervals.

Cow 1 received supplement of Fe, Co, Ni, Zn and Mn (but no Cu) during first eight tests.

* Calving between these two dates; Cow 2 calved on day of sampling.

+ Summer period (December to March) during which samples were not collected.

The blood copper levels of cows also range within fairly wide limits (0.69 to 1.66 mg. per litre). No seasonal variation was noted in these figures.

Schultze *et al.* (1936) have made studies on the blood of copper-deficient pigs and reached the conclusion that a minimum blood copper level of 0.20 mg. per litre was necessary for haemoglobin formation. Their experiments are hardly comparable with those given in the present paper, but our findings in copper-deficient sheep and cows are as follows: The blood copper level can remain at 0.2 to 0.3 mg. per litre for long periods without any occurrence of anaemia. There is no absolute correlation between low blood copper levels and the development of anaemia, but if the blood copper level falls below 0.1 to 0.2 mg. per litre for any length of time anaemia generally develops. Further discussion is unwarranted in view of our limited knowledge of the function of copper in haemoglobin formation.

SUMMARY.

The copper content of the blood of normal Western Australian sheep varies between 0.4 and 1.6 mg. Cu per litre; most figures however lie between 0.6 and 1.2 mg. per litre. Haemoglobin figures for the same sheep lie between 9.3 and 17.4 gm. per 100 ml.

Normal cows show blood copper values between 0.7 and 1.7 mg. Cu per litre, and haemoglobin figures between 8.6 and 16.8 gm. per 100 ml.

In copper-deficient sheep and cows there appears to be no absolute correlation between low blood copper levels and the development of anaemia, but anaemia

generally occurs if the level falls below 0·1 to 0·2 mg. Cu per litre for any length of time.

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The Liver Copper Levels of Foetal and Newly Born Marsupials and Whales

In many mammalian species the liver copper level of the newly born animal is much higher than that of the mother. Such increased levels have been recorded for man (Griffith, Butt and Walker, 1954), the ox and pig (Cunningham, 1931), the horse (Seekles, 1950), the rat (McFarlane and Milne, 1934; Lorenzen and Smith, 1947) and the guinea-pig and rabbit (Lorenzen and Smith, 1947). In the sheep alone there seems to be little difference between the liver copper levels of the mother and the newly born (McDougall, 1947). The physiological significance of the foetal stores of copper is not completely understood and possible explanations have been discussed by Underwood (1956).

TABLE I
Liver copper levels of maternal and young marsupials

Species	Details	Liver copper (p.p.m. Cu on dry matter)	
		Maternal	Young
<i>Macropus agilis</i> .. (Wallaby)	Fitzroy River : joey almost ready to leave pouch	15	160
<i>Macropus robustus</i> (Euro)	Ord River : joey	15	250
	Shaw River : joey, one-third grown	14	180
<i>Macropus ocydromus</i> (Grey Kangaroo)	Margaret River : pouch embryo, hairless, ca. 7" long	19	295
<i>Macropus rufus</i> .. (Red Kangaroo)	Wiluna : pouch embryo, ca. 2" long	15	170
" "	Wiluna : pouch embryo, ca. 3" long	14	155
" "	Wiluna : pouch embryo, ca. 4" long	17	230
<i>Setonix brachyurus</i> ("Quokka")	Rottnest Is. : pouch embryo, small and hairless	13	270

During investigations on the liver copper levels of vertebrates (Beck, 1956) it was found that increased liver copper levels occur in the newly born of two groups of mammals not previously studied.

Table I shows such data for a range of Western Australian marsupials. The persistence of a high liver copper level in the fully developed joey of the wallaby is rather surprising as there appears to be a rapid fall in liver copper levels of most species after birth. Recently Barker (1960) has made observations on *Setonix brachyurus* and has shown that there is a rapid fall of liver copper levels after the young leave the pouch.

Table II gives liver copper levels for hump-back cow whales and near-term foetuses. These samples were collected by Dr. G. Chittleborough (Division of Fisheries, C.S.I.R.O.) at Carnarvon, Western Australia in July 1953. The length of the cow whales were 41 to 47 feet. Whale calves are usually 15 feet long at birth.

It is possible that the marked elevation of late foetal liver copper levels is a characteristic of mammalian physiology rather than of viviparity. Only one set of samples was obtained from non-mammalian viviparous

TABLE II
Copper content of livers from whale cows and foetuses (*Megaptera nodosa*)

Length of Foetus (Feet)	Sex of Foetus	Liver copper (p.p.m. Cu on dry matter)	
		Maternal	Foetal
14.4	♀	13	425
14.0	♀	11	395
15.4	♂	15	395
15.3	♀	13	455
14.1	♂	13	295
12.8	♀	11	490

animals. A lizard (*Trachysaurus rugosus*) containing two fully developed foetuses showed a liver copper level of 20 p.p.m. (on fat-free dry matter). The livers of the foetuses were only slightly higher (24 and 32 p.p.m. on fat-free dry matter).

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SECTION 3

SECTION 3

COPPER DEFICIENCY IN STOCK - FIELD STUDIES

This Section is made up of six papers, four of which are primarily concerned with the animal, while two relate to studies on the pastures which have caused deficiency symptoms in stock.

Sub-Section (a)

Animal Studies

- 3:8 "Enzootic ataxia and copper deficiency in sheep in Western Australia", H.W. Bennetts and A.B. Beck : Council for Industrial Research, 1942: Bulletin 147 (52 p.p.).
- 3:9 "Falling disease of cattle in the south-west of Western Australia : Part 2 : Studies of copper deficiency in cattle", H.W. Bennetts, A.B. Beck, R. Harley and S.T. Evans. Aust. Vet. J., 1941, 17, 85.
- 3:10 "The pathogenesis of 'Falling Disease'" : H.W. Bennetts, A.B. Beck and R. Harley. Aust. Vet. J. 1948, 24, 237.
- 3:11 "Enzootic marasmus: The relation of copper to incidence and treatment" : E.J. Underwood and A.B. Beck, Aust. Vet. J., 1941, 17, 155.

Section 3

- 2 -

Sub-Section (b)

Pasture Studies

- 3:12 "A survey of the copper content of Western Australian pastures". A.B. Beck, J. Agric. W. Aust., 1941, 18, 285.

This paper was revised slightly in 1951 and reprinted as W. Aust. Dept. of Agriculture, Leaflet 678. The revised version is included in this Thesis.

- 3:13 "The levels of copper, molybdenum, and inorganic sulphate in some Western Australian pastures." A.B. Beck, Aust. J. Exp. Agric. Anim. Husbandry : 1962, 2, 40.

Sub-Section (a)

Animal Studies

The candidate entered the field of copper metabolism late in 1937 when he joined Dr. H.W. Bennetts of the Western Australian Department of Agriculture in the investigation of enzootic ataxia of lambs. At that time methods of analysis of biological material for copper were not standardized and grateful acknowledgment is made

Section 3

- 3 -

for the co-operation and patience of Dr. Bennetts as satisfactory techniques and methods were worked out under appalling laboratory conditions.

In the investigations in this Sub-Section, the candidate acted as analytical biochemist with full responsibility for chemical aspects, but the planning of the projects was essentially done by Dr. Bennetts in Papers 3:8 to 3:10, and by Dr. Underwood in paper 3:11. In Papers 3:9 and 3:10, Mr. R. Harley was responsible for the supervision of field experiments, and Mr. S.T. Evans completed the analytical work when the candidate left Western Australia.

Sub-Section (b)

Pasture Studies

While the work of an enzootic ataxia and "falling" disease" was being carried out, it became obvious that information was required concerning the copper status of pastures which allowed normal health in stock. Accordingly pasture samples were collected from a wide range of Western Australian soil types and an attempt was made to correlate these levels with animal health. The conclusions are set out in Paper 3:12.

Section 3

- 4 -

The original survey of copper content of Western Australian pastures was the candidate's own project. The survey of the molybdenum and sulphate levels reported in Paper 3:13 was carried out partly at the suggestion of Dr. L.B. Bull, former Chief of the Division of Animal Health, C.S.I.R.C. All analytical work was carried out by the candidate, or by his assistants.

COMMONWEALTH  OF AUSTRALIA

Council for Scientific and Industrial Research

BULLETIN No. 147

Enzootic Ataxia and Copper
Deficiency of Sheep in
Western Australia

By

H. W. BENNETTS, D.V.Sc.
(Department of Agriculture, Western Australia)

and

A. B. BECK, M.Sc.
(Division of Animal Health and Nutrition)

MELBOURNE, 1942

Registered at the General Post Office, Melbourne, for transmission by post as a periodical

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War time economy requires reduction in the Council's expenditure on publication. It is hoped that the adoption of the photo-lithographic process will be for a brief period only.

ERRATA.

- age 3, line 2: for "(Dunlop et al., 1939)"
read "(Innes and Shearer, 1940)"
- line 4: after "Dunlop and Wells (1938)"
insert "and Dunlop et al. (1939)"
- line 7: before "Shearer, Innes, and
McDougall"
insert "Innes and Shearer, 1940".
- age 20, Table 2: "Group 2 (Control)" alter
figures to "25 - 25 - 24 - 96".

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S U M M A R Y .

1. Enzootic ataxia is a disease of the unweaned lamb characterised clinically by ataxia affecting the hind limbs, and pathologically by demyelination of the nervous system, a typical degeneration of the spinal cord being pathognomonic. Extensive brain lesions may occur in the more acute cases.
2. Enzootic ataxia is due to copper deficiency. It is prevented by the administration of copper supplements to the mother, and in the lamb the course of the disease is arrested by copper treatment.

A low copper status of the mothers (liver, blood, and milk) and of the affected progeny (liver and blood) has been constantly demonstrated in all cases investigated.

Pasture analyses from a number of widely separated affected areas have shown a low copper status throughout. The distribution of the disease, moreover, coincides with that of other stock, cereal, and pasture problems known to be associated with copper deficiency.

3. Adult sheep maintained on affected properties show signs attributable to copper deficiency. These are "stringiness" of wool and, in breeding ewes, loss of physical condition and diarrhoea during the late gestation and the lactation periods; a severe anaemia is commonly exhibited concurrently. Copper supplements or a change to "healthy" country are curative.
 4. Severe anaemia in the ewe near to parturition is directly correlated with an acute form of ataxia in the progeny. Ataxia, however, may occur in the progeny of ewes that do not develop anaemia.
 5. It has been demonstrated experimentally, and extensively under practical farming conditions, that the benefits of copper supplementation are not confined to the prevention of ataxia. Copper promotes optimal growth in lambs and has a marked beneficial effect on the adult members of the flock, anaemia and other signs of ill health being absent in the breeding ewe and wool growth being normal throughout.
 6. Copper supplements, under practical conditions, are administered in lick form or by topdressing of pastures with copper compounds. It has been demonstrated that the copper status of mixed pasture may be raised to "normal" levels by this means. There is some evidence that topdressing is the more beneficial.
- An investigation to determine the optimal rate of topdressing and the period of effectiveness is now in progress.
7. The pathogenesis of enzootic ataxia has been discussed in some detail, but pending the results of fundamental studies of copper metabolism in the sheep and a more complete knowledge of the normal process of myelination in the lamb no really satisfactory explanation is possible.

Enzootic Ataxia and Copper Deficiency of Sheep in Western Australia

I. INTRODUCTION.

During the first decade of the twentieth century the increasing incidence of "Gingin rickets" in lambs as well as of a less well-defined malaise in horses and cattle, all on an area of Cretaceous country at Gingin and Dandarragan, began to constitute a real problem. A similar problem was experienced early on coastal country, notably in the Busselton district.

In the affected areas it was found by experience that a change to "sound" country during the gestation period or the annual repurchase of breeding stock from healthy areas constituted an effective means of controlling the problem, notably in sheep with which such measures offered a more or less practicable solution. For this reason, and because of the apparently restricted area affected, the problem was at first not deemed to be of serious economic importance. However, as conditions became progressively worse and the control measures adopted by stockowners less effective, scientific investigation became necessary.

Subsequently it became evident that the problem, apart from its obvious fundamental importance, was of much more moment than at first appeared, since copper deficient areas are much more widespread than was at first evident.

Earlier work comprised the collection of data from affected areas, a geological survey of the Gingin district carried out by the Assistant Government Geologist F. Foreman, occasional clinical and pathological examinations of affected lambs, and the carrying out of the analyses of soil, pasture, water, ewe's milk, and lamb's blood at the Government Chemical Laboratory.

In 1932, one of us (H.W.B.), who was at that time and until 1935 seconded to the Council for Scientific and Industrial Research, commenced field experiments at Gingin and at the Pardelup Prison Farm, Mt. Barker, two areas in which ataxia of lambs was enzootic. A series of field experiments has been carried out at Gingin and observations continued at Dandarragan and in other areas where ataxia was becoming apparent.

In 1936, F. E. Chapman of the Government Chemical Laboratory became associated with the investigation, and the conclusion was reached, based on experimental and chemical data, that the disease was due primarily to copper deficiency as reported by Bennetts and Chapman (1937). The work was continued and since the end of 1937 has been carried out jointly by the present authors.

Although seasonal and other factors have repeatedly interfered with the investigation and have sometimes prevented clear-cut results from experiments, the additional information obtained, notably the demonstration that the copper status of affected pastures is low throughout the year, affords ample confirmation of the original claims regarding the etiological significance of copper.

During the course of the investigation it was found that adult sheep, particularly breeding females, showed definite clinical and pathological evidence of copper deficiency. These data are included in Section III.

The results of the investigation have already received extensive application in the field, and on account of the almost universal use of copper, administered as a lick or as a topdressing, it is impracticable to continue experimental investigations at Gingin except pasture trials to determine the optimal rate and periodicity of topdressing with copper.

The general problem of copper deficiency in Western Australia is receiving further consideration: a study of the extent of its distribution is proceeding and includes an investigation of copper deficiency in cattle in the South-west in connection with the associated problem of "falling disease" in cattle which has been described elsewhere by Bennetts and Hall (1939), and by Bennetts *et al.* (1941).

II. ENZOOTIC ATAXIA OF LAMBS.

1. Definition and Synonyms.

Enzootic ataxia is a disease of the unborn or the unweaned lamb, due primarily to copper deficiency and characterised pathologically by destruction of myelin in the nervous system. A typical degeneration in the spinal cord always occurs and is responsible for the ataxia which frequently only affects the hind limbs. Nutritional disturbances generally precede and almost invariably accompany the ataxia. The enzootic disease in Western Australia rarely gives rise to lesions in the brain, but lambs so affected die *in utero*, or are affected within a few days of birth by a rapidly fatal type of the disease in which there are signs of cerebral involvement.

The disease has been known popularly as "Gingin rickets", Gingin being the district in which it was first recognised and where its incidence was high. The name enzootic ataxia was first applied by Bennetts (1932). A disease occurring in South Australia has been described by Bull *et alii* (1938) under the name "ataxia in young lambs". The clinical and pathological description coupled with the findings regarding the etiological significance of copper leave no doubt that this disease is identical with enzootic ataxia.

Diseases of lambs with similar clinical features have been described from other parts of the world under various names:- "renguera" in Peru by Gaiger (1917) and Tabusso (1935); "paralysis of lambs" in Sweden by Magnusson (1920); "swing back" in South Africa by Dunning (1933). The pathology of these diseases has been only incompletely studied and their etiology is undetermined. A much more detailed study has been made of a similar disease occurring in Great Britain where it is known popularly as "sway-back", "swing-back", "warfa" etc. Stewart (1932) investigated this condition which he designated ataxia. He demonstrated the occurrence of spinal cord degeneration; the distribution of affected nerve fibres described and illustrated was identical with that found in cases of enzootic ataxia by Bennetts (1932). Stewart's clinical and pathological descriptions left little doubt of the identity of the two diseases. Subsequently Innes (1934-35, 1936) made a much more extensive and detailed investigation of the neuropathology of "sway-back". Although he constantly finds a spinal cord degeneration as described by Stewart and ourselves, he regards this as secondary and considers a diffuse symmetrical demyelination of the cerebral white matter as the characteristic lesion. He finds that the essential clinical features of the disease are "spastic paralysis particularly of the hind limbs, inco-ordination of movement, blindness in some cases, a progressive course unassociated with fever, and a general fatal termination caused in some cases by an intercurrent infection". The main differences between Innes' findings and those of the Australian workers are attributable to the fact that 29 of the 32 cases he described had been affected at birth or within a few days of it. He was thus dealing almost exclusively with a form of the disease, with early and severe manifestations, which is encountered only rarely in Western Australia and which has not been described elsewhere in Australia. Subsequent to earlier publications, we have investigated subjects of this type, undoubtedly cases of enzootic ataxia, in which the pathological findings were similar to those described by Innes who, in fact, confirmed

the identity of the lesions in material forwarded to him (Dunlop *et al.*, 1959). Earlier confirmation of the identity of the two diseases was afforded in the demonstration by Dunlop and Wells (1938) "that the administration of copper to pregnant ewes has a remarkable beneficial effect on prevention of the disease in the progeny". More recent work by Shearer, Innes, and McDougall (1940) suggests that "sway-back", unlike enzootic ataxia, is not due to copper deficiency *per se*, although related, apparently, to a disturbance in copper metabolism.

It appears probable that all the diseases referred to will be shown ultimately to be identical, although it is apparent that the pathogenesis may vary somewhat with locality.

2. Distribution and Economic Importance.

The disease was described originally by Bennetts (1932) as being confined to a relatively small area of Cretaceous country at Gingin and Dandarragan, and to one property in the Mt. Barker district which is of entirely different geological origin. Gingin lies some 50 miles north of Perth, the area affected being approximately 30,000 acres. The Dandarragan district, 50 miles further north, comprises some 200,000 acres.

Ataxia has been responsible for serious economic loss in the Gingin and Dandarragan districts where sheep raising and later fat-lamb breeding comprised a large part of the farm production. Apart from the actual loss of a percentage of lambs, the measures adopted to prevent the disease, viz:- change of country for ewes for from 4 to 8 weeks during the gestation period or the annual purchase of fresh breeding stock, were costly and inefficient. Retarded growth of lambs and deterioration of wool quality also constituted a direct loss. The use of copper supplements is removing these disabilities.

It is now known that the disease is much more widespread. Coastal country, derived from wind-borne shell fragments, has been found by Bennetts (1940) to be affected. Ataxia has been recorded as far north as the vicinity of Broome, at various points along the west coast to the south, and on the southern coast it is known to occur as far west as Doubtful Island Bay. In these areas cobalt deficiency is a complicating factor.

The disease is not confined to the calcareous littoral but occurs further inland on the coastal plain on country of a different type. This area extends in a discontinuous belt from about 20 miles below Perth for a distance of some 150 miles in a southerly direction and inland for a distance up to 20 miles from the coast. Similar areas inland from the south coast are undoubtedly affected, although little direct evidence is available. There is abundant evidence, however, that the distribution of ataxia corresponds with a low copper status of plants and animals, ataxia having been recorded at different points throughout these coastal and semi-coastal copper-deficient areas where sheep breeding has been attempted. A survey of this area is projected to determine the copper status of animals, and thus to define more clearly areas which are copper deficient. This, apart from giving more complete information regarding the potential occurrence of ataxia in country which as yet is

unstocked or only lightly stocked with sheep, may also give further information regarding the actual distribution of the disease.

The total economic importance of ataxia is difficult to assess. The occurrence of the disease is merely one phase of a general problem of copper deficiency with various manifestations in the animal and plant life of the affected areas. It was, however, the solution of the etiology of ataxia which was responsible mainly for the initiation of several investigations which are showing that this general problem is of serious moment in Western Australia.

One very important aspect of the general problem is that the development and most economic use of large tracts of coastal and semi-coastal country was impeded on account of a deficiency of copper, associated in coastal country with a cobalt deficiency. It would appear that these vast tracts in an assured-rainfall area should be capable now of great development.

3. Type of Country and Method of Sheep Husbandry.

(a) The Cretaceous Area.

Gingin and Dandarragan are derived from the same Cretaceous series, and the type of country and method of farming are essentially the same in both districts. Some account of the physiography, geology, and climatic features and a detailed soil survey of the Gingin area have been given by Hosking and Greaves (1935-36). Both are old-established pastoral districts renowned for fattening of sheep and cattle for which purpose they have been largely used. They were at one time noted for the quality of the thoroughbred horses bred there but, mainly through the increasing incidence of "rickets" in foals, breeding to a large extent was discontinued.

The Western Australian lupin (*Lupinus varius*) grows to perfection in the deep coarse sand on the lighter country, the fertility of which is thereby considerably improved. Adequate recognition of the value of this plant of recent years has revolutionised the stock practices in these districts. The dry shed seeds will fatten up to five sheep to the acre during the summer months, they form an ideal ration for flushing ewes, and in the absence of green feed are a suitable ration for lambing ewes. The relatively high copper content of lupin seeds may have significance in regard to improvement in Hb. values of ewes during the summer months, but the ration obtained under natural grazing conditions during the summer is inadequate to prevent the incidence of ataxia in the progeny some months later.

A brief reference to cereal crops is pertinent. These are grown only for stock feed. Rye has been found the most satisfactory crop for feeding-off by sheep and cattle and is used extensively for this purpose, particularly in the early winter. Oats, grown for hay production, is a more uncertain crop. In view of the demonstrated value of copper supplements for stock, one of us (H.W.B.) suggested that copper as a soil fertilizer (20 lb. copper sulphate per acre) might be of value. The suggestion was adopted in 1938 on one property at Dandarragan with apparently very successful results throughout. C. S. Piper of the

Waite Institute, in 1938, recognised symptoms of typical reclamation disease in oat plants forwarded from an untreated crop. Teakle, Turton, and Throssell (1940) have since reported a very striking demonstration, under careful experimental conditions, of the value of copper as a soil fertilizer in the growth of oats in this area. Wheat is not grown.

(b) Coastal Country.

This comprises a strip of country, which extends inland for a maximum of 11 miles, derived largely from wind-borne shell fragments piled up into sand dunes with, frequently, extensive flats between the ranges of coastal hills. The soil is a fine sand rich in lime which in older formations may consolidate into limestone. This country is practically treeless and carries a good cover of natural perennial grasses (*Poa caespitosa* and *Danthonia* spp.), cyperaceous plants, and low herbage much of which is edible. It is very similar to the South Australian littoral described by Marston *et al.* (1938).

Owing to the incidence of coast disease and ataxia, very little attempt has been made to breed or maintain stock on coast country. It is used almost exclusively by settlers farther inland as change country for their cattle and, to a less extent, sheep. The general practice is to graze it from March to June.

(c) Semi-coastal Country and Mt. Barker.

The affected area is, as yet, somewhat undefined except in the lower South-west where the copper status of animals and pastures is receiving much attention. A somewhat detailed account of the country and its problems has been given in connection with "falling disease". Although it is largely a dairying zone, occasional flocks of sheep are carried. Ataxia is known to be widely distributed in this area bounded by Ludlow in the north and Forrest Grove in the south, and extending inland for about 10 or 20 miles.

Ataxia was observed during 1939 and 1940 in the progeny of a small experimental flock carried on a property at Jindong leased for the purpose of investigating "falling disease".

There is some evidence supported by results of pasture analyses that the "stalling" of subterranean clover, prevalent throughout, is associated with copper deficiency (Elliot, 1939).

Further inland the heavily timbered country to the west of Mt. Barker is not dissimilar in type to the South-west area referred to above. The earliest pasture development in this area was at the Pardelup Prison Farm where the disease first appeared in 1930. Subsequently other properties have been developed for sheep, and ataxia has been reported from some of these.

A more detailed account of the main features of these copper-deficient areas, referred to only briefly here, will be published elsewhere.

4. Incidence.

The disease is confined to the progeny of ewes which have been depastured continuously for at least six months on "affected" country. It does not spread to healthy districts despite frequent transfers of stock from affected districts. On account of the measures adopted to control the disease, viz., change to healthy country or purchase of fresh breeding ewes each season, it has been impossible, at least in the Gingin and Dandarragan districts, to obtain accurate data of the natural incidence of the disease during recent years. Past records from these districts, some experimental evidence, and observations made in areas where the disease occurred later and was not known to be preventable, viz., at Mt. Barker and in the South-west, clearly indicate that the incidence progressively increases with time and with the increased rate of stocking which follows clearing of the country, the elimination of natural scrub, and development of improved pastures. Also, the longer the same breeding flock is maintained on "affected" country, the heavier is the incidence and the more acute is the course of the disease. At Gingin and Dandarragan it became manifest only after the country had been stocked for some 15 or 20 years and it was not until about 30 years ago that the disease became at all prevalent. Subsequently, with increased stocking which followed improvement, the incidence increased. Instead of only a relatively small proportion being affected at the second successive lambing on "affected" country, and nearly all at the third, the maximal figure was reached at the second lambing. This was verified in experimental flocks at Gingin during 1932-37; there was, however, a low incidence in 1936. But for the discovery of measures of control, sheep breeding would have proved impossible. Even here, of recent years, the "margin of safety" decreased. It was found that if ewes were purchased from healthy country earlier than November for mating for the subsequent May-June lambing, or if lambing were delayed until July, a proportion of the progeny developed ataxia. The experience at Mt. Barker in the South-west is very similar. The property purchased in 1927 to establish the Pardelup Prison Farm was reported to have carried sheep satisfactorily, in its virgin state, for a period of some 80 years. The disease first made its appearance in 1930; and in 1931, 35 of 86 lambs from second-season mothers developed ataxia. In 1933 all the progeny of 213 third-season ewes were affected. A number were born dead and the majority developed the disease within a few days of birth.

The South-west country in its virgin state was used by the settlers as effective change country to prevent ataxia and "rickets" in foals. After settlement for dairying some 20 years ago, this country is now in its improved state very definitely copper-deficient: as recorded by Bennetts and Hall (1939) cattle are affected and the progeny of ewes maintained for the second successive lambing season develop ataxia.

(a) Seasonal Occurrence.

There is no definite seasonal occurrence. Lambs born at any period of the year may develop the disease; nevertheless there is some evidence at Gingin that lambs dropped in the late spring, i.e. October to mid-November, or in the dry summer months, are less liable to develop the disease than those born at the usual time, between May and July, although the mothers may have been brought on to the "affected" country at the same time. This may be related to the slower growth rate of summer lambs.

(b) Yearly Variation and Climatic Influence.

Despite the qualifications made in the introductory paragraph of this section, there is some evidence that the incidence of the disease shows a variation from year to year. Stockowners are definite that seasons which favour a luxuriant growth of pasture and especially a rank growth of capeweed also favour the incidence of the disease. Observations and experimental results at Gingin, detailed later, support this contention. Bull *et al.* (1938) have made a similar observation regarding the disease in South Australia.

(c) Species Incidence.

Enzootic ataxia is, so far as we are aware, confined to lambs.

A "posterior paralysis" of young pigs with similar clinical signs and, in two cases we have examined, identical neurological lesions and with a low copper status occurs in the same areas but is not confined to them. The etiology of this condition is undetermined, but a lick containing limonite and copper* is prophylactic and to a certain extent curative.

Young foals and calves bred in areas where ataxia is enzootic develop pathological conditions the clinical manifestations of which are entirely different from the disease affecting lambs. The essential clinical features are malnutrition and abnormalities in structure and posture of the limbs; there are never any indications of ataxia or any signs of involvement of the central nervous system. The pathology has been studied only incompletely and no controlled experimentation has been practicable. There is evidence, however, that copper supplements are of benefit. It seems very probable that these diseases are actually manifestations of copper deficiency.

(d) Sex and Breed Incidence.

Sex is of no significance. Ataxia has been observed in Merinos, Border Leicesters, English Leicesters, Corriedales, and in the progeny of these breeds or their Merino crosses sired by Southdown, Dorset Horn, Shropshire, and Merino rams.

It is considered by some stockowners that the progeny of the Merino ewe is somewhat more susceptible than that of the crossbred but there is little direct evidence.

(e) Age Incidence.

Affected lambs may be born dead or be affected at any age up to four months. The disease is rarely observed in this State before lambs have reached the age of from 3 to 6 weeks. Lambs do not develop the disease after they are weaned, which is generally not later than at four months. In younger lambs weaned on to cow's milk or, if old enough, on to the same pasture when showing early signs of the disease, the course is arrested. The reason for this would appear to be the resultant check in growth rate, which would lower the copper requirement, and the consumption of greater quantities of pasture which though subnormal in copper content is more adequate in this respect than milk.

* Denmark No. 1 lick the formula of which is given by Filmer and Underwood (1936).

(f) Condition.

Other things being equal, the rapidly growing and fattening lamb is most susceptible. This conclusion is based on field observations, but no actual experimental evidence is available. Observations on experimental ewes at Gingin appear to indicate that, in general, mothers in good strong condition at the beginning of the gestation period are less likely to produce ataxic progeny than those in poorer condition.

5. Clinical Description.

Most commonly, lambs are affected when from 1 to 2 months old. If carefully observed, the first signs are an appearance of unthriftiness and an apparent check in growth rate. If lambs in this early stage of the disease are driven for a distance of from a half to one mile, an inco-ordination of gait affecting the hind limbs became apparent. This is accentuated by further driving. The condition becomes progressively worse and in the course of a few days ataxia will be manifest after the animal has progressed only a short distance. The hind legs are at first drawn up together when the lamb runs, the limbs are excessively flexed and "knuckle over" at the pasterns, the gait shows inco-ordination of movement, the hind quarters wobble and ultimately sway to one side and the lamb falls over. If left quietly it gets up again and can proceed normally for a short distance but with each successive compulsory advance the progress becomes shorter and more difficult and the period of rest longer. Heart rate and respiration are greatly accelerated by the exertion, and respiration occasionally becomes very noisy. The disease progresses more or less rapidly until the lamb is unable to walk at all without exhibiting an ataxic gait; finally the fore legs also appear to be affected and the animal remains in a position of decubitus. Appetite is maintained throughout but the animal is unable to suck or graze. The condition becomes steadily worse and the lamb dies usually within three or four weeks from malnutrition, myiasis, intercurrent infections, etc. Many lambs, particularly the older ones, that survive until September or October then commence to thrive and thereafter recover, although some ataxic gait generally persists and the hind quarters remain somewhat atrophied. In lambs that do not develop the disease until they are 3 or 4 months old, the course is generally much less severe; the check in growth and condition may not be very apparent, the ataxic gait is less marked and may only become evident after driving. Such lambs almost invariably survive and are sometimes marketable as fat lambs.

The condition appears to be essentially one of ataxia without any true paralysis. There appears to be no definite loss in power. Affected lambs become uncomfortable if sat up on their hind quarters and will kick quite vigorously even when the disease is advanced. We have previously reported a diminution in sensory response in the hind limbs (Bennetts, 1932) but since have been unable to assure ourselves that this definitely exists.

We have more rarely observed a much more severe form of the disease which affects lambs from birth or within a few days of it. This form of the disease was observed at Mt. Barker in 1933 in the progeny of third-season ewes and

in the experimental flock at Gingin in 1937, a year that was especially favourable to the occurrence of the disease. It is considered that this form of the disease occurs when conditions bring about an exceptionally low copper status of the mother. Lambs may be carried to full term and be born dead without apparent reason, but a pathological examination confirms the existence of the disease. Some lambs may be undersized at birth, e.g. weigh only 2 lb. and be too weak to stand or suck. There may be signs of cerebral involvement. Lambs may exhibit a general inco-ordination: they are unable to locate the direction of their mother's bleat, are apparently blind, and make erratic movements, circling etc. They generally die within three or four days but the less severely affected may live longer and show the typical ataxic gait. There are intermediate stages between this severe form of the disease and the more typical one which has been described in some detail.

6. Pathology.

(a) Macroscopic Appearances.

The external appearance varies with the course of the disease and the stage at which the examination is made. Some degree of malnutrition and evidence of arrested development is almost invariably present. Occasionally this is very marked: a four months old lamb the live weight of which, under normal conditions, should be at least 60 to 70 lb. may weigh only from 17 to 20 lb. If animals have been affected for some weeks they are frequently anaemic. The carcass usually shows some degree of emaciation, the skeletal system is poorly developed, and shafts of long bones may show only a thin layer of compact bone. In one lamb aged three months the weight of the femur was only 17.0 g. whereas the femur of a "normal" lamb of the same age and breed was 30.5 g. The bones are brittle, and fractures of ribs, presumably resulting from effects of falling over, are not uncommonly present. In long-standing cases little red marrow may be present in the long bones. In the very acute cases or the milder chronic ones anaemia is absent, and there may be relatively little evidence of malnutrition.

Macroscopic lesions in the central nervous system are generally absent. The spinal cord invariably appears normal. Gross brain lesions as described by Innes (1934-35) have been observed infrequently, viz. in 4 of the 35 cases in which the brain was examined, and occur only in the very acute type of the disease. Fluctuant softening and cavitation of the cerebral hemispheres, particularly in the occipital poles, were observed in four cases from the 1937 experimental control group, all of which were affected at birth. Brains from three of these, one of which showed external evidence of cavity formation, were forwarded intact to Innes who reported that the macroscopic lesions were identical with those found by him in Great Britain in cases of "sway-back" (Innes and Shearer, 1940). The brain from the fourth lamb, which was born dead, showed a more advanced lesion than any described by Innes; cavity formation and internal hydrocephalus had advanced so far that the cerebral hemispheres consisted merely of a thin transparent membrane enclosing approximately 60 ml. of a clear amber-coloured fluid. No macroscopic lesions were recorded in four acute cases examined at Mt. Barker in 1933. All of these lambs had been affected since birth and were killed when less than nine days old. Thus

macroscopic brain lesions were found in only 4 of 10 acute cases and in none of the remaining 25 typical subacute cases examined; 19 of the total 35 were examined after the publication of Innes' findings, in the hope of confirming them. It would appear, therefore, that macroscopic brain changes occur only in the acute cases of ataxia which are rare in Western Australia and that they are not constant even in these.

There are no macroscopic lesions that may be regarded as characteristic of the disease.

(b) Microscopic Appearances.

There are no constant microscopic lesions in any tissue other than in the nervous system. In the liver various changes, cloudy swelling, fatty changes, and occasionally necrosis of small groups of liver cells, have been noted. A degree of haemosiderosis is commonly observed.

(i) Nervous System. We have examined spinal cords from 57 cases, nerves including the sciatics and their branches and the femorals from 22 cases, lumbar sympathetic ganglia from 7 cases, portions of the brain especially medulla and cerebellum from 21 cases, and the brain more comprehensively from 11 subjects. The material was fixed in Zenker's and Muller's fluids, or latterly in neutral formol-saline solution. Sections were stained with Mallory's eosin-methylene blue, Giemsa, or with haematoxylin and Van Gieson (Freeborn's formula) for histological examination. Marchi preparations were used for the study of degeneration. More comprehensive neurological methods were impracticable.

Spinal cord. Characteristic lesions were constantly present in 56 of the 57 cases examined. These lesions, as described by Bennetts (1932 and 1933), consist of degeneration of the myelin sheaths of nerve fibres in the cord, the microscopic appearance being essentially the same in all cases examined. The distribution of the degenerated fibres is similar throughout the mid-dorsal to mid-lumbar portions but is much less marked in the more caudal portions. In one case in which the cervical portion of the cord was examined, the distribution of degeneration was similar to that in the mid-dorsal region. In acute cases there is a tendency to a more diffuse distribution, the usual zoning being less clearly marked.

The typical distribution illustrated by Bennetts (1933) corresponds closely with that figured by Bull et al. (1938). We are in entire agreement with their statement:- "In all cases, the degeneration has been most intense in areas corresponding to the direct cerebellar and Lissauer's tracts. The lesions do not, however, appear to be definitely systematized, and, in the absence of more exact information on the development of well-defined tracts in the cord of sheep, we do not believe that a dogmatic opinion on the distribution of the degenerated fibres can be expressed. It appears probable that both motor and sensory fibres are involved". In support of this opinion we may advance the evidence that both dorsal and ventral nerve roots included in the sections of dorsal and lumbar portions of the cord not infrequently show some degenerated nerve fibres.

There was no evidence of degeneration of nerve cells in the grey matter in any of the cases examined.

In eight animals affected for from 5 to 13 months a

definite sclerosis of the cord was found; scar tissue was replacing the degenerated fibres, although these were still more or less evident in the usual situations except in one case, this being the only one in the total of 57 examined where demyelination of nerve fibres was not demonstrated.* This animal in company with others of this group had been depastured for several months on healthy country. Apart from the lesions in these very long-standing cases no inflammatory changes were recognised.

Lumbar ganglia. Occasional vacuolated or shrunken nerve cells were observed in one or more ganglia from three of the seven cases examined. Marchi preparations were made from ganglia from one side of the cord in three cases. In two there was evidence of degenerated fibres in one or more ganglia; no other abnormalities were detected.

Peripheral nerves. A proportion of degenerated fibres was found in the sciatic nerves or their branches or in the femoral nerves in 13 of 22 cases in which Marchi preparations were made. The degree of degeneration and proportion of fibres affected varied. Usually fibres exhibited osmic staining only occasionally, and frequently the sheath merely showed dotted areas of degeneration. In some cases, however, from 20 to 30 per cent. were affected and the whole fibre appeared intensely stained. No cellular changes were demonstrable in Giemsa-stained sections.

Brain. No constant or extensive changes were observed in the brain. In this respect our findings are more in agreement with those of Bull, who failed to find any evidence of degeneration or inflammatory reaction, than with Innes. As already pointed out, however, this may be due to the difference in the types of the disease examined. We have had no opportunity of attempting to verify Innes' findings in acute cases. Our methods, moreover, have been much more limited. His report on the histological and macroscopic appearances of the Western Australian material forwarded to him for examination leaves no doubt, however, of the identity of the lesions occurring in acute cases of "sway-back" and ataxia.

Nevertheless, our findings indicate that cerebral lesions do not occur in the typical subacute cases of the disease generally observed here, although spinal cord lesions have been constantly demonstrated by us in Australia and by Innes in England.

In nine typical subacute cases, the brain after fixation in neutral formol-saline was sliced by vertical transverse sections from the olfactory lobes to the medulla. A large series of sections, particularly from the occipital poles of the cerebral hemispheres, claimed by Innes to be the primary situation of the pathological process, were then prepared for microscopic examination. No evidence of inflammatory reaction or cell degeneration was detected. In no case was there evidence of any degeneration in the white matter of the cerebral hemispheres except that in three cases occasional nerve fibres in the centrum ovale of the occipital lobes showed slight osmic staining.

* This was one of two cases previously reported by Bennetts (1932) as having given negative results in Marchi preparations. Typical degeneration was subsequently demonstrated in further preparations from the other case.

Complete brains from two further cases were examined by the Weigert-Pal method. No evidence of demyelination was detected in the cerebrum.

In the medulla, however, degeneration is generally quite marked. It was very definite in 15 of the 21 cases examined, the distribution corresponding with that reported by Innes. Some degeneration was commonly observed in the *corpus medullare* of the cerebellum.

(ii) Haemosiderin. Bull *et al.* (1938) reported that haemosiderosis of the liver and pancreas was a constant feature in cases of longer standing examined in Victoria and South Australia. We have examined only a limited number. Haemosiderin was present in the liver in eight of nine affected lambs over the age of five weeks. No haemosiderin was demonstrable in liver, pancreas, spleen, or kidney of one four months-old lamb affected for at least three weeks. Haemosiderin was present in the spleen of two cases and in the kidney of one case.

(c) Haematology.

Anaemia recorded in an earlier communication by Bennetts (1932), although commonly present in subacute cases, is not now considered a constant or essential feature of the disease. As, however, the blood picture in lambs is so distinct from that exhibited by the copper-deficient anaemic mothers, a summary of available data is warranted. Methods, indices etc., except where specified, are identical with those used and detailed for haematological studies in adult sheep. Prior to 1937, haemoglobin (Hb.) estimations and red cell (R.B.C.) counts were carried out on 19 ataxic lambs from Gingin and Mt. Barker. Their ages varied from one day to seven months, the majority being from one to three months old. In these cases Hb. was estimated with a Sahli haemoglobinometer standardised against iron determination.

Excluding four lambs from one to eight days old, the Hb. range was 5.6 - 12.6, mean 9.0. The corresponding R.B.C. values were 10.0 - 17.0, mean 14.0; and for colour-count ratio (C.C.R.) were 0.54 - 0.83, mean 0.64.

The four acute cases aged from one to eight days examined at Mt. Barker in 1933 showed no evidence of anaemia, although rather low blood figures were observed in the eight-days-old lamb; Hb. values were 9.1 - 14.5, mean 12.9; and R.B.C. 9.0 - 13.2, mean 11.9; C.C.R. was 1.0 - 1.2

Recently a comparison was made between blood figures of 12 healthy lambs aged from 8 to 18 weeks obtained in fours from three "sound" localities, and of three ataxic lambs of from 6 to 12 weeks old. Haemoglobin was estimated by the Newcomer method. The results are shown in Table 1.

Healthy lambs from an affected property at Gingin showed no evidence of anaemia, and the C.C.R. was within the normal range.

Earlier, Filmer had carried out determinations of mean cell diameter on 13 smears from 9 ataxic lambs by the method of Pijper (1929). The values obtained, average variation 3.7 - 5.8, mean diameter 4.4, are somewhat lower than those found by Filmer (1933) for 7 normal lambs. viz. 4.0 to 6.3, mean 4.9.

TABLE 1.

Blood Values of "Normal" and Affected Lambs.

Subjects	Hb.	R.B.C.	Cell Vol. %	Mean Cell Volume (in c. μ)	Colour Count Ratio	Colour Volume Ratio
12 Healthy Lambs from "sound" localities	13.2-17.6 (15.7)	14.0-16.9 (15.0)	41.3-47.5 (44.5)	27.0-32.1 (29.6)	0.88-1.2 (1.05)	0.29-0.43 (0.35)
3 Ataxic Lambs	5.9-13.2 (8.5)	9.4-17.1 (12.1)	18.8-37.5 (25.4)	20.0-21.9 (20.8)	0.60-0.77 (0.68)	0.30-0.35 (0.33)

Note: Upper figures = range.

Lower figures in brackets = mean.

Blood smears were made from the majority of lambs examined; some anisocytosis was commonly observed and there was generally some evidence of hypochromasia. Stippled cells were rarely observed and were never numerous.

It is not possible to draw definite conclusions from the limited data available, particularly in view of the known influence of the age of the lamb on haematological findings. It is clearly evident, however, that a definite anaemia is frequently present in subacute ataxia and that this is characterised by a diminution of haemoglobin relative to erythrocytes, which tend to be decreased in size. Indications are then that the anaemia is of the microcytic hypochromic type, the type encountered in experimental copper deficiency of animals.

7. Etiology.

A considerable amount of preliminary investigational work which has been recorded by Bennetts (1932, 1933, 1935) failed to give any clue as to the cause of the disease but narrowed the field of enquiry. Chemical data obtained in 1936 indicated that a copper deficiency might possibly be the cause of the disease. A preliminary account of these findings was recorded, Bennetts and Chapman (1937). An account of the subsequent investigations is now presented.

(a) Field Experiments with Sheep.

A series of field experiments was carried out at "Whakea" Gingin. Previous observations and experiments had indicated that a disease incidence of about 100 per cent. could be expected in the progeny of ewes from their second consecutive lambing on this property and dependence was, therefore, placed on this supply of experimental animals.

(1) First Series of Experiments (1937). This series of experiments was carried out to determine (a) if a copper supplement administered to the ewes during pregnancy would lower or prevent the incidence of ataxia in the lambs; (b) if either commercial or pure ammonium chloride had any real and regular prophylactic value*; (c) if the disease were due to a dietetic deficiency or to the excretion of a toxic principle in the ewe's milk. For these purposes experimental ewes were selected from the "Whakea" farm flock all the members of which had come from the same healthy area twelve months previously. They had all borne lambs on "Whakea" in the previous winter (May and June) and under normal farm practice would have been sold and not allowed a second lambing season on the affected country. The ewes were Merino and were mated in December with Southdown rams. In addition "sound" ewes from a healthy district, Beverley, were transferred to "Whakea" in May when within from one to three weeks of lambing.

On February 12th, the "Whakea" ewes were divided at random into five groups. Experimental groups were depastured under rotational grazing so that all groups, as far as possible, received identical general treatment.

Experiment No. 1. was designed to test the prophylactic value of copper on the one hand and of ammonium chloride on the other. Opportunity was taken to test the curative value of copper in the early stages of ataxia.

Group 1 consisted of 12 ewes each of which received a daily drench of 60 ml. of a 0.1 per cent. solution of cupric sulphate (B.D.H., A.R.) representing a daily dose of 15 mg. of Cu.

Group 2 consisted of 25 ewes which received no treatment and served as controls.

Group 3 consisted of 12 ewes which received a daily drench of 60 ml. of a solution of commercial ammonium chloride (I.C.I.) to give a weekly intake of 60 g. of the salt.

Group 4 consisted of 12 ewes which received, in the same manner as those of Group 3, 60 g. of ammonium chloride (B.D.H., A.R) per week.

The treatments commenced on February 16th and were discontinued on May 3rd, 1937. Lambing commenced in May and was complete by mid-July, the majority of the lambs being born in the first two weeks of June. In all four groups lambing was 100 per cent. The results of the experiment are summarised in Table 2.

The outstanding features of the experiment were the complete absence of ataxia in the group treated with copper and the unusually acute course and very high incidence of the disease in the groups that received no copper. The disease, under the seasonal conditions prevailing, took the very acute form which had not commonly been observed previously. Several lambs were born dead and two of these in the control group showed gross brain lesions to which reference has already been made. In Group 4 with few exceptions the course of the disease was very acute. In Group 3 the incidence of ataxia was somewhat lower and the course was very much less severe. Only one

* Ammonium chloride administered to ewes as a "deleading" agent in earlier experiments had given suggestive although inconsistent results.

of the lambs showed the acute form and the others did not exhibit signs of the disease until they were about three or four weeks old. Subsequent development of the disease was slow. In this group three ewes died before lambing, thus reducing the numbers. It seems evident that the administration of commercial ammonium chloride had a beneficial influence on the course of the disease. Similar samples of commercial ammonium chloride had been shown previously (Bennetts and Chapman, 1937) to contain traces of copper as an impurity. The results obtained in Group 1, treated with copper, were in sharp contrast, as all the lambs developed normally and on slaughter were classed as high grade fat lambs.

TABLE 2.

Showing the Grouping of the Animals in Experiment 1 and the Incidence of Ataxia in the Lambs.

Group (Treatment)	No. Animals in Groups		Incidence of Ataxia		History, Growth, and Development of Lambs
	Ewes	Lambs	No.	%	
1 (Copper)	12	12	0	0	12 developed normally.
2 (Control)	25	24	23	96	1 late lamb showed no ataxia. 2 with ataxia successfully treated with Cu. 8 with ataxia "re-moth-ered" on to normal ewes. 14 with ataxia died.
3 (Am. Chlor. Commercial)	9	9	7	86	2 developed normally 7 with ataxia died.
4 (Am. Chlor. Pure)	12	12	12	100	3 with ataxia successfully treated with Cu. 9 with ataxia died.

The therapeutic value in the early treatment of ataxia was determined. The dose given was 7.5 mg. of Cu daily administered by giving 30 ml. of a 0.1 per cent. solution of cupric sulphate (B.D.H., A.R.). Five lambs, one showing a very advanced stage of the disease, were successfully treated. Three of these were affected in June when the course of the disease was rapid in untreated lambs. A sixth lamb died of pneumonia in the early stages of treatment. The observations are summarised in Table 3.

TABLE 3.Showing the Results of Treatment of Ataxia with Copper.

Lamb No.	From Group No.	Age and Clinical Condition at Commencement	Period of Treatment	Results of Treatment
W35	2	7 days Affected since birth. Unthrifty; ataxic gait.	3 months	Good condition. Slight residual ataxia.
59	4	3 weeks Affected about 1 week. Unthrifty; ataxic gait.	3 months	" "
W50	2	4 weeks Affected about 2 weeks. Unthrifty; ataxic after driving.	2½ months	" "
44	4	3 months Affected about 4 weeks. Very unthrifty; advanced ataxia.	24 days	Good progress
61	4	3 months Affected about 4 weeks. Unthrifty; advanced ataxia.	24 days	Good growth and condition. Slight ataxia.
37	4	1 month Affected 2 weeks. Unthrifty; advanced ataxia.	1 day	Died of pneumonia.

Two lambs Nos. W35 and 59 made such good progress that they were graded for export on October 31st. 1937; the remainder were kept under observation until six months old.

Experiment No. 2 was carried out concurrently with No. 1 and was designed to determine if the disease is due to the ingestion of a mineral poison which is excreted in the mother's milk and which may be eliminated from the animal body during a sojourn on healthy country. The fifth lot of experimental "Whakea" ewes was used in this experiment.

Group 1 consisted of 14 "Whakea" ewes which were removed to "Avondale", Beverley, on March 3rd. Seven of these ewes received a daily dose of 20 g. of finely divided Gingin soil ("Whakea sand") administered as a drench for a period of three months from March 3rd to May 10th. The other seven ewes were not drenched and served as controls. All were returned to Gingin on May 13th. Two of the ewes died during the experiment and eleven lambs were obtained from the remaining twelve. Ataxia did not develop in any of these lambs which grew normally.

Group 2 consisted of "Whakea" ewes and their lambs as well as normal ewes from Beverley which were brought to

the experimental area within from one to three weeks of lambing. The lambs from the "Whakea" ewes were changed over to the Beverley ewes and those from the Beverley to the "Whakea" ewes. The object was to determine if the "Whakea" ewes excreted a toxic substance in their milk capable of producing ataxia in lambs and if the milk of normal ewes would prevent or cure ataxia in lambs from "Whakea" ewes. Ultimately lambs were changed over from eight pairs of ewes when three to five days old. The "Whakea" ewes were selected from Group 2 of Experiment 1 and they were matched with Beverley ewes according to the coincidence of lambing. The change-over or re-mothering was effected by confining the ewes and foster-lamb in a small yard, so narrow that ewes were unable to turn around, until the lamb was adopted. Subsequently the Beverley and "Whakea" ewes with their foster-lambs were run in separate paddocks to prevent lambs returning to their true mothers.

All the lambs from the "Whakea" ewes showed early signs of ataxia at the time of re-mothering. Their clinical appearance as well as the incidence and course of ataxia in Group 2 of Experiment 1, left little doubt that none of the lambs could have lived if left on their mothers. After adoption by their foster-mothers they remained unthrifty and development was sub-normal for three or four weeks but thereafter improved. All of them survived and seven of the eight fattened and were ultimately slaughtered for consumption.

Of the eight lambs from the Beverley ewes, two died within three days of changing as a result of difficulty in fostering and five were disposed of as fat lambs.

There seems to be little doubt that in this experiment no evidence was obtained of the presence of a toxic substance in the milk of the "Whakea" ewes. The evidence, however, is consistent with an assumption that an essential constituent for normal development is deficient in the milk of these ewes, that this constituent can be supplied to the lambs of these ewes by the milk of normal ewes, and that a short change to normal or "healthy" country enables these "Whakea" ewes to supply this normal constituent to their lambs.

(ii) The Second Series of Experiments (1938). The object of this experiment was to confirm and extend the observations on the prophylactic value of copper supplements. For the purpose copper was given as a drench, in lick form, or through topdressed pasture. The experimental animals available were predominantly Merino with a proportion of Comeback and Corriedale ewes. They were matched on type and weight. With the institution of group treatment, experimental groups were depastured under rational grazing so that all were, so far as possible, under identical general conditions. Groups on copper-topdressed pasture were, however, retained on the same paddock. The accommodation available for experimental groups consisted of four small paddocks, the same as used in the 1937 experiments. These were all of similar type, the soil throughout being predominantly the "Whakea Sand" of Hosking and Greaves (1935-36).

Experiment No. 3 was made with four groups each of 25 matched ewes.

Group 1 received no treatment but had free access to a lick of common salt and acted as a control.

Group 2 received the equivalent of 15 mg. of Cu daily given as a drench of copper sulphate solution thrice weekly. Dosing commenced on March 16th and was concluded on August 15th with an intermission between June 8th and July 7th. Free access to a lick of common salt was given.

Group 3 was depastured from May 6th to September 12th on a pasture which had been topdressed during April with copper sulphate at the rate of 20 lb. to the acre. The topdressing was delayed on account of unfavourable seasonal conditions which influenced the whole of this experiment throughout. Free access to a lick of common salt was given.

Group 4 had free access to a lick containing 0.5 per cent. of copper sulphate in common salt between March 11th and August 25th. The average weekly consumption per head was 90 g. and thus the daily consumption was equivalent to 16 mg. copper.

The experiment failed to yield any information on the prophylactic value of copper as only one case of ataxia developed in the control group, Group 1, and a very mild case in the copper lick group, Group 4.

(111) The Third Series of Experiments (1939). The object of this experiment was the same as for Experiment 3. The experimental animals were selected from a flock of 73 crossbred ewes which had been depastured at "Whakea" continuously since their purchase from healthy country in November, 1937. These were the only experimental animals available and unfortunately had had access to a salt lick containing copper for four months, from May until August, in 1938. The ewes were mated with a Southdown ram on December 17th, 1938. As some ewes had not mated, rams were reintroduced to the groups later and consequently lambing was somewhat prolonged.

Experiment No. 4 was made with three groups, as it was impracticable to include a trial of copper administration by drench. On March 7th, 60 ewes were selected and matched in threes on weight and haemoglobin values obtained on two occasions with an interval of four weeks. They were randomised into three groups each of 20. On March 9th, groups were separated, put into their paddocks, and supplied with lick.

Group 1 received no treatment but had access to a lick of common salt and acted as a control.

Group 2 had free access to a lick of common salt containing 0.5 per cent. of copper sulphate between March 11th and August 19th. The average weekly consumption was 67 g. per head, and their daily consumption was equivalent to 12 mg. copper.

Group 3 was depastured on the same paddock as Group 3 in Experiment No. 3; the paddock had been topdressed with copper in 1938. The group remained there throughout, except that from May 19th to May 30th it was run with Group 1 on less restricted grazing in order to reduce the risk of pregnancy toxæmia. Two ewes died early in May from this disease and death had been ascribed to a check resulting from the comparatively low nutritive value of the early winter pasture, although ewes were all in excellent

condition. The two ewes were replaced by two previously rejected but nevertheless a good match.

Again striking results were not obtained as the incidence of ataxia was once more very low in the control group in which only 3 out of 29 lambs became affected. Of these three cases one was a typical slowly developing case, whereas the other two were mild and did not develop ataxic gait until October when at the marketable age. Ataxia did not develop in any one of the 26 lambs in Group 2 or in any one of the 32 lambs in Group 3.

The progeny of the two groups receiving the copper supplement as well as showing no ataxia made better growth and development than those of the control group. Although this offers no direct evidence on etiology, growth and development are an expression of health, and the results of the experiment are consistent with those of Experiment 1 in which striking evidence was found of a copper deficiency in the animals and of its part in the production of ataxia. This improved growth in the lambs in the two groups on a higher copper intake are summarized in Table 4.

TABLE 4.

Showing the Relative Speed of Development of Lambs in Three Groups in Experiment No. 4.

Group	No. of Lambs Born June and July	Lambs Marketable (Export) on 28/9/39			Lambs Marketable (Export) on 30/10/39		Percentage Marketable (Export)
		No.	%	Mean Weight lb.	No.	Mean Weight	
1	18	4	22	76.5	4	77.8	44
2	19	11	58	73.7	5	78.2	84
3	11	7	64	77	4	83.4	100

The lambing had been prolonged in these groups, most markedly in Group 3, but this consideration which is confined to the earlier lambs is illustrative of the general trend of growth in each group.

It will be seen later that the blood Cu status of Groups 2 and 3 was much better than that of Group 1.

Discussion. In 1937 the disease in the control group had a high incidence and appeared in a very acute form which previously had not been observed to be common at Gingin. Clear-cut results were not obtained during the following two seasons owing to an extremely low incidence of ataxia in the control groups. This low incidence is contrary to all past experience on "Wakaia". The results of analyses (vide Table 6) indicated that the copper content of the pasture was low throughout, although somewhat higher in 1938. Past experience and the results of Experiment 1 had, however, been obtained with Merinos, whereas Crossbreds or Comebacks were used in the later experiments. There is some evidence that British breeds of sheep and their crosses with the Merino possibly have a higher avidity for copper and a higher capacity for its storage

than the Merino according to the findings of Albiston *et al.* (1940). Further, the sheep used in 1939 in Experiment 4 had access to a salt-copper lick until within four months of mating, and this probably increased their copper storage which may have taken some time to deplete. However, in 1938 the experimental sheep were predominantly Merino and had had no access to a copper lick. It seems, therefore, that some other explanation of the sharp contrast between the results must be sought.

Accidental copper contamination of the pastures is a possible explanation. The farm flock had had access to licks containing copper sulphate since 1937, and although topdressing of their pastures with copper sulphate was carried out in 1938 and more extensively later, considerable care was taken to avoid any contamination of the paddocks upon which experimental groups were depastured. Possibly the experimental paddocks may have been contaminated to some degree with copper excreted by experimental sheep which had received copper in drenches and in licks in these paddocks during the previous seasons. Although pasture analyses had shown low figures for copper, it is conceivable that copper might have become concentrated on small areas, such as sites of old camps etc., and that the control sheep or lambs may have thus acquired a certain amount of copper supplementation.

It is considered possible, however, that seasonal factors were predominantly responsible for the variation in the yearly incidence. In 1937 the early winter rainfall was above normal and dull days were frequent, whereas in the two succeeding seasons the rainfall for the corresponding period, as for the whole year, was unusually low, and warm sunny days predominated. These seasonal differences were associated with differences in plant growth and in species distribution in the pastures.

(iv) The Correlation of Anaemia and Low Blood Copper in Ewes with Ataxia in their Progeny. During the conduct of the field experiments blood examinations were carried out on the ewes. The subject of the blood changes in ewes is dealt with more fully in Section III and only the more salient features will be dealt with here.

Haematology. Reference has been made by Bennetts and Chapman (1937) to the preliminary results obtained in the several groups in Experiment 1 and 2 when individual determinations on all ewes were carried out at monthly intervals. In June and July, higher haemoglobin and red blood cell values were obtained in the group receiving copper and in the ewes that had been on healthy country than in the other groups. For example, in June the mean values were as follows:-

	Copper Group	Control Group	Am. Cl. (Contt.) Group	Am. Cl. (A.R.) Group	Healthy Country Group
Haemoglobin	9.7	6.5	7.0	7.0	10.8
Red Blood Cells	9.7	5.9	6.8	6.4	9.5

In all groups these were the lowest values obtained during the observations, presumably because the majority of the ewes had lambed about this time. During August and September there was a relative improvement in the values from the ewes in the control group and those receiving ammonium chloride, presumably because almost all of them had lost their lambs and the strain of lactation had been removed. By October the values in all the groups were within "normal" range, i.e. Hb. 9.7 to 11.2; R.B.C. 9.7 to 11.5.

A striking correlation was found between anaemia in the late stages of pregnancy or the early lactation period in the ewe and the development of ataxia in the lamb. In the control group, for example, 18 of 20 ewes whose lambs were ataxic showed definite anaemia either before parturition or from three to four weeks later. Thus, seven ewes showed Hb. or R.B.C. values below 5.0, five ewes below 6.0, and six ewes below 7.0. The lowest values obtained for any ewe were Hb. 3.5; R.B.C. 2.7. The other two ewes showed no anaemia at any time, i.e. values did not fall below 8.0.

In the two ammonium chloride groups the position was comparable. Of a total of 19 ewes with affected progeny, 15 were anaemic; seven showed values below 5.0, three below 6.0, four below 7.0, and one below 8.0. Four ewes with affected lambs and two with healthy lambs showed no evidence of anaemia.

In the copper group, two of the twelve ewes with healthy lambs became anaemic, one of them three weeks and the other two months after parturition, values being then below 6.0. It seems probable that anaemia would not have developed in this group if the copper supplement had been continued at least throughout the gestation period.

Among the animals that had been on healthy country, two ewes that had lambed in June showed a drop in Hb. two months later when values below 5.0 and 6.0, respectively, were found. One of the Beverley ewes also showed values below 6.0 in June.

Similar observations were carried out in the second series of experiments in 1938. A random sampling of 25 ewes before the groups were formed gave a mean Hb. value of 14.2, range 10.9 to 16.8. These are unusually high values, with the exception of the three approximating the minimum. By March 9th when regular monthly haematological examinations on ten matched sheep from each group were commenced, haemoglobin had apparently dropped somewhat. The mean figures were then:- Group 1, 12.4; Group 2, 12.0; Group 3, 13.4; Group 4, 13.0. Haemoglobin and red cell counts remained relatively normal throughout the period of observation, viz. until August 24th. The lowest mean figures were obtained in July:- Hb. 10.4, R.B.C. 8.5, results of the same order being obtained in all groups in June and July.

Only one of the ten controls tested showed any evidence of anaemia, the Hb. values being 6.6 in July, 7.4 in August, and 6.8 in September, but there was no evidence of anaemia before parturition which had occurred before the June determination which was 9.3.

These results, in a season when ataxia was practically absent (1 case in 25 controls - the mother of this lamb not being amongst the 10 sampled for Hb.), are in sharp contrast

with those of the previous season and give further confirmation of the observation that anaemia of the mother is related to ataxia in the progeny.

In the third series of experiments carried out in 1939, haemoglobin estimations and group counts were made on ten matched ewes from each group at four-weekly intervals from February to November. As in the previous season, haemoglobin values were high in the early part of the year. In February, mean values for the 10 matched ewes in each group were as follows:- Group 1 = 14.7, Group 2 = 14.5, Group 3 = 14.9; the range over the whole series was 11.3 to 17.6. The lowest figures for all groups were obtained in September when the means were 9.9, 10.8, and 11.2 respectively. Throughout the period of observation there were no significant differences in the blood figures for the three groups.

In contrast to the 1937 experience, there was little evidence of any correlation between anaemia in the mother and ataxia in the progeny. We may recall, however, that the course of the disease was unusually acute in 1937 and very mild in the three cases to develop in 1939. Of their mothers, one showed no evidence of anaemia at any stage and another showed only a slight fall in haemoglobin to 8.9. Yet another, however, showed a definite anaemia after lambing in July when the Hb. value was 6.3; it remained low until September when it was 5.4 but reached a normal figure of 9.7 in October. All, however, showed low blood copper figures (0.02 mg. per cent.) which fell still further after lambing.

Blood Copper. Blood copper determinations were carried out on only four of the experimental ewes during 1937. These were all mothers of ataxic lambs born from 3 to 7 weeks previously. The values were exceedingly low, one being 0.01 mg. per cent. and the remainder below this.

During 1938 monthly blood copper determinations were carried out on 6 matched sheep from each group, with the control sheep between July and October, with Group 2 between July and September, and with the other two groups in July and August. The results are briefly summarised here:-

With the controls the results were extremely variable. One ewe had normal values of 0.13 to 0.07 mg. Cu per cent. throughout the period of testing, whereas another had 0.01 mg. per cent. throughout the same period. The majority of the values, however, lay between 0.02 and 0.05 mg. per cent. A point that should be noted is that with one exception none of the sheep showed any evidence of anaemia, although this might have been expected from the low blood copper figures.

The blood copper figures for Group 2 (copper-drench group) showed very little individual variation at any one test. In July the values lay between 0.10 and 0.13, in August between 0.08 and 0.11, and in September between 0.06 and 0.08 mg. per cent.

Group 3 showed very variable values of 0.04 to 0.14 mg. per cent. but there was little individual variation on the two occasions of testing. Samples of pasture on which these sheep were grazing were found to contain 14.8 p.p.m. Cu (dry basis) in July, and 11.5 p.p.m. in September.

Group 4 showed very variable results. In July the range was 0.03 to 0.15, mean 0.07; and in August 0.02 to 0.08, mean 0.045 mg. per cent. It is assumed that the

low values were from sheep not receiving an adequate intake of copper-salt lick.

During 1939, monthly blood copper determinations were made on 10 controls and on 5 matched ewes from each of the other groups during the period from March to November inclusive. Estimations were also made at intervals on 6 additional controls. Significant differences were revealed. All the 10 controls showed low values down to 0.02 mg. per cent. at some stage, and 5 were below this by July. A consideration of the blood copper values for the 5 matched animals in the three groups showed that a significantly higher level was maintained in the groups receiving supplement than in the control group, although three animals in Group 2 showed figures below 0.05 from July onwards. In Group 3, almost consistently normal values of 0.08 or higher were maintained throughout; one animal, however, showed a rather variable blood copper, and values as low as 0.03 were recorded in June and July.

A consideration of the detailed values reveals no definite correlation between haemoglobin and blood copper. Generally speaking, however, low haemoglobin values were not noted until the blood copper fell to levels between 0.01 and 0.02 mg. per cent. In many cases the copper fell to these levels without a fall in the haemoglobin value (e.g. control No. 260 in July, Hb. = 12.7, blood Cu = 0.01); when the haemoglobin fell it was usually some time after the copper level had fallen.

The data reviewed above would indicate that ewes are able to rear healthy lambs despite blood copper values as low as 0.02 mg. per cent., but that when values of 0.01 or lower obtain ataxia may be expected in the progeny. It is regrettable that the extensive blood copper data were obtained only during the two seasons when the incidence of ataxia was very low.

Supporting evidence was obtained in a small flock of experimental ewes which was depastured at Jindong, an affected property in the South-west used for another investigation. The flock was bled at monthly intervals for haemoglobin and blood copper estimations over a two-year period. Results were similar to those obtained at Gingin; ataxia developed in the progeny of ewes which showed blood copper values of 0.01 mg. per cent. or lower, and Hb. values below 6.0 or 7.0 either before or within 3 or 4 weeks of lambing; low values subsequent to this were not correlated with the incidence of ataxia.

(b) The Copper Status of Ewes and Lambs.

The investigation of the copper content of blood, liver, and milk, begun by Chapman, was continued. His methods and the results obtained by him, which will be referred to only briefly here, were published in the previous communication.

The methods followed by us since 1937 are as follows:-

(i) Methods.

Blood Copper. The method of Tompsett (1934) with trichloroacetic acid was used.

Liver Copper and Milk Copper. The samples were analysed by a dry-ashing method which is essentially a combination of

the methods of Sylvester and Lampitt (1935) and Van Niekerk (1937). The method used is as follows:- The sample (usually 5 g.) was ashed with 1.5 to 2 g. magnesium nitrate (added as 20 per cent. solution). After ashing at incipient red heat the ash was dissolved in hydrochloric acid, the silica dehydrated on a steam bath, and the residue treated with HCl. In the first analyses, the carbon-silica residue was volatilized with HF (cf. Piper, 1939) and the residue oxidised with perchloric acid, but it was later found that all the copper in the carbon-silica residue could be liberated by treatment with perchloric acid and a little nitric acid. This procedure has been adopted in all the 1939 samples. Ten ml. of a citrated buffer (10 per cent. citric acid neutralised with NH_3) are added to the combined solution, which is brought to pH 3 using bromphenol blue as indicator (Piper and Evans, 1940). The solutions are extracted with dithizone, the complex decomposed with sulphuric and perchloric acids, and the copper estimated as the dithiocarbamate complex in amyl alcohol as usual.

(ii) Blood copper.

The results of systematic blood copper determinations on experimental ewes during the 1938 and 1939 seasons have been reviewed in the previous section. Even in these seasons, when the incidence of ataxia was extraordinarily low, values of 0.02 mg. per cent. or less were commonly encountered in ewes in the control groups during the gestation and lactation periods, whereas values below 0.05 mg. per cent. were rarely met with in groups receiving copper supplements, except for occasional animals in the lick group which, presumably, were not consuming adequate quantities. In general, values for ewes in the copper groups approximated 0.10 mg. per cent., whereas values of over 0.05 mg. per cent. were relatively rare in the control ewes.

Mothers of three ataxic lambs showed extremely low values of 0.01 or less, and these were comparable with those found for the mothers of four ataxic lambs in 1937. It would appear that ataxia may be expected in the progeny when values of this order obtain near to parturition, although such ewes may rear normal lambs.

It may be pointed out also that the blood copper values of lambs from the control group in September, 1939, were significantly lower than those in groups that had received copper and where development of lambs was better (Table 4). The mean values and range for Control Group, Copper Lick Group, and Copper Topdressed Pasture Group were respectively 0.035, 0.004 - 0.07; 0.085, 0.06 - 0.13; 0.09, 0.07 - 0.11 mg. per cent.

There was evidence of some positive correlation between blood copper values of the lambs and their mothers. Values of 0.01 - 0.02 mg. per cent. were obtained from 4 ataxic lambs.

For healthy animals in Western Australia the values found by us are as follows:-

Ewes, 0.05 - 0.16 mg. per cent. (more detailed information is given by Beck (1941a).

Lambs, 0.07 - 0.10 mg. per cent.

Albiston *et al.* (1940) found the normal range for Australian sheep to be from about 0.08 to 0.20 mg. per cent.

(iii) Liver copper.

Liver copper values for sheep and for ataxic lambs from Gingin and other affected areas, invariably, are very considerably lower than those found for sheep and lambs from healthy areas in the State. These are tabulated:-

TABLE 5.

The Copper Content of Livers from (a) "Normal" Lambs in Healthy Areas, and (b) Affected Lambs.

No.	Age and Source	Cu content of Liver	
		P.p.m. dry basis.	mg. in whole liver.
(a)	<u>"Normal" Lambs</u>		
1	Roleystone - 7 days old.	300	2.73
2	do. - 6 weeks old.	350	7.54
3	Meckering - 1 hour old.	180	4.15
M1	do. - 3 weeks old.	140	7.68
M2	do. - do.	120	3.77
(b)	<u>Affected Lambs</u>		
W48	"Whakea" - born dead.	8.0	-
W46	do. - do.	6.0	0.06
57	do. - 1 month old.	4.0	0.14
W26	do. - 1 day old.	6.0	0.05
W27	do. - do.	8.0	0.05
SL3	do. - 11 months old.	13.1	-
	Affected 7 months.		
278	do. 3½ months old.	2.5*	-
237	Jindong Experimental Farm (Busselton District) - 3 months old.	4.4	-
254	do. - 7 weeks old.	1.0†	-
236	do. - 6 weeks old.	3.4†	-
SL4	Cowaramup (Busselton District) - 5 months old.	2.9*	-

* Blood Cu = 0.02 mg. per cent.

† " " = 0.01 mg. per cent.

A four weeks old lamb, the progeny of a "Whakea" flock ewe depastured on copper topped pasture, had a liver Cu value of 164 p.p.m.

"Normal" values. The following values were found for livers from seven healthy Western Australian sheep from three "sound" districts:- 113 to 380, mean 216 p.p.m. Cu.

One exceptionally low value, 22 p.p.m.,* was obtained from a sheep from "Avondale", Beverley, although values of 113 to 188 p.p.m., included above, were obtained from four other sheep from the same property where the copper status of pastures is known to be very satisfactory.

Nine sheep from a selected area, Harvey, where the Cu status of the pastures is particularly high (10 to 14 p.p.m. dry basis) gave much higher values than the above viz. 240 to 800, mean 504 p.p.m. (Blood Cu values of these sheep were 0.06 - 0.10 mg. per cent.).

* Underwood and Beck (1941) have recorded low values of this order from sheep in the Denmark district where, however, the copper status of pastures is marginal (Beck, 1941b).

TABLE 6.

The Copper Content of Liver and Blood of Ewes from
Affected Properties, 8 Ewes ex "Whakea",
2 Ewes ex Jindong.

No.	Time after Lambing	Ataxia in lamb.	Cu Content of Liver (p.p.m. dry basis)	Blood Cu (mg. per cent.)
W38	1 month	+	3.0	< 0.01
W27	15 days.	+	4.0	< 0.01
181	2 months.	-	6.9	0.01
164	2 do.	-	7.3	0.01
133	4 do.	-	9.2	0.06
250	4 do.	-	10.1	0.01
278	4 ¹ / ₂ do.	+	9.5	0.04
271	3 do.	-	14.9	0.05
254	7 weeks.	+	3.7	0.01
236	6 do.	+	3.0	0.02

Note:- These ewes were from experimental groups 1937-1940 and had been depastured for at least 12 months on "Whakea" (Nos. W38 to 271) or Jindong Experimental Farm (Nos. 254 and 236).

The data presented in Tables 5 and 6 call for little comment.

Liver copper values for lambs and ewes from affected properties are constantly below 15 p.p.m., whereas corresponding values for healthy lambs are 120. to 350 p.p.m. and for healthy adult sheep, with the one exception, lie between 113 and 800 p.p.m. These latter values are of the same order as those recorded for "normal" sheep by Cunningham (1931), Dunlop Young (1937), Moore (1938) and Albiston *et al.* (1940).

The copper concentration in the livers from ewes from affected properties is low in all cases, but with one exception (278) twice as high a concentration was found in those from ewes the lambs of which were free from ataxia as in those from ewes the lambs of which developed ataxia. As lambing had occurred about four months before these examinations were made, it is possible that the copper status may have been higher during pregnancy and that the differences may have been greater. It will be noted that there is a general positive correlation between liver and blood copper values. This, however, is by no means absolute. In three experimental ewes from Gingin (Nos. 133, 278, and 271), relatively normal blood values are associated with low liver values. The samples, however, were obtained late in October, by which time of the year blood figures and the general health of ewes show marked improvement. It would appear probable that, as a result of the weaning of the progeny and better nutritional conditions, higher blood Cu values may be maintained notwithstanding a low liver status. It is of interest to note that although two of these ewes showed very low blood copper values (0.01 to 0.02) throughout the late gestation and early lactation periods (May-July), one (No. 271) showed high values (0.09 to 0.18).

(iv) Milk copper.

As reported in the previous communication, Chapman found that the milk of three mothers of ataxic lambs contained only 0.03 to 0.05 mg. of Cu per litre, whereas that of two Beverley

ewes which had been depastured on the same copper-deficient property for only the previous two months contained 0.12 and 0.19 mg. per litre. Samples were obtained within 3 weeks of lambing in all cases. This observation, which suggested that lowered copper intake in sheep causes a very marked lowering of the milk copper, has been further investigated.

The results as recorded by Beck (1941) show that the copper content of the milk of normal ewes falls progressively from values lying between 0.20 and 0.64 mg. per litre in early lactation to between 0.04 and 0.16 mg. per litre several months later, and that sheep from copper-deficient areas show reduced levels of copper in the milk, values between 0.01 and 0.02 mg. per litre being obtained in most cases. He found no corresponding fall in blood copper levels with the fall in milk copper.

(c) The Copper Content of Pastures.

Material for analysis was obtained by taking a composite from a number of small samples collected at random over the paddock concerned. Unless specified to the contrary in the following tables, samples were mixed clover-grass pastures.

TABLE 7.

The Copper Content of "Whakea" Pastures.

Sample No.	Date Sampled	Origin of Sample	Copper p.p.m. dry basis
<u>1937 Season.</u>			
84	17.6.37	Limestone paddock	3.1
85	17.6.37	Fern paddock	3.8
98	5.8.37	Limestone paddock	2.6
154	4.10.37	" "	1.7
155	4.10.37	Fern and top field	2.5
261	16.12.37	Long paddock	1.7
262	16.12.37	Limestone paddock	1.3
263	16.12.37	"Whakea" paddock	1.3
195	November	Lupin seed	7.9
<u>1938 Season.</u>			
255	28.7.38	Willow field, top end	3.5
256	28.7.38	" " bottom end	3.9
257	28.7.38	"Whakea" paddock	2.6
258	28.7.38	Limestone paddock	6.1
259	28.7.38	Long paddock	2.3
277	11.8.38	Windmill field, Capeweed	4.6
308	20.9.38	Long paddock	2.1
310	20.9.38	Limestone paddock	2.1
311	20.9.38	Willow field	3.1
331	14.10.38	Willow field	3.8
333	14.10.38	Cultivation paddock	3.9
377	24.1.39	Limestone paddock dry pasture heavily grazed.	1.1
378	24.1.39	Willow field	2.5
386	8.2.39	Midland paddock, Lupin seeds	5.0
<u>1939 Season.</u>			
396	5.4.39	"Whakea" paddock, Dry mixed pasture	2.0
399	31.5.39	Orchard paddock - <i>Romulea rosea</i>	4.1
397	1.6.39	Heavily grazed green mixed pasture Limestone paddock	2.4
398	31.5.39	Hollow paddock	2.6
446	15.8.39	Cow paddock	2.3
447	15.8.39	Willow field	2.5
448	15.8.39	Cultivation paddock	2.0

The samples were dried and then crushed in a Wiley mill with iron screens. The analytical method was identical with that used for livers and milk.

The results for pastures collected in the 1937, 1938, and 1939 seasons from "Whakea" Gingin are given in Table 7.

The soil of all paddocks with the exception of the "limestone", which consists entirely of "Gingin clay", were predominantly of the "Whakea sand" type of Hosking and Greaves (1935-36). Experimental sheep were depastured only on the following paddocks:- "Willow field", "Cultivation paddock", "Cow paddock", and "Top field".

The "Top field" adjoining the two former was topdressed with copper sulphate in April, 1938, at the rate of 20 lb. per acre. It was used to depasture the appropriate experimental groups in 1938 and 1939.

The results of pasture analyses are tabulated:-

TABLE 8.

Copper Content of Copper Topdressed Pasture -
"Top field" "Whakea".

Sample No.	Date Sampled	Copper p.p.m. dry basis.
254	28.7.38	14.8
309	20.9.38	11.5
332	14.10.38	10.3
379	24.1.39	4.4
400	31.5.39	10.4
431	28.7.39	11.3
575	19.9.40	7.6

This paddock was heavily stocked with the experimental animals, and it is considered that the low copper content of sample No. 379 is due to this cause (cf. also No. 377, Table 7). The herbage of higher copper content, such as clovers and leafy material, had been eaten out first, so that this sample was comprised largely of reject material, such as stems, which has a low copper content.

In addition to those from Gingin, samples have been taken at different periods of the year from other areas where ataxia occurs. These include one affected property at Jindong where the copper content of the pastures was consistently low (1.6 to 2.0 p.p.m.). Seven other properties, also in the Busselton-Margaret River area, where the geology, soil types, and plant growth are very similar, were sampled. On all these, cattle show evidence of copper deficiency but sheep are not carried. The copper content of these pastures ranges from 1.1 to 3.2 p.p.m.

Two samples have been taken from Rockingham on the affected calcareous littoral. The copper content was 2.8 and 3.2 p.p.m.

More recently, pasture samples were obtained from the Pardelup Prison Farm, Mt. Barker, where the disease had been investigated earlier. Pasture from two paddocks where the incidence had been high in experimental groups during 1932-33

gave copper values of 2.8 and 2.9 p.p.m. Pasture from a more recently "improved" paddock gave a higher value, viz. 4.4 p.p.m.

A large number of pasture samples taken throughout the agricultural areas of this State have been analysed for copper. The results of this survey, which will be published elsewhere, show that ordinary mixed pastures from areas where stock remain healthy contain between 5 and 14 p.p.m. It will be noted that almost all the values cited previously fall well below these "normal" values. Examination of the complete data indicates that the copper content must fall below levels of about 3 p.p.m. before ataxia in lambs or evidence of copper deficiency in cattle develops. Levels of between 3 and 5 p.p.m. are regarded as being marginal.

Although a marked improvement is observed in the health of all stock in the late spring, and although lambs born in this period are less susceptible to ataxia, yet it should be noted that the copper content of pastures at this period is not significantly higher.

(d) Summary of Etiological Studies.

Earlier work, described in previous communications, had narrowed the field of enquiry. The demonstration by Chapman in 1936 of the low copper status of ataxic lambs led in 1937 to field experiments and further chemical investigation to determine the possible etiological significance of copper deficiency. At the same time other experiments designed to give further evidence as to whether the disease was of toxic or dietary origin were continued. These experiments, which have been detailed, definitely incriminated copper deficiency as an etiological factor and, in association with Chapman's chemical determinations, disposed of any conception of a toxic origin, especially of the lead-intoxication hypothesis.

Subsequent work was restricted to the confirmation of the claim that ataxia is due to copper deficiency.

It has been shown that the administration of copper to the mother during the gestation period prevents the occurrence of the disease. Copper administered to the lamb will also arrest the progress of the disease, even in the later stages and when the course is rapid in untreated controls. Treated animals, apart from the persistence of some ataxic gait, recover.

The low copper status of the mothers (liver, blood, and milk) and of the affected progeny (liver and blood) has been conclusively and constantly demonstrated in all cases investigated.

Regular four-weekly haematological examinations of the mothers during the gestation and nursing periods have clearly indicated a positive correlation between severe anaemia present during the late gestation and early lactation periods, and the more acute form of ataxia in the progeny. Mothers of the more subacute cases may show no evidence of anaemia. The nature of the anaemia which rapidly responds to copper therapy will be discussed in a later section.

There is also evidence of a positive correlation between low blood copper in the mother during the same periods and the occurrence of ataxia in the progeny. Unfortunately the data, here, are less complete on account of the very low incidence of ataxia in the control groups in the 1938 and 1939 field experiments, when systematic blood copper determinations were carried out. In the last year's experiments, there is evidence of some correlation between the blood copper values of mother and progeny; blood coppers were much lower in control ewes and lambs than in those in copper-treated groups.

It has been demonstrated under experimental conditions that copper supplements in the form of lick or as a top-dressing prevent the occurrence of ataxia and promote optimal growth of lambs. Indications are that topdressing is the more effective means of administration.*

A large number of analyses of pasture from widely separated affected districts have shown a low copper status throughout.

The distribution of the disease, moreover, coincides with problems in other stock, cereals and pasture associated with copper deficiency, viz., "falling disease" and other conditions in cattle, coast disease, "reclamation disease" of cereals, and one type of "stalling" of subterranean clover.

The data here reviewed provide conclusive evidence that ataxia is a manifestation of copper deficiency.

8. Pathogenesis.

The occurrence of ataxia is associated constantly with a low copper status of the pastures and the animal, both mother and progeny. Other experimental data confirm this evidence that the disease is a manifestation of copper deficiency.

Any account of pathogenesis, however, must be very incomplete and provisional in the present state of our knowledge of normal processes. No satisfactory explanation of the development of the essential lesion, a demyelination of the central nervous system, can be expected until more is known about myelination in the lamb. This question, according to Innes and Shearer (1940), is receiving consideration.

It has never been shown that copper plays any role in the process of myelination, and the significance of this element in animal nutrition generally is, as yet, very incompletely understood.

Our present knowledge is derived from experiments with animals maintained on a copper-deficient ration, notably milk, which apart from its low iron and copper content forms an adequate diet for the young animal, and from the investigation of diseases of farm animals occurring under conditions of natural copper deficiency. In the latter category are diseases of cattle, notably "Lecksucht" in Holland (Sjollema, 1933), "Saltsick" in Florida (Becker et al. (1931)),

* During the past three seasons an extensive application of these methods by stock owners at Gingin and Dandarragan has been an unqualified success, and field observations have further confirmed the experimental results.

and the milk anaemia of pigs which responds to iron and copper therapy, although there is little experimental evidence that the copper addition is necessary. It is remarkable that none of the conditions described are essentially analogous clinically or pathologically to ataxia in lambs, and that bovines and equines, in this State, maintained in areas where ataxia is enzootic, show manifestations of disease that are quite distinct. The conditions encountered in cattle are, however, in some respects similar to those described in Holland and Florida.

It would appear, therefore, that the copper metabolism of sheep may differ in some respect from that of most other species*, and that comparative studies under conditions of controlled copper intake are of fundamental importance.

Our present knowledge of the functions of copper in the animal organism has been reviewed recently by Underwood (1939-40) and will be dealt with only briefly here.

Following the original work of Hart *et al.* (1928), evidence has accumulated demonstrating beyond doubt that copper is essential for the mobilisation of iron for haemoglobin synthesis. A certain minimal level of copper in the blood is also necessary for rapid haemopoiesis (Schultze *et al.*, 1936).

The claim of Shearer, Innes, and McDougall (1940) that, "although it has been shown in other animals that Cu deficiency does interfere with normal haemopoiesis this is not the case in the ewe", is based on insecure evidence.

It has been recognised too that copper plays a part in certain of the oxidation-reduction enzyme systems of the cell, although no attempt has yet been made to relate this finding to the study of metabolic disturbances occurring in different species of animals as a result of copper deficiency.

Having reviewed the general knowledge available we are now in a position to attempt to explain the sequence of events in the occurrence of ataxia of lambs.

Experimental data presented in this communication indicate that when sheep are maintained in the "affected" areas the copper stores of the body are depleted and the liver Cu reaches levels below 15 p.p.m., whereas the corresponding values found for healthy Western Australian sheep are 113 to 800 p.p.m. These are of the same order as the "normal" values reported by other workers.

The occurrence of appreciable quantities of copper in the liver of animals, particularly the young, has been recognised for some time. The level of storage necessary for normal function in the sheep and other animals is, however, unknown.

Limited experimental evidence here, suggests that the male and non-breeding female sheep is able to maintain normal health even when the copper content of pastures is below 5 p.p.m. Following depasturage on "affected" country for periods of several years, the only clinical signs in these non-breeding animals are "stringiness" of the wool. After one or two years sojourn blood copper levels may be very low, e.g. 0.01 mg. per cent., but anaemia is only rarely encountered.

* Reference was made on p. 12 to a condition, "posterior paralysis", affecting sucking pigs which has a striking clinical and pathological resemblance to ataxia. There is some evidence that this condition may also be due to copper deficiency.

In the breeding ewe the position is quite different. As a result of the demand for copper by the developing embryo (cf. Wilkerson, 1934) the ewe is, apparently, unable to maintain a copper reserve adequate for normal functional purposes during the later gestation and early lactation periods. This is sometimes apparent even during the first gestation period on "affected" country and is generally very obvious during the succeeding ones. Blood copper values of below 0.01 mg. per cent. are not uncommon, and a severe anaemia may supervene. The fall in haemoglobin content may not occur till some time after a marked depression in blood Cu values and is sometimes absent altogether. "Stringiness" of the wool is marked, and the limp ragged appearance of the fleece accentuates the wretched appearance of the ewe, particularly during the wet months of July and August when some loss of condition and continued diarrhoea, both controllable by copper supplementation, are evident. This diarrhoea which is associated particularly with the ingestion of *Cryptostemma calendulaeum*, may cause decreased absorption of copper. The high moisture content of the plant may also result in decreased intake of dry matter and hence of copper.

The mothers, after lambs are weaned, regain their normal state of health although, of course, wool growth remains affected throughout.

Marked signs of ill health in the gestating ewe are almost invariable precursors of ataxia, in its more acute form, in the progeny. The more common subacute ataxia, however, may occur in the progeny of ewes which have shown little evidence of copper deficiency other than low blood copper values and low reserves in the liver.

Ataxia is evidently due to a copper starvation of the developing embryo following a depletion of the copper reserves of the mother*; gross lesions of the central nervous system may be present in lambs affected at birth or born dead, and the copper content of their livers is extremely low. In the more frequently encountered cases in which no signs of the disease are apparent until the lamb is several weeks old, low liver and blood copper values obtain, again indicating that the supply of copper available during intra-uterine or possibly early extra-uterine period is inadequate.

On pathological grounds it appears extremely unlikely that the condition is one of myelin aplasia, although as suggested by Innes and Shearer it is "feasible that with the onset of demyelination normal myelination would be inhibited".

A further knowledge of the genesis of the lesions in lambs could be obtained only by a careful study of the development of the central nervous system of healthy embryos and of embryos from ewes depastured for prolonged periods on "affected" country and, if practicable, from experimental ewes maintained on a appropriate copper-deficient ration.

The difficulty of correlating the incidence of ataxia and the health of the mothers with the copper content of the pastures, expressed on a dry basis, under similar experimental

* The deposition of haemosiderin in the liver, kidney, and spleen of the mothers and their ataxic progeny is further evidence of a suboptimal copper status, appropriate tests (Bennetts and Chapman, 1937) having shown that there is no evidence of excessive blood destruction.

conditions, was discussed earlier. It would appear that, under the conditions of suboptimal copper intake prevailing throughout the affected areas, the margin of safety for the developing embryo is very narrow and that a very slight alteration in the copper status of the mother is able to determine whether the progeny shall develop normally, suboptimally, or become affected with ataxia. It should be noted also that the liver copper content of ewes which have reared healthy lambs may be quite low, and that the progeny of ewes showing very low blood copper values during the gestation and lactation periods do not invariably develop ataxia.

An investigation of experimentally produced copper deficiency, and fundamental studies of copper metabolism in the sheep, should throw some light on this and many other baffling features of the disease.

9. Differential Diagnosis.

No other condition clinically resembling ataxia has been encountered in Western Australia.

The occurrence of a disease of young lambs with clinical characteristics as described, particularly if associated with other evidence of copper deficiency in animals or plants, would justify a provisional diagnosis of ataxia. Confirmation is afforded by the demonstration of the spinal cord lesions which are pathognomonic.

10. Methods of Control.

These have been referred to previously and will be considered very briefly.

(a) Farm Management.

Ataxia may be prevented by using only breeding ewes introduced from healthy districts immediately prior to mating. If breeding ewes are introduced much earlier, affected lambs may result.

A breeding flock may be maintained on affected properties provided ewes are transferred, during their gestation, to known healthy country. The minimum effective period varies, according to locality, from 4 to 8 weeks. Alternatively, a change to healthy country after parturition may be found effective unless the course of the disease is very acute.

The incidence and course of the disease in a flock may be checked by this procedure, and the response is usually very rapid.

Now that the etiology of the disease is elucidated the above methods of control evolved by stock owners are of historical rather than practical interest.

(b) Copper Supplements.

It has been shown both experimentally, and, since 1938, extensively under field conditions that the administration of copper sulphate to the mother provides an efficient method of preventing ataxia in the progeny. A daily intake of from 10-20 mg. of Cu during the gestation period is

effective. The minimal amount required has not been determined in this State, but investigations in South Australia would indicate that a daily intake of 5 mg. of Cu is adequate even when pastures are extremely copper-deficient. Under Western Australian conditions the supplement can be conveniently administered in lick form, since salt licks containing copper sulphate are relished by sheep and readily taken by young lambs. Licks currently used contain from 0.25 to 0.5 per cent. copper sulphate, the concentration being adjusted to aim at an average daily consumption in the vicinity of 10 mg. of Cu. Although supplementation for only a portion of the gestation period may prevent ataxia, it is recommended that licks be available to the flock throughout the year, since regular consumption results in marked improvement in the health and production of the adults and in optimal development in the lambs.

Topdressing of pastures with copper compounds has been shown to raise the copper status of the pastures. Flocks maintained on such pastures under experimental and field conditions have done remarkably well. Ataxia is effectively controlled*, and limited experimental evidence suggests that this procedure, which ensures that all members of the flock receive a regular and adequate intake of copper, will result in better production that is obtainable by means of lick feeding.

It is at present recommended that pastures be top-dressed with copper sulphate at the rate of from 10 to 20 lb. per acre, but experiments to determine the optimal rate of topdressing and the period for which it is effective are not complete.

The value of copper compounds other than the sulphate is also being investigated.

Drenching with solutions of copper sulphate may occasionally form a valuable supplementary means of control, e.g. in the case of gestating ewes that have not taken lick or lambs that show early signs of the disease.

III. COPPER DEFICIENCY IN ADULT SHEEP.

In earlier publications, reference was made to the health of adult sheep in flocks affected with ataxia, but for clarity the discussion of this phase of the investigation has been limited in this communication to a consideration of the health of the mother in its more direct relationship to the health of the progeny. Since there is little information on the effects of copper deficiency in sheep and since this problem is so closely related to ataxia, general observations made on adult sheep merit a more detailed description.

The clinical evidence of copper deficiency most widely distributed throughout affected flocks is a characteristic type of wool growth ("stringiness") exhibited particularly

* As an example of the efficacy of copper supplementation under field conditions, the following experience may be cited:— 750 ewes of the "Whakea" farm flock, depastured on copper-dressed country, lambed in May to June 1940. They were remated and lambed again in November to December of the same year. None of the progeny developed ataxia. Forty per cent. of these mothers had been on "Whakea" continuously since December 1938 and sixty per cent. since December 1939.

by sheep of the Merino breed. Other signs - anaemia, diarrhoea, and loss of bodily condition - are exhibited by the breeding ewes. All respond to copper supplementation. Haemosiderosis of the liver, kidney, and spleen has been demonstrated.

1. The Effect on Wool Growth.

After a few months sojourn on copper-deficient country, sheep exhibit characteristic wool changes. The wool becomes "stringy", that is, the crimp disappears and the wool has a limp, glassy and straight appearance. There is an absence of "bulkiness". These changes are most evident in Merino fleeces and are exhibited by sheep of any age and either sex.

The reported distribution of this condition is identical with that of ataxia and of low copper status of flocks and pastures.

Following a change to healthy country the wool resumes its normal type of growth.

It has been demonstrated experimentally that "stringiness" is prevented by the administration of copper supplements.

(a) Observations in the Experimental Groups at "Whakea", Gingin.

Observations were carried out during 1937 and 1938 on ewes from the experimental groups detailed in Section II of this communication.

In September, 1937, an examination of fleeces at shearing gave definite indications of response to copper. The fleeces of the groups not receiving copper or change of country were in general markedly "stringy", whereas normal quality was very evident in wool growth corresponding with the period when copper was administered. This was particularly marked in the fleeces of two very anaemic control ewes (Nos. W32 and W44) which responded to experimental copper treatment.

Dr. J. E. Nichols, at that time Professor of Agriculture of the University of Western Australia, kindly undertook determinations of weight variations from base to tip of fleece samples from these two sheep and from two "stringy" controls. The result indicated that there was no significant difference in weight or fibre diameter as a result of copper treatment, it being concluded that the obvious difference in quality resulted from greater uniformity of wool growth in the treated animals.

Results obtained the following season were confirmatory. A wool classer was present at the shearing in September, 1938, to class fleeces from experimental ewes. The weight, yield, and count of each fleece were assessed and general remarks made on the wool from each group. There were no significant differences in wool weights, but in the control group fleeces tended to be "stringy" and tender, whereas in the copper-drenched and copper-topdressed pasture groups "stringiness" was absent, and the wool was sound and showed more body and condition.

(b) Experiments with Merino Sheep Selected for Wool Quality

The object of this experiment was to determine whether good quality merino wool could be grown at Gingin with the aid of copper supplements. It was considered that more significant differences should be observed in animals selected for wool quality rather than in those selected for the breeding of fat lambs, which were the only ones available for general experimental purposes from the flock on "Whakea".

1938 Experiment. For the purpose six animals (cull Merino rams), selected for high wool quality, were provided. They were received at Gingin on March 30th, 1938, and were paired on wool type and randomised into two groups of three, one to receive copper, the other to serve as controls.

The sheep in the copper group received a drench of copper sulphate thrice weekly, the Cu intake being equivalent to 15 mg. daily. The supplement was administered from May 5th until September 1st, 1938. The sheep were shorn and fleeces classed on September 13th. The details are shown in Table 9:-

TABLE 9.

Wool Yields of Control and Copper Treated
Merino Sheep, 1938.

Controls							Copper Drenched						
No.	Weight (lb.)				Count	Yield	No.	Weight (lb.)				Count	Yield
	Belly	Pieces	Fleece	Total				Belly	Pieces	Fleece	Total		
A25	1	1 $\frac{1}{2}$	11	13 $\frac{1}{2}$	64	48	A24	1 $\frac{1}{2}$	1 $\frac{1}{2}$	10	12 $\frac{1}{2}$	60-64	46
A21	1	1	10	12	64	48	A20	1	1 $\frac{1}{2}$	12 $\frac{1}{2}$	15	60-64	46
A23	1	1 $\frac{1}{2}$	10	11 $\frac{3}{4}$	64	48	A22	1	1 $\frac{1}{2}$	11	13 $\frac{1}{2}$	60-64	46

The report of the classer was as follows:- "Copper-drenched group have definitely better grown fleeces with more bulk and heavier condition. They are slightly broader in quality and there is a complete absence of "stringiness" whereas in the control group "stringiness" is very marked in one fleece (A23) and noticeable in the others"

1939 Experiment. This was a repetition of the previous year's experiment and the same sheep were used. The groups were as in 1938 except that the numbers of one pair (A22 and A23) were reversed so that A23 now received copper and A22 acted as a control.

Copper treatment, discontinued on 1/9/38, was recommenced on 4/3/39 and continued until 23/8/39, the dose and method of administration being as in 1938.

The sheep were shorn on 15th September, 1939.

Details of weights and classing are shown in Table 10:-

TABLE 10
Wool Yields of Control and Copper Treated
Merino Sheep, 1939.

No.	Weight (lb.)				Count	Yield	Remarks
	Belly	Pieces	Fleece	Total			
<u>Controls</u>							
A25	1 $\frac{1}{2}$	1	9 $\frac{3}{4}$	12	60-64	57	Stringy, no lock. Very tender, short.
A21	1	1 $\frac{1}{2}$	8 $\frac{1}{2}$	11	64	58	Stringy, thin, no lock, very tender, fair length.
A22	$\frac{3}{4}$	1 $\frac{1}{2}$	9 $\frac{1}{2}$	11 $\frac{3}{4}$	64	56	Stringy, no lock, tender
<u>Copper Drenched.</u>							
A24	1 $\frac{1}{4}$	1 $\frac{1}{2}$	10 $\frac{1}{4}$	13	64	62	Sound, warp, good length.
A20	1	1 $\frac{1}{2}$	10 $\frac{1}{2}$	13	64	59	Sound, half warp, fair lock, average length.
A23	1	1	9 $\frac{3}{4}$	11 $\frac{3}{4}$	64	58	Sound, no lock, fair length.

The difference in fleeces of animals in both groups was most marked, particularly in the pair A25 and A24. Control fleeces were all definitely "stringy" and tender, whereas in the copper-drenched group all fleeces showed complete absence of "stringiness" and were quite sound. The results although not quite so striking, probably on account of the six months' period when no copper supplement was administered, definitely confirm those of last year.

During the past two seasons, the woolbrokers have reported very favourably on the marked improvement of clips from copper-deficient country which, to our knowledge, coincides with the use of copper supplements administered as a lick or through topdressing of pastures. From two properties in particular where investigations have been carried out by us, the 1939 and 1940 clips showed a complete absence of the "stringiness" which predominated prior to the use of copper.

There is very definite evidence, therefore, that good quality Merino wool can be produced in the affected area by the use of copper supplements.

2. The Health and Weight of Experimental Sheep.

In experimental flocks at Gingin it was noticed repeatedly that breeding ewes during the wet winter months lost condition and showed persistent diarrhoea, particularly

during the 1937 season. Two of the control ewes (Nos. W32 and W44), which had apparently lost much condition, were severely anaemic, and were persistently voiding liquid faeces, showed a very marked response to the administration of 15 mg. of Cu daily. This, together with other observations in the treated and untreated group, suggested that copper supplements exerted a very favourable influence on the health and condition of breeding ewes.

This was confirmed by repeated observations that diarrhoea did not occur in sheep receiving copper supplements, and by the results in experimental groups during 1939 when ewes in the two groups receiving copper supplement appeared to thrive better than the controls and maintained their body weight better.* At the conclusion of the experiment in October, the mean loss of weight in the controls was 8.4 lb., whereas the mean gain, not allowing for the weight of wool removed in September, was 1.9 lb. in Group 2 and 4.3 lb. in Group 3.

Sheep which are not rearing lambs rarely show diarrhoea and loss of condition. Marked improvement in the condition of the entire flock, both ewes and wethers, becomes apparent with the advent of the warm spring weather in September; this is related to improved general nutrition and is not accompanied by any significant change in the copper status of the pastures (see Table 7).

3. Anaemia of Breeding Ewes.

The results of regular haematological examinations on ewes in experimental groups during 1937 - 1939, which demonstrated the frequency of a severe anaemia during the gestation and lactating periods in groups not receiving copper supplement, were reviewed in the previous section.

A more complete haematological examination of a number of anaemic ewes from the experimental groups was carried out in 1937. As a result of this the anaemia was classified in the preliminary communication as macrocytic and hyperchromic.

Subsequent investigations, comprising the haematological examination of a large number of normal sheep from different districts and of the few anaemic ewes available in experimental flocks at Gingin and Jindong (Busselton district) in 1939 and 1940, have led to a revision of the previous conclusions.

The haematological findings will now be considered in some detail:-

(a) "Normal" Values.

In the previous communication (Bennetts and Chapman, 1937), as a result of a limited number of determinations, the values Hb. 11.5, and R.B.C. 11.0, were accepted as "normal" and were used for the calculation of colour index.

Subsequently we had occasion to carry out regular Hb. determinations on pregnant and non-pregnant ewes aged from 2 to 6 years and on wethers from several healthy areas. Some hundreds of determinations indicated that

* All ewes in experimental groups were weighed at monthly intervals.

blood figures for healthy sheep may show extremely wide variation which apparently is not influenced by sex, age within the range mentioned, gestation, or lactation. The range for Hb. was 9.0 - 16.8 and for R.B.C. 7.6 - 13.3.

It appears impracticable, therefore, to fix any "normal" value for the Hb. and R.B.C. of sheep. It is reasonable, however, to accept Hb. figures of below 8.0 as evidence of anaemia.

Values for cell volume per cent., mean cell volume, and indices, determined for 10 healthy sheep, showed much less variability (see Table 11).

(b) Methods.

All examinations were carried out on blood obtained from the jugular vein and collected into bottles containing the appropriate quantity of oxalate from solution evaporated to dryness. Smears were fixed immediately with methyl alcohol and stained subsequently with Giemsa.

(i) Haemoglobin (Hb.). The Newcomer acid haematin method was used, the apparatus being standardized against iron determination and oxygen capacity estimated by the gasometric method. Results are expressed in grammes per 100 ml. (1% Hb. = 1.34 vol. % O₂).

(ii) Red Cell Count (R.B.C.). Individual counts were carried out by the usual method, two standardized Thoma haemocytometers being used. Toisson's or Hayem's fluid was the diluent. Group counts were made by a flask dilution method, equal quantities of blood from each individual being added to Hayem's solution in a 100 or 200 ml. volumetric flask to make a final dilution of 1 - 1000, or 1 - 500 if a low count was to be expected. All ruled squares on the slide were counted. Duplicate counts were made on two haemocytometers and the mean taken.

In some cases, notably for M.C.V. determinations, the flask dilution method was used for individual counts. A very good correlation was obtained for both methods. Counts are expressed in millions per c.mm.

(iii) Mean Corpuscular Volume (M.C.V.). This was estimated by the method described by Whitby and Britton (1937) except that, for convenience, 20 ml. of oxalated blood was centrifuged in a 25 ml. graduated tube instead of 10 ml. as recommended. It was found that the corpuscular volume became constant after centrifuging for about 1 hour at 3,500 revolutions per minute and readings were made at 1½ hours.

Potassium oxalate was used as an anticoagulant in 1937 but subsequently the mixture (potassium and ammonium oxalate) of Heller and Paul (1933-34) which does not cause shrinkage or expansion of red cells was substituted.

The red cell counts were made accurately in duplicate, and were repeated if discrepancy occurred. M.C.V. determinations were made within three hours of bleeding, on samples collected in the field or obtained from sheep brought to the Perth laboratory for the purpose.

(iv) Reticulocyte Counts. These were carried out in the field or laboratory by the method recommended by Todd and Sanford (1928).

(v) Indices. In view of the great variability in Hb. and R.B.C. values for healthy sheep, the use of colour index has been discontinued and the colour count ratio utilized by Marston and McDonald (1938) substituted. Colour volume ratio adopted by them also seems a more appropriate term than mean corpuscular haemoglobin to describe an identical ratio.

These indices are calculated as follows:-

$$\text{Colour Count Ratio (C.C.R.)} = \frac{\text{Hb. in grammes per cent.}}{\text{R.B.C. in millions per c.mm.}}$$

$$\text{Colour Volume Ratio (C.V.R.)} = \frac{\text{Hb. in grammes per cent.}}{\text{C.V. per cent. (Volume of packed R.B.C. per 100 ml. of blood.)}}$$

(c) Anaemia in Adult Sheep.

The anaemia to be described is apparently exhibited only by the gestating ewe, particularly during the later stages of pregnancy and for several weeks after parturition. Recovery is generally rapid following cessation of lactation. Thereafter blood values in the higher limits of the normal range are found commonly until towards the end of the next gestation period. Unmated ("dry") ewes do not become anaemic, and a number of determination on male sheep from Gingin and Jindong indicate that neither rams nor wethers become affected when depastured for from 2 to 3 years under the same conditions, although somewhat low Hb. values (8.3) may be observed during the winter and early spring.

The anaemia of ewes will now be considered in detail:-

(i) Haemoglobin. Haemoglobin is considerably decreased, commonly to below 6.0. Values as low as 3.7 were observed on more than one occasion in 1937.

(ii) Red Cells. There is a corresponding gross reduction in red cell count. This, too, is frequently below 6.0 and counts as low as 2.7 have been recorded.

When counts are relatively normal, i.e. above 7.0, no marked changes are usually observed in blood smears; there is, however, frequently evidence of anisocytosis, and macrocytes may be present. With lower counts, notably those below 6.0, abnormalities of cells are very evident. Anisocytosis is marked, macrocytes are numerous, and red cells exhibit considerable variation in shape. Stippled cells are generally numerous, varying from less than 1 per cent. to 11 per cent. Red cells showing polychromasia may be common. Jolly bodies are only rarely encountered. Normoblasts, usually uncommon, are sometimes numerous; in one film 49 were noticed when counting 100 white cells. Megaloblasts were observed in two cases but were not numerous.

Red cells for the most part appear fully haemoglobinized. Reticulocytes are generally numerous in the more anaemic animals. For 17 counts carried out on 9 subjects with Hb. 6.5 or lower, the range was from below 1 to 11.3 per cent., mean = 4.2 per cent. With Hb. 7.0 or higher, counts of less than 1 per cent. were consistently obtained.

TABLE 11.

(a) "Normal" Sheep from Healthy Areas

Sheep No.*	Date	Hb.	R.B.C.	C.V.%	M.C.V. (in μ)	C.C.R.	C.V.R.	Blood Cu (in mg.%)
3M	25/3/38	10.5	7.7	30.0	39.0	1.4	0.35	-
5M	"	11.2	10.1	34.1	33.8	1.1	0.33	-
6M	"	9.8	7.9	30.0	38.0	1.2	0.33	-
10M	"	11.4	9.4	31.3	33.3	1.2	0.37	-
1W	28/3/38	13.9	11.0	40.0	36.4	1.3	0.35	-
2W	"	12.3	9.9	37.5	37.9	1.2	0.33	-
3W	"	13.9	12.6	42.5	33.7	1.1	0.33	-
4W	"	14.0	11.4	37.5	32.9	1.2	0.37	-
V40	8/9/39	16.8	13.2	48.8	36.9	1.3	0.35	-
S74	"	14.5	11.1	33.8	30.4	1.3	0.43	-
Range		9.8- 16.8	7.7- 13.2	30.0- 48.8	30.4- 39.0	1.1- 1.4	0.33- 0.43	-
Mean		12.8	10.4	36.5	35.2	1.2	0.35	-

* M Sheep = pregnant Merino ewes ex Meckering.
 W Sheep = Merino wethers ex Wagin.
 V40 and S74 = non-pregnant ewes ex Perth.

(b) Sheep without Anaemia "Whakea" Gingin.

Sheep No.	Date	Hb.	R.B.C.	C.V.%	M.C.V. (in μ)	C.C.R.	C.V.R.	Blood Cu (in mg.%)
W32*	14/7/37	9.2	9.2	32.8	35.7†	1.0	0.28	-
178	15/2/38	13.4	12.7	41.0	32.3	1.1	0.33	-
113	"	13.7	11.5	40.5	35.2	1.2	0.34	-
198	"	10.9	8.5	35.0	41.2	1.3	0.31	-
282	15/8/39	9.4	6.7	20.0	29.8	1.4	0.47	0.01
275	24/8/39	8.3	6.7	28.0	41.8	1.2	0.30	0.01
289	"	8.5	7.1	27.5	38.7	1.2	0.31	0.01
133	6/9/39	10.8	9.6	30.2	31.4	1.1	0.35	-
"	21/9/39	9.0	8.9	28.6	32.1	1.0	0.32	-
266	6/9/39	11.5	9.8	36.8	37.5	1.2	0.31	0.01
260*	"	12.3	9.1	34.1	37.4	1.4	0.36	0.01
269	21/9/39	9.9	8.5	31.0	36.5	1.2	0.32	0.01
F	"	15.8	12.2	42.5	34.8	1.3	0.37	0.08
A21	18/7/40	13.7	12.3	40.0	32.5	1.1	0.34	0.08
"	21/8/40	14.4	11.7	40.0	34.2	1.2	0.36	0.08
"	18/9/40	14.2	13.1	40.0	30.5	1.1	0.36	0.08
A22	18/7/40	10.9	8.8	35.0	39.8	1.2	0.31	0.06
"	21/8/40	13.0	9.7	40.0	41.2	1.3	0.33	0.04
"	18/9/40	11.7	9.9	40.0	40.4	1.2	0.29	0.03
A25	18/7/40	10.1	9.1	35.0	38.5	1.1	0.29	0.03
"	21/8/40	12.8	10.6	37.8	35.6	1.2	0.34	0.03
"	18/9/40	12.8	12.3	37.8	31.2	1.0	0.34	0.03
Range		8.3- 15.8	6.7- 13.1	20.0- 42.5	29.1- 41.8	1.0- 1.4	0.28- 0.47	0.01- 0.08
Mean		11.7	9.9	35.2	35.8	1.2	0.33	0.04

* after sheep No. indicates that progeny was affected with ataxia.

† = values corrected for shrinkage due to use of potassium oxalate as an anticoagulant (factor = 1.05).

Note: With the exception of F, subjects are all from experimental flock - Nos. F, W32, 113, 198, and A22 received some copper supplementation. With the exception of A21, A22 and A25, which were cull Merino rams used in the wool growth experiment, all are breeding ewes.

TABLE 11.

(c) Anaemic Sheep from Affected Areas (Gingin and Busselton Districts).

Sheep No.	Date	Hb.	R.B.C.	C.V.%,	M.C.V. (in c. μ)	C.C.R.	C.V.R.	Blood Cu (in mg.%)
W27*	2/7/37	4.0	2.7	15.8	58.8†	1.5	0.25	<0.01
W35*	14/7/37	4.7	3.9	18.4	47.2†	1.2	0.27	-
W40*	"	5.5	4.9	22.3	45.5†	1.1	0.25	0.01
59*	"	5.4	4.7	24.9	53.0†	1.1	0.22	<0.01
Range 1937		4.0-5.5	2.7-4.9	15.8-24.9	45.6-58.4	1.1-1.5	0.22-0.27	
Mean 1937		4.9	4.05	20.7	51.1	1.2	0.25	
234*	15/8/39	7.2	4.6	22.5	48.9	1.6	0.32)	0.01 on
"	24/8/39	6.5	4.0	21.3	53.2	1.6	0.30)	22/8/39
"	6/9/39	6.3	4.9	20.5	41.8	1.3	0.31	and 19/9/39
133	15/8/39	6.8	7.4	22.5	30.4	0.9	0.30	0.01 on
"	24/8/39	7.3	7.4	24.5	33.1	1.0	0.30	30/5/39 & 26/7/39 0.06 on 19/9/39 & 17/10/39
229*	26/10/39	5.3	4.5	17.1	38.0	1.2	0.30	0.02
237*	"	3.9	3.7	15.5	41.8	1.1	0.24	0.01
254*	31/8/40	7.5	7.2	23.8	33.0	1.0	0.31	0.01
236*	"	6.5	5.8	22.5	38.8	1.1	0.29	0.02
Range Whole Series		3.9-7.5	2.7-7.4	15.5-24.9	30.4-58.5	0.9-1.6	0.22-0.32	
Mean Whole Series		5.9	5.05	20.9	43.3	1.2	0.27	

* after sheep No. indicates that progeny was affected with ataxia.

† = values corrected for shrinkage due to use of potassium oxalate as an anticoagulant (factor = 1.05).

All subjects are breeding ewes from experimental flocks at "Whakea" and (Nos. 229, 237, 254 and 236) from Jindong. Ewes lambed June and July. None received any copper treatment.

The range and mean values for 1937 are given separately as it was on these 1937 determinations that the anaemia was defined previously as of the macrocytic type.

(iii) Mean Cell Volume (M.C.V.) and Indices. Determinations were carried out on a number of ewes with and without anaemia, on some males from affected areas, and on normal sheep from healthy areas. These together with Hb., R.B.C., and indices calculated therefrom are presented in tabular form (Table 11). As the anaemia was previously classified on the examination of four anaemic ewes in 1937, the mean and range values for these are given separately for comparison with those obtained subsequently.

It will be noted that the M.C.V. of normal sheep shows relatively much less variation than Hb. and R.B.C. The values for range 30 to 39 c. μ and mean 35 c. μ correspond very closely with those found by Josland (1936-37) for New Zealand sheep and by McDonald (1938) who for 23 determinations for South Australian sheep gave the values 34 to 42, mean 36.3 c. μ . The values for sheep not showing anaemia at Gingin are almost identical with those of our normals.

In all four of the very anaemic sheep examined in 1937, the M.C.V. is significantly above the normal range. In two determinations (No. 234 in 1939) high M.C.V. values were obtained. In other cases examined in 1939 and 1940, however, values were well within or not significantly higher than the normal range.

It will be noted also that C.C.R. values for anaemic sheep, with the possible exception of Nos. W27 and 234, are not significantly higher than the normal values given in the table, these being representative of a much larger number of determinations. It is apparent that the ratio of 1.0 accepted as a normal in 1937, and which led us to classify the anaemia as hyperchromic, was too low and that the anaemia cannot now be classed as of this type. A consideration of the C.V.R. values, moreover, indicates that the Hb. saturation of corpuscles is in general somewhat incomplete in the anaemic animals. Our "normal" range, 0.33 to 0.43, is of the same order as that of Josland, viz. 0.31 to 0.38 (his values being expressed as M.C.H. per cent. (31 to 38%)).

Leucocytes. No abnormal white cells were noted in smears. Counts carried out on eleven anaemic subjects gave values from 4,800 to 11,300 with a mean of 7,400 per c.mm. These figures are within the normal range given by Frazer (1929-30). Differential counts were carried out on two very anaemic sheep, with the following results:-

	No. 30	No. W27
Polymorphonuclears	31 per cent.	39 per cent.
Lymphocytes	61 "	55 "
Monocytes	6 "	4 "
Eosinophiles	2 "	2 "

In the one case 6 and in the other 49 normoblasts were noted when counting one hundred white cells.

Compared with the finding of Norris and Chamberlain (1929) for Australian sheep, these figures indicate a relative lymphocytosis.

(iv) The Type of Anaemia. Recent haematological work has failed to confirm the previous claim that the anaemia of lambing ewes is macrocytic and hyperchromic. It is evident that in severe cases macrocytes may be sufficiently numerous to raise M.C.V. values significantly above normal. This, however, is irregular. Although the colour-count ratio may be equivalent to or even higher than the normal range based on recent determinations, erythrocytes tend to be incompletely saturated with haemoglobin.

A classification of the anaemia is not possible without further extensive investigations which are beyond the scope of the present inquiry.

Etiology. The results of systematic blood copper and haematological studies and other data reviewed previously clearly incriminate copper deficiency as the cause of the anaemia. In all cases where chemical investigations have been carried out, the anaemia is associated with a low copper status (blood 0.01 to 0.02 mg. per cent.; liver below 15 p.p.m.) of the affected animal. It is apparent, however,

(see Tables 6 and 11), that very low blood copper figures and low liver values may be present without evidence of anaemia. Generally, the Hb. level falls some time after blood copper has fallen to the levels indicated. In the non-breeding animal the same very low blood copper levels may exist for several months without any corresponding fall in Hb. to values below 8.3.*

After the completion of the gestation and lactation periods, blood copper values of the anaemic ewe return to normal earlier than do the Hb. values, but low liver copper values still continue.

As we have seen earlier, the anaemia is preventable by "pure" copper supplements. Controlled experiments reported in detail by Bennetts and Chapman (1937) clearly indicate that the anaemia responds to this treatment. A reticulocyte crisis was demonstrated from the 5th to the 7th day, when an increase to 19 per cent. was recorded. Hb. and R.B.C. values at the outset below 6.0 and in one case below 4.0 showed an almost immediate rise. Thereafter the blood picture steadily improved, reticulocytes and other abnormal red cells disappeared from the circulation, and Hb., R.B.C., and M.C.V. etc. approached normal values, which were recorded after four weeks' treatment. During the same period the control ewes became somewhat more anaemic.

It is clearly evident that in sheep as in other species copper is necessary for normal haemoglobin formation and erythropoiesis. It would appear, however, that this function may be carried out, in the sheep, under conditions of copper deficiency when the blood and liver copper values of the animal are very low, provided that it is not called upon to produce and rear progeny. In this event, owing to the drain on the mother's reserves for the embryo a breakdown may occur and anaemia supervenes.

In view of the inference drawn from the South Australian work on coast disease by Shearer, Innes, and McDougall (1940) that the anaemia previously described by us is "merely a symptom coincidental with cobalt deficiency" it must be pointed out that none of our experimental work on ataxia has been carried out on the calcareous littoral where a complicating deficiency of cobalt does occur. There is no clinical evidence of cobalt deficiency at Gingin. The cobalt content of 5 sheeps' livers from Gingin was 0.06, 0.10, 0.12, 0.13, and 0.17; mean 0.12 p.p.m. on dry basis. These results show no evidence of cobalt deficiency. Further, the anaemia described by Marston *et al.* (1938) differs in many respects, and the anaemia described by Filmer (1933) from an area of uncomplicated cobalt deficiency is of an entirely different type to that described by us.

4. Haemosiderosis of Ewes.

Ewes that had shown marked clinical evidence of copper deficiency showed at autopsy no constant changes except some evidence of anaemia and malnutrition and the presence of haemosiderosis. The occurrence of haemosiderin was investigated in six experimental ewes from Gingin. Nos. W27 and W28, which were very anaemic, in poor condition, and had given birth to ataxic lambs, were killed in June and July, 1937.

* A Hb. value of 6.6 was obtained in October, 1941, from a wether maintained for the previous four years on the very Cu deficient pastures of the Jindong Experimental Farm.

Blood copper in both cases was low. The most marked haemosiderosis was noted in these ewes; in both cases heavy deposits were present in the liver and kidney, particularly in the proximal convoluted tubules. The spleen of W27 showed extensive deposits of haemosiderin, but there was little in W28.

Four were killed in 1939. No. 133 reared a healthy lamb in the third successive lambing at Gingin; it had been very anaemic and blood copper had been low but at the moment of slaughter haemoglobin and blood copper figures were "normal" although it was still in poor condition. No. 278 was the mother of an ataxic lamb but was not anaemic and in fair condition. No. 250 had shown no anaemia, had reared a normal lamb, and was in good condition when killed; blood copper, however, had been very low for three months before death. No. 271 reared a healthy lamb and had shown no evidence of anaemia or low blood copper values throughout the period of observation; it was in good condition when killed.

The occurrence of haemosiderin in the four ewes examined in 1939 was very variable. No. 278 showed a moderate quantity in the liver only; Nos. 250 and 271 showed large amounts in the spleen only, and No. 133 showed very large amounts in the kidney, moderate amounts in the liver, and a small amount in the spleen.

In two ewes from Jindong (Nos. 254 and 236) which were mothers of ataxic lambs, haemosiderin was very abundant in the liver and kidney but there were only slight deposits in the spleen.

In none of the cases examined was there any evidence of haemosiderin in the pancreas.

In these animals there appeared to be some correlation between the incidence of haemosiderin and the condition of the blood. Haemosiderin was most abundant, particularly in the liver and kidney, in subjects that had been affected with an anaemia of some months duration associated with low blood copper figures.

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numbered from 71 onwards.

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71. Investigations on Irrigated Pastures
72. Varieties of Wheat in Australia
73. A Soil Survey of the Nyah, Tresco, Tresco West, Kangaroo Lake (Vic.), and Goodnight (N.S.W.) Settlements
74. Observations on Soil Moisture and Water Tables in an Irrigated Soil at Griffith, N.S.W.
75. *Nigrospora Musae* n.sp. and its Connexion with "Squirter" Disease in Bananas
76. A Soil Survey of the Hundreds of Laffer and Willalooka, South Australia
77. Studies on the Phosphorus Requirements of Sheep.—I.
78. Methods for the Identification of the Light-coloured Woods of the Genus *Eucalyptus*
79. The "Lucerne Flea" *Smynturus viridis* L. (Collembola) in Australia
80. The Establishment, Persistency, and Productivity of Selected Pasture Species on an Irrigated Reclaimed Swamp
81. A Comparative Study of *Lolium perenne* and *Phalaris tuberosa* at Varying Stages of Growth
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83. Natural Pastures: Their Response to Superphosphate
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85. Studies on the Phosphorus Requirements of Sheep.—II. (*Out of print*)
86. A Soil Survey of the Berri, Cobdogla, Kingston, and Moorook Irrigation Areas, and of the Lyrup Village District, South Australia
87. Radio Research Board: Report No. 6
88. Radio Research Board: Report No. 7
89. Radio Research Board: Report No. 8
90. The Identification of the Principal Commercial Australian Timbers other than Eucalypts
91. Further Investigations into the Transport of Bananas in Australia
92. The Apple-growing Soils of Tasmania. Part 1: A General Investigation of the Soils. Part 2: A Soil Survey of Part of the Huonville District.
93. Studies on Contagious Pleuro-Pneumonia of Cattle.—I.
94. Fertility in Sheep: Artificial Production of Seminal Ejaculation and the Characters of the Spermatozoa contained therein
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96. Observations on Myxomatosis Cuniculi (Sanarelli) made with a View to the Use of the Virus in the Control of Rabbit Plagues
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108. The Basaltic Soils of Northern Tasmania

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143. Production of Dried Grapes in Murray Valley Irrigation Settlements. 1.—Viticulture.
144. Interference in a Wind-Tunnel of Octagonal Section.
145. Friction and Lubrication: Report No. 1.
146. An Analysis of the Outbreaks of the Australian Plague Locust (*Chortoicetes terminifera* Walk.) during the Seasons 1937-38 and 1938-39
147. Enzootic Ataxia and Copper Deficiency of Sheep in Western Australia

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"FALLING DISEASE" OF CATTLE IN THE SOUTH-WEST
OF WESTERN AUSTRALIA. 2.

STUDIES OF COPPER DEFICIENCY IN CATTLE.

By H. W. BENNETTS, D.V.Sc., A. B. BECK, M.Sc.,
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STUDIES OF COPPER DEFICIENCY IN CATTLE.

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Introduction.

A PREVIOUS communication by Bennetts and Hall (1939) gave an account of a disease of cattle known popularly as “falling disease”, this name being retained pending the determination of ætiology.

The disease is enzootic in the south-western corner of this State,* and is characterized by a seasonal occurrence and by sudden death almost invariably without premonitory signs. Glomerulo-nephritis and marked hæmosiderosis are regarded as pathognomonic. The disease was shown to be associated with the occurrence of a severe anæmia in affected herds during the season of incidence. A low copper status of animals and pastures was also demonstrated. The anæmia resembled that occurring in copper deficient lambing ewes as described by Bennetts and Chapman (1937), and a preliminary experiment showed that a “complete” mineral supplement containing copper prevented its occurrence.

Although on *prima facie* evidence copper deficiency could not be regarded as the actual cause of death it was considered that it might be a factor contributing to the ætiology of “falling disease”. Controlled experiments were designed to throw light on the possible ætiological part played by mineral deficiency and the ingestion of a high proportion of clover, notably *Trifolium cernuum*.

Owing to the absence of “falling disease” in the experimental herds during the past three seasons it has not been possible to relate its occurrence to copper deficiency under experimental conditions. During 1939 and 1940, however, the results of the extensive administration of copper-containing supplements in the affected area in association with experimental data provide strong presumptive evidence that this deficiency is a factor predisposing to “falling disease”. Information from a number of herds, kept under close observation for several seasons, indicates that copper supplements, apart from preventing the occurrence of the disease, have significantly improved the health, production and fertility of the milkers and have exerted a favourable influence on the growth of young stock.

The ætiology of the disease, however, has not been elucidated further and is still being investigated.

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* Note.—“Falling Disease” has been reported from King Island by Dumaresq (1939), pathological identity being confirmed by the examination of specimens kindly provided by him.

"FALLING DISEASE" OF CATTLE.

The purpose of the present article is to present data relating to copper deficiency in cattle, which have been obtained from experimental and commercial herds during the course of the investigation. In view of the paucity of published data on naturally occurring uncomplicated copper deficiency in cattle it was thought that this information might be of assistance to other workers.

Clinical Appearances.

Observations, over a four-years period, on a large number of dairy herds in the copper deficient areas of the south-west have resulted in the recognition of a syndrome attributed to an inadequate intake of copper. The evidence for this conclusion is the constant demonstration of a very low copper content of pastures, a low copper status (blood and liver) of animals in affected herds and the response to copper alone or in association with other supplements, notably limonite. The possibility that a mineral deficiency other than copper may be contributory has not been entirely eliminated, but experimental evidence indicates that the response to copper alone is as good as that obtained from it in combination with Co, Zn, Mn, Ni and Fe. The cobalt status of animals in the area is being investigated and will be reported on later.

The clinical symptoms in adult cattle are especially evident during the flush growth of pastures in from August to October, when, despite this, there is some loss in condition, a rough staring coat and evidence of anæmia. Depraved appetite is common and is manifested, generally, by ingestion of subsoil adhering to roots and butts of upturned trees. The syndrome somewhat resembles that described by Sjollem (1938) and Brouwer *et al.* (1938) observed in copper deficient areas in Holland, but diarrhœa, although sometimes present, cannot be regarded as characteristic of the disease in Western Australia. There is also evidence that the suppression of œstrum and temporary sterility are associated with the deficiency here. Difficulty is frequently experienced in getting cows in calf at the customary time (April-July). Many cows served during the autumn and winter do not conceive and œstrum is suppressed until November, when conception readily occurs and the anæmia and other signs also tend to disappear.

In calves growth is suboptimal and they develop marked appearances of malnutrition. The limbs show abnormalities; pasterns are straight and calves tend to stand up on their toes. Bony prominences are noted just above the metatarso-tarsal joint and to a less extent on the metacarpo-carpal joints particularly on the lateral aspects. These resolve as animals grow older. Diarrhœa and anæmia are commonly present. The animals grow very slowly and have a very stunted, undeveloped and unthrifty appearance. On some holdings they cannot be reared into profitable members of the herd and are destroyed. Maximum size is not attained in adults on badly affected properties and each successive generation bred tends to be smaller in frame.

Pathology.

General.—The pathology has been studied only incompletely. Observations on adults have been confined largely to cows dead of "falling disease" and to organs obtained from animals killed for meat.

A number of affected calves and yearlings, however, have been slaughtered for post-mortem examination. No definite changes have been encountered;

"FALLING DISEASE" OF CATTLE.

a degree of hæmosiderosis, especially marked in the liver, kidney and spleen of animals dead of "falling disease", is, however, generally present.

The nature of the processes affecting the limbs in young animals has not yet been studied in detail.

Hæmatology.—(i) Source of Material: From 1937 to the present time periodical hæmoglobin surveys of cows from affected private herds and regular four-weekly hæmatological examinations of cows on an experimental property at Jindong have been carried out. A summary of the data from the experimental herd is presented.

More detailed determinations were made from time to time on anæmic animals in the various herds under review. Values obtained from these and from healthy cows are compared in Table 1.

Systematic investigations have been confined largely to milking cows, but there is abundant evidence that non-lactating cows as well as calves and yearlings may show a severe degree of anæmia.

(ii) The Anæmia of Cattle: The anæmia, as exemplified later, is of seasonal occurrence, being especially prevalent in September and October. It is associated with low blood copper values, although as in the case of sheep low values may obtain without a corresponding fall in hæmoglobin. During the summer months hæmoglobin values may be normal without any corresponding rise in blood copper. Copper supplements administered regularly are prophylactic. The anæmia, briefly referred to in the previous communication, where methods were also given, may now be described in more detail.

Hæmoglobin is considerably decreased, frequently to below 6.0 g. *per cent.*, while values in the vicinity of 4.0 g. are not uncommon. Values of the same order have been obtained in young stock also. There is a corresponding reduction in red cell count, values from 1.9–3.5 millions per c. mm. being frequently encountered. In smears the changes observed are anisocytosis, macrocytes being common, poikilocytosis, polychromasia, punctate basophilia; occasional Jolly bodies and rarely normoblasts are present. Reticulocytes were not numerous in the few cases examined, the highest count obtained being 1.4 *per cent.*

A comparison of values obtained from 12 anæmic cows* from herds in the affected Busselton-Margaret River areas and from 10 healthy cows from unaffected districts is made in Table 1.

The results obtained clearly indicate that in anæmic animals the mean cell volume (M.C.V.) value is constantly considerably higher than normal. The colour count ratio (C.C.R.) also tends to be higher, generally significantly so. The cell volume *per cent.* (C.V.%) and colour volume ratio (C.V.R.) values, however, are almost constantly below the normal range. In addition to the values tabulated, cell fragility tests were carried out on a number of subjects, a wide range of values being obtained from both healthy and anæmic bloods. The Van den Bergh test was completely negative in the case of seven anæmic subjects tested.

No abnormal leucocytes were encountered in smears. White cell counts gave values of 2,700 to 6,400, mean 3,800, these being somewhat lower than those given by Frazer (1929-30) for normal dairy cows.

* Animals available for these more complete hæmatological examinations, unfortunately, did not include any with the extremely low hæmoglobin figures referred to above.

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The anæmia described is of the macrocytic hypochromic type, for although the C.C.R. tends to be high, erythrocytes are incompletely saturated with hæmoglobin.

TABLE I.
Comparative Blood Values for "Normal" and "Affected" Dairy Cows Three to Eight Years of Age.

	Hb. Gm. %.	R.B.C. 10 ⁹ c.mm.	C.V. %	M.C.V. CCL.	C.C.R.	C.V.R.	Bld. Cu. Mg. %.
<i>Normal values :</i>							
Range ..	11.0-16.7*	5.6-9.4*	30.5-46.3	46.7-55.9	1.7-2.1	0.36-0.41	0.07-0.17**
Mean ..	13.3±0.65	7.2±0.41	36.1±1.9	50.6±0.9	1.86±0.07	0.37	—
<i>Anæmic cows ex affected area :</i>							
	7.0	3.6	27.5	76.4	2.0	0.25	0.01
	6.9	2.4	25.0	104.1	2.9	0.28	0.01
	—	2.4	28.8	120.0	—	—	—
	7.8	3.5	27.5	78.6	2.2	0.28	0.01
	4.6	2.4	17.5	72.9	1.9	0.26	0.01
	5.9	2.0	22.5	112.5	3.0	0.26	0.01
	6.5	2.8	22.5	80.4	2.8	0.29	0.01
	6.8	2.7	18.0	66.7	2.5	0.38	0.02
	6.2	2.4	21.3	88.8	2.6	0.29	0.02
	7.7	2.7	23.8	88.2	2.9	0.32	0.01
	7.9	3.2	25.0	78.6	2.5	0.32	0.02
	6.8	3.1	22.5	72.6	2.2	0.30	0.02
Range ..	4.6-7.4	2.0-3.6	17.5-28.8	66.7-120.0	1.9-3.0	0.25-0.38	0.01-0.02
Mean ..	6.7±0.29	2.8±0.14	23.5±1.0	86.7±4.96	2.5±0.11	0.29±0.01	—

* Representative of a larger series.

** Beck (1941c).

$$\text{C.C.R.} = \frac{\text{Hb.}}{\text{R.B.C.}}$$

$$\text{C.V.R.} = \frac{\text{Hb.}}{\text{C.V.}}$$

Experimental.

The Seasonal Variation in Blood Values and the Response to Copper Supplements in Experimental Dairy Cows.

Data were collected from an experimental herd used for the purpose of investigating "falling disease", and these are now briefly reviewed.

A property at Jindong, which had a history of "falling disease", was made available entirely for experimental purposes by officers of the Agricultural Bank who also supplied cows, derived from the affected area, and other facilities.

In 1938, 1939 and 1940 cows matched on hæmoglobin and, in the latter two seasons, on blood copper values also, by the use of a table of random sample numbers (Tippett, 1927), were allocated to groups designed to determine the effects of different mineral supplements particularly as regards blood values and the incidence of "falling disease".

In the last two seasons' experiments controlled groups comprised only cows which had been originally in groups not receiving supplements containing copper. Mineral supplements* were given as a daily drench (20 ml. solution). Four-weekly individual hæmoglobin and group red cell counts were carried out throughout the year and in the last two seasons regular blood copper determinations also, the trichloroacetic acid method of Tompsett (1934) being used. In a number of cases where values were low, copper was estimated by a dry ashing method using magnesium nitrate.

1938 Season.—Two groups of five cows each were carried: (1) a control group, and (2) a test group which from February to December received

* The minerals were given in the form of Ferrous sulphate, Cupric sulphate, Cobaltous chloride, Manganous sulphate, Nickel sulphate and Zinc sulphate. "Pure" grades were used.

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mineral salts to give a daily intake of Fe 250 mg., Cu 15 mg., Co 5 mg., Zn 10 mg., Mn 25 mg., and Ni 15 mg.

Analyses of blood figures obtained showed a sudden drop in hæmoglobin and R.B.C. values in the control group from normal in September to low in October with improvement in November and a return to normal in December; values for the test group remained normal throughout. In October mean and range values for hæmoglobin and group red cell counts were as follows:

Control Group.—October: Hb. 8.6 (6.9-11.0), R.B.C. 3.8. December: Hb. 11.7 (9.7-13.0).

Test Group.—October: Hb. 12.6 (11.0-13.2), R.B.C. 6.3. December: Hb. 14.6 (13.8-15.0).

Although the mineral supplement had, apparently, prevented the appearance of anæmia in the test group, low blood copper values were obtained; for example, November values were: controls 0.01-0.02 mg. *per cent.*, test group 0.02-0.03 *per cent.*, indicating that the rate of copper supplementation was suboptimal.

1939 Season.—A further experiment was carried out in order to separate the effects of minerals and to test the efficacy of copper alone. In view of marked hæmosiderosis common to cows in the area, and because of other evidence that the iron intake was adequate, Fe was omitted. The copper dosage was increased to 60 mg. daily. With these exceptions minerals were given in the same amounts as previously. Supplements were not commenced until May 1 and were discontinued in December.

Four groups of five cows each were allocated as follows:

Group 1: Controls.

Group 2: "Complete" mineral supplement (Cu, Co, Zn, Mn, Ni).

Group 3: "Complete" mineral supplement minus copper.

Group 4: Copper alone.

In groups 1 and 3 not receiving copper a definite drop in hæmoglobin and R.B.C. values was noted in October with a return to normal in December; this was more marked in group 1 than in group 3.

Blood copper values were low for all groups at the beginning of the experiment. Values fell still lower in groups not receiving copper, whereas in copper-treated groups values of over 0.05 mg. *per cent.* were reached in almost all cases by July, and thereafter good values were maintained throughout. In October the range values were as follows:

Group 1 = 0.01-0.02 mg. *per cent.*

Group 2 = 0.06-0.08 mg. *per cent.*

Group 3 = 0.01-0.02 mg. *per cent.*

Group 4 = 0.06-0.10 mg. *per cent.*

It appeared evident, therefore, that an intake of copper at the rate of 60 mg. daily both prevented anæmia and was adequate for the maintenance of normal blood copper values. Cows in group 2 were in no respect better than those in group 4, although on appearance cows in both groups were healthier than those in groups not receiving copper.

In view of this evidence that copper alone promoted optimal response the possible influence of the other minerals was not investigated further.

1940 Season.—The experimental herd was divided into two groups of seven cows each—one to serve as a control and the other to receive the copper supplement. The daily dose of copper for the test group was increased to 100 mg. Drenching commenced in May.

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The results obtained from the experiment confirmed the previous findings that a pure (B.D.H., A.R.) copper supplement prevented anæmia, maintained good blood copper values and, apparently, promoted optimal health in an experimental dairy herd on a representative property in the affected area.

The results are summarized in Table 2 where it is shown that the hæmoglobin and R.B.C. values were significantly lower in the control group during the period from September to November. Very low blood copper values were recorded in this group from September onwards, yet despite maintenance of these low figures the hæmoglobin and R.B.C. values show marked improvement during December (January, 1941 values are of the same order in both groups).

A similar seasonal improvement during the late spring and early summer months has also been noted by Bennetts and Beck (1941) in sheep depastured on copper-deficient areas north of Perth, but in neither case can any satisfactory explanation be offered.

Liver Copper.

A number of livers from cattle from the known copper-deficient areas in the south-west (the Busselton-Margaret River District, Northcliffe and Walpole), as well as from healthy areas in Western Australia, have been analysed by a modification of the method of Sylvester and Lampitt (1935), either dry ashing (Van Neikerk, 1937) or wet ashing (Piper, 1939) being used.

The results obtained clearly indicate a low liver copper status of cattle in the affected area, although our normal values are higher than 70 p.p.m., the value given by Cunningham (1931) for the adult bovine.

The following values were obtained in this State:

Affected Areas: 6 adult cows dead of "falling disease"; 1-6, mean 2.1 p.p.m. Cu (dry basis); 4 adult cows, 1 yearling and 5 calves; 1-5, mean 3.7 p.p.m. Cu (dry basis).

Non-affected Areas: 6 healthy cows from 5 districts; 37-231*, mean 122 p.p.m. Cu (dry basis).

In addition, values of the order found in cattle in non-affected areas were obtained from seven bovines (cows, calves and yearlings) affected with a variety of pathological conditions.

Bennetts and Beck (1941) have recorded very low copper values (2.9-4.4 p.p.m.) for sheep from the affected south-west areas including the experimental property at Jindong.

Milk Copper.

Beck (1941a) has published elsewhere data from Jindong and another property in the same district showing that the milk copper level is also reduced. Values down to 0.01 to 0.02 mg. copper per litre were obtained in most cases, whereas values for healthy districts generally lay between 0.05 and 0.20 mg. per litre.

Pasture Copper.

A large number of pasture samples, including mixed pasture and in some instances the single plant species *Cryptostemma calendulacum* and *Trifolium cernuum*, were collected from affected properties throughout the south-west

* 37 = only result below 94 p.p.m.

TABLE 2.
Seasonal Variation in Hb., Blood Cu. and R.B.C. Values in Dairy Cows with and without Copper Supplements (1940).

Group.	—	April.	May.	June.	July.	August.	September.	October.	November.	December.
Control.	Hb.:									
	Range	10.4-11.4	9.2-11.4	8.9-10.6	9.8-11.5	8.6-11.1	4.7-8.9	5.6-10.2	9.0-13.1	9.4-14.0
	Mean	10.9±0.19	10.4±0.27	9.9±0.20	10.5±0.26	10.1±0.38	5.9±0.59	7.6±0.57	10.3±0.58	11.9±0.76
	Bld. Cu.:									
	Range	0.01-0.04	0.03-0.06	0.02-0.04	0.02-0.04	0.02-0.03	0.01-0.04	<0.01-0.01	<0.01	0.01
R.B.C.:										
Group count ..	—	5.3	5.0	5.6	5.8	2.4	2.4	3.6	5.8	
Test Group. (Cu. 100 gm. daily from May 17th.)	Hb.:									
	Range	10.6-15.5	9.6-16.6	9.6-15.8	10.2-15.0	10.1-14.5	8.1-12.2	9.8-16.1	11.9-16.3	13.5-16.5
	Mean	12.0±0.75	11.6±0.90	11.7±0.83	11.8±0.64	11.4±0.62	10.3±0.50	12.0±0.85	13.6±0.57	15.3±0.47
	Bld. Cu.:									
	Range	0.03-0.05	0.03-0.07	0.03-0.06	0.06-0.08	0.06-0.08	0.06-0.10	0.08-0.09	0.06-0.07	0.06-0.08
R.B.C.:										
Group count ..	—	6.0	5.5	6.4	5.9	4.4	5.3	5.1	6.8	

Hb. gm. per cent.

Blood Cu. in mg. per cent.

R.B.C.=group red cell counts in 10⁴ per c.mm.

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and were analysed by the method referred to above using the dry ashing technique. Very low values were obtained constantly, the range being from 1.1 to 3.2 p.p.m. Cu dry basis. Values for several samples from the experimental property at Jindong were all below 2 p.p.m.

The results of a survey of the agricultural areas of this State, carried out by Beck (1941b), show that ordinary mixed pastures from areas where stock remain healthy contain between 5 and 15 p.p.m. of Cu.

Experimental work carried out by Elliot (1939) indicates that the "stalling" of Subterranean clover and its replacement by *T. cernuum*, a phenomenon characteristic of the "falling disease" area, is associated with the problem of copper deficiency.

Summary.

1. A very low copper status of pastures and of animals is constantly associated with the occurrence of "falling" disease. The aetiology of the disease remains obscure, but the investigation of the rôle played by copper has led to the accumulation of experimental and other data relative to what appears to be an uncomplicated copper deficiency disease of cattle in the south-west of this State.

2. Clinically the manifestations of copper deficiency are malnutrition, anæmia, a depraved appetite, and in cows frequently as temporary sterility. Young animals show marked evidences of malnutrition and abnormal development; intermittent diarrhœa and anæmia are frequently present.

3. The pathology of this disease has been studied only incompletely. The most marked features are anæmia and hæmosiderosis, the anæmia in cows being of the macrocytic, hypochromic type.

4. In an experimental dairy herd optimal response was obtained by the administration of pure copper supplements; the addition of other minerals had no appreciably beneficial effect.

5. Under field conditions the widespread use of mineral supplements containing copper has resulted in a marked improvement in health and production of the entire herds, and apparently in the prevention of any occurrence of "falling disease" in cows receiving such supplements.

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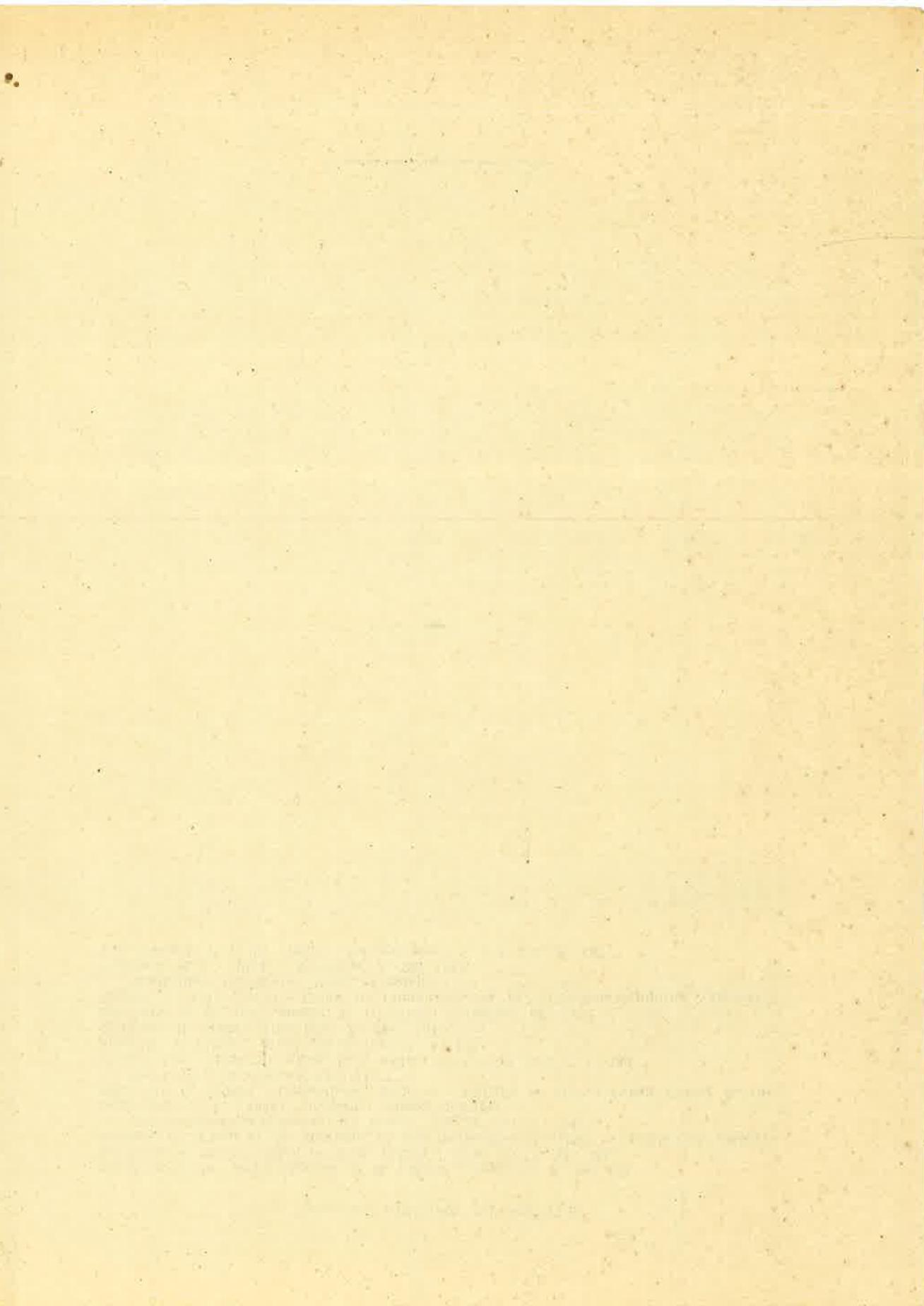
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THE PATHOGENESIS OF "FALLING DISEASE."

STUDIES ON COPPER DEFICIENCY IN CATTLE.

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Introduction.

A condition known popularly as "falling disease" and which is characterized, clinically, by sudden death, has been described by Bennetts and Hall (1939). Subsequent investigations reported by Bennetts *et al.* (1941 and 1942) showed that the disease was constantly associated with a very low copper status of the pastures and of the animals, and that its occurrence could be prevented by the use of copper supplements. The low copper status of dairy cows in the affected region resulted also in seasonal anæmia, a high incidence of infertility, decreased production, and retarded growth and development of young stock. Experimental and field observations showed that these manifestations also were controllable by copper supplements.

Since these investigations were reported, the use of copper fertilizers has become almost universal throughout the affected region. This has resulted not only in the complete control of "falling disease," but also in marked improvement in the quality and bulk of the pastures as reported by Jones and Elliott (1944), and also in a great increase in butter-fat production.

In the earlier papers it was claimed that "falling disease" was a terminal manifestation of severe copper deficiency. Death was attributed to heart failure resulting from myocardial fibrosis, which lesion was almost constantly found in fatal cases and was commonly present in non-fatal cases of copper deficiency.

It was suggested that the heart lesion was essentially a starvation atrophy of myocardial tissue due to the direct or indirect effects of copper deficiency or both, with replacement of this highly specialized tissue by the less specialized fibrous tissue. On the evidence available, however, no complete and well-considered account

of the pathogenesis of "falling disease" could be given.

The purpose of this paper is to describe the results of an experiment which was designed primarily to throw further light on the development of the heart lesion in bovines maintained on an "affected" property until slaughtered for pathological examination. One of us (H. W. B.) was responsible for the general direction and pathological studies in the investigation, one of us (A. B. B.) for the chemical and biochemical studies, and the other (R. H.) for the field studies.

Experimental Animals. Grouping and Management.

The location of the experiment was the property at Jindong, in the Busselton District, where the previous investigations had been carried out, and which, since 1938, had been available entirely for experimental purposes. As reported earlier the copper status of these pastures was consistently below 3 p.p.m., D.B.

The experimental animals comprised two groups, which may be described as follows:—

Herd 1.—This consisted of 14 female calves, six of which were the progeny of cows in Herd 2, born at Jindong between 20/10/41 and 2/5/42. The remaining eight were purchased as available from suitable herds in the neighbouring districts and were transferred to Jindong, when only a few days old, during the period 20/6/42 to 16/9/42. There was therefore a considerable variation in the ages of the experimental animals, but this was unavoidable. This group was used primarily to study the progressive development of cardiac lesions. They were slaughtered for pathological examination at intervals during the period from 1/4/43 to 20/3/46, when the experiment was concluded.

One animal died early in the experiment from an undetermined cause, and two others died of "falling disease," one in 1945 and one in 1946.

Herd 2.—This consisted of 14 cows retained as a producing herd. Initially the herd included ten cows remaining from the experiments concluded in 1941 and reported by Bennetts *et al.* (1942). Of these animals, seven derived from the copper group and three from the "control" group which had received no supplement throughout; these three animals subsequently died of "falling disease."

This herd is of importance to the present discussion principally because electrocardiographic studies

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TABLE 1.

A Summary of Data on Experimental Animals, Herd 1. Animals Arranged According to Length of Time on Jindong Pastures.

Identification No. of Animal	Age at Death.	Date, Birth and Source.	Date of Death.	Histopathology.			Inorganic Constituents of Tissues.						
				Heart.	"Hæmosiderin"		Parts per million—dry basis.						
							Tissue	Cu	Co	Fe	Ni	Zn	Pb
9	9 months	18/6/42 Rosa Brook	1/4/43 Killed for examination	Occasional small areas of cellular infiltration throughout.	Liver Kidney Spleen	+ — ++	Liver	10	0.09	830	—	—	—
4	10 months	9/2/42 Born Jindong	28/12/42 Died	No specimens obtained.			—	—	—	—	—	—	—
3	1 year 5 months	20/10/41 Born Jindong	1/4/43 Killed for examination	Normal.	Liver Kidney Spleen	+ — ++	Liver	4.3	0.07	1,800	—	—	—
2	1 year 5 months	24/10/41 Born Jindong	1/4/43 Killed for examination	Occasional areas of cellular infiltration throughout.	Liver Kidney Spleen	+++ ++ +++	Liver	5.3	0.08	2,860	—	—	—
1	2 years	19/11/41 Born Jindong	24/11/43 Killed for examination	Occasional small areas of cellular infiltration throughout—fibrous tissue replacement in three sections, one involving main conduction branch in R.V.	Liver Kidney Spleen	— + ++	Liver	2.1	0.07	370	ca 0.05	90	1.5
5	2 years	26/11/41 Born Jindong	24/11/43 Killed for examination	Very occasional small areas of cellular infiltration.	Liver Kidney Spleen	++ +++ +	Liver	2.3	0.09	1,230	ca 0.05	80	1
13	2 years 4 months	1/5/42 Cowaramup	21/9/44 Died Falling Disease (Calved 18/9/44)	Small areas of fibrosis. Passive congestion.	Kidney	+++	No Samples Available.						
7	2 years 4 months	1/5/42 Manjimup	6/9/44 Killed for examination	Occasional small areas of cellular infiltration throughout.	Kidney Spleen	+++ +++	Liver	1.4	0.05	3,100	—	—	—
8	2 years 4 months	2/5/42 Manjimup	6/9/44 Killed for examination	Occasional small areas of cellular infiltration throughout.			Liver	2.4	0.05	3,400	—	—	—
14	3 years 5 months	1/5/42 Cowaramup	24/10/45 Killed for examination	Extensive fibrosis L.V. chiefly affected. Main branches of conduction system free, but some Purkinje fibres affected.	Liver Kidney Spleen	++ + +	Liver Heart	1.8 11.0	0.07 —	7,500 210	— —	120 —	2 —
10	3 years 5 months	1/5/42 Margaret River	23/10/45 Died Falling Disease (in calf)	Extensive gross fibrosis throughout. Conduction system involved. Passive congestion.	Liver Kidney	— —	Liver	2.9	0.04	1,400	—	—	—
11	3 years 3 months	25/7/42 Margaret River	25/10/45 Killed for examination	Small areas fibrosis and cellular infiltration throughout.	Liver Kidney Spleen	+ + +++	Liver Heart	2.0 11.0	0.10 —	2,320 180	ca 0.05	— —	— —
12	3 years 5 months	8/9/42 Rosa Brook	20/3/46 Killed for examination	Gross and extensive fibrosis (recognisable macroscopically) L.V. particularly affected.	Liver Kidney Spleen	++ — +++	Liver Heart	5.7 11.9	0.14 —	2,400 210	<0.05 —	170 —	1 —
6	3 years 9 months	10/6/42 Manjimup	20/3/46 Killed for examination	Fibrosis widely distributed in L.V.—R.V. little affected. Left conduction system involved.	Liver Kidney Spleen	++ — +++	Liver Heart	5.5 12.7	0.14 —	2,400 190	— —	— —	— —

were carried out on cows from the original copper and control groups.

All cows were bled several times a year for hæmoglobin and blood-copper determinations until July, 1944, after which only occasional examinations were made.

A small flock of sheep was also run on the property to study the effects of extreme copper deficiency on these animals. Biochemical data on the livers of some of these animals are included in this paper to confirm the conclusion that copper deficiency is the only significant abnormality in the "falling disease" areas.

Pathological Studies.

Specimens of heart, liver, kidney and spleen were obtained from eleven animals in Herd No. 1; these were slaughtered for examination at the times indicated in Table I. Tissues were fixed immediately in Lendrum's (1941) fixative for subsequent embedding in paraffin. Duplicate heart specimens were fixed in 10 per cent. neutral formal-saline solution so that staining for fat could be carried out on frozen sections.

From subjects Nos. 10 and 13 in Herd No. 1 and two of the cows (Polly and Kinsella) in Herd No. 2, which died of "falling disease," the complete heart and portions of kidneys were fixed in 10 per cent. formalin solution.

In all instances the same ten regions of the heart were sampled. Five sections were taken from each ventricle. These comprised, for each, a complete section of the ventricular wall at base and apex, and three sections to include the main branches of the conduction system, the distribution of which has been described by Cohn (1912-13), together with the adjacent heart muscle to a depth of approximately $\frac{1}{4}$ -inch below the endocardium.

The sections of the left and right divisions of the auriculo-ventricular bundle were taken near their site of entry into the respective ventricles; the left branches to the anterior and posterior muscle and the right branches to the septal and posterior papillary muscles were, in all instances, sectioned at the same selected position.

Paraffin sections of heart muscle were stained with hæmatoxylin and eosin for general histological examination, by Mallory's aniline blue stain for connective tissue, and by Krajian's (1940) modified silver method for reticular fibres. Frozen sections of heart muscle were in every instance stained with Sudan IV, and in some cases also with osmic acid and with Nile blue sulphate, for examination for evidence of fatty degeneration of muscle fibres.

Paraffin sections of kidney, liver and spleen were stained for hæmosiderin. Hæmatoxylin and eosin stained sections of kidneys were examined for evidence of the presence of the lesions described by Bennetts *et al.* (1942) as being characteristic of fatal cases of copper deficiency. These lesions were consistently absent in animals slaughtered for examination.

The occurrence of hæmosiderin is indicated in Table I where brief reference is made also to the cardiac findings which must now be discussed in more detail:—

Heart Lesions.—In the present investigations, it has been possible to follow the progressive changes and to make a more detailed study of the pathological histology than was practicable in fatal cases where the suddenness of death makes it very difficult to obtain material in good condition for examination.

The findings in this series are consistent with the views expressed in the earlier publications. It appears that the heart lesion is essentially an atrophy of heart muscle with a progressive replacement with fibrous tissue.

(a) *Macroscopic Appearances.*—As noted previously, the myocardium is paler than normal, has a parboiled appearance, and is soft and flabby suggesting considerable loss of muscular tone. In one fatal case (Heifer No. 10) the ventricle was mottled with lighter areas.

In one animal (Heifer No. 12) a gross fibrosis of the myocardium was obvious to the naked eye, but this was an exceptional finding.

(b) *Histopathology.*—There has been no evidence of degeneration or necrosis of muscle fibres in any of the specimens examined, and in frozen sections there was in no case any evidence of the presence of fat other than in the normal situations. In some regions, however, fibres or groups of fibres appeared to be atrophic, being rather shrunken with prominent lipochrome granules at the nuclear poles. In silver-stained sections patchy areas of varying size were observed where fibres, seen in cross section, were considerably reduced in diameter and were surrounded by a thickened argyrophil reticulum giving a much closer and more well-defined mesh than that seen in unaffected areas. It is not clear whether the thickening of the reticulum is due to increase in the number of reticular fibres or to contraction of existing fibres.

The replacement of atrophic muscle fibres appears to begin with small foci of infiltration with lymphocytes, histiocytes and fibroblasts either in the interstitial tissue or between individual muscle cells. This is followed by the separation of muscle fibres with delicate strands of collagenous fibres arborizing from the interstitial tissue or from the zones of cellular infiltration. These strands increase in extent and in coarseness until large or relatively large areas of myocardium are replaced by a mass of

collagenous tissue in which remnants of atrophic muscle fibres can be recognized.

It appears that the myocardial atrophy and fibrous tissue replacement is a progressive process. In general, the animals killed earlier showed little change apart from some indications of muscular atrophy, and the presence of occasional small foci of small round-celled infiltration, whereas those killed when three years or more old showed extensive and widespread lesions of fibrosis, the affected areas being at different stages of development, suggesting a progressive involvement.

It is notable that the degree of fibrosis of the myocardium, in the regions examined, was much greater in at least three of the animals killed for examination than in Heifer No. 13 which died of "falling disease." In this connection, we have previously reported very extensive lesions in other experimental animals slaughtered for examination.

In the specimens there was evidence of some involvement of the conduction system in four subjects, Nos. 1, 6, 10 and 14. In No. 1 there was a large area of dense fibrosis immediately below the main right branch near to its site of entry into the ventricle, but the Purkinjie fibres showed no abnormalities; in No. 6 one of the smaller branches in the depth of the wall of the left ventricle was surrounded by fibrous tissue and the Purkinjie fibres appeared atrophic; in No. 10, which died from "falling disease," the branch to the papillary muscle of the left ventricle appeared to be compressed by an extensive fibrotic lesion immediately below its course; in No. 14 the main branch to the left ventricle did not appear to be involved, but there was an extensive fibrosis in this region and one of the deeply situated Purkinjie fibres was surrounded by fibrous tissue.

No definite abnormalities were seen in the Purkinjie fibres throughout the series except that vacuolation was common, particularly in fibres constituting the main branches of the conduction system. Identical changes, however, were commonly seen also in normal tissues prepared by that same method; the vacuolation was therefore regarded as an artefact.

Although we have been unable to obtain any direct evidence on the point, it does appear possible that death, in some cases at least, follows involvement of the conduction system. On the other hand there is evidence that the aborizations of the Purkinjie network are more extensive in the ox than in man and some other

species of animals. The experiments of Alfredson and Sykes (1940) indicate that the bovine can, in consequence, tolerate interference with the conduction system to a much greater extent than other species; these workers have shown that the cutting of either left or right bundle branches produces only minor alteration in the normal electrocardiogram; on the other hand Sykes and Alfredson (1940) and Sykes and Moore (1942) showed that a ration low in potassium induced degenerative changes in the Purkinjie fibres of the calf's heart accompanied by alternations in the electrocardiogram.

It would appear, however, that heart block is a much less likely cause of death in the bovine than in man where, as stated by Boyd (1945), "any lesion which interferes with the conduction bundle in any part of its course may result in heart block."

Electrocardiographic Studies.

These studies were made possible by the interest of two Perth medical practitioners, Drs. H. S. Lucraft and C. Fortune, who, with a portable apparatus, made electrocardiographic records of the seven cows in Herd 2 at Jindong, and of three normal cows in the Claremont Hospital herd during September, 1942. All cows were aged (approximately 8-11 years).

The cows were bailed, and electrocardiograms taken at rest, no difficulty being experienced. The three standard leads were employed; namely, lead I, right fore leg and left fore leg; lead II, right fore leg and left hind leg; lead III, left fore leg and left hind leg.

Subsequently the electrocardiographic records were compared with those described by Alfredson and Sykes (1942) for a large number of normal bovines, using the same standard leads. These workers found the bovine electrocardiogram very variable and described four main types.

Our records, in every case, conformed to one of their normal types, the distribution being as follows:—

		Type according to Alfredson and Sykes.
"Normal" Cows, Claremont—	Cow No. 1	IA
	" " 2	IB
	" " 3	III
Original "Control" Group, Jindong. (No Cu supplement at any stage.)	Polly	III
	Kinsella	IB
	Hoppitt	IV
Original Copper Group, Jindong. (Cu supplement had been discontinued in Nov., 1941.)	Cows No. 1 and 2	IB
	Cow No. 3	II
	" " 4	IV

It is interesting to note the fate of the cows in the control group. Polly died of "falling disease" on 18th October, five weeks after the electrocardiograms were taken; Hoppitt died on November 14, and Kinsella in September, 1945, both from the same cause.

The hearts of Polly and Kinsella both showed extensive and long-standing myocardial fibrosis; that of Hoppitt was not examined. It seems surprising that the electrocardiograms of the first two cows particularly showed no departure from the recognized normal.

Biochemical Studies.

Although earlier studies had shown conclusively that the disease was associated with the low copper status of the animals concerned, it was considered desirable to confirm this finding with the present series of animals and to extend the work to some of the other "trace elements."

In an endeavour to find some more definite biochemical basis for the role of copper in the disease, it was considered that copper deficiency might interfere with synthesis of the vitamin B complex in the rumen. Follis *et al.* (1943) had reported myocardial necrosis in pigs following thiamin deficiency, and, although the lesions reported differed entirely from those occurring in "falling disease," it was considered worthwhile to investigate the thiamin status of the animals as indicated by blood pyruvate levels.

Analytical Methods.—The method of analysis of the tissues for Cu, Co, Fe, Ni, Zn, Pb was essentially as described by Beck (1945); slight modifications were necessary for samples high in iron.

The figures for "zinc" represent the dithizone-extractable metals after removal of copper (and bismuth), nickel and cobalt and will include lead, cadmium and thallium. However, as the samples contain only small amounts of lead and no cadmium, and as it is highly improbable that any thallium is present, it is considered that the "zinc" figures give a true indication of zinc levels. Lead was determined by the mixed colour dithizone method of Clifford and Wichmann (1936). Nickel was extracted by chloroform as the dimethylglyoxime complex and subsequently determined by the method of Rollet (1926).

Two methods were used for plasma-iron determinations. For some samples, the plasma was digested with nitric, sulphuric and perchloric acids; for others, the plasma proteins were first precipitated with trichloroacetic acid and allowed to stand twenty-four hours before filtering. Although satisfactory recoveries of added iron were obtained with the trichloroacetic acid method on Jindong blood samples it is now realized that this method is not reliable.

In both cases the iron was determined with dipyrityl. In samples from Jindong the total iron values were from 1.1 to 1.5 times greater than those obtained by the trichloroacetic acid method; in the case of the "normals" the ratio was over 2.

When taking blood samples for pyruvate determinations, care was taken to ensure that the animals had as little movement as possible both before and during sampling. The blood was taken from the jugular vein into a cooled syringe; 2 ml. were squirted into 18 ml. of an ice-cold tungstate precipitation mixture (Van Slyke and Hawkins, 1928). After standing on ice for about 15 minutes the mixture was filtered and estimated by the dinitrophenyl-hydrazine method of Klein (1941).

Results.—Throughout the experiments, hæmoglobin and blood copper values showed the same general trends as those reported in earlier experiments. In the producing cows (Herd II) very low blood Cu values, of the order of 0.1-0.2 mg. per l. were reached and maintained after 1 to 2 year's depasturage at Jindong. The seasonal fluctuation in hæmoglobin values was very definite throughout the period, values of about 7 g. per 100 ml. being common during the periods August to October.

In the young animals (Herd I) the initial blood Cu values were rather variable, due presumably to variation in source and liver storage of the calves, but by September, 1943, all animals showed very low values, the majority being below 0.2 mg. per l. Seasonal anæmia was not evident in this herd until the spring of 1944, when the eight survivors, during the first breeding season, showed hæmoglobin values of 6.3 to 8.9 g. per 100 ml. It seems evident from this and earlier data that pregnancy and maximum milk production are factors contributing to the seasonal anæmia; no anæmia was noted in non-producing animals showing very low blood Cu values during the spring of 1943.

The analysis of the liver samples is given in Tables 1 and 2.

As found in earlier investigations, the copper content of livers is extremely low. The values for heart copper were consistently higher.

The liver cobalt values are extremely variable and in some cases suggest a deficiency of this element, but as reported earlier, we have had no clinical evidence of cobalt deficiency at Jindong, and we were unable to show experimentally that cobalt supplements were of any demonstrable value. It seems evident therefore that cobalt deficiency is not a factor contributing to the pathogenesis of "falling disease."

TABLE 2.
Chemical Data on Livers of Sheep Grazing on Jindong.

Age of Sheep, etc.	Period on Jindong.	Parts per million, dry basis, Inorganic Constituents of Liver.					
		Cu	Co	Fe	Zn	Ni	Pb
Lamb, 6 months, still on ewe, good condition.	6 months	3.9	0.07	1,300	97	< 0.05	0.6
Mature Ewe from Gingin (Cu-deficient area), died pregnancy toxæmia, liver cont. 48% fat, results expressed on fat-free basis.	4.5 months	6.7	0.19	630	131	ca 0.1	1
Mature Ewe, slaughtered.	2.4 years	3.3	0.13	2,120	110	ca 0.05	2
Lamb of above, ataxic, age 8 weeks.	8 weeks	1.6	0.03	660	50	< 0.05	1
Mature Ewe, slaughtered.	2.4 years	2.0	0.15	6,700	70	ca 0.05	2
Lamb of above, ataxic, age 6 weeks.	6 weeks	2.0	0.06	280	95	< 0.05	1
Mature Wether, slaughtered.	3.3 years	3.8	0.04	14,400	110	0.38	3

There seems to be no definite correlation between the histologically assessed degree of hæmosiderosis and total iron content of the liver.

The determinations for lead content were made because of the demonstration of high lead values in "sway-back" sheep in England (Innes and Shearer, 1940). The values are much lower than those obtained by the English workers and give no suggestion that lead is in any way involved in "falling disease."

Zinc levels were determined, as Smith and Larson (1946) have shown a possible interrelation between this element and copper. The liver samples, however, all show normal levels of zinc. This is in agreement with the earlier finding that zinc supplements had no significant effect on the prevention of the seasonal anæmia.

No explanation can be offered for the high nickel level obtained in the liver of the wether. The possibility of contamination of the sample prior to analysis cannot be ruled out, but we do not consider this likely.

It has been shown (Dick and Bull, 1945) that a high intake of molybdenum may cause a decreased copper storage in sheep. It is intended to carry out molybdenum determinations on "falling disease" pastures at a later date, but as the soils of the area are highly leached and acidic, it is probable that extremely low levels will be found.

The values obtained for plasma iron are given in the following table:—

TABLE 3.

Date.	Herd No.	No. of Analyses	Mean	Range	Method of Analysis
21/9/43	1	10	1.7	1.0-2.2	Cl ₂ CCOOH
21/9/43	1	4	2.3	1.8-2.5	Wet digest
19/10/43	2	10	1.5	0.9-2.3	Cl ₂ CCOOH
19/10/43	1	10	1.5	0.9-2.1	Cl ₂ CCOOH
5/9/44	1	8	1.5	0.9-1.9	Cl ₂ CCOOH
5/9/44	2	6	1.6	1.3-2.0	Cl ₂ CCOOH
12/4/48	"Normals"	10	1.1	0.7-1.5	Cl ₂ CCOOH
12/4/48	"Normals"	5	2.4	2.0-3.2	Wet digest

Although the methods used for analysis were not entirely satisfactory, the results show that there was no very great increase in plasma iron as might be expected from the high levels of iron in the liver.

In 1943 the pyruvate determinations were not done until after the hæmoglobin values had returned to normal so the test was repeated in 1944. The results set out in the following table show that all animals had normal pyruvate levels.

TABLE 4.
Blood Pyruvate Values for Normal and Copper Deficient Cows (mg. pyruvic acid per 100 ml. blood).

Normal.*	Copper-deficient cows.	Jindong.†
23/10/43.	25/10/43.	6/9/44.
0.87‡	0.90‡	0.60
0.71	0.66	0.81‡
0.79	0.61	0.46
0.63	0.59	0.60
0.53	0.66	0.60
0.70‡	0.73	0.52
		0.84
		0.56

*Claremont herd: Inadequate facilities were available for taking the samples and generally the animals moved about more than those at Jindong. Hæmoglobin determinations were not done.

†Hæmoglobin values—reading from top of the columns:—

1943—10.8, 9.6, 12.0, 10.6, 10.3, 12.1 gm. per 100 ml.
1944—6.4, 7.7, 6.3, 7.7, 7.7, 9.1, 6.3, 7.8 gm. per 100 ml.
‡These animals struggled slightly.

Discussion.

As a result of investigations completed earlier it was claimed that "falling disease" was a terminal manifestation of severe copper deficiency, that the essential lesion was atrophy of the myocardium with replacement fibrosis, and that the sudden death, which clinically characterises the disease, resulted from heart failure.

The first point had been clearly established. The complete control which has resulted from the use of copper supplements has provided further confirmation of the etiological significance of severe copper deficiency. The extremely low copper status of animals grazing "affected" pastures is again indicated in the further analyses in Tables 1 and 2.

It seems quite evident that a severe degree of deficiency is needed to induce the fatal termination, "falling disease." It has been encountered, in Western Australia, only in the most acutely deficient areas where the Cu values of the pastures lie below 3 p.p.m. and are generally of the order of 2 p.p.m., D.B. So far as we are aware no corresponding condition has been encountered in known copper deficient areas in other parts of the world except that Dumaresq (1942) reported and confirmed the occurrence of "falling disease" on King Island and Neal and Ahmann (1937) reported myocardial fibrosis in experimental cattle fed on forage from "salt sick" country in Florida. In both these last-mentioned areas, cobalt deficiency is a complicating factor, but we have given reasons for our belief that cobalt deficiency does not contribute to the pathogenesis of "falling disease" in Western Australia.

The more detailed study of the progressive development of the heart lesion in a series of experimental female animals, has confirmed the second claim. The nature of the lesion is essentially myocardial atrophy and replacement fibrosis. It is evident that the morbid process is a progressive one commencing with the presence of occasional areas of small celled infiltration and proceeding to the replacement of relatively large and widely distributed areas of atrophied myocardium with dense collagenous tissue.

As we have obtained no further evidence regarding the genesis of the myocardial atrophy which, on evidence discussed by Bennetts *et al.* (1942), was attributed to the direct and indirect effect of severe copper deficiency, there is no point in adding to that discussion.

In the publication referred to, an explanation of the heart lesion alternative to the one now accepted, was suggested. We had then expressed the view, deduced from the common occurrence of massive deposits of hæmosiderin, that circulating iron might reach a sufficiently high level to induce toxic changes in heart muscle. The studies now under consideration have failed to provide any evidence in support of such an hypothesis.

On the third point, it cannot be claimed that the immediate cause of death in fatal cases of copper deficiency has yet been established, but as further evidence has been adduced, some further discussion might be profitable. We see no reason to modify the view previously expressed that the immediate cause of death is heart failure and that the fatal termination is the direct result of the cardiac lesions. Death generally occurs when the effects on the animal are accentuated by the coincident occurrence of seasonal anæmia, pregnancy or maximum production.

From the data now available, however, it is evident that the extent of the fibrotic lesions does not necessarily determine a fatal issue, death having occurred in cases where the demonstrable lesions were relatively inconspicuous, whereas other animals affected with extensive and long-standing fibrotic lesions did not succumb.

It is possible that the distribution of the lesions may be of more significance than their extent. In order to obtain definite information on this point it would be necessary to examine a much larger series of experimental animals and in much greater detail than has been possible in this study. In this connection we had suggested earlier that death possibly resulted from heart block due to involvement of the conduction system. In view of experimental evidence of Alfredson and Sykes (*loc. cit.*) that the bovine can tolerate considerable interference with the conduction system, this explanation seems an unlikely one. In the subjects we examined, there was evidence of some involvement of the conduction system both in animals which did and in those which did not die from "falling disease."

We have shown, moreover, that quite extensive fibrotic lesions may occur without any abnormality being detected in the electrocardiogram even within a few weeks of death.

There is a striking discrepancy between the macroscopic evidence of loss of tone of the myo-

cardium of copper deficient bovines, particularly when anæmic, and the absence of correspondingly gross histological changes in the muscle. It is possible that after all the heart failure is unrelated either to the extent or distribution of the fibrotic processes, but results from the inability of the atrophic, anoxic myocardium to respond to the requirements of the animal.

In this connection Boyd (1945), commenting on the absence of myocardial lesions adequate to explain cardiac failure in the acute stages of rheumatic disease in man states: "It is evident that the technique of the pathologist is inadequate for estimating the functional capacity of the myocardium."

We must conclude, therefore, that although the clinical characteristics of the disease and the constant occurrence of passive congestion in fatal cases leave little room for doubt that the immediate cause of death is heart failure, the actual mechanism has not yet been elucidated.

Summary.

An account is given of a further study of the nature and development of myocardial lesions in experimental female cattle maintained from calfhood on the severely copper-deficient pastures at Jindong.

These more comprehensive studies have confirmed the earlier claim that the heart lesion is essentially a myocardial atrophy with replacement fibrosis. The morbid process is progressive.

The etiological significance of severe copper deficiency is quite clear, but no further evidence has been obtained regarding the genesis of the myocardial atrophy. Analytical data on livers of experimental animals give no suggestion that elements other than copper are involved.

The possible reasons for the heart failure, considered to be the primary cause of the fatal termination, "falling disease," are discussed in the light of further clinical and pathological evidence.

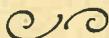
Acknowledgments.

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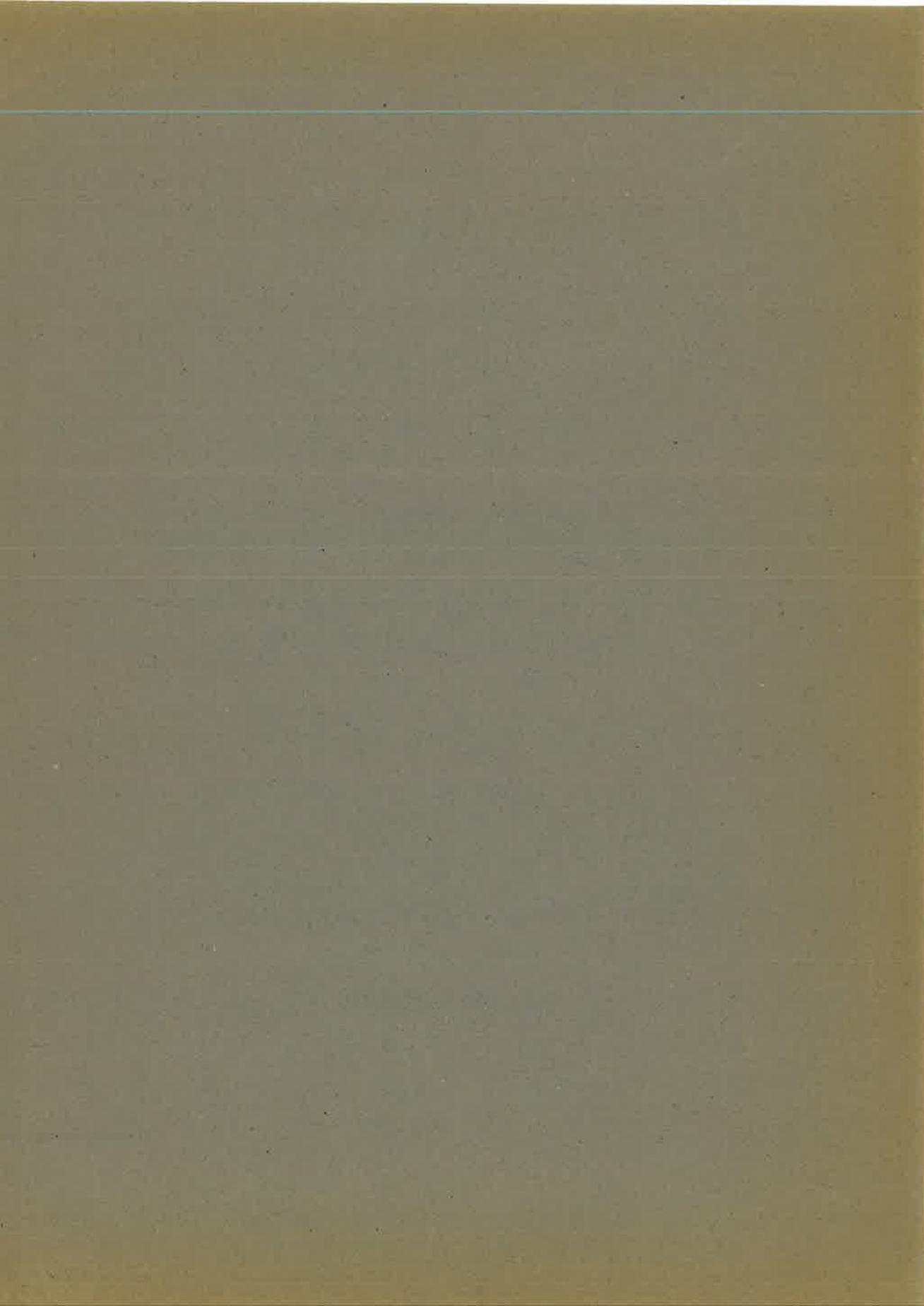
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ENZOOTIC MARASMUS: THE RELATION OF COPPER
TO INCIDENCE AND TREATMENT.

By E. J. UNDERWOOD, B.Sc. (Agric.), Ph.D., and A. B. BECK, M.Sc.



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Introduction.

PREVIOUS experiments (Underwood and Filmer, 1935; Filmer and Underwood, 1937) have shown that small regular doses of cobalt chloride alone would cure enzootic marasmus and maintain good health in sheep and cattle in "affected" areas at Denmark for periods up to sixteen months. Furthermore, in a field trial extending over twelve months, cobalt chloride was found to be as effective, in promoting growth of calves and maintaining condition of mature cattle, as a limonite lick supplying a number of elements besides cobalt, including copper. These results, together with the analytical data on the cobalt content of soils, pastures and animal organs obtained by Underwood and Harvey (1938), make it perfectly clear that the disease must be regarded as being due only to a deficiency of cobalt in the diet. If secondary deficiencies of trace minerals exist it would seem that they must do so to a very minor degree.

The possible significance of copper in the aetiology of the disease was suggested by the finding of low levels of copper in the livers of several sheep from affected areas which were being examined by the senior author for another purpose.* Accordingly it was decided to carry out an experiment

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* The original observation was made by Mr. F. E. Chapman, A.A.C.I., of the Government Chemical Laboratory, Perth, to whom the authors are grateful for drawing their attention to this matter.

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at Denmark where enzootic marasmus was known to occur, in which a direct comparison of the effects of supplying cobalt plus copper and of cobalt alone could be made. At the same time it was thought advisable to obtain data on the copper status of the pastures of the district generally, so that comparison could be made with results from work on copper deficiency in other parts of Western Australia.

Experimental Procedure.

Twenty Border Leicester x Merino weaner wethers (aged 7-8 months) were obtained from a sound area outside the Denmark district and sent to an affected holding at Denmark on 1/2/39. Cross-bred weaner wethers were chosen for this purpose since such young rapidly growing animals are known to be the most susceptible to the disease. They were immediately bled for hæmoglobin and blood copper determinations. During the next nine months they were given no treatment other than a single dosage of carbon tetrachloride against worm infestation. Most of the sheep began to fall rapidly in live weight as they became affected with enzootic marasmus after from 4 to 6 months on the affected grazing.

It was intended to allow all twenty sheep to become badly affected with the disease and then divide them into ten matched pairs, one of each pair to receive treatment with cobalt only and the other with cobalt plus copper. Unfortunately, however, ten of the sheep were lost through accident and exposure, four of the worst affected animals dying just after treatment had begun. The loss of the four sheep immediately after the matching of pairs, three of which were from the cobalt plus copper treated group, made rematching very difficult and only three of the pairs can be regarded as satisfactorily matched. In the other two pairs (A1 and B17; A6 and B11) the two "A" sheep (Co + Cu treated) were definitely larger framed animals than their pairs.

Treatment commenced on 7/11/39, just nine months after being brought on to the holding, and was as follows:

Group A: 2 mg. Co + 10 mg Cu as a drench thrice weekly.

Group B: 2 mg. Co thrice weekly.

Cobalt chloride was used as a source of cobalt and copper sulphate was the source of copper.

Treatment was continued for a period of thirteen months until 12/12/40, when the animals were killed and their livers, spleens, kidneys and pancreas obtained for iron and copper determination.

The live weights and the hæmoglobin and blood copper levels were obtained for all the sheep at frequent intervals.

Samples of the pasture on which the experimental sheep were grazed, together with samples from other parts of the Denmark district, were taken from time to time for copper determinations.

Results.

Live Weights.—The individual and mean live weights of the experimental sheep from the time of commencement of treatment are presented in Table I.

The very large increase in live weight of all sheep over the period, irrespective of treatment, is apparent at a glance. Not only was there a complete restoration of the wasted muscles and organs, but the animals showed an excessive deposition of fat at slaughter. No difference in this

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TABLE I.
Live Weights of Sheep in Pounds.

Sheep No.	Group.	7/11/39.	5/12/39.	28/12/39.	7/2/40.	12/3/40.	17/4/40.	29/5/40.	3/7/40.	16/8/40.	25/9/40.	8/11/40.	12/12/40.
15	A	62	67	81	87	93	98	99	112	119	131	146	153
10	B	69	66	80	84	89	92	93	101	110 ¹	120	128	138
18	A	74	75	92	90	102	104	105	115	124	128	134	141
9	B	72	74	87	92	97	100	101	113	117	123	136	143
7	A	79	88	96	91	101	101	103	114	121	125	132	135
2	B	79	90	99	100	110	108	109	119	127	126	134	143
1	A	—	73 ^a	84	89	98	104	106	119	128 ¹	139	149	153
17	B	55	70	79	83	88	92	96	102	109	115	123	130
6	A	—	75 ^a	76	86	90	93	98	114	125	137	152	166
11	B	58	70	76	79	85	91	94	105	110	120	131	141
Mean	A	—	76	86	89	97	100	102	113	123	128	138	147
Mean	B	—	74	84	88	94	97	99	108	115	125	135	142

¹ Treatment of these two sheep was changed over on this date, i.e. from 16/8/40 to 12/12/40. Sheep 10 was given "A" treatment and sheep 1 "B" treatment.

^a Treatment of these two sheep did not commence until 6/12/39.

respect was evident between those sheep receiving the cobalt plus copper supplement ("A" treatment) and those receiving the cobalt only ("B" treatment).

The mean live weight figures in Table 1 show little difference in the growth of the sheep on the two treatments and make it clear that the absence of a copper supplement has at no time limited growth in these cross-bred sheep. The slightly greater mean increase of the Co+ Cu treated sheep compared with the Co only is not significant and in any case is brought about largely by the appreciably higher live weights of sheep 1 and 6 which, as mentioned earlier, were very much larger framed animals than their pairs.

It should be pointed out that the sheep remaining on the "B" treatment throughout have had no copper in their diet other than that obtained from the affected grazing for a total period of twenty-two months. Even in this length of time, although rapid growth has taken place in the period, the absence of a copper supplement has not placed the sheep at any apparent disadvantage.

On 16/8/40, after nine months' treatment, the effect was tried of adding copper to the treatment of one of the "B" group sheep (No. 10) and removing copper from the treatment of one of the "A" group (No. 1). Both sheep continued to develop at approximately the same rate and to maintain the same position relative to the live weight of its pair as before the change. No difference in appearance or activity was evident between sheep on the different treatments either in the case of these two sheep or any other of the experimental animals.

Hæmoglobin Levels.—The individual and mean hæmoglobin levels of the blood of the experimental sheep for the treated period are given in Table II. The Newcomer-disc method of hæmoglobin determination was used after previous standardization with oxygen-capacity determinations (1 per cent. Hb. = 1.34 vols. per cent. O₂).

Although at the commencement of treatment the animals were all showing emaciation and weakness characteristic of the disease, none showed

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TABLE II.
Hæmoglobin Levels of Sheep.
Expressed as grammes per 100 ml. blood.

Sheep No.	Treatment.	7/1/39.	5/12/39.	28/12/39.	7/2/40.	12/3/40.	17/4/40.	29/5/40.	3/7/40.	16/8/40.	26/9/40.	8/11/40.	12/12/40.
15 10	A	8·6	7·8	12·6	15·8	13·7	—	14·1	14·9	12·8	13·5	16·3	14·4
	B	10·3	6·2	11·4	14·4	12·4	11·2	12·1	12·6	10·8 ¹	12·3	16·5	14·8
18 9	A	13·5	12·5	17·6	17·6	15·5	13·2	15·5	14·4	12·0	14·4	16·1	13·3
	B	9·9	9·0	13·2	16·3	12·6	12·5	13·3	13·0	11·4	11·3	16·1	13·3
7 2	A	11·1	11·8	15·1	—	14·4	13·1	14·0	13·2	11·2	13·9	14·4	13·6
	B	11·6	9·8	12·8	15·1	13·4	13·2	14·0	14·4	11·9	12·6	15·8	12·8
1 17	A	—	7·3	10·2	15·8	12·1	12·6	13·2	13·0	10·5 ¹	11·5	15·2	12·2
	B	9·1	8·7	12·5	14·4	12·8	11·0	13·9	—	11·8	11·8	—	15·8
6 11	A	—	7·9	8·2	12·2	12·2	—	11·8	11·7	11·5	11·0	16·0	13·2
	B	9·1	8·8	13·1	14·4	13·2	12·6	12·5	11·3	10·8	12·0	16·3	13·3
Mean Mean	A	—	9·5	12·7	15·3	13·6	13·0	13·7	13·4	—	—	—	—
	B	—	8·5	12·6	14·9	12·9	12·1	13·2	12·8	—	—	—	—

¹ Treatment of these two sheep was changed over on this date, i.e. from 16/8/40 to 12/12/40 sheep 10 was given "A" treatment and sheep 1 "B" treatment.

² Treatment of these two sheep did not commence until 6/12/39.

hæmoglobin values which can be regarded as really low, and it is interesting to note that some showed lower values at the end of a month's treatment. Only in the following month are rises to values close to normal apparent, and by the end of the next month all the hæmoglobin levels are high and in several cases exceptionally high. No significant difference is evident at this time between the animals treated with the Co + Cu and the Co only, either in the levels reached or the rate at which such levels are attained.

For the succeeding eleven months, that is, to the termination of the experiment, the hæmoglobin levels are seen to fluctuate widely between 10·5 and 16·5 g. Hb. per 100 ml., though a high proportion of the values lie between 12 and 14 g. Similar wide variations in the hæmoglobin levels of normal sheep are reported by Beck (1941). No suggestion of lower values in the sheep receiving the supplement of Co only is evident right up to the end of the experiment, though they have subsisted on the affected grazing without any copper supplement for a total period of twenty-two months.

A further point which deserves mention is that although the hæmoglobin levels fluctuate widely after several months of treatment, in a number of cases almost up to polycythemic levels, at no time is there any sign of low values indicative of even the mildest anæmia. In previous studies (Filmer and Underwood, 1937), transitory but large falls in hæmoglobin which rose spontaneously to normal were obtained in almost all the sheep treated with various levels of cobalt only.

Blood Copper Levels.—The levels of copper in the blood of the experimental sheep, obtained at frequent intervals during the period of treatment, are given in Table III.

All the sheep show high values at the commencement of treatment while affected with enzootic marasmus, as well as during the following four to five months while recovering from the disease and growing rapidly. The copper intake during the nine months preceding treatment (that is, while becoming

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TABLE III.
Blood Copper Levels.
Mg. per 100 ml.

Sheep No.	Treatment.	7/11/39.	5/12/39.	28/12/39.	7/2/40.	12/3/40.	17/4/40.	29/5/40.	3/7/40.	16/8/40.	26/9/40.	8/11/40.	12/12/40.
15	A	0.13	0.13	0.10	0.08	0.08	0.09	0.07	0.11	0.08	0.10	0.05	0.07
10	B	0.13	0.10	0.07	0.07	0.05	0.05	0.01	0.05	0.05 ¹	0.04	0.08	0.06
18	A	0.08	0.07	0.06	0.07	0.06	0.06	0.05	0.05	0.07	0.07	0.07	0.05
9	B	0.09	0.06	0.06	0.07	0.05	0.05	0.02	0.03	0.02	0.04	0.04	0.05
7	A	0.09	0.09	0.08	0.08	0.08	0.10	0.10	0.10	0.10	0.13	0.09	—
2	B	0.10	0.09	0.08	0.08	0.07	0.07	0.04	0.06	0.05	0.04	0.07	0.07
1	A	— ²	0.08	0.09	0.09	0.10	0.09	0.09	0.07	0.05 ¹	0.03	0.04	0.04
17	B	0.11	0.10	0.08	0.08	0.08	0.09	0.07	0.06	0.04	0.05	—	0.05
6	A	— ²	0.10	0.12	0.11	0.08	0.10	0.07	0.12	0.08	0.09	0.08	0.08
11	B	0.06	0.04	0.05	0.05	0.03	0.04	0.01	0.03	0.01	0.02	0.02	0.04
Mean	A	0.10	0.09	0.09	0.09	0.08	0.09	0.08	0.09	0.08	0.09	0.07	0.06
Mean	B	0.10	0.08	0.07	0.07	0.06	0.06	0.03	0.05	0.03	0.03	0.04	0.05

¹ Treatment of these two sheep was changed over on this date, i.e. from 16/8/40 to 12/12/40. Sheep 10 was given "A" treatment and sheep 1 "B" treatment.

² Treatment of these two sheep did not commence until 6/12/39.

affected) and during the following five months (that is, while recovering) cannot therefore be regarded as a limiting factor. For several months following, however, two (Nos. 9 and 11) of the five sheep of the "B" group (Co only) show definite and consistently low blood copper levels, though the other three members of this group do not fall to nearly the same extent; all five sheep in the "A" group (Co + Cu) maintain high values throughout. A further point of interest is the small spontaneous rise in the levels of the sheep of the "B" group during the final two months of the experiment, although no copper treatment was given. A similar tendency for the blood copper levels of sheep to rise in the summer when the grazing is dry has previously been observed by Bennetts and Beck (unpublished data).

Copper Content of Animal Tissues.—At the conclusion of the present experiment the sheep were killed and their livers, kidneys and spleens examined chemically for copper. In addition the copper content of the livers of a number of other animals from previous experiments were obtained. The method of Sylvester and Lampitt (1935), after wet digestion, was used. These results are presented in Table IV.

The copper contents of the kidney and spleen of the copper-treated sheep are closely similar to those of the sheep which have received no copper, other than that obtained from the grazing, throughout the experimental period and for a period of nine months prior to the commencement of treatment. Significant differences are evident, however, in the case of the livers. The livers of the "B" group (Co only) sheep are very much lower than those of the "A" group and are definitely much below the levels which are found in the livers of sheep grazing on normal healthy pastures in sound areas. Even the copper-treated animals have not, with the exception of Nos. 18 and 7, liver copper levels which can be regarded as normal in this respect.

The liver copper levels from sheep used in earlier experiments in the same district are very similar to those mentioned above. Apparently the

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TABLE IV.
Copper Content of Liver, Kidney and Spleen of Sheep.
Expressed as p.p.m. on dry basis.

Sheep No.	Treatment.	Period of Treatment.	Liver.	Kidney.	Spleen.
15	Co, 2 mg., plus Cu, 10 mg., thrice weekly.	7/11/39-12/12/40	87	15	7
18	" " "	" "	222	17	5
7	" " "	" "	144	15	5
6	" " "	" "	45	22	5
10	" " "	16/8/40-12/12/40	65	14	4
9	Co, 2 mg., thrice weekly.	7/11/39-12/12/40	15	14	9
2	" " "	" "	28	14	5
17	" " "	" "	22	14	5
11	" " "	" "	10	14	3
1	" " "	16/8/40-12/12/40	21	14	5
101	Co, 2 mg., per day.	22/1/35-16/5/36	18	—	—
107	" " "	" "	31	—	—
139	Co, 0.05 mg., per day.	27/4/35-16/5/36	16	—	—
110	Limonte lick supplying Co plus Cu.	19/1/35-7/5/35	203	—	—
114	" " "	" "	218	—	—
4	No treatment—badly affected with enzootic marasmus.	—	22	—	—
8	" " "	—	14	—	—
20	" " "	—	15	—	—

unsupplemented grazing in this area is not capable of sustaining large reserves of copper in the livers of sheep for very many months. The results from the three sheep badly affected with enzootic marasmus, which are of the same low order as those healthy sheep treated with cobalt only, are of great interest. A much lower copper requirement by these animals might be expected, since their growth and haemoglobin formation is being limited by a deficiency of cobalt. It would not be unreasonable to anticipate a banking up of ingested copper leading to high liver copper levels in such cases. The absence of such high levels and in fact the occurrence of levels well below normal for a sheep's liver point strongly to a low copper intake from grazing.

In the North Island of New Zealand, on the other hand, figures obtained by Aston (1911) for the livers of twenty sheep affected with bush-sickness, apparently an uncomplicated cobalt deficiency, show very high values. These vary from 120-1,210 p.p.m. Cu on the dry basis with a mean of 370 p.p.m.

Iron Content of Animal Tissues.—It was considered that an examination of the iron content of certain organs from the experimental sheep might provide data of interest. Previous findings had indicated that the excessive stores of iron in the tissues of affected animals (Underwood, 1934) are used up during recovery from the disease while being treated with cobalt (Filmer and Underwood, 1937). Accordingly such determinations were made on the livers, kidneys and spleens using the thiocyanate colorimetric method after wet digestion. The results are presented in Table V, together with the mean values for each group and the mean values for such tissues obtained in a previous study.

It will be noted that the iron content of the three tissues of both the cobalt treated and the cobalt plus copper treated sheep examined are of the same order of magnitude and conform fairly closely in the case of the liver and kidneys to the values found for normal sheep in a previous investigation

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TABLE V.
Iron Content of Liver, Kidney and Spleen of Sheep.
Expressed as p.p.m. on dry basis.

Sheep No.	Treatment.	Period of Treatment.	Liver.	Kidney.	Spleen.
15	Co, 2 mg. + Cu, 10 mg., thrice weekly.	7/11/39-12/12/40	310	356	1,028
18			415	575	1,077
7			461	378	1,370
6			302	289	1,825
10			226	258	1,005
Mean			343	371	1,261
9	Co, 2 mg., thrice weekly.	7/11/39-12/12/40	302	652	1,485
2			572	442	2,451
17			299	222	1,450
11			386	833	1,027
1			576	750	1,245
Mean			427	578	1,532
Mean of 6 normal sheep	See Underwood (1934).		430	460	3,800
Mean of 9 sheep affected with en- zootic marasmus			2,380	2,320	10,100

(Underwood, 1934). The figures for the iron content of spleens are, in both groups, of the same order as the figure 1,100 p.p.m. given by Moore (1938) as the mean of five normal sheep. Although lower than the mean figure of Underwood (1934), the present figures lie within the same range. It is obvious further that in none of the tissues examined in the present investigation does the iron content reach the very high levels obtained previously (Underwood, 1934) for animals affected with enzootic marasmus. In other words, no gross banking up of iron in the liver, kidney and spleen has occurred in the animals treated with Co + Cu or with Co only. Apparently a supplement of cobalt is all that is necessary to secure an effective mobilization of the iron reserves of affected animals in these areas, and therefore cobalt deficiency alone, *i.e.*, without the necessity of including copper, can logically explain the high levels in diseased animals. The higher mean levels of iron in the tissues of the sheep receiving the supplement of cobalt only are not statistically significant.

Copper Content of Pastures.—Samples were taken periodically from the pastures being grazed by the experimental sheep with the object of obtaining information on their actual copper intake throughout the experimental period. The results for nine such samples are given in Table VI, together with a brief description of the principal plant species involved in each case.

The copper levels are seen to fall within a narrow range (2.9 to 4.2 p.p.m. Cu on dry basis) in spite of the variation in plant species making up the samples and the different times of the year at which they were collected. It is apparent that for the greater part of the experiment the grazing must have supplied between 3 and 4 p.p.m. of copper.

It was also thought advisable to examine samples from other parts of the district in order to obtain information as to the copper status of the pastures of the Denmark area generally for comparison with the particular pastures where the experiment was carried out and with the pastures in other parts of the State. Some of the samples were obtained from holdings sound with

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TABLE VI.
Copper Content of Pastures Grazed by Experimental Sheep.

Sample No.	Date.	Cu Content, p.p.m. on dm.	Botanical Notes.
394	28/3/39	4.4	Sub-clover, yorkshire fog and kikuyu grass.
401	7/6/39	3.9	Cocksfoot, yorkshire fog and kikuyu.
402	7/6/39	4.2	Cocksfoot, yorkshire fog and kikuyu.
471	5/9/39	2.9	Mainly sub-clover with yorkshire fog and silver grass.
530	8/11/39	3.8	Mainly sub-clover with yorkshire fog and silver grass.
551	28/12/39	3.7	Sub-clover, paspalum, kikuyu.
626	16/8/40	2.9	Mixed pasture.
641	12/12/40	4.1	Paspalum, kikuyu, sub-clover.
Mean	3.7	

respect to the incidence of enzootic marasmus, and others from definitely affected holdings. For convenience, however, they are grouped according to the soil types on which the pastures were grown, using the soil survey data of Hosking and Burvill (1938). All the samples are predominately subterranean clover with varying amounts of such annual grasses as silver grass and perennial grasses as yorkshire fog, perennial rye, cocksfoot and paspalum. The results are presented in Table VII.

TABLE VII.
Copper Content of Denmark Pastures.

Sample No.	Year.	Soil Type.	Cu p.p.m.
—	1937	Wakundup.	3.5
—	1937	"	4.4
344	1938	"	3.9
346	1938	"	3.5
347	1938	"	2.2
348	1938	"	3.1
349	1938	"	3.3
525	1939	"	2.9
527	1939	"	3.3
533	1939	"	4.2
535	1939	"	4.5
536	1939	"	6.5
537	1939	"	4.0
656	1940	"	2.5
Mean	3.7
—	1937	Alluvial.	22.2
345	1938	Scotsdale gravelly loam.	10.6
528	1939	" " "	10.7
581	1939	" " "	5.9
532	1939	" " "	4.3
526	1939	Alluvial soil.	5.8
529	1939	" "	9.6
Mean	9.9

The much more variable and in general much higher copper content of the pastures from the alluvial and Scotsdale soil types compared with those from the Wakundup soil type is immediately evident. This last soil type occurs in both sound and affected holdings, but it is the predominant type on affected holdings. The alluvial and Scotsdale gravelly loam soils also occur to some extent on affected holdings, but predominate on holdings unaffected with the disease. It is, therefore, to be expected that areas where enzootic marasmus occurs will generally have a lower copper status than

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adjacent sound areas of the Denmark district. The experimental sheep were grazing on pastures growing on the Wakundup soil type, and it may be noted that the mean value for these samples (3.7 p.p.m. Cu) is identical with the mean obtained for the other pastures from this soil type. It is apparent that on many farms in the Denmark district, and particularly where enzootic marasmus occurs, sheep and cattle will obtain 3 to 4 p.p.m. Cu from the grazing for most of the year. On farms where such soils as the Scotsdale gravelly loam and alluvial types predominate a much higher copper intake would be expected.

Discussion.

The weight figures and hæmoglobin levels obtained from the experimental sheep show quite clearly that cross-bred wether sheep can be maintained for as long as twenty-two months on pasture typical of the affected areas without showing any clinical evidence of copper deficiency and in fact without any observable disability other than that of enzootic marasmus, which is completely preventable by cobalt therapy alone. Moreover, perfectly normal wool, with no sign of stringiness, was obtained from the sheep in two successive shearings, although stringy wool is highly characteristic of copper-deficient areas and, according to Bennetts and Beck (1941), develops in both male and female sheep within six months of being depastured on copper-deficient grazing.

On the other hand, the blood copper levels of the sheep receiving no additional copper were, during the final 6 to 8 months of the experiment, consistently below normal levels, that is, levels shown by sheep on a known adequate copper intake, while the levels for two of these sheep, for several months at least, were comparable with those shown by sheep on a known inadequate copper intake. The liver copper levels of sheep receiving cobalt only (mean of 8 sheep 20 p.p.m. (range 10-31)) are also lower in every case than those receiving copper in addition to the cobalt and very much lower than the levels shown by animals from normal areas, which usually range from 200-400 p.p.m. Whether such high values are in any way essential to the wellbeing or productivity of sheep or whether they merely reflect a high copper intake, only a portion of which is actually required, is not at present known. Some figures, however, are available from other areas which suggest that levels at least as low as 30 p.p.m. are quite consistent with perfect health in sheep. Values of 22, 37 and 56 p.p.m. were obtained from the livers of three wethers from areas which must be regarded as perfectly sound. Values of 16, 52 and 54 p.p.m. were also found by one of us for the livers of three copper-treated ewes from the deficient area at Robe, S.A. These sheep, which had been receiving 5 mg. Cu and 1 mg. Co daily for two years, were in perfect condition, had dropped healthy lambs and gave blood copper values which, although slightly low on one occasion (0.04 mg. per 100 ml.), were generally within the normal range (0.07-0.11 mg. Cu per 100 ml.). The results of the present investigation suggest that liver copper levels even of the order of 20 p.p.m. (dry basis) do not necessarily imply copper deficiency, at least with the male animal. This level is approximately twice that found by Moore (1938) in the livers of ewes and weaners affected with coast disease, a known copper deficiency, and almost three times the mean level (7.2 p.p.m.) found by Bennetts and Beck (1941) for 10 ewes from areas in Western Australia where copper deficiency is clearly established.

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The results of the pasture analyses indicate a somewhat similar position, namely, that the copper content of pastures which predominate on affected holdings is definitely low. In a survey of pastures in other parts of Western Australia (Beck, 1941) such values were found in areas where copper-deficiency symptoms in stock were occasionally encountered. These pastures were tentatively classed as "marginal—probably unsound". The values were lower than those obtained for areas known to be healthy for stock (5-15 p.p.m.), but higher than the levels of 1-3 p.p.m. obtained for pastures definitely associated with falling disease (Bennetts and Hall, 1939) and enzootic ataxia (Bennetts and Beck, 1941), both of which are prevented by copper therapy. This would suggest that the Denmark pastures, with the exception of those from the heavier soil types, must be regarded as sub-optimal with respect to copper.

The absence of falling disease in these areas or of any regular or consistent suppression of œstrus or disturbance of the œstrus cycle of cows, such as is reported by Bennetts *et alii* (1941) to occur in copper-deficient areas, even where cobalt alone has been fed as a supplement for as long as three years, suggests that 3-4 p.p.m. can be regarded under Western Australian conditions at least as a critical level. The position in regard to breeding ewes and the possible incidence of enzootic ataxia in these areas is at present unknown, since sheep breeding is not carried out to any extent in the district. There is no doubt that breeding ewes reveal signs of copper deficiency much more rapidly than dry ewes or male sheep (Bennetts and Beck, 1941). There is not sufficient published evidence to state conclusively, however, that they will suffer such disabilities on the level of copper intake provided by the Denmark pastures, although evidence from elsewhere suggests that this is very likely to be the case.

A further point to be noted in the present experiments is that they were carried out with Border-Leicester x Merino sheep. It is possible that some more definite evidence of copper deficiency might be shown under the same conditions by pure Merino sheep, since Bull (private communication) has some evidence which suggests that the English breeds and their crosses more readily absorb and store copper than the Merino. Merinos also show evidence of stringy wool more readily than do the English breeds or their crosses. Although the evidence provided by the present work supports the conclusion that a considerable proportion of the area affected with enzootic marasmus is marginal for dry sheep and cattle, and sub-optimal for breeding ewes, it should be emphasized that such a position does not in any way disturb previous findings that this disease is an uncomplicated cobalt deficiency. In no case either in the present or previous experiments has cobalt plus copper treatment of affected animals resulted in any better response than cobalt treatment alone. Moreover, practical field evidence, as well as experimental trials, show clearly that cows and male sheep can be maintained in apparently perfect health and condition for as long as two years with no special treatment other than that of a regular cobalt supplement. This distinguishes enzootic marasmus clearly from coast disease which has been shown conclusively (Marston and McDonald, 1938) to be due to a dual deficiency of cobalt and copper.

Summary.

An experiment designed to test the possible significance of copper, in addition to cobalt, supplements in the treatment of enzootic marasmus and

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the maintenance of health in young cross-bred wethers in the Denmark district is described.

No effect on growth or hæmoglobin levels from the use of the copper was found either during recovery from the disease or in the latter stages of treatment, although by this time the sheep receiving the cobalt supplement alone had been for twenty-two months without copper, other than that obtained from the grazing. Significant falls in the blood copper levels were found, however, during the last 6 to 7 months of treatment—in two cases to very low levels. No sign of stringy wool was found in these sheep either in the first or second shearing.

The iron content of the livers, kidneys and spleen at the end of treatment was found, in all cases, to lie within normal limits, but the copper content of the livers of the sheep receiving no copper supplement was well below normal levels usually reported for healthy sheep, though appreciably higher than the values reported for the livers of sheep from areas known to be highly copper deficient.

The copper content of pastures growing on the principal soil types on affected holdings was found to average 3 to 4 p.p.m. on the dry basis, although the pastures on some of the other soil types were found to contain much higher concentrations.

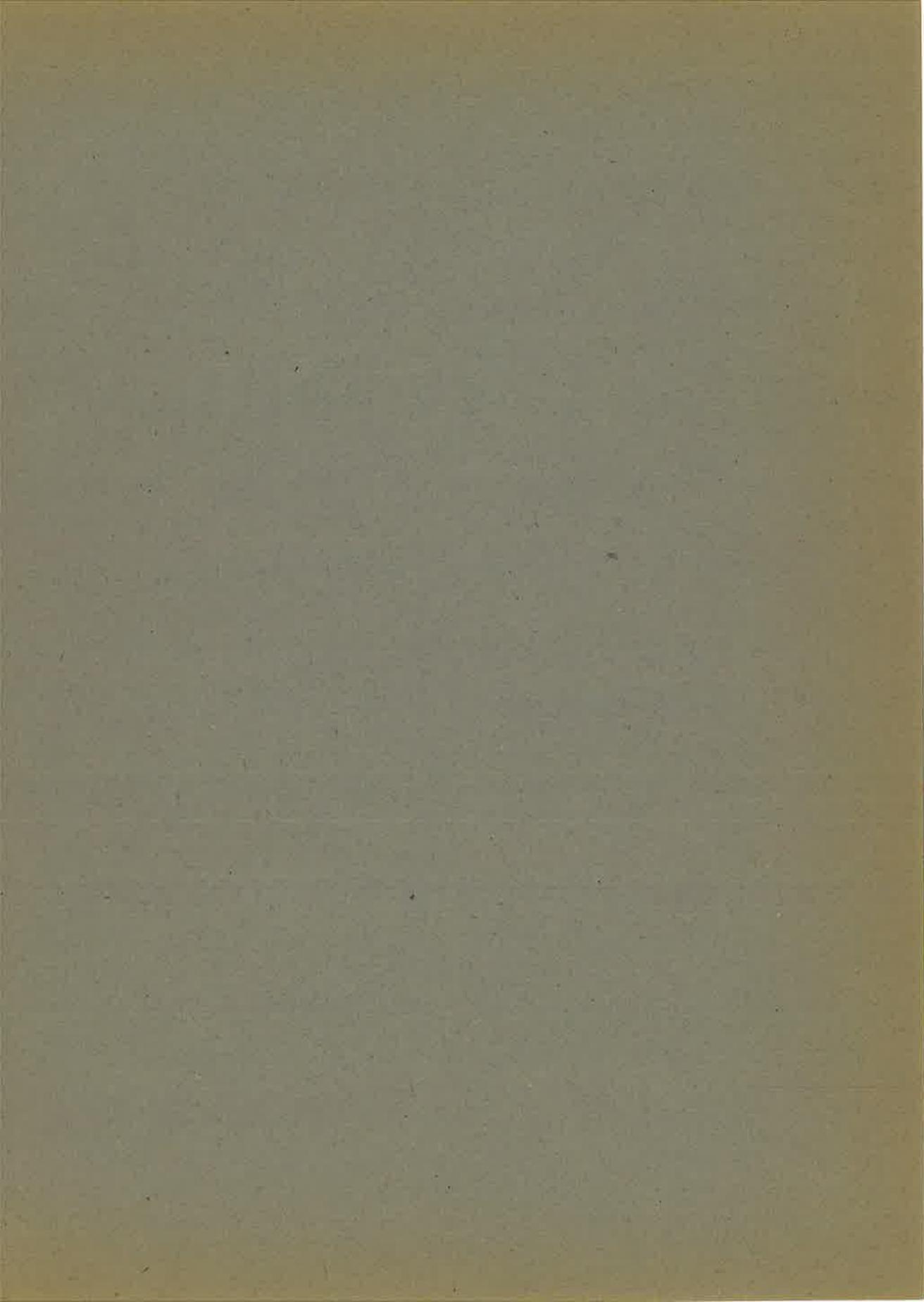
The significance of these findings and their relation to other investigations is discussed. It is concluded that enzootic marasmus is due to a deficiency of cobalt uncomplicated by lack of copper. The copper status of the affected areas, however, must be regarded as marginal even for dry sheep and cattle, and evidence from elsewhere indicates that it is sub-optimal for the breeding of sheep.

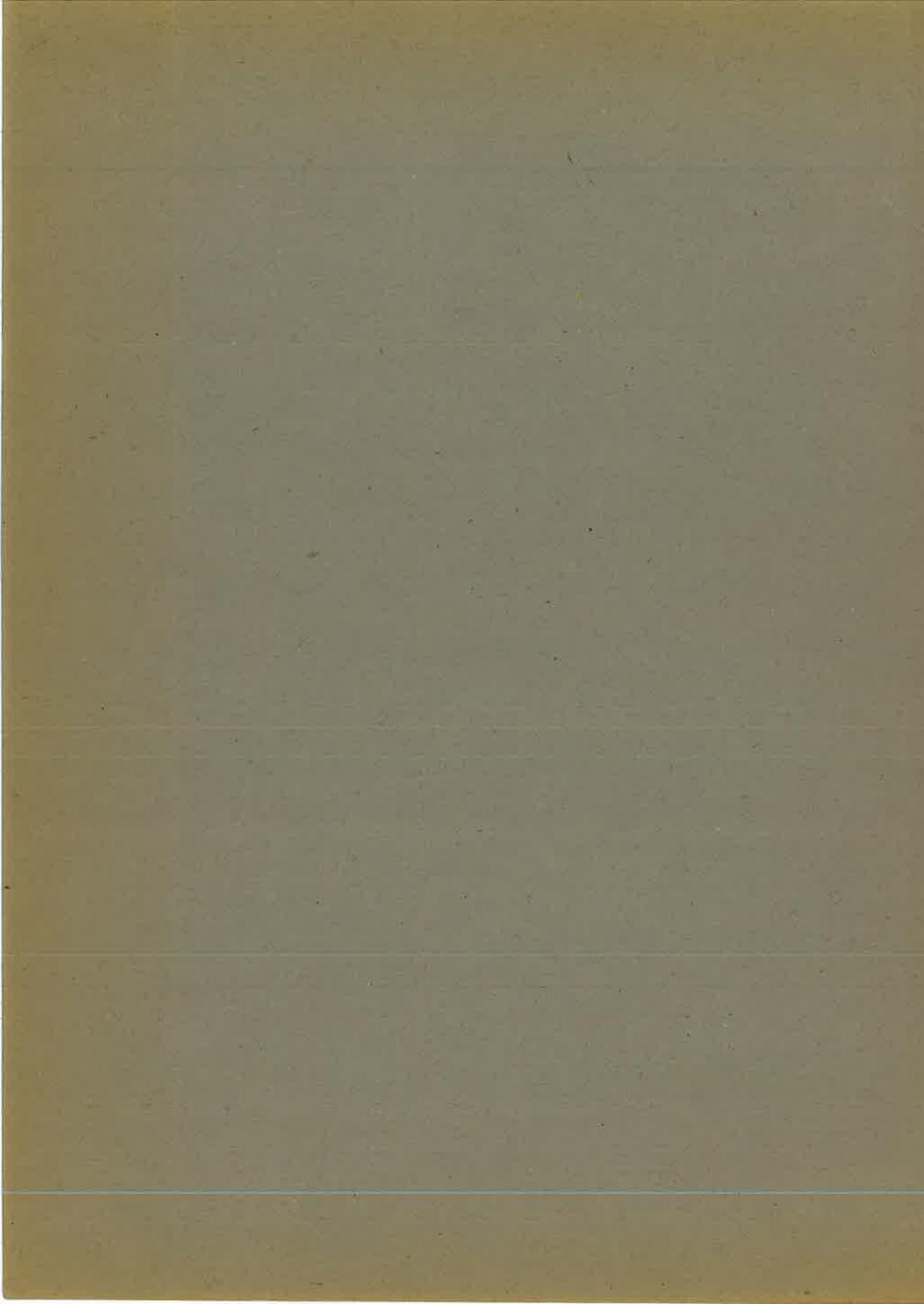
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Western



Australia

DEPARTMENT OF AGRICULTURE.

A Survey of the Copper Content of Western Australian Pastures

By A. B. BECK.*

SUMMARY.

Approximately 270 samples from Western Australian pastures have been analysed for copper content.

The analyses of mixed grass-clover pastures collected during the growing period seem to be satisfactory for giving an indication of the copper status of an area. Samples from areas where severe copper deficiency symptoms occur in stock contain under 3 parts per million (dry basis). Samples from sound areas contain over 6 p.p.m., usually 7 to 12 p.p.m. Values between 3 and 6 p.p.m. are regarded as marginal.

There is a variable fall in the copper content of a pasture as it approaches maturity. Heavy grazing of dry pastures causes a large fall.

The chemical analysis of capeweed (*Cryptostemma calendulaceum*) does not appear to give always a correct indication of the copper status of mixed pastures.

The occurrence of copper deficiency in the various soil zones of Western Australia is discussed. This deficiency is quite widespread, although in some localities it may be confined to small pockets of certain soil types.

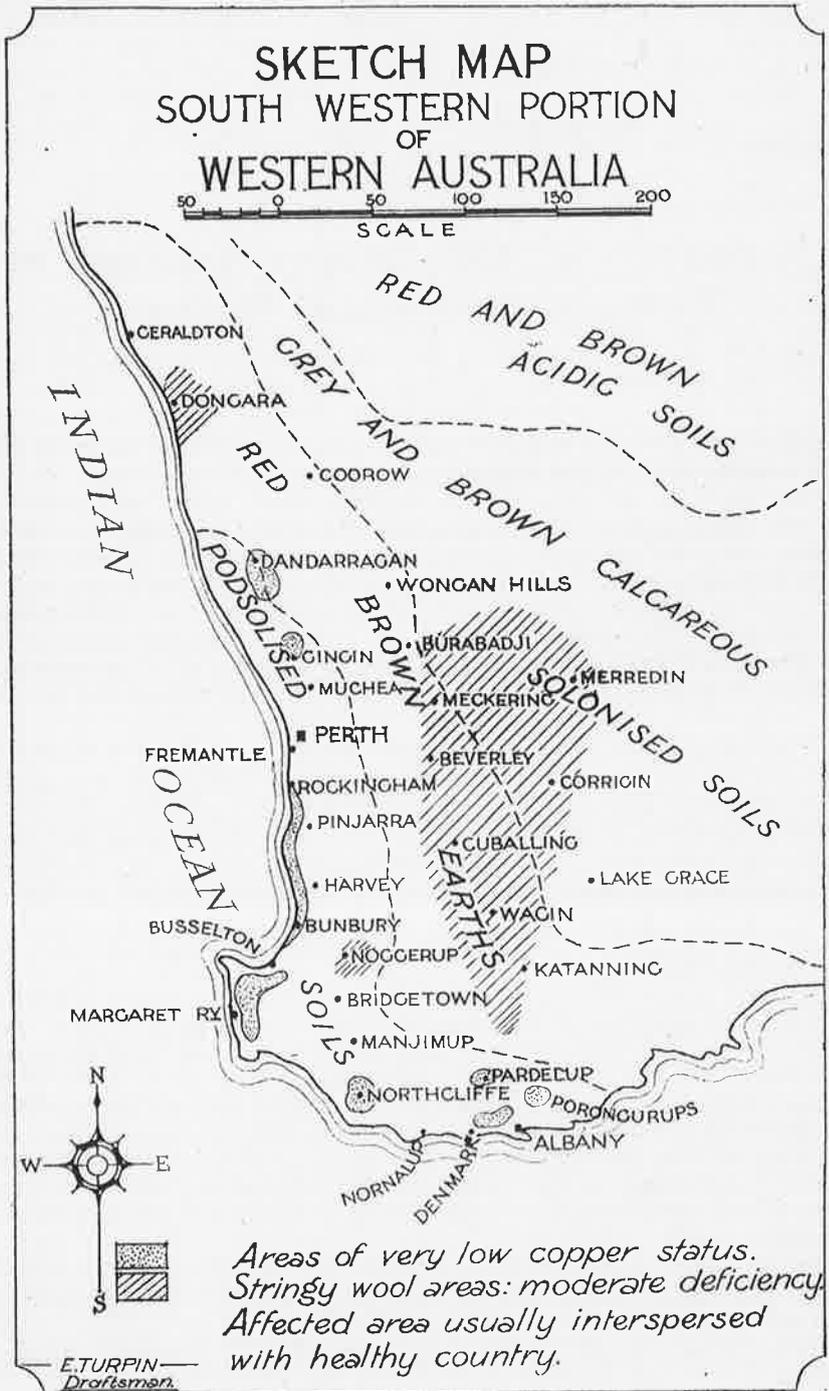
1. Introduction.

In 1937 Bennetts and Chapman showed that the livers and blood of lambs affected with "Gingin Rickets" or enzootic ataxia were extremely low in copper. This present investigation was originally intended to confirm and extend their observations by a study of the copper content of pastures from Gingin. As later work by the Stock and Plant Nutrition Branches of the Department showed that the existence of copper deficiency was quite widespread in Western Australia, the scope of the investigation was widened in an attempt to map out both "healthy" and deficient areas by the analysis of pasture samples.

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† The results set out in this Leaflet were originally published in the Journal of the Department (Beck, 1941). As it was necessary to reprint the Leaflet, the opportunity was taken to incorporate additional results obtained during the period 1941 to 1950. The findings and conclusions given in the original paper are essentially unchanged.

As problems of animal health were being investigated, the majority of the samples analysed were mixed pastures, selected as far as possible to represent the material actually grazed by the animal.



There is however definite evidence that the different species of a mixed pasture have different copper-collecting powers and for certain purposes of the analysis of a suitable single species is desirable. A number of botanically pure samples of capeweed (*Cryptostemma calendula-ceum*) have been analysed; this has been chosen as one of the few plants growing throughout the wide range of rainfall conditions of the State. Samples of subterranean clover were collected in 1947 from a series of soil types but these were all from areas where there was no evidence of deficiency symptoms in animals.

2. The Copper Content of "Normal" Pastures.

Published data give but little indication of the range of copper content of "normal" pastures. Rusoff, Rogers and Gaddum (1937) review data which suggest that values between 5 and 30 parts per million (p.p.m.) copper on a moisture-free basis can be regarded as "normal." Innes and Shearer (1940) report values of 12-24 p.p.m. for apparently normal English pastures, while Eden (1941) states that the copper content of Northumbrian hillside pastures is 6-10 p.p.m.

Cunningham (1946) states that the mean copper content of fifty-two normal New Zealand pastures was 11.0 with a range of 8.1—18.7 p.p.m.

The data set out in the Appendix have been correlated with the health of stock grazing on the pasture concerned. The following criteria have been adopted for "unsound" and "marginal" pastures:— (1) Ataxia occurs in lambs or "falling disease" in cattle (Bennetts *et al* 1941); (2) "stringy" wool is grown by sheep (Bennetts 1932); (3) where the growth of cattle is improved by copper supplements; and (4) liver analysis. Samples from localities where stock are not run have been omitted unless unequivocal evidence is available from the work of Teakle and his collaborators. The following standards have been adopted for mixed grass-clover pastures, collected during the growing period. As in all the figures which follow, the copper content has been calculated on a moisture-free basis.

Below 3 p.p.m.	Unsound.
3 to 4 p.p.m.	Marginal: probably unsound.
4 to 6 p.p.m.	Marginal: probably sound, except for stringy wool.
Above 6 p.p.m.	Sound.

"Falling disease" pastures usually contain below 2 p.p.m., while most normal Western Australian pastures contain 7-12 p.p.m. In a detailed study of the Denmark area, Underwood and Beck (1941) have obtained further confirmation of the hypothesis that pastures containing 3-4 p.p.m. should be classed as marginal.

Stringy wool appears to be the first symptom of copper deficiency in merino sheep and where the copper intake is "marginal," such wool may occur without any other upset of animal health (Marston and Lee, 1948).

It should be stressed that the above figures apply only in relation to animal health. No attempt has been made to deduce from the analyses whether or not a given pasture would be expected to give increased plant growth after the application of copper-containing fertilizers.

3. The Availability of Copper in Plant Material.

The standards of "soundness" as set out in the previous section require a certain amount of qualification. It has been observed both in this State and also in South Australia (Bull *et al.*, 1938) that the incidence of ataxia in lambs is greatly increased in seasons which favour a good growth of pasture. In experimental animals at Gingin (Bennetts and Beck, 1942) there was a high percentage of ataxia in 1937 and practically none in 1938. There was a tendency for a higher copper content in the 1938 pastures, but the difference was small and was not considered to be sufficient to explain the marked difference in the incidence of the disease. It would appear that there are one or more factors which operate in certain seasons and influence the availability of the plant copper to the animal. This point has been discussed more fully elsewhere (Bennetts and Beck, 1942) but it should be mentioned that, at Gingin, a season favouring ataxia is usually one in which there is a rank growth of capeweed. Sheep grazing on such pastures show bad scouring which may upset the absorption of copper.

Some support for the hypothesis that the copper in rank green pastures is less available, is given by the fact that symptoms of deficiency are less marked in the summer months even although the copper content of the dry summer pastures is lower than in the winter and spring.

A marked seasonal variation has been noted in the amounts of copper stored in the livers of sheep grazing at the Merredin Research Station. Experiments are being carried out by the writer to find the cause of this variation.

Lee (1950) reports that on the incipiently deficient soils of South Australia, stringy wool only occurs in certain years.

Recent work in Australia and overseas has shown that the presence of moderate amounts of molybdenum in pastures may greatly influence the utilisation and storage of copper by sheep. The effect of small amounts of molybdenum in "normal" pastures is at present under investigation at the Merredin and Bramley Research Stations. A number of the samples listed in the Appendix have been analysed for molybdenum content and the results will be published elsewhere. It may be pointed out, however, that with the possible exception of one of the Dongara samples (No. 455, molybdenum content 5.4 parts per million) none of the pastures have shown levels of molybdenum which can be classed as high by overseas standards.

Marston and Lee (1948) have made detailed investigations on sheep grazing on pastures of the calcareous soils at Robe, South Australia. These pastures contain about 3 p.p.m. copper but a daily supplement of between 7.5 and 10 mg. copper (equivalent to a total pasture copper of 10.5 to 13 p.p.m.) is necessary to maintain normal blood copper levels and to produce good quality wool. Apparently there are factors in these pastures (possibly due to molybdenum or to the highly calcareous nature of the soil) which depress copper absorption in the animal. Normal blood copper levels and wool growth are obtained on Western Australian pastures containing levels of copper above about 6 p.p.m.

The results of Innes and Shearer (1940) on "Swayback" in lambs calls for brief comment. This disease is pathologically identical with ataxia, and the livers of "swayback" lambs and their mothers have a copper content similar to that encountered at Gingin. The disease also responds to copper therapy (Dunlop and Wells, 1938) but by Western Australian standards the copper content of "swayback" pastures is extremely high (14-27 p.p.m.). No satisfactory explanation for the mechanism of this disease has been put forward. Either the copper in the pastures is not available or else the copper metabolism of the animals is deranged.

The experiments of Eden (1940) show that, even under conditions of high intake, copper sulphate, when mixed with the food prior to consumption, is only very slightly absorbed by sheep. Similar results have been reported by Lee (1950) who found that about 5 per cent. of a heavy oral dose was stored in the liver. It is probable therefore that, even under the best of conditions only a small part of the total copper of pastures is available for use by the animals, and that under the field conditions there are unknown factors which may decrease the availability still further.

4. The Copper-collecting Powers of Different Botanical Species.

On certain newly-reclaimed sand and moor soils in Holland it was found (Hudig and Meyer, 1926) that unless copper sulphate was applied to grassland pastures, the sown species quickly disappeared leaving only Yorkshire fog (*Holcus lanatus*). Early experiments at Nornalup in this State (Baron Hay, 1933) showed that Yorkshire fog together with *Lotus major* and Cocksfoot (*Dactylis glomerata*) were the only species which could be established on sandy peat soils now known to be copper deficient. Teakle and Morgan (1939) have made similar observations at Bornholm. Chemical analysis of samples of *Lotus major* and Yorkshire fog (Nos. 499 and 506), taken from an excellent stand on land shown to be copper deficient for potatoes, shows very low values (0.8 and 1.0 p.p.m. respectively) and supports the field evidence that both have a very low copper requirement.

Certain types of "stalling" of subterranean clover and the subsequent replacement by drooping flowered clover (*Trifolium cernuum*) are associated with the different copper requirements of the two species (Elliott, 1939). The consistently low copper content of the latter clover, and its frequent association with deficiency symptoms in stock suggest that it has a low copper requirement. Samples 475 and 476 were taken from inside and just outside a small area, about one chain in diameter, where the subterranean clover was being replaced by drooping flowered clover. The extremely low values for both samples (1.2 and 1.1 p.p.m.) suggest that the copper status of the particular area was too low to support the subterranean clover but was sufficiently high for the drooping flowered clover. The fact that a composite sample (largely subterranean clover), taken from other parts of the paddock had a rather higher content (2.9 p.p.m.) suggests that the most acute deficiency was localised in the area where the subterranean clover had "stalled."

Although the copper content of the young oat, wheat and barley plants may vary with the copper status of the soil, the mature plants show very little differences in copper content even after heavy dressings

with copper fertilisers (Riceman *et al*, 1940, Teakle, Thomas and Turton, 1941, Teakle and Turton, 1943). Apparently in the later stages, the growth rate of the plant is much greater than the rate of absorption of copper from the soil.

Teakle and Turton (1943) have reported that subterranean clover gives satisfactory results as an indicator plant for chemical analysis.

Results from separated samples collected at Merredin show that the medics have a consistently higher copper content than barley grass growing on the same area.

The general impression gained from the results of this investigation and from other published data is that fully grown leafy plants (e.g. clovers, medics, capeweed, wild geranium, etc.) will have a higher copper content than grasses when growing on soils of moderate to high copper status. On soil of low copper status little or no difference is noted. The position may be different for very young plants, but insufficient figures are available to draw any definite conclusions.

The case of capeweed has been investigated in more detail. Table 1 gives the results for samples from areas whose "soundness" is definitely known. Where the copper status is at all doubtful the figures are bracketed. Only plants collected in the preflowering or early flowering stage are included, as there is some evidence from the figures in the Appendix that very young plants may be relatively high in copper even when growing on areas known to be deficient.

TABLE 1.

Copper Content of Capeweed Samples.

A.—Western Australian Samples.

"Sound" Areas.		Deficient Areas.	
Sample No.	Cu. Content.	Sample No.	Cu. Content.
91	13.4	277	4.6
97	10.0	290*	2.8
294†	10.0	434	(4.8)
295	(7.8)	436†	(4.0)
304	7.6	573	4.2
305	13.5	574	2.9
430	8.4	A 76	3.0
502	13.3	A127	1.3
504	(5.2)	914j	1.4

B.—South Australian Samples.

A 81	12.8	A 77*†	4.9
A 82	9.3	A 78*	3.3
A117	11.8	A 79*	2.1
		A 80*	2.0

* Calcareous soil. † Rather small plants.

The results for the above samples seem to give a good correlation for areas whose copper status is definite, but in some other cases high values have been obtained for samples from localities which might be expected to be copper deficient. The sample from "Perth Sand"

(No. 282) showed a normal value (9.9 p.p.m.) and samples from two South Australian farms where ataxia occasionally occurred gave values of 5.5, 8.6 and 11.2.

Underwood, Robinson and Curnow (1943) give figures which show that the copper absorption by capeweed may vary with the soil type. Samples taken at Gingin from areas receiving 0, 7.5 and 15 lb. bluestone per acre showed that in each case the copper content of the capeweed was identical (within the experimental error) with that of subterranean clover and Wimmera rye grass growing on the same plot. As a contrast to these results, capeweed growing on podsolised sand of low-lying jarrah bush at Crawley showed a much higher copper content than subterranean clover and Wimmera rye grass from the same area. In the present investigation samples of capeweed from the highly calcareous littoral at Rockingham gave approximately the same value as grass pastures growing on the same area.

In certain areas of the Riverina (N.S.W.), capeweed (and two other species) has been found to contain about twice the copper content of ordinary pasture plants (Australian Wool Board, 1940).

Teakle, Thomas and Turton (1941) have published the analysis of seven capeweed samples from deficient and copper-treated areas. The results do not in any way correspond with those obtained in the present investigation. None of the "deficient" samples are low in copper and the treated samples tend to be lower than the non-treated. No satisfactory explanation can be offered for the different conclusions reached in the two investigations.

It has not been possible to investigate further the copper relationships of capeweed. It would seem that under conditions of extreme copper deficiency the copper content will be low, but under certain circumstances on normal soils, this species is able to absorb more copper than most others. For these reasons it is probably not generally suitable for indicating the availability of soil copper to other plants.

5. Seasonal Variation in the Copper Content of Pastures.

In a number of cases samples of mixed pasture have been taken throughout the growing season from the same area. These results are set out in Table 2.

TABLE 2.

Stage of Growth.	Wagin.				Gingin.		Merredin.
Very short	15.2	3.6	3.6	4.1	3.1	3.5	10.6
Middle of growing period	12.0*	3.1	3.0	4.6	2.6	3.1	6.0
Mature but not dry	10.3	2.5	3.5	3.7	1.7	3.8	5.0†
Dry	9.8	2.8	3.1	2.5	1.3	2.5	5.2

* Rather heavily grazed.

† Almost completely dry.

The figures indicate that in general there is a rather variable fall in copper content as the plant approaches maturity.

In actual practice, the seasonal variation is complicated by the effect of grazing. No data have been obtained to show the effect of heavy grazing on the copper content of green pastures, but two Gingin samples of dry pasture (Nos. 377 and 379) which had been heavily grazed showed a considerable fall in copper content. It is assumed that the sheep selectively graze the leafy material leaving the stalks and stems of lower copper content. Because of this variable drop in copper content during the growing period and the effect of grazing on the copper content of dry pasture, the writer considers that, when pasture analysis is used for diagnostic purposes, the samples preferably should be taken during the green period, and dry, grazed pastures carefully avoided.

Except where noted, the pastures listed in the Appendix had only been very lightly grazed, if at all.

6. Top Dressing with Copper-containing Fertilisers.

A discussion on this subject is outside the scope of this leaflet, but it may be stressed here that, for normal soils, the copper applied is retained for long periods. If copper fertilisers are applied too frequently the copper status of soils may be increased to such an extent that sheep might show copper poisoning when grazing on the pastures.

Underwood, Robinson and Curnow (1943) showed that 15 lb. per acre bluestone on Gingin soils maintained its effect on the pasture for three years. These soils are of a very open texture and a rapid leaching of the copper appears to have occurred.

Limited data from farms in the South-West on soils with a gravelly surface and clay subsoil show that 10-20 lb. bluestone will maintain the pastures at 7-10 p.p.m. for at least six years. More accurate information on this point will be obtained from experiments to be carried out at the Bramley Research Station.

Unpublished results from the C.S.I.R.O. Field Station at Robe, South Australia, show that the application of heavy dressings of bluestone to highly calcareous soils may not raise the copper content of the pastures to levels which are adequate for animal health. Apparently the high pH of the soil prevents proper absorption of the copper. Similar results may be expected on the shelly sands bordering the coastline in this State.

7.—The Distribution of Copper Deficiency in the Soil Zones of Western Australia.

The distribution of pasture copper levels in the various soil zones is set out in Table 3. Capeweed samples are included in this table. It should be pointed out that many of the samples analysed were from properties on which copper deficiency was suspected and accordingly, the table does not represent a random distribution over all soil zones.

TABLE 3.

The Copper Content of Western Australian Pastures. Distribution according to Soil Zones.

Soil Zones and Regions.	Copper Content. (Parts per Million.)									
	Unsound.		Marginal.			Sound.				
	0·0- 1·9.	2·0- 2·9.	3·0- 3·9.	4·0- 4·9.	5·0- 5·9.	6·0- 6·9.	7·0- 7·9.	8·0- 8·9.	9·0- 9·9.	Above 10·0.
A.—Podsolised Soils—										
1. Swan Littoral Region :										
(a) Talus Soils	1
(b) Alluvial Soils	1	1	2	2	5
(c) Sandhill Soils	2	1	2	1	1	1
(d) Gingin Soils	5	14	7	2	1	1	2
(e) Falling Disease Areas	23	8	4	5	1	2
2. Darling Penoplain	4	1	5	1	2	1
3. Frankland Region	5	10	11	11	4	1	2	1	5
B.—Red-Brown Earths—										
1. Irwin Region	2	2	2	2	1	2	1	2
2. Dwarda Region	1	3	1	1
3. Avon Region	5	12	5	3	2	3	1	5	11
C.—Calcareous Solonised Soils—										
1. Corrigin Region	2	3	8	2	1	1
2. Merredin Region	2	3	4	5	2	2	2	1
D.—Tropical Soils	1	1

The samples from the alluvial soils at the foot of the Darling Ranges were generally taken from good agricultural areas and the copper content is normal. The samples from the Harvey Irrigation Area show some of the highest figures encountered in this survey.

Abutting on to the westward side of these soils are the poor sandhill soils which in some cases produce pasture of low copper content. Much of this area is at present undeveloped agriculturally, but it will be surprising if large portions are not deficient in copper. At Muchea, maize and Sudan grass give increased growth with copper-containing fertilisers (Teakle and Burvill, 1941).

The Gingin pastures are uniformly low in copper. The relation of these figures to ataxia has been discussed elsewhere (Bennetts and Beck, 1942). Although no pasture samples have been obtained from the similar cretaceous area at Dandaragan, definite evidence of copper deficiency has been reported both in stock (Bennetts and Beck, 1942) and in plants (Teakle, Turton and Throssell, 1940).

With two exceptions the samples from the "falling disease" areas were taken from soil types which consist essentially of a grey sandy surface with a yellowish subsoil which often contains ironstone gravel. Pasture sample No. 474 was from a brown karri loam and No. 478 was from a lateritic gravel soil. The pasture analyses show that originally, most of the area must have been acutely deficient in copper. Even properties which were regarded by local farmers as being sound (e.g. No. 482) had a copper status at least as low as Gingin. Field observations (Bennetts *et al.*, 1941) showed the widespread occurrence of copper deficiency symptoms in stock, even where "falling disease" was not reported. The absence of "falling disease" on some properties growing pastures of extremely low copper content is ascribed to the fact that copper-containing licks were fed to stock.

Since the discovery of copper deficiency in these areas most properties have received relatively heavy dressings with copper-containing fertilisers and apart from one isolated case in 1946 no "falling disease" has been reported since about 1941.

The lateritic soils of the Darling Ranges have been but slightly developed agriculturally and so little information is available concerning a possible deficiency of copper in either plant or animals. Most of the samples taken from plants growing on these soils show a low copper content, and as this area is being opened up for pastures, crops and animal production, the probable development of copper deficiency problems should not be overlooked.

Ataxia in lambs has been investigated at the Pardelup Prison Farm near Mount Barker. This locality was originally placed by Teakle (1937-38) in the Frankland Region but subsequent investigations by him (private communication) have shown that it more appropriately belongs to the Darling Peneplain Region. The soils at Pardelup consist largely of immature granitic types and lateritic gravelly types.

There is evidence from the occurrence of stringy wool and of low copper levels in cow livers and in pastures that parts of the Wilga-Noggerup areas have a low copper status but no systematic examination of the district has been made.

The soils of the Frankland Region are probably subject to more intense leaching than those in other parts of the State. Some normal pastures have been found in this region but generally speaking the figures are low. The Denmark area has been subject to a separate investigation (Underwood and Beck, 1941). These authors found that the copper status of properties where "wasting disease" occurred was "marginal." Pastures from healthy properties in the district generally showed higher copper levels. In certain areas near Walpole and Nornalup, various difficulties have been experienced in stock raising. On clinical grounds these have been associated with copper deficiency and the analyses of the limited number of pasture samples give confirmation of this.

The effect of copper fertilisers on plant growth on the soils between Albany and Denmark has been discussed by Teakle and Morgan (1939) and Teakle, Morgan and Turton (1941). The samples were mostly collected by Dr. Teakle and the analyses bear out the results of field trials. The pastures from the peaty sands are low in copper whereas those from the brown karri soils have a higher copper status. The sample from Kronkup was from a farm where ataxia has been reported.

A bad outbreak of ataxia in lambs occurred at Millinup (approximately 20 miles north of Albany) in 1948. Analysis of liver samples from affected animals and of pasture samples showed low copper levels. Pasture samples from neighbouring properties (Nos. 865-870) showed fairly low copper levels.

The samples from Dongara were forwarded to the writer following complaints by farmers that sheep were "not doing well." The description in the tables in inverted commas indicate the assessment of the paddock by the farmer. The copper content of the "unsound"

pastures is definitely low. Ataxia has been confirmed in this area (Bennetts, private communication) and there is some evidence of trace element deficiencies in wheat (Teakle, Thomas and Turton, 1941).

A low copper content has been obtained for some of the capeweed samples from the Wongan Hills Research Station. If these figures actually represent copper deficiency, as the writer believes they do, it must be very localised as samples 511 (high) and 512 (low) were separated only by about half a mile, and under normal farming methods, sheep would not be expected to show deficiency symptoms. This also applies to the sample from Burabadji. There is no field evidence of copper deficiency in stock in these areas.

The pasture samples from the Meckering, Muresk and Beverley districts were collected from areas where basic intrusions were common in the granite. The samples all showed normal copper levels but eastward of these localities stringy wool has been reported on some of the light soils.

The question of the fertility of "light land" at Wagin has already been discussed by Stewart and Teakle (1939). Responses to copper fertilisers were obtained by these writers and the pasture samples analysed in the present work were for the purpose of obtaining further information concerning this area. The 1939-40 series were from plots of several square chains which had been fenced with the object of taking the samples, grazing down with sheep, and then shutting up the plots until the next sampling. This, however, was not done and with the exception of sample 493, the plots received very little grazing. The figures for this series illustrate the extreme variation of copper status with the different soil types. The second and third class land are within a mile of the first class basic red loam, yet they are definitely suboptimal and perhaps even deficient in copper. In view of the remarks in Section 4 concerning the "stalling" of subterranean clover it should be noted that an excellent stand of this clover was growing on the areas where the lowest copper content of the Wagin series was recorded. Apparently the copper supply was sufficient to meet the immediate needs of the clover stand.

The widespread occurrence in the Corrigin region of soils of "low" and "marginal" copper status is shown by the copper content of the pasture samples listed in the Appendix.

Some of the samples from the heavy gimlet (*Eucalyptus salubris*) soils of the Merredin Research Station show rather low levels of copper. As mentioned in Section 3 there is evidence of a very marked seasonal variation in the copper content of livers of sheep grazing on these pastures. The low copper content of the capeweed growing on the Wodjil soil probably indicates copper deficiency. Teakle, Thomas and Turton (1941) applied copper fertilisers to wheat growing on this soil type but apparently their rate of application was too heavy as impaired germination, delayed growth and depression of yield were noted.

Stringy wool has been noted in 1948 at the Merredin Research Station and on other properties further south on the lighter country. It would seem that the whole area must be classed as "marginal" in copper status.

8. The Value of Pasture Analysis in Delineating Areas of Copper Deficiency.

From the data obtained in this investigation it would seem that a careful interpretation of pasture analyses will give an excellent indication of areas on which copper deficiency symptoms in stock may be expected, particularly if animals are continuously grazed for a number of years.

When considering the analytical figures it is necessary to remember that the development of gross deficiency symptoms is influenced by other factors, e.g. (1) seasonal factors not fully understood; (2) whether the animals have previously had access to "sound" pastures; (3) the effect of species and breed, e.g., horses are less susceptible than sheep and cows, and the English breeds of sheep and their crosses are less susceptible to copper deficiency than merinos. In all cases, particularly where the pastures contain a "marginal" copper content, animal experiments with biochemical control are the only conclusive means of finding whether an area is "sound" or not. This will involve experiments for a period of at least two years, and preliminary pasture analyses will give a rapid method for indicating whether such experiments might be expected to give positive, negative, or uncertain results. The analysis of mixed clover-grass pastures, collected during the growing period would seem to be quite satisfactory for general diagnostic purposes.

Where the soil type varies on any property, it is essential that pasture samples be taken from all soil types as a single sample might give quite misleading results.

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APPENDIX.

1. Collection of Samples and Analytical Methods.

In collecting samples care was taken to avoid soil contamination; in the case of capeweed the samples were usually washed with water to remove adhering soil particles. After drying, samples were crushed in mills fitted with iron or stainless steel screens.

In all earlier samples the analyses were done by the method of Sylvester and Lampitt (1935) after dry ashing with magnesium nitrate (Bennetts and Beck, 1942). In later samples the more accurate wet digestion method of Eden and Green (1940) was used. Many of the earlier samples have been re-analysed by wet digestion method and good agreement has been obtained with the two methods. The more recent figures are given in the tables.

2. Results.

For convenience, samples are generally grouped according to their soil zones (Teakle, 1937-38) and are arranged in geographical sequence. Soil details are given only when the soil type is not typical of the particular region, or when soil types vary within the locality. The names of properties are given only in cases where Teakle and his collaborators have carried out experiments.

The Gingin and Denmark results have been published elsewhere in detail but are summarised here for sake of completeness. Most of the samples from Denmark and from the Corrigin region were analysed by Mr. S. T. Evans.

The copper content of the pastures is expressed as parts per million, calculated on a moisture-free basis.

The following abbreviations have been used for the pasture species:—

Abbreviation.	Common Name.	Systematic Name.
<i>Clovers—</i>		
Clover 1	White Clover	<i>Trifolium repens</i>
„ 2	Subterranean Clover	<i>T. subterraneum</i>
„ 3	Cluster Clover	<i>T. glomeratum</i>
„ 4	Hop Clover	<i>T. procumbens</i>
„ 5	Woolly Clover	<i>T. tomentosum</i>
„ 6	Drooping Flowered Clover	<i>T. cernuum</i>
„ 7	Suckling Clover	<i>T. dubium</i>
Medic 1	Burr Medic	<i>Medicago denticulata</i>
„ 2	Goldfields Burr	<i>M. minima</i>
<i>Grasses—</i>		
Grass 1	Wimmera Rye Grass	<i>Lolium sp.</i>
„ 2	Perennial Rye Grass	<i>L. perenne</i>
„ 3	Cocksfoot	<i>Dactylis glomerata</i>
„ 4	Paspalum	<i>P. dilatatum</i>
„ 5	Silver Grass	<i>Vulpia myuros</i>
„ 6	Barley Grass	<i>Hordeum marinum</i>
„ 7	Barley Grass	<i>H. maritimum</i>
„ 8	Yathroo Oat	<i>Avena barbata</i>
„ 9	Yorkshire Fog	<i>Holcus lanatus</i>
„ 10	Kikuyu	<i>Pennisetum clandestinum</i>
„ 11	Prairie Grass	<i>Ceratochloa catharticus</i>
„ 12	Madrid Brome	<i>B. madritensis</i>
„ 13	Couch Grass	<i>Cynodon dactylon</i>
<i>Other Species—</i>		
....	Capeweed	<i>Cryptostemma calendula- ceum</i>
....	Wild Geranium	<i>Erodium botrys</i>
....	Guildford Grass	<i>Romulea rosea</i>
....	Sorrel	<i>Rumex acetosella</i>
....	Wodjil	An association of <i>Acacia</i> spp., <i>Casuarina camp- estris</i> , <i>Hakea multi- lineata</i> , and <i>Dodonaea</i> sp. growing on lateritic soil.

A.—THE ZONE OF THE GREY, YELLOW, AND RED PODSOLISED SOILS.

I.—THE SWAN LITTORAL REGION.

Sample No.	Date Sampled.	Locality.	Details.	Copper Content.
(a) Talus Soils.				
423	17-7-39	Armadale	Guildford Grass, <i>Bromus</i> sp., Clovers 2, 7	7.6
(b) Alluvial Soils of the Flats.				
435	7-9-39	Guildford	Guildford Grass	4.9
523	4-11-39	Keysbrook	Clover 2	7.3
563	1939 Cut	Pinjarra	Meadow Hay; Clovers 2, 7; Grass 5, 1	7.8
198	28-1-38	Harvey	Clover 1; Grasses 2, 3	8.8
199	do.	do.	Grasses 3, 4, 13	5.4
559	17-6-40	do.	Very short. Clover 2; Grasses 4, 2	14.0
560	do.	do.	Short. Grasses 2, 4; Clover 1	12.4
561	do.	do.	Clovers 1, 2	12.3
562	do.	do.	Clover 2; Grasses 2, 4	8.8
305	16-9-38	Waterloo	Capeweed, flowering	12.6
549	5-12-39	Dardanup	Grass 1; various Clovers	10.2
(c) The Sandhill Soils.				
267	30-7-38	"Mimegarra," Dandaragan	Medic 1; Grasses 5, 6. Soil—Strictly an alluvial, derived from "Gingin Clay"	6.3
268	do.	do. do.	As No. 267, with Capeweed	2.3
872	2-10-48	Bullsbrook	Grass 5, Clover 7	1.9
434	2-8-39	West Subiaco	Capeweed	4.8
170	8-11-37	do.	Lupin seeds	10.2
282	23-8-38	King's Park	Capeweed	9.9
436	9-8-39	Maida Vale	Capeweed. Soil—Deep white sand	4.0
290	5-9-38	Rockingham	Capeweed. Soil—Calcareous sand	2.8
291	do.	do.	Adjacent to 290. <i>Bromus</i> sp. Grasses 13, 10, 5	3.2
(d) Falling Disease Areas.				
A67	9-6-41	Quindalup*	Capeweed, small plants	3.3
314	28-9-38	Vasse*	Clover 6	2.6
317	27-9-38	Jindong*	Clover 6, 7; various Grasses	1.9
391	7-3-39	do.*	<i>Hypochoeris</i> sp.; Sorrel; Grass 9	1.4
412	19-6-39	do.*	Short Clover 6; Grass 9; Capeweed	1.8
A127	11-9-41	do.*	Capeweed	1.3
748	21-9-43	do.*	Mainly Clover 6	1.8
330	13-10-38	Yelverton	Clover 2	7.8
315	27-9-38	Metrioup*	Clover 6; Grass 9; Clover 7	2.2
316	do.	do.*	Clover 6	1.3
337	31-10-38	do.*	Clover 6; some Grasses	1.3
574	3-9-40	do.*	Capeweed	2.9
A126	6-8-41	do.*	Capeweed; small Plants	3.7
336	1-11-38	do.	Clover 2; some Grasses	3.5
343	24-11-38	do.	Clover 2	4.6
472	1938 Cut	do.	Meadow Hay; Clover 2; Grasses	3.6
318	27-9-38	Cowaramup*	Clover 6	1.7
389	8-3-39	do.*	Dry Grasses; Sorrel; Clover 6	1.2
390	do.	do.*	Grass 4; Rushes; <i>Lotus</i> sp.	2.3
A65	9-6-41	do.*	Capeweed; small Plants	7.2
914a	23-10-50	Bramley Research Station	Grass 5	1.6
914g	do.	do. do.	Sorrel	1.5
914h	do.	do. do.	<i>Lotus</i> spp.	0.9
914j	do.	do. do.	Capeweed	1.4

* Properties on which Falling Disease has occurred.

A.—THE ZONE OF THE GREY, YELLOW, AND RED PODSOLISED SOILS—*continued*.
I.—THE SWAN LITTORAL REGION—*continued*.

Sample No.	Date Sampled.	Locality.	Details.	Copper Content.
(d) <i>Falling Disease Areas—continued.</i>				
914m	do.	do. do.	Clovers 4 and 7	1.1
414	20-6-39	Margaret River	Capeweed; Clover 2	2.9
475	5-9-39	do. do.	Clover 6	1.1
476	do.	do. do.	Clover 2. (See note in text)	1.2
478	do.	do. do.	Clover 2; Grass 5; Capeweed	1.8
473	do.	Osmington	Clover 2; Capeweed; <i>Lotus</i> sp.; Grass 1	4.8
483	do.	do.	Clover 2; Grass 5	4.0
477	do.	Group 74	Clover 2, 7, 6	2.1
481	do.	do.	Clover 6	1.4
A66	9-6-41	Witchcliffe *	Capeweed; small Plants	6.6
388	8-3-39	Forest Grove	Dry Clover 2; Grass 5	2.5
482	4-9-39	do.	As 388 (green)	2.2
387	8-3-39	do.*	Dry Grasses; Clover 6, 4; Grass 4 (green)	1.4
413	20-6-39	do.*	Capeweed; Clover 6; Grass 1	1.7
415	1938 Cut	do.*	Meadow Hay	1.5
479	4-9-39	do.*	Short Clover 6; Grass 5	1.3
480	do.	do.*	Clover 6; Grass 1	1.1
573	3-9-40	do.*	Capeweed	4.2
474	5-9-39	do.	Short Clover 2; Grass 5	4.6

* Properties on which Falling Disease has occurred.

(e) *Gingin Cretaceous Area (Ataxic).*

Group 1:—"Gingin Clay."

Sample No.	Date.	Copper Content.	Sample No.	Date.	Copper Content.
84	17-6-37	2.9	258	28-7-38	6.1
98	5-8-37	2.6	310	20-9-38	2.2
154	4-10-37	1.7	377	24-1-39	1.1
262	16-12-37	1.4	397	1-6-39	2.4

Mixed pastures. Predominating plants Grasses 8, 6, Medic 1. Samples 262 and 377 dry 377 and 397 heavily grazed. For soil details see Hosking and Greaves (1935-36).

Group 2:—"Whakea Sand."

Sample No.	Date.	Copper Content.	Sample No.	Date.	Copper Content.
85	17-6-37	3.8	311	20-9-38	3.1
155	4-10-37	2.2	331	14-10-38	3.4
261	16-12-37	1.7	333	14-10-38	3.9
263	16-12-37	1.3	378	24-1-39	2.5
255	28-7-38	3.5	396	5-4-39	2.0
256	28-7-38	3.9	398	31-5-39	2.6
257	28-7-38	2.6	446	15-8-39	2.2
259	28-7-38	2.3	447	15-8-39	2.5
308	20-9-38	2.2	448	15-8-39	2.0

Mixed pastures. Predominating plants Grass 5, Capeweed, Clovers 7, 2, Medic 1, *Poa annua*, Grasses 12, 8, 6. Samples 261, 263, and 378 dry.

A.—THE ZONE OF THE GREY, YELLOW, AND RED PODSOLISED SOILS—*continued.*
 I.—THE SWAN LITTORAL REGION—*continued.*

Group 3: "Whakea Sand."
 Miscellaneous Samples.

Sample No.	Date.	Details.	Copper Content.
399	31-5-39	Guildford Grass	4.1
195	1937	Lupin seeds	7.9
386	1938	do.	5.0
277	11-8-38	Capeweed—Large plants	4.6
A68	16-6-41	do. Small plants	7.1
A76	21-7-41	do. Large plants (Same area as A68)	3.0
A69	16-6-41	do. Small plants	5.8
A70	16-6-41	do. do.	7.7

II.—DARLING PENEPLAIN REGION.

Sample No.	Date Sampled.	Locality.	Details.	Copper Content.
514	27-9-39	Bindoon	Chittering Valley : Grasses and Clovers. Soil—Alluvial red sandy loam	6.9
913a	21-10-50	Werribee	Clover 4	3.9
913b	do.	do.	Clover 2, Grasses 1, 12	6.4
508	26-9-39	Beechina	Short growth. Clovers 2, 3 ; Capeweed	4.1
509	do.	Wooroloo	Clover 2 ; Grass 5	2.7
421	17-7-39	Karragullen	Capeweed	2.7
422	do.	do.	Clover 2 ; Grasses ; Capeweed	4.0
916A	Sep., 1950	Noggerup	Clover 2	4.2
916B	do.	do.	do.	5.5
497	20-9-39	Mayanup	do.	4.3
304	16-9-38	Bridgetown	Capeweed. Soil—Heavy red loam	7.6
634	12-9-40	Pardelup	Clover 2 ; Grasses	2.9
635	do.	do.	Clovers 7, 6 ; Grasses 5, 1	2.8
633	do.	do.	Clover 7 ; Grass 5	4.4

All samples except Nos. 304 and 514 were from lateritic gravel soils. Soil details of No. 916 not available.

III.—FRANKLAND REGION.

Sample No.	Date Sampled.	Locality.	Details.	Copper Content.
502	19-9-39	Jardee	Capeweed	13.3
539	9-11-39	Northcliffe	Small Clovers ; Grasses	2.1
501	19-9-39	Walpole	Capeweed	8.0
550	14-12-39	do.	Grasses ; Clover 7	2.8
516	17-9-39	Nornalup	Clovers 6, 7 ; Grasses	1.8
499	18-9-39	Young's Siding	K. Martin's <i>Lotus major</i> . Soil— Black peaty sand	0.8
506	do.	do. do.	Grass 9, adjacent to No. 499	1.0
498	do.	Bornholm	J. Wolfe's. Grass 2 ; Clover 2. Soil —Karri slope	10.2
503	do.	do.	J. Wolfe's. Grass 9. Soil—Peaty sand	1.9
504	do.	Horton's Siding	Cake Bros. Capeweed. Soil—Brown karri slope	5.2

A.—THE ZONE OF THE GREY, YELLOW, AND RED PODSOLISED SOILS—*continued.*III.—FRANKLAND REGION—*continued.*

Sample No.	Date Sampled.	Locality.	Details.	Copper Content.
432	31-7-39	Kronkup	Medic 1 ; Grasses 11, 1	3.0
500	18-9-39	Torbay	Rutherford's. Clover 2 ; Grass 9. Soil—Peaty sand	4.5
505	do.	do.	Grayson's. <i>Medicago</i> spp., <i>Sonchus</i> sp., annual grasses. Soil—Brown karri slope	7.9
848	15-8-48	Millinup, Porong- orups	Grasses ; Clovers 2, 7	2.8
849	do.	do. do.	Grasses ; Clover 7	2.3
850	do.	do. do.	Clover 6 ; Grasses	1.6
865	21-9-48	Porongorups	Clover 2 ; Grasses	3.3
866	do.	do.	Grasses	5.5
867	do.	do.	do.	7.3
868	22-9-48	Porongorups	Capeweed ; Grasses	4.2
869	do.	do.	Capeweed ; Clover 6	2.8
870	do.	do.	Grasses	4.4

Denmark Pasture Samples—

(a) From experimental holding, Wakundup soil type (Hosking & Burvill, 1938): 8 samples 23rd March, 1939, to 12th December, 1940. Range 2.9-4.4, Mean 3.7 p.p.m.

(b) From other properties on Wakundup soil: 14 samples, 1937-1940. Range 2.2-6.5, Mean 3.7 p.p.m.

(c) From properties on Scotsdale gravelly loam and alluvial soils: 7 samples, 1937-1939 Range 4.3-22.2, Mean 9.9 p.p.m.

B.—THE ZONE OF THE RED BROWN EARTHS.

I.—DWARDA REGION.

Sample No.	Date Sampled.	Locality.	Details.	Copper Content.
788	1-10-44	Kojonup	Grass 5 ; Clover 2 ; <i>Bromus</i> sp.	10.7
789	do.	do.	do. do. do.	6.6

II.—IRWIN REGION.

Sample No.	Date Sampled.	Locality.	Details.	Copper Content.
852	26-9-48	Lynton	Mixed native herbage ; Calcareous soil ; pH 9.1	4.0
853	do.	do.	Grasses, <i>Erharta</i> sp., <i>Bromus</i> sp. ; soil as above	3.1
863	19-9-48	Ajana	Grass 7 ; <i>Erharta</i> sp. ; sandy loam pH 6.2	6.3
864	do.	do.	<i>Stipa</i> sp. ; soil as No. 863	5.6
747a	Sep., 1943	Northampton	Grass 6 ; trace Capeweed	5.4
747b	do.	do.	Grass 6	4.0
178	26-11-37	Greenough Flats	Lupin Seeds	11.5

B.—THE ZONE OF THE RED BROWN EARTHS—*continued.*II.—IRWIN REGION—*continued.*

Sample No.	Date Sampled.	Locality.	Details.	Copper Content.
453	23-8-39	Dongara	Capeweed; Grasses 6, 5; Medic 1. "Sound Country"	8.9
454	do.	do.	Capeweed and various small Clovers. "Sound Country"	8.8
455	do.	do.	Grasses and Medic 1. "Sound Country"	10.6
456	do.	do.	Grasses 6, 5; small Clovers. "Possibly Sound"	3.2
457	do.	do.	Grass 6; Medic 1. "Probably Unsound"	2.9
458	do.	do.	Grass 6. "Probably Unsound"	2.2
218	3-5-38	Coorow	Grass 6; very short	9.5
233	18-5-38	do.	Grass 6; short	11.2

III.—AVON REGION.

Sample No.	Date Sampled.	Locality.	Details.	Copper Content.
511	26-9-39	Wongan Hills Research Station	Capeweed. Soil—Tamar country, greyish sand, clay subsoil	12.0
512	do.	do. do.	Capeweed. Soil—Poorer smokebush country, yellow sand, clay and gravel subsoil	2.2
513	do.	do. do.	Capeweed. Soil—Poor mallee country, light grey sand, gravel subsoil	3.3
510	do.	Burabadji	Capeweed	4.4
97	28-7-37	Meekering	Capeweed	10.0
274	9-8-38	Muresk	Clover 2; Capeweed; Grasses	8.5
275	do.	do.	<i>Stipa</i> sp.; Grasses 5, 6	9.3
91	22-7-37	Beverley	Capeweed	13.4
249	14-7-38	do.	Grass 7	7.5
276	10-8-38	do.	Clover 2; Wild Geranium; Capeweed; Grasses	9.0
....	Sep., 1942	Brookton West	Capeweed; Sandy soil; Stringy wool	3.1
....	do.	do. do.	Capeweed; Heavier soil, Jam (<i>Acacia acuminata</i>) country; Stringy wool	4.2
....	do.	do. do.	Clover; Soil as above; Stringy wool	5.1
....	do.	Aldersyde	Capeweed; Stringy wool	2.5
....	do.	Pingelly East	Capeweed; Sandy soil; Stringy wool	5.4
294	13-9-38	Cuballing	Capeweed	10.0
841	22-3-48	Highbury	1947 Meadow Hay; Clover 3, and mixed Grasses; Stringy wool	5.2
295	do.	Wagin	Capeweed. Soil—Light grey sand, clay subsoil	7.8
296	do.	do.	Clover 2; Wild Geranium. Soil—Light gritty granitic	6.7
....	Sep., 1942	Tambellup	Capeweed; Sandy soil; Stringy wool	3.3
....	do.	do.	do. do. do.	4.4
....	do.	do.	Clover 2; Sandy soil; Stringy wool	3.9

III.—AVON REGION—*continued.*

WAGIN SERIES, 1939-40.

Date.	Group 1.		Group 2.		Group 3.		Group 4.	
	Sample No.	Copper Content.						
22-8-39	451	15.2	450	3.6	449	3.6	452	4.1
19-9-39	493	12.0	494	3.1	496	3.0	495	4.6
24-10-39	519	10.3	517	2.5	520	3.5	518	3.7
14-3-40	552	9.8	555	2.8	553	3.1	554	2.5

All samples dry on 14th March, 1940.

- Group 1: "First Class Country."—Red brown loam on diorite ridge.
Pasture Species.—Grasses 6, 7, 12; Capeweed; Clovers 3, 4.
Sample 493 rather heavily grazed.
- Group 2: "Second Class Country."—Light gritty soil.
Pasture Species.—Clover 2; Wild Geranium; Clovers 3, 5.
- Group 3: "Second Class Country."—Light grey sandy surface, clay subsoil.
Pasture Species.—Clover 5; Grasses 1, 5.
- Group 4: "Third Class Country."—Yellowish grey sand, gravel subsoil; original vegetation mallet, mallee, and poor wandoo.
Pasture Species.—Clovers 3, 5; Capeweed; Grass 5; some Clovers 2, 4.

IV.—"CLOVER BELT," DWARDA AND AVON REGIONS.

All samples of Subterranean Clover, "Dwalganup" strain, collected during early flowering, September, 1947. Leaf and petiole sampled.

Sample No.	Locality.	Soil Type and pH.	Copper Content.
821	Northam	Light sandy; 5.82	6.9
824	do.	Heavy; 6.64	14.7
822	Burges' Siding	Heavy; 6.34	12.5
813	Dale Bridge	5.94	9.0
811	West Dale	Heavy; 6.32	12.7
812	do.	Light sandy; 5.22	7.1
818	Wandering	Light sandy; 5.74	4.0
826	Narrogin	Light lateritic; 6.30	3.7
809	do.	Heavy; 6.19	14.0
808	Highbury	Light; 6.32	9.1
814	Kojoonup	Light lateritic; 6.30	9.4
815	do.	Light; 5.66	6.8
829	do.	Light sandy; 6.10	6.3

C.—THE ZONE OF THE GREY AND BROWN CALCAREOUS SOLONISED SOILS.

I.—CORRIGIN REGION.

All Stringy wool properties except No. 283.

Sample No.	Date Sampled.	Locality.	Details.	Copper Content.
....	Sep., 1942	Tammin	Capeweed ; " Wodjil " soil	4.1
....	do.	North Bungulla	Capeweed ; " Sand plain "	6.6
....	do.	Kellerberrin	Capeweed ; Sandy soil	4.5
....	do.	do.	Capeweed ; Heavier soil	8.5
....	do.	Doodlakine	Capeweed ; Sandy and wandoos (<i>E. redunca</i> var. <i>elata</i>) country	4.8
....	Aug., 1942	Kwolyin	Capeweed	3.3
....	do.	do.	do.	4.2
....	Sep., 1942	do.	Capeweed ; Clover 7	3.3
....	do.	Pantapin	Capeweed	4.1
....	do.	Yoting	do.	2.6
....	Aug., 1942	Lomos	do.	2.4
....	do.	Corrigin	do.	4.5
....	do.	Bullaring	do.	3.0
....	do.	do.	do.	4.2
749	Sep., 1943	Yealering	do.	4.0
750	do.	do.	do.	6.1
283	24-8-38	Lake Grace	Grass 6	7.0

II.—MERREDIN REGION.

Sample No.	Date Sampled.	Locality.	Details.	Copper Content.
430	24-7-39	Merredin Research Station	Capeweed	8.4
429	do.	do. do.	Short Grass 6	10.6
461	30-8-39	do. do.	Grass 6 with Medic 2, 1	6.0
522	3-11-39	do. do.	Grass 6 ; Dry	5.0
556	27-3-40	do. do.	do. do.	5.2
887	27-7-49	do. do.	Grass 6	7.8
888a	6-9-49	do. do.	do.	6.0
888c	do.	do. do.	Medic 1	9.0
893a	3-10-49	do. do.	Grass 6	4.0
893b	do.	do. do.	Clover 3, 5 ; Medic 1, 2	6.6
901a	25-7-50	do. do.	Short Grass 6	6.4
901b	do.	do. do.	Medic 2	9.9
909a	19-9-50	do. do.	Grass 6	5.0
909b	do.	do. do.	Medic 2	7.0
912c	17-10-50	do. do.	Grass 6 ; Dry	4.2
912d	do.	do. do.	Clover 5 ; Medic 1	8.3
427	24-7-39	do. do.	Capeweed ; Wodjil soil	4.7
459	30-8-39	do. do.	do. do.	3.5
856	6-9-48	Merredin-Colgar	Capeweed ; Light soil, pH 7.0	5.9
857	do.	do. do.	Grass 6 ; Heavy soil, pH 7.5	6.3
859	do.	do. do.	Capeweed ; Clover 3 ; Grass 5 ; Light soil, pH 6.9	3.9

All samples from Merredin Research Station except 427 and 459 from a brown sandy clay loam with calcareous subsoil ; pH 7 to 7.5.

D.—THE ZONE OF THE BROWN TROPICAL SOILS.

KIMBERLEY RESEARCH STATION, ORD RIVER.

Sample No.	Date.	Details.	Copper Content.
895c	28-10-49	Elephant Grass (<i>Pennisetum purpurea</i>)	5.5
895e	do.	Flinders Grass (<i>Iseilema fragila</i>)	3.5

Sample 895c from Ord River sandy loam ; 895e from Cununurra clay.



The levels of copper, molybdenum and inorganic sulphate in some Western Australian pastures a contribution to the study of copper deficiency diseases in ruminants

A. B. Beck

Summary—Levels of copper, molybdenum, and inorganic sulphate are reported for samples of Western Australian pastures from areas where copper deficiency diseases in ruminants have occurred and from unaffected areas.

The copper contents of the pastures were determined previously. It was found that the copper deficiency diseases commonly occurred where pastures contained less than 3 p.p.m. Cu in the dry matter during the growing period, while such diseases were absent where the pastures contained more than 6 p.p.m. Cu. Values between 3 and 6 p.p.m. Cu were classified as marginal.

The molybdenum and inorganic sulphate contents of the original pasture samples have now been determined. The molybdenum contents of the three classes of pasture were found to lie within the same range (0.1 to 4 p.p.m. Mo in the dry matter, with the majority less than 1 p.p.m.). These values are similar to those recorded by overseas workers for normal pastures. Inorganic sulphate contents of the three classes of pasture also lay within the same range (0.1 to 0.9 per cent SO_4 , with the majority between 0.2 and 0.4 per cent).

The results for the pastures examined suggest that in Western Australia the low copper level is the constant and the most significant factor associated with enzootic ataxia in sheep and falling disease in cattle.

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The levels of copper, molybdenum and inorganic sulphate in some Western Australian pastures

a contribution to the study of copper deficiency diseases in ruminants

A. B. Beck

During the period 1937 to 1948 it was shown by Bennetts and his co-workers (Bennetts and Chapman 1937; Bennetts and Hall 1939; Bennetts *et al.* 1941; Bennetts and Beck 1942; Bennetts, Harley and Evans 1942; Bennetts, Beck and Harley 1948) that enzootic ataxia in lambs and falling disease in cows were characterized by low levels of copper in the blood and liver of affected animals and that these disorders could be prevented by the use of copper supplements. Other elements used as supplements had no beneficial effects and as both diseases were invariably associated with a low level of copper in the pastures grazed, it was concluded that copper was the only element influencing the incidence of the disease syndrome.

An assessment of the copper content of Western Australian pastures in relation to animal health (Beck 1941, 1951) had shown that typical disease syndromes developed in stock grazing on pastures which contained less than 3 p.p.m. Cu in the dry matter during the growing period, but not in those which contained more than 6 p.p.m. Pastures with concentrations between 3 and 6 p.p.m. were classed as marginal.

Since these earlier investigations were carried out, however, it has been shown that copper metabolism in sheep may be markedly altered by differences in the amounts of molybdenum and of inorganic sulphate in the diet (Dick and Bull 1945; Cunningham 1950; Dick 1953, 1954; Wynne and McClymont 1956). However, Allcroft and Lewis (1956) conclude that molybdenum and inorganic sulphate are not the main factors which cause conditioned copper deficiency in stock in England.

The present paper reports the copper, molybdenum and inorganic sulphate contents in a number of pastures from the Western Australian agricultural areas, particularly from those on which copper-deficiency syndromes commonly occurred in sheep and cattle.

Methods and material

Determinations of copper were made after wet digestion by the method of Eden and Green (1940). With samples of low molybdenum content the dithiol method of Piper and Beckwith (1948) was used without modification. With other samples, the copper was first determined as the diethyldithiocarbamate complex in amyl alcohol. The aqueous phase (after removal of the copper) was then boiled to remove any remaining amyl alcohol and most of the excess ammonia. The solution was then acidified to pH 0.5 or less, the molybdenum extracted with cupferron and estimated as in the method of Piper and Beckwith. This modification gave quantitative recoveries of microgram amounts of molybdenum.

Inorganic sulphate was estimated by the benzidine method of Dick and Bingley (personal communication). In this method the sulphate was extracted from the plant material by boiling 0.01N HCl and protein precipitated with alcoholic trichloroacetic acid. In order to ensure quantitative precipitation of benzidine sulphate, 1.77 mg Na₂SO₄ was added to each of the test solutions. Correction was made for non-specific material precipitated by the alcoholic solution of benzidine. The precipitated benzidine sulphate was titrated with 0.03N NaOH at 100°C.

All values, both for pastures and for livers, are reported on the moisture-free basis.

In many cases the samples were separated into mixed grasses and a single legume. Where no such classification is given the pastures are generally mixed grass-clover pastures. A few separated samples of

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capeweed (*Cryptostemma calendulaceum*) are included in the tables as this species is an important constituent of some Western Australian pastures. Some of the samples from the original investigations were no longer available for determination of molybdenum and inorganic sulphate content.

Botanical details and soil pH of many of the areas are given by Beck (1941); soil types are described by Teakle (1937-38).

Origin of pasture samples

Gingin

These samples were from one property on which the nature and cause of enzootic ataxia were investigated by Bennetts and Beck (1942). In 1937 a high incidence (96 per cent) of ataxia occurred here in experimental lambs, whereas in 1938 only 4 per cent were affected. The natural incidence of ataxia on the property was not determined in 1939; the occurrence of 10 per cent of cases in the progeny of ewes which had access to copper-containing licks in the previous year, indicated that this would have been high.

The results for samples from Whakea Sand and Gingin Clay (Hosking and Greaves 1935-36) have been combined as there were no significant differences in the pasture levels of any of the constituents examined.

Porongorups and South Merredin

These samples were collected from properties at times when there was a high incidence of ataxia in lambs. The pastures at South Merredin were heavily grazed and the sample collected may not represent exactly the material eaten by sheep.

Falling disease areas

The pastures of this region, which lies between Busselton and Margaret River, have been classed as severely deficient in copper (Bennetts *et al.*, 1939, 1941, 1942, 1948) and have recently been shown to have a low cobalt status (Harley and Beck, 1958). Copper fertilizers have been used for many years throughout the area. The samples from the Bramley Research Station (near Margaret River) were from an untreated area of a property on which falling disease had occurred in 1938.

Merredin Research Station

Sheep grazing on the pastures sampled have shown liver copper values of 50-300 p.p.m. On the basis of unpublished values for about 1200 Western Australian

sheep, these levels are classed as low normal to normal. Values for individual sheep showed considerable variation from year to year but the reason has not been found. Blood copper levels have remained within the normal range (Beck 1956).

Beverley, Kojonup and Wokalup Research Stations

Normal levels of liver copper (110-580 p.p.m.) have been found in sheep and cattle on these properties.

Great Southern Region

These samples were collected in connection with other investigations and the copper status of animals grazing on these pastures is generally not available. However, clinical signs of copper deficiency are generally absent in this region and a limited number of liver samples have shown normal copper levels.

Toodyay

The samples were from a property on which chronic copper poisoning in sheep had occurred. Histological evidence suggested that this was of hepatogenous origin, (Anon. 1956) but no plant with a hepatotoxic principle has been found.

Results and discussion

The analytical data are set out in tables 1 and 2.

In addition to these data, two small areas of pasture have been found in which the molybdenum content was moderately high. One was on certain alluvial soils at Dongara where the pasture contained up to 5.4 p.p.m. Mo. The other was on a peat swamp at Mandogalup, near Fremantle, where values up to 12 p.p.m. Mo were obtained. Steely wool was reported in sheep grazing at Dongara and some scouring occurred in cattle at Mandogalup, but the areas were quite small and atypical.

Animal relationships

An examination of the molybdenum levels shows that they are similar to those found in normal pastures in New Zealand and England (Cunningham 1954; Allcroft 1952), and are generally very much lower than those found in areas of New Zealand where "complicated" copper deficiency occurs in stock (Cunningham, 1950). There is no evidence that levels are higher in pastures from areas where ataxia and falling disease have occurred.

The daily intake of molybdenum for sheep grazing on the pastures listed will rarely be outside a range of

TABLE 1

Copper, molybdenum, and inorganic sulphate contents of pasture samples from areas where copper deficiency has occurred in stock.

Sample details				Copper content			Molybdenum content			Sulphate content		
District	Year	Month	Type of pasture†	No. of Samples	Mean	Range	No. of Samples	Mean	Range	No. of Samples	Mean	Range
				<i>p.p.m. (dry matter)</i>			<i>p.p.m. (dry matter)</i>			<i>% (dry matter)</i>		
Gingin				5	2.6	1.5-3.8	3	0.22	<0.10-0.37	3	0.33	0.25-0.40
	1937	June-Oct.		11	3.4	2.2-6.1	6	0.20	0.11-0.27	4	0.33	0.28-0.36
	1938	July-Oct.		6	2.7	2.0-4.1	3	0.37	0.14-0.63	1	0.36	—
	1939	June-Aug.		6	1.6	1.1-2.4	5	0.18	<0.10-0.47	2	0.15	0.09-0.21
	1937-39	Dec.-Apr.	Dry									
Porongorups				3	2.2	1.6-2.9	3	0.75	0.40-1.09	2	0.38	0.32-0.44
	1948	Aug.										
South Merredin				1	3.9	—	—	0.20	—	—	—	—
	1948	Sept.	Heavily grazed									
Busselton-Margaret River‡				6	2.2	1.3-4.6	6	0.55	<0.10-1.25	4	0.33	0.20-0.50
	1938-43	July-Nov.		1	2.3	—	—	0.34	—	—	0.72	—
	1939	March	Green summer	3	1.3	1.1-1.5	3	0.91	0.67-1.4	2	0.15	0.15-0.16
			Dry summer	1	7.9	—	—	0.17	—	—	0.23	—
	1938	Oct.	Sub clover §									
Bramley Research Station				13	3.1	1.8-4.7	13	1.76	0.86-3.8	—	—	—
	1950-51	July-Aug.	Grasses	11	1.8	1.4-2.5	11	0.39	<0.10-1.10	—	—	—
			Legumes	2	2.4	—	2	0.70	0.66-0.75	—	—	—
			Capeweed	16	2.3	1.6-3.3	16	1.03	0.36-3.4	—		—
		Sept.-Oct.	Grasses	14	1.5	0.9-2.7	14	0.53	0.29-1.9	—	—	—
			Legumes	3	1.7	1.4-2.4	3	0.46	0.28-0.76	—	—	—
			Capeweed	14	2.1	1.4-4.6	14	0.64	0.21-1.28	14	0.28	0.18-0.62
	1953-55	Nov.	Grasses	18	1.9	1.0-3.1	18	0.28	<0.10-1.1	18	0.20	0.10-0.48
			Legumes									

† All pastures green except where specified otherwise.
 ‡ Falling disease properties.
 § Healthy property in falling disease area.
 || A bulk sample of 5 of these grasses contained 0.37 per cent inorganic sulphate.

values of 0.2 to 1.5 mg Mo. Dick (1954) describes a pen experiment with sheep on a daily intake of 10 mg Cu and an unspecified intake of inorganic sulphate. An increase of the molybdenum intake from 0.4 to 1.4 mg had no effect on liver storage of copper under the conditions of the experiment. The effect of such variations under conditions of low copper intake and at known levels of sulphate intake has not been investigated. However, as published data (Cunningham, 1950) suggest that the ratio of Mo to Cu is more

important than absolute amounts, it is possible that the levels of molybdenum in some of the low copper pastures may be sufficient to cause some depression of copper storage in grazing stock.

There are few data available in the literature to assess the levels of inorganic sulphate in pasture which are normal with respect to animal health. Recently, Barker (1961) has published a survey of the copper, molybdenum and inorganic sulphate levels in the herbage of Rottneest Island, Western Australia, but

TABLE 2

Copper, molybdenum, and inorganic sulphate contents of pasture samples from areas where signs of copper deficiency have not been observed in stock.

Sample details					Copper content		Molybdenum content		Sulphate content	
District	Year	Month	Type of pasture†	No. of Samples	Mean	Range	Mean	Range	Mean	Range
					<i>p.p.m. (dry matter)</i>		<i>p.p.m. (dry matter)</i>		<i>% (dry matter)</i>	
Merredin Research Station ‡										
	1953	April	Grasses	3	8.2	7.7-8.6	1.34	1.26-1.42	0.23	0.20-0.27
		June	Grasses	3	8.5	7.7-9.4	1.09	0.85-1.35	0.25	0.23-0.26
		Aug.	Grasses	3	5.6	5.2-5.8	0.53	0.40-0.64	0.16§	0.15-0.17
			Medics	3	10.3	9.5-11.1	0.91	0.65-1.21	0.15§	0.14-0.17
		Oct.	Grasses (dry)	3	2.5	2.2-2.6	0.47	0.41-0.50	<0.10	—
	1954	June	Grasses	5	9.9	8.0-11.7	1.04	0.73-1.41	0.30	0.20-0.43
		Aug.	Grasses	6	5.7	5.0-6.7	1.15	0.80-1.94	0.27	0.19-0.36
			Medics	3	9.3	8.1-10.0	0.55	<0.20-1.28	0.24§	0.21-0.28
		Sept.	Grasses	8	4.6	3.6-7.5	1.17	0.76-1.86	0.25	0.16-0.33
			Medics	5	8.2	7.4-9.1	0.54	0.33-1.81	0.22	0.17-0.27
		Oct.	Grasses (dry)	6	3.1	2.5-3.8	0.74	0.42-1.14	0.18	0.11-0.24
			Medics (dry)	3	6.3	5.6-6.7	1.34	1.24-1.45	0.22	0.16-0.28
	1955	July	Grasses	6	8.1	6.9-11.1	0.49	0.29-0.77	0.17	0.14-0.20
		Sept.	Grasses	6	5.9	5.1-6.5	0.75	0.66-0.82	0.18	0.13-0.23
			Medics	5	9.5	9.0-10.0	1.37	0.82-1.88	0.27	0.20-0.40
		Oct.	Grasses (mature but green)	6	4.5	4.0-5.0	0.55	0.41-0.70	0.18	0.15-0.20
			Medics	5	7.9	7.1-8.5	1.68	1.13-2.73	0.20	0.15-0.22
Beverley (Avondale Research Station)										
	1953-54	Sept.	Grasses	17	5.9	3.8-9.6	1.48	0.45-2.42	0.23	0.11-0.44
			Sub. clover	16	8.2	2.8-13.0	0.56	0.17-1.48	0.24	<0.10-0.90
			Capeweed	2	9.0	6.5-11.4	0.97	0.96-0.98	0.17	0.14-0.21
Kojonup (C.S.I.R.O. Field Station)										
	1959	Sept.	Grasses	4	6.4	5.8-6.7	0.23	0.15-0.45	0.25	0.18-0.31
			Sub. clover	3	6.4	5.4-7.2	<0.10	—	0.27	0.18-0.40
			Capeweed	4	6.3	4.9-9.5	0.11	<0.10-0.15	0.28	0.14-0.38
Wokalup Research Station										
	1954	Sept.	Grasses	6	7.3	4.3-8.5	0.25	<0.10-0.62	0.36	0.26-0.55
			Legumes	7	11.1	6.7-13.7	0.10	<0.03-0.45	0.20	0.15-0.25
			Capeweed	1	15.8	—	0.19	—	0.23	—
Great Southern Region (Northam to Kojonup)										
	1947	Sept.	Sub. clover	14	8.7	3.7-14.7	0.17	<0.03-0.32	—	—
Toodyay										
	1956	Aug.-	Grasses	6	6.7	4.3-8.6	1.37	0.62-2.26	0.19	0.13-0.34
		Sept.	Capeweed	5	8.3	5.4-14.4	1.60	0.49-2.61	0.19	0.17-0.22
	1957	Aug.	Capeweed	4	11.5	9.1-14.0	0.63	0.49-0.94	0.28	0.17-0.49

† All pastures green except where specified otherwise.

‡ The 1953 samples are from 3 sites in one paddock ; those of 1954-55 are from 6 sites in another paddock of similar soil type.

§ Only 2 samples analysed.

domestic stock are not carried in this area. Allcroft and Lewis (1956) give a range of 0.29 to 0.96 per cent for 34 pasture samples from swayback and non-swayback areas, and from two non-swayback farms in a high molybdenum area. There was no significant difference in the inorganic sulphate levels of pastures from the three areas. Studies on copper deficiency in stock in Greece (Spais 1956), showed that the usual pastures of that country contained between 0.2 and 0.8 per cent inorganic sulphate, while some halophytic species contained up to 4 per cent. Moule, Sutherland and Harvey (1959) found levels of 0.01 to 1.7 per cent in herbage from north-western Queensland where copper deficiency had occurred in sheep. In Western Australian pastures the levels of inorganic sulphate are generally lower than those quoted above. The levels in some of the copper deficient pastures tend to be slightly higher than in normal pastures but generally there is little difference.

The findings of the present investigation give no reason for altering earlier conclusions, that, in Western Australia, enzootic ataxia and falling disease were primarily due to the low level of copper in the pastures grazed. From other observations there is a strong suggestion that there are factors in the spring pasture of these areas which exert a marked seasonal interference with copper utilization but as yet there is no evidence to associate these with molybdenum or inorganic sulphate.

Plant relationships

Although the primary object of this work was to relate the molybdenum and inorganic sulphate levels in herbage to copper metabolism in animals, several interesting plant relationships have emerged from the data. The most striking is that the relative amount of copper in grasses and subterranean clover is apparently related to the amount of available copper in the soil. Figure 1 shows the results for 38 pairs of samples of grasses and subterranean clover growing side by side on a wide range of soil types. Statistical examination of the results shows that the correlation coefficient between the copper levels of clover and grass is 0.898 ($P < 0.001$). The regression equation is $y = -2.84 + 1.752x$ where y and x are the concentrations of copper in the clover and grass respectively. At the point 4.0 ± 0.4 (5 per cent fiducial limits) the concentration of copper will be the same in both species. If the subterranean clover has a copper level less than 4 p.p.m., thus approaching the deficient classification, associated grasses will be found to contain a higher level

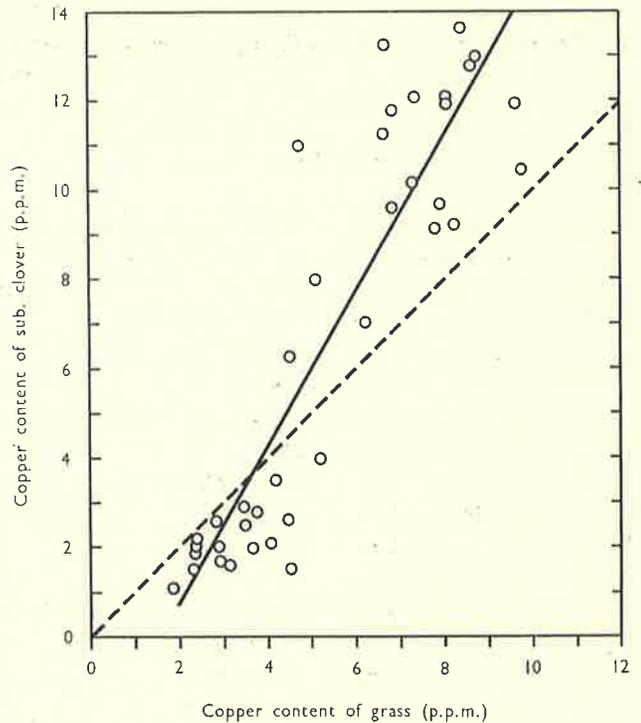


Figure 1—Copper content of grasses and subterranean clover growing together.

— Calculated regression line.

----- Line representing equal concentrations in both species.

of copper. If the level of copper in the clover is greater than 4 p.p.m., and thus approaching the normal classification, the grasses will be found to contain less copper.

The samples of subterranean clover showed quite low levels of molybdenum with the exception of those from the Avondale Research Station, Beverley. Grasses were found always to contain more molybdenum than subterranean clover growing on the same area. A summary of the molybdenum levels is set out in table 3, together with comparable data of Dick, Moore and Bingley (1953) for the Murray Valley of Eastern Australia.

The high levels of molybdenum recorded for some of the grass samples at the Bramley Research Station were due to the presence of a large proportion of Yorkshire fog (*Holcus lanatus*). The ability of this species to absorb molybdenum preferentially is shown in the data for separated species given in table 4. Similar observations have been made by Lewis (1943) and by Walsh, Neenan and O'Moore (1953).

There is little difference between the inorganic sulphate levels in grasses and in legumes.

TABLE 3

Molybdenum content of grasses and subterranean clover.

Source of samples	Number of samples	Molybdenum content	
		Mean	Range
		<i>p.p.m. (dry matter)</i>	
Beverley (W.A.)			
grass	17	1.48	0.45-2.42
clover	16	0.56	0.17-1.48
All other areas (W.A.)			
grass	110	0.87	<0.10-3.80
clover	40	0.16	<0.03-0.62
Murray Valley (N.S.W., Vic.)*			
grass	97	0.96	0.18-4.36
clover	22	0.69	0.25-1.70

* Data from Dick, Moore and Bingley (1953).

TABLE 4

Molybdenum and copper contents of different species growing together at Bramley Research Station, October, 1953.

Species	Molybdenum content	Copper content
	<i>p.p.m. (dry matter)</i>	
<i>Holcus lanatus</i>	2.55	3.7
<i>Anthoxanthum odoratum</i>	2.00	4.0
<i>Vulpia myuros</i>	1.30	4.3
<i>Lotus uliginosus</i>	0.64	1.5
<i>Bromus gussonii</i>	0.45	4.5

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SECTION 4

SECTION 4

COPPER TOXICITY IN MAN AND IN SHEEP

This Section comprises two papers -

- 4:14 "Aspects of copper metabolism in Wilson's disease" :
J.B. Stokes, A.B. Beck, D.H. Curnow, J.G. Topliss
and E.G. Saint. Aust. Annals Medicine, 1955, 4,
36.
- 4:15 "Copper poisoning in sheep in Western Australia".
A.B. Beck and H.W. Bennetts, J. Roy. Soc. W. Aust.,
1963, 46, 5.

The first paper describes observations made on a patient suffering from Wilson's Disease in 1953-54. This disease is extremely rare, but the etiology has been extensively studied in other countries. The primary metabolic upset is apparently still uncertain, but the disease is always characterized by a profound alteration of copper metabolism leading to the massive accumulation of copper in the liver and brain. The results obtained in the present investigation were similar to those obtained by other workers elsewhere.

In the above investigation the candidate was responsible only for the chemical and balance studies.

Section 4

- 2 -

The second paper describes two outbreaks of copper poisoning of sheep in Western Australia. In the first case the poisoning was probably due to ingestion of plants high in copper, but the etiology of the second outbreak was not determined. The preliminary diagnosis of copper poisoning was made by Dr. Bennetts, who also did much of the histological examination. The design of the investigations and most of the field observations were carried out by the candidate, and chemical work was done either by the candidate, or his assistants.

Aspects of Copper Metabolism in Wilson's Disease

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ASPECTS OF COPPER METABOLISM IN WILSON'S DISEASE¹

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HEPATOLENTICULAR DEGENERATION, first described by Kinnier Wilson in 1912, is an uncommon disease (only one other case has hitherto been diagnosed in this State, with a population of 600,000), characterized by the clinical association of cirrhosis of the liver, neurological symptoms and signs attributable to degenerative changes in the lenticular nuclei (muscle rigidity, dysarthria, athetoid movements and emotional lability), and the appearance of a peripheral rim of greenish-brown corneal pigmentation (Kayser-Fleischer rings). A comprehensive study of the pedigrees of many patients has produced evidence that the disease is inherited as an autosomal recessive (Bearn, 1953). That a disorder of copper metabolism plays an important role in the pathogenesis of Wilson's disease was evident from the finding by Glazebrook (1945) of a high copper content of both brain and liver, and by Mandelbrote and his colleagues (1948) of a high level of excretion of copper in the urine. Amino-aciduria was discovered by Uzman and Denny Brown (1948) to be a further detectable biochemical anomaly. Since an increase in the level of serum amino acids has not been found in cases of Wilson's disease (Cooper *et alii*, 1950), defective tubular reabsorption of amino acids must be presumed, and in this respect Wilson's disease falls into line with other recently identified familial aminoacidurias (see review of Brick, 1952).

It is a progressive fatal disorder, death occurring in the second or third decade usually from the complications of cirrhosis. Mobilization of copper from the brain and the liver has been a major object of treatment. BAL (2:3 dimercaptopropanol) was used as the copper-mobilizing agent by Denny Brown and Porter (1951), who noted a striking improvement in the neurological signs in many of their patients treated with short courses of the drug. Calcium ethylenediamine tetraacetate ("Versene") is a recently discovered

powerful chelating agent (Sidbury *et alii*, 1953); its use in the treatment of Wilson's disease has not been previously reported.

In this communication the clinical, pathological and biochemical features of a typical case of hepatolenticular degeneration are described. The mobilization of copper following the administration of BAL and "Versene" has been studied quantitatively, and a full copper balance study has been carried out over a thirteen day period. An attempt has been made to ascertain whether chelating substances are capable of removing more copper than gains entrance to the body under ordinary physiological circumstances.

METHODS OF STUDY

Tests of liver function and of serum electrolytes were performed by standard laboratory techniques.

Urinary amino acid nitrogen was estimated by the method of Peters and Van Slyke (1932), modified by Frame *et alii* (1943). Individual amino acids were identified by filter paper chromatography.

The copper content of urine, blood, tissues and food was determined by the diethyl-dithiocarbamate method after wet digestion of the material with nitric, sulphuric and perchloric acids (Eden and Green, 1940). All specimens were collected in stainless steel metal containers, or in glass washed in acid and glass-distilled water.

During the thirteen days of the copper balance study the patient received a diet to which he was accustomed, containing approximately 150 grammes of protein and 500 milligrammes of sodium per day. Much of this protein was provided in the form of milk foods reinforced with proprietary protein hydrolysates. All food was weighed and aliquots were taken for copper analysis. Returned food was weighed and analysed.

CASE REPORT

A young man, aged twenty-two years, was first admitted to the Royal Perth Hospital for investigation in March, 1953. No family history of Wilson's disease was elicited. As a child the patient had been regarded as delicate, being subject to frequent bilious attacks.

¹ Received on October 5, 1954.

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His scholastic achievement was slightly above average. At the age of sixteen years he was noted to be jaundiced and to have moderate oedema of the ankles. The jaundice persisted for many months and was attributed to chronic infectious hepatitis. A year later he began to feel tired and became conscious of slight clumsiness of his fingers, finding difficulty in doing up his buttons. Slight indistinctness and monotony of speech were also commented on at this time by close relatives. He was of a cheerful disposition, and was able, despite these minor disabilities, to do part-time work as a reporter.

In May, 1952, he had an attack of pneumonia. It was noticed then that his dysarthria was considerably worse. He had become aware of increasing stiffness of gait and of a tendency for saliva to trickle from the angles of his mouth.

On admission to hospital the patient was noted to be a youthful looking man, with a smooth chin and pronounced facial acne. He had a fixed smile exposing the upper teeth. Axillary and suprapubic hair was scant. Bilateral gynecomastia was present; numerous spider naevi were present over the arms and chest. The sclerae were slightly icteric. A rim of greenish-brown pigmentation (Kayser-Fleischer rings) was present in the limbus of each cornea. On the skin of the legs and other exposed areas pronounced melanotic pigmentation was seen. Pitting oedema was present below both knees. The heart and lungs were normal. The spleen was palpated five centimetres below the left costal margin, but the liver could not be felt and the area of hepatic dullness was diminished. Generalized plastic hypertonia was present. Pronounced tremor of pill-rolling type was present, made worse by voluntary movement and emotional excitement. His speech was monotonous and indistinct, sentences tending to tail off into inaudibility. There was a notable poverty of facial expression, the fixed smile being unwavering. Choreiform or athetoid movements were not a feature of his illness. His gait was of shuffling character. The deep reflexes were all brisk and the plantar responses were flexor. There were no signs of sensory loss.

Investigations

The results of investigations were as follows. The haemoglobin value was 15.1 grammes *per centum*; the leucocytes numbered 5100 per cubic millimetre. The fasting blood urea content was 38 milligrammes per 100 millilitres; the result of the urea clearance

test was 110% of normal. The urinary excretion of 17-ketosteroids was five milligrammes per twenty-four hours; the total urinary uroporphyrin excretion (method of Sveinsson *et alii*, 1949) was 6.0 microgrammes per twenty-four hours (normal, nil to a trace); the total coproporphyrin excretion (method of Schwartz *et alii*, 1951) was 32.9 microgrammes per twenty-four hours (normal, 52 to 250 microgrammes). The total amino acid nitrogen excretion was 0.615 gramme per twenty-four hours.

In six controls the range of total amino acid nitrogen was from 0.282 to 0.504 gramme per twenty-four hours, with a mean of 0.352 gramme. The following amino acids were identified by filter paper chromatography: ? aspartic acid "+", ? glutamic acid "+", cystine "+++", glycine "+++", lysine "+++", alanine "++++", tyrosine "+", valine "++", methionine "++", leucine "trace".

Serial estimations of tests of liver function and serum electrolyte contents were performed (see Table I). The most notable abnormalities seen were the reversal of the albumin-globulin ratio, a rising and falling positive thymol flocculation, and slight hyperchloraemic acidosis with hypokalaemia. A bromsulphthalein excretion test on one occasion showed 21% retention of dye at forty-five minutes.

A barium bolus X-ray examination of the oesophagus failed to reveal the presence of oesophageal varices.

Treatment and Progress

The downhill progress of the patient was observed in six prolonged periods in hospital.

March-April, 1953.—During the period March and April, 1953, preliminary observations were carried out on the patient, and a diet of high protein and low sodium content was given to control the oedema.

June-July, 1953.—By June, 1953, the oedema had almost completely subsided. His speech was noticeably less distinct and his gait more strikingly shuffling in character. On this occasion a ten-day copper balance study was performed prior to a first course of treatment with BAL. The regime recommended by Denny Brown and Porter (1951) was carried out as follows: two millilitres of a 10% solution of BAL in peanut oil were given intramuscularly twice daily for ten days. There was no noticeable improvement in his neurological signs during or after this course of treatment.

TABLE I

Results of Serial Tests of Liver Function in a Case of Wilson's Disease. Courses of BAL were Given in June and August, 1953. In October, 1953, a Course of "Versene" was Given

Date	Serum Bilirubin Content (Normal, <0.5 Milligrammes per 100 Cubic Millimetres)	Serum Alkaline Phosphatase Content (Normal, 3 to 13 King-Armstrong Units)	Thymol Turbidity (Normal, 0 to 3 Units)	Thymol Flocculation (Normal, Nil)	Zinc Sulphate Turbidity (Normal, 0 to 6 Units)	Serum Albumin Content (Normal, 3.5 to 5.0 Grammes per 100 Millilitres)	Serum Globulin Content (Normal, 2.0 to 3.5 Grammes per 100 Millilitres)	Serum Sodium Content (Normal, 140 to 152 Milliequivalents per Litre)	Serum Potassium Content (Normal, 4.0 to 5.2 Milliequivalents per Litre)	Serum Chlorine Content (Normal, 98 to 106 Milliequivalents per Litre)	Serum Carbon Dioxide Content (Normal, 23 to 34 Milliequivalents per Litre)
10. 3.53	1.6	15.7	2	0	7	2.7	4.0			106	20.0
9. 4.53	1.0	15.2	2	++	5	3.1	3.1	148	3.1	III	21.0
5. 6.53	1.0	21.6	2	+	7	2.8	3.2				
3. 7.53	1.1	16.9	2	+	6	3.3	2.9				
15. 7.53	0.4	18.1	3	+++	8	4.1	3.3				
31. 7.53	1.3	23.2	2	+++	9	4.4	3.1				
7. 8.53	1.4	17.1	2	++	9	4.2	2.8				
23.10.53	0.2	9.2	2	0	6	2.1	3.1	146	2.9	107	17.0
22. 4.54	4.0	15.7	2	0	11	1.4	3.9	139	3.0	109	21.5
27. 4.54	—	—	—	—	—	1.2	3.7				

August, 1953.—In August, 1953, he was given a second course of treatment with BAL. All observers agreed that far from improving, he began to deteriorate at this time. His speech became less distinct, his tremor more agitated and his gait more shuffling, and in addition he became emotionally excited. He roamed the wards all night and slept little during the day. It became necessary to transfer him to a mental institution, where unfortunately facilities for giving him a special diet were not available.

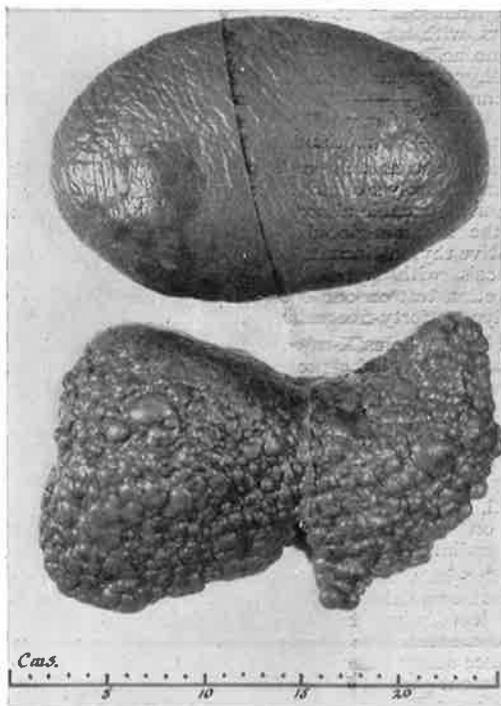


FIGURE I

Atrophic cirrhosis and congestive splenomegaly in Wilson's disease

October, 1953. In October, 1953, his equanimity had returned. A staphylococcal infection of the leg was associated with high fever. He became drowsy and was readmitted to the Royal Perth Hospital in a semi-comatose state. Oedema had reaccumulated. With penicillin treatment he improved, and after a period of normal dietary intake a six-day course of "Versene" (calcium ethylenediamine tetraacetate) was given. One gramme was given intravenously over a period of two hours each day. On the fourth day he developed a purpuric rash on the right forearm, and had three small epistaxes. No alteration was noted in the bleeding or clotting times or in the level of circulating platelets, but the serum calcium content was found to have fallen from 9.4 to 8.4 milligrammes per 100 millilitres; calcium gluconate was given intramuscularly. The two remaining daily doses of "Versene" were given without further complications. No objective signs of improvement were noted during or after the course of treatment. For the following three months he was nursed at home. He remained emotionally placid, and took a lively interest in people and books, but became virtually anarthric and could barely walk. His intention tremor was so gross that he had to be fed.

March, 1954. In March, 1954, he became once more excitable and hostile to friendly restraining influences, and was readmitted to hospital for a short period before transfer to a mental institution.

April, 1954.—In April he was readmitted to hospital in a semi-comatose condition. He was febrile (no obvious source of infection was detected) and jaundiced. Dependent oedema and ascites were present. He regained consciousness quite suddenly without active treatment. Hypertonicity had increased to the extent of causing virtual immobility. His nocturnal excitement required sedation, and his tolerance for barbiturates, paraldehyde and latterly morphine was remarkable in view of the advanced state of malnourishment and hepatic decompensation at which he had by this time arrived. He lapsed once more into coma and died on May 8, 1954.

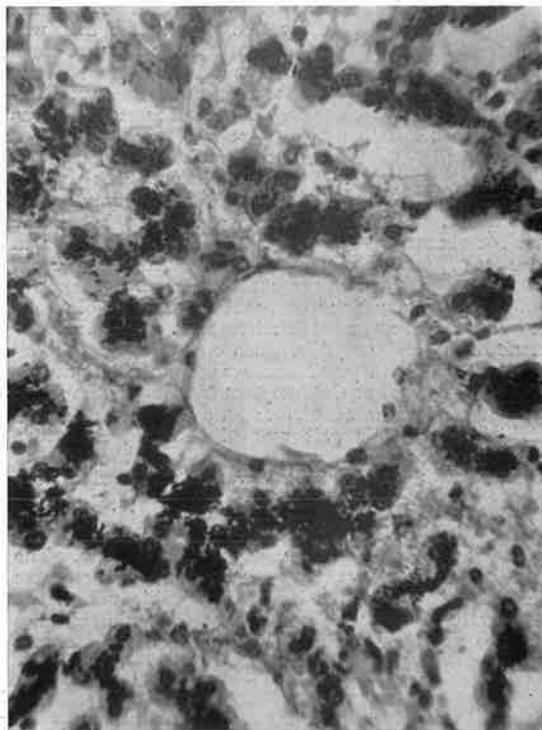


FIGURE II

Histochemical demonstration (rubeanic acid) of abundant copper-containing pigment present in the polygonal cells of the cirrhotic liver in Wilson's disease. ($\times 450$)

Autopsy Findings

The body was examined two hours after death. The heart and blood vessels were normal. Hypostatic congestion of the lower lobes of both lungs was present. The spleen was greatly enlarged, weighing 1200 grammes. It was firm and plum-coloured; at the lower pole on the lateral surface there was a recent infarct two centimetres in diameter. The liver weighed 900 grammes. It was brownish in colour, the surface having a sheen like burnished copper. It was coarsely and irregularly nodular, the largest nodules having a diameter of one centimetre (see Figure I). The pancreas was normal.

No oesophageal varices were present, but between the peritoneal layers of the lesser omentum near the cardia there were a number of large tortuous dilated veins, up to 1.5 centimetres in diameter. Similar though smaller vessels were present in the lienorenal ligament and adjacent retroperitoneal tissues. There was also a large tortuous vein (0.75 centimetre in diameter) to be seen on the free edge of the falciform ligament extending from the liver to the umbilicus, where it anastomosed with dilated hypogastric vessels.

The brain weighed 1200 grammes. No obvious macroscopic abnormality was present. The basal ganglia were not appreciably shrunken when sections were examined.

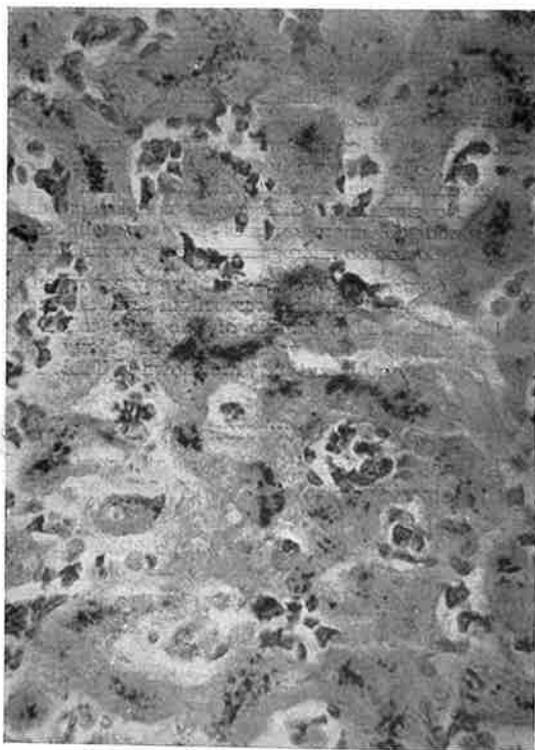


FIGURE III

Deposits of haemosiderin present in the liver in Wilson's disease. (Stained with Prussian blue reagent, counterstained with eosin. $\times 200$)

Microscopically no abnormality was seen in the adrenals, pancreas, thyroid or pituitary. Active spermatogenesis was absent in the seminiferous tubules, and hyperplasia of the ductal and connective tissue of the breast was seen. In the myocardium and kidneys no abnormal changes were found. The pigment of the skin was melanin present in excess in the cells of the *stratum germinativum*.

In the spleen congestive changes only were found. In the liver the changes of advanced cirrhosis were present. Pseudo-lobules of varying size were interspersed between broad bands of fibrous tissue containing many small bile ducts. There was evidence of much recent necrosis of parenchymal cells. Most of the polygonal cells contained greenish-brown pigment, some of which was thought to be bile pigment and lipofuscin. In addition it was found that some of the

pigment gave a positive Prussian blue reaction. The distribution of this haemosiderin was patchy. The majority of the polygonal cells contained material which gave a strongly positive staining reaction for copper with rubeanic acid, after pretreatment with hydrogen peroxide (modification of the method of Okamoto and Utamura, suggested by Gomori, 1952). Both haemosiderin and copper-containing pigment were confined to the polygonal cells (see Figures II and III). The microscopic changes in the basal ganglia were not striking. Neurons were reduced in number; in some early degenerative changes were present, but no neuronophagia was seen. Cystic changes were absent, though there was some diffuse increase in glial fibres.

Results of Special Studies

Urinary Copper Excretion.—The patient excreted 50 to 100 times the normal amount of copper in the urine (mean, 1150 microgrammes; range, 640 to 1490 microgrammes in twenty-four hours; 14 and 22 microgrammes in two controls). Values obtained by other investigations are shown in Table II.

TABLE II
Urinary Copper Excretion in Wilson's Disease

Authors	Copper (Microgrammes) Excreted in Twenty-four Hours
Present authors	1490, 1420 (mean of 13 days) 1060, 630
Bearn and Kunkel (1954), 16 cases	703 \pm 318
Sullivan, Martin and McDowell (1953), two cases	318, 438
Porter (1949), four cases	207 to 716
Uzman (1953)	17 to 640

Copper Balance.—The copper balance study was divided into two periods, eight days and five days, which made possible the collection of pooled samples of urine, faeces, solid food, milk mixtures and drinking fluids. The results are shown in Table III. Over

TABLE III
Result of Thirteen-Day Copper Balance in a Case of Wilson's Disease¹

Period of Study	Copper Content (Milligrammes)			Balance
	Food	Faeces	Urine	
Period I (8 days)	31.4	16.2	9.1	+6.1
Period II (5 days)	22.8	10.7	8.7	+3.4

¹ Mean daily copper intake, 4.2 milligrammes; mean daily faecal excretion, 2.1 milligrammes; mean daily urinary excretion, 1.4 milligrammes; mean daily copper balance, +0.7 milligrammes.

the period of observation approximately 50% of copper in the diet was absorbed by the gut. Despite a daily urinary copper excretion of approximately 1.4 milligrammes the patient was in positive daily copper balance of 0.8 milligramme (800 microgrammes).

Effect of BAL on Urinary Copper Excretion.—During the balance study the mean daily excretion of copper in the urine was 1400 microgrammes. This study was followed immediately by the first course of BAL, 400 milligrammes being given daily for ten days. The total urinary copper excretion was estimated on the sixth and tenth (last) days of the course. The

amounts excreted, 1610 and 1045 microgrammes, were not greatly in excess of those observed under basal conditions. The hourly excretion of copper in urine after the administration of BAL was observed on the first day of the second course. The total amount of

copper in the urine appeared within the first hour (4.56 microgrammes per millilitre) and thereafter the excretion of copper fell sharply. A total increment of 550 microgrammes of copper over the previous twenty-four-hour excretion was noted (Figure IV).

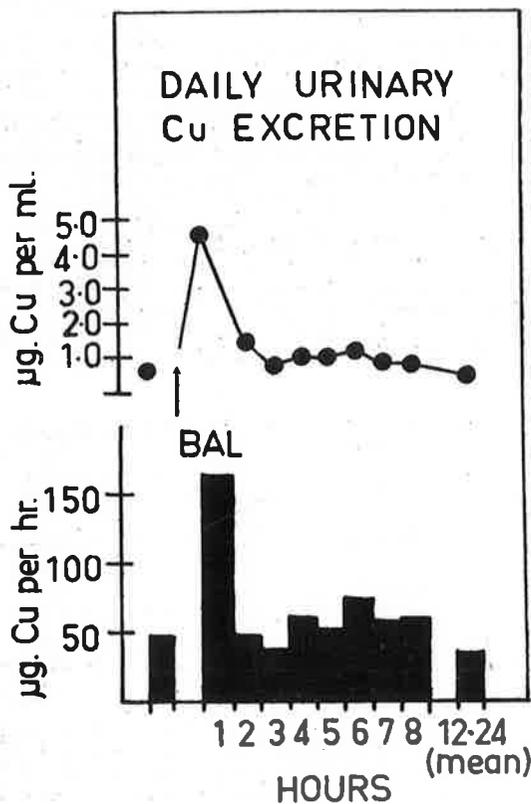


FIGURE IV

Hourly excretion of copper in urine after administration of 200 milligrammes of BAL

copper excreted on the previous day was 1060 microgrammes (0.66 microgrammes per millilitre). The bladder was emptied at 6 a.m. and two millilitres of BAL were given. Urine was collected thereafter at hourly intervals for seven hours. The increment of

Effect of BAL on Blood Copper Levels.—Blood levels of copper were estimated one hour and twelve hours after the administration of BAL on the fifth and tenth days of the first course. On the first occasion a sharp rise was noted from 62 to 93 microgrammes per 100 millilitres, but no significant change was observed on the tenth day (56 to 48 microgrammes per 100 millilitre).

Urine and Blood Copper Content during Calcium ("Versene") Treatment.—The urinary copper output was studied on the first, third and sixth days of the course of "Versene". The increment of copper excretion was small (630 microgrammes rising to 930 and 890 microgrammes). It is not known why at this stage of the patient's illness he had a lower baseline of daily copper excretion.

Blood levels of copper were estimated on the first day at nil, one and two hours after "Versene" had been given, and on the sixth day at nil and twelve hours. No significant change was noted (first day, 57, 58 and 60 microgrammes per 100 millilitres; sixth day, 60 and 80 microgrammes per 100 millilitres).

Copper Content of Organs.—At autopsy a portion of the liver, the spleen, the cerebellum and the right basal ganglia, and a sample of bile were taken for copper analysis. The values obtained, along with those given by other authors, are shown in Table IV.

DISCUSSION

This case, which in all respects conformed to the classical clinical descriptions given of hepatolenticular degeneration, was interesting in that symptoms and signs of hepatic insufficiency—jaundice and oedema—preceded by many years the typical neurological manifestations. In older literature it has been stated that cirrhosis in Wilson's disease is often difficult to diagnose; but in two-thirds of a total of 30 cases studied by Sweet *et alii* (1941), by Herz and Drew (1950), by Homburger and Kozol (1946) and by Franklin and Bauman (1953), one or more abnormalities were revealed by biochemical tests of liver function. Of

TABLE IV

Copper Content of Organs and Bile in Wilson's Disease

Authors	Copper Content (Milligrammes per 100 Grammes Dry Weight)						Copper Content of Bile (Milligrammes per Litre)
	Cortical White Matter	Cerebellum	Caudate Nucleus	Putamen and Globus Pallidus	Liver	Spleen	
Cummings (1948): normal figures	1.1 to 8.2	?	3.4 to 9.4	6.1 to 18.8	3.7 to 17.2	?	0.3 to 2.0
Present authors	—	24.2	25.2	31.6	49.0	1.1	1.3
Cummings (1952):							
First case	10.9	—	10.1	8.4	156.4	—	—
Second case	14.7	—	31.8	31.0	55.0	—	—
Third case	12.9	—	13.8	39.9	39.4	—	—
Spillane <i>et alii</i> (1952) ..	18	—	27	17 to 37	33 to 37	1	—

the 11 patients studied by Franklin and Bauman, six died in liver failure.

The high level of copper excretion in the urine, the aminoaciduria, and the low blood copper level were also pathognomonic biochemical findings. The normal blood level of copper is about 100 microgrammes per 100 millilitres (range, 69 to 117 microgrammes—Lahey *et alii*, 1953). The mean of several estimations in this case was 68 microgrammes per 100 millilitres. The range of serum copper levels in the 16 cases of Wilson's disease studied by Bearn and Kunkel (1954) was 60 ± 15.4 microgrammes per 100 millilitres (controls, 108 ± 9.8 microgrammes per 100 millilitres. (Serum copper levels are approximately 10% greater than whole blood levels.) Copper in serum is present in the form of a metalloprotein, caeruloplasmin (Gubler *et alii*, 1953). This copper-protein has been shown to have oxidase activity (Holmberg and Laurell, 1951) and Bearn and Kunkel (1952, 1954) have shown that "copper enzyme activity" is greatly diminished in Wilson's disease.

The range of copper excretion in urine in this case was 1060 to 1500 microgrammes in twenty-four hours. This was higher than in most cases reported by other authors. The amount of α -amino nitrogen in the urine was increased in comparison with controls, but did not reach the high levels observed by others (Uzman and Denny Brown, 1948; Porter, 1951). The degenerative changes in the liver and basal ganglia in Wilson's disease cannot be directly related to the aminoaciduria, for this seems to be the most variable of the biochemical anomalies; at least two authentic cases of the disease without aminoaciduria have been recorded (Stein *et alii*, 1954; Cooper *et alii*, 1950), and on the other hand siblings of patients with Wilson's disease have been shown to have aminoaciduria without developing clinical manifestations of the disorder. It was suggested by Matthews *et alii* (1952), who showed that copper excretion was increased after the ingestion of large amounts of alanine and glycine, that copper was excreted in the form of amino acid-copper chelates; but this has not been confirmed by subsequent investigators. This perplexing paradox concerning the relation of cupruria and aminoaciduria appears to have been solved by Uzman (1953), who noted that the urine of patients with Wilson's disease contained abnormal oligopeptides with terminal dicarboxylic acids. Chromatographic spots of these oligopeptides reacted strongly with the sensitive copper reagent rubeanic acid. It is believed by Uzman that copper is excreted as a peptide-chelate, and that competition for the

tubular resorption of amino acids and this chelate occurs. The finding in this case of an inordinately high level of cupruria with only moderate aminoaciduria would seem to support Uzman's observations.

The balance study performed on this patient appears to confirm the tacit assumption that has been made that in Wilson's disease increased alimentary absorption of copper is taking place. Nearly 50% of the total dietary copper was absorbed, and despite the pronounced cupruria the patient was in considerable positive copper balance. It is fully realized that longer balance studies on controls and on patients with Wilson's disease are desirable, for there is a paucity of information on this aspect of copper metabolism. Nevertheless, the situation is indeed truly analogous to the absorption of iron in relation to the genesis of haemochromatosis, in which a much higher proportion of the iron "load" presented to the duodenal mucosa is absorbed than by normal patients. It is possible that the same chelating peptide with an avidity for copper present in urine in Wilson's disease is present also in intestinal mucosal cells, as well as in tissues where copper is deposited in excessive amounts.

The results of treatment with BAL have been variable. Cumings (1952) and Schechter and Jones (1953) did not meet with the success that favoured Denny Brown and Porter (1951), and the present patient failed to show any clinical improvement. It is of probable significance that Denny Brown's and Porter's cases were of the more chronic "pseudosclerotic" variety of hepatolenticular degeneration. Perhaps in such cases the daily positive copper balance is less considerable than in the present case, in which there was evidence that neither BAL nor "Versene" was able to put the patient in negative balance. Moreover, the curve of urinary excretion and blood copper levels following BAL administration seems to indicate that only a small amount of copper is available for binding with thiol groups, the bulk being probably more firmly bound to peptide linkages. The continued increase in copper excretion attending the use of "Versene" suggests that it may be a more efficient copper-binding agent; but its avidity for other metals renders it of limited therapeutic value when used over long periods of time is to be considered.

The limitation of alimentary copper intake by modifications of the diet is difficult to effect. Copper is of ubiquitous distribution in food (McCance and Widdowson, 1946), and the problem is complicated by the necessity for a high intake of protein in the presence of impaired liver function. The protein supplements given

to our patient accounted for his comparatively high daily intake of copper (four milligrammes); yet when he did not receive this extra source of nourishment, progressive deterioration of hepatic function occurred.

On the evidence presented it is doubtful whether BAL and "Versene" can be expected to modify the natural history of severe hepatolenticular degeneration. The importance of Uzman's discovery of copper-chelating peptides is to turn attention away from the minutiae of copper metabolism towards the fundamental disorder of body protein metabolism which presumably accounts for all the biochemical abnormalities in Wilson's disease. The full understanding of abnormal protein metabolism may explain why these patients develop cirrhosis and degeneration of the lenticular nuclei.

SUMMARY

A case of Wilson's disease in a man, aged twenty-two years, with tremor, rigidity, Kayser-Fleischer rings and signs of hepatic insufficiency, is described. The patient died in hepatic coma six years after the onset of his first symptom, jaundice.

Signs of gross portal hypertension were present at autopsy. Examination of the liver revealed severe atrophic cirrhosis, and histochemical study demonstrated the presence of excessive amounts of copper-containing pigment. Chronic degenerative changes were seen microscopically in the lenticular nuclei.

General aminoaciduria of moderate degree was present. Blood copper levels were low, and urinary copper excretion was approximately fifty times normal.

A thirteen-day balance study showed that on a daily dietary intake of four milligrammes, 50% of the copper was being absorbed, and that the patient was in a daily positive copper balance of 0.7 to 0.8 milligramme.

After the administration of BAL there was a rapid rise in urinary copper excretion, which was not sustained; this indicates that much of the copper stored is not available for combination with the -SH groups of BAL. The increase of urinary copper excretion was sustained for longer periods with "Versene". It is improbable that either substance promoted the excretion of copper in greater amounts than were normally absorbed each day in the alimentary tract.

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ADDENDUM

Since this paper was submitted for publication a further case of Wilson's disease has been studied. The patient was a girl, aged ten years, weighing 22 kilograms, who presented with choreo-athetoid movements of all four limbs seven months prior to her death. These neurological manifestations were rapidly progressive; four months later she had become severely dysarthric, and plastic rigidity of the limbs was present. Both the liver and spleen were enlarged on clinical examination, although liver function tests gave results normal in all respects. After a severe melæna she developed ascites. Balance studies were not performed owing to urinary and faecal incontinence. She was excreting 400 microgrammes of copper daily (150 microgrammes per litre of urine). A ten-day course of BAL caused no really significant clinical improvement. In the middle of the course of BAL the daily urinary copper excretion rose to about 1900 microgrammes *per diem* (670 microgrammes per litre). Filter-paper chromatograms showed a generalized aminoaciduria which was not investigated in detail. The patient died a week after the termination of the course of BAL therapy. Cirrhosis of the liver with considerable fatty infiltration, splenomegaly, œsophageal varices and gliosis of the basal ganglia were noted at the autopsy. The liver contained approximately 80 milligrammes of copper per 100 grammes and the brain 24 milligrammes per 100 grammes.

The striking clinical features of the case were the rapidity of development of neurological signs and symptoms and the lack of immediate response to treatment with BAL, despite an apparent fourfold increase in urinary excretion of copper.

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PAPER 4:15

COPPER POISONING IN SHEEP IN WESTERN AUSTRALIA

By

A. B. BECK and H. W. BENNETTS

2.—Copper Poisoning in Sheep in Western Australia

By A. B. Beck* and H. W. Bennetts†

Manuscript received—16th October, 1962

Investigations were made to ascertain the cause of high-copper status of sheep in the Wiluna area of Western Australia. It was concluded that this was due mainly to the ingestion of plants naturally high in copper.

An occurrence of copper poisoning in sheep in the Toodyay area was also investigated. Here the copper content of pastures generally was not high. Although the factors responsible for the high copper status of sheep were not determined, histological evidence suggested that this was associated with a hepatotoxic principle of plant or fungal origin.

Introduction

During investigations relating to the deficiency of copper in stock in Western Australia and in the course of routine laboratory diagnosis, we have encountered numerous cases of copper poisoning in sheep. In some instances the cause has been due to an over-generous use of copper-containing licks or fertilizers, often to both. In other cases the poisoning has been associated with lupinosis when the damaged liver accumulates excessive amounts of copper, particularly if copper supplements have been fed. Investigations into this disease and the significance of the storage of heavy metals in the liver are being continued by the Department of Agriculture at the present time.

In eastern Australia, copper poisoning is commonly associated with plants containing pyrrolizidine alkaloids, particularly heliotrope (*Heliotropium europaeum*) and "Pattersons Curse" (*Echium plantagineum*) (Bull 1961; St. George-Grambauer and Rac 1962). The first plant is rare in southern Western Australia and no cases of copper poisoning due to either plant have been reported.

The purpose of the present paper is to record the results of detailed investigations of the cause of the high copper status of sheep at Wiluna in the North Eastern Goldfields pastoral region, and at Toodyay.

The High Copper Status of Sheep in the Wiluna Area

In 1951 one of us (H.W.B.) visited the Eastern Goldfields area of Western Australia to enquire into possible causes of sheep losses in the 1950-51 drought. Some pastoralists had considered that the losses were unduly severe and could not be attributed solely to the effects of drought. On some stations it was reported that sheep had

lost their appetite for top feed which should have been adequate for their requirements. As a result of these observations it was decided to investigate the remote possibility that cobalt deficiency was responsible for this reported anorexia. During the analysis for cobalt content it was noted that the livers contained large amounts of copper. Shortly afterwards chronic copper poisoning was diagnosed at "Albion Downs" Station. The present investigation was then carried out to ascertain the reasons for the high copper status of sheep at Wiluna and the area of country affected.

Because of the remoteness of the region and of the large areas of the properties concerned, the scope of the investigation was restricted to the determination of copper in the livers of sheep from nine station properties, to the analysis of herbage from three properties where sheep showed high copper status and to the determination of copper in well waters on one property. Histological examination was made on many of the liver samples to check for the possible effects of hepatotoxic plants.

Materials and Methods

At "Albion Downs" Station, every species likely to be eaten by sheep was collected in the 1953 sampling. The sampling was much less comprehensive at later dates and on other stations.

Soil contamination was avoided in the collection of samples but in some short and semi-prostrate species it was not possible to avoid this entirely. In these cases an iron determination was done to obtain some indication of soil contamination.

As levels of molybdenum, manganese and inorganic sulphate are known to influence copper metabolism in sheep, determinations of these constituents were made on many of the herbage samples. Copper, molybdenum and inorganic sulphate were determined as described previously (Beck 1962), manganese by the periodate method (Willard and Greathouse 1917) and iron by the thioglycolic acid method (Mayer and Bradshaw 1951).

Except for one sheep which died from copper-poisoning, all liver samples were from healthy sheep killed for rations. The livers were preserved in copper-free alcohol or alcohol-formalin mixture.

The analysis of all samples is reported on the dry-matter basis. No correction was made for the fat content of livers, but no obvious fat was noted in any of the samples.

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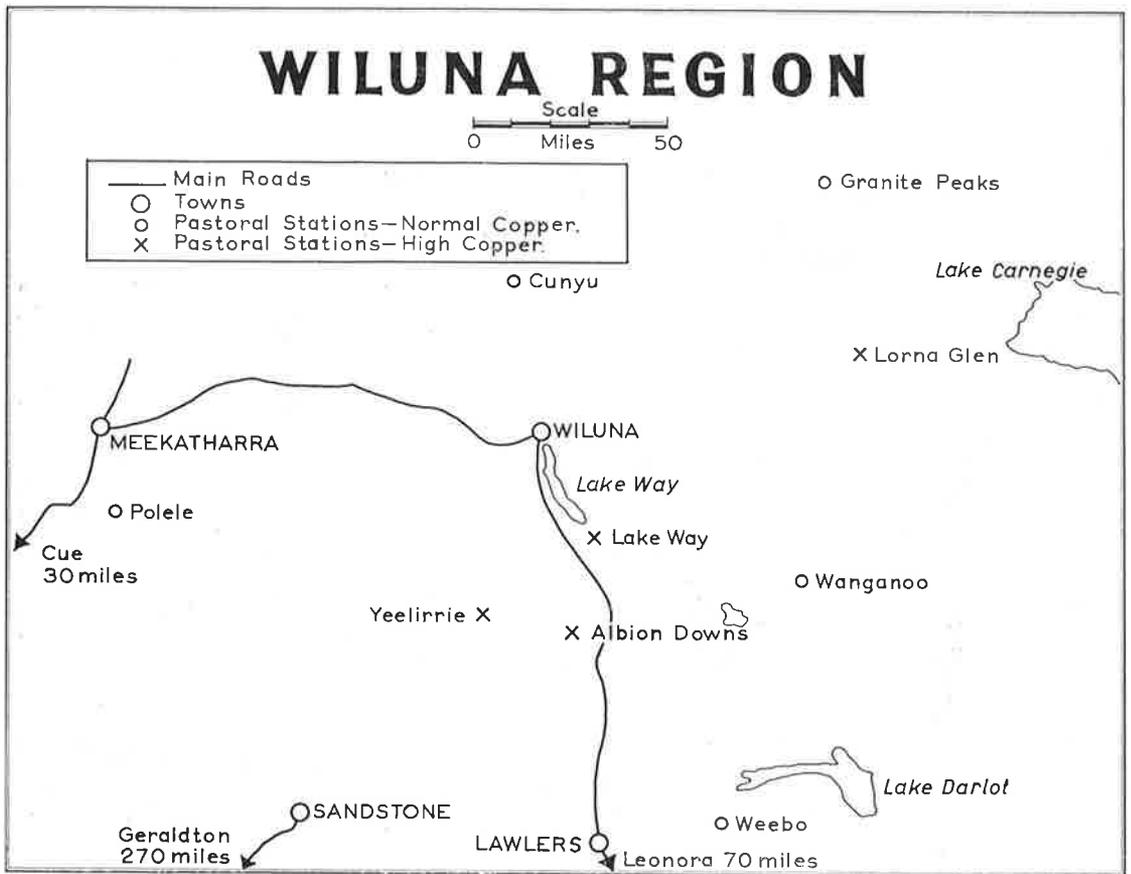


Fig. 1.

Results

An examination of well waters was made on "Albion Downs" Station in 1953 but the copper levels were less than 0.04 mg per litre and would not be responsible for the high levels of copper in liver.

The analytical data for liver samples and for herbage material are set out in Tables I and II respectively. The livers of normal Western Australian sheep contain 50 to 400 p.p.m. copper and as sheep with levels over 1000 p.p.m. are liable to develop copper poisoning under conditions of stress, the percentage of such samples is indicated in Table I. The geographical distribution of properties is shown in the accompanying map.

With *Goodenia Mueckeana* and *Eremophila leucophylla* there was little difference in copper content at the different times of sampling, but in all other species the levels in the 1953 samples were appreciably higher. There was no obvious difference in other constituents of species at different sampling times or on the different properties.

A number of livers from ration sheep have been examined histologically for evidence of ingestion of hepatotoxic plants. Some of

the high-copper samples showed small areas of megalocytosis but there was no significant liver damage in any of the specimens.

Prior to the outbreak of copper poisoning in 1952 it had been noted that the affected sheep had been grazing heavily on *Goodenia eremophila*. This species did not reappear in appreciable amounts until 1959 when a sample was forwarded to the C.S.I.R.O. Chemical Research Laboratories, Melbourne, for alkaloid determination. The analysis showed a very low alkaloid content (0.015 per cent. tertiary base, on assumed molecular weight of 300; N oxides were absent) which would be unlikely to cause any liver damage.

Discussion

From the data obtained it would seem that the occurrence of high copper status in merino sheep is restricted to an area within 100 miles of Wiluna.

It seems probable that the development of the high liver copper levels was primarily due to the ingestion of herbage of high copper content. Data from the agricultural areas of Western Australia (Beck 1941, 1962) indicate that in those regions, levels of copper in pastures

TABLE I
Copper Content of Wiluna Sheep Livers
 Values as p.p.m. Cu on dry liver

Property	Date	No. of Samples	Liver Cu		Details
			Mean and Range	Percentage of values above 1000 p.p.m.	
"Albion Downs"	Sept.-Nov., 1952	7	1010 300-1760	43	One sheep died Cu poisoning
	Jan.-April, 1953	6	1410 380-1990	83	Aged wethers
	July, 1953	2	860 780-940	0	
	Sept., 1953	3	1600 1190-2100	100	Aged ewes
	Sept., 1953	7	520 440-600	0	Young sheep 12-30 months
	Aug.-Sept., 1954	8	810 360-1390	37	
	Sept., 1955	12	860 390-1940	33	Mixed age ewes and wethers
	Sept.-Oct., 1956	6	1250 300-2140	83	Aged wethers
	May-Oct., 1959	6	620 220-1440	17	Aged ewes and wethers
	"Yeelerrie"	Jan., 1954	6	1820 790-2730	67
Feb.-Aug., 1955		8	780 210-1900	25	Wethers 3-6 years
"Lorna Glen"	March-April, 1954	5	1220 520-1850	60	Aged sheep
"Lake Way"	Nov., 1951-Feb., 1952	3	870 310-1750	33	
	Oct.-Nov., 1952	6	720 350-2000	17	
	Jan.-March, 1953	6	800 450-1380	33	Wethers 3-4 years
"Wanganoo"	July-Aug., 1956	6	450 180-740	0	Wethers 3-7 years
"Granite Peaks"	Aug.-Nov., 1952	6	380 150-820	0	Wethers 3-5 years
"Cunyu"	Aug.-Sept., 1956	6	300 140-520	0	Wethers 3-5 years
"Polele"	Oct.-Nov., 1952	3	290 210-440	0	Wethers 4 years
"Weebo"	Aug.-Sept., 1954	6	290 100-830	0	

rarely exceed 10-12 p.p.m. (dry basis). Dick (1954) has shown that cross-bred sheep will store dangerous amounts of copper when receiving more than about 10 p.p.m. copper in the diet, although merinos can, apparently, tolerate somewhat higher levels. The copper content of *Goodenia Mueckeana* was consistently high (14-22 p.p.m) and other species, at least in certain seasons, supplied amounts of copper well above normal. Seasonal variations in liver copper levels could be explained by the preferential grazing of species of lower copper content in some years.

The storage of copper in sheep is also controlled by factors other than the copper intake. Molybdenum above about 5 p.p.m. in the diet causes depression of copper storage provided adequate sulphate is present (Dick 1954). Very low molybdenum levels have been found in some of the species and this may have favoured copper storage. Sulphate levels were generally similar to those found elsewhere in Western Australia (Beck 1962). High levels have been observed in *Hibiscus pinonianus*, *Bassia* spp. and in three salt bushes, but the area of salt lake country was quite restricted on the properties where the investigations have been carried out. Similar high levels of sulphate have been found in halophytes elsewhere (Spais 1956; Barker 1961).

There is some evidence that manganese interferes with the limitation of copper storage imposed by molybdenum and sulphate (Anon. 1957-58) and it has been shown that very high levels of manganese cause an increase of copper storage in liver of the rat (Gubler *et al.* 1953). However, it is not known what effect the moderately high manganese levels of the Wiluna herbage would have on the copper storage of the grazing sheep.

Although the histological studies have given no definite evidence, it is still possible that hepatotoxic alkaloids may have contributed to the development of high copper levels in some instances.

The occurrence of liver copper levels up to 2700 p.p.m. in clinically healthy sheep indicates the very high concentrations which can be tolerated by sheep in the absence of stress. As it is well known that starvation will readily precipitate a fatal haemolytic crisis in such sheep, it is highly probable that some unexplained losses, reported during mustering and shearing, may have been due to copper poisoning.

Copper Poisoning in Sheep in the Toodyay Area.

In August, 1955, reports of heavy sheep losses were received from a 1,100 acres property some 10 miles north of Toodyay. A clinical diagnosis

of copper poisoning was confirmed by pathological examination and chemical analysis.

Copper fertilizers and supplements were not used and the sheep were bred on the property. The soils and pastures resembled those of a large belt of agricultural country where no cases of poisoning had been reported. At first it was thought that the poisoning might be similar to that encountered in sheep grazing on subterranean clover in certain seasons in Western Victoria (Anon. 1956). Analysis of the Toodyay pastures showed moderately high copper levels but the liver histopathology was quite distinct and suggested that a hepatotoxic plant was implicated (Bull, personal communication).

Geology and Soil Types

No geological survey of the area has been made but the rocks appear to be mainly biotitic gneisses and quartzite with dolerite intrusions.

The soils are red brown clay loams and sandy clay loams with non-calcareous subsoils.

History

The history was not very satisfactory. Losses from what was apparently copper poisoning were reported to have occurred on this property for many years. Young sheep were not affected. Losses were seasonal and occurred usually in August and September. In 1955, losses began earlier and the mortality was reported to be 50 in a flock of 1,700 Corriedale sheep. In 1956 there were no losses, in 1957 one or two cases and none in subsequent years. Occasional deaths from what appeared to be a similar condition have been reported from elsewhere in the district, but investigations have been confined to the one property.

TABLE II

Inorganic Constituents of Wiluna Herbage

Values expressed as mean and range on dry matter basis

Figures in brackets indicate number of samples analysed when less than the total number

Species	Date	Stations* Sampled	No. of Samples	Cu p.p.m.	Mo p.p.m.	Mn p.p.m.	Fe p.p.m.	SO ₄ %
<i>Goodenia Mueckeana</i>	July, 1953	a c	4	19.9 17.1-20.9	<0.1	900 790-1100	0.15 (2) 0.14-0.16
	Feb.-Aug., 1955	a b	4	19.1 13.8-21.7	<0.1	760 570-1000	190 (2) 150-230	0.11 0.08-0.18
<i>G. eremophila</i>	Oct., 1952	a	1	15.7
	July, 1953	a	3	11.8	<0.1	430
	Sept., 1954	a	1	10.0-13.3	<0.1-0.16	340-500	0.36
	Aug., 1955	a b	2	6.8 8.3 8.0-8.7	<0.1	490 1100 810-1400	0.16 0.08-0.25 0.14
<i>Helichrysum Davenportii</i> (Everlasting)	Oct., 1959	a	1	7.9	0.11	370
	July, 1953	a	1	20.3	0.53
	Sept., 1954	a	1	15.3	<0.1	0.40
	Aug., 1955	a b	2	11.8	0.12	390	160 (1)	0.36-0.43 1.2 (3) 0.9-1.5 0.62 0.51-0.74
<i>Hibiscus pinonianus</i>	July, 1953	a b	4	14.6 12.3-16.5	0.27 (3) 0.15-0.47	92 (2) 82-103
	Aug., 1955	a b	2	9.4 7.3-11.6	0.38 0.22-0.55	97 95-100	225 (1)
<i>Didiscus glaucifolius</i> <i>Trichinium obovatum</i> (Cotton bush)	July, 1953	a	1	13.6	<0.1
	July, 1953	a	1	13.5	0.28	100	0.16
	Aug., 1955	a b	2	5.6 5.5-5.8	0.72 0.31-1.13	57 50-65	165 (1)	0.15-0.17
<i>T. exaltatum</i> <i>Myriocephalus</i> (Billy button)	July, 1953	c	1	10.1	0.86
	July, 1953	a	2	12.7	0.28 (1)	300 (1)	1.10 0.73-1.47
	Aug., 1955	a b	2	10.8-14.6 9.4 7.5-11.4	0.19 0.08-0.30 0.15 (3)	450 200-600 600	445 (1)	0.25-0.40 0.21 0.15-0.27 0.26 0.22-0.30 0.34 (2) 0.24-0.44
<i>Eremophila leucophylla</i> (Poverty or flannel bush)	July, 1953	a	4	12.3 9.8-13.9	0.13-0.17 0.22	400-740 450
	Aug., 1955	a b	3	12.8 10.3-14.5	0.13-0.29 0.17 (5)	190-870 130 (1)	160 (2) 150-170	0.22-0.30 0.34 (2) 0.24-0.44
<i>Erodium cymnorum</i>	July, 1953	a c	6	11.1 9.3-14.3	0.33 <0.1-0.37	0.14 0.24
<i>Kennedia</i> sp.	July, 1953	a	1	10.3	0.31 (2)
<i>Sida corrugata</i>	July, 1953	a	1	9.8	0.30-0.32
<i>Danthonia bipartita</i> (Wandarric grass)	July, 1953	a	4	9.6	0.15 (3)	290 (2)	0.12
<i>Ruellinia tozophylla</i>	July, 1953	a c	3	7.7-10.4 9.0 6.9-11.0	0.10-0.25	250-330 275 (1)	0.08-0.15 0.37
<i>Halorrhagis</i> sp.	Aug., 1955	a	1	8.8	<0.1	2400	207	3.3 (4)
<i>Bassia</i> spp.	July, 1953	a c	5	8.6 7.3-9.8	0.42 (2) 0.27-0.58	0.8-6.0 0.79 (1)
<i>Codonocarpus cotinifolius</i> (Native poplar)	July, 1953	a	2	8.6 7.9-9.3	0.62 (1)	360 (1)
<i>Brachysema Chambersii</i> (flowers only)	July, 1953	a	2	6.3
<i>Acacia genistoides</i> Salt Bushes	July, 1953	a	1	3.3	0.10
<i>Kochia pyramidata</i>	July, 1953	a	1	8.2	5.0
<i>Atriplex pulchosa</i>	July, 1953	a	1	4.9	1.0
<i>Lycium australe</i>	July, 1953	a	1	9.3	2.0

* "a" indicates "Albion Downs," "b" "Yeelirrie" and "c" "Lorna Glen."

TABLE III
Copper, Molybdenum and Inorganic Sulphate Levels of Toodyay Pastures
 Values expressed as mean and range on dry matter basis

Date	No. of Samples	Details	Cu p.p.m.	Mo p.p.m.	SO ₄ %
Oct., 1955	2	Capeweed	14.4 14.2-14.6	0.65 0.60-0.70	0.30 0.28-0.32
	1	Sub. clover	14.6	0.40	0.24
Aug., 1956	1	Mixed sub. clover and capeweed	9.7	0.50	0.33
	6	Grasses	6.7 4.3-8.6	1.37 0.62-2.26	0.19 0.13-0.34
	4	Capeweed	6.7 5.4-8.3	1.87 1.0-2.6	0.19 0.17-0.22
	2	Mixed pasture	0.7 9.2-10.2	1.10 0.95-1.25	0.17 0.19
Sept., 1956	1	Capeweed	14.4	0.49	0.19
	1	<i>Plantago cretica</i>	9.2	0.49	0.28
Aug., 1957	1	Mixed pasture	11.0	0.60	0.18
	4	Capeweed	11.5 9.1-14.0	0.63 0.48-0.94	0.28 0.17-0.49
	1	Grasses	8.4	0.61	0.17
	2	Sub. clover	12.0 10.2-13.0	0.16 0.14-0.18	0.12 0.07-0.18

Pasture Investigations

The pastures were of the annual Mediterranean type common to the 20-25 inch rainfall belt. The main species were *Trifolium subterraneum*, capeweed (*Cryptostemma calendula*), wild geranium (*Erodium botrys*) and annual grasses (*Bromus* spp. *Vulpia myuros* and *Wimmera* rye grass, *Lolium* spp.). Surveys were carried out by the former Government Botanist (Mr. C. A. Gardner) in August, 1955, and September, 1956, but the only unusual species found was *Plantago cretica*. An examination of dry paddocks in March, 1957, showed no unusual plants and none considered likely to cause toxic effects. *Echium plantagineum* ("Pattersons curse") and lupins (*L. varius* and *L. angustifolius*), known to cause liver damage, were completely absent from the property.

Analysis of pasture samples is set out in Table III. Levels of molybdenum and inorganic sulphate were determined as these are known to affect copper metabolism. Two samples (August, 1956) were analysed for manganese content but normal levels were found (72 and 85 p.p.m. on dry matter).

As the histology described in the following section had suggested the action of a hepatotoxic alkaloid, determinations for alkaloid content were made on *P. cretica* at the C.S.I.R.O. Chemical Research Laboratories, Melbourne. As the manager of the property had stated that cases of poisoning only occurred in years of rank capeweed growth, this species was also examined, even though there was no suggestion that it caused trouble elsewhere. Both species showed very low alkaloid content (*P. cretica*, 0.018 per cent. tertiary base, quaternary and weak bases and N oxides absent; capeweed, 0.014 per cent. tertiary base and 0.004 per cent. N oxide).

Animal Studies

Chemical.—The liver of the sheep dying of copper poisoning in August, 1955, showed 900 p.p.m. copper (dry basis); a similar sheep in July, 1957, showed 600 p.p.m. These values are rather lower than those usually found in cases of haemolytic jaundice due to copper poisoning.

In June, 1956, liver samples for chemical and histological examination were collected at the abattoirs from 30 sheep from the property. The sheep had been without food for at least 18 hours and consequently the livers contained more fat than usual. There had been no losses from haemolytic jaundice during yarding. Some of the livers were macroscopically abnormal; two were small, five were yellowish and one rather fibrous. Chemical analysis showed the following results which are expressed as the means with range of values in parenthesis; the values for fat are on the dry material and values for iron and copper on the dry, fat-free material:—copper 1500 p.p.m. (490-3120), iron 990 p.p.m. (280-2410), fat 23 p.c. (15-40). Seventy-three per cent. of the livers contained over 1000 p.p.m. copper. Livers from normal Western Australian sheep contain 50-400 p.p.m. copper, 200-800 p.p.m. iron and less than 10 per cent. fat.

Histology.—Sections were available from the two moribund animals and the thirty abattoirs animals mentioned above.

In the liver from the first affected animal (August, 1955) the interstitial tissue in the portal tracts was slightly increased and abnormally cellular. A slight fine diffuse fibrosis was present. Excess bile pigment was present in the ducts and canaliculi. There was some new bile duct formation. Ceroid and protein inclusion globules were plentiful, but megalocytosis and central necrosis were absent. The kidney tubules were laden with haemoglobin casts and degradation products. The second liver (July, 1957) showed marked portal tract fibrosis infiltrated with lymphocytes and polymorphs. These reactive cells were also significantly increased in numbers, both diffusely and focally throughout the liver, presumably as a reaction to necrosis of liver cells. There was a great variation in nuclear size. The reticulo-endothelial cells were increased in numbers, swollen and packed with degenerating red blood cells and bilirubin. There was some small bile duct proliferation in the portal tracts.

The livers of many of the abattoirs sheep showed cellular reaction in the portal tracts

with fibrosis and bile duct damage. The Kupffer cells frequently showed yellow-brown granules.

Discussion

Copper levels of pasture were at times moderately high but it is not considered that these alone could have caused a dangerous accumulation of copper in sheep livers. Molybdenum and inorganic sulphate levels were normal.

The histological data on livers were limited but suggested that two processes were involved. The first consisted of fibrotic and bile duct changes probably leading to some degree of excretory obstruction. This had probably been acting for some time before deaths occurred, and could have been due to ingestion of a plant containing a hepatotoxic alkaloid or to recovered facial eczema due to fungal toxicity. The absence of megalocytosis indicated a different type of poisoning from that due to *Heliotropium* (Bull 1961). The second change in the livers was due to an acute haemolytic process which caused the actual deaths. Although the clinical findings indicated that this was due to copper poisoning, the histological picture gave some suggestion that it may have been due to difficulty in the excretion of bilirubin normally produced. The relatively low liver copper liver values for the two sheep which died also gave some support to the idea that copper toxicity was not the primary cause of death.

It is not possible to give a satisfactory explanation for the massive accumulation of copper in the thirty abattoirs sheep but it was probably consequent on the liver damage as in heliotrope and lupin poisoning.

Acknowledgments

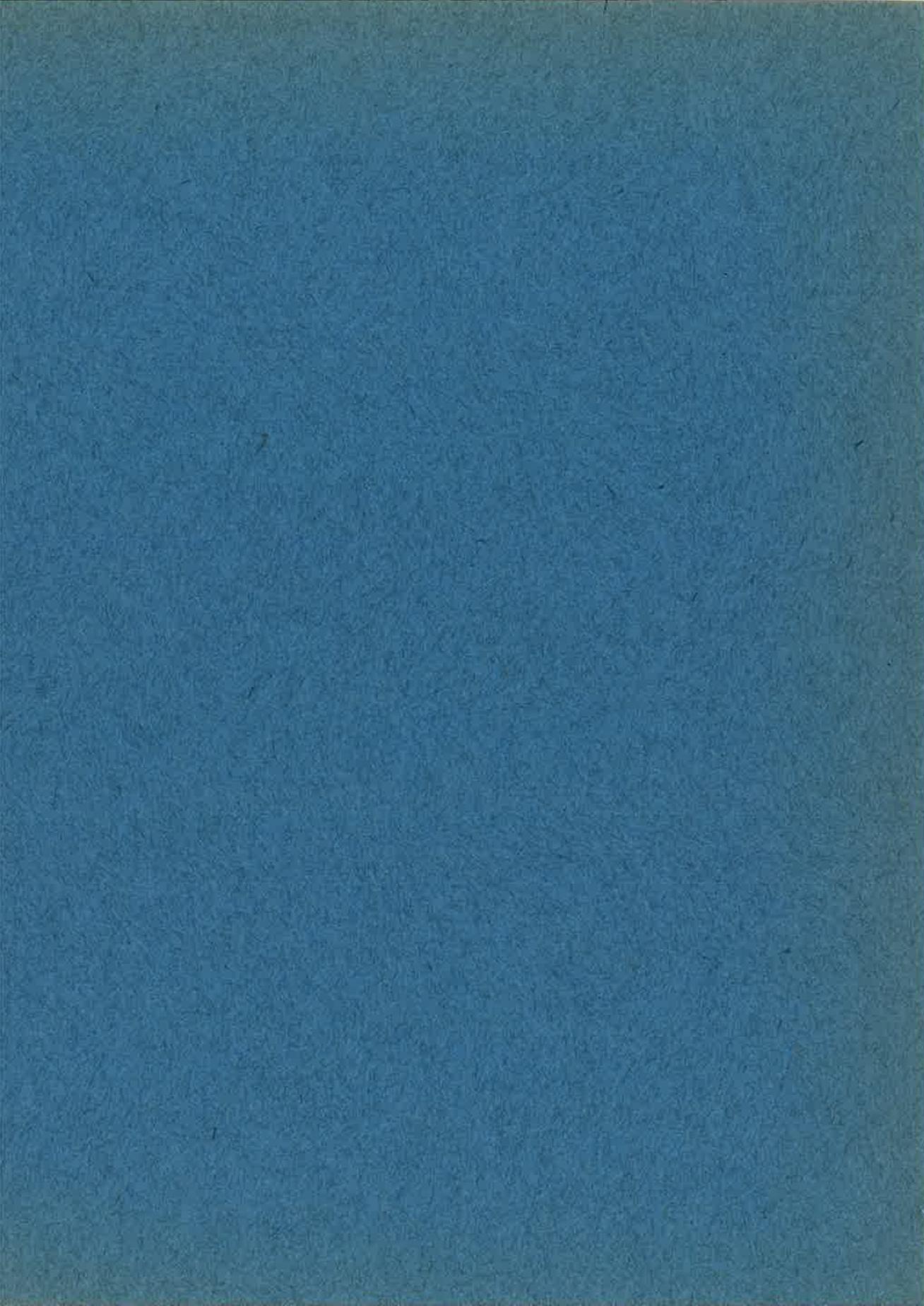
This investigation was carried out cooperatively by the Division of Animal Health, C.S.I.R.O. and the Western Australian Department of Agriculture.

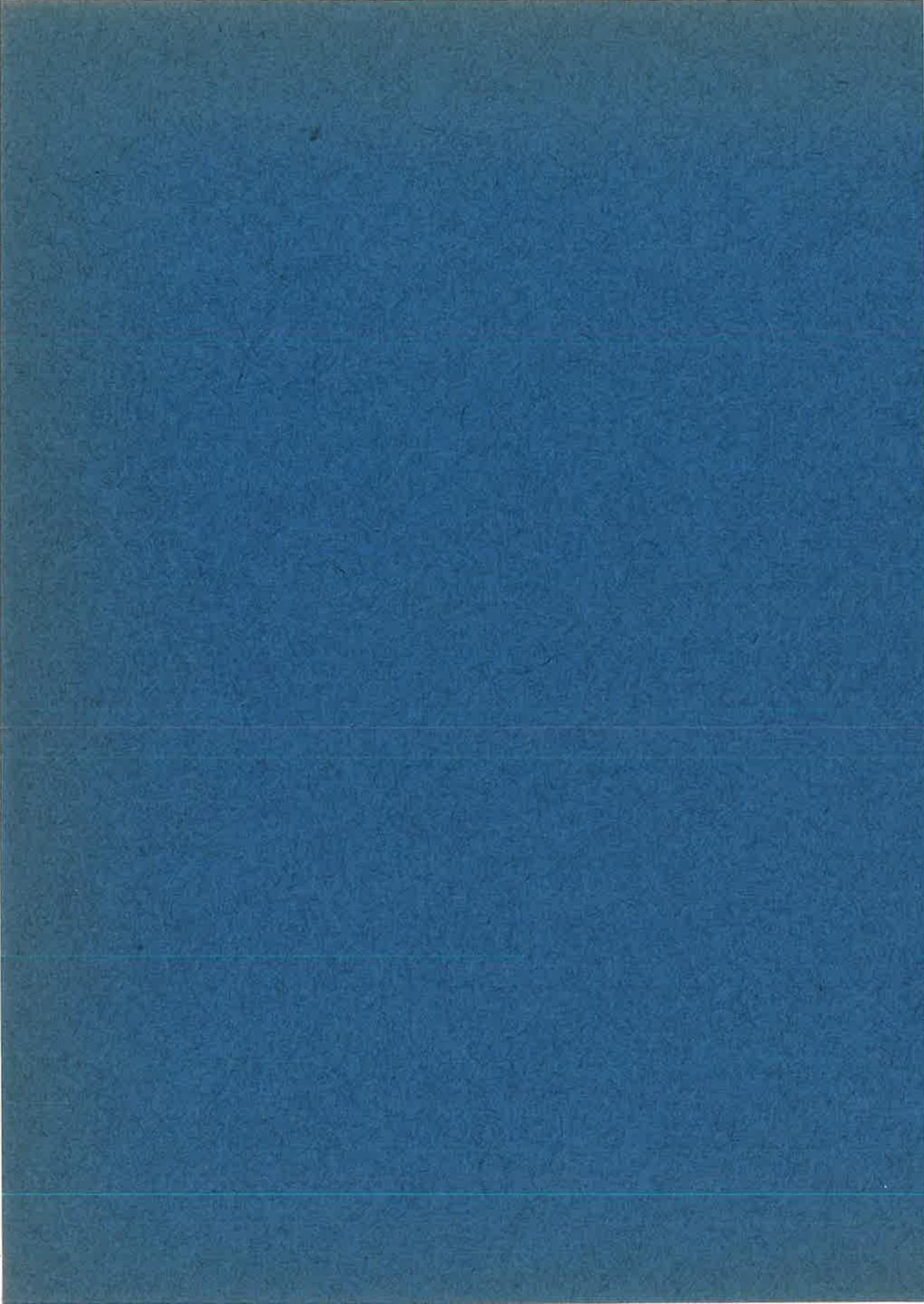
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SECTION 5

THE COPPER METABOLISM OF WARM-BLOODED ANIMALS

A review of aspects related to the
findings given in Papers 1:1 - 4:15

It was originally planned to complete the first part of the Thesis (Sections 1-4) by reviewing the papers therein and relating the findings to those of other investigators. This, however, has already been done very completely by Underwood (1962) and accordingly it was decided to alter somewhat the scope of this Review. It is intended to discuss broadly those aspects of copper metabolism which have appeared important to the candidate while these investigations were being carried out. Gaps in existing knowledge will be pointed out and in some cases the weaknesses of current concepts will be discussed. No attempt will be made to cover all phases of copper metabolism as this has already been done by Underwood (1962) and by Scheinberg and Sternlieb (1960). Sufficient data are not yet available to make possible a comprehensive survey of the copper metabolism of warm-blooded animals, but as wide a range of species as possible will be discussed.

In this Review the word "metabolism" is used to

cover absorption, transport, storage, cellular function and excretion. It must be stressed that more knowledge of all these aspects is essential before a satisfactory and comprehensive picture of copper metabolism will be obtained.

I DIFFICULTIES INHERENT IN THE STUDY OF COPPER METABOLISM

Some of the confusion in our understanding of copper metabolism is due to the technical difficulties attached to such studies and it has been considered worthwhile to summarize some of these.

(a) Radio-copper studies.-- Investigations have been handicapped by the fact that the only available radio-isotope, Cu^{64} , has a relatively short half life of 12.8 hours. This allows for experiments up to about four days, which is too short for many purposes.

Frierson, Hood and Whitney, and Comar (1952) have shown that, after 10 half-lives, other long-life contaminants (mainly Zn^{65} and Ag^{110}) may cause serious errors in studies with radio-copper. This work suggests that prior purification of radio-copper is essential.

The use of radio-copper can be a valuable technique for laboratories near to a nuclear reactor, but

it is limited in its application and many problems of copper metabolism will have to be investigated by other means.

(b) Errors in chemical estimation.- While most investigators are aware of the danger of contamination by extraneous copper, the errors inherent in chemical analysis of biological material are often overlooked. Many earlier methods were not entirely specific for copper and losses due to adsorption were probably common. Even with new reagents and techniques, the error of determination of 10 ug copper in pure solution is probably $\pm 0.5\%$ and under ordinary working conditions is probably nearer $\pm 1\%$. In balance studies there must be added errors due to sampling of feeds and faeces, and possible errors due to variable excretion of faeces. In certain types of material, e.g. faeces, there is danger of loss of copper due to adsorption during wet digestion (see Paper 1:2, page 743).

The writer's experience, mentioned in Paper 1:3 (page 136) is that the total errors of estimation are almost certainly greater than the retention or loss of copper in an adult animal under physiological conditions. The consequences of this fact are discussed further in the section on "Absorption".

(c) The pathological effects of excess dosage of copper.-

Copper salts are quite toxic and the importance of this toxic effect has often been overlooked in some studies where relatively massive doses have been given to study physiological effects.

Necrosis of the proximal convoluted tubular epithelium of the kidneys has been reported by Vogel (1960) in mice following intraperitoneal injection of 0.37 mg copper as the albumin complex; this dose is equivalent to about 15 mg Cu per kg body weight. It seems likely that similar necrosis has occurred in the experiments of Gitlin, Hughes and Janeway (1960), and in some of the sheep injection experiments reported in Paper 1:3. The injection doses of 0.5 mg Cu per kg body weight used in fowls and rabbits (Papers 2 and 3) were the lowest that could be given to obtain satisfactory elevation of liver copper levels. Ideally, it is almost certain that reduction to about one fifth of this amount would have been desirable to reduce pathological changes to a minimum, but the use of radio-copper would be essential for the evaluation of such low doses. In various published experiments where 1 mg of radio-copper has been given to adult humans (equivalent to 0.01 - 0.02 mg per kg body weight) pathological changes must be negligible.

(d) Direct or indirect effects in copper deficiency.- In studies on copper deficiency signs it has often been very difficult to decide whether the curative effects of copper supplements are due to copper per se, or whether they are due to an inactivation of unknown toxic factors by copper. A simple analogy is in the treatment in cattle of "teart", a diarrhoea due to excess molybdenum. This disease is effectively cured by copper salts, but the curative effect is not due to the elimination of copper deficiency, but rather to some obscure copper-molybdenum interaction in the gut.

The possible influence of phylogenous factors in diseases attributed to copper deficiency (enzootic ataxia, "falling disease", and bone disorders) is discussed in Section 5 of this Review.

(e) The non-specific avidity of copper for proteins.- This problem has not been encountered in the present investigations, but it has been a source of confusion in the literature. Copper has a great avidity for many proteins and possibly for other biological compounds. The presence of copper in biological preparations is not necessarily a sign that it is an essential component. Copper was originally reported as a component of butyryl coenzyme dehydrogenase and aminolevulinic acid dehydrase, but

subsequent work has shown that the copper may be removed from both without affecting enzyme activity (Steyn-Pavre and Beinert 1958 and Wilson et al 1959).

The role of copper in cytochrome oxidase has been quite obscure, but a recent paper by Morrison, Horie and Mason (1963) may help to clarify the position. These workers claim that part of the copper is loosely bound and not essential for oxidase activity. The remainder is tightly bound and cannot be removed without loss of activity.

II ABSORPTION

For the purpose of discussion it is desirable to consider separately the monogastric animals and the ruminants.

(a) Monogastric animals.-- The net retention of copper under physiological conditions in adult animals must be extremely small, as there is no evidence of any marked secular changes in the storage of copper in the liver or elsewhere in the body. Many of the published data from balance studies on adult humans which show a strong positive balance must be disregarded completely (see references listed by Cartwright 1950, page 279; also Suzuki and

Hayakawa 1956). If these balances were real some of the individuals would double their liver storage in a few days. The experience of the candidate suggests that these positive balances may be due to errors of determination of copper in faeces, where losses from adsorption can readily occur (Paper 1:2, page 743).

When considering copper absorption, it is important to remember that active secretion of copper occurs continuously through the bile and possibly to some extent through the intestine wall. Under these conditions the small net retention could be due either to a small absorption, or to a small difference between a moderate absorption and an almost equal excretion into the intestine.

Investigations in humans with Cu^{64} (Bush et al 1955) have suggested that between 6 and 43% (mean 32%) of an oral dose of 1 mg Cu was absorbed. This order of absorption is similar to that which might be expected from a consideration of the excretion of copper in bile. Gubler et al (1957) report normal bile copper levels for man as 0.24 - 5.4 mg Cu per litre, while Cummings (1948) reports 0.3 - 2.0 mg per litre. If it is assumed that the mean daily secretion of bile is 750 ml, then between 0.2 and 4 mg Cu is excreted daily through the bile. The

excretion through the urine is negligible and the amount secreted into other parts of the intestine is considered to be small. The average daily intake is between 2 and 3 mg Cu (Underwood 1962, and Paper 4:14) and it must accordingly be assumed that the absorption of such dietary copper in man is relatively high, probably in the range of 25 to 50 percent.

The question of absorption of copper in humans with Wilson's Disease is of considerable interest. In the investigation described in Paper 4:14 some 25% of the dietary copper appeared in the urine and a total of about 50% appeared to be retained in the body. Uzman (1953) claimed that abnormal copper-chelating peptides occur in the urine of Wilson's Disease patients and it has been postulated in Paper 4:14 that these peptides might also be present in intestinal secretions and thus lead to excess absorption. The occurrence of a high copper absorption in Wilson's Disease has been suggested by the observations of Zimdahl, Hyman and Cook (1953), Bearn and Kunkel (1955) and Bush et al (1955). However, this aspect has generally been overlooked and the occurrence of increased absorption has been queried by Gitlin, Hughes and Janeway (1960). These workers made studies with mice and obtained results which could be interpreted in the following terms: "the

amount of copper excreted in the bile by the normal animal increases with increasing copper loads". The large doses used in some of their experiments have caused abnormalities in urinary excretion and these animals could hardly be classed as "normal" (see Section 1C of this Review). However, the conclusion is probably valid and agrees well with the results for fowls and rabbits in Papers 1:2 and 1:3. In Wilson's Disease all investigations have shown that there is a great increase of hepatic copper, but no increase in the concentration of copper in the bile. Gitlin, Hughes and Janeway (loc. cit.) argue from this that the elevation of tissue copper is probably "due to a defect in biliary copper excretion, rather than to an increase of copper absorption". The results of Jensen and Kamin (1957) do suggest that the excretion through the bile is not increased above normal amounts and it is probable that this defect is responsible for the elevation of tissue copper. However, it does not seem valid to argue from this that no increase of absorption has occurred. The total amount of copper absorbed is equal to at least the normal biliary excretion to which must be added the relatively large amount appearing in the urine. It is obvious that in Wilson's Disease the copper absorption must reach very high levels and, in the

candidate's opinion, this aspect needs further investigation.

The absorption of copper by the rat and mouse presents some rather curious features. Observations by Cunningham (1931) and Lindow, Peterson and Steenbock (1929) on the urinary excretion of copper by the rat suggest that, on diets low or normal in copper content, a relatively high absorption has occurred. On the other hand, Cunningham showed that the consumption of a diet very high in copper had no effect on growth or reproduction in the rat.

Some unpublished observations were made on the mouse by the candidate in 1957. These experiments were originally designed to raise the liver copper levels for subsequent studies on the rate of loss of copper. Groups of ten mice (age ca. 8 weeks) were fed for eight weeks on the following diets:-

- (a) Laboratory ration (protein 16.5%, Cu 7.8 p.p.m. Mo 0.36 p.p.m., inorganic sulphate 0.66% all on dry basis).
- (b) Laboratory ration with CuSO_4 added to give 540 p.p.m. Cu (dry basis).

- (c) Laboratory ration with Cu-glycine added to give 540 p.p.m. Cu (dry basis).

At the end of the feeding period, the animals were slaughtered. The mean copper content of the control livers was 18 p.p.m., for the CuSO_4 group 21 p.p.m., and for the copper-glycine group 39 p.p.m. (all values on dry liver). The concentration of copper in the livers of the glycine group was significantly higher than the other two groups which did not differ significantly.

Since this work was done McCall and Davis (1961) have made further studies on rats. With a feeding period of 30 days and a supplemented copper intake of 2500 p.p.m. Cu, they found that the copper stored in the liver was related inversely to the protein content of the diet. With the protein level at 10% the mean liver copper level was 918 p.p.m., with 17.5% protein, 347 p.p.m., while at 25% protein, the liver copper levels were only 36 p.p.m.; not significantly higher than those found in rats on an unsupplemented diet. High levels of protein caused but little depression of copper storage at normal levels of copper intake, and the writers comment that the effect of protein at high levels of copper intake cannot be due only to an inhibition of absorption due to unavailable copper-protein complexes. The candidate considers that the

results obtained may have been related to toxic effects of the dietary copper on the intestinal mucosa. The diet low in protein would be expected to contain a relatively large amount of ionic copper which is known to cause inflammation and necrosis of the mucosa. In the diet high in protein, a larger proportion of the dietary copper would be present in the form of copper-protein complexes which would cause less damage to the mucosa. All experiments with rats and mice suggest that the intact mucosa is an effective barrier against copper absorption and it is suggested that in a damaged mucosa this barrier might be broken down. The question of copper absorption in the rat and mouse is obviously not completely understood, but it would seem that these species have a very effective mechanism for the prevention of storage of excess copper in the liver.

(b) Ruminants.-- The absorption of copper in the ruminant is a problem of great complexity. Not only do all the problems of non-ruminant digestion apply, but there is also the effect of rumen organisms on the copper in different plant species at varying stages of growth. The qualitative changes in the herbage ingested will almost certainly influence the type of organisms within the rumen and it is probable that the availability of copper to the

sheep varies continuously throughout the growing season of the plants.

Mills (1954) showed that a large proportion of the copper in herbage was insoluble in water and in organic solvents. Further investigations (Mills 1956 - 1957) showed that the copper in freeze-dried grass was considerably more effective in the treatment of copper deficiency in rats than was an equivalent amount of ionic copper and that the "active" copper was associated with the water-soluble fraction. Fractionation of water extracts of grass showed that most of the copper was present in a neutral or anionic complex and that these fractions were highly effective in the treatment of copper deficiency in rats. Mills suggests that absorption of copper in the ruminant under physiological conditions probably occurs as complexes, either preformed in the food or as complexes formed by interaction with specific complex-forming ligands in the digestive tract. One rather surprising fact emerged from Mills' studies. The copper in herbage from "swayback" areas seemed equally effective in overcoming copper deficiency in rats as that in normal herbage. Swayback pastures cause a serious upset of copper metabolism in sheep.

Australian experience has indicated that there is

a marked difference in the availability of copper in green pasture as compared with dry pasture, at least under conditions of low copper intake.

Dick (1954) has published data for the storage of copper in the livers of crossbred sheep which were fed dry lucerne and oaten hay to which was added varying amounts of copper sulphate. Storage of copper was observed at all levels of intake and extrapolation of the results indicated that the sheep would be in copper balance on a daily intake of about 0.5 mg Cu. This figure is in marked contrast to that obtained by the writer (Paper 3:12) for sheep grazing on green pastures. If the copper content of green herbage drops below about 6 p.p.m. (on dry matter) defects are likely to appear in the wool, and once the level falls below 3 p.p.m. anaemia may occur in the ewe and gross pathological changes may occur in lambs born to ewes grazing such pastures. These concentrations of copper are equivalent to daily intakes of approximately 6 and 3 mg Cu respectively, for an adult sheep. The difference in the occurrence of copper deficiency symptoms on green and dry pastures was noted early by Bennetts (see Paper 3:9, page 11). Unpublished studies have been made by the candidate on the changes of the liver copper of sheep grazing on dry summer pastures at Merredin, Western

Australia. The summer grazing is largely dry grasses with copper content of 2.5 - 4 p.p.m. Cu. Liver samples were taken by aspiration biopsy from one group of sheep during the summer of 1951-52, and from another group of sheep in the summers of 1952-53 and 1953-54. Very little change occurred in the liver copper levels in spite of the very low copper content of the grazing. There are suggestions from other investigations that the copper of dry feed is more available than that in green feed, but no detailed studies have been made (Hunter, Eden and Green 1945, page 25).

The most spectacular effect of certain green pastures is noted in sheep and cattle grazing copper deficient pastures. At Gingin (Paper 3:8) and in the "falling disease" areas (Paper 3:9) a very profound anaemia develops in certain seasons in late August or early September. This persists until late October and then spontaneously disappears. These changes are shown in the figures given in Paper 3:9, page 91. Copper levels in the pastures of these areas show a slight decrease during the period. Blood copper remains at a very low level throughout. At both Gingin and in the "falling disease" areas the anaemia is associated with lush pasture and, as the pasture becomes more fibrous, the anaemia disappears.

At Gingin (Paper 3:8, page 12) a rank growth of capeweed seemed to favour the development of anaemia and ataxia. The anaemia seems to be almost invariably associated with pregnancy and particularly with lactation both for sheep and for cows.

In 1945 it was shown by Dick and Bull that molybdenum could interfere with copper metabolism. Subsequently Dick (1953) showed that the presence of adequate inorganic sulphate was necessary for the action of molybdenum. Paper 3:13 gives the levels for molybdenum and inorganic sulphate in Western Australian pastures, and particularly in those from copper deficient areas. The findings were rather inconclusive. It is probable that molybdenum does depress the availability of copper in some of the "falling disease" areas, but the writer does not consider that molybdenum is the factor responsible for the very dramatic interference with copper metabolism of animals on the lush spring pastures of these areas.

No satisfactory explanation can be given for the seasonal anaemia. It would seem that the availability of copper in these lush pastures must be extremely low, at least to the sheep and the bovine. The sudden onset of the anaemia suggests to the writer that there may be factors

which actually decrease the availability of copper already in the animal tissues. There is no evidence as to the nature of these factors, nor is there any evidence to indicate whether they are present in the pastures as such, or whether they are elaborated in the rumen as the result of the particular conditions set up by the ingestion of such pastures.

III TRANSPORT

In Paper 1:1 the total amounts of copper in the blood of various species were recorded. It has been shown, at least in man and in the pig, that "total blood copper" is a composite figure made up of three major components: (a) the copper in the red cells, (b) a small amount of so-called "direct-reacting" copper in the plasma, and (c) a stable copper-containing protein (caeruleo-plasmin) in the plasma. In man, the main, if not the only copper compound in the red cells, is a protein which has been named "erythrocuprein" (Markowitz, Cartwright and Wintrobe 1959); in the ox a different copper-protein (haemocuprein) was reported by Mann and Keilen (1938). The "direct-reacting" copper is so-called as it reacts directly with the dithiocarbamate ion. It appears to be a complex of serum albumin and copper, and is probably the only true "transport

copper" in blood.

The copper in caeruloplasmin will not react with dithiocarbamate until the protein moiety is denatured and it is thus referred to as "indirect-reacting" copper. Caeruloplasmin levels in man vary widely in disease and pregnancy and, although this compound has oxidase activity, its function is not understood.

Although much work has been done on the forms of transport copper in man and in the pig, very little work has been done on other species. McCosker (1961) has made studies on several species using the oxidation of paraphenylene-diamine as a measure of caeruloplasmin content. The oxidase activity varies widely between species and does not correlate well either with total or with indirect-reacting copper. The results indicate the need for further studies on the forms of transport copper in different species. Studies are also needed on the foetal and maternal blood copper of different species.

Various workers have now shown that there is more than one component in caeruloplasmin and this aspect should be kept in mind in future studies (see Richterich 1961; also McAlister, Martin and Benditt (1961).

During early investigations on copper deficiency in sheep and cattle it was found that very low

liver copper levels always resulted in low blood copper levels, but more or less normal blood levels were sometimes encountered in animals with fairly low liver levels. This aspect has recently been investigated in cattle by Haag and Adams (1958). These workers found that plasma copper was constant once the liver copper was above 33 p.p.m. (dry basis), but below this level there was fairly good correlation between blood and liver copper levels. Under Western Australian conditions, 33 p.p.m. is considered to indicate sub-optimal intake of copper.

IV STORAGE

It is usually assumed that the liver is the storage organ for copper in the animal body. It is true that copper can be stored in the liver, and in the ruminant this undoubtedly does occur as a normal physiological process. In monogastric animals, however, the liver probably functions more as an excretory organ than as a storage organ. In most monogastric animals a small to moderate increase of dietary copper apparently will cause no increase in liver copper levels (Paper 1:3). Although experimental evidence is lacking, it seems reasonable to assume that at least part of the increased dietary copper is absorbed, and this is compensated by an increased

excretion from the liver through the bile. In Papers 1:2 and 1:3 it was shown that copper injected into the fowl or rabbit was temporarily stored in the liver and then rapidly eliminated. In the case of the duck (Paper 1:2) it was not possible to detect excess copper in the liver 24 hours after injection. Bile copper levels were, however, raised and it must be assumed that the elimination of copper from the liver was extremely rapid. If dietary copper is sufficiently high it seems that all species will store copper in the liver. Although experimental proof does not seem to be available, it is likely that, under these conditions, the absorption of copper has exceeded the excretory capacity and the liver is functioning as an organ where the excess copper can be stored in a non-toxic form, until it can be excreted.

The form in which copper is stored in the liver does not seem to be known, but it would appear to be in a very stable chemical combination. The relatively large amounts of copper in sheep liver do not stain with rubeanic acid and in Paper 1:2 no staining copper was noted in the livers of fowls which had been raised to 200 p.p.m. Cu after the injection of copper sulphate. Paper 1:3 gives data which suggest that the form of liver copper in sheep is the same whether it is received orally or by

intravenous injection.

It was pointed out in Paper 4:15 that sheep can tolerate very high levels of copper in the liver with no apparent ill effect, provided they are not subject to physiological stress. Recent work by Todd and Thompson (1963) suggests that there may be damage to the liver, although no histological or clinical change is noted. These workers studied sheep being fed high levels of copper sulphate which eventually caused a haemolytic crisis and death. Six to eight weeks before the crisis occurred, marked increases were noted in levels of serum lactic dehydrogenase and serum glutamic-oxalacetic transaminase. No studies have yet been made on apparently normal sheep with high liver copper levels which do not terminate in a fatal crisis.

In most species on which studies have been made the values for liver copper found by the candidate were similar to those obtained by other workers (Paper 1:1). It would seem that the concentrations of copper found in the liver of any one species is relatively constant under different environmental conditions. However, the candidate found a mean liver copper level for guinea pigs to be 77 ± 6 p.p.m. (range 29 - 205), while other workers had found levels of about 20 p.p.m. No explanation can be

offered for this difference.

Unpublished studies have been made by the candidate which show an enormous variation in the individual liver copper levels of sheep grazing together on pastures at Merredin, Western Australia. In April 1954, a group of 40 two-year-old sheep showed a range of 100 to 425 p.p.m. (dry basis). In November 1957 another group of 70 sheep showed a range of 23 to 286 p.p.m., and in August 1958 the same sheep showed values of 11 to 310 p.p.m. Blood copper levels are consistently normal in this region.

The data in Paper 1:1 suggest that a higher variability of liver copper is noted in species which normally show a high level. No satisfactory explanation can be given for these observations. In the sheep, which is sensitive to variations of dietary copper, the variation may be related to individual differences in absorption of copper. This explanation would not apply to the duck which shows no increased copper storage with increased dietary copper. The variations may possibly represent individual differences of copper-binding capacity of the liver tissues.

V THE FUNCTION OF COPPER IN THE ANIMAL BODY

Until recently our knowledge of copper metabolism in warm-blooded animals has been derived from two main sources: (a) biochemical and pathological observation made on copper-deficient animals, and (b) studies relating to Wilson's Disease in humans. In more recent years more studies have been made on the role of copper in enzyme systems. Although the results obtained from enzyme studies have been rather meagre so far, there is no doubt that considerable progress in this field will be made in the future.

In this section discussion is confined largely to data obtained from copper-deficient animals.

(a) Blood formation.— Copper deficiency signs vary widely in different species and the only feature common to all is anaemia. Even the anaemia varies considerably in type between species and it would appear that it is generally a late development in the deficiency syndrome.

Underwood (1962, page 72-75) has summarized our knowledge of the role of copper in blood formation and concludes that "neither the manner in which copper influences erythropoiesis, nor the stage at which its

effects are exerted are known".

The seasonal anaemia in the copper-deficient areas of Western Australia is discussed in Section 2 (Absorption) of this Review.

(b) Bone formation.-- Copper is apparently essential for normal bone formation, as bone pathology has been noted in copper-deficient animals of a number of species (Underwood 1962, page 75). Such changes, however, have not been a common feature in copper deficiency in Western Australia. The field studies of G.K. Davis and his colleagues on copper deficiency in Florida (see Comar, Singer and Davis 1949) suggests that the bone changes there are related to molybdenum excess and some other factor, as well as to copper deficiency. These causes could hardly apply, however, in the case where bone changes have been produced on experimental diets. The role that copper plays in normal bone formation is quite unknown.

(c) Hair and wool formation.-- Copper is essential for the normal formation of keratin and for the formation of hair pigments. All species studied (except the pig) show achromotricia in copper deficiency. This is presumably due to lack of copper-containing enzymes of the polyphenol oxidase type which convert tyrosine to melanin.

In the copper-deficient areas of Western Australia cattle show poor coat colours and black sheep fail to produce the normal black wool pigment.

Copper appears to be essential for the production of normal wool. Under conditions of even mild deficiency the normal crimp is lost. This is apparently due to a lack of enzymes which catalyse the oxidation of sulphhydryl groups to disulphide linkages (Marston 1949, page 386). Other changes occur in copper-deficient wool; the arrangement of peptide chains is apparently altered (Burley and de Kock 1957), and there is a decrease in the amount of high-sulphur proteins in the wool fibre (Gillespie 1964). The loss of crimp in wool is widespread in the copper-deficient areas of Western Australia.

(d) Copper and the central nervous system.- The effect of copper in the prevention of enzootic ataxia in lambs was first reported in 1937. A considerable amount of investigational work has been carried out since then, but the mechanism causing demyelination of the C.N.S. in copper deficiency is still quite obscure. Two concepts are current and the evidence seems about equally divided between these two theories.

(i) The first concept is that the demyelination is only secondary to copper deficiency. According to this

theory the actual demyelination agent is an unknown factor present in the herbage. In the presence of adequate dietary copper, this is detoxicated either in the rumen or in the animal body. As a precedent for this type of action one can note the protective action of cobalt salts against the toxic principle of Phalaris tuberosa (Lee et. al. 1957), and of Vitamin B₁₂ against the pyrrolizidine alkaloids (Dick et. al. 1963).

- (ii) The second concept is that the demyelination will be explained solely in terms of derangement of enzyme systems under conditions of copper deficiency.

These concepts have been discussed by Underwood (1962, pages 76-80), but some further comments seem desirable.

Demyelination due to copper deficiency is almost entirely confined to sheep. It has been reported occasionally in goats (Schultz et. al. 1951). Cunningham (1950) has reported occasional cases of ataxia in calves, but the candidate considers that these possibly may have been due to some factor other than copper deficiency. In our studies on "falling disease", conditions of extreme deficiency were produced in cattle, but no signs of ataxia

were noted; sheep did, however, develop ataxia under these conditions. An ataxia in pigs associated with low liver copper levels was noted in "falling disease" areas, but this was not studied (Paper 3:8, page 12; see also McGavin, Ranby and Tammemagi 1962). Horses grazing on the enzootic pastures of Gingin, W.A., showed pathological changes, but no ataxia. No nervous lesions have been produced experimentally in copper-deficient rats, pigs or dogs.

It has been noted by various workers that the severity of the nervous lesions does not seem to be related to the copper status of the ewe or lamb as judged by liver copper levels.

This conclusion is strengthened by our own unpublished observations in the "falling disease" areas. Two ewes were run for 13 months on severely deficient pastures at Jindong, W.A. They were then mated and were continued on the same pastures during pregnancy. Both gave birth to apparently normal lambs, but after about six weeks, mild ataxia was noted in the lambs. The copper content of the livers of the lambs was found to be 2 p.p.m. (dry matter) and, of the livers of the ewes, 3 and 2 p.p.m. At Gingin, where ataxia was much more acute, liver copper levels of lambs were 2.5 - 8 p.p.m., and of ewes 3 - 15 p.p.m.

A possible explanation of the above facts may be found in the observations of Mills and Williams (1962). These workers found that the incidence of swayback in lambs could be correlated with the copper content of the brain, but not of the liver. A level of 3 p.p.m. Cu on dry brain tissue seemed to be the critical level below which the ataxia would occur. Unfortunately, no brains from ataxic sheep were analyzed in our investigations. It is interesting to note that S.T. Evans (1943, unpublished data) analyzed brains from some acutely deficient cows in the "falling disease" areas. These contained between 3 and 11 p.p.m. Cu (on dry matter).

The work of Mills and Williams does open up a more rational approach to the connection between ataxia and copper deficiency. On the other hand, their findings complicate the picture because it seems necessary to postulate the existence of some chemical factor in "swayback" pastures, which causes a preferential depletion of brain copper reserves as compared with those in the liver. To explain results quoted above it must also be assumed that this factor (or a similarly acting factor) is present to some extent in pastures causing enzootic ataxia at Gingin, but only to a very slight extent in

those at Jindong. The variable occurrence of such a factor might also explain why ataxia has been reported in cattle in New Zealand, but not in Western Australia.

Conflicting reports on attempts to produce ataxia under experimental conditions will probably be resolved in the light of Mills and Williams' work, but unfortunately brain copper determinations do not appear to have been made by the workers concerned. New Zealand workers (Anon 1957-58), Victorian workers (Anon 1961-62), and Butler and Barlow (1963) have failed to produce ataxia in lambs by depleting copper reserves of the ewes with molybdenum and sulphate. On the other hand, Mills and Fell (1960) have produced ataxia in lambs by this technique. In a private communication Mills (1963) has pointed out that the grass used in his experiments did have some peculiarity with respect to copper metabolism, of which they were not aware when the experiments were carried out. Lambs born to ewes grazing this herbage are clinically normal and show normal liver and brain oxidase activities, but liver copper levels (mean 12 p.p.m.) are much lower than would be expected from the copper content of the herbage (4.8 - 5.5 p.p.m.). It could easily be that the hypothetical factor in herbage causing depletion of brain copper levels was present in moderate amounts just insufficient to

cause ataxia. The general copper-depleting effect of the molybdenum and sulphate would then cause brain copper levels to fall below the critical level.

(e) Copper and fibrosis of the bovine myocardium (Papers 3:9 and 3:10).- As in enzootic ataxia, there is the possibility that the cardiac lesion in "falling disease" may be due to some other factor which is detoxicated by the animal in the presence of adequate copper. The candidate considers that the evidence for such a toxic factor is less strong than in the case of enzootic ataxia.

The limited distribution of "falling disease" cannot of itself be taken to indicate a second factor. The intensity of the copper deficiency in the "falling disease" areas is probably greater than in any other part of the world, at least when judged by liver and blood copper levels. Furthermore, it would appear that identical histological and clinical signs have been observed in cattle on King Island, eastern Australia (Dumaresq 1942), and in Florida (Neal and Ahmann 1937).

Under conditions occurring in the "falling disease" areas, greatly decreased cytochrome oxidase levels might have been expected in the heart muscles of affected cows (Lemberg, Newton and Clarke 1962; Mills,

Williams and Poole 1963). On this was superimposed the severe seasonal anaemia and it is probable that the resulting anoxia was responsible for the heart lesion.

(f) Copper and enzyme systems.- In spite of intensive investigation, the role of copper in enzyme systems is still relatively obscure and only three such systems will be discussed here.

Caeruloplasmin has been discussed in Section 3 of this Review. It has been pointed out that this compound has oxidase activity and that concentrations in the blood increase in infections in various species, and in pregnancy in humans. It might be expected that the function of caeruloplasmin is to catalyse the oxidation of toxic metabolites, but there is no evidence to support such a concept. In copper-deficient sheep and cows, caeruloplasmin must fall to exceedingly low levels, but clinical observations in Western Australia do not suggest that such animals are more prone to the effects of infection than are copper-sufficient animals.

It has been shown by Gallagher, Judah and Rees (1956) that phospholipid synthesis is impaired in the copper-deficient rat, but the exact point at which the copper acts was not ascertained. Lemberg, Newton and

Clarke (1962) consider that the effect is secondary to loss of cytochrome oxidase. It is likely that this finding will be important in the understanding of enzootic ataxia in sheep (Section 5:(v)) but, as has been pointed out by various workers, demyelination of the C.N.S. does not occur in the rat under conditions of copper deficiency.

Low cytochrome oxidase activity has been demonstrated in tissues of most species under conditions of copper deficiency and this enzyme system is the only one in which copper is clearly implicated. After years of controversy it seems certain that copper is an integral part of the cytochrome oxidase molecule (Morrison, Horie and Mason 1963), but it would also appear that the depletion of the enzyme under conditions of copper deficiency is primarily due to a defect in the synthesis of the haem a component of the molecule (Lemberg, Newton and Clarke 1962). Other haemo proteins containing a different haem (e.g. myoglobin, cytochrome c and catalase) do not appear to be reduced in moderate copper deficiency and it seems that the primary defect is in the synthesis of haem a.

VI EXCRETION

Under physiological conditions, the excretion of copper through the urine is extremely small in man, the

sheep and the rabbit. In Paper 4:14 the daily excretion for two healthy men was reported to be 14 and 22 ug Cu. In Paper 1:3 the mean daily urinary excretion for rabbits in metabolism cages was 7.4 ug Cu. This figure is undoubtedly in excess of the true value, as some degree of faecal and dietary contamination occurred in these samples. The candidate has obtained copper concentrations of less than 10 ug Cu per litre from a number of sheep urine samples collected directly from the vulva to avoid contamination. Reference has already been made in Section 2 of this Review to the published data which suggest that a rat excretes an appreciable proportion of its dietary copper in the urine. This fact may be linked in some way with the fact that rat milk is higher in copper than that of most species. It should be pointed out that the data showing high urinary copper in the rat were obtained in 1929 and 1931 before modern analytical methods were available. These figures do not seem to have been repeated by recent investigators.

Because of the very low concentration of copper in urine, the form of the urinary copper does not appear to have been studied.

In humans and in sheep, small but significant amounts of copper are lost in the milk (Papers 2:4, 2:5).

Copper deficiency anaemia in sheep and cows is almost always associated with lactation under field conditions. However, the loss of copper through the milk must be extremely small, as the concentration of copper in the milk of such animals is exceedingly low.

In 1935 Judd and Dry suggested that the main channel of excretion of copper was through the bile. This has been confirmed by many investigators, and yet virtually no work has been done to study the details of bile excretion. This aspect was the next to be studied when it became necessary to terminate these studies in 1961. Two major questions are unanswered:-

- (a) A characteristic of bile copper concentration for any one species is its extraordinary variability; this is true for man (Section 2 of this Review), for the sheep, pig and the cow (Paper 1:3), and to a lesser extent for the duck and fowl (Paper 1:2). The reason for this could either be in a variation in the amounts of copper excreted by the bile; alternatively, it could be due to variations of bile volumes with a relatively constant copper excretion.

(b) No studies seem to have been made to determine the chemical form in which copper occurs in bile. It would seem likely that relatively simple procedures such as chromatography, electrophoresis and solvent extraction, would give information on this aspect.

A number of investigators have obtained results which suggest that some copper is secreted directly into the intestines, but this amount of copper must be relatively small. There is evidence that small but significant amounts of copper are excreted into the caeca of the domestic fowl (Paper 1:2).

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SECTION 6

SECTION 6

MISCELLANEOUS PUBLICATIONS

The papers dealing with subterranean clover have been grouped together, otherwise the papers are arranged chronologically. Details of assistance received from co-authors and others is indicated below. Where no such acknowledgement is given, the planning and carrying out of the project was done solely by the candidate.

Papers relating to subterranean clover

- 6:17 "The oestrogenic isoflavones of subterranean clover." Aust. J. Agric. Res. 1964, 15: 223.
- 6:18 "Studies on the oestrogenic extracts prepared by the action of alcoholic alkali on chloroplast material of subterranean clover." Unpublished (1952-1960). With C. Kowala. Mr. Kowala carried out purification and identification of compounds isolated. Bioassays, bulk extractions and bulk chromatography were done by the candidate.
- 6:19 "The composition of the Dwalganup strain of subterranean clover." J. Agric. W.A. 1952, 1 (3rd series): 257.

All analyses in this paper were carried out by the W.A. Government Chemical Laboratories.

- 6:20 "Studies on the oestrogenic substance in subterranean clover." Aust. J. Exp. Biol. Med. Sci. 1951, 29: 273.
With A.W. Braden. Dr. Braden carried out all the bioassay work of this investigation.

General Papers

- 6:21 "Studies on the excretion of oestrogens by pregnant ewes." Aust. J. Agric. Res., 1950, 1: 322.
This project was suggested by a committee directing research into clover disease in sheep.
- 6:22 "The copper and nickel content of the blood of the W.A. marine crayfish (Panulirus longipes) and of seaweeds." Aust. J. Exp. Biol. Med. Sci., 1949: 27: 307.
With K. Sheard. Dr. Sheard suggested the project and organized the collection of material.
- 6:23 "The quantitative extraction of cobalt and iron from ashed biological material." Aust. J. Exp. Biol. Med. Sci., 1945, 23: 311.
- 6:24 "The relation between colour and chemical composition in soils." J. C.S.I.R., 1939, 12: 128.

6.3

This project was suggested by and directed by Professor J.A. Prescott of the Waite Institute, S.A.

6:25 "The composition of capeweed (Cryptostemma calendulaceum) from Meckering and Beverley." With R.G. Lapsley. J. Agric. W.A. 1938, 15: (2nd series), 422. Mr. Lapsley assisted in the analytical work in this investigation.

6:26 "Report on the effect of lead salts and alkalis in cyanidation." Proc. Aust. Inst. Mining and Metall. 1935, 499. With H.W. Gartrell.
Mr. Gartrell was Officer-in-Charge of the Mining Department of the S.A. School of Mines, where the investigation was carried out. The project was the candidate's own.

6:27 "Notes on the occurrence of Azotobacter in some South Australian soils." Aust. J. Exp. Biol. Med. Sci., 1935, 13: 127.

PAPER 6:17

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THE OESTROGENIC ISOFLAVONES OF SUBTERRANEAN CLOVER

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THE OESTROGENIC ISOFLAVONES OF SUBTERRANEAN CLOVER

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Summary

A number of strains of subterranean clover has been examined for isoflavone content in an attempt to explain the differences of oestrogenic potency in sheep and cattle under field conditions.

A method of thin-layer chromatography has been developed which allows a rapid semiquantitative estimation of the main isoflavones present in this species.

The isoflavones are present in a combined form, probably as glycosides, and the free isoflavones are rapidly released after crushing the leaf.

The percentages of formononetin, genistein, and biochanin A in the leaves are very much higher than reported by earlier workers and vary widely between strains. Moreover, the ratios of the isoflavones differ greatly among strains. The relevance of these plant data to the oestrogenic potency in the animal is discussed.

I. INTRODUCTION

In recent years there has been a resurgence in Western Australia of the reproductive abnormalities of sheep associated with the ingestion of subterranean clover (*Trifolium subterraneum* L.) (Bennetts 1946, 1947; Bennetts, Underwood, and Shier 1946). Field evidence obtained in this State with sheep and cattle has suggested that certain strains of the clover (e.g. Dwalganup and Yarloop) have a considerable oestrogenic potency, while others (e.g. Mount Barker) have little or none. Earlier studies on strains of clover by guinea-pig bioassay (Alexander and Watson 1951), or by chromatographic estimation of genistein (Curnow and Rossiter 1955), had not given results which would explain the observed field differences.

As a result of these facts, the problem of strain differences is being re-investigated. Preliminary observations with wethers as test animals (McKeown 1962) and with ovariectomized ewes (Davies and Bennett 1962) have already been published. Interpretation of these results was not possible because of lack of knowledge of the concentrations in the clovers of the isoflavones presumed to be responsible for the oestrogenic stimulation.

The present paper presents the results of a preliminary investigation made to ascertain the levels of formononetin (7-hydroxy-4'-methoxyisoflavone), genistein (5,7,4'-trihydroxyisoflavone), and biochanin A (5,7-dihydroxy-4'-methoxyisoflavone) in commercial strains of subterranean clover.

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II. EXPERIMENTAL

(a) Extraction of Isoflavones from Plant Material

Up to the end of 1962, extracts were prepared by placing combined leaf and petiole in cold ethanol. Some discordant results led to a re-examination of the method and it was found that the isoflavones of the clover were present in a combined form. Only a partial hydrolysis of the combined form occurred after immersion in ethanol. The extraction technique was modified (see below) to allow for complete hydrolysis. The subsequent purification procedure is similar to that used by Wong (1962).

A sample of 3 g green leaf or 5 g green leaf and petiole was crushed with about 10 g acid-washed sand and a little water. The resulting sludge was allowed to stand 30 min at 37°C; 150 ml ethanol was then added and the mixture was heated nearly to boiling. After standing overnight the solution was filtered and the residue was re-extracted similarly with a further 150 ml ethanol. The combined filtrates were concentrated under vacuum to about 100 ml, and water added to make the solution approximately 70% (v/v) ethanol. Allowance was made for the water in the original sample and for that added during crushing. Lipids were then extracted with two portions of 70 ml petroleum ether (b.p. 50–70°C).

Difficulty was initially experienced in some samples in the final extraction of the isoflavones with ether (compare Wong 1962), but the following procedure gave satisfactory results. The 70% ethanol solution was evaporated under vacuum to about 10 ml to remove all ethanol, and a further 10 ml water was added. About 70 ml of peroxide-free ether was added and the mixture transferred to a separating funnel. Usually only a small amount of pigment passed into the ether phase at this stage, and sodium chloride was added in small amounts with shaking until a marked transfer of colour to the ether phase occurred. Excess sodium chloride was undesirable. The satisfactory extraction of formononetin into the ether phase appeared to be dependent on the time of contact as much as on the amount of shaking, and a minimum of 2 hr was allowed. A total of four portions of 70 ml ether was used; each portion was allowed to stand in contact with the aqueous phase for at least 30 min, and the funnel was shaken intermittently. A small amount of brownish sludge usually remained, but this contained no isoflavones. The ether extracts were combined, care being taken to avoid any contamination with the aqueous phase, and were evaporated to dryness. Last traces of solvent were removed by heating under vacuum on a water-bath, and the dried extract was weighed. The dried extract was dissolved in benzene-ethanol (50% v/v) to give a convenient concentration, usually 7–15 mg/ml. It was necessary to use sufficient solvent to dissolve completely all formononetin, and to warm solutions which had been stored at low temperatures before use.

(b) Chromatographic Methods

Wong (1962) has recently published a method for the separation of isoflavones in red clover by paper chromatography, but it appeared that thin-layer chromatography would be more rapid and would give better separation of components (see reviews by Wollish, Schmall, and Hawrylshyn 1961; Russel 1963). Silica gel

(Kieselgel G—Merck) plates were used and some 60 solvents and solvent mixtures were tested. The methanol–chloroform system adopted was the only one to give satisfactory results.

After the present method was developed it was noted that Grisebach and Brandner (1961) had separated formononetin and biochanin A by thin-layer chromatography, using 8% (v/v) ethanol in benzene. The methanol–chloroform system gives a better spread of the isoflavones, and allows for easier observation on other unidentified compounds with similar R_F values.

Standard silica gel plates (20 by 20 cm) were used with an adsorbent thickness of 0.25 mm. Before use, the plates had to be activated by heating to 100° for 1 hr, or to 120° for 30 min. For comparison with the clover solutions, reference spots of 1, 2, and 3 μg formononetin, and 3, 6, and 9 μg genistein and biochanin A, were used. The chromatograms were developed in 11% (v/v) methanol in chloroform.

A tank with a saturated atmosphere (Russel 1963) was adopted for genistein estimations, but an incompletely saturated atmosphere was preferred for biochanin A and formononetin, as this gave a greater separation of the spots. The solvent was allowed to go the full distance on the plate (c. 16 cm).

Subsequent investigations have shown that for most samples it is preferable to use a shorter solvent run (10–12 cm) in an incompletely saturated tank.

The plate was allowed to dry in a stream of air for a short time and the intensity of formononetin spots compared with the standards under u.v. light (257 $m\mu$). The genistein and biochanin A spots were made visible by spraying with diazotized sulphanic acid (Bray and Thorpe 1954) and exposing to ammonia fumes. The R_F values for biochanin A, formononetin, and genistein were approximately 0.85, 0.75, and 0.55 respectively in an incompletely saturated tank, and approximately 0.65, 0.60, and 0.50 in a saturated tank.

The estimation of each isoflavone was repeated six times. Individual determinations were usually well within $\pm 20\%$ of the mean. Results were calculated on the dry weight of the leaf.

All solvents were distilled before use. The chloroform used was virtually free from ethanol; if ordinary chloroform is used the percentage of methanol in the developing mixture should be reduced. It is essential that the chloroform be free from oxidizing material. The oxidation of diphenylthiocarbazon (dithizone) is a convenient and sensitive test for such impurities.

(c) *Compounds Present in Clover Extracts*

The greater part of the extracts consists of unknown compounds of low R_F values. Genistein and formononetin were first shown to be present in clover extracts by Bradbury and White (1951). Although biochanin A had previously been found in subterranean clover (Beck, Kowala, and White 1956; Guggolz, Livingston, and Bickoff 1961) it was not anticipated that it would be the predominant isoflavone in some strains. It was accordingly considered desirable to verify the identity of the chromatographic spot considered to be biochanin A. An extract was prepared, by the methods described above, of a large sample of a Mount Barker strain showing a

strong biochanin A spot. Biochanin A was separated by column chromatography and was then recrystallized from aqueous ethanol to constant melting point. The properties of the isolated biochanin A were generally identical with those of the synthetic product: m.p. 215° (corr.), not depressed by admixture with the synthetic compound; λ_{max} in ethanolic solution 262.5 m μ , and in 0.01N ethanolic sodium hydroxide 276 m μ . The value of $E_{1\%}^{1\text{cm}}$ at 262.5 m μ was 1230, identical with the value reported by Wong (1962). This value was slightly lower than that obtained with our synthetic compound (1260), a discrepancy probably related to the fact that the synthetic material had a very pale buff tint not removed by chromatography and recrystallization.

Traces of daidzein (7,4'-dihydroxyisoflavone) have been found in subterranean clover by Guggolz, Livingston, and Bickoff (1961), and by Wong (1963). This compound has a slightly lower R_F value than genistein and is easily recognized by a strong fluorescence under u.v. light. Traces of fluorescence were noted in the daidzein position in some of the samples, but the amounts present were too small to be measured accurately by the method used.

Some strains, particularly Mount Barker, showed a strong spot with an R_F value between genistein and formononetin. This compound has not yet been identified, but it is probably the isoflavone pratensein (Wong 1963).

Guggolz, Livingston, and Bickoff (1961) have reported small amounts of coumestrol in subterranean clover. This compound is about 30 times as active in mice as is genistein and could contribute significantly to the total oestrogenic potency of the clover. Coumestrol has the same R_F value as genistein in the methanol-chloroform system used, but a good separation from the four isoflavones may be achieved by the use of the following solvent system: 18 vols. methanol, made to 1.0N with ammonia gas, plus 82 vols. chloroform. The intensity of the coumestrol spot is greatest if the plate is viewed under u.v. light before drying, and 0.05 μg coumestrol, or less, is visible under such conditions. The solvent system used for coumestrol does not give complete separations of biochanin A from formononetin, or of daidzein from genistein.

Most extracts contained a small amount of yellow pigment with the same R_F value as biochanin A in the solvent system used. The pigment is readily separated from biochanin A with other solvent systems, and tests have shown that it leaves virtually no colour after treatment with diazotized sulphanilic acid and ammonia.

III. RESULTS AND DISCUSSION

During investigations concerning the nature of the combined isoflavone, the following points were established:

(1) In all the strains of subterranean clover which were tested (and also in one sample of red clover) extremely low levels of isoflavones were found if intact green leaves were dropped into boiling ethanol. If, however, the green leaves were first crushed and then placed in either cold or boiling ethanol, high levels of isoflavones were found. These facts indicate that the isoflavones of all strains were in a combined form and that the strains examined contained a hydrolytic enzyme.

Isoflavones usually occur in plants as glycosides, and as the combined forms in steam-inactivated clover leaves were partly hydrolysed by acid and β -glucosidase, it is considered that they are glycosides.

(2) The rate of hydrolysis of the combined forms after crushing was extremely rapid. Maximum yields of genistein were obtained in the time necessary to crush leaves and to transfer to alcohol (<1 min); about 70% yields were obtained with formononetin and biochanin A under these conditions. All samples from West River were incubated for both 15 and 60 min, but no significant differences in the isoflavone levels were noted.

(3) Little or no loss of isoflavones occurred when green clover was stored in polyethylene bags for 2 weeks at 4°C (Carnamah, Clare, and Yarloop), or for 24 to 48 hr at 22° (Dwalganup, Mount Barker, and Yarloop). No significant hydrolysis of the combined forms occurred in intact leaves stored for 24 hr at 22° (Dwalganup).

(4) The enzyme of green clover leaves was inactivated by dropping the leaves into boiling water. Such leaves contained no free isoflavones. They were then crushed with fresh green leaves and the mixture incubated for 30 min at 37°C. These experiments were carried out with Dwalganup and with Carnamah leaves. Cross-hydrolysis was also done on boiled Yarloop leaves (high formononetin) with intact *Bacchus Marsh* leaves (low formononetin). Recoveries of 70–100% of the isoflavones in the boiled leaves were obtained.

By contrast, if boiled Dwalganup or Carnamah leaves were crushed with the corresponding green petioles, virtually no hydrolysis occurred. It is concluded that the petioles contain little or no hydrolytic enzyme, and that analyses of petioles alone will give no indication of the true isoflavone content.

Table 1 gives the percentage of isoflavones in the leaf fraction of nine strains of subterranean clover. Most of these strains have been described by Quinlivan (1962) and are used commercially; the very early-flowering strain, Carnamah, has been described by Rossiter and Millington (1961). The samples were collected from experimental plots at two widely separated localities—West River (25 miles southwest of Ravensthorpe) and Bakers Hill (45 miles east of Perth). At West River, the plants germinated after early rains in February and were collected in mid May; at Bakers Hill, the plants germinated after normal rains in May and were collected early in July. All strains were still in the vegetative growth stage, and were at approximately the same level of production.

One of the most important findings in the present investigation is that very high levels of isoflavones occur in subterranean clover. The strains tested showed total contents of the three isoflavones varying from 2.5 to 5.6% in the dry matter of the leaf. The very much lower values reported by earlier workers (Curnow 1954; Guggolz, Livingston, and Bickoff 1961) seem due, at least in part, to the failure to allow complete hydrolysis of combined isoflavones. A recent paper (Wong 1963) gives isoflavone levels in the Mount Barker strain similar to those in Table 1, even though full hydrolysis of the combined forms might not be expected with the extraction technique used.

Another important finding is that the ratios of the different isoflavones vary widely between different strains (see Table 2). The widely different environments at West River and Bakers Hill had little effect on these ratios. During 1961 and 1962 a large number of samples from different localities in the agricultural areas of Western Australia were extracted by the earlier method (see Section II), which did not allow of complete hydrolysis of the combined isoflavones. The levels obtained were between one-quarter and one-half of those reported in Table 1, but the ratios of the isoflavones in corresponding strains were similar. The results suggest that, within the Western Australian environment, each strain contains a characteristic ratio of the three isoflavones.

TABLE 1
ISOFLAVONE CONTENT IN LEAVES OF STRAINS OF SUBTERRANEAN CLOVER

Strain	Locality*	Isoflavone (%) on Dry Matter		
		Formononetin	Genistein	Biochanin A
Yarloop	WR	1.7	2.4	0.93
	BH	1.6	3.5	0.48
Dwalganup	WR	1.6	2.1	0.93
	BH	1.6	2.1	0.88
Geraldton	WR	1.0	0.65	0.82
	BH	1.5	0.61	1.3
Bacchus Marsh	WR	0.29	0.86	2.7
	BH	0.20	1.2	3.6
Woogenellup	WR	0.36	3.1	0.69
	BH	0.20	3.3	1.0
Clare	WR	0.22	3.5	<0.1
	BH	0.29	4.3	0.26
Dinninup	WR	2.1	1.5	1.4
	BH	1.6	1.7	2.0
Mount Barker	WR	<0.10	0.96	2.1
Carnamah	BH	1.1	2.2	0.74

* WR, West River, collected 19.v.63. BH, Bakers Hill, collected 2.vii.63.

The relatively uniform "genistein" levels found in different strains by Curnow and Rossiter (1955) were apparently due to the fact that the method used (Curnow 1954) did not differentiate between genistein and biochanin A.

The estimation of isoflavones in petioles will not be possible until satisfactory enzyme preparations are available. Calculation of the percentage amounts by difference between leaf and leaf plus petiole has been made for the strains grown at Bakers Hill, but the results are not very reliable owing to the relatively high errors of estimation. The petioles of some strains appear to contain a significant concentration of isoflavones.

All samples in Table 1 were examined for coumestrol content, but the amounts present were too small to be measured with any accuracy. The maximum amount

noted was of the order of 15 p.p.m. (dry matter basis) and many samples showed no signs of coumestrol.

The correlation of isoflavone levels with biological activity in sheep has not been possible, as earlier determinations were done on partially hydrolysed samples. Davies and Bennett (1962) published data on the estimation of oestrogenic potency of Dwalganup, Yarloop, and Mount Barker strains with ovariectomized ewes as test animals. These observations were extended in the 1962 season to include Clare, Dinninup, Geraldton, Gingin, Bacchus Marsh, and Woogenellup strains (Davies, Boundy, and Beck, unpublished data). The Mount Barker strain (biochanin A >1.2%) and Clare (genistein >1.8%) caused little, if any, increase in uterine weights. All samples showing strong oestrogenic potency contained high levels of both formononetin and genistein. At the same time Millington, Francis, and McKeown (unpublished data) studied some of the same strains using the increase of teat length

TABLE 2
RATIOS OF ISOFLAVONES IN STRAINS OF SUBTERRANEAN CLOVER
Means of values from samples from West River and Bakers Hill

Strain	Ratios		
	Formononetin	Genistein	Biochanin A
Geraldton	2.0	1.0	1.7
Dinninup	1.2	1.0	1.0
Dwalganup	0.8	1.0	0.4
Yarloop	0.6	1.0	0.3
Carnamah	0.5	1.0	0.3
Bacchus Marsh	0.3	1.0	3.1
Mount Barker	<0.1	1.0	2.2
Woogenellup	<0.1	1.0	0.3
Clare	<0.1	1.0	<0.1

of wethers as a measure of oestrogenic potency. They found that both Clare and Mount Barker had some degree of activity, and that overall activity was correlated with formononetin content. While it seems clear that high oestrogenic activity is always associated with high formononetin content, the reverse may not always apply. In the unpublished experiments of Davies, Boundy, and Beck, the Geraldton strain caused very little increase in uterine weight and yet this strain is moderately high in formononetin. In certain areas of Victoria, the clover disease has not been observed in sheep grazing for a number of years on Yarloop-dominant pastures. Partly hydrolysed samples from three such pastures showed formononetin levels similar to Western Australian samples extracted in the same manner. It would seem desirable at this stage not to exclude the possibility that the clover disease in sheep is due, in part, to factors other than formononetin.

In assessing field reports of sheep infertility on different strains of subterranean clover, it is essential to have accurate botanical identification. "Certified seed" may contain up to 5% of other varieties, and if the environment is unfavourable for the

main strain, this may be rapidly replaced by other strains which were originally present only as contaminants (Quinlivan and Rossiter, personal communications). Furthermore, in Western Australia an appreciable area has been sown with uncertified "Mount Barker" seed which was, in fact, the Dinninup strain; these two strains are entirely different in oestrogenic potency (Davies, Boundy, and Beck, unpublished data).

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PAPER 6:18

STUDIES ON THE OESTROGENIC EXTRACTS PREPARED BY
THE ACTION OF ALCOHOLIC ALKALI ON
CHLOROPLAST MATERIAL FROM
SUBTERRANEAN CLOVER

A Summary

Unpublished Data

by

A.B. Beck and C. Kowala

The results published in Paper 6:20 by Beck and Braden had shown that when clover was treated with alcoholic sodium hydroxide, the extracts showed higher oestrogenic potency in mice than extracts made with alcohol alone. Bradbury and White (J.C.S. 1951, 3447) subsequently isolated genistein (5 7 4' trihydroxy isoflavone) from a chloroplast preparation of clover. No other oestrogenic compound was found in the clover chloroplast, by these workers.

The apparent increase of activity due to alkali has been investigated. The results have not been published owing to the fact that many of the compounds isolated have not yet been identified. The investigations did indicate, however, that some highly active compound, not genistein, was present in the saponified material.

The present summary has been included in this Thesis to indicate the way in which the findings of Beck and Braden were followed up. It is hoped that in the near future a further attempt will be made to isolate and identify the oestrogen produced by the alkali treatment.

The bulk extractions, the large scale chromatography, and the mouse bioassays, were done by the candidate and the purification and identification of compounds by Mr. Kowala.

I EXPERIMENTAL

(a) Extraction techniques.-- The chloroplast material as prepared by Bradbury and White (loc. cit.) was used, although it is fairly clear now that this material contains only a small proportion of the isoflavone content of the original clover.

A very curious fact was observed in the extraction with alkali. It was found that the highly active compound was not present in the first extraction by alcoholic sodium hydroxide. In one test, chloroplast was extracted consecutively with 2N alcoholic NaOH, 0.5N NaOH and then ethanol alone. The extracts were assayed by the bioassay method described in Paper 6:20. The mean uterine weights were as follows:

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- 3 -

<u>Fraction from</u>	<u>Dose equivalent to g. of original chloroplast</u>	<u>Mean Uterine Weight</u>
2N NaOH	0.06	3.9
0.5N NaOH	0.06	13.6
Ethanol	0.30	15.5

Control mice were not run with this test, but these regularly gave uterine weights of between 2.5 and 3 mg in other tests.

Not only is the activity of the first extract very much lower, but the oestrogenic response was qualitatively different.

The following dose-response figures were obtained with another batch of first extract:

<u>Dose equivalent to g. chloroplast</u>	<u>Mean Uterine Weight</u>
0.00	2.6
0.03	3.1
0.09	4.6
0.18	5.0
0.27	5.1

The very flat dose-response curve will be noted.

Another batch of first extract was fractionated by column chromatography and no high-activity fraction was found.

No explanation can be given for the absence of high activity in the first fraction. It may be noted that in all cases a large excess of NaOH is present.

In preparation of large scale extracts the chloroplast preparation was boiled with 1N alcoholic NaOH and this alcoholic extract discarded. The solid residue was boiled with 0.5N alcoholic NaOH and then with 0.2N alcoholic NaOH. The second and third extracts were combined, brought to pH 6.5 with alcoholic sulphuric acid and the resultant sodium sulphate filtered off and discarded.

(b) Purification of extract.— The neutral alcoholic solution was evaporated to dryness under vacuum. The dry residue was dissolved in a sodium carbonate-bicarbonate mixture and the phenols extracted by ether as described by Beck and Braden (Paper 6:20). Fatty acids were then removed by esterification with methanol.

(c) Chromatographic fraction of extracts.— The first gross separations were done on silica gel columns (100-200 mesh) using increasing concentrations of ether in petroleum ether. The pattern of elution is as follows:

<u>% Concentration of ether</u>	<u>Approximate % of original matter eluted</u>	<u>Composition and biological activity</u>
50	trace	Inactive oil
50-55	25	Ononetin fraction. Inactive at 1 mg per mouse
55-57	10	Biochanin A fraction (4' methoxy genistein) Almost inactive at 1 mg per mouse
57-60	10-20	Yellowish oil: highly active at 0.2 mg per mouse
+60	35	Genistein fraction: first fractions highly active but activity decreases as elution progresses

There is an appreciable amount of dark material which remains on the column and is not eluted by ether. This can be extracted by acetone and has been shown to have very low oestrogenic potency.

It is interesting to note that biochanin A was not detected in the original investigation by Bradbury and White (loc. cit.).

The fractions listed above have been examined further with the following results:

(i) Ononetin fraction.-- The ononetin in this fraction is formed by the action of alkali on the large amounts of formononetin present in the original chloroplast. A second chromatographic separation with ether-petroleum ether readily separates a small amount of a second white compound of low molecular weight. This compound has no oestrogenic activity.

(ii) Genistein fraction.-- The first fractions from the column are highly active, but repeated crystallization from aqueous alcohol leaves the genistein in a fairly pure form. On evaporation, the mother liquors are distinctly oily, and contain all of the highly active material. These were combined with the "yellowish oil" for fractionation. The "purified" genistein contains some formononetin.

(iii) Yellowish oil (active).-- By use of gradient elution chromatography with acetone in methylene chloride on a silica gel column, it was possible to isolate in almost pure form a compound which crystallizes in bright yellow crystals. This compound has not yet been identified and shows no oestrogenic activity. The remaining phenols are oily and show a high degree of oestrogenic activity at doses of 40 ug per mouse. Attempts at purification by paper chromatography produced more active fractions, but no pure

crystalline products were obtained. The most active showed a mean mouse uterine weight of 15.8 mg at a dose of 10 ug per mouse. Control uteri were 2-3 mg and genistein at a dose of 1 mg per mouse gave a mean uterine weight of 7.7 mg. The active oestrogen is thus more than 200 times as active as genistein. This activity is, however, still only about one three hundredth of that of oestradiol.

II DISCUSSION AND CONCLUSIONS

The treatment of chloroplast material from subterranean clover with ethanolic sodium hydroxide gives rise to some compound of relatively high oestrogenic potency. This compound is not genistein and was not obtained when genistein was treated with alcoholic sodium hydroxide.

The active compound has not been found in the original chloroplast material and is almost certainly an artefact formed by the alkali.

The increase of activity of whole clover after alkali treatment is presumably due to the formation of the same compound, and whole clover may prove to be a more convenient raw material in future investigations of the oestrogen.



WESTERN AUSTRALIA

DEPARTMENT OF AGRICULTURE
1952

**THE COMPOSITION OF THE
DWALGANUP STRAIN OF
SUBTERRANEAN CLOVER**

By A. B. BECK

(From Animal Health and Nutrition Laboratory, Department of Agriculture and the
Government Chemical Laboratories, Western Australia)

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1952

The Composition of the Dwalganup Strain of Subterranean Clover

By A. B. BECK*

(From Animal Health and Nutrition Laboratory, Department of Agriculture and the Government Chemical Laboratories, Western Australia)

SUBTERRANEAN clover is now the major pasture species in Western Australia and according to Elliott (1948) nearly 90 per cent. of the sown pastures of this State have a subterranean-clover base or were originally sown with this species. In spite of its great economic importance, no data have been published showing the chemical composition of the clover growing in this State. In order to fill this gap in our knowledge, series of samples of the Dwalganup strain were collected from three localities. These have been analysed by the Government Chemical Laboratories and the results are presented in this paper.

The samples collected were of leaf and petiole only and the analyses have been done by standard methods. The results are presented in Table 1. The figures for "crude protein" are calculated on the basis of 6.25 times the nitrogen content and the "crude fat" is a petroleum ether extract.

DISCUSSION

The results set out in Table 1 show trends similar to those found in all pasture species. The percentage protein content which is high in the young plants, falls slowly until the beginning of seeding when the level drops rapidly. Part of this apparent loss of protein is due to dilution by carbohydrate material synthesised by the plant but probably the major loss is due to a transfer of protein to the seeds which are not included in the samples analysed. No samples of seed were collected from the experimental areas, but a sample of cleaned commercial "Dwalganup" seed has been analysed. The results, together with some figures recorded by Shapter (1935) and Franklin and Powning (1942) are given in Table 2.

The crude fibre changes are also similar to those of other pasture species. There is generally a slow rise during

growth with a sharp increase as the plant begins to dry off. The Kojonup samples (Series 3) are rather unusual in that there was no apparent change in crude fibre content between June and September.

The samples of dried leaf and petiole all show a rather high content of crude fibre but the protein content is sufficiently high to make this material a valuable feeding-stuff. The additional value of the seed is to be taken into account when considering these figures.

The total ash figures do not show any obvious trends but in all cases the phosphorus levels decline steadily during growth and the calcium content increases, at least up to the wilting stage. Beck (1938) has noted similar increases in the calcium content of capeweed throughout the growing period. Figures for the zinc, copper and manganese content of subterranean clover are given by Teakle and Turton (1943) but it is probable that many of the samples were of the "Midseason" (Mt. Barker) strain. A few figures for the copper content of "Dwalganup" samples are given by Beck (1951).

In considering the analytical figures as indicators of the feeding value of subterranean clover, it should be remembered that the Dwalganup strain

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has certain properties which make it different from most other pasture species. Together with other clover strains it is relatively unpalatable in the early stages of growth and throughout the growing period contains appreciable amounts of an oestrogenic substance. Because of its unpalatability there is a tendency for it to become the dominant species of the pasture. If other species are present in small amounts only and sheep are thus forced to consume a high proportion of clover, serious breeding abnormalities will occur as described by Bennetts, Underwood and Shier (1946). It is probable that sheep grazing on a Dwalganup clover pasture containing a good proportion of grass will only begin to eat appreciable amounts of the clover from flowering time onward. For this reason the later analytical figures are the ones which have most significance from the viewpoint of animal nutrition. The clover is oestrogenic from flowering up to the wilting, at which stage the activity becomes extremely low (Braden 1950). It is unlikely however that sheep grazing on a well-balanced clover pasture will show any untoward effects in this period, as the presence of adequate grass in the pasture will "dilute" the clover and the period of oestrogenic stimulation is probably too short to cause any trouble. Cattle do not seem to show any ill effects from grazing green clover.

The importance of subterranean clover lies not only in its feeding value but also in the increase of the nitrogen status of soils when the pasture is grazed by animals. This makes it possible to establish in the second phase of pasture development good stands of grasses or cereals which are palatable and non-oestrogenic throughout the growing period.

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Table 1.

COMPOSITION OF SUBTERRANEAN CLOVER, DWALGANUP STRAIN.

(All figures given as percentage on a moisture-free basis.)

SERIES 1.—INSTITUTE OF AGRICULTURE, NEDLANDS, 1947.

	May 29.	June 27.	July 29.	Aug. 28	Sept. 29.	Oct. 28.	Nov. 13.	Nov. 29.	Dec. 28.
Crude protein	26.1	26.7	23.1	19.2	14.3	13.9	9.7	11.3	11.5
Crude fibre	11.1	12.9	15.1	16.8	19.1	18.2	24.9	30.6	37.5
Crude fat	3.4	2.7	2.6	5.2	2.3	3.2	2.8	1.7	1.6
N-free extractives	49.9	46.4	50.3	50.7	54.6	54.5	52.8	48.8	43.5
Total ash	9.5	11.3	8.9	8.1	9.7	10.2	9.8	7.6	5.9
Calcium (Ca)	1.45	1.56	1.19	1.45	2.04	2.69	3.50	2.66	1.86
Phosphorus (P)	0.27	0.30	0.27	0.21	0.15	0.13	0.10	0.09	0.09

Germination began on April 20th. Flowering on July 25th. Sample of November 13th mostly dry: Completely dry on November 29th. Soil type:—grey sand.

Table 1—continued.

COMPOSITION OF SUBTERRANEAN CLOVER, DWALGANUP STRAIN—continued.

SERIES 2: BEVERLEY, 1948.

	Aug. 10.	Aug. 25.	Sept. 28.	Oct. 21.	Dec. 12.
Crude protein	28.9	27.4	22.9	13.2	10.0
Crude fibre	12.4	14.3	17.8	21.4	34.4
Crude fat	2.7	2.6	1.6	1.9	1.3
N-free extractives	47.2	47.4	48.5	52.5	48.3
Total ash	8.8	8.3	9.2	11.0	6.0
Calcium (Ca)	0.96	0.88	1.11	1.7	1.46
Phosphorus (P)	0.25	0.22	0.15	0.08	0.04

Germination rain on June 5th, but growth very slow on account of cold weather.

No flowering August 25th, full flower September 28th, wilting October 21st, completely dry December 12th.

Soil type—brown sandy loam.

SERIES 3—"GLEN LOSSIE" C.S.I.R.O. FIELD STATION, KOJONUP, 1950.

	June 23.	July 20.	Aug. 18.	Sept. 1.	Dec. 1.
Crude protein	27.8	26.7	23.1	24.1	15.1
Crude fibre	12.0	11.8	12.5	11.8	29.4
Crude fat	2.3	3.4	2.5	2.1	1.6
Total ash	13.1	10.6	9.6	9.1	11.1
N-free extractives	44.8	47.5	52.3	52.9	42.8
Calcium (Ca)	1.22	1.25	1.54	1.43	1.59
Phosphorus (P)	0.32	0.29	0.25	0.22	0.16

Germination rain on May 8th: flowering commenced Aug. 12th: Wilting at end of October.

Soil type.—gravelly sand.

Table 2.

COMPOSITION OF THE SEED OF SUBTERRANEAN CLOVER.

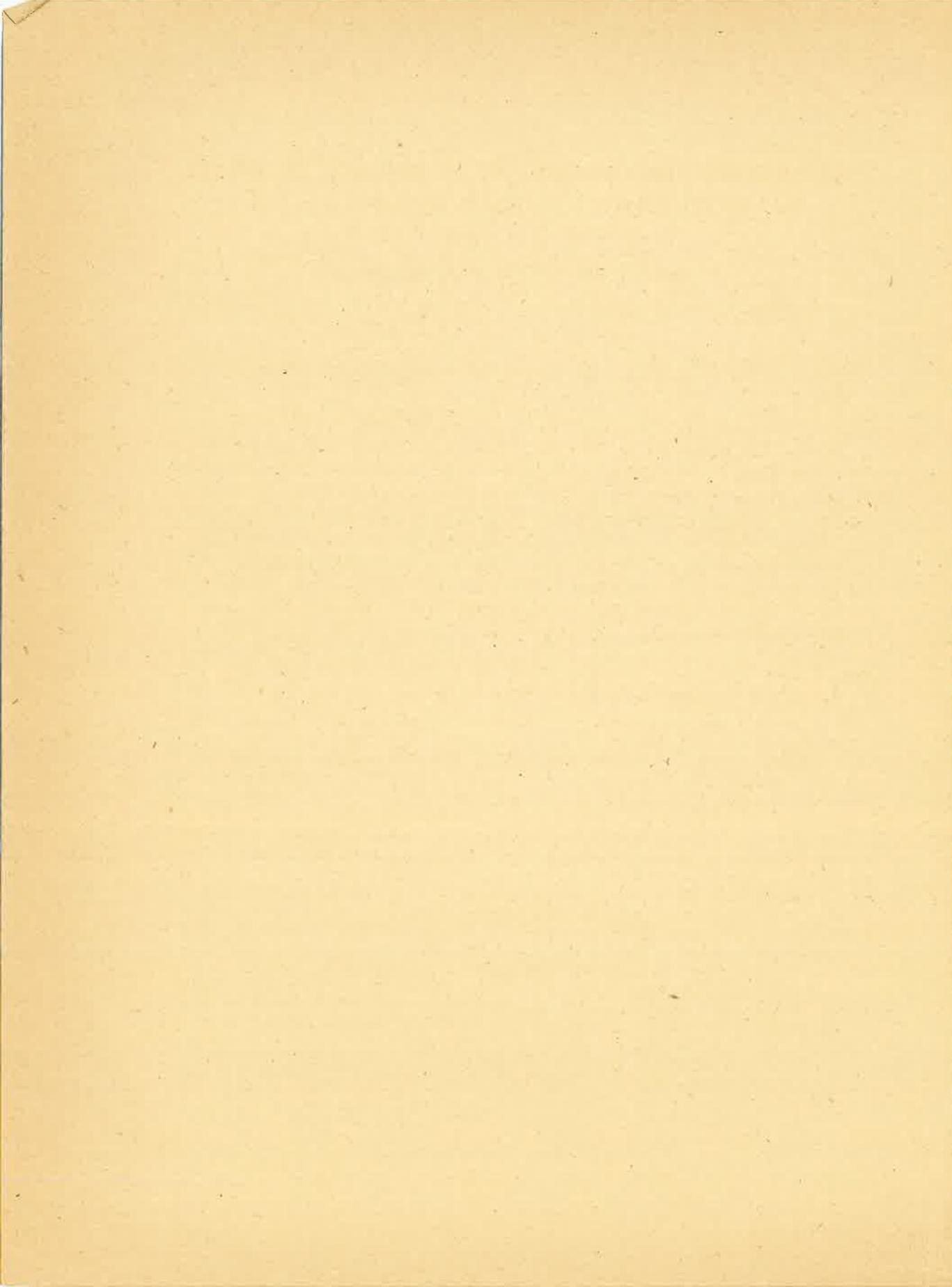
(All figures given as percentage on a moisture-free basis.)

Sample.	Crude Protein.	Crude Fat.	Crude Fibre.	N-free extract.	Total Ash.	Ca.	P.
W.A. Commercial (Dwalganup Strain)	28.1	17.3	8.3	42.2	4.1	0.16	0.55
Franklin and Powning (1942) (Dwalganup Strain)	41.6	0.17	0.77
Franklin and Powning (1942) (Dwalganup Strain)	38.7	0.18	0.73
Shapter (1935) (Strain not given)	39.6	15.2	9.0	31.6	4.6	0.72

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STUDIES ON THE OESTROGENIC SUBSTANCE IN SUBTER-
RANEAN CLOVER: (*TRIFOLIUM SUBTERRANEUM*
L. VAR. DWALGANUP)

by A. B. BECK AND A. W. BRADEN



STUDIES ON THE OESTROGENIC SUBSTANCE IN SUBTERRANEAN CLOVER: (*TRIFOLIUM SUBTERRANEUM* L. VAR. DWALGANUP)

by A. B. BECK¹ AND A. W. BRADEN²

(From Department of Agriculture, Animal Health and Nutrition Laboratories, Nedlands, Western Australia).

(Accepted for publication 7th May, 1951.)

Earlier investigations (Bennetts, Underwood and Shier, 1946; and Bennetts, 1946, 1947) on a breeding problem of sheep had shown that the disease was probably due to the presence of some oestrogenic substance in the subterranean clover (*T. subterraneum* L. var. Dwalganup) which was the main species in the pastures grazed. Subsequent work by Curnow, Robinson and Underwood (1948) and Robinson (1949) had demonstrated the presence of considerable oestrogenic activity in the extracts of this clover. The present paper gives the results of experiments designed to obtain further information on the chemical nature of the oestrogen, the preparation of large scale concentrates and the method of assay.

METHODS.

Preparation of concentrates and extracts.

Robinson (1949) used alcohol for the extraction of clover but this method was not found satisfactory for large scale work.

Recent observations by English workers (Legg, Curnow and Simpson, 1950; and Curnow, 1950) had shown that the oestrogenic activity was associated with the "chloroplast" fraction of the press juice of clover. The following procedure, based on these observations, was used on twenty-five hundredweight of clover harvested at flowering in 1949. The freshly cut clover was crushed in a hammer mill and the juice separated by mechanical pressure. The residue was discarded. The juice showed no apparent loss of activity after several weeks when stored at 4° C. and preserved with 0.25 p.c. chloroform. The chloroplast fraction was first separated by centrifuging at 30,000 r.p.m. in a Sharples super-centrifuge. The solid material was dried in a stream of air at 55° C. The juice, from which the bulk of the chloroplasts had been removed, was then heated to 70° C., the precipitate filtered and dried in a similar manner. The two products had similar levels of activity and kept well in the cold.

As some larger scale extractions (100 gm. lots) had given poor recoveries, the finely ground, dried products were extracted in small lots (less than 20 gm.) with normal alcoholic sodium hydroxide. Three extractions were given with 150-200 ml. portions of NaOH and the mixture refluxed for 10 minutes each time. After neutralization and evaporation, the

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oestrogen was separated by ether extraction from a sodium carbonate-bicarbonate solution as described under the method for assay. The oestrogenic material was separated from neutral substances by extraction from ethereal solution with 0.1 N sodium hydroxide. This product, which represents about 3 p.c. of the original chloroplasts, is referred to as the "crude phenols."

The "crude phenols" contain a fairly large proportion of fatty acids and towards the end of the investigation it was found that these acids could be separated from the oestrogen by esterification with methyl alcohol and sulphuric acid.

The "crude phenols" were refluxed for 30 minutes with anhydrous methyl alcohol containing 1 p.c. (w/v) sulphuric acid at the rate of 100 ml. of methyl alcohol per gram of extract. After cooling, the mixture was neutralized with alcoholic sodium hydroxide (pH 6.7, brom-thymol blue) and evaporated to dryness under vacuum. Water was added to dissolve the sodium sulphate, the solution acidified, and the mixture extracted with ether. The ether extract was evaporated to a convenient volume and the phenols extracted three times with equal volumes of 0.1 N NaOH. The neutral substances contained no oestrogenic activity and were discarded. The alkaline extract was acidified and extracted with ether which was washed with freshly prepared, saturated NaHCO_3 , then with water until neutral and evaporated to dryness. This extract, which represents 25-40 p.c. of the "crude phenols", was a clear brown viscous oil and is referred to as the "purified phenols" fraction.

Method for assay of clover and clover extracts.

Attempts have been made to improve further Robinson's (1949) method for the preparation of extracts for assay of clover samples. The following is the best method obtained. Green clover (50-100 gm. fresh weight) is collected directly into absolute alcohol (400 ml.) and stored at 4° C. until extracted. The clover is boiled with fresh alcohol for one hour. The two extracts are bulked, concentrated under vacuum to about 250 ml. and boiled for 10 minutes with one fifth volume of 3 N alcoholic NaOH. Following alcoholic extraction, the clover leaves are disintegrated in a high speed macerator, boiled for 10 minutes with 0.5 N alcoholic NaOH, filtered and boiled again with alcohol. The combined extracts are neutralized with 20 p.c. alcoholic H_2SO_4 to pH 6.7 (brom-thymol blue) and allowed to stand overnight at 4° C. After filtration, the solution is evaporated to dryness under vacuum. Purified "white spirit" (Beck, 1950) is added as anti-frothing agent if necessary. The dried residue is allowed to stand with 50 ml. freshly prepared, saturated NaHCO_3 and 50 ml. ether, any remaining solid residue dissolved in a minimum amount (10-20 ml.) of 4.5 p.c. Na_2CO_3 and a further 30 ml. of NaHCO_3 added. The oestrogen is extracted with 6 additional lots of 100 ml. ether. If any solid material separates out during the extraction it is redissolved as above. The ether extract is concentrated and the active fraction removed by three extractions with equal volumes of 0.1 N NaOH. The alkaline solution is acidified and the oestrogen re-extracted with ether. After washing with water the ether phase is evaporated to dryness.

In order to determine the loss of oestrogenic activity during the extraction and purification processes, two lots of a non-oestrogenic species (*Medicago sativa*) were extracted in the usual way. To one a measured amount of standardized chloroplast extract was added before extraction and to the other the same amount was added after the extraction and purification were complete. The activities of the two extracts were then compared and it was found that the loss of activity was 27 p.c. (Limits 14 and 39 p.c., $P = 0.05$).

The method of assay of the clover or chloroplast extracts was a modification of that described by Evans, Varney and Koch (1941). Entire immature female mice (7.5-10.5 gm.) were randomized into groups so that littermates were distributed evenly. Groups of six were generally used but greater accuracy was obtained when this was increased to ten. The log. dose-response line of the clover extracts was found to be significantly different from those of

oestrone and oestradiol so that it was necessary to prepare a large amount of "crude phenols" extract for use as a reference standard. This was done by extracting small batches (10-20 gm. dry weight) of the chloroplast material by the method detailed above. This standard is best kept in the dry state under nitrogen, as alcoholic solutions show a slow loss of potency.

Generally extracts to be compared were assayed at two or three dose levels of both standard and unknown. It was necessary to have doses differing by a factor of 2 or 3 as the slope of the log-dose response line is rather low. The mice were injected subcutaneously twice daily for three days with 0.05 ml. of a peanut oil solution of the extract under test. They were killed 18 hours after the last injection and the uteri dissected out and fixed in Bouin's fluid for 24 hours. The uteri were pressed between filter papers and weighed.

The mean uterine weight of the untreated controls was 2.5-3.5 mg. and the maximum obtainable by clover extracts a little over 20 mg., but the dose-response line is only linear in range 6-15 mg., and hence it is necessary to adjust the doses so that the responses will fall within this range. Another feature of the responses obtained with clover extracts is that, if the doses are increased above a certain level, the responses progressively decline though not to the control level. This phenomenon has also been noted by Costello and Lynn (1950) in assays of oestrogenic licorice root extracts by the Allen and Doisy (1923) vaginal smear technique. Thus it seems that this phenomenon is related to the crude nature of plant extracts rather than to the assay method.

The results obtained by the above assay method were analysed by the statistical methods described by Emmens (1948). The fiducial limits of error found were often rather wide, and this must be kept in mind in interpreting the results. These limits, however, could be narrowed by the use of larger numbers of animals per test.

RESULTS.

Although the extracts are relatively crude, it is possible to obtain a considerable amount of information on the nature of the oestrogen by phase distribution studies and by following the chemical distribution of biological activity after treatment with a number of comparatively mild chemical reagents.

TABLE 1.

Partition coefficients of clover oestrogen.

Phases	Percentage in aqueous phase		
	Clover oestrogen	Oestrone; oestradiol	Oestriol
Ether:water	4	(0)	4
Ether:NaHCO ₃ (Sat.)	0	0	0
Ether:Na ₂ CO ₃ (9 p.c.)	78 (64-88) 88* (85-90)	2 —	20 —
Ether:0.1 N NaOH	96	33	97
Ether:N NaOH	98.	62	100
Benzene:Water	6.5 (5-8) 8.7* (7-11)	0 —	78 —

* These separations done on "purified phenols", all others on "crude phenols".

Partition coefficients of the clover oestrogens.

The results obtained from phase separations done on "crude phenols" and in two cases on the "purified phenols" are set out in Table 1. Where the results were suitable for analysis, the statistical limits of error ($P = 0.05$) have been calculated from the figures and are given in brackets below the respective means. Corresponding partition coefficients for oestrone, oestradiol and oestriol (Bachman and Pettit, 1941) are given for comparison.

Chemical studies on the clover oestrogen.

The activity is not precipitated with digitonin and accordingly the oestrogen is not a 3β hydroxy steroid. Condensation with Girard's reagent T leaves the activity in the non-ketonic fraction. The fact that concentrated hydrochloric acid does not dissolve the activity from the "crude phenols" suggests that the γ pyrone ring is absent.

Treatment of the "crude phenols" with acetic anhydride and pyridine results in the conversion of at least 80 p.c. of the activity into a form no longer extracted from ether by 0.1 N sodium hydroxide. This indicates that the acidic groups of the oestrogen are phenolic. Continued shaking of the ethereal solution with 0.1 N NaOH extracts a considerable amount of activity, indicating a fairly rapid hydrolysis. The acetylated product has an activity as great as, or slightly greater than the original but further investigations were not carried out because of its ease of hydrolysis and because of the large amount of ether-insoluble and oil-insoluble material present.

Benzoylation by the standard Schotten-Baumann reaction gives an alkali-insoluble product showing a low oestrogenic activity but the product is largely insoluble in ether and oil.

The "crude phenols" react slowly with diazomethane to give a product of lower oestrogenic activity in which the active fraction is much less alkali-soluble than in the original material. Methylation of this material in acetone solution with methyl sulphate and sodium hydroxide gives a three to four fold rise in oestrogenic activity (one batch gave a ratio of 3.6 with limits of 2.4 and 5.3, $P = 0.05$) and the activity is now in the alkali-insoluble fraction. A product of identical properties and biological activity is obtained by direct methylation of the "crude phenols" with methyl sulphate.

The "purified phenols" react similarly with diazomethane but further methylation with methyl sulphate gives a smaller rise in activity. Direct methylation of the "purified phenols" with methyl sulphate gives a product of low activity. It is suggested that the acids present in the "crude phenols" exerted a protective action against the strong alkali used in methylation and that some destruction of the oestrogen has occurred with the purer material.

The changes in alkali solubility after methylation may be explained on the hypothesis that the oestrogen is a dihydric phenol. Diazomethane would appear to methylate completely one phenolic group with incomplete methylation of the other group while methyl sulphate reacts with both groups to give the dimethyl ether.

An accurate comparison of the oestrogenic potency of the original and methylated products is not possible as the slopes of the log. dose-response lines are different. Fig. 1 shows the dose-response curves of a batch of "purified phenols" and the product obtained by methylation of the same material first with diazomethane and then with methyl sulphate. Curves for oestradiol and for another batch of "crude phenols" are given for comparison. These last two lines were obtained at different times from the first two and so may not be strictly comparable. The curves of the "purified phenols" and to a lesser extent of the methylated product have flattened out at somewhat lower levels than usually occur with "crude phenols". It will be noted that with the particular assay method, the methoxy compound appears to have a lower relative activity than the parent phenol at low levels of injection but this is reversed at higher levels.

Data quoted by Solmssen (1945) suggest that the conversion of the partly methylated product to the fully methylated compound should be accompanied by a decrease of oestrogenic activity. The observed rise may be due to the action of the methyl sulphate or sodium hydroxide, or both on some group other than the phenolic hydroxyl groups. On the other hand the increased activity of the fully methylated product may be due to a decreased absorption rate or to a greater resistance to inactivation in the body of the assay animal.

As diazomethane normally methylates only carboxylic acids and phenols, an attempt was made to ascertain whether any free alcoholic groupings were present in the methoxy compound formed by diazomethane. The standard condensation with succinic anhydride and pyridine (Pincus and Pearlman, 1941) was carried out but no activity was found in the succinic half-ester (alcohol) fraction.

A study of the methoxy derivatives suggests that a lactone group is not present in the original oestrogen. The methoxy compound formed by diazomethane is refluxed for 10 minutes with 2.5 N alcoholic NaOH and then poured into boiling 2.5 N aqueous NaOH. This treatment would normally open a lactone ring and give a sodium salt insoluble in benzene. However, the bulk of the oestrogenic potency can be extracted from the alkaline solution with benzene. If the benzene-extracted solution is acidified and extracted with ether, the small amount of oestrogen obtained behaves like a phenol i.e. it is extracted from ether by 0.1 N NaOH. If it were a lactone the acidification would be expected to close the ring and the product would not be extracted by NaOH. Furthermore, in the methylation by methyl sulphate, the strong alkali used (40 p.c.) would normally open a lactone ring and the methylated product would give a free carboxylic acid on hydrolysis. Tests have shown that after hydrolysis the oestrogenic fraction is completely insoluble in alkali. This fact also gives conclusive evidence that a carboxylic acid group is absent.

The methyl ether formed by methyl sulphate is not volatile in steam.

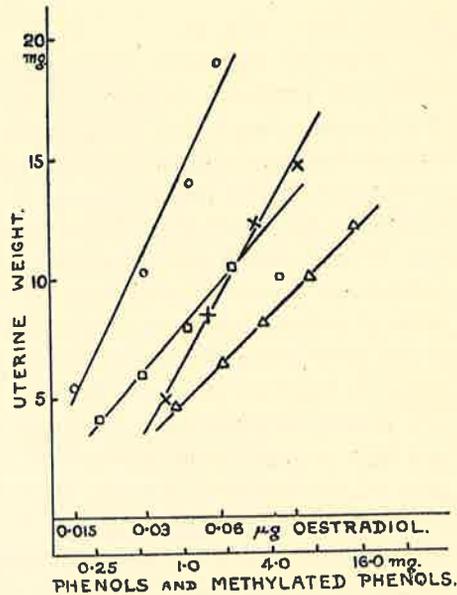


Fig. 1. Dose-response lines for oestradiol, and for oestrogenic preparations from subterranean clover.

—○—○— Oestradiol:

$$E = 21.17X + 106.9.$$

—△—△— "Crude phenols":

$$E = 6.182X + 4.74.$$

—□—□— "Purified phenols":

$$E = 7.315X + 7.91 \text{ (omitting 4.45 mg. dose).}$$

—×—×— "Purified phenols" after complete methylation:

$$E = 10.98X + 6.41.$$

Where E = observed uterine weight in mg.

$$X = \log_{10} \text{ of total dose in mg. per mouse.}$$

DISCUSSION.

Work by Robinson (1949) on whole clover showed that alcoholic sodium hydroxide produced extracts of higher oestrogenic potency than alcohol alone. Furthermore, saponified extracts are more soluble in the oil used for injection into the test animals, and because of these facts, we have used saponified material throughout in this investigation. It is pertinent to consider here whether such treatment may have altered the oestrogen molecule.

We have confirmed Robinson's observation on the lower activity of alcoholic extracts as compared with those of alcoholic sodium hydroxide; in one test the ratio of activity was 0.46 (limits of 0.28 to 0.76, $P = 0.05$) but this difference is not great when the limitations of the assay method are taken into consideration. It has been shown by Emmens (1939) that palmitic and other fatty acids can greatly increase the oestrogenic activity of oestriol and it is probable that the high proportion of fatty acids in saponified extracts may explain at least part of the increased activity of such extracts. It is also possible that in the clover plant the oestrogen is linked to other compounds which are split off during hydrolysis, and this also could account for the change in activity.

It would seem that the "crude phenols" are relatively stable toward boiling 0.5 N alcoholic sodium hydroxide, as the oestrogenic potency does not alter significantly if the time of hydrolysis is increased from 10 minutes to 30 minutes.

The relatively small change in activity which occurs during hydrolysis suggests that such treatment does not produce any radical change in the oestrogen molecule.

Although this investigation has not given sufficient evidence to suggest the identity of the clover oestrogen, it would appear that the compound is not one of the common steroid oestrogens. The partition coefficients show definitely that it is not oestrone or oestradiol. Although there is a slight resemblance to oestriol, the clover oestrogen appears to be more acidic and less hydrophylic than this compound. The evidence from the methylation studies suggests that at least two phenolic groups are present. The partition coefficient between benzene and water shows that the number of hydroxyl groups per molecule must be limited and if a comparison with oestriol is valid, the number will be less than three. It is accordingly suggested that the molecule contains two phenolic groups. A fairly large range of oestrogenic, dihydric phenols is recorded in the literature, but unfortunately, no partition coefficients seem to be available for comparison with the clover oestrogen.

Before further progress in identification can be made, it will be necessary to effect very considerable purification of the so-called "purified phenols".

SUMMARY.

Details are given for the large scale preparation of oestrogenic extracts from subterranean clover (*T. subterraneum* L. var. Dwalganup). The process involves the preparation of press juice, the separation of chloroplasts and of the heat-precipitable fraction of the juice. The dried chloroplast and heat-precipitated materials are extracted with alcoholic NaOH, and the oestrogenic material extracted with ether from an aqueous Na_2CO_3 - NaHCO_3 solution of the extract.

Phase distribution studies, methylation and acetylation tests on the purified extract have indicated that the oestrogen probably contains two phenolic groups. Tests have failed to show the presence of ketone, lactone, carboxylic, or alcoholic groupings.

Some details of the assay method are given.

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STUDIES ON THE EXCRETION OF OESTROGENS BY
PREGNANT EWES

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Summary

Adaptations of standard methods are described for the preparation of oestrogenic extracts from the urine and faeces of sheep. The extracts were separated into strong phenols, non-ketonic weak phenols, and ketonic weak phenols. The concentration of oestrogen in these fractions was determined by bioassay. Special consideration was given to the accuracy of the methods in light of the findings of Friedgood, Garst, and Haagen-Smit (1948).

Four ewes were examined for urinary excretion only and seven ewes for both faecal and urinary excretion throughout pregnancy. Little or no excretion occurred until the last three or four weeks of pregnancy and even then the levels were consistently low. The faecal levels were higher than those of the urine. With one or two possible exceptions no strongly phenolic oestrogen was detected. Generally the ketonic oestrogen (calculated as oestrone) of both urine and faeces was higher than the non-ketonic oestrogen (as oestradiol). The daily levels observed during the last few weeks of pregnancy lay within the following ranges: urinary "oestradiol" $< 0.2-3 \mu\text{g.}$, "oestrone" $< 1.5-20 \mu\text{g.}$; faecal "oestradiol" $1-20 \mu\text{g.}$, "oestrone" $1-100 \mu\text{g.}$ The faecal oestrogen was not conjugated.

Exploratory tests were made on ovaries, bile, and placentae. In all cases the oestrogen levels were low and both ketonic and non-ketonic activity was noted. In the bile most of the activity was found in the conjugated form.

No pregnanediol was found in the urine samples.

I. INTRODUCTION

The studies reported in this paper were undertaken because of the almost complete absence of information on oestrogen excretion by normal pregnant ewes. Figures are given for the excretion of oestrogens in the urine and faeces of ewes throughout the gestation period. Attempts have been made in exploratory tests to throw further light on the findings by a study of the oestrogen content of bile, placentae, and ovaries.

II. EXPERIMENTAL

(a) *Experimental Animals and Collection Methods*

The Merino ewes used in the 1945 experiments were mated at the Merredin Research Station and brought to the Institute of Agriculture, Nedlands, near Perth, where the collections of urine were made.

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In the 1946 and 1947 experiments, normal Merino ewes at the Avondale Research Station, Beverley, were mated with raddled rams to obtain the service date. Except for the collection periods, these ewes were run with the station flock. Different sheep were used each year.

Urine and faeces were collected in metabolism crates similar to those described by Marston (1935). Twenty-four-hour collection periods were used with ewes after lambing, otherwise forty-eight-hour periods were used. The urines were preserved with toluene and were hydrolysed and extracted within 48 hours of collection. The faeces were roughly crushed and preserved in excess ethanol until extracted.

The samples of ovaries, bile, and placentae were from Merino ewes unless otherwise specified.

(b) *Chemical Methods*

The preparation of the crude extract from urine was done by the standard method, HCl hydrolysis followed by ether extraction. With faeces, an alcoholic extraction was used (Levin 1945).

Owing to the tendency of extremely persistent emulsions to form, it was necessary to purify further the crude faecal extract. This was done by a partition between petroleum ether and aqueous alcohol (Allen and Meyer 1933) and a further extraction of the phenols from ether by NaOH.

The strong and weak phenols were extracted from benzene solutions of the crude urinary extracts and of purified faecal extracts by the procedure of Bachman and Pettit (1941).

Ketones were separated from the weak phenols by the procedure of Talbot *et al.* (1940).

After the separations had been completed, Friedgood, Garst, and Haagen-Smit (1948) published a paper in which they severely criticized some of the Bachman and Pettit separations. The effect of these criticisms was subsequently investigated and will be discussed later.

The following technique was used for all samples of urine and faeces. In conformity with the practice adopted by recent workers, the symbol *D* will be used for non-ketonic weak phenols (oestradiol-like), *O* for ketonic weak phenols (oestrone-like), and *T* for strong phenols (oestriol-like).

1. *Urine*

(i) *Hydrolysis and Extraction.*—The urine samples were hydrolysed by boiling for exactly 10 minutes with 25 vol. per cent. constant boiling HCl. This acid was used as most of the commercially "pure" HCl contained oxidizing material (KI test) which was removed in the first runnings during distillation. After cooling, the urine was extracted with four lots of ether (peroxide-free) of one-fifth of the volume of the urine plus the acid. Emulsions were readily broken up by treating the emulsified ether with solid NaCl.

The ether extract was washed once or twice with a small quantity of freshly prepared saturated NaHCO_3 , then twice with a small quantity of water. After the suspended water had settled out, the ether was evaporated on a water bath and the last traces of ether and water were removed under vacuum. The dried residue was dissolved in 0.5 ml. alcohol and 30-35 ml. benzene were added.

(ii) *Separation of the T Fraction (Strong Phenols).*—The benzene was extracted with one equal volume and then two half volumes of 0.9 per cent. Na_2CO_3 (see Section II(d)) and finally washed with one half volume of water. The benzene phase was kept for subsequent extraction of weak phenols. The combined aqueous extracts were acidified ($\text{pH} < 6$) and extracted three times with half volumes of ether. The ether was washed with several ml. of saturated NaHCO_3 and then water. After evaporation of the ether and drying under vacuum, the residue was dissolved in 0.5 ml. alcohol and 50 ml. benzene added. This extract is referred to as "Crude T". After washing with one ml. saturated NaHCO_3 the oestriol-like substances were extracted from the benzene by shaking with three equal volumes of water. The benzene was discarded. The aqueous phase was acidified, nearly saturated with NaCl , and extracted with four lots of 40 ml. ether. After evaporation of the ether the residue was dried under vacuum, dissolved in absolute alcohol, and transferred quantitatively to a small bottle calibrated at four ml.

(iii) *Separation of Combined O plus D Fraction (Weak Phenols).*—The benzene from which the strong phenols had been removed was extracted with four equal volumes of N NaOH . (It was originally intended to investigate the neutral steroid fraction of the urines, hence the sulphuric acid washing of Bachman and Pettit was omitted; it is probable, however, that such a treatment would have given cleaner extracts.) The NaOH was neutralized with HCl to $\text{pH} < 6$ and the phenols extracted with four lots of 50 ml. ether. The ether was washed with saturated NaHCO_3 and with water. After evaporation, the extract was transferred with ether to a small Quickfit boiling tube calibrated at four ml. The ether was blown off in a stream of air and the residue dried under vacuum.

(iv) *Separation of O Fraction (Ketones) from D Fraction (Non-ketones).*—This separation with Girard's Reagent T was done according to the method of Talbot *et al.* (1940). Electric heaters were used instead of water baths, which were unsatisfactory. The *O* and *D* fractions were dissolved in absolute alcohol and transferred to small bottles calibrated at four ml.

2. Faeces

The partly crushed faeces were allowed to stand with alcohol for several days and then filtered and washed with 80 per cent. (v/v) alcohol. Absolute alcohol should not be used for washing or subsequent extraction as a considerable amount of resinous material is dissolved. The residue

was re-extracted by boiling with 80 per cent. alcohol containing 1 vol. per cent. of 20 per cent. HCl. The mixture was allowed to stand overnight in a cool place. After filtering, the residue was washed with 80 per cent. alcohol and then discarded.

The combined alcoholic extracts were then evaporated on a water bath under vacuum until all the alcohol was removed and the sludge reduced to a convenient volume. Bad frothing usually occurred when the bulk of the alcohol had distilled off, but this could be prevented by the addition of purified "white spirit". This anti-frothing agent was prepared by fractionally distilling commercial "white spirit low in aromatics". The fraction boiling at 170-175°C. was treated with concentrated H₂SO₄ to remove the bulk of the aromatic hydrocarbons, washed, and distilled under vacuum (water pump).

It is essential that all alcohol be removed, otherwise stable emulsions are formed in the subsequent extraction; the disappearance of "rings" from inside the condenser indicates that all alcohol has been removed. The aqueous sludge was transferred to a separating funnel and nearly saturated with NaCl; any lumps were crushed with ether. The sludge was then extracted very cautiously with ether. Two extractions with one quarter volume ether were given, the sludge was then made acid (pH < 6), and two further extractions made. In cases where the sheep were on green pasture the number of extractions was usually increased. The ether extracts were combined and washed with NaHCO₃ to remove free acids. If the volume of NaHCO₃ was large, it was back-extracted with ether which was added to the main extracts. The ether was then washed with water until the washings were neutral (brom-thymol blue), stood overnight to allow suspended water to settle, evaporated to dryness, and dried under vacuum.

As the work of Levin (1945) had shown that the oestrogen of cow faeces is not conjugated, it was decided to ascertain if further oestrogen could be liberated by hydrolysis. This was done by evaporating the ether from the extracted sludge, adding 25 vol. per cent. of 20 per cent. HCl, and heating on a boiling water bath for 15 minutes. After cooling, the solution was extracted with three or four lots of one quarter volume of ether and the ether washed with NaHCO₃ and water as above. Any lumps were crushed with ether. The "hydrolysed" and "non-hydrolysed" extracts were treated separately but purification procedure for both was identical.

The dried residue was warmed with 30 ml. absolute alcohol until dissolved, 30 ml. of petroleum ether (b.p. 40-60°C.) and then 30 ml. water added. After cautious shaking the mixture was allowed to stand several hours, preferably overnight. The petroleum ether phase was re-extracted with three times its volume of 50 per cent. (v/v) alcohol and then discarded. The combined aqueous alcohol extracts were evaporated to dryness under vacuum and then gently agitated with a mixture of 30 ml. ether

and 30 ml. N NaOH. The two phases were allowed to settle out and the ether extracted with four additional lots of NaOH, after which the ether phase was discarded. The aqueous extracts were then acidified and the phenols re-extracted with ether. After evaporation of the ether the residue was dissolved in 0.5 ml. alcohol and 30-35 ml. benzene. The separation of the *T*, *O*, and *D* fractions was then carried out as for the urines, except that 9 per cent. Na_2CO_3 was used to extract the *T* fraction as the stronger solution decreased the tendency to form emulsions. Unless great care was taken in all extractions, extremely stable emulsions formed.

3. Ovaries, Placentae, and Bile

The ovaries and placentae were macerated with alcohol, then refluxed with alcohol containing 15 vol. per cent. of 20 per cent. HCl. After filtration, the residue was re-extracted with 80 per cent. alcohol. The extracts were bulked, neutralized to pH 7, and evaporated to dryness under vacuum. The residue was shaken with water and extracted with ether. Phospholipids were then precipitated with acetone. Fatty material was removed by a partition between petroleum ether and 50 per cent. (v/v) alcohol. The aqueous alcohol was evaporated to dryness under vacuum and the residue dissolved in ether from which the phenols were extracted with six equal volumes of N NaOH. The separation of the *T*, *O*, and *D* fractions was carried out as described in Section II(b)1 except that 0.2M Na_2HPO_4 was used for the separation of *T* instead of 0.9 per cent. Na_2CO_3 (Friedgood, Garst, and Haagen-Smit 1948).

The bile was extracted by the methods of Pearlman *et al.* (1947, 1948), and the separation of the *T* fraction was done using 0.2M Na_2HPO_4 as above.

(c) Assay of Extracts

Tests carried out on the 1945 samples showed that chemical methods for the assay of oestrogenic substances would have been impossible owing to the low oestrogen content and to the extremely large amount of coloured phenols present in the extracts. Bioassays were therefore adopted.

Ovariectomized rats were used as test animals and vaginal smears as the criteria of reaction. The extracts were injected subcutaneously.

The *D* and *O* fractions, oestrone and oestradiol, were given in a single injection in 0.15 ml. peanut oil. The *T* fractions and oestriol were given in two injections each of 0.1 ml. oil at 9.30 a.m. and 4.30 p.m.

Vaginal smears were taken at 48, 54, and 60 hours after the first injection. Originally, smears were taken at 72 hours as well, but this was later abandoned as the leucocytic invasion had invariably occurred in this smear.

In interpretation of the smears, the presence of nucleated or cornified epithelial cells, or both, with the complete absence of leucocytes was taken as positive. A system of scoring similar to that described by Robson (1938)

was used for those smears in which there were obvious signs of activity but which showed increasing proportions of leucocytes.

The doses necessary to produce 50 per cent. cornification were approximately as follows: oestradiol 0.1 $\mu\text{g.}$, oestrone 1 $\mu\text{g.}$, oestriol 7 $\mu\text{g.}$

Rats were injected each week and, unless full vaginal cornification was obtained, were primed on the following week with 0.5 $\mu\text{g.}$ oestradiol.

Many of the urine *T* and *D* fractions were relatively toxic, in some cases one-quarter of a 48-hour extract was sufficient to kill a rat. A few urine extracts contained large amounts of solid phenol which was almost insoluble in oil. Attempts were made to purify some of the urine *T* and *D* fractions by steam distillation of the volatile phenols, but the residue obtained was insoluble in oil and the method was abandoned. The method might have been more useful if the original crude extract had been steam-distilled and the separations made on the residue. The extracts from the faeces contained a very much smaller proportion of extraneous phenols than those from urine.

In the assay of the small amounts of oestrogen in the bile, ovaries, and placentae, more sensitive methods were required. The intravaginal injection in 50 per cent. glycerine was tried with ovariectomized rats but the method was abandoned as unsatisfactory. The method of Bulbring and Burn (1935) as modified by Robinson (1949) was adopted and found to give a fairly high degree of sensitivity and reasonable accuracy.

(d) Accuracy of Results — Recovery Tests

The results obtained in this investigation are subject to considerable errors. However, it should be pointed out that the variation of oestrogen excretion between individual ewes is far greater than the errors due to separations and assays. The following factors may contribute to the total error of the results.

(i) *The Identification of Sheep Oestrogens as Oestradiol and Oestrone.*—In the calculation of oestrogen levels, it has been assumed that the active principles of the *D* and *O* fractions are oestradiol and oestrone. The following facts suggest that this assumption is reasonable. The cow shows a rather similar type of oestrogen excretion and crystalline oestrone has been isolated from pregnant cow bile (Pearlman *et al.* 1947). Several samples (ovaries *D*, faeces *D* and *O*) have been assayed at several dose levels by the immature mouse uterus method (Bulbring and Burn 1935, modified by Robinson 1949). This method has the advantage that the effective dose-response curve covers a wider range (two- to four-fold). The slopes of the dose-response curves were identical (within the limits of error) with those of pure oestrone and oestradiol and the level of oestrogen obtained by this method agreed closely with the results from the vaginal smear method.

(ii) *Daily Variation in Faecal and Urinary Excretion.*—No figures are available for the variation in urinary volume but the daily excretion of faeces varies considerably. Unpublished figures show that the weight excreted in 48-hour periods may vary by as much as 25 per cent. from the figure calculated from the weekly mean.

(iii) *Incomplete Extraction from Urine and Faeces.*—The losses from urine samples are probably negligible. The same applies to faeces from sheep on dry feed, but with sheep grazing on young green pastures (i.e. late pregnancy) it is probable that complete extraction is not easily obtained.

(iv) *Bioassay.*—The low concentration of oestrogen in most of the extracts made it impossible to use the standard methods of assay with 20 rats per test group. For the purpose of the investigation, however, the order of the results was the main consideration. To obtain the maximum degree of accuracy with a small number of rats the following precautions were observed.

The rats used were selected for uniform sensitivity and a close check was kept on the sensitivity of individual rats as well as on the group sensitivity. The maximum range of oestrogen necessary to give 50 per cent. cornification varied at different times between 0.08 and 0.12 μg . oestradiol and 0.75 and 1.1 μg . oestrone (15-20 rats per group).

The usual procedure was to inject doses of 1/4, 1/8, 1/16, etc., of the 48-hour extract into one or two rats. It was usually possible to obtain full cornification on one level and little or no reaction on the next level below. Additional rats were then used at these two levels and if sufficient of the extract was available another was tested at an intermediate level. A standardization with the corresponding oestrogen was usually made with each batch but if not, the figures of 0.10 μg . oestradiol and 0.9 μg . oestrone were used for 50 per cent. response.

The following were the approximate numbers of rats used at each of two levels in the assay of the 1946 and 1947 samples. These levels will differ by a factor of 2 for low concentrations and about 1.5 for the higher concentrations.

0.2 μg . *D* or 2 μg . *O* per day, 2-3 rats,

0.6 μg . *D* or 6 μg . *O* per day, 3-5 rats,

1.0 μg . *D* or 10 μg . *O* per day, 5 rats (usually at 3 levels),

1.5 μg . *D* or 15 μg . *O* per day, 5-8 rats (usually at 3 levels).

In the recovery tests described later, two levels of the unknown were used (15 rats per group) straddling a standard oestrogen group giving approximately 50 per cent. response.

(v) *Losses during Chemical Separations.*—The work of Friedgood, Garst, and Haagen-Smit (1948) suggested that such losses might be much greater than was usually assumed. Two of their findings which concerned the present investigation were the loss of oestradiol into the "crude *T*"

fraction (but probably not into the purified *T* fraction) and the incomplete extraction of oestrone from ether by NaOH in the faeces extracts. Accordingly it was thought desirable to repeat earlier recovery tests on both urine and faeces and to examine in some detail the distribution of added oestrogens.

The samples used for the tests were a bulk 48-hour urine sample from four ewes early in pregnancy and a bulk 48-hour faeces sample from four ewes late in pregnancy. With the urine the oestrogen was added to the sample before hydrolysis but with the faeces the addition was made to the alcohol extract.

The results obtained are set out in Tables 1 and 2.

TABLE 1
RECOVERIES OF OESTROGEN FROM SHEEP URINE
(Values expressed as micrograms)

Addition	<i>D</i>	<i>O</i>	<i>T</i>	"Crude <i>T</i> not <i>T</i> "**
Control	<0.3	<2	<30	<0.8 <i>D</i>
20 µg. <i>D</i>	18.0	<2	<30 <i>T</i> or <0.4 <i>D</i>	2 <i>D</i>
30 µg. <i>O</i>	<0.3	25	<30 <i>T</i> or <4 <i>O</i>	<8 <i>O</i>
200 µg. <i>T</i>	0.4†	<2	400	200 <i>T</i>

* Activity assayed and calculated as oestradiol for control and as added oestrogen for others.

† Activity assayed and calculated as oestradiol.

No biological activity was noted in those samples where the value is given as < a certain figure.

The following points emerge from the results:

There is a definite loss of oestradiol into the "crude *T*" fraction but the subsequent purification generally separates this from the final *T* fraction. As discussed in Section III(b), it would seem that under some circumstances small amounts of oestradiol may pass into the purified *T* fraction.

The separation of oestrone from other oestrogens seems to be complete.

The extraction of oestrone from ether by N NaOH is complete under the conditions of the experiment. The difference between our findings and those of Friedgood, Garst, and Haagen-Smit (1948) may be due to the fact that these workers did not reach equilibrium between the two phases. In the case of faecal extracts, emulsions form and may take hours to settle out. There is thus a very intimate mixing of the two phases and ample time is available to reach equilibrium.

The presence of relatively large amounts of oestriol in the "hydrolysed" faeces is almost certainly due to incomplete extraction from the "non-hydrolysed" portion.

The extraneous phenols in the urine *T* fraction have greatly enhanced the biological activity of the added oestriol.

The investigation suggests that the values presented in the subsequent section do in fact give an approximately true indication of the level of

TABLE 2
RECOVERIES OF OESTROGENS ADDED TO FAECAL EXTRACTS
(Values expressed as micrograms of oestrogen)

Addition	Fraction	Direct Extract	After Hydrolysis of Residue	Total	Recovery (%)	Residual Activity	
						Fraction after Removal of <i>T</i> (expressed as <i>D</i>)*	Residual Activity in Ether after NaOH Extraction*
Control	<i>D</i>	8	<0.3	8	—	2.0	—
	<i>O</i>	20	<2.0	20	—	—	—
	<i>T</i>	c. 50	<14.0	c. 50	—	—	—
15 μ g. <i>D</i>	<i>D</i>	20	0.2	20	80	4.0	<0.2
	<i>O</i>	20	<2.0	20	—	—	<2.0
	<i>T</i>	<30	<14.0	<40	—	—	—
80 μ g. <i>O</i>	<i>D</i>	8	<0.2	8	—	3.5	<0.2
	<i>O</i>	44	<2.0	44	80	—	<2.0
	<i>T</i>	c. 30	<14.0	c. 30	—	—	—
200 μ g. <i>O</i>	<i>D</i>	8	1.0	9	—	2.0	<0.2
	<i>O</i>	140	10.0	150	65	—	<3.0
	<i>T</i>	c. 30	<14.0	c. 30	—	—	—
1,000 μ g. <i>T</i>	<i>D</i>	Lost	1.0	—	—	5.0	—
	<i>O</i>	17	2.0	19	—	—	—
	<i>T</i>	600	150.0	750	70	—	—

* These figures apply to the direct extract. No activity was found in any of the corresponding "after hydrolysis" extracts.

No biological activity was detected in those samples where the value is given as < a certain figure.

oestrogen likely to be present in both urine and faeces. There will be some loss of oestradiol, but as oestriol probably does not occur to any extent in sheep urine or faeces the limitations of the method for this oestrogen will not affect the results.

III. RESULTS

The results for the urinary and faecal excretion for the 1946 and 1947 collections are set out in Tables 3 and 4. The figures for the *D* fractions are not corrected for loss of *D* into the "crude *T*" fraction. This loss is probably between 10 and 30 per cent. for urine samples and somewhat higher (probably 20 to 40 per cent.) for the faeces.

(a) Urinary Excretion of Oestrogen

The 1945 samples were not sufficiently numerous to warrant detailed publication but the values were similar to those obtained in the 1946 and

1947 series. The daily excretion of four of the ewes during the last fortnight of pregnancy was 1 and 2, 1 and 3, 4 and 2.5, and 0.4 and 2.0 μg . *D* and *O* respectively.

Oestrogenic activity was not detected in any of the strong phenol fractions (*T*) of the 1946 ewes or of ewes 250 and 251 (1947), but in several of the 1945 late pregnancy samples and in ewes 252 and 253 (April 27, 1947), slight activity was noted in this fraction. In most cases the activity was very small. The activity from ewe 253 (c. 35 μg . *T* per day) is of particular interest in that no activity was detected in either the *D* or *O* fraction at this sampling. A portion of this *T* fraction was re-purified by extracting from benzene with water and it was found that the activity parted into the water phase. It is, therefore, assumed that this activity was actually due to some oestriol-like compound, although the excretion of such compounds by sheep would seem to be rather uncommon.

(b) Faecal Excretion

The amount of oestrogenic activity in the "hydrolysed" fraction of the faeces (i.e. after hydrolysis of the ether-extracted sludge) has been found to be very small (usually < 10 per cent.) compared with the activity of the plain extract (cf. Table 2). In one or two cases where higher activity was noted, the faeces were from sheep on young green pastures and the extracts contained large amounts of extraneous material. Here the values for "hydrolysed" oestrogen almost certainly represent oestrogen left behind after the first extractions. Accordingly it is concluded that, as with the cow (Levin 1945), the faecal oestrogen of sheep is not conjugated. In Tables 3 and 4 the faecal values represent the total of "hydrolysed" and "not hydrolysed" oestrogen.

No potency was obtained in the *T* fraction of any of the 1947 faeces samples but some activity was noted in two of the 1946 samples and in the faeces used for recovery tests (Table 2). Two of these extracts were again purified by extraction from benzene with water. It was found that the activity remained in the benzene phase and hence it is concluded it was probably due to some *D* carried into the purified *T* fraction. The results accordingly suggest that oestriol-like substances are not excreted in the faeces of pregnant ewes.

As pointed out earlier, the samples for the 1948 recovery tests (Table 2) were from ewes late in pregnancy. The mean daily excretion was 5 μg . *D* and 12 μg . *O* per ewe; these values are of the same order as for corresponding samples in the 1946 and 1947 series.

(c) Ovaries

Abattoirs material (mainly from Merino ewes) was used in these exploratory tests and the samples were taken during the breeding season. In the first batch the ovaries showing active follicles were separated and

TABLE 3
URINARY AND FAECAL EXCRETION OF 1946 MERINO EWES
(Values expressed as micrograms per 24 hours)

Details		Pre-mating								Post-lambing
		18.xii.45	15.i.46	18.ii.46	12.iii.46	6.iv.46	30.iv.46	7.v.46	13.v.46	
C192	Urine <i>D</i>	<0.2	<0.2	<0.2	0.3	0.5	<0.5*	1.5	0.5	—
Mated 21.xii.45	Urine <i>O</i>	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	3.0	3.0	—
Lambd 16.v.46	Faeces <i>D</i>	>0.2	0.15	(0.1)	0.6	1.0	2.0	10.0	5.0	—
	Faeces <i>O</i>	(1)	<1.0	<1.0	<1.0	4.0	2.0	2.0	1.5	—
C193	Urine <i>D</i>	11.xii.45	15.i.46	18.ii.46	12.iii.46	6.iv.46	30.iv.46	13.v.46	20.v.46	30.v.46
	Urine <i>O</i>	<0.2	<0.2	<0.2	0.5	3.0	1.2	3.0	—	<0.4
Mated 24.xii.45	Urine <i>O</i>	<1.0	<2.0	<2.0	<2.0	3.0	5.0	20.0	>8.0	—
Lambd 23.v.46	Faeces <i>D</i>	0.4	<0.1	0.5	—	1.0	>7.0	6.0	20.0	<0.4
	Faeces <i>O</i>	<2.0	<1.0	<1.0	—	(1)	>30.0	20.0	100.0	<2.0
C182	Urine <i>D</i>	11.xii.45†	—	2.ii.46	5.iii.46	31.iii.46	24.iv.46	17.v.46	27.v.46	11.vi.46
	Urine <i>O</i>	<0.2	—	<0.2	‡	0.2	0.4	0.2	0.3	(0.1)
Mated 11.i.46	Urine <i>O</i>	<1.0	—	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0
Lambd 3.vi.46	Faeces <i>D</i>	<0.2	—	0.2	0.2	1.0	2.0	3.5	5.0	(0.1)
	Faeces <i>O</i>	<1.0	—	<1.0	<1.0	<1.0	3.0	4.0	9.0	<1.0

* Much oil-insoluble material in extract.

† C182 not in sheep first tested. Figures on 11.xii.45 from another sheep in same group.

‡ Extract insoluble in oil.

Values given as < a certain value indicate that no biological activity was observed.

Values in brackets show activity at highest level only, i.e. with only one rat.

the *D* fraction assayed separately. The results are set out in Table 5.

An attempt to assay the combined *O* fraction of Batch 1 by intra-vaginal application showed that activity was present but the method was unsatisfactory and quantitative interpretation was not possible.

TABLE 4
URINARY AND FAECAL EXCRETION OF 1947 MERINO EWES
(Values expressed as micrograms per 24 hours)

Details		17.ii.47	17.iii.47	13.iv.47	27.iv.47	Post-lambing 8.v.47
Ewe 250	Urine <i>D</i>	<0.16	<0.2	0.25	0.4	4.0
Mated 7.xii.46	Urine <i>O</i>	<1.5	<1.5	<1.5	<1.5	10.0
Lambled 7.v.47	Faeces <i>D</i>	<0.1	0.6	1.4	7.0	2.0
	Faeces <i>O</i>	<0.6	<2.0	<1.5	16.0	4.0
						12.v.47
Ewe 251	Urine <i>D</i>	<0.3	(Solid)	2.0	2.5	<0.8
Mated 7.xii.46	Urine <i>O</i>	<1.5	<3.5	8.0	8.0	<2.5
Lambled 11.v.47	Faeces <i>D</i>	(0.1)	1.2	5.0	6.0	0.9
	Faeces <i>O</i>	<2.0	(1)	4.0	8.0	<2.0
						8.v.47
Ewe 252	Urine <i>D</i>	<0.4	0.5	0.3	2.5	<0.4
Mated 7.xii.46	Urine <i>O</i>	<1.5	<1.5	<1.5	<1.5	<3.0
Lambled 7.v.47	Faeces <i>D</i>	0.2	0.8	5.0	4.5	3.2
	Faeces <i>O</i>	<1.0	<1.0	3.0	5.0	6.0
						8.v.47
Ewe 253	Urine <i>D</i>	<0.4	<0.8	0.5	<0.2	<0.3
Mated 7.xii.46	Urine <i>O</i>	<1.5	<1.5	1.5	<1.5	<3.0
Lambled 1.v.47	Faeces <i>D</i>	<0.2	0.5	1.7	0.8	<0.3
	Faeces <i>O</i>	<2.0	Lost	(1)	(1)	<2.0

Values given as < a certain value indicate that no biological activity was observed.

Values in brackets show activity at highest level only, i.e. with only one rat.

No activity was detected in the *T* fractions by the mouse uterus method.

(d) *Bile*

The first tests were done on abattoirs material by direct injection into rats, but no activity was noted in any of the samples. Extracts were then prepared from new samples and assayed by the mouse uterus method. In all cases the ewes were within three weeks of lambing. The results are set out in Table 6.

Activity was not observed in any of the *T* fractions.

The Tooradin bile was assayed by direct injection into immature mice (0.1 ml. bile per mouse per day). A small but definite increase in uterine weight was obtained. The biliary oestrogen is largely conjugated and comparisons with pure oestrogens are, therefore, not valid. If a comparison is made with pure oestrone dissolved in inactive bile, the concentration of "oestrone" in the Tooradin bile would be of the order of 50 $\mu\text{g.}$ per litre.

TABLE 5
THE OESTROGEN CONTENT OF SHEEP OVARIES

Material	Date	Fresh Weight	Oestrogen Content ($\mu\text{g.}$)	
			D	O
Batch 1 active	25.i.49	120	0.4	—
Batch 1 inactive	25.i.49	214	0.2	—
Batch 2 mixed	5.iv.49	374	1.6	0.4

(e) *Placentae*

Three placentae from ewes in the last three weeks of pregnancy were separated into the maternal and foetal components and the whole of the tissue extracted. The oestrogen levels are set out in Table 7.

TABLE 6
THE OESTROGEN CONTENT OF THE BILE OF PREGNANT EWES

Source of Material	Oestrogen Content ($\mu\text{g./litre}$)			
	Free		Conjugated	
	D	O	D	O
Beverley, W.A.: experimental sheep; 4 Merinos; bulked 3.v.49	<0.5	<1	2.1	26
Melbourne Abattoirs: 5 ewes unknown breed; bulked 23.vi.49	1.3	Lost	1.3	10
Tooradin, Vic.: experimental sheep; 4 Merinos; bulked 26.ix.49	c.1	2	0.6	25

(f) *Pregnanediol*

A number of 1945 and 1946 urine samples were examined by the method of Astwood and Jones (1941) but no pregnanediol was detected.

IV. DISCUSSION

Our findings support the conclusion reached by Whitten (1943) that in pregnancy, urinary excretion of oestrogen occurs only in the last few

weeks. Faecal oestrogen is also absent until fairly late in pregnancy and is generally greater than in the urine. The levels observed are much lower than those reported for other species but, as discussed in Section II(d), there is no reason to doubt that the figures give a reasonably accurate picture of the excretion by the ewe.

TABLE 7
THE OESTROGEN CONTENT OF SHEEP PLACENTAE

Tissue	Wet Weight (g.)	Total Oestrogen Content (μ g.)	
		D	O
Foetal	1030	1.0	2
Maternal	2155	0.5	1

One noteworthy feature of both the faecal and urinary levels is the wide variation between individual ewes, e.g. ewe 253 (1947) showed no oestrogen excretion two weeks before lambing, whereas ewe 193 (1946) showed relatively high levels. The ratio of ketonic to non-ketonic oestrogen also varies considerably.

In the bile samples the total amount of oestrogen found is very much lower than other published values for sheep (Council for Scientific and Industrial Research 1946). The reason for this is not clear, but it does not seem to be due to loss of activity during chemical separations of oestrogen from bile, which Pearlman *et al.* (1947) have shown to be appreciable. The combined oestrogenic potency of the separated fractions of the Tooradin bile (Table 6) is approximately 50 per cent. of original bile and hence it is assumed that no abnormal loss of potency has occurred during the separations of the samples listed in Table 6. As Pearlman *et al.* (1947) have found for the cow, the sheep biliary oestrogen is mainly ketonic, but in contrast is almost entirely in the conjugated form.

Cantarow *et al.* (1942) quote evidence that in other species the oestrogen in bile is largely inactivated in the intestines. Our findings suggest that this may not be so in the sheep. If we accept the figures quoted by Dukes (1935) for the biliary secretion of the sheep (*c.* one litre per day), our findings would indicate that the daily biliary oestrogen (O plus D) is of the same order as the daily faecal oestrogen output. This may mean that in the sheep the intestinal organisms hydrolyse conjugated oestrogen and reduce some oestrone to oestradiol but do not otherwise attack the oestrogen molecule.

The ovarian levels found in sheep are very much lower than corresponding values recorded for the sow by Sealey and Marlow (1941). Our finding that portion of the ovarian oestrogen is ketonic is of interest in

the light of the observations of Westerfeld and Doisy (1937) which showed that ketonic oestrogen is present in small amounts in sow ovaries but is absent from cow ovaries.

Parkes and Bellerby (1927) have assayed crude extracts from sheep placentae collected at 3-4 months. Their values indicate a considerably higher level of oestrogen than that given in Table 7 but quantitative comparison is not possible because of the different type of extract and the different method of assay.

A comparison with the results of other workers is difficult because of the wide individual variation which may occur in oestrogen excretion in the sheep. Furthermore, no figures are available for breeds other than the Merino. The present investigation strongly suggests that the pregnant Merino ewe secretes very much smaller amounts of oestrogen than other species, but more critical work is needed before any final conclusions may be drawn concerning the oestrogen metabolism of the ewe.

V. ACKNOWLEDGMENTS

This investigation was part of a cooperative investigation into sheep infertility being conducted mainly in Western Australia.

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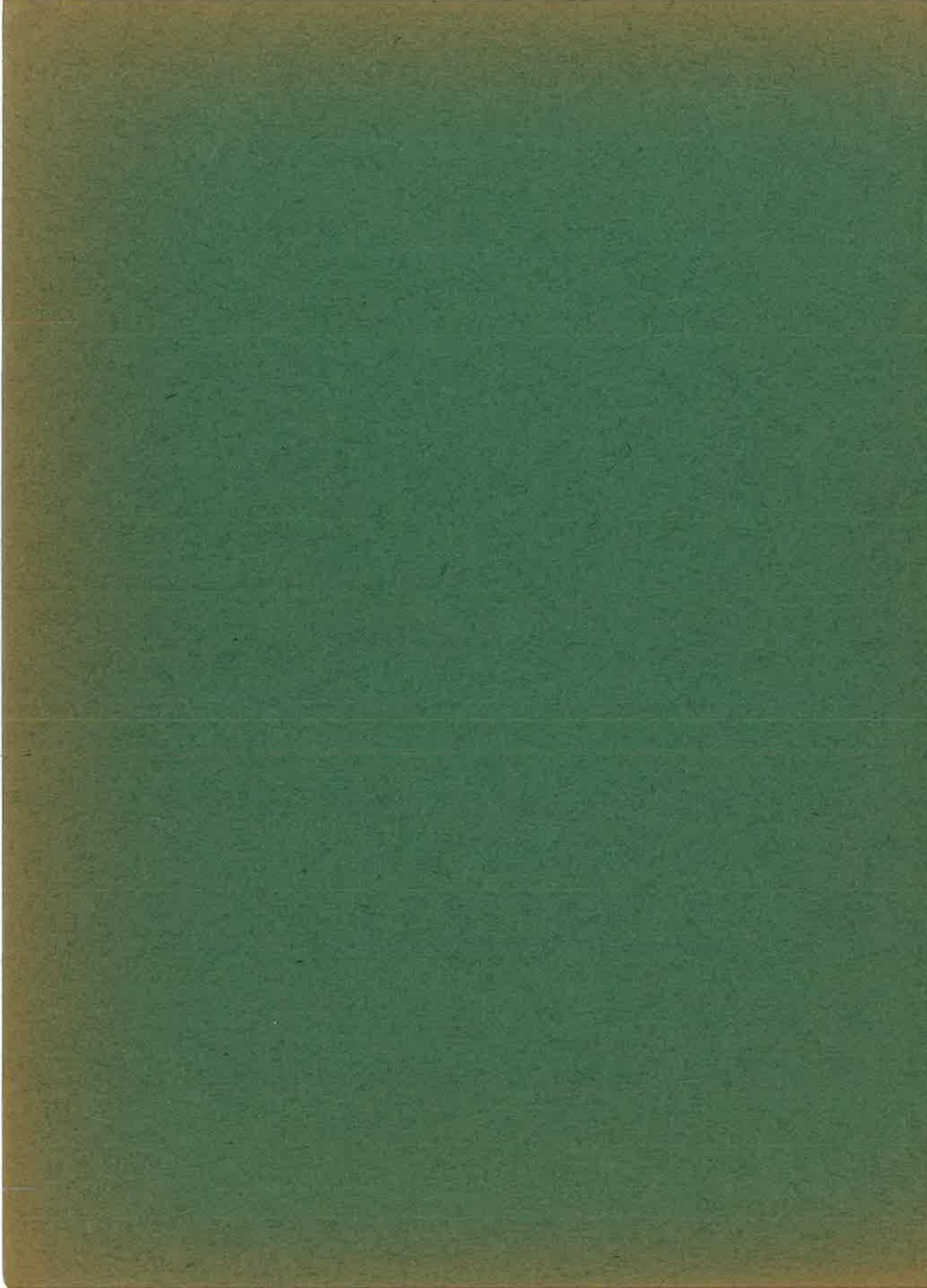
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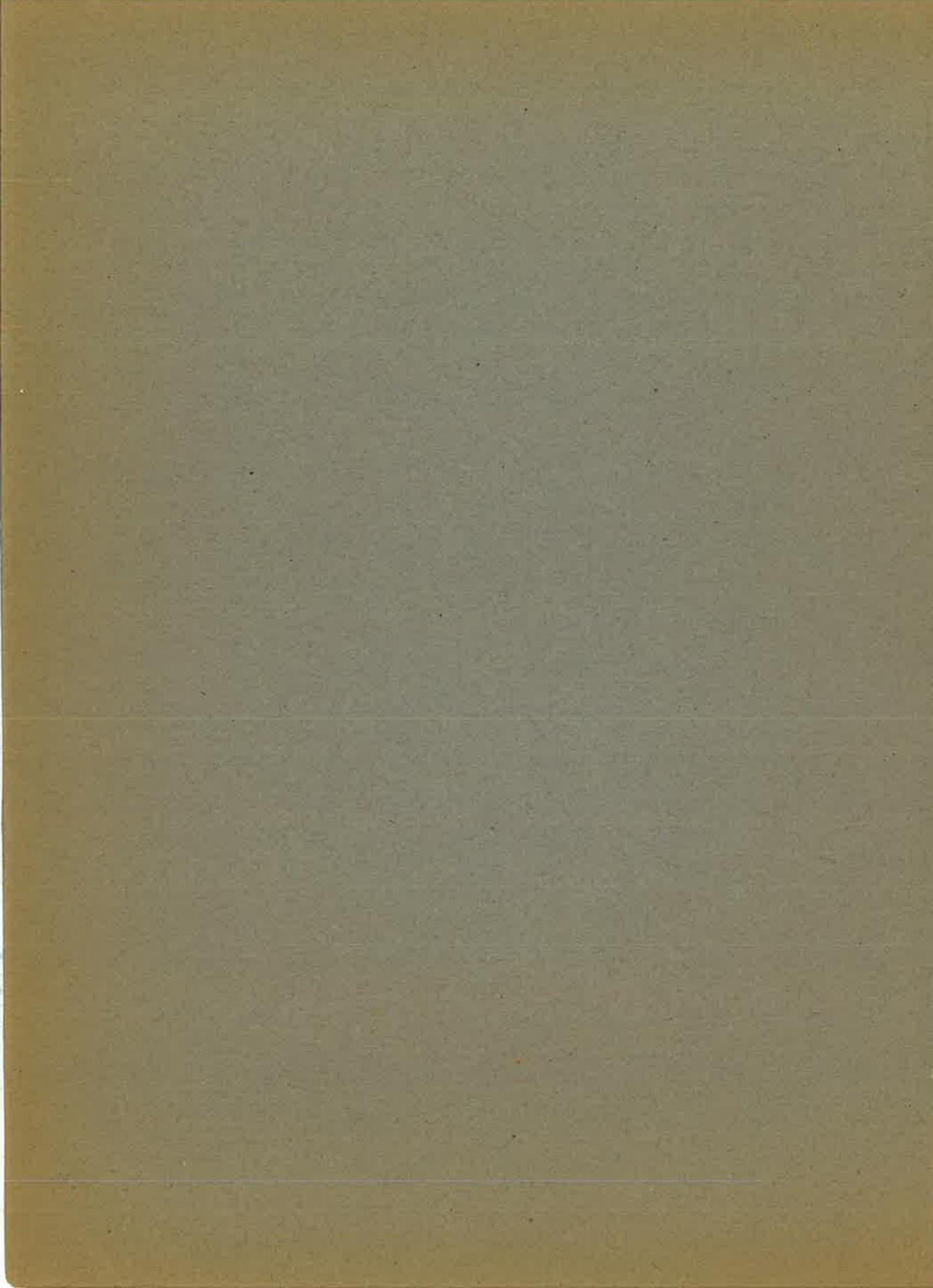




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THE COPPER AND NICKEL CONTENT OF THE BLOOD OF
THE WESTERN AUSTRALIAN MARINE CRAYFISH
(*PANULIRUS LONGIPES* MILNE EDWARDS) AND OF
SEAWEEDS

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by A. B. BECK¹ AND K. SHEARD²

(Accepted for publication 23rd December, 1948.)

In the processing of the flesh of the Western Australian marine crayfish (*Panulirus longipes*) much difficulty has been experienced due to a darkening of the canned or frozen product. Similar observations have been made on the Pacific Coast commercial crab by Fellers and Parks (1926). The darkening was apparently due to the slow formation of copper sulphide from the haemocyanin of the blood, and could be prevented by a preliminary treatment of the flesh with a faintly acid, very dilute solution of aluminium chloride (200-400 parts per million $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ by weight was optimum for commercial practice). Provided that the subsequent processing technique was efficient, this treatment was generally successful in producing a satisfactory product, but it was not effective in the case of a certain number of specimens. To ensure complete absence of darkening, a greater concentration of aluminium chloride (600 p.p.m.) was found necessary.

This work was undertaken partly to ascertain the range of blood copper, partly to check whether the suspected variation was related to crayfish size, sex, gross inter-ecdysal stage or to periods of gonad activity, and partly to provide a basis for future work on the biochemistry of this and other species of crayfish and crabs. At the same time, one of the writers (A.B.B.) was interested in the possible rôle of nickel in animal metabolism. As the occurrence of nickel had already been reported in the haemocyanin of the gastropod *Busycon canaliculatum* (Montgomery, 1930), a few nickel determinations were done on crayfish blood; several vertebrate blood samples were also analysed for comparison.

A few samples of seaweed from the area were also analysed for copper and nickel content to obtain some indication of the possible intake of these elements by the crayfish.

Time was not available to analyse a sufficient number of blood samples to give a complete picture of the copper and nickel levels, and the results given here are to be regarded as a preliminary survey only.

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METHODS.

Most of the crayfish were from the Abrolhos Islands some 250 miles north of Perth, Western Australia. Six samples were obtained from Lancelin Island, 70 miles north of Perth. The measurements of the carapace length were taken from the posterior edge of the base of the rostral horns (supraorbital spines) to the posterior end of the carapace approximately in the mid-dorsal line. The samples for copper determinations were taken with a glass syringe and stainless steel needles; for nickel determinations glass needles were used, and the blood was aspirated directly into bottles containing oxalate. It was not possible to obtain samples from crayfish which had shed their exoskeleton (moulted) recently, and were thus soft shelled, as the blood clotted almost instantly.

In taking samples, the animals were placed in a walking position near the edge of a box with the abdomen hanging over the edge. The needle was then inserted under the posterior edge of the carapace along the mid-dorsal line immediately under the shell until it pierced the dorsal abdominal artery at its point of emergence from the heart (1.0 to 1.5 inches).

As an anticoagulant 4-5 mg. potassium oxalate (purified) were used per ml. of blood. Sodium citrate was not effective.

Some trials were done in which the blood was squirted directly from the syringe into 6 p.c. trichloroacetic acid solution. This treatment appears to liberate the copper quantitatively, but it was found to be more convenient to measure 5.00 ml. of blood directly from the syringe into a small oxalated bottle; the whole of the blood sample was subsequently digested with nitric, sulphuric and perchloric acids. After digestion the copper was determined directly on aliquots with sodium diethyldithiocarbamate, the copper complex being dissolved in amyl alcohol. Sodium pyrophosphate was added as a routine measure although iron was virtually absent. In a number of cases the direct copper determination was checked by doing a preliminary separation of the copper at pH 3 with dithizone and estimating as before. Results by the two methods agreed very closely.

Where nickel was to be determined larger blood samples were necessary. Both bloods and seaweeds were digested as before and copper was first removed by extraction with dithizone at pH 3 in the presence of citrate. The pH was then raised to 8 with ammonia and the nickel extracted as the dimethyl-glyoxime complex in chloroform. The nickel was then determined by a modification of Rollet's method (1926). In the case of the bloods the colours were read in 19 cm. Nessler tubes.

The seaweeds were rinsed with glass-distilled water after collection and allowed to dry in the air. Samples prepared in this way contain a large percentage of NaCl. In order to present the copper and nickel figures on a basis comparable with land plants, a rough chloride determination was done by shaking a weighed amount of the material with a large excess of water, filtering and doing a standard chloride titration (Volhard's method) on an aliquot of the filtrate. The figures were then corrected for the salt content.

In the collection, handling and analyses of all material, standard "trace element" precautions were taken against contamination.

EXPERIMENTAL.

Blood Copper Levels.

The blood copper values are set out in Table 1. In this Table the gross phase of the inter-ecdysal period has been coded as follows: "A" represents crayfish which had recently moulted but of which the integument had hardened; "B", those approximately in the middle

TABLE 1.

*Blood copper of Panulirus longipes (Milne-Edwards).*Maximum analytical error ± 3 p.c.

Values expressed as micrograms Cu per ml. blood.

Serial number	Carapace length, inches	Intermoult stage	Sex and gonad condition	Month	Fishing ground	Blood copper
38	4.3	B	♂ spent	October	Lancelin Island	43
7	3.8	A	♀ spent	April	Southern group	70
24	4.3	B	♂ active	July	Easter group	81
12	3.4	B	♂ active	July	Easter group	88
10	5.1	C	♂ inactive	April	Southern group	91
27	3.0	B	♂ active	July	Easter group	94
28	2.9	B	♂ active	July	Easter group	95
36	4.0	B	♂ spent	October	Lancelin Island	103
34	4.7	B	♂ spent	August	Wallabi group	105
26	3.5	B	♂ active	July	Easter group	114
8	5.2	B	♂ inactive	April	Southern group	114
5	3.0	A	♀ inactive	April	Southern group	122
40	4.0	B	♀ ovigerous	October	Lancelin Island	122
3	4.2	B	♂ inactive	April	Southern group	127
2	4.4	B	♂ inactive	April	Southern group	128
22	3.3	B	♂ inactive	July	Easter group	146
31	4.4	B	♂ spent	August	Wallabi group	146
35	4.0	B	♀ ovigerous	October	Lancelin Island	149
13	4.0	B	♂ active	July	Easter group	150
9	3.4	A	♀ inactive	April	Southern group	150
4	3.8	A	♀ inactive	April	Southern group	150
29	2.6	B	♀ active	July	Easter group	152
20	3.2	B	♀ active	July	Easter group	153
6	2.9	A	♀ inactive	April	Southern group	154
17	3.6	B	♂ active	July	Easter group	154
18	3.9	B	♂ active	July	Easter group	157
33	4.2	B	♂ spent	August	Wallabi group	157
23	2.9	A	♂ active	July	Easter group	158
37	4.0	B	♀ ovigerous	October	Lancelin Island	160
14	4.0	B	♂ active	July	Easter group	161
11	3.5	B	♂ active	July	Easter group	162
39	4.0	B	♀ ovigerous	October	Lancelin Island	163
21	3.5	B	♂ active	July	Easter group	171
1	3.5	A	♂ inactive	April	Southern group	177
25	3.5	B	♂ active	July	Easter group	181
15	3.1	B	♂ active	July	Easter group	187
19	3.5	B	♂ active	July	Easter group	198
32	4.5	B	♂ spent	August	Wallabi group	208

Mean 138 μg . per ml.; σ M, ± 6.0 ; σ , ± 37 .

of the inter-ecdysal period and "C" those which were closely approaching an ecdysis. Crayfish with serial numbers 1-29 were taken directly from the sea, while those numbered 31-40 had been taken out of the water from 6-8 hours before sampling.

The wide range of values (43-208 $\mu\text{g. per ml.}$) indicates a possible reason for the varying tendency of crayfish flesh from different individuals to darken under uniform processing conditions.

As can be seen directly from the Table there is no obvious correlation between the blood copper levels and any one of the following factors: crayfish size, as measured by carapace length; period of the year; gonad activity; fishing area, or the early or middle inter-ecdysal period. The number of samples is too small to draw any definite conclusions concerning the effect of sex, but it should be noted that 9 out of the 10 female values lie between 120 and 170 $\mu\text{g. per ml.}$, whereas the proportion of males in this range was only 12 out of 28.

Examination of the grouping of the figures, together with the negative skewness of the grouped frequency curve, suggests that some unknown factors may have been operating which tended to produce a particular type of distribution.

The wide range of values observed raises several questions concerning the metabolism of copper in the crayfish which are not answered by the present investigation:

(a) What is the optimum blood copper level? The relatively low efficiency of haemocyanin for oxygen transport might suggest that the level would be as high as possible with the limiting value probably the toxic threshold. The results suggest that the upper limit is in the neighbourhood of 200 $\mu\text{g. per ml.}$ and the optimum may lie between 140 and 180 $\mu\text{g. per ml.}$ It should be stressed that the values in Table 1 are for total copper and although it is likely that this value is the same as for oxygen-carrying copper, the relationship has not been investigated.

(b) Has the crayfish any storage depot for copper, either localized or general, and if so does the circulating copper vary with the stored copper? In vertebrates, e.g. sheep and cows on low copper intake, the blood copper gives a rough indication of the storage of copper in the liver.

(c) Are the blood copper levels determined solely by balance between intake and excretion, and a low blood level merely indicates that the animal had been grazing for a period on seaweeds and corals which happened to be low in copper?

Copper and Nickel Content of Seaweeds.

The results of the analyses of some common seaweeds known to enter into the diet of the marine crayfish are given in Table 2. Both the copper and nickel content are given in parts per million on an H_2O -free and NaCl -free basis. In the case of the marine flowering plant, *Cymodocea antarctica*, there was insufficient material for the determination of NaCl but the figure was low and the corrected figures are calculated on the assumption of 10 p.c. NaCl .

TABLE 2.

Copper and nickel content of seaweeds.
Easter Group, Abrolhos Islands, July, 1947.

Species	NaCl content dry basis p.c.	Copper and nickel (p.p.m. on H ₂ O-free and NaCl-free basis)	
<i>Cladophora</i> sp.	30	10.0	4.4
<i>Polysiphonia</i> sp. plus <i>Hypnea episcopalis</i>	25	8.4	8.8
<i>Caulerpa</i> sp.	60	3.8	2.2
<i>Sargassum</i> sp.	30	1.6	1.6
<i>Cystophyllum muricatum</i>	15	2.8	4.0
<i>Cymodocea antarctica</i> (leaves and tops of stems only)	(10)	5.6	25.0

These samples from the area occupied by the crayfish showed copper levels similar to those of land plants, but the nickel content was much higher.

The Nickel and Copper Content of Blood.

The results obtained are set out in Table 3. The vertebrate blood was obtained from the carotid artery after decapitation. The lower limit of estimation on 30 ml. of blood is about 0.01 µg. Ni per ml.

TABLE 3.

Nickel and copper content of blood.
Microgram per ml.

Species	Details	Nickel	Copper
<i>Panulirus longipes</i>	series number 38	<0.01	43
<i>Panulirus longipes</i>	from Table 1. 34	0.01	105
<i>Panulirus longipes</i>	32	0.015	208
<i>Panulirus longipes</i>	36	0.03	103
<i>Panulirus longipes</i>	40	0.03	122
<i>Panulirus longipes</i>	35	0.03	149
<i>Panulirus longipes</i>	31	0.04	146
<i>Panulirus longipes</i>	33	0.05	157
<i>Panulirus longipes</i>	37	0.05	160
Domestic fowl (<i>Gallus bankiva</i>)	white leghorn ♀ mature	∞0.01	0.20
	white leghorn ♀ mature	∞0.01	0.26
Guinea pig (<i>Cavia porcellus</i>)	bulk sample ♂♂ adult	∞0.01	0.53
Merino Ewe (<i>Ovis aries</i>)	ovariectomized, mature	∞0.01	—
	ovariectomized, mature	∞0.01	—
Rabbit (<i>Oryctolagus caniculus</i>)	mature ♀ mixed breed	<0.01	0.70

The amounts of nickel found in the crayfish blood are variable and extremely small, but they are definitely higher than those found in the vertebrate bloods tested. It will be noted that there is some tendency for the higher nickel levels to be associated with the higher copper values. On the other hand, the high level of nickel found in the seaweeds tested suggests that the blood nickel may be adventitious.

SUMMARY.

Thirty-eight samples of blood from the Western Australian marine crayfish *Panulirus longipes* have been examined for copper content. A wide range of values (43–208 $\mu\text{g. Cu. per ml.}$) was obtained, with a decided grouping in the sub-range (140–170). There was no indication that this variation was related to size, period of the year, fishing area, gonad activity or to the early or middle inter-ecdysal stage. The number of samples was insufficient to draw any definite conclusions as to the effect of sex.

Seaweed samples from the area showed copper levels similar to those of land plants (1.6 to 10.0 p.p.m.), but the nickel levels were quite high (1.6 to 25 p.p.m., both on NaCl-free and water-free basis).

Traces of nickel were noted in *Panulirus* blood. The amounts were variable and very small, but were higher than the levels noted in certain vertebrate bloods.

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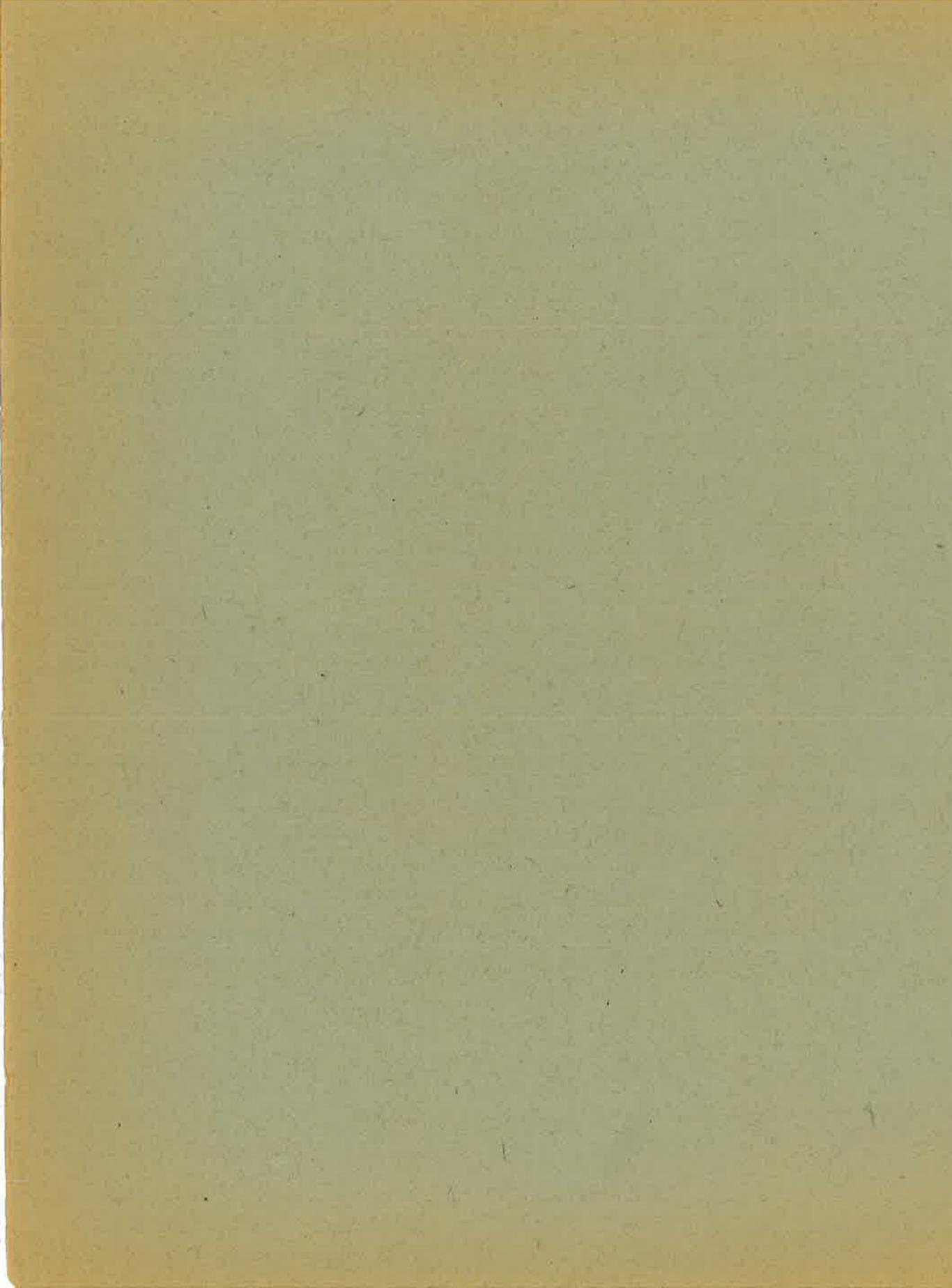
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THE QUANTITATIVE EXTRACTION OF COBALT AND IRON
FROM ASHED BIOLOGICAL MATERIAL

by A. B. BECK



THE QUANTITATIVE EXTRACTION OF COBALT AND IRON FROM ASHED BIOLOGICAL MATERIAL

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(From the Division of Animal Health and Production, Council for Scientific and Industrial Research).

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In the investigation of "trace element" deficiencies in plant or animal material it is an obvious advantage to be able to determine the concentrations of a number of elements on one sample; most existing methods allow for the determination of only one or perhaps two. The degree of separation of these elements by solution of organic complexes in immiscible solvents is influenced by pH, by the concentration of sulphate ions (from wet digestion) and of citrate ions (added to prevent precipitation in alkaline solutions), but complete data on the effects of these factors are not given in existing literature.

In 1943 an investigation was begun to obtain further information on these points and also to evolve simple, yet strictly quantitative separations of a number of elements. This investigation has had to be suspended for an indefinite period on account of more urgent work, but it is considered that the results obtained so far are of value, particularly where it is required to separate cobalt quantitatively from other elements.

The conditions for the quantitative extraction of iron by solution of the cupferron complex in chloroform, and the possibility of separating iron from copper and cobalt by this method, have been investigated.

The extraction of cobalt both as dithizone and 1-nitroso-2-naphthol complexes has been examined, and as a result of these investigations a scheme is suggested for the quantitative separation of copper, cobalt, zinc, lead and iron from solutions obtained after the ashing of biological material. The scheme has been used for the examination of samples and has given completely satisfactory results.

EXPERIMENTAL.

General.

A visual colorimeter was used for comparison with standard solutions in most of the estimations made. Tall Nessler tubes (19 cm.) were used for amounts of less than 1 microgram of cobalt, which were determined by a slight modification of the nitroso-R-salt method of Marston and Dewey (1940) and Bayliss and Pickering (1941). Small amounts of zinc and lead were estimated by the mixed-colour dithizone method similar to that used for lead by Clifford and Wichmann (1936). Larger amounts of zinc were estimated by the dithizone colorimetric method (Hibbard, 1937), copper as the diethyldithiocarbamate complex in amyl alcohol (Sylvester and Lampitt, 1935) and iron as the dipyriddy complex (Jackson, 1938).

For the determination of extraction curves, aqueous solutions containing the element as sulphate or chloride were used: 3 ml. H_2SO_4 (representing the maximum amount likely to be present after wet digestion) and varying amounts of citric acid (as ammonium citrate purified by dithizone) were added in each case. The approximate pH was obtained by the addition of ammonium hydroxide, using internal indicators, and the volume made to 100 ml. After extraction of the element, the exact pH was determined with the glass electrode. All extractions were done at 22–23° C. The quantities of reagents, times of shaking, etc., had to be decided arbitrarily, and where possible are on the same basis as those used by other workers.

In the colorimetric estimation of cobalt, the same amount of sulphuric acid was added to the standards as to the actual estimation to ensure comparable conditions. It was found that in all grades of sulphuric acid available (including glass distilled) there was a small amount of some

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material which caused decreased colour formation. Digestion of the sulphuric acid with a little perchloric and nitric acids completely removed the interfering substance.

Chloroform was purified according to the procedure of Bambach and Burkey (1942).

Extraction of Iron with Cupferron.

Estimation of iron with dipyriddy directly on aliquots from a wet digest sometimes gives low results (presumably due to phosphates). This can be overcome by separation of the iron as sulphide (Jackson, 1938) or by checking the colour intensity again after twenty-four hours (Koenig and Johnson, 1942). The second method is not considered desirable, while the first, although giving excellent results, is cumbersome and not selective. A simpler quantitative separation would be desirable not only for accurate estimation, but also because iron interferes in certain colour reactions, and may produce precipitates which absorb other elements. In these cases citrate is usually used to depress the ionization of iron, but complete removal would be preferable.

Cronheim (1942), in the estimation of relatively large amounts of cobalt (above 20 micrograms), suggests that iron could be precipitated first from a strongly acid solution as the cupferron complex but he gives no details. This complex is readily soluble in chloroform, but as far as is known, no studies have been made to determine the possibilities of using this property for quantitative separation of iron from biological ashes, although the method is widely used in general analyses (Baudisch, 1909).

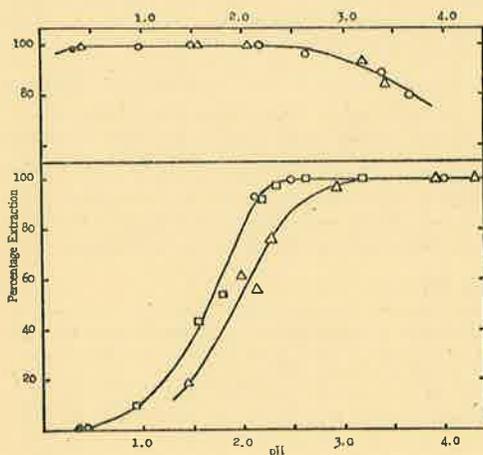


Fig. 1. Top curve. Extraction of iron as cupferron complex in CHCl_3 .

- No citric acid.
- △—△— 0.75 p.c. citric acid.
- 4.0 p.c. citric acid.

Bottom curves. Extraction of copper as cupferron complex in CHCl_3 .

- No citric acid. 200 p.c. excess cupferron.
- △—△— 0.75 p.c. citric acid. 50 p.c. excess cupferron.
- 0.75 p.c. citric acid. 200 p.c. excess cupferron.
- 4.0 p.c. citric acid. 200 p.c. excess cupferron.

also dissolve the Fe-cupferron complex and presumably would give similar extraction curves. There is a definite time factor involved in the reaction of iron with cupferron at pH values above 3; longer standing gives increased complex formation.

Extraction of Copper and Cobalt with Cupferron.

The extraction of copper from a solution of CuSO_4 containing 2 mg. Cu per 100 ml. was studied under exactly the same conditions. With 25 p.c. in excess of the theoretical amount of cupferron, poor recoveries were obtained, so excesses of 50 p.c. and 200 p.c. were used. The results are shown in Fig. 1. It will be noted that in the most acid solution containing 3 ml. H_2SO_4 per 100 ml., 0.8 p.c. of the copper is still extracted. Therefore, the quantitative separation of iron from copper does not appear to be possible.

The extraction of cobalt by cupferron has not been studied completely. With 20% Co in the presence of 3 ml. H_2SO_4 , 0.75 gm. citric acid per 100 ml. and ammonia to the required pH, no cobalt is extracted using 50 mg. cupferron and chloroform at pH values below 2. At pH 3-4 small traces (0.1%) are extracted. Using 200 mg. cupferron, a small amount of Co is extracted even at pH 1.4 and at pH 3-4 a large percentage of the cobalt passes into the chloroform. If, however a cupferron separation is done on solutions of pH 0.5 to 1.5 and containing 5 mg. Fe and 4% Co (with citrate and sulphate), incomplete recoveries of cobalt (around 90 p.c.) are obtained; this indicates that co-precipitation and subsequent extraction occur even at low pH values.

These experiments indicate that cupferron is not satisfactory for separating iron in the presence of copper or cobalt. Iron however, may be quantitatively separated from the wet digest after the removal of dithizone-extractable metals, by reacidifying, forming the cupferron complex, and extracting with $CHCl_3$ or CCl_4 . In this way iron may be separated from all materials which are likely to interfere with the determination by dipyriddy.

Extraction of Cobalt with Dithizone.

In the dithizone method of Marston and Dewey (1940), sulphate must be absent and the citrate concentration below 0.03 M. In the method of Parks *et al.* (1943) sulphate is absent and the citrate concentration is about 0.2 M, yet quantitative recoveries are obtained. Sylvester and Lampitt (1940) extract cobalt quantitatively in the presence of both sulphate and citrate. This difference in the effect of these two ions in the different methods has been confirmed. The only essential difference is that Marston and Dewey use sodium salts for neutralization and buffering, whereas the other workers use ammonia salts, and the difference in extractions must be due to this fact.

Although Marston and Dewey give complete pH-extraction curves for their technique, no curves seem to have been reported for the extraction using ammonium hydroxide and citrate for neutralization and buffering. Twenty % of Co were taken in a solution containing 3 ml. sulphuric acid, 1.0 or 4.0 gm. citric acid, and ammonia to the required pH. The volume was made up to 100 ml. and the solution shaken by hand for 2 minutes with 5.00 ml. of 0.025 p.c. purified dithizone in CCl_4 which had been standardized by extractive titration against silver sulphate (Fischer, Leopoldi and Uslar, 1935). After standing, a suitable aliquot of the CCl_4 was pipetted off for analysis. The results are shown in Fig. 2. It is apparent that quantitative recoveries are possible in the presence of sulphate and 0.2 M citrate, provided ammonia is used for neutralization. Small amounts of cobalt are extracted even at pH 4, although the quantities are much smaller than those recorded by Marston and Dewey. It should be pointed out, however, that the curves shown in Fig. 2 represent extractions with two-minute shakings, whereas the above workers give values approximating to equilibrium. The position of points below maximum extraction on the acid side will be raised by more intense or more prolonged shaking. A few extractions have been done using 0.025 p.c. dithizone in chloroform and the results confirm the findings of Marston and Dewey that the extraction curve is shifted considerably to the right, and that no cobalt is extracted at pH 3 if chloroform is used as the solvent, even with more concentrated solution of dithizone.

Parks *et al.* (1943) separate zinc from cobalt by shaking the alkaline dithizone extract with 0.02 N hydrochloric acid. The cobalt remains in the CCl_4 phase, but as has been pointed out by Walkley (1942) the decomposition of zinc dithizone by dilute acid is slow and incomplete. The use of stronger acid (up to 0.1 N) gives better recoveries of zinc without removal of cobalt. Although not strictly quantitative, the separation is sufficiently complete for ordinary use. The nitroso-naphthol separation described in the next section is preferred however, as it is more specific.

Separation of Cobalt with 1-Nitroso-2-Naphthol.

Although it has been known for many years that the cobalt complex of 1-nitroso-2-naphthol is soluble in chloroform, this fact does not seem to have been used to separate cobalt from biological ashes, apparently because iron and copper form similar complexes. The following scheme was evolved for separating cobalt from the alkaline-dithizone extract, which may contain Zn, Pb, Co and probably Ni, Bi and Cd.

Digest the dithizone extract with H_2SO_4 and $HClO_4$, dilute, add 10 p.c. citric acid and ammonia to pH 6 (brom-cresol purple), warm to 60° C. or more, transfer to a separating funnel and add 0.2 ml. of 2.5 p.c. nitroso-naphthol in acetone. Allow to stand for thirty minutes or more, and extract the cobalt complex with chloroform. This method is slightly cumbersome, but the separation is specific for cobalt and the reagent concentrations and pH are not critical.

This procedure can also be used with success on biological ashes, provided copper is first removed at pH 3 by dithizone. In the presence of adequate citrate the reaction with iron is extremely slight. There is some evidence that incomplete oxidation of the organic matter (as

sometimes occurs with liver tissue) may cause low results, but no difficulty has been experienced with well digested samples.

At this stage of the investigation a paper by Alexander and Taylor (1944) was noted in which the writers carry out a preliminary separation of cobalt, prior to zinc determination, by shaking the solution (containing citrate) at pH 8.2-8.4 with 0.05 p.c. nitroso-naphthol in chloroform. This technique appeared to be more convenient than the method then in use, so the extraction curve was studied. A solution containing forty γ Co, 3 ml. H_2SO_4 and 4.0 gm. citric acid was brought to the required pH with ammonia and the volume made to 100 ml. After saturating with chloroform and removing excess, 10.0 ml. of freshly prepared 0.05 p.c. nitroso-naphthol in chloroform were added and the separating funnel vigorously shaken for two minutes. After standing, a suitable aliquot of the $CHCl_3$ was pipetted off and the cobalt content determined. The results are shown in Fig. 3.

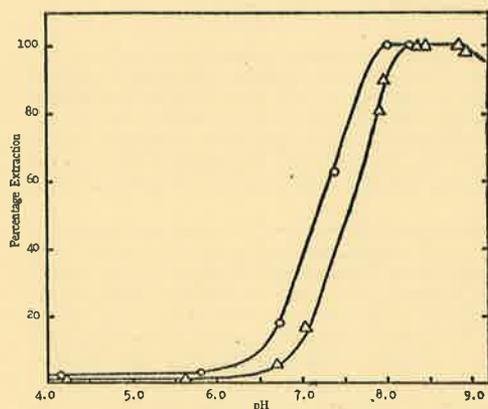


Fig. 2. Extraction of cobalt by dithizone in carbon tetrachloride.

—○—○— 1.0 p.c. citric acid.

—△—△— 4.0 p.c. citric acid.

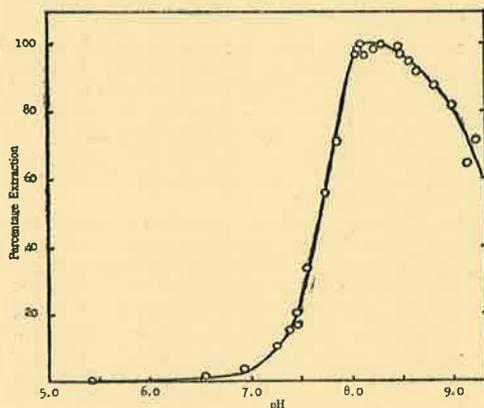


Fig. 3. Extraction of cobalt by 0.05 p.c. nitroso-naphthol in chloroform.

It will be noted that the extractions above about pH 8 tend to be erratic. The cobalt remaining after a single shaking reacts only partially when a second extraction is made. Apparently cobalt-ammonia complexes are formed which react very slowly with nitroso-naphthol at room temperatures. For maximum recoveries the pH must be close to 8.2 and it is practically impossible to obtain such accuracy with wet digest solutions using internal indicators. It is considered that although cobalt can be extracted by the method in its present form, the conditions for complete extraction are too critical for ordinary use.

It should be emphasized that these findings in no way invalidate the claim by Alexander and Taylor that their technique quantitatively separates cobalt from zinc. Any cobalt not extracted by nitroso-naphthol will remain in the CCl_4 phase when the zinc and cobalt dithizonates are decomposed by dilute acid.

Carbon tetrachloride gives a maximum extraction at a lower pH, but it is not satisfactory as the solubility of the cobalt complex in this solvent is extremely low.

SUGGESTED SCHEME FOR THE SEPARATION OF TRACE ELEMENTS.

A method for the estimation of 12 elements on one sample has been put forward by Parks *et al.* (1943), but their scheme for the trace elements has not been followed for two main reasons. As has already been pointed out, the separation of zinc and cobalt is not quantitative. These writers also do a number of their estimations with selective reagents in the presence of relatively large amounts of other elements. This technique is time-saving, and in some cases

cannot be avoided, but it may not always give reliable results, particularly on materials of unknown composition. In such cases it is highly desirable to separate the element to be estimated from the other elements and radicals present.

The following tentative scheme of separation makes no claim to cover all trace elements, but is adequate for most deficiency investigations. The separations are quantitative and the reagent concentrations and pH values are not critical. As far as the method has been tested it has given completely satisfactory results. All reagents must of course be "free" from the elements to be tested and "blanks" should be done with each batch.

Digest the sample (usually 10 gm.) with 3 ml. H_2SO_4 , 2-3 ml. $HClO_4$ (70 p.c.) and nitric acid (about 70 ml. for 10 gm. samples). Add additional HNO_3 and $HClO_4$ until oxidation is complete. With liver samples the digest frequently takes on a brownish tint after the perchloric acid has been fumed off; it is usually necessary to drop a mixture of HNO_3 and $HClO_4$ (2:1) on to the fuming sulphuric acid in order to oxidize completely the last traces of organic matter.

If the material is very high in iron (above about 10 mg. Fe) dilute with 8 N HCl and extract the bulk of the iron with isopropyl ether saturated with HCl. Boil off the HCl and take down to fumes. This stage is not necessary with ordinary materials.

Dilute with a little water and add a few drops of SO_2 solution to remove any material likely to oxidize dithizone. Boil to remove excess SO_2 . Add a slight excess of bromine water, allow to stand a few minutes then boil off excess bromine. If the material is high in Ca, or if Pb is to be estimated, add 5 ml. 20 p.c. HCl to assist solution of the sulphates. To the warm solution add 10 p.c. citric acid (as ammonium citrate), sufficient to prevent precipitation at pH 8; for unknown material 40 ml. is used, but if this can be reduced to 20 or even 10 ml. more rapid extraction of cobalt and zinc will be obtained. Add a minimum amount of brom-phenol-blue indicator and partly neutralize with ammonia. Warm to ensure complete formation of ferric citrate, and filter through an acid-washed filter paper. Bring to pH 3 with ammonia (check externally with brom-phenol-blue). Cool if necessary and extract copper completely with 0.2 p.c. dithizone in chloroform. Evaporate the chloroform, digest the residue with H_2SO_4 and $HClO_4$ and estimate the copper as the dithiocarbamate complex.

If cobalt and iron only are to be extracted, brom-cresol-purple indicator is added to the solution which is brought to pH 6 (definite purple) with ammonia. If however, zinc and lead are to be extracted afterwards, this indicator will mask the phenolphthalein colour change at that stage; in this case the indicator must be used externally (between 6.5 and 8.5 ml. of 5 N ammonium hydroxide will be needed to bring the solution to pH 6). Warm to 60° C. or more, transfer back to the separating funnel, add 0.2 ml. of 2.5 p.c. nitroso-naphthol in acetone. Allow to stand 30 minutes or more and extract with three lots of 5 ml. $CHCl_3$ (one minute shakings). Combine extracts, evaporate, digest thoroughly with 0.5 ml. H_2SO_4 and a few drops of $HClO_4$ and HNO_3 ; estimate cobalt as the nitroso-R-salt complex. To the aqueous solution after the removal of cobalt, add ammonia to pH 8.2-8.4 (phenolphthalein). It is probable that nickel can be extracted quantitatively at this stage using dimethyl-glyoxime and chloroform (Alexander and Taylor, 1944) but this point was not investigated. Extract zinc and lead with dithizone in CCl_4 ; these elements may be estimated by standard procedures. Acidify slightly and wash out excess dithizone with $CHCl_3$ or CCl_4 ; add thymol blue indicator and HCl or H_2SO_4 to pH 1-2. Boil up with a little bromine water to ensure that all iron is in the ferric state. Cool thoroughly, add excess cupferron, allow to stand 10 minutes or more; extract with $CHCl_3$ or CCl_4 . Add more cupferron to ensure complete complex formation, allow to stand and re-extract. Evaporate the solvent and digest the residue cautiously with 0.5 ml. H_2SO_4 and a few drops of $HClO_4$ and HNO_3 . If a preliminary isopropyl ether extraction has been done, combine extracts and estimate the iron by dipyriddy or other standard method.

If cobalt only is to be estimated, dithizone is the more convenient reagent, provided there is no danger of precipitates forming at pH 8.4 and provided that the quantities of zinc or lead are not excessive. If these conditions exist, nitroso-naphthol should be used.

SUMMARY.

The extraction of copper, iron, and cobalt by solution of the cupferron complex in chloroform has been studied at pH values between 0.4 and 4. It was not found possible to separate quantitatively iron from copper and cobalt.

Data are given for the extraction of cobalt by dithizone in carbon tetrachloride and by 1-nitroso-2-naphthol in chloroform at various pH values.

A scheme for the separation of "trace elements", largely based on existing procedures, is given. Copper is extracted by dithizone in chloroform at pH 3, cobalt at pH 6 by nitroso-naphthol and chloroform, and zinc and lead at pH 8.4 with dithizone in carbon tetrachloride. After reacidifying to pH 1-2, iron is extracted by chloroform or carbon tetrachloride as the cupferron complex.

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The Relation between Colour and Chemical Composition in Soils.

By A. B. Beck,* M.Sc.

Summary.

An attempt has been made to study the relationship between the colours of 32 soils, and the calcium carbonate, humus, and free iron oxide contents.

The method of Drosdoff and Truog for the estimation of free iron oxide does not appear to give an absolutely sharp distinction between free iron oxide and iron combined as silicate.

Calcium carbonate has the effect of darkening the colour due to humus in certain soils.

The yellow colour of some podsollic subsoils cannot be removed by a treatment which removes free ferric oxide, and appears to be intrinsic to the mineral colloid of the soil.

In grey, black, and white soils, the free iron oxide is probably very low, while in normal brown and red soils, free ferric oxide and humus are the predominating colouring materials.

In certain red soils of basaltic origin, the colour seems to be due in part to the presence of a brown complex iron silicate.

1. Introduction.

It is generally considered that the colours of soils are due to two main factors, the so-called "humus," which is responsible for a black or dark-brown colour, and free ferric oxide, which is assumed to give a red or yellow colour according to the degree of hydration. Certain other minor constituents such as magnetite, pyrolusite, and ferrous compounds probably contribute to the colour of certain soils, and it is also recognized that some soils with a high lime status have rather a darker colour than would be expected from their humus content. It has apparently been tacitly assumed that iron silicates are of very minor importance in determining the colour of soils. Any attempts in the past to correlate soil colour and chemical composition have been hindered by the lack of a chemical method of estimating free ferric oxide, most methods being too drastic and resulting in the decomposition of silicates.

In 1935, Drosdoff and Truog (1935) brought forward an entirely new method for the estimation of iron oxide based on its reaction with H_2S to give an easily soluble iron sulphide, a reaction which has been applied for many years in the purification of coal gas. The writers claimed that iron silicates remained unattacked. The present project was made possible by the application of this new method, and was an attempt to correlate the chemical composition with the colour of 23 soils which had been chosen by the Division of Soils as representing a range of typical colours likely to be encountered during soil surveys. A number of additional soils were also investigated to obtain further

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information on the analytical method for free iron oxide. The soils used had all been taken as type samples in soil surveys, and a considerable amount of chemical and physical data was already available. The present investigation was confined to the estimation of free ferric oxide, humus, and, in some cases, manganese oxide.

2. Experimental.

In preliminary experiments on Waite Institute soils, it was found that, using Drosdoff and Truog's original method in which the soil was allowed to react with the H_2S for 30 minutes, the residues were still definitely red to red-brown in colour. A second treatment brought out a considerable amount of ferric oxide, and the residues were of a very pale colour. It was considered that the residual red colour after the first extraction was due to unattacked ferric oxide, and, accordingly, it was decided to increase the time of reaction with H_2S to six hours. This period is more or less arbitrary, but, from the experiments with this type of soil, an extraction of six hours was necessary to give reasonably pale residues and hence a complete extraction of the free ferric oxide. It was realized that decomposition of complex silicates containing iron might also be taking place, but, as it was desired to obtain the colour of a soil free from iron oxide, it was considered better to have all the ferric oxide removed even if small proportions of the silicates were decomposed. In some cases where the residue was still strongly coloured after six hours, a second extraction was given. The significance of the residual colours is discussed later.

The following modified method was adopted for the determination of free ferric oxide:—5 gm. of the air-dry soil was treated in the cold with sufficient N/20 HCl to decompose carbonates and humates. With soils rich in carbonates, N/10 acid was used. The acid was decanted off after centrifuging, and the residue washed several times with water. The residue was transferred to a beaker and sufficient 100 vol. hydrogen peroxide added to make the suspension equivalent to 20 vols. per cent. The suspension was allowed to stand on a steam bath for one hour (or longer if necessary) to decompose and bleach the organic matter. The suspension was centrifuged and washed once, potassium nitrate being added as a coagulant if necessary. The residue was then treated on a steam bath with 150 ml. of 2 per cent. sodium carbonate to remove colloidal silica. The suspension was then centrifuged and washed several times with the sodium carbonate solution; the soil was left as the sodium-complex so as to facilitate dispersion. After centrifuging, the residue was hand dispersed with a rubber pestle and mortar, 2 ml. of N ammonia added, and the volume made up to about 200 ml. The suspension was dispersed by shaking overnight in end-over-end shakers and transferred to a 500 ml. Erlenmeyer flask, saturated with H_2S (20 mins.), and agitated for six hours. The iron sulphide thus produced was dissolved with 0.1 N hydrochloric acid as described by Drosdoff and Truog, and the iron estimated by cupferron or permanganate. When a second extraction was to be done, the residue was hand dispersed with 4 ml. N ammonia and 200 ml. water, saturated with H_2S , and allowed to shake overnight. The iron sulphide was dissolved as before. The sulphur in the residue was removed by means of an alcoholic solution of carbon disulphide.

The humic matter of the soils was determined by the method of Eden (1924) who extracts an acid-treated soil with hot 10 per cent. caustic soda. A solution of Merek's "Acidum Humicum", as standardized by the method of Hoffmann (1933), was used for comparison. It will be noted in the following tables that the percentage of humic material, hereafter simply termed "humus," represents only a small proportion of the total organic matter as indicated from the figures for organic carbon.

The results, together with chemical and physical data compiled from the Division's records, are set out in Tables 1-4.

The standards of colour and the corresponding Ridgway standards have been briefly discussed by Taylor (1935). The colours of the samples, and also of the residues, after the various treatments were assessed by Mr. J. K. Taylor.

The term "off" after a colour indicates that the colour is not quite the same as the standard colour.

In the other columns, the figures in the "Free Fe_2O_3 " column represent the ferric oxide from each extraction as numbered. The colour given in the "Colour after removal of free Fe_2O_3 " column represents the colour after removal of CaCO_3 , humus, and Fe_2O_3 . All analytical figures are for air-dry material.

3. Discussion of Results.

The first point of interest is that appreciable amounts of silica are extracted from the soil along with the iron oxide. Two parallel estimates were made on soil U153, in one the complete extraction was done, and in the other the treatment with H_2S was omitted, the dispersed soil being simply treated with N/10 HCl . No iron was extracted in this second treatment, but the amounts of silica and alumina extracted in both cases were the same, hence in this case at least, the silica dissolved arose from the action of the dilute hydrochloric acid on the aluminium silicates and not from decomposed iron silicates.

Considering the data of the grey and black soils (Table 1), perhaps the most outstanding feature is the effect of the small percentage of calcium carbonate on the colour of soils 876 and 2328 as set out in Table 4. These soils were originally dark grey and grey-black respectively, and after the removal of calcium carbonate alone the colours become light grey and grey respectively. The intensifying effect of the carbonate is shown by a comparison of soils 207 and 876. The first, an acid podsol, has five times as much humus as soil 876, and yet the first is only a "grey" as compared with a "dark grey" of the calcium carbonate soil. Robinson (1936) mentions the effect but offers no suggestion as to the cause. This may be due either to an increased dispersion of the humus, or to an increased ionization of the humic acid as the calcium salt, or to both reasons. The fact that the residues, after removal of calcium carbonate and humus, have no visible red or brown tint, and the observations that small amounts of ferric oxide have a strong tinctorial effect, suggest that with the soils of Table 1 the iron oxide extracted by the treatment is primarily derived from silicates.

The yellow soils (Table 2) gave some interesting results. The treatment of soils 4242 and 2014, both podsollic subsoils, brought out definite amounts of iron oxide without appreciably altering the colour. Soil 3941 showed a similar effect, although the presence of fine limonite renders this observation of less value. It is considered that the yellow colour in these soils is intrinsic to the soil colloid, and is not due to free hydrated ferric oxide. Further work on the separated mineral colloid of yellow podsollic subsoils would give further information along these lines. It is considered that the iron oxide extracted from 4242 and 2014 is derived from the decomposition of silicates.

Passing on to red and brown soils in Table 3, we have soils in which the colour is probably largely due to free ferric oxide. The ferric oxide causing the colour is apparently present as a film over the soil particles, and the greater the percentage of clay and hence the greater the surface area, the greater the amount of oxide necessary to give it a definite colour. Thus 2299, a coarse sand, has only 0.71 per cent. ferric oxide, 1231, a loamy sand with 11 per cent. clay, has 1.42 per cent., and 1016, a heavy clay soil with 62 per cent. clay, has between 14 and 18 per cent. free iron oxide, and yet the colour of these three soils is substantially the same. It is probable, too, that once the coating of ferric oxide reaches a certain thickness, the colour will be unaffected by increased ferric oxide.

The colours of the residues after removal of free iron oxide call for comment. In most cases the colours are very pale and, where red or brown, are probably due to veins of ferric oxide running through sand grains and hence not accessible to the H_2S . This is true of all the soils derived from sedimentary deposits, but with some of the soils of basaltic origin (1016 and 1982) there is a very definite residual brown colour which is apparently due to some complex iron silicate. In soils 1218 and 3902 (also of basaltic origin), the fact that the humus free residues have little red in their colour makes it probable that most of the iron oxide extracted is from silicates.

4. Acknowledgments.

The writer wishes to acknowledge his indebtedness to Professor J. A. Prescott of the Waite Institute, who suggested the above investigation, and who gave much helpful advice and criticism; also to Mr. J. K. Taylor for the assessment of the colours of the soils and the residues after treatment.

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TABLE 1.—WHITE, GREY, AND BLACK SOILS.

Soil No.	Colour.			Humus.	Free Fe ₂ O ₃ .	Clay.	pH.	Other Data.
	Original.	After Removal of—						
		CaCO ₃ and Humus.	Free Fe ₂ O ₃ .					
2519	Very light grey to white	*	Very light grey ..	% 0·10	% 0·11	% 49·9†	..	McGillivray, Kangaroo Island. 30–42". CaCO ₃ 62·2 per cent.
207	Grey	Very light yellow grey	White (off) ..	1·06	0·56	14·2	5·2	Myponga, S.A. 0–9". Contains unhumified organic matter
1917	Light grey ..	White (off) ..	White to very light grey	0·18	0·24	4·1	5·7	Kuitpo, S.A. 0–12". N. 0·03 per cent. Contains unhumified organic matter
876	Dark grey ..	White, slightly yellow*	White	0·22	0·49	56·0	8·6	Wimmera, Vic. 0–12". Org. C. 0·50 per cent; CaCO ₃ 3·7 per cent.
2328	Grey black ..	White (off)* ..	White (off) ..	0·35	0·23	30·4	8·9	Madras, India. Surface. Org. C. 0·32 per cent.; CaCO ₃ 5·2 per cent.; Mn ₂ O ₄ 0·053 per cent.
1473	Black	Light grey ..	White (off) ..	3·0	0·42	59·0	8·4	Glenlossie Swamp, River Murray. 0–12". Mn ₂ O ₄ 0·11 per cent.; CaCO ₃ 0·06 per cent.

* See Table 4 for the effect of removal of CaCO₃ alone.

† NaBrO method.

TABLE 2.—YELLOW AND LIGHT BROWN SOILS.

Soil No.	Colour.			Humus.	Free Fe ₂ O ₃ .	Clay.	pH.	Other Data.
	Original.	After Removal of—						
		CaCO ₃ and Humus.	Free Fe ₂ O ₃ .					
4242	Light yellow ..	Very light yellow	(1) Light yellow (2) Very light yellow	0·50*	(1) 0·47 (2) 0·48	39·2	4·9	Denmark, W.A. 24-36"
2014	Yellow ..	Yellow brown to yellow	(1) Yellow to light yellow (2) Yellow to light yellow	0·02	(1) 0·78 (2) 0·78	36·6	5·7	Kuitpo, S.A. 16-27"
3941	Light brown, some yellow	Light yellow brown	Light yellow ..	0·19	3·17†	27·3	6·3	Denmark, W.A. 19-27"
2598	Grey yellow ..	Light yellow to yellow	(1) Very light grey yellow (2) Light grey, slightly yellow	0·13	(1) 0·86 (2) 0·67	52·9	7·5	Willalooka, S.A. 6-20". Contains unhumified organic matter
3711	Yellow-brown ..	Light yellow brown	(1) Very light yellow (2) .. (3) Very light grey to white	0·09	(1) 1·40 (2) 0·98 (3) 0·28	42·6	8·4	Marabel, S.A. 14-27". Org. C. 0·22 per cent.
1827	Light brown ..	Light brown ..	Buff	0·06	0·54	21·7	8·6	Renmark, S.A. 12-27". CaCO ₃ 9·8 per cent.
4550	Light grey brown (slightly off)	Very light yellow brown	White, slightly yellow	0·01	0·50	24·0	8·3	Curlwaa, N.S.W. 18-30". CaCO ₃ 0·85 per cent.

* Soluble in acid 0·13 per cent. Soluble in alkali 0·37 per cent.

† This soil contained much nodular limonite. The coarser fraction was sieved out before using, but the sample used still contained much fine limonite.

TABLE 3.—RED AND BROWN SOILS.

Soil No.	Colour.			Humus.	Free Fe ₂ O ₃ .	Clay.	pH.	Other Data.
	Original.	After Removal of—						
		CaCO ₃ and Humus.	Free Fe ₂ O ₃ .					
1231	Red	Light red brown to red brown	Light grey brown	0·28	1·42	11·1	5·8	Roto, N.S.W. Mallee: Surface
1016	Red, stronger than 1231	Red brown to red	(1) Brown, slightly red (2) Brown	0·65	(1) 14·2 (2) 4·5	63·4	5·4	Basaltic loam. Woolongbar, N.S.W. 18–27". Mn ₃ O ₄ 0·08 per cent.; org. C. 1·44 per cent.
	Red	(1) (2) Brown, slightly red	..	(1) 13·4 (2) 5·9	100·0	..	Clay separated from 1016. Total Fe ₂ O ₃ 29·6 per cent.
2299	Red brown to red	Red brown to red	Light brown ..	0·06	0·71	7·7	..	Gibson's Desert. Surface. Coarse sand, 62·0 per cent.
3816	Dark red to red	Light red ..	Very light brown	0·35	3·02	41·8	7·4	Yarcowie, S.A. 5–14". Org. C. 0·58 per cent.
1982	Chocolate ..	Dark red brown*	(1) Brown .. (2) Brown ..	1·04	(1) 7·3 (2) 2·8	31·3	8·4	Atherton Tableland, Q. Surface. N. 0·015 per cent.; Mn ₃ O ₄ 1·78 per cent.
2776	Light red brown	Light brown, slightly red	Very light yellow brown to buff	0·31	0·52	12·9	..	Berri, S.A. 33–57". CaCO ₃ 1·19 per cent.
2818	Dark red brown	Light red brown	Very light grey brown	0·26	1·03	10·9	8·1	Berri, S.A. 0–15"
3496	Brown ..	Brown to light brown	Buff	1·50	1·62	17·4	6·4	Waite Institute. Surface: N. 0·115 per cent.
3467	Grey brown ..	Light brown to light yellow brown	White (very pale buff)	0·07	0·62	49·1	..	Mirrool, N.S.W. 27–42"

* Also after removal of Mn₃O₄.

TABLE 3.—RED AND BROWN SOILS.—*continued.*

Soil No.	Colour.			Humus.	Free Fe ₂ O ₃ .	Clay.	pH.	Other Data.
	Original.	After Removal of—						
		CaCO ₃ and Humus.	Free Fe ₂ O ₃ .					
3635	Dark brown ..	Brown to light brown	Light grey to light grey brown	0·73	1·23	27·0	..	Mirrool, N.S.W. 0–10". CaCO ₃ 2·13 per cent.
3902	Dark grey brown	Light grey to grey*	Very light grey ..	3·1	2·10	48·1	..	Bundaberg, Q. 0–6". Mn ₃ O ₄ 0·03 per cent.; N. 0·19 per cent.; CaCO ₃ 0·65 per cent.
1218	Very dark brown	Grey brown, slightly light brown*	(1) Light grey brown (2) Grey to light grey brown	3·6	(1) 4·9 (2) 1·4	41·5	7·3	Ilparran, N.S.W. 0–9". Alluvial, derived from basalt. Mn ₃ O ₄ 0·37 per cent.; org. C. 3·06 per cent.
U151	Dull brown ..	Brown to light brown	(1) (2) White to very light grey	1·53	(1) 1·62 (2) 0·52	18·0	6·0	Waite Institute profile, 0–4". Org. C. 1·32 per cent.
U152	Dull brown ..	Brown to light brown	(1) (2) Very light grey	1·02	(1) 1·88 (2) 0·62	21·8	5·8	4–9". Org. C. 1·07 per cent.
U153	Brown ..	Light red brown to brown	(1) (2) Very light grey	0·59	(1) 3·71 (2) 0·97	47·4	6·3	9–18". Org. C. 0·76 per cent.
U154	Brown, slightly red brown	Light red brown to brown	(1) (2) Very light grey	0·41	(1) 3·89 (2) 1·28	59·9	6·7	18–27". Org. C. 0·67 per cent.
U155	Brown ..	Light red brown to brown	(1) (2) Very light grey	0·45	(1) 3·70 (2) 1·02	59·2	7·2	27–36". Org. C. 0·66 per cent.; CaCO ₃ 0·13 per cent.
U156	Dull brown ..	Brown to light brown	(1) (2) Very light grey	0·34	(1) 2·30 (2) 1·12	40·7	8·4	36–45". Org. C. 0·41 per cent.; CaCO ₃ 4·8 per cent.
U157	Dull brown ..	Brown to light brown	(1) (2) Very light grey	0·26	(1) 2·22 (2) 1·14	37·2	8·4	45–54". Org. C. 0·30 per cent.; CaCO ₃ 3·96 per cent.

* Also after removal of Mn₂O₃.

TABLE 4.—SOILS SHOWING COLOUR CHANGES AFTER THE REMOVAL OF CALCIUM CARBONATE ALONE.

Soil.	Colour.			CaCO ₃ Per cent.
	Original.	After removal of—		
		CaCO ₃ .	CaCO ₃ and Humus.	
876	Dark grey ..	Light grey ..	White, slightly yellow	3·7
2328	Grey black ..	Grey	White (off) ..	5·2
2519	Very light grey to white	Grey to light grey	62·2
2776	Light red brown	Brown to light brown, slightly golden	Light brown, slightly red	1·2

NOTE.—In all other calcareous soils and No. 1473 no colour change was noted on the removal of the CaCO₃.

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LEAFLET No. 569



DEPARTMENT OF AGRICULTURE.

1939.

**THE COMPOSITION OF CAPEWEED (*Cryptostemma calendula-
ceum*) FROM MECKERING AND BEVERLEY.**

A. B. BECK¹ and R. G. LAPSLEY.²

During the investigations of toxic paralysis carried out over the past three years, it has been noticed that with sheep, the occurrence of depraved appetite, and hence of toxic paralysis, is definitely associated with a low plane of nutrition due to the poor quality of the summer grazing. At Meckering, where the toxic paralysis investigations were carried out, the main plants of unimproved pastures are capeweed, silver grass and cluster clover. The analysis of the capeweed samples was undertaken to see if there was any abnormality in the chemical composition which might be correlated with the development of depraved appetite. For the purpose of comparison, plants were also analysed from the Avondale Research Station, Beverley, an area of heavier soils and better pastures, where depraved appetite is unknown. The work was also undertaken as a part of a wider investigation. Owing to the short growing season in the wheat belt areas, the natural pastures consist entirely of early maturing annuals. The quality of these pastures is of the greatest importance, in that the dry feed which the sheep receive in the summer is usually composed entirely of the dry residues of annual plants which have grown during the winter and spring. As a continuation of the pasture investigations commenced between 1933 and 1935 (Underwood, Shier, and Harvey, this Journal, December, 1937, page 442), it is the intention of the Animal Nutrition Branch to carry out a "long range" investigation of the subject in order to provide a basis on which to improve our pastures, and so to improve the nutrition of our sheep.

Samples were taken at intervals of from three to four weeks so as to ascertain the changes in composition during the growing season.

The samples from Meckering were obtained from a large paddock of about 100 acres in the Research Station property; half of this paddock had been cleared and cropped in past years. In the cleared area, from which all the samples were taken, there were two distinct soil types, a red sandy loam which comprised the larger part of the area, and also a small area of 4-5 acres of deep, grey sand. Normally when sheep were running in this paddock they had access to both areas. The capeweed

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was the main plant in the sand area, and was more sparse, although larger, on the loam. Except where specified to the contrary in the following tables, the sample analysed was a composite sample taken from both areas.

At Beverley two series of samples were taken. The first was from around an oat crop on typical York gum soil; this, as is usual, formed a large succulent growth and probably represents the best type obtainable. The second series was obtained from a paddock under grazing. This paddock was of a more sandy type of soil than that of the oat crop, and because of this and the grazing the plants kept quite small.

The sample from Merredin was taken from the typical heavy salmon gum and gimlet soil at the Research Station.

Experimental Methods.

The collection, sampling and analysis of the samples presented many rather peculiar problems, and standard methods had to be more or less arbitrarily adapted for this work.

The sample for analysis was obtained by picking a large number of plants from the area concerned until a sample of one to two lbs. green weight was obtained. The plants were picked up at random places over the area and were selected as average samples from that particular place.

Samples were obtained by cutting off whole plants with curved scissors just above the ground. In most cases capewood grows close to the ground and the dense felt of hairs on the back of the leaves collects and retains the sand from the soil with great tenacity. Sieving the dried plants does not remove this sand, so for all the samples prior to the commencement of flowering, the plants were washed to remove the sand. It is realised that this procedure is open to the objection that there will be a certain loss of cell sap by diffusion, but as the washing only takes a very short time, it was considered that the error from this cause would be very small and certainly very much less than the error caused by the presence of the sand.

After washing, the plants were allowed to air dry to as near as could be judged to their original condition and then weighed in order to give their original moisture percentage. The samples were then dried at 60-70° C. and then allowed to come to an "air-dry" condition for crushing and analysis.

The earlier samples were crushed in a small coffee mill, but later a Wiley mill was installed and this was then used. In some cases during the air-drying process after oven drying, the plants took up so much moisture that it was necessary to use dried samples for crushing. In both types of crushing there was a certain amount of segregation of the woolly fibres from the back of the leaf, but with the product from the Wiley mill this was small.

Standard methods of analysis were used. "Moisture" is the loss on drying at 100° C. overnight. Ashing was done at a dull red heat, and the portion insoluble in 1:1 hydrochloric acid was termed "insoluble ash" (see later). The "ether extract" was done on moisture-free samples using ethyl ether. The crude fibre was filtered on a No. 54 filter paper. The appearance of the "crude fibre" from the younger samples was quite remarkable, the fibre consisting of the hairs from the back of the leaves and appearing as a felt-like mass. Phosphorus was determined volumetrically and in some cases colorimetrically as well; the agreement was not always as close as might be desired.

As is seen in the following table the "insoluble ash" of some of the samples is quite high. A microscopic examination of the product suggested that it consisted entirely of coarse sand grains, and so it was decided to correct all the analytical figures for this sand, using the assumption that the "insoluble ash" was entirely adventitious sand. This assumption is open to two possible errors; firstly the "insoluble ash" may contain plant silica, this is considered unlikely as the appearance of

this ash showed nothing but sand and further an analysis showed that the "soluble silica" of capeweed ash is very low (about 1 per cent. of the total ash). The high base content of the ash would further tend to give acid soluble silicates during ashing. The second objection is that the insoluble ash may represent a part only of the adventitious sand and clay, as the high alkali would tend to convert the finer portions of these into soluble silicates. This objection, however, is not considered to be serious and it is considered that the method of tabulation of results will probably give the truest picture of the composition of the plants. Another reason in favour of this method is that the presence of coarse sand in the lighter capeweed makes sampling very difficult and even if the sand content varies the corrected figure will be unaltered.

In the following tables the "moisture" figure is of the sample as collected from the field, corrected for "insoluble ash." The "insoluble ash" figure is for the sample actually analysed, calculated on a moisture-free basis. All other figures are for the "moisture-free assumed ash" basis. These have been calculated from the actual analytical figures (moisture-free) as follows:—

$$\frac{\text{"moisture-free assumed ash" figures}}{\text{figures}} = \frac{\text{actual analytical "moisture-free" figures}}{\text{figures}} \times \frac{100}{100 - \% \text{ "acid insol." ash actual dry basis.}}$$

As an example of the method of calculation, Sample No. 100 as analysed contained 13.3 per cent. moisture, 3.0 per cent. insoluble ash and 20.6 per cent. crude protein. Correcting these for moisture, the insoluble ash and protein then become 3.5 per cent. and 23.8 per cent. respectively. To correct the protein figure for the insoluble ash it is multiplied by $\frac{100}{100 - 3.5}$; the protein content on a "moisture-free assumed ash" basis then becomes 24.7 per cent.

In all cases except where specified to the contrary the analyses refer to whole plants collected as previously described.

TABLE 1.
MECKERING.

Date.	Sample No.	Moisture.	Assumed Ash Dry-Basis Figures.							Insol. Ash.	Remarks.
			Crude Protein.	Ether Extract.	Crude Fibre.	N.F.E.	Assumed Ash.	CaO.	P ₂ O ₅ .		
8-6-37	77	92.6	27.5	3.8	13.2	39.3	16.2	2.15	0.55	1.4	Specially selected; ungrazed; best growth available.
2-7-37	89	93.3	30.2	4.9	13.9	32.1	18.9	1.75	0.98	2.2	
23-7-37	97	89.7	17.2	4.4	13.9	49.2	15.3	1.87	0.70	0.2	Sample from red loam area only.
17-8-37	132	86.5	11.4	3.0	13.0	62.1	10.5	1.79	0.43	0.6	
17-8-37	133	86.9	11.5	3.3	14.5	59.8	10.9	1.42	0.36	1.3	Sample from grey sand area only.
31-8-37	140	82.1	7.9	2.4	12.7	68.1	8.9	1.58	0.48	3.6	Some flowers out; many flowering heads.
13-9-37	142	84.2	8.0	3.7	14.4	65.6	8.3	1.65	0.65	2.9	In full flower; bottom leaves drying off.
29-9-37	152	81.0	6.9	6.0	19.3	57.0	10.8	2.14	0.50	1.7	Leaves drying; still in flower. No seed heads shed.
13-10-37	160	79.8	5.9	4.9	22.7	54.7	11.8	2.14	0.35	1.9	Flowering finished; many seed heads, large proportion shed; bottom leaves dry.
3-11-37	169	10.0	6.2	5.4	25.2	57.8	5.4	2.34	0.32	6.6	All dry.
31-8-37	139	84.8	10.6	5.5	14.7	60.1	9.1	1.76	0.71	1.9	Flowers with about 1/2 in. stalk; badly infested-red mite.
13-10-37	161	82.7	11.2	5.0	15.9	59.3	8.6	1.55	0.68	0.5	Flowers; all small.
Nov., 1936	176	7.5	13.2	1.41	1.22	Seeds.
13-10-37	162	10.4	11.3	3.9	8.4	1.53	0.97	0.7	Seeds.

Other analysis—No. 97: K₂O 3.45; Na₂O 2.74; MgO 0.59 per cent.

TABLE 2.
 BEVERLEY OAT CROP SAMPLES.

Date.	Sample No.	Moisture.	Assumed Ash Dry-Basis Figures.						Insol. Ash.	Remarks.	
			Crude Protein.	Ether Extract.	Crude Fibre.	N.F.E.	Assumed Ash.	CaO.			P ₂ O ₅ .
22-7-37	91	93.1	25.1	4.8	16.4	37.2	16.5	1.34	0.67	0.1	Stalks cut off largest samples. Contained a few flowers. A few flowers in sample. In flower. A few flowers, but mostly seed heads with seeds; bottom leaves showing slight signs of drying off. Stems moist, leaves dry. Plants quite dry. Flowering heads and about 2 in. stalk only. Seeds only.
13-8-37	100	92.7	24.7	4.0	14.6	42.5	14.2	1.63	0.71	3.5	
30-8-37	137	92.9	16.5	3.9	17.8	44.4	17.2	1.50	0.59	0.2	
16-9-37	150	91.0	15.2	5.7	16.7	48.2	14.2	1.69	0.43	0.4	
6-10-37	156	89.7	10.2	7.2	19.0	46.9	16.7	2.21	0.38	0.2	
19-10-37	165	87.4	10.0	3.5	20.6	50.3	15.6	2.00	0.35	1.2	
8-11-37	174	36.7	5.6	5.3	27.9	47.2	14.0	2.11	0.55	5.3	
23-11-37	177	7.2	6.5	3.8	27.0	50.0	12.7	2.70	0.31	1.7	
16-9-37	149	86.6	26.8	3.6	16.4	44.7	8.5	1.37	0.74	0.2	
19-10-37	164	8.8	11.1	6.3	1.74	0.91	0.1	

Other analyses.—No. 137: MgO 0.60 per cent.
 No. 100: Na₂O 3.50 per cent.; K₂O 4.86 per cent.

 TABLE 3.
 BEVERLEY STUBBLE PADDOCK SAMPLES.

Date.	Sample No.	Moisture.	Assumed Ash Dry-Basis Figures.						Insol. Ash.	Remarks.	
			Crude Protein.	Ether Extract.	Crude Fibre.	N.F.E.	Assumed Ash.	CaO.			P ₂ O ₅ .
21-5-37	76	93.1	26.0	4.3	14.4	37.7	17.6	1.85	0.88	9.6	Showing slight signs of grazing. Not grazed recently; flower heads showing. Some plants showing signs of grazing. Plants all flowering. Most seed heads empty; bottom leaves dry. Apparently all dry.
24-6-37	86	92.5	27.0	4.7	14.8	36.7	16.8	2.50	0.73	4.1	
22-7-37	96	88.8	17.0	4.2	15.8	49.7	13.3	2.22	0.55	3.1	
13-8-37	99	86.8	12.2	3.3	11.2	58.1	15.2	2.15	0.60	0.5	
30-8-37	138	85.7	9.5	3.4	15.0	62.1	10.0	1.90	0.50	3.7	
16-9-37	151	86.8	9.4	3.5	16.4	62.2	8.5	2.14	0.55	3.7	
6-10-37	137	86.9	10.3	4.9	18.4	55.5	10.9	2.16	0.47	1.3	
19-10-37	163	70.2	6.2	3.5	21.2	58.4	10.7	2.71	0.42	11.5	
8-11-37	175	24.0	5.7	4.5	23.5	56.9	9.4	2.40	0.45	9.4	

Other analyses.—No. 99: K₂O 2.18 per cent.; Na₂O 1.72 per cent.

MERREDIN SAMPLE.—No. 135, 27-8-37: Moisture 90.4 per cent, Crude Protein 19.8, Ether Extract 3.1, Crude Fibre 12.2, N.F.E. 50.5, Assumed Ash 14.4, Insoluble Ash 1.1, CaO 1.88, P₂O₅ 0.64, MgO 0.66 per cent.

Discussion of Results.

The analyses of the Meckering plants show no abnormality which would account for the development of depraved appetite in this area.

The most prominent feature of the analyses is the very high water content of the plants, which persists up to the time of flowering. This high moisture content probably explains the scouring and also the loss of weight that the sheep undergo when on the first green feed at Meckering. If the pasture were pure capeweed containing 92 per cent. water it would be necessary for a sheep to eat 25 lbs. green weight to obtain a dry matter intake of 2 lbs. The sheep apparently leave the dry grazing once the green feed appears and the sparseness of the growth of the early green feed makes the loss of weight easily understood.

If, however, we consider the dry matter only it is obvious that for the greater part of the season capeweed is a high quality feed. The high protein content in the early growth is quite outstanding and, even in the dry plants after the seeds have

been shed, the percentage of protein is as high or even higher than that of average wheaten chaff. Up to the time of drying off the fibre content is relatively low and even in the most fibrous sample (No. ~~100~~¹⁷⁴) can hardly be regarded as excessive.

The assumed ash forms a high percentage of the dry matter. It should be remembered that, in the samples analysed the bulk of the adhering sand has been removed, whereas in the case of the material eaten by the animal the product will usually contain a relatively high percentage of sand. The percentage of sand is greatest in the young growth, particularly after rain.

The high percentage of calcium, sodium, and potassium contribute further evidence against any suggestion that depraved appetite in sheep might be due to acidosis caused by alkali deficient foods. The calcium content is high and the increase of calcium content in the older samples is worthy of notice. In most samples the phosphorus content tends to be on the low side as judged by overseas standards.

It is hoped at a later date to do a feeding trial with some sheep using dry capeweed alone and thus to obtain further data on the feeding value. In many parts of the Western Australian wheat belt capeweed is apparently regarded as a valuable fodder. The rapid early growth makes it valuable as a first green feed even although it causes scouring. It is regarded as a fair to good feed during growth, but not fattening until it has dried off somewhat. These observations are strongly supported by the analytical data which indicate that the high moisture content give it a low feeding value in the green state, but on a dry basis it should be regarded as a fairly high quality feed.

As a contrast to Western Australian experience it is interesting to note that in South Australia capeweed is generally regarded as being unpalatable to sheep.

It is probable that the main advantage of capeweed as a pasture plant lies in the fact that it can flourish under conditions which prohibit the growth of better quality pasture plants. Although it has many advantages as a pasture plant, particularly for lighter soil areas, it also has several distinct disadvantages.

There is no evidence that the felt-like hairs or fibres on the back of the leaves cause any digestive troubles, but in some localities the internal fibres of the leaf and stem get between, and loosen the teeth of the sheep. The very succulent nature of the growth results in a low yield of dry matter per acre and the bulkiness of the plants enables it to crowd out more desirable species. When dry the capeweed tends to break up and blows away easily. Another important disadvantage has been suggested by the observations of Mr. K. R. Norris (private communication) which support the contention that capeweed favours the increase of Earth Mite (*Halotydeus destructor*, commonly known as red mite).

Although capeweed forms the bulk of the grazing on the lighter soils of the wheat belt and the analyses show that, on a dry basis, it is of good quality, yet, taking all facts into consideration it cannot be regarded as a good pasture species. Pasture improvement, including the introduction of legumes such as early subterranean clover, should do much to improve the present low-producing capeweed pastures in the better rainfall areas of the wheat belt.

Australasian Institute of Mining & Metallurgy
(INCORPORATED)

Report on the Effect of Lead
Salts and Alkalis in Cyanidation.

②

BY A. B. BECK AND H. W. GARTRELL.

②

(Proceedings New Series, No. 100 1935)



REPORT ON THE EFFECT OF LEAD SALTS AND
ALKALIS IN CYANIDATION.*

BY A. B. BECK† AND H. W. GARTRELL.‡

During investigations of the treatment methods for ore from the Bird-in-Hand gold mine carried out at Kalgoorlie¹ and at these laboratories,² it was found that excess alkali caused a lowering of extraction by cyanide, the cause of this being attributed to the presence of a small amount of oxidised lead compounds in the ore. The subject has been discussed by various writers,³ but no satisfactory explanation of existing facts has been put forward. The experiments described in this report have been carried out with the object of obtaining some insight into the chemical reactions underlying the effect of lead salts and alkalis in cyanidation.

The formation of cyanide-insoluble coatings on gold during roasting with lead compounds is well known, but the adverse effect of alkalis in cyanidation of ore is

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¹W. G. Clarke and B. H. Moore. C.S.I.R. Report, Kalgoorlie, 6th April, 1935.

²A. B. Beck and H. W. Gartrell, C.S.I.R. Report, Adelaide, 28th May, 1935, also S.A. Mining Review 62, 1935.

³C. M. Harris. Chem. Eng. Min. Rev., June, 1935, p. 327; V. T. Edquist *ibid* July, p. 360; R. J. Lemmon *ibid* July, p. 359.

apparently not common; two cases are reported by the Canadian Department of Mines,⁴ and the effect with an ore containing lead chromate is discussed by V. T. Edquist.³ An important contribution to our knowledge of the subject was made by Leaver, Woolf and Jackson.⁵ These writers find that above pH 11, alkali soluble lead compounds inhibit cyanidation, calcium hydroxide having a much greater effect than sodium, magnesium and ammonium hydroxides; if the pH is kept below 11 when using these last alkalis the lead compounds have no effect, but even below this pH lime inhibits cyanidation (normal 0.1% potassium cyanide has a pH of 10.3—10.4). The effect of lead salts is discussed by M. Mladenovic and V. Stajic,⁶ who attribute the decreased extraction to the formation of insoluble lead cyanide complexes.

Recently Barsky, Swainson and Hedley,⁷ while working with the rate of solution of pure gold foil in cyanide solution, have found that above pH 12 sodium hydroxide decreases the rate of solution, while lime has marked effects even below pH 11. The effect of calcium hydroxide is due to the combined effect of calcium and hydroxyl ions, as calcium chloride and sulphate have little effect.

The first tests carried out were cyanidations of table sands and slime (Bird-in-Hand ore) using various amounts of lime. These two products assayed 1.9 and 3.4 dwt. per ton gold, 0.21% and 1.60% lead and 0.02% and 0.19% copper respectively.

⁴Can. Dept. of Mines. Branch Reports, 1932, 736, 204, and 1934, 743, 111.

⁵E. S. Leaver, J. A. Woolf and T. A. Jackson. Amer.I.M.E., 1933, Contribution 5.

⁶Milutin Mladenovic and Vojislav Stajic, Bull. Soc. chim. roy. Yougoslav 1933, 4, 179. See American Chem. Abs., 1934, 28, 3035.

⁷G. Barsky, S. J. Swainson, N. Hedley, Trans. Amer.I.M.E., 1934, 112, 675.

TABLE 1.

CaO added.	% CaO in final solution.	Final alkalinity (normality)	CaO consumed, lb/ton	Condition of slime.	Assay tails, dwt.	Test No.
Sand:						
0 lb./ton	—	—	—	Peptized	0.1	52
1.5 "	0.007	0.003	1.26	Coag.	0.2	53
4 "	0.034	0.012	1.73	Coag.	0.6	82
Slime:						
0 lb./ton	—	—	—	Peptized	0.1	49
2 "	0.002	0.001	1.87	Coag.	0.1	50
4 "	0.002	0.001	3.84	Coag.	0.2	79
11 "	0.050	0.019	7.62	Coag.	0.6	44

Lemmon³ has suggested that slime precipitation may enter the question, but the fact that 2 lb. lime coagulates the slime without affecting the extraction disproves this hypothesis. In view of the more complex nature of the slime, no further tests were carried out on this product, all tests described in later sections being with the table sands.

The next experiments carried out were to ascertain whether the effect of the lime was due to the calcium or hydroxyl ions (c.f. 7). Samples were cyanided with caustic soda equivalent to 1.5 and 4 lb. lime per ton of sand, and with calcium sulphate equivalent to 4 lb. lime. The results are given in the following table:—

TABLE 2.

Added.	CaO Equivalent.	Normality of final solution.	Condition of slime.	Assay tails, dwt.	Test No.
NaOH . .	1½ lb.	0.005	Peptized	0.2	77
NaOH . .	4 lb.	0.017	Peptized	0.2	80
CaSO ₄ . .	4 lb.	—	Coag.	0.1	81

The fourth column gives the condition of the small amount of slime left in the sands; the fact that calcium sulphate coagulates this without affecting the extraction, further disproves Lemmon's hypothesis.

These results are in good qualitative agreement with those of Barsky, Swainson and Hedley,⁷ but subsequent results from cyanidation of material that had been acid leached indicated that it was the presence of the 0.21% lead which was the chief cause of the poor extraction.

A number of tests were carried out in order to ascertain the method of formation and nature of the residual 0.6 dwt. when using 4 lb. CaO/ton. These are given in the following table:—

TABLE 3.

No. Test	Procedure.	Alkalinity (normality) of final solution.	Assay tails (dwt.).
85	24 hrs. Cyanidation, 4 lb. CaO ..	0.013	0.6
86	48 hrs. Cyanidation, 4 lb. CaO ..	0.011	0.4
82	Ordinary KCN, 4 lb. CaO, 18 hrs. agitation. Initial 0.096% KCN. Final solution 0.079%	0.012	0.6
95	Strong KCN, 4 lb. CaO, 18 hrs. agitation Initial solution 0.151%. Final 0.134% KCN.	0.013	0.4
89	18 hrs. ordinary KCN, no CaO. 4 lb. CaO added and further 1 hr. agitation	0.013	0.1
83	As 89, but 18 hrs. agitation after CaO added	0.013	0.1
84	Cyanided 18 hrs. 4 lb. CaO. Solution removed and sand washed (Residue assumed 0.6 dwt.), then cyanided with KCN alone ..	—	0.1
90	200 g. sand cyanided 4 lb. CaO 18 hrs. (Note initial KCN accidentally too strong 0.139% KCN) then a further 200 g. and extra 1 lb. CaO added, but no KCN, and agitated further 18 hrs.	0.014	0.4

Summarizing we see that:—

- (1) The residual 0.6 dwt. is very slowly soluble in alkaline (CaO) cyanide;
- (2) Small variations in KCN strength will have no marked effect;
- (3) Once the gold is in solution, the addition of lime has no effect;
- (4) The residual 0.6 dwt. is readily soluble if no alkali is present;
- (5) After the first 1.3 dwt. has been dissolved the solution is still capable of dissolving more gold.

It was thought that these results might be explained by the presence of a calcium aurate coating the gold, but this was disproved by the following experiment. 800 gm. of the sand (assay 1.9 dwt.) were leached with hot normal hydrochloric acid; the product still assayed 1.9 dwt., but after cyaniding a portion of this product with 4 lb. lime per ton, residues were obtained assaying 0.1 dwt. Iron, zinc and lead were the only metals noted in any appreciable quantities in the leach liquor, so it is considered that the removal of the lead is responsible for the almost complete extraction. Of the total lead in the sands 57% is soluble in ammonium acetate, this portion apparently being present as anglesite and the remainder as galena. Although a large proportion of the lead is present as the sulphate, the amount found in solution after agitating 100 gm. samples (1:3 pulp) for 18 hrs. with lime (4 lb.), caustic soda (equivalent to 4 lb. CaO), and with lime plus cyanide and caustic soda plus cyanide, was in each case too small to be estimated by ordinary methods.

A number of tests were then carried out in order to check Leaver, Woolf and Jackson's observations and to obtain further information concerning the effect of lead salts.

TABLE 4.

Lead salt added.	% by weight of feed.*	Alkali added.	% by weight of feed.	Final solution normality	Assay tails (dwt.)	Test No.
Original Sand						
— Unleached.						
Hydroxide .	0.66	—	—	—	0.3	88
Hydroxide .	0.66	NaOH	0.08	0.004	1.2	98
Hydroxide .	0.66	NaOH	0.26	0.016	1.8	87
Sulphate ..	0.66	NaOH	0.38	0.009	1.7	97
Leached Sand.						
Hydroxide .	0.17	NaOH	0.07	0.004	1.4	106
Hydroxide .	0.17	CaO	0.05	0.004	1.7	108
Hydroxide .	0.17	CaO	0.09	0.007	1.6	110
Hydroxide .	0.17	NaOH	0.16	0.010	1.7	113
Hydroxide .	0.66	NaOH	0.08	0.005	1.7	105
Dioxide ..	0.17	NaOH	0.26	0.018	0.1	111
Dioxide ..	0.17†	NaOH	0.65†	0.045	0.2	114
Hydroxide .	0.17	NaOH	0.16	0.008	0.1	116
		Plus	Plus			
		H ₂ O ₂	0.62%			
Hydroxide .	0.17	NaOH	0.16	0.010	0.1	118
		Plus	Plus			
		H ₂ O ₂	0.15%			
Hydroxide .	0.17	CaO	0.09	0.005	0.2	120
		Plus	Plus			
		H ₂ O ₂	0.15%			

*These figures are expressed as percentage of metallic lead.

†The NaOH and PbO₂ were fused together with a few drops of water to form Na₂PbO₃.

A point that should be noted here is that the gold in the leached sands is very rapidly soluble in potassium cyanide solution. When cyaniding with 0.18% lime (4 lb./ton) for 3 hours, residues were obtained assaying 0.1 dwt. As it seems obvious that the decreased extraction is caused by the deposition of a cyanide-insoluble lead compound from the solution on to the surface of the gold, it is apparent that the rate of solution of the lead hydroxide or

sulphate and the subsequent coating of the gold particles must be very rapid; the first reaction is probably the slower, and hence is the one governing the decrease of extraction. The lead will be present in the solution as calcium or sodium plumbite.

Several facts are obvious from the results given in Table 4; the presence of calcium hydroxide is not necessary to give the depression of extraction, as caustic soda alone gives the effect (c.f. 8). Calcium hydroxide appears to have a greater effect than an equivalent amount of sodium hydroxide (Tests Nos. 106 and 108). Cyanidation tests with lead dioxide have been previously reported by I. I. Andreev,⁹ and it would seem that only divalent alkali-soluble lead compounds can have any effect in cyanidation. Leaver, Woolf and Jackson showed that the presence of oxidising agents (sodium and hydrogen peroxides) can eliminate the effect of lead salts and alkalis, and from these observations suggest that absorption of oxygen may be the cause of the decreased extraction. The action of hydrogen peroxide has been confirmed, but the effect of lead salts must be attributed to causes other than oxygen depletion, for oxygen determinations on the pregnant solutions in tests Nos. 108, 110 and 113, using the hydrosulphite method of Weinig and Bowen¹⁰ showed that in each case the solutions were almost completely saturated with oxygen (8.6, 8.5 and 8.1 mg. per litre respectively).

It would seem unlikely that lead hydroxide is the only hydroxide that can depress the extraction of gold by cyanide, but 18-hr. agitation tests on the leached sands with bismuth, mercuric and stannous hydroxides with caustic soda gave residues of 0.2 dwt. in each case. The

⁸R. J. Lemmon, Chem. Eng. Min. Rev., Aug., 1935, p. 404.

⁹I. I. Andreev, Z. Elektrochem., 1913, 19, 667.

¹⁰A. J. Weinig and M. W. Bowen. Trans Amer.I.M.E., 1925, 71, 1018.

choice of an hydroxide for this purpose is limited: the solubility in alkalis must be sufficiently great so that it may dissolve and coat the gold before the latter is completely attacked by the cyanide, and further, it must not have a tendency to form complex cyanides. The present method of testing, however, is quite incapable of showing up any slight effect, and it will be necessary to use sensitive physico-chemical methods to study the question further.

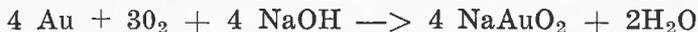
DISCUSSION.

The fact that gold can be coated by roasting with lead compounds, suggests that the presence of potassium cyanide is not necessary for the formation of a coating with lead hydroxide and solutions of lime or caustic soda (c.f. 6). Further, the fact that the alkalinity must be above pH 11 indicates that the hydroxyl ion plays an important part in the reaction.

A theory that seems at first sight to be very attractive, is that in the normal solution of gold by cyanide solutions, the first stage is the oxidation of the gold by oxygen in solution to form one of the oxides of gold. This oxide then dissolves with the formation of potassium aurocyanide and hydrogen peroxide according to Bodlaender's equation. In the presence of lead salts this oxide is "fixed" as an almost insoluble lead aurate, which prevents further action. Such a film would be limited to a few molecules thickness in view of the extremely efficient protection it affords against the action of cyanide. Theories that an oxide of gold was the intermediate product in cyanidation were put forward

by Engler and Weissberg¹¹ and also by D. Reichinstein,¹¹ who suggested that the oxidation and solution were slow and fast reactions respectively. On thermodynamic grounds, however, the spontaneous formation of auric oxide from gold and oxygen is impossible,¹² and it is doubtful whether other oxides have been prepared. Against these last facts we have the observation of J. Strohacker,¹³ who showed by direct weighing that air is absorbed on the surface of gold to give a unimolecular film, which is removed by a vacuum of 10^{-5} mm. only after 30 hours. Further, Muller and Low,¹⁴ with the aid of a reflection polarization microscope, show that, on standing in air, gold becomes covered with films which are distinctly visible after four hours, and which become very pronounced after 27 hours. The last workers assume that the film is an oxide or hydroxide, so further information on the composition of this film may be of great importance in a complete understanding of the facts under discussion.

An alternative but similar hypothesis which seems more probable is that the first stage in cyanidation is the formation of sodium aurate thus:—



the aurate thus formed dissolving to form $\text{NaAu}(\text{CN})_2$ and H_2O_2 as before, and in the presence of lead salts reacting

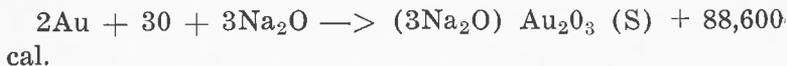
¹¹C. Engler and J. Weissberg. *Kritische Studien uber die Vorgange der Autoxydation*, Braunschweig, 100, 1904, see Mellor. *Comprehensive Treatise of Inorg. and Theor. Chem.*, Vol. 3, 502. D. Reichinstein, *Z. Elektrochem.*, 1913, 19, 674. See Mellor 3, 501.

¹²R. H. Gerke and M. D. Rourke *J. Amer. Chem. Soc.*, 1927, 49, 1855. T. F. Buchrer and W. E. Roseveare, *ibid*, 1989.

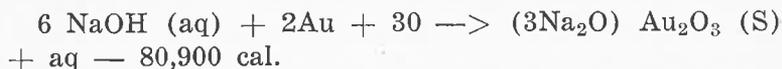
¹³J. Strohacker, *Z. Physik*, 1930, 64, 248.

¹⁴W. J. Muller and E. Low, *Ber.*, 1935, 68, 989.

to form lead aurate. The existing thermal data for this equation is quite unsatisfactory. Mixer¹⁵ and Rengade¹⁶ give:—



$\text{Na}_2\text{O} + \text{aq} \longrightarrow \text{Na}_2\text{O aq}$ (i.e. 2NaOH) + 56,500 cal. respectively, and by combining these equations we get



The usually accepted formula for sodium aurate, however, is $\text{Na}_2\text{O Au}_2\text{O}_3$, and substituting this in the above equations we get a heat of reaction of + 32,100 cal., which would indicate that the reaction should be possible. This hypothesis necessitates the presence of hydroxyl ions for the solution of gold by cyanide, a fact which has been confirmed by Mikhailenko and Mescheryakov,¹⁷ although this may be capable of other interpretation. The action of alkalis in promoting the formation of oxide films on gold has been fully discussed by Schutt and Walton.¹⁸

The above discussion has been confined to the case of caustic soda and lead salts, but it is possible that lead films on gold may be of three types; the Pb-Au-S type as formed by roasting gold in the presence of lead and sulphur compounds, the Pb-Ca-Au type formed during cyanidation in the presence of lime, and the Pb-Au type formed where caustic soda is used. Oxygen is probably present in each type of film.

¹⁵W. G. Mixer, Amer. J. Sci. 4th Series, 1911, 32, 202.

¹⁶E. Rengade, Bull. Soc. Chim. (4), 3, 194. See Amer. Chem. Abs., 1908, 2, 1522.

¹⁷Mikhailenko and Mescheryakov, J. Russ. Phys. Chem. Soc., 1912, 44, 567.

¹⁸W. J. Schutt and A. Walton, Trans. Faraday Soc., 1933, 29, 1209.

SUMMARY.

The effect of alkalis in causing decreased cyanide extraction of Bird-in-Hand table sands has been investigated, and is attributed to the presence of a small amount of oxidised lead minerals.

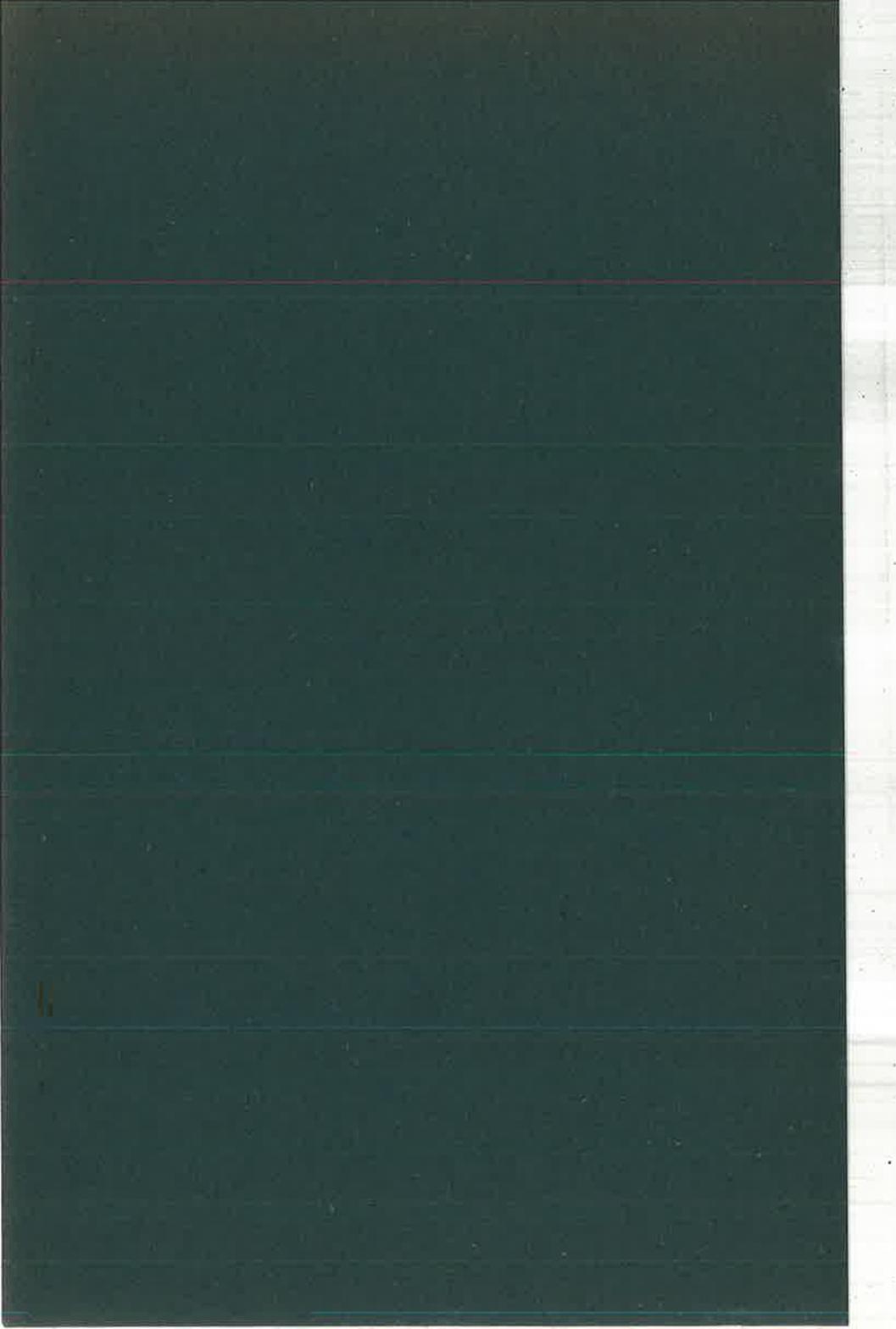
Cyanide tests carried out by adding definite amounts of lead hydroxide with lime or caustic soda to lead-free sands showed that 0.17% lead as hydroxide with a very low alkalinity (0.005N) can almost completely stop solution of the gold, calcium hydroxide having a greater effect than sodium hydroxide; the addition of hydrogen peroxide can counteract the effect of the lead hydroxide. The results confirm the observations of Leaver, Woolf and Jackson,⁵ but the suggestion of these writers that oxygen depletion is the cause of decreased extraction is disproved by oxygen determinations on the solutions. Lead in the tetravalent state has no effect.

Bismuth, mercuric and stannous hydroxides were tested in the same way, but gave no decrease of extraction.

An hypothesis is put forward that the first stage in the normal solution of gold by cyanide is the formation of sodium aurate by oxidation in the presence of hydroxyl ions. This dissolves with the formation of sodium aurocyanide and hydrogen peroxide as in Bodlaender's equation. In the presence of lead salts the aurate is "fixed" as an insoluble lead aurate, which coats the gold and prevents further action by the cyanide.

6th January, 1936.

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NOTES ON THE OCCURRENCE OF AZOTOBACTER
IN SOME SOUTH AUSTRALIAN SOILS

by

A. B. BECK

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(Submitted for publication 12th March, 1935.)

Although the distribution of *Azotobacter* in other parts of the world has received considerable attention, there is only one paper referring to its occurrence in South Australia. H. K. Lewcock (1925) published a paper dealing with the stimulating effects of phosphates on nitrogen fixation, and in it he refers to unpublished work which showed the "universal distribution of a vigorous *Azotobacter* flora in South Australian soils ranging in type from a light calcareous sand, carrying virgin mallee scrub, to a heavy, black, alluvial, truck soil. In every instance also a dense *Azotobacter* film was formed in Ashby man-nite media within 6 days . . . ". Unfortunately the details of these experiments are no longer available.

During 1934 the writer carried out an investigation of the occurrence of *Azotobacter* in a number of South Australian soils, and the observations made are offered as a contribution to our limited knowledge of this subject as far as Australia is concerned.

Lewcock utilized Ashby's liquid culture medium for his investigations, but the more recent development of Winogradsky's (1926) silica-gel plate method allows a more satisfactory technique to be employed. This method consists of inoculating finely divided soil on to a silica-gel plate impregnated with a suitable nitrogen-free culture medium, and by counting the colonies appearing on the plate, a quantitative estimation may be made of the number of *Azotobacter* colonies occurring in the soil. The gels for this method were prepared in 5-inch petri dishes. The amount of soil used varied with the *Azotobacter* content, a trial count being usually done with a 3 inch dish in order to obtain some idea of the quantity required to give a reasonable number of colonies on the plate; with soils of very low *Azotobacter* content up to 2 gm. of soil were used. The dish was kept at 30°C., and counts made daily for 7 days.

Although the method is described as quantitative, it is only roughly so, and the counts on any given area of constant chemical and botanical features will depend on the depths at which samples were taken, the time of the year, climatic conditions of existing and past seasons, and the degree of fineness of the sample used. The last condition is rather important (Batchelor (1933), Curie (1931)), and the writer used samples which had been passed through a 30-mesh sieve. At

least two counts have been done on each sample; with *Azotobacter* colonies the agreement is quite good (the difference is usually less than 20 p.c.), but with the small type of colony the error may be very much larger. Counts have been done on 33 soils from various parts of the State, and the results together with the details of the soils under consideration are given in Table 1.

Colonies of unidentified organisms, easily distinguished from *Azotobacter* by their extreme smallness, appeared on the plates with some of the samples, and the counts obtained are also given in the table. These colonies varied in size from about 1 mm. to 0.5 mm. diameter or even smaller, and were best observed on fairly moist (but not wet) gels. Microscopic examination was difficult on account of the size and rigidity of the colonies, but no *Azotobacter* cells were observed, and probably several types of organisms are included in the group. They all occurred in barren soils, and had an incubation period (at 30°C.) of three days or longer. Very little work has been done on these organisms, but it may be mentioned that the colonies from No. 30 produced vigorous but heterogenous growth on mannite-agar slants, but no growth on Ashby's liquid mannite media.

TABLE 1.

No.	Locality.	When sample taken.	Age of sample when count done.	Count per gram.		Growth on Ashby's medium.	pH	Remarks.
				Azotobacter.	Other species.			
ADELAIDE PLAINS.								
1.	Lockleys	Jan.	8 months	0	6	—	6.3	Sandy soil.
2.	West Mitcham	Aug.	1 month	138	0	Strong white	7.1	Originally derived from soil similar to 3. Under cultivation for past 10 years.
3.	West Mitcham	Feb.	5 months	0	110	Very slight film	6.2	Red-brown earth. Grazed annually. No cultivation for at least 25 years.
4.	Dublin	Jan.	7 months	45	0	—	7.2	Sandy soil used for wheat.
12.	Findon	March	3 months	560	0	Strong white	7.0	P ₂ O ₅ .30%.
13.	Findon	March	8 months	7	0	—	6.9	P ₂ O ₅ .15%.
14.	Findon	March	4 months	18	0	—	7.0	P ₂ O ₅ .14%.
17.	Direk Siding	April	5 months	0	0	—	6.2	Fallow land.
27.	Oaklands	May	2 months	0	0	—	5.9	From a vineyard.
47.	Marino	Sept.	2 months	0	0	—	7.4	Calcareous soil taken about 100 yards S.W. of railway station.
MOUNT LOFTY RANGES.								
33.	Littlehampton	July	3 months	0	0	—	5.0	Subterranean clover land under sheep.
48.	Eden	Nov.	1 month	0	0	—	6.3	Fairly rich soil from side of gully.
SOUTH EAST.								
20.	Mount Gambier	April	5 months	0	0	Very slight film	5.0	Sandy soil.
28.	Near Lake Leake	July	3 weeks	0	0	—	5.7	Sandy soil P ₂ O ₅ .14%.

No.	Locality.	When sample taken.	Age of sample when count done.	Count per gram.		Growth on Ashby's medium.	pH	Remarks.
				Azoto-bacter.	Other species.			
MURRAY LANDS.								
49.	Wanbi	Dec.	1 week	0	0	—	7.0	Land under wheat, sand.
50.	Wanbi	Dec.	1 week	0	0	—	7.0	Fallow land, sand.
YORKE PENINSULA.								
9.	Corny Point	Feb.	5 months	0	0	—	7.1	Manganese deficient.
MID NORTH.								
6.	Appila	Jan.	7 months	0	2	Very slight film	7.3	Heavy red clay.
29.	Quorn	July	3 weeks	0	0	—	7.8	Red sand. Wheat land fallow 2-3 miles north of town.
31.	Seven Hills	July	3 weeks	0	0	—	6.2	Sandy Eucalyptus and Acacia scrub land. 1 mile south of town.
FAR NORTH.								
30.	Wilpena Pound	July	2 months	0	33	Very slight film	6.7	Euc. rostrata and grass country just inside the pound.
32.	Hawker	July	1 month	0	0	—	7.7	Approx. $\frac{1}{2}$ mile N.W. of railway station. Wheat crop just up.
36.	Blinman	July	2 months	0	0	—	7.3	Just north of town before mines.
37.	Ferguson's Gorge	July	3 months	0.4	0	Slight film	7.4	About 2 miles west of Angorichina Hostel, from side of creek.
38.	Koonamore	Aug.	2 weeks	0	0	—	7.7	University reserve. Silt flat carrying annual herbage.
39.	Koonamore	Aug.	1 month	0	26	Very slight film	7.3	Sand hill carrying mulga and annual herbage.
40.	Koonamore	Aug.	2 months	0	0	—	7.7	Salt bush soil.
41.	Pandi	Aug.	1 month	4	16	Strong white	7.0	Flood plain adjacent to Diamantina.
42.	Pandi	Aug.	2 months	0	0	—	7.0	Sand hill on Diamantina flood plain.
43.	Karatunka	Aug.	1 month	26	7	—	7.1	On Diamantina, 35 miles from Birdsville. Sandhill.
44.	Diamantina Flood Plain	Aug.	3 weeks	52	0	—	7.0	From along cattle creek.
45.	Gibber Plain	Aug.	1 month	2	3	—	7.0	At 7-mile bore.
46.	Clayton Bore	Aug.	6 weeks	0	0	—	above 8	Highly saline.

Two types of *Azotobacter* colonies were observed, white, raised colonies (the "colonies seches" of Winogradsky) and the flat, more fluid type ("colonies fluides"), the distribution of the two types being apparently quite haphazard. The growth in the case of No. 37 was much more flat and watery than the usual "fluid colony" type. The growths from all soils containing *Azotobacter* have been examined microscopically, using either gentian-violet (Winogradsky, 1926) or carbol-fuschin as staining agent. Although there was some variation in form,

all showed the typical coccus or oval form, 1.8 to 3.0 μ long and 1.2 to 1.8 μ broad, diplococci and tetrads being common.

As no soils have been obtained from many important areas, no statements can be made concerning the distribution of *Azotobacter* in this state, but the results show that Lewcock's claim for a universal distribution cannot be substantiated. Further, it seems probable that even in the majority of soils containing *Azotobacter*, the numbers present are so small that their effect on the nitrogen cycle will be almost negligible. Reasons for the absence of *Azotobacter* in the soils examined are not obvious. An interesting point is the limited number of areas in which the bacteria have been found, namely the Adelaide Plains, the Diamantina cattle track, and the isolated instance of Ferguson's Gorge, but the most important fact is that only those soils of pH between 6.9 and 7.4 (approximately) contain *Azotobacter*; this is surprising, as workers in other parts of the world find that pH limits are from 6.0 to about 8.0. Chlorides have a toxic effect (Lipman, 1911), but except with No. 46, are probably not responsible. Two other factors which may be of importance are lack of sufficient carbohydrate material and inadequate aeration of the soil in its natural state. The low phosphate content of South Australian soils by itself will probably have no effect on their distribution, as it has been shown by Greene (1933) that in Arizona the *Azotobacter* flora have adapted themselves to somewhat similar conditions.

In view of the discrepancy between these results and those of Lewcock, a number of soils have been tested in Ashby's liquid media, the results being included in Table 1. In the cases where a "very slight film" is recorded, the result was due to anaerobic action at the bottom of the flask. This film, which only appeared in 6 to 10 days, was extremely thin and of a dirty brown colour, and could not possibly be confused with the result given by Nos. 2, 12, and 14, which gave a definite white *Azotobacter* film in 4 to 5 days.

One quantitative estimation on the nitrogen fixed in liquid media was carried out on No. 12 using 2 gm. mannite, 1 gm. calcium carbonate, 10 gm. soil, and 100 ml. of Ashby's solution. Two flasks (with blanks) were incubated; 0.02 gm. potassium phosphate was added to the first flask, and the second contained no phosphate apart from that introduced by the soil sample. After 7 days' incubation at 30°C., the film on the first flask was jet black, while that on the second was orange brown and much thinner. The nitrogen fixed per gm. of mannite for the flask containing potassium phosphate was 8.5 mg., and for the other flask 5.0 mg.

SUMMARY.

Thirty-three South Australian soils have been examined for *Azotobacter* content by Winogradsky's silica-gel plate method. Only ten soils were found to contain *Azotobacter*, the average count for the active soils being 85 colonies per gm., with a maximum count of 560 per gm.

As far as can be ascertained the only factor governing the distribution of *Azotobacter* is the soil reaction, the pH of all the active soils lying between 6.9 and 7.4.

ACKNOWLEDGMENTS.

The writer wishes to acknowledge his indebtedness to the Adelaide Chemical and Fertilizer Co. for providing facilities for carrying out this work; to Professor Prescott and other members of the Waite Agricultural Research Institute, and to those who have assisted in this work by providing soil samples from various parts of the State.

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