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SOMATIC VARIABILITY IN MAMMALIAN PIGMENTATION PARTICULARLY HUMAN, WITH AN ATTEMPT TO RELATE THE PHENOMENA OBSERVED TO CERTAIN INHERITED CONDITIONS IN MAN

by

E. M. Nicholls

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PREFACE

The existence of mutation in the genes of germinal cells has been agreed upon and we have gone a long way towards an understanding of this process in chemical terms.

We believe that the genes of somatic cells are not fundamentally different from the genes of germinal cells, and that mutations must occur in these genes. If frequencies of gene mutation in somatic cells are in any way comparable with those which have been studied in germinal cells, then millions of somatic mutations must occur every day in any individual.

If this is so then there are two possibilities. The first possibility is that despite the frequency of this phenomenon there is no part of our physiology which is upset by it, and in fact biologically it is unimportant. The second possibility is that in the multicellular diploid organism there are potentially serious consequences of frequent somatic mutation, that the body may develop mechanisms to combat these serious consequences, and that occasionally the organism as a whole suffers from a failure of these mechanisms.

Geneticists as a class are very wary of discussing somatic mutation because it does not lend itself to the mathematical rigors which were foreshadowed by the work of Mendel. This seems to be a somewhat negative approach - if it is there it should be studied; a phenomenon which can be inferred to occur in every living person must be as worthy of attention as for instance the acquiring of photographs of the reverse side of the moon. If eventually somatic mutation can be proved not to occur then a major

biological discontinuity has been proved - between germinal and somatic cells. If technological advance makes it more and more convincing that somatic mutation occurs and is a fundamental biological phenomenon, then the techniques of conventional genetics will undoubtedly be enriched by a greater understanding of the mechanisms involved.

ACKNOWLEDGMENT

The work recorded here commenced at an observational level whilst the writer was in general medical practice in South Australia. It was continued in Sydney, N.S.W. at the Red Cross Blood Transfusion Laboratories at 1 York Street, Sydney, whilst the writer was a member of the Department of Human Genetics of the University of N.S.W., headed by Professor R. J. Walsh. Help with the techniques employed and obtaining material has come from many sources both inside and outside the Department and detailed acknowledgment will be appended to the appropriate chapters.

STATEMENT - THE PERFORMANCE OF THE WORK

The work recorded in this thesis was performed entirely by the author, except as regards introduction to techniques and provision of materials in some instances. The most important external contribution was made by those who punched data cards and prepared programmes for use in electronic computation of population data.

Introduction to the Subject Matter of the Thesis

"Somatic variability in mammalian pigmentation" in the title of this thesis means several things - it means the patterns of skin and coat colour of animals, it means pigmentary mosaicism with which an individual is born, and it means changes in skin colour or hair colour of a discontinuous nature which may develop during extrauterine life. The particular cell system under study is the melanocyte system, although only to a very limited extent at the microscopic level. The production of a pigmentary change is assumed to have a basis in altered biochemical activity of a melanocyte or a group of melanocytes. A limited number of sections of pigmented tissue were studied microscopically, but in the main reliance was placed on the very well documented light microscopic and electron microscopic studies which have been and are still being made in various parts of the world.

Somatic variability of pigmentation in many animals obviously has adaptive significance (e.g. giraffe, zebra) and one can infer that evolution has brought this about by mechanisms of gene activation or gene inactivation of a sort which at the present time are only just beginning to be understood.

Setting aside this group of phenomena the thesis here recorded considers that some mosaicism is not adaptive. A model system has been built on the hypothesis that somatic and germinal mutations occur at about equivalent frequencies, and are probably subject to much the same laws e.g. in relation to ionizing radiation. The model has been applied to certain of the mosaic phenomena in humans, and has been extended to include the malignant cell which is a cell of aberrant behaviour arising from one

or another normal tissue.

The particular phenomena studied are freckles, birthmarks and moles, and significant relations between the occurrence of these on individuals have been shown. Red hair has been implicated by the hypothesis that the red haired individual is in general a homozygote, and the brown haired and freckled individual a heterozygote of genes at one particular locus. The occurrence of red hairs on a brown haired person and brown hairs on a red haired person has similarly been related to individual gene action. The biochemistry of marsupial, higher mammalian, and human hair pigments have been studied in order to provide a biochemical basis for the genetic model. Work has been done on the free pigments which are found on the skin and amongst the hair of marsupials, and efforts to synthesize some of these pigments from 3-aminotyrosine are recorded.

Finally a number of inherited human disorders involving multiple tumour formation are recorded and a discussion of how these might fit the model is made. A broader implication of the problems of malignancy is made.

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Chapter I Review of the Literature.

As suggested in the Introduction this thesis is concerned with pigmentation, somatic mutation and cancer. Studies of pigmentation have a long history; interest in somatic mutation has been possible only in this century; and studies of cancer which have been made at an observational level for centuries have in the last few decades become highly experimental, and overwhelming in content. The three topics will be reviewed separately, and also a brief review of specific interest to the material of the individual chapters will be made at the beginning of each chapter.

In the case of the review of cancer literature the review will be very selective. There is no possibility of reviewing all that has been written or even a small fraction of it, nor even of trying to adequately summarize all the concepts as to causation which have been put forward. It may well be that those who support viral, and those who support genetic points of view, for example, will find considerable accord with each other in the ultimate understanding.

Pigmentation

The modern study of pigmentary phenomena began last century with the application of light microscopy to skin and other pigmented tissues. Heusinger (1823) reported pigment granules in Malpighian cells, Waldeyer (1871) was the first to give a description of the melanocyte which is now regarded as the basic cell in the pigmentary system. Riehl (1884) first discovered melanocytes present in the hair bulb, and in 1885 Aeby demonstrated that the melanocyte transmits pigmented granules to the Malpighian

BIOCHEMICAL PATHWAY TO MELANIN

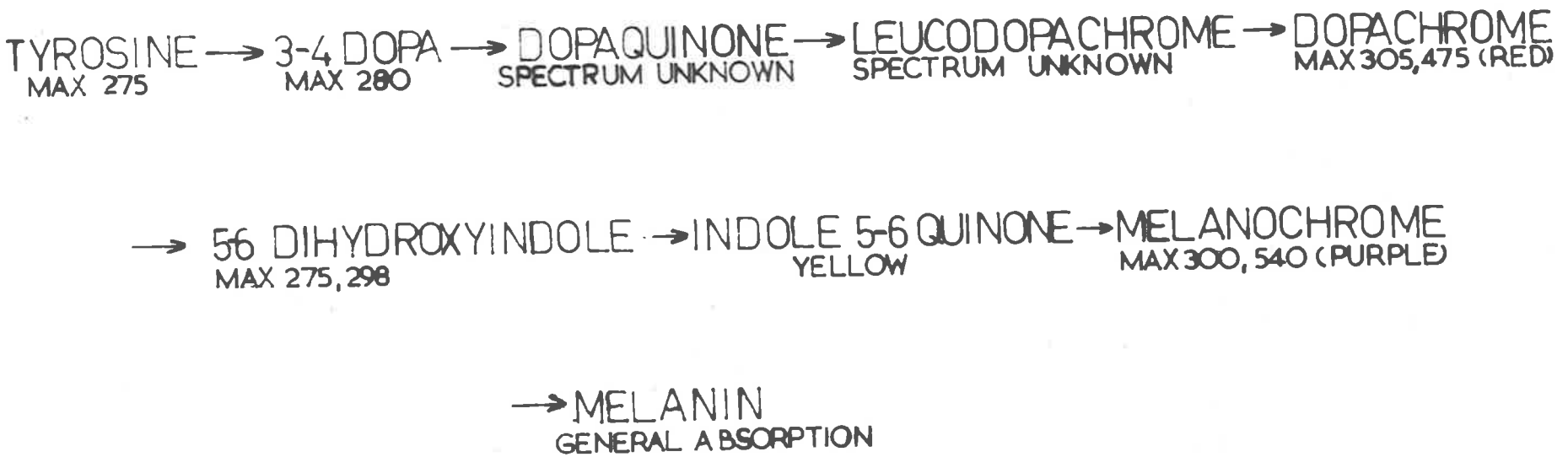


Fig. 1. The biochemical steps leading to the production of melanin.
(Fitzpatrick & Szabo 1959)

cells. Bizzozero (1906) studied melanocytes and Langerhans cells and introduced staining by silver impregnation techniques.

In 1917 Bloch and Ryhiner found that by treating sections of skin with Dihydroxyphenylalanine (DOPA), a substance related to tyrosine, they could produce darkening of the dendritic melanocytes, and this became the basis of the "DOPA reaction" of melanocytes. This reaction also stained leucocytes which have a polyphenoloxidase enzyme system, and in 1950 Fitzpatrick, Becker, Lerner and Montgomery introduced tyrosine to replace dopa, and the reaction, while slower, is more specific.

The first significant chemical advance was made by Bourquelot and Bertrand in 1895 who found that a colourless substance in a toadstool (Russula nigricans) was acted on by an enzyme to produce first a red and then a black pigment. They showed that the pigment precursor was tyrosine, and named the enzyme tyrosinase. Raper (1928) worked with potato tyrosinase in its relations with tyrosine, and elucidated the main chemical steps. These, taken from Fitzpatrick and Szabo (1959) are shown in Fig. 1.

Thus a strong case for tyrosine as the precursor of melanin has been built up. Direct proof of this hypothesis even at the present day seems nearly impossible because both tyrosine melanin and natural melanin are very inert chemically, insoluble in most solvents, and since they are complex polymers difficult of meaningful chemical analysis. However, Kukita and Fitzpatrick (1955) showed incorporation of radioactive labelled tyrosine into human hair follicles in vitro. A number of such studies have been carried out, e.g. Coleman (1962) who showed incorporation of radioactive labelled tyrosine into mouse hair which correlated with the

genotypic status of the mouse, giving results according to the degree of melanization.

The study of the behaviour of melanocytes as an organized tissue has progressed greatly. Billingham (1949) discussed the syncytial nature of the monolayer of melanocytes at the dermoepidermal junction. Szabo (1954) reported on melanocyte counts in different races, where the number is essentially the same regardless of pigmentation; and regionally on the body, where the greatest difference is that the face may have two to three times as many melanocytes/sq. mm. as some other parts of the body. Fitzpatrick and Szabo (1959) produced data on the same topic, giving some characteristic values as follows - 2,310$\pm$ 150 melanocytes per sq. mm. on the face, and 1160$\pm$ 40 on the upper limb. On the face there are three or four epithelial cells for each melanocyte. Billingham and Silvers (1964) studied the phenomenon of "pigment spread" from coloured to white skin, and from eumelanic to phaeomelanic skin in guinea pigs. It is found that a zone of pigmented skin 1 cm. in width may underlie a white patch of hair in one of these animals, and the present view is that this is due to a gradual encroachment onto the less pigmented area by the pigment producing melanocytes. The hair follicles in the area do not take up the pigment producing melanocytes, hence the clear distinction into three zones (colourless hair, colourless skin; colourless hair, coloured skin; coloured hair, coloured skin). This phenomenon appears to be of considerable theoretical importance and will be referred to again later.

Studies have been made on the establishment of the melanocyte system

by cells migrating from the neural crest, differentiating to become melanoblasts and eventually moving to the dermoepidermal junction and maturing to become melanocytes (Rawles, 1940).

Hormonal effects on pigmentation have been observed in sexual dimorphism in some animals, and colour change related to cyclical changes in sexual function. In women one well known phenomenon is the darkening of the face and other body areas in chloasma gravidarum. Lee and Lerner (1952) and Lerner and Case (1959) studied polypeptides which have been defined as α & β MSH (melanocyte stimulating hormones) which increase melanization in certain experimental animals. Melatonin, a 5-dihydroxyindole derived from the pineal gland of cattle aggregates melanin within the melanophores of some lower animals (Lerner and Case, 1959). An interesting and unexplained phenomenon is that thyroidectomy causes the production of "red melanin" in some otherwise black breeds of poultry. (Blivaiss, 1947)

The last 20 years has seen a great extension of knowledge in defining the ultrastructure of the melanocyte, as has occurred in most other fields of histology, through the introduction of the electron microscope. Many papers have been written and the melanosome has become of paramount importance as the fundamental organelle of the melanocyte. Differences between melanosomes have been detected in many genetical and pathological situations (Mishima 1965) and the specific interest of melanosomes in black and brown hair and skin, and red hair and skin will be discussed later.

Genetical knowledge of pigmentation of animals is extensive.

Commercial breeding of large animals and laboratory breeding of small animals have contributed greatly to knowledge. In humans there are special problems as in much of human genetics. A great number of rare pigmentary disorders are known with some genetic determination, which may or may not be as yet clearly defined. Population studies have been made in skin pigmentation using techniques of matching colours or reflectance spectrometry. Edwards and Duntley (1939) first studied skin spectrometry, and on a population basis studies have been made by Weiner (1951), Harrison (1957), Walsh (1963, 1964) and others. Walsh (1964) reports that mean reflectance values are much smaller for forehead than for chest wall or forearm, and this difference applies for babies as well as for adults. Harrison and Owen (1964) suggest that the differences between Europeans and Africans could be explained by an effective difference of 3-4 gene pairs.

The genetics of pigment differences at the biochemical level has been greatly impeded by lack or clear biochemical knowledge of the nature of pigments. The word melanin is derived from Greek "melas" black, and there are a very few substances, which include dopa and tyrosine which produce a black pigment under the influence of tyrosinase. However, tyrosinase acts on a larger number of phenolic derivatives to produce coloured pigments (Yasonubu 1959) which are referred to as melanins by many workers. In animals we have infinite gradations of colour, but also clearly definable black, brown, blue, yellow and red colours. These colours could come about by differences in the basic chemical nature of the polymers, or by differences

in physical distribution or physico-chemical state. It seems that genetic understanding will be facilitated and consolidated by advances in biochemical understanding, as it has been, for example, in the case of many flower pigments.

The only human pigment which lends itself to genetical interpretation based on presumed chemical identity at the present time is the pigment of red hair. Distinctions in man and animals have been made between eumelanins (black and brown) and phaeomelanins (yellow and red) but this does nothing to relieve the confusion in genetic understanding, and must be further defined.

Somatic Mutation

The causes of mosaicism in tissues have been discussed by various authors. A number of mechanisms are possible: chromosomal deletion or rearrangement giving rise to position effects; gain or loss of a chromosome due to mitotic non-disjunction; somatic crossing over between homologous chromosomes so revealing otherwise undetected recessive genes; X chromosome inactivation; gene activation or inactivation by operator-regulator systems; introduction of embryonic tissue from a dizygotic twin; and somatic mutation at the gene level involving some chemical change in the polynucleotide chain, possibly by loss or gain of a small segment or alteration of base sequences.

Authors writing on mosaicism do not always clearly define which of these mechanisms is operating, generally because they, in fact, cannot do so. Certain changes are now recognized to be due, for example, to inactivation of an X chromosome; other changes may have an adaptive significance

as in the coat colour of animals and one might believe that an operator-regulator effect was responsible for the mosaicism. One feels that the most interesting, as likely to be the most general mechanism, is the last one listed, and it is this mechanism which is to be the theme of this thesis and relevant to this section of the review of literature.

Mutant forms of normal genes in germinal material were studied at a very early stage following the rediscovery of genetics in 1900, and perhaps the person most worthy of mention is Garrod (1909) in his study of inborn errors of metabolism. The first vigorous experimental attack on gene mutation was that of Müller who studied the effects of irradiating Drosophila. Estimates of radiation induced and natural mutation rates have been made in Drosophila and other organisms, and numerous estimates of natural mutation rates in Man have been made. Müller was able to show that the mutant types which he produced bred true for the new character and this remains the one great advantage which the study of germinal mutation has over the study of somatic mutation, at least in animals.

In plants variations frequently occur in sectors of a plant so that a change of flower colour, leaf shape or some other character shows up on a small area of a large plant. This sector of the plant may produce seed and East (1917) and others have shown that such variations can breed true for the new character, and where the character is definable in simple genetic terms show that it behaves as a genuine change at the gene level.

Müller (1927) in the course of his studies on radiation effects on Drosophila observed mosaic effects and suggested that mutations in somatic cells might cause cancer.

Wright and Eaton (1926) reported mosaicism in guinea pigs and suggested that this might be due to somatic mutation. Dunn (1934)studied a black male mouse spotted with light tan and found that the animal segregated for three distinct alleles of the albino series in breeding tests, so suggesting that there was a mosaicism involving both germinal and somatic tissues and probably arising by a mutation. Bittner (1932) studied seven mosaic guinea pigs and was able to show that one of these must be the possessor of both a somatic and a germinal mosaicism. Bhat (1949) described a germinal and somatic mosaicism in a mouse involving the agouti locus.

Russell and Major (1956) irradiated female mice after 10 days of pregnancy, administering 100r of irradiation to each mouse. These animals were each heretozygous for four coat colour genes, and as judged by the known effects of these genes, a somatic mutation was expected to cause visible change if any of the appropriate genes was affected. They obtained 11% of mosaic animals as against 0.5% in controls. They estimated somatic mutation rates on the assumption that if, say, 100 cells with an ultimate pigment producing capacity were present at the time of irradiation, and an appropriate gene of one of these cells mutated, the fraction of skin surface affected on the animal would be about 1/100th of the total. They arrived at the figure of measurable change per cell/100 r of 3.1×10^{-4}. Their results were consistent to the extent that extremes of the range of effect were separated by a calculated six cell divisions.

Attwood & Pepper (1961) studied mosaicism for A. and B. antigens in human red blood cells and attempted to relate the findings to a somatic

mutation hypothesis. The finding of non-A and non-B carrying cells in people of blood group AB fits the suggestion that the genes responsible for the A and B antigens have mutated or are mutating continuously in a small proportion of cells. In people genotypically "BB" a smaller frequency of non-B cells were found of the order of 5 to 10 times smaller than in the AB individuals. However, this frequency is still some 50-200 times greater than would be expected to occur if the mutations of the two B genes in each cell were independent events with a combined frequency equalling the square of the frequency of the single event. The occurrence of anti-A antibodies in group B people, and anti-B antibodies in group A people has similarly been attributed to antibody stimulation by mutant cells, although other reasons have also been put forward.

Many observations have been made on cancer cells in tissue culture, and in the passage of cancers from one animal to another. These have involved showing stepwise and permanent change in behaviour of a cell or a group of cells. Strong (1958) produced cancers in inbred strains of mice. These cancers could be transferred to an F1 from two such inbred strains. In the F_2, however, the majority of cancers did not "take", and at first behaved as though there were 5 incompatibility factors operating, these being antigens present in the tumours "recognized" by the new host. As the cancers were passaged through more generations the number of these incompatibility factors became reduced in stepwise fashion until the tumours would grow in all F_2, and eventually in foreign strains as well.

Law (1952, 1958) using a culture of mouse leukaemia and causing inhibition of growth using folic acid antagonists found that the laukaemic cells adapted to and became dependent on the antimetabolite in random, stepwise fashion. He adapted the fluctuation test of Luria and Delbrück, used to study mutation in bacteria, to show that a similar type of random change was occurring in the cultured cells. Szybalski and Hunter Szybalska (1962) used a leukaemic human culture obtained by sternal puncture, and showed a similar stepwise development of resistance to various agents introduced into the culture. Gowen (1958) studying leukosis in poultry noted sudden changes to greater lethality which appeared to happen as a random process within large groups of cells. Apropos of such sudden changes may be the well known clinical situation in humans whereby cancers can be graded according to malignancy depending on cell type, and that a tumour of low malignancy may be found to have small areas which have undergone a change to greater malignancy and more anaplastic growth.

Much work experimentally and statistically has been done on the effects of irradiation. Early observations on miners exposed to radio-active ores, and on factory workers exposed to radio-active compounds as in the use of such materials in luminous paints, showed a high incidence of cancer in particular organs. Many of the early radiologists developed skin cancers particularly of hands and fingers as a result of injudicious exposure to radiation. Radiation has been used to produce cancers in experimental animals. More recently atomic energy in war and peace has

has provided a large body of material for study. Some of many studies in the field will be mentioned here.

Court Brown, Buckton and McLean (1965) reported chromosomal aberrations in cultured lymphocytes many years after exposure to radiation. Conard and Hicking (1965) reported incomplete recovery of peripheral blood in people exposed to radiation. Curtis and Crowley (1963) studied the effects of irradiation on experimental animals and found that there was a shortening of life span. They found that there were a variety of chromosomal changes produced, and concluded that these could be due to "multihit" events but that there was some return to normal after chromosome damage. Bender and Gooch (1963) examined human blood cultures 42 months after radiation exposure and found that there were chromosomal abnormalities present.

A number of studies have been made of victims of atomic bombing of Hiroshima and Nagasaki. Angevine and Jablon (1964) reported that there were no greatly increased frequencies of malignancies in autopsies from Hiroshima and Nagasaki. Jablon, Ishida and Beebe (1964) found that leukaemia was increased in both Hiroshima and Nagasaki, and that there was an increase in deaths from aplastic anaemia. They also claimed an increased death rate in females from cancer of the stomach and uterus in Hiroshima but no other general increase. Lewis (1957) discussed leukaemia in atomic bomb victims, the frequency being inversely proportional to the distance from the hypocentre at the time the bombs were dropped. He also discussed leukaemia in people exposed to radiation for ankylosing spondylitis and thymic enlargement, and data on leukaemia in American radiologists. He

found increased leukaemia in all these groups, and concluded that radiation was a cause of leukaemia and that there was no apparent threshold to the effect. On the contrary Court Brown and Doll (1958) found that in British radiologists there was no shortening of the life span and no significant increase in cancer.

The study of clones of cells with specific capacities to produce proteins (antibodies) has been given great impetus by Burnet in work extending over a number of years. Burnet (1962) suggests that the clones of cells could arise by somatic mutation, and in 1964 he states "that no greater mutability may be required than in any other type of functioning somatic cell". Thus somatic mutation has been implicated in this most important of biological functions, and techniques are being used to identify cells with specific antibody producing functions and to study their behaviour within the body.

Various people have studied aging processes and suggested that somatic mutation is implicated in this phenomenon. Failla (1958) made observations on irradiation effects in man and animals and concluded that somatic mutation could explain aging. He suggested that a "4 hit random process" could explain mortality data available and gave a gene mutation rate of $12 \times 10^{-5}/30$ years based on this hypothesis. Crowley and Curtis (1963) studied the effects of irradiation on long lived and short lived strains of mice and found that the frequency of chromosomal abnormalities in liver cells was inversely proportional to the life expectancy.

A number of the authors already quoted suggest that somatic mutation is the basic phenomenon in the neoplasia that they studied. Perhaps the most specific such theory is that of Nordling (1953). He considered data on cancer deaths from several countries, and also gave consideration to work indicating that carcinogenic agents were in many cases known mutagenic agents, and the views of such authors as Strong (1949) and Bauer (1959) who claimed that the cancer cell was a mutated cell. He observed that in males, and in females if one excluded breast and uterus, the death rate from cancer increased according to the 6th power of the age. By plotting the age against the logarithm of the death rate he obtained a linear relationship. He found discrepancies in women in breast and uterus which he suggested was due to a changing hormonal status at the time of the menopause. On the basis of his statistical study he propounded the theory that cancer could result from sequential mutations, which would be speeded up by mutagens, and that in a particular case approximately seven mutations would be necessary in a cell lineage to produce cancer.

Cancer

In this thesis it is hoped to provide some evidence in support of sequential somatic mutation, as required by the theory of Nordling, and to show how this sequential change may be related to known familial incidence of neoplasia and those genetically determined disorders where a high frequency of benign, and a lower frequency of malignant neoplasms is found.

This review of cancer literature is therefore largely a series of references, not exhaustive, to a very limited part of the total picture of research into cancer, with greatest emphasis on the familial aspects of the subject. There is a continuing problem of differentiating between the effects of exogenous influences such as viruses, and purely endogenous influences which suggest that cancer cells behave the way they do because of some spontaneous alteration in cell heredity. The subject of viruses and cancer, great and important as it is, must be largely ignored. Chapter VIII contains a review encompassing the heredity of simple tumours, and very little of this will be discussed at this point.

Literature on heredity and cancer can be divided into four groups. The first group considers cancer as a racial characteristic, and is perhaps the least satisfactory because it is very apparent that environment has as much to do with the situation as might some innate hereditary racial characteristic. Nevertheless there are some cases which suggest a true racial predisposition. The second group is a broadly statistical one, comparing the relatives of cancer sufferers with the relatives of normal controls. The third group are the comparisons between identical and non identical twins in development of cancer. The fourth and numerically largest group of reports concerns individual families where rare cancers have occurred in more than one individual of a family. The influence of environment in these latter cases may be as important as it is in the cases of racial predisposition, but some of the examples are very compelling; for example families reported in which three members have suffered from malignant melanoma. The

expected frequency of this disease is of the order of 1 per 10,000 to 1 per 100,000, and to have three cases in the same family suggests a very strong family predisposition, even when environmental factors are considered.

Some references to recent work in these fields will be given.

Lilienfield (1963) reported on breast cancer and commented that the frequencies of occurrence in the U.S. had been very stable since 1930. However, in Japan there is a very low mortality and in Japan and Finland also a very low mortality in cancer of the male breast. He points out that the Japanese women nurse for about two years, and that this may have its effect through suppression of hormonal action, but does not explain the very low male death rate. Svoboda, Kirchner and Shammugaratnam (1965) and Jung (1965) discuss nasopharyngeal cancer which has a very high incidence among certain Eastern people. Li, Kao and Wu (1962) discuss oesophageal cancer said to have a high frequency in North China. Burrell (1962) investigated oesophageal cancer in the Transkei, now a common disorder but said to be much rarer at the beginning of the century. He suggests unknown carcinogens and Oettlé (1964) gives a list of possible carcinogenic influences operating in different parts of Africa. Lancaster (1956) has reported on a high and increasing incidence of malignant melanoma as one moves from South to North in Australia, and Pack, Davis and Oppenheim (1963) report on the incidence of melanoma amongst Whites and Negroes in the U.S.A., with a much lower frequency amongst Negroes.

Studies of frequency of cancer amongst relatives of affected individuals

and controls include the following: Tokuhata and Lilienfield (1963) studied lung cancer probands and found excess lung cancer among relatives but not among spouses. They concluded that there were genetic factors as well as the relation to smoking. Spranger and Verschuer (1964) decided that there was no general hereditary predisposition to cancer but that cancer localization was partly determined by genetic factors. Hörnecke and Berndt (1965) studying 3159 cancer patients found a higher incidence of cancer in relatives of breast cancer, cystic mastitis, and cancer of the stomach, colon, and rectum cases than they did among the relatives of normal controls. Contrary to Tokuhata and Lilienfield they found no increase in the relatives of lung cancer cases. Lilienfield (1963) found a greater incidence among relatives of breast cancer cases, both female and male, and also among the relatives of patients with benign breast conditions. He states that six former surveys have shown familial aggregations and two have not. Bucalossi, Pandolfi, Veglia and Veronesi (1962) found a greater incidence of cancer in relatives of cancer patients but there was no correlation between types of cancer. Court Brown (1962) states that the incidence of cancer of the breast and leukaemia is increased in relatives of sufferers.

The study of twins and their cancer experience tends to give conflicting results, as do some of the reports recorded in the last group.

Jarvik and Falek (1962) in a small series of twin pairs taken from a group of cancer sufferers, found that there was greater concordance as to cancer in general and type of cancer in particular among monozygotic twins

than among dizygotic twins. Harvald and Hauge (1963) found 1038 cases of cancer among 6893 pairs of twins, but did not find any greater concordance amongst monozygotic twins than amongst like sexed dizygotic twins. Pack (1964) found neoplasms more common in both members of monozygotic pairs than in both members of dizygotic pairs.

Reference must now be made to reports of cases occurring in families, either as an unusually high incidence of a fairly common cancer, or a lesser incidence of a rare cancer.

In the gastrointestinal tract we have reports by Covington and Bulls (1964) of cancer of the tongue in identical twins; Terquem, Rougemont, Scemama and Wiel (1962) cancer of the stomach in two sisters; Jones (1964) of five or more cases of cancer of the stomach in a Maori family; and Kaplan and Cole (1965) of hepatomas or hepatocellular cancer in three brothers. Cancer of the bowel has been particularly noted in familial aggregations, apart from the familial incidence which accompanies polyposis. Recent reports include Mathis (1962) where 11 of 24 individuals were affected in three generations with single or multiple tumours; Heinzelman (1964) recorded 85 individuals in five generations of a family, amongst whom there were 13 cases of cancer, 10 being in the bowel; Kluge (1964) found seven cases of colonic cancer in four generations among 11 individuals; Bieler and Heim (1965) found cancer of both bowel and uterus in two sisters, and in the same family there were nine other cases of carcinoma of the colon in 34 members; and Aure and Nilsson (1965) reported five of seven in a

family with carcinoma of the colon and one with carcinoma of the stomach; the mother and uncle also had carcinoma of the stomach and the mother's father had a carcinoma of the colon.

Cases of cancer of the breast in families are of doubtful significance when taken individually. However, Lilienfield (1963) reports two brothers with cancer of the breast. There have been a number of reports of cancer developing in the second breast after mastectomy for cancer. Missakian, Witten and Harrison (1965) 25 cancers of the breast in 397 post mastectomy patients; Leis, Mersheimer, Black and Chabon (1965) primary cancer of the second breast in 7.5-10% of a series of cases, five times the frequency in the general population; and Robbins and Berg (1964) state that seven patients per year per thousand post mastectomy patients develop cancer in the second breast. These data are of interest; it is impossible to say that hormonal or environmental effects are not of great importance here, but one might also expect that there is some intrinsic factor in the breast cells bringing about this high incidence of cancer in the second breast. Another observation to note is that in Klinefelter's syndrome the incidence of cancer is the same as in women; Jackson, Muldal, Ockey and O'Connor (1965) say that the frequency is 665 times the expected incidence in normal males.

Leukaemia has been frequently implicated as having a higher incidence than expected in families. One must first mention the Philadelphia chromosome which is of very regular occurrence in chronic myeloid leukaemia (Nowell 1962, Le Jeune, Berger, Caille and Turpin, 1965). Whang, Frei, Tjio, Charbone and Brecher (1963) claim that the deletion of chromosome 21

is found also in red cell precursors and megakaryocytes, and this is also supported by Tough, Jacobs, Court Brown, Baikie and Williamson (1963). Goh and Swisher (1965) found that one of identical twins had chronic myeloid leukaemia and a Ph' chromosome, whereas the other was normal with a normal karyotype. It seems, therefore, that the Philadelphia chromosome and resultant chronic myeloid leukaemia from this cause are unlikely to be found as a familial condition, although the closely allied Christchurch Chromosome (Gunz and Fitzgerald, 1964) was found as a familial character, and two individuals bearing it were sufferers from lymphoid leukaemia. Of other familial cases, Kolmeier and Bayrd (1963) reported leukaemia in mother and son; Heath and Moloney (1965) reported five cases of acute leukaemia in three generations of a family; and Pearson, Grello and Cone (1963) reported acute leukaemia in identical twins aged 3½ years. There are reports of this nature dating back many years. One would be inclined to accept this as being very likely a genetic predisposition except for at least one report which should make one cautious, and that is the so called "leukaemia cluster" of eight cases in a confined district at Niles, Illinois (Schwartz, Greenspan, and Brown 1963). Claims of "leukaemogenic" viruses causing this situation have not been fully substantiated.

Further reports of unusual malignancies in families include that of Stewart and Bagshaw (1965) of malignant testicular neoplasms in 37-year old identical twins; of Wilms tumour in brother and sister aged less than one year reported by Zunin and Soave in 1964; of intestinal carcinoid in a brother and sister reported by Eschbach and Rinaldo (1962); and of Ewings sarcoma in siblings reported by Hutter, Francis & Foote in 1964.

Chapter II - The Statement of the Thesis.

In summary the thesis is that sequential somatic mutation can explain the phenomena of neoplasia.

This concept is the same as that expressed by Nordling in 1953. Nordling arrived at his theory through a statistical study of data on the incidence of cancer, and certain general considerations on the subject of mutation and allied matters. The statement in this chapter will cover in very general terms what this writer believes are the essential steps in understanding such a theory.

The fact of mutation of genes in the germinal cell line has been accepted as proven. Higher and lower organisms have been observed for mutations both spontaneously occurring and brought about by various experimental agencies. In humans mutation rates for various genes have been calculated from data on sporadic cases of genetic disease. These rates are in many cases of the order of one mutation per gene per 10,000 germ cells.

The first hypothesis is that mutation in somatic cells occurs as in cells of the germinal cell line, and that they have frequencies of the same order as those of the germinal cell line. Let us postulate for further discussion a somatic mutation rate of one mutation in 100,000 genes in each unit of time (which could be a year, or it could be 10 years). The actual figure may be subject to cell division rates, but since cells divide with greatly varying rates according to the tissue under consideration it is easier to consider a unit of time.

What happens to a cell in which a somatic mutation has occurred?

Since there are many thousands of genes with different known or presumed functions, it is reasonable to assume that a mutation in a gene for say haemoglobin production is unlikely to have any importance in a melanocyte or a brain cell. The second hypothesis is therefore that most somatic mutations have no effect whatever in the cell lineage in which they occur.

What, then, happens to a cell in which a gene active in the biochemical functioning of that cell mutates? Genes produce enzymes or proteins which all have a part to play in the organism. A cell in a diploid organism which has two effective genes at a particular locus can presumably continue to function, perhaps not as well as previously, by the loss of function of one of these genes. If both genes cease to function at the particular locus, and if this locus determines a specific function of the cell, the cell must lose either immediately or after a suitable lag period this particular function. It has been shown that cytoplasmic particles have a degree of autonomy in some cases in carrying out the work of a nuclear gene and one must allow for the possibility of a gene's function in effect outliving the gene for a period. One would advance the hypothesis that the cell ceases to have its original specific function after losing its specific genetic effectors at the particular locus, but that this loss does not necessarily affect the viability of the cell.

What function then does the cell take on? Most biochemical pathways have been shown, where knowledge is adequate, to be complex, depending on a number of steps, each thought to be controlled by an enzyme. When a step at the end of the biochemical chain is missing, what function does the cell have? Presumably a modified function related to its original function, but not the same as it. What physiological responses will the

cell have in relation to the other cells in the tissue? Will it respond to normal stimuli by an increased rate of division, or by an unaltered rate of division, or by a reduced rate of division, presuming that it is one of the many cells in the body which is still capable of division?

The phenomenon of "pigment spread" in mammals may be used as an example here. Where a eumelanin producing melanocyte is adjacent to a phaeomelanin producing melanocyte, or either type is adjacent to a melanocyte producing no pigment, then the cell producing the lesser type or amount of pigment gradually becomes ousted by the one producing the greater type or amount. Since the cells are in a monolayer and numbers of cells can be accurately determined, this must mean that the cell producing eumelanin must respond to physiological stimuli with a greater reproductive performance than the cell producing phaeomelanin, and this latter again has a reproductive advantage over the non pigment producing cell. Taking the example of the melanocyte further - the change in pigment producing ability could be in either direction, and a mutant cell might be quickly eliminated if it stopped producing any melanic product. One would therefore advance the hypothesis that a mutant change in a cell is capable of altering the reproductive potential of the cell, either leading to an increased cell division rate or a reduced cell division rate; alternatively the rate may remain unchanged.

If a mutant cell is produced having an increased rate of division (and this may well be a very rare phenomenon in relation to the frequency of somatic mutation as a whole), then in the course of time a clone of cells descendant from the mutant cell will be built up. Such a clone may be eventually in excess of a million cells. If we return to our original

postulate of one mutation per 100,000 genes per unit of time this gives a high theoretical probability, supposing the postulate is correct, that further mutations will occur at specific loci within this group of cells. Alternatively if there is no reproductive advantage the chance of a further mutation at a particular locus is only 1 in 100,000. If there is a reproductive disadvantage this cell line will sooner or later be eliminated. If a clone of cells is established capable of further somatic mutation, one can extend the hypothesis of added reproductive advantage in some of the mutant cells to produce the next hypothesis - that is that sequential mutations acting as a selective force in successive clones of cells may produce cells with successively greater rates of multiplication.

What are then the ultimate possibilities in sequential somatic mutations occurring in successive clones of cells? The cells of a developing embryo have a very high rate of multiplication and a very low level of specialization. It is apparent that many of the cells of the developing embryo have not developed their full biochemical potency as they will have when they become mature cells, and this means that the biochemical potential of many genes is not being put to use; on the other hand it must be axiomatic that biochemical pathways leading to growth and reproduction of the cells must be very active in the embryo. Do the biochemical activities leading to specific functions of a cell have an inhibiting effect on the biochemical activities leading to growth and multiplication? As a small chemical factory a cell can't be "all things at all times" - a cell active in the production of haemoglobin cannot also be active in building cytoplasm and nuclear material preparatory to a cell division. The final

hypothesis then is that cells which lose genetic components leading to differentiation and definitive function are potentially freed to adopt a more primitive behaviour with greater rate of cell division, greater accent on nonspecific cytoplasmic increase, and increased nuclear size and activity.

There is a final rider to these hypotheses which I feel is very important, and is fundamental to this thesis. All higher organisms are diploid. A somatic mutation at a particular locus will, on the presumption that the changed gene no longer produces protein effective in the enzymatic pathway, leave the cell effectively haploid in relation to this gene. Using the frequency of somatic mutation of 1/100,000 per unit of time, this means that in a tissue effectively diploid there will be one cell in 50,000 with only one effective gene at this locus at the end of the unit of time and one cell in 50,000. x 100,000 or 5 x 10^9 with no effective genes at this locus. If a reproductive advantage does not occur until both genes at the locus are ineffective it can be seen that clones of cells resulting from the double event are going to be rare, unless some extraneous force greatly increases the mutation rate. However, if the organism has only one effective gene at the locus, in the first place, then the expected rate of mutation in the normal gene will produce one cell in every 100,000 with the deficient function. If this is sufficient to confer a reproductive advantage on the cell then, as cell populations go, a very large number of cells will have this reproductive advantage.

Where then does one find individuals who may have only one effective gene at the particular locus? The concept of dominance in genetics at

times has proved to be unfortunate. It describes certain situations extremely well, but leads to unfortunate habits of mind in other directions. To cite two examples - many supposedly recessive genes can be detected by refined investigations in heterozygotes, which reduces the relevance of the recessive-dominant distinction; and many "dominant" conditions in man have never been observed in homozygotes and it is suspected that quite different and in many cases non-viable phenotypes would arise in such a homozygote. If an individual shows marked phenotypic differences due to the possession of a gene which is not contributing any active enzymatic product, then this heterozygous individual will be detected by the occurrence of the phenotype due to the defective gene, and this gene will have credited to it the importance of being a "dominant" gene, even though it is one which in fact does nothing worthwhile.

The subsidiary hypothesis is therefore put forward that many dominant genes may in fact be genes which produce no active enzymatic product, and that the organism may remain phenotypically normal until the remaining normal gene at the locus undergoes mutation in particular somatic cells, producing somatic variability of that tissue, and that this variability may express itself as multiple tumour formation. Expressed more briefly the hypothesis is that those disorders in which multiple tumours occur are likely to be dominantly inherited.

Chapter III Freckles, birthmarks and moles.

A. Literature.

Freckles have rated a small but definite scientific interest over the years, Heusinger (1823), one of the earliest microscopists to study pigmentation discussed freckles, "liver spots" and moles. Simon (1840) commented on the pigment in the rete malpighii in freckled persons, and Henle (1843) discussed the freckles of blondes. The study of freckles, birthmarks and moles continued throughout the latter half of the century and Blaschko (1901) and Darier (1902) discussed various phenomena. Darier stated that he regarded freckles as in every way comparable with naevi.

In the second and third decades of the twentieth century there were contributions to freckling by German authors. Much effort went towards the study of the heredity of freckling. Hammer (1912) concluded that freckling behaved as a dominant character and found 151 freckled individuals out of 222 where one parent was freckled. Meirowsky (1919) also studied segregation ratios, - where one parent was freckled he observed 10 freckled and six unfreckled children; where both parents were freckled he observed 42 freckled and 14 unfreckled. He studied five generations in one family with 62 members freckled, eight of whom had red hair. Scheidt (1925) published two pedigrees of freckling each showing freckling through three or four generations. Decking (1926) considered uniovular and binovular twins. In presumed uniovular twins he found no freckles in 22 pairs, traces in nine pairs, medium amounts of freckling in 18 pairs, and heavy freckling in four pairs, whereas there were minor differences between members of a pair in eight pairs. In binovular twins the situation was "totally

different". Of 30 pairs six pairs were freckle free, five pairs were freckled to a similar degree, but in sixteen pairs there were distinct differences, in three pairs lesser differences. He attributed freckling to heredity and environment, the latter being in particular exposure to sunlight. Siemens (1924) made studies of a very similar nature. He had 46 pairs of uniovular twins - both freckled in 30 pairs, both unfreckled in 15 pairs, one freckled on the nose and one unfreckled in one pair. For binovular twins he had twenty pairs of which two pairs were unfreckled, both were freckled in 14 pairs, and five pairs were dissimilar. He reported a strong correlation between freckling and red hair. Meirowsky (1942) in reviewing some of the older work stated that the former distinctions between freckles and naevi were not justified and that freckles represented the simplest form of pigmented naevus.

Prosser Thomas (1960) discussed freckles and stated that a freckle was "a small flat pigmentary spot of serrated outline and a yellowish colour", and that it "can occur occasionally in covered parts", this type being referred to as a cold freckle. He said that freckles do not "as is sometimes supposed, disappear in winter".

The first application of a modern technology to the problem of freckling was introduced by Breathnach (1958 and 1963). He found (1958) that there were fewer tyrosine positive cells per unit area than in surrounding skin (901 $\pm$ 8 and 1441 $\pm$ 40 respectively). The melanocytes are larger and more dendritic, and the freckles manifest themselves as small spots which spread slowly. He stated that the boundary between a freckle and the surrounding skin lies "within the territory of a single

melanocyte", and suggested that there could be two genetically different types of melanocyte. In 1963 he discussed the melanosomes of freckles and surrounding skin. In the former he found elongated rod like melanosomes 150 mμ x 370 mμ in greatest dimensions, with a 90 Å striation. In the latter he found that the cells have a "peculiarly empty appearance", elongated melanosomes were not found, but rounded ones 250 mμ or less in diameter were found in small numbers. These have a finely granular internal structure and are similar in size and shape to the melanosomes of the melanocytes of red hair. He felt that there were "differences of kind rather than of degree" and suggested that "randomly distributed melanocytes undergo somatic mutation into the type found in freckles", which are the type found in the skin of non red haired people. He quoted an unpublished opinion of Fitzpatrick that freckling might be due to somatic mutation due to untraviolet irradiation.

The origin and genetics of birthmarks has been the subject of much speculation. At this point it seems appropriate to try to define certain terms, as the terminology appears to reflect some of the confusion in a science in which there are still no clearcut guideposts. The word naevus (L born) implies something that one is born with, but it has been applied to almost any pigmented, or depigmented, vascular or avascular, lipomatous, fibromatous or epithelial object found on the surface of the skin, and often by inference in deeper organs as well, regardless of whether one is born with it or not. In this thesis a simple English terminology is used wherever possible - "freckle" as defined by Prosser Thomas, which is produced by a monolayer of melanocytes, as is the pigment of normal

skin; "mole" which is a collection of melanocytes of more than one layer in thickness, and pigmented birthmark which can have the microscopic characters of either a freckle or a mole. A birthmark is further essentially something present at birth (this question will be discussed later in this chapter) and may be pigmented, vascular etc., although as stated the interest here is in pigmented birthmarks.

At the end of the last century writers were impressed by the way in which birthmarks, pigmented and otherwise, were oriented on the surface of the body. Meirowsky (1942) quoted several authors - Baerenstrung (1861 and 1863) who supposed "a connection with the system of nerves", Simon (1873) with the "lines of cleavage" and Blaschko (1901) who found that they were arranged "always in the same system of lines".

Meirowsky (1942) discussed the inheritance of various types of birthmarks. Certain of these hereditary situations are now regularly included as part of certain phenotypes which will be discussed later, e.g. neurofibromatosis, Sturge-Weber disease, Lindau's disease. Some of the reports quoted e.g. of parent and child with a pigmented naevus in much the same area of the body, obviously draw on chance association to a great degree - such observations would have to be related to the frequency of the lesions concerned in the particular area and the degree of agreement observed.

On the histology of the flat pigmented birthmarks Lorincz (1959) stated that they are indistinguishable from freckles.

There is one interesting phenomenon which has been observed by a number of authors and this is the "divided naevus" occurring on the eyelids.

Harrison (1960) describes one such case and says that about a dozen cases have been reported. A pigmented birthmark is found which is complete and oval in outline when the eyes are shut - when they are opened it is seen to be partly on the upper lid and partly on the lower. Embryologically the lids fuse at eight weeks and then begin to separate again at about four months. This means that the developmental accident causing the divided naevus must occur during this time.

Finally one must say something about the voluminous literature on moles and melanomas. The background to the behaviour of the melanocyte has been discussed in preceding parts of this work (Chap. 1) and a few aspects not yet touched on will be discussed.

As in the study of freckling in the early part of the century, so also with moles, certain German authors worked out correlations between twins with moles. Siemens (1925) studied 45 uniovular and 23 binovular twin pairs. On the basis of counts of moles he found a correlation of 0.82 ± 0.05 for uniovular and 0.4 for binovular twins. Lenz (1926) reported data of cases collected by Meirowsky on 150 uniovular and 150 binovular twin pairs and found a correlation of 0.78 ± 0.36 for uniovular and 0.31 ± 0.09 for binovular twin pairs.

The histology of moles is more complex than that of freckles, where as already stated the melanocytes form a monolayer as in normal skin. There are certain aspects of the histology of moles which are important in assessing potential malignancy and in studying the changes which can occur on a temporal basis. Pathologists distinguish between junctional naevus, where the main proliferation of cells is at the dermoepidermal

junction, intradermal naevus where as suggested the naevus cells have moved into position in the dermis away from the dermoepidermal junction, and compound naevus in which a transitional stage may be seen.

Electronmicroscopic studies have greatly extended the scope of histological investigation and much information has been gained in recent years. Mishima (1965) deals with these matters in detail. He distinguishes melanosomes in intradermal naevi, melanosis of Dubreuilh, melanotic and amelanotic malignant melanoma, and junctional naevi. In the latter the melanosomes are an average 500 mμ x 150 mμ. In the intradermal naevus there are three distinguishable cell types - the A type which are superficial, consisting of large epithelioid naevus cells which contain spherical melanosome granules 200 mμ in diameter, with internally a fine granular appearance. The B type are found in the mid corium and are small round cells with fewer melanosome particles. The C type are in the deep corium and are elongated spindle shaped cells often forming "neuroid" structures, but with no evidence of melanosomes and no tyrosinase activity. The change from one type of cell to another is not abrupt. The amount of cholinesterase activity increases as one progresses from A to B to C.

Other aspects of moles and melanomas concern differences in frequencies according to race, differences according to exposure to sunlight, differences according to complexion in Europeans, frequency of change from benign to malignant lesions, and aggregations of melanomata within families. Lancaster and Nelson (1957) showed that 26.6% of melanoma patients and 21.4% of skin cancer patients had been excessively exposed

to sunlight whereas the percentage of a control group was 16.2. They showed that there is a greater frequency of melanoma and skin cancer among males than there is among females, and that Australians in coastal areas have a higher incidence than those inland, suggesting that the habit of sunbathing at the beach has undesirable effects. Pack, Davis and Oppenheim (1963) similarly wrote about racial differences which suggested that European populations, especially when living in hot climates were subject to a much greater incidence of moles and melanomata than people of dark skin such as negroes. People of mixed race had a high frequency of melanomata. He gives the figure of 14.6 moles per person amongst Europeans which contrasts with a figure of 40 per person quoted by Stegmaier (1964). This latter discrepancy suggests that there has been no generally accepted standard for the clinical assessment of moles, and this will be discussed further shortly.

Lancaster (1956) published data for Australia which showed that the frequency of death from malignant melanoma increases from 8 per million per year in Tasmania to 23 per million per year in Queensland. This he attributed to increasing exposure to sunlight in these climates. Regional data have also been obtained in the U.S.A. showing the same effect. The other aspect of exposure to sunlight is the distribution of moles and melanomata according to anatomical site. McComb (1965) says that the distribution of moles and melanomas shows a preponderance on exposed parts of the body. McGovern and Mackie (1959) observed the great importance of sunlight in producing moles on exposed parts, showing a high rate of melanoma of the face in either sex and of the trunk in males and the

lower limb in females. Pack et al (1963) also claims a greater frequency of moles on exposed areas of the skin.

The question of frequency of melanoma according to pigmentation likewise has been studied. Lancaster and Nelson (1957) find an excess in red haired and fair haired people, and those with blue or grey green eyes. Pack et al (1963) show that 3% of Americans have red hair, but amongst melanoma cases there are 15% with red hair. Miller and Pack (1962) state that among melanoma cases 51.6% have blonde or red hair, 67.6% have blue or hazel eyes, 68.5% have a fair or sandy complexion, against controls which are 11.6%, 46% and 38.5% respectively.

The factors premonitory to a melanoma are interesting. Many melanomata seem to arise more or less de novo, rather than from preexisting moles. McComb (1965) states that a pigmented lesion is present before the melanoma commences in 86% of cases. Allyn, Kopf, Kahn and Witten (1963) in a survey of moles of hands and feet found that plantar naevi are present in 9%, palmar naevi in 5.8%, and conjunctival naevi in 0.8% of people and point out that malignant melanoma often starts on the hands and feet. They are unable to say what percentage of palmar and plantar melanomata arise from preexisting naevi. Negroes have a high incidence of palmar and plantar melanoma and also of benign naevi. Stegmaier (1964) states that 25% of melanomata arise in benign naevi. These percentages one feels must be accepted with the greatest reserve as patients are notoriously unreliable when certain types of information are requested.

Finally some discussion on familial melanoma is indicated. Miller and Pack (1962) point out that melanoma affects one person per 100,000 per annum so that multiple cases in a family represent a very unlikely random

chance. The figure of 1 per 100,000 may not be very accurate, but suppose it was halved or doubled the chance of more than one case per family would still be very small. Despite this, quite a number of cases with familial aggregation have been reported. Schoch (1963) in describing three cases in one family lists a number of previous reports. Recent reports include Salamon, Schnyder and Storck (1963) who had four families with three cases, two cases, three cases and two cases respectively, and they state that in three of the families there were many freckled individuals. Bowen, Brady and Jones (1964) describe intraocular melanoma in a woman and her daughter. Turkington (1965) reported that among 250 melanoma cases 15 had another affected family member. Relevant to this situation are the reports of multiple primary melanomas, e.g. McGovern and Mackie (1959) in describing 151 cases said that six had multiple primaries, and one individual had four primaries.

B. The phenomenon of freckling.

A freckle is composed of a group of melanocytes which produce a darker melanin than their neighbours. The melanocytes of a freckle form a monolayer beneath the epidermis whereas a mole is multilayered. The cells of a freckle are flattened and dendritic whereas those of a mole are usually nearly spherical and less likely to be dendritic.

It has been assumed in this thesis that all small macular pigmented skin lesions must be either freckles or moles. Clinical differentiation has been related to the histological difference. A freckle must give no impression of thickening relative to the rest of the skin either visually

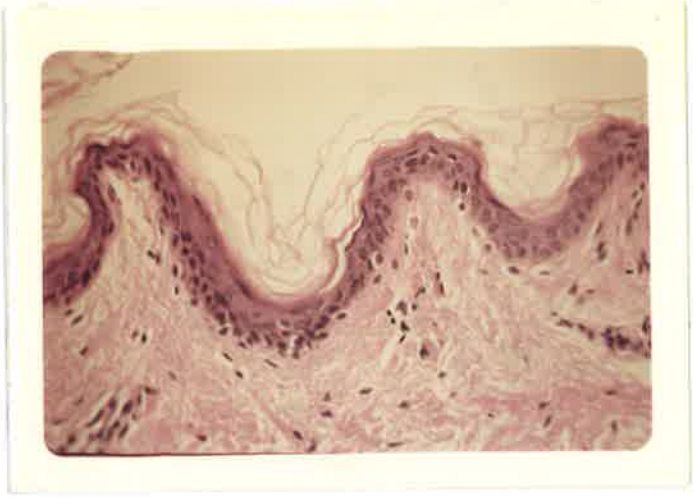


Fig. 2.

Skin of European boy showing no visible pigment in the Malpighian layer. H & E Stain.

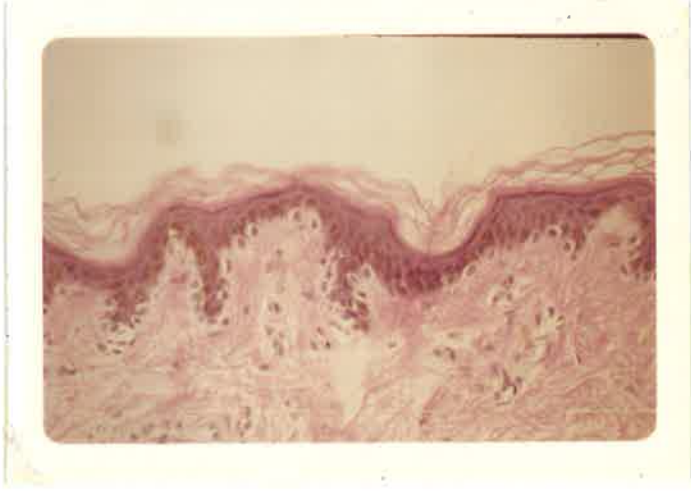


Fig. 3.

Birthmark of same subject as in Fig. 2, showing pigment in the basal layers of the epidermis. Freckles on this boy were histologically the same as seen in this microphotograph.

Fig. 4.

Low power view of a small mole showing masses of naevus cells and distortion of the reticular pattern of the epidermis.

or to palpation. Even very small moles which are not palpably thickened are seen on close inspection to distort the normally regular reticular pattern of the skin surface (Fig. IV). For the sake of numeration of moles no mole with diameter less than 2 mm was counted, and there is generally no confusion between moles and freckles above this size. Other distinguishing features between larger freckles and moles is that the largest freckles which may be 1 cm or more in diameter have an irregularly crenated border, whereas larger moles, even if irregular in shape, still have a relatively smooth border. In relation to degree of pigmentation, a mole is more often more darkly pigmented than a freckle but some individuals, particularly if red haired, can have a range of colours from very pale yellow brown to jet black amongst their freckles, the lighter shades being the most numerous and the jet black freckles usually being very infrequent, at most perhaps 1 - 5 on an individual. The individuals showing this phenomenon seldom have more than one or two moles. The colours in these cases are broadly definable in separate steps - pale yellow brown, light brown, brown, dark chocolate brown and black; for each definable step there are numbers of freckles which can be clearly allocated to the particular level of pigmentation, and particularly with the darker colours there is no overlap or intermediacy in colour.

Fourteen hundred and fifty-six school children were surveyed and for purposes of quantitation two classifications of freckling were used (A1, A2, B1, B2 in the first series of children; A, B, C, D and E in the second.) The purposes of these divisions was related to the distinct differences in degree of freckling discernible between different individuals. In the

Fig. 5. Freckling at the age of 8 years.

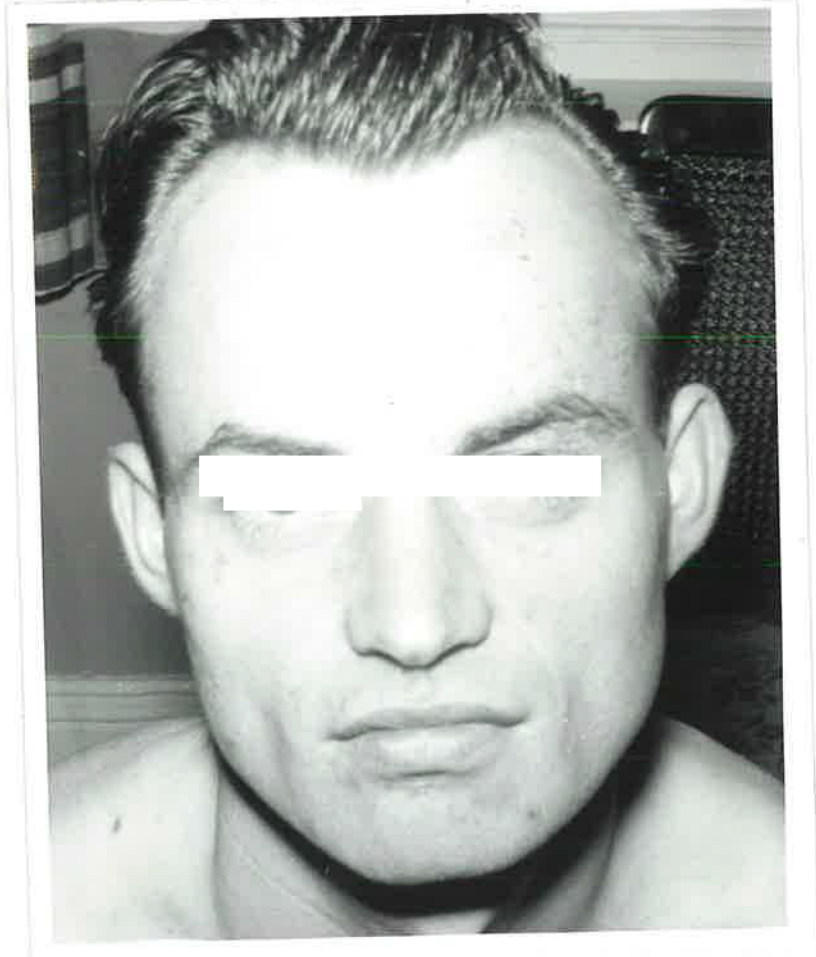


Fig. 6. Unilateral freckling, L forehead.

first classification A1 individuals had no freckles or such a small number of very indistinct freckles as to be regarded as unfreckled. A2 had a small number usually confined to the nose and cheeks, and a few on the arms. B1 had numerous freckles on close inspection, and included individuals who would ordinarily be described by the layman as freckled, although some in this group would not be so described. At this stage one was inclined to search rather diligently for freckles and include many individuals who later would have been excluded from the freckled category. B2 were excessively freckled. In the second series the altered classification was intended to eliminate as far as possible this difficulty. Class A became those with no detectable freckles whatever, B included those with minimal numbers, C included those who were freckled to close scrutiny but not to a degree sufficient to earn this description by the layman, D were the obviously freckled individuals and E were the excessively freckled ones. To give these distinctions a numerical basis the numbers of freckles per square inch in each category were counted and A individuals have 0 per square inch, B 1-5 per square inch, C 5-30 per square inch, and D more than 30.

The difficulties involved in this apparently straightforward method of assessment are apparent when one considers the progression of freckling on different parts of the body at different ages. Any count which is made must be made in an area where the freckles are clearly definable as distinct spots without blending, and usually in the age groups studied the cheek, forearm or shoulders provided a suitable site for counting.

In the freckled person, freckles first appear on the end of the nose between two and three years of age. By four years of age the nose is

Fig. 7. Freckling of the buttocks in heavily freckled adult.

Fig. 8. Multicoloured freckles. Light brown, brown, dark brown and black freckles are visible.

covered in freckles and there are a few on the "butterfly" area of either cheek lateral to the bridge of the nose. By seven years there is a distinctive area crossing the bridge of the nose and spreading widely over each zygomatic bone; meanwhile the end of the nose has become freckled to the point where individual freckles are no longer discernible. By ten years of age most of the face is freckled, and forearms, shoulders and legs are also freckled. By 15 years of age blending of the freckles over the nose, butterfly area of the face, forearms and lower legs may have occurred, but a greater area of the back, and some of the chest, upper arms and upper legs have now become freckled. By the age of 20 years the freckles have largely disappeared from ordinarily visible parts. Meanwhile a greater area of back, chest and upper legs and arms has become freckled. From this point onwards the evolution of change is slower - the back becomes less obviously freckled by the age of 40 years, but freckling can be observed still on upper arms, upper legs and around the waist. As old age is approached the freckling of many areas becomes "senile" in type without clearout pigment spots but a more diffuse appearance seemingly of many pigmentary and other changes which include depigmentations, vascular changes, and degenerative changes in the skin. The place where obvious freckles may still be seen in a "freckler" in the 60 and over age range is on the buttocks where sunlight ordinarily never has an effect.

One must say that this description of the natural history of freckling applies to Southern Australia at the latitude of Sydney and Adelaide, and

probably bears a strong relationship to the behaviour of Australians in these cities, which includes the habit of going hatless in a sunny climate, and the frequent exposure of large areas of the body surface at the beaches. The production of freckles in a freckler is very sunlight dependent, although not exclusively so, and an individual might have the genetic capacity to freckle without ever doing so, due to lack of exposure. The sequence of events may be modified by particular habits, and the outline given can be regarded only as an average expectation at the different ages.

At this point one must return to the classification and point out that A individuals and B individuals will be definable as such at any time in life. The frequencies of freckles in C and D individuals refer in particular to the school children who made up the major part of this study. With the lesser type of freckling (C) the freckles are usually smaller, less heavily pigmented, and of later development and evolution through various phases, and blending on exposed parts appears to occur before very large numbers of obvious freckles are detectable. The E class of freckling refers to a very dense freckling which appears to involve a large part of the body of an adolescent, and may produce the multicoloured freckles already referred to, and after general blending of the freckles can still be determined by inspection of the bathing trunk area, which because of the frequency of freckling in this area in this class of person refutes the commonly held notion that freckling must be due to exposure to sunlight. Another idea which is very erroneous is that freckles disappear in winter - various authors agree that the colour lightens in winter but certainly by no means to the point of disappearance.

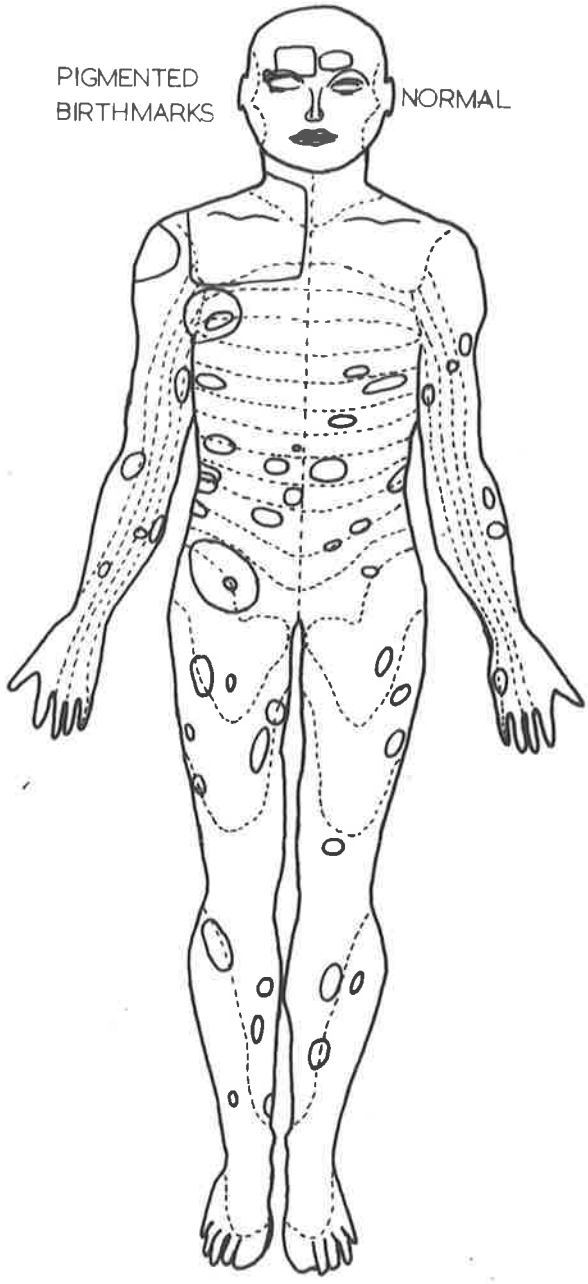


Fig. 9. Outlines of birthmarks seen on normal school children drawn on figure showing segmental nerve supply (dotted lines).

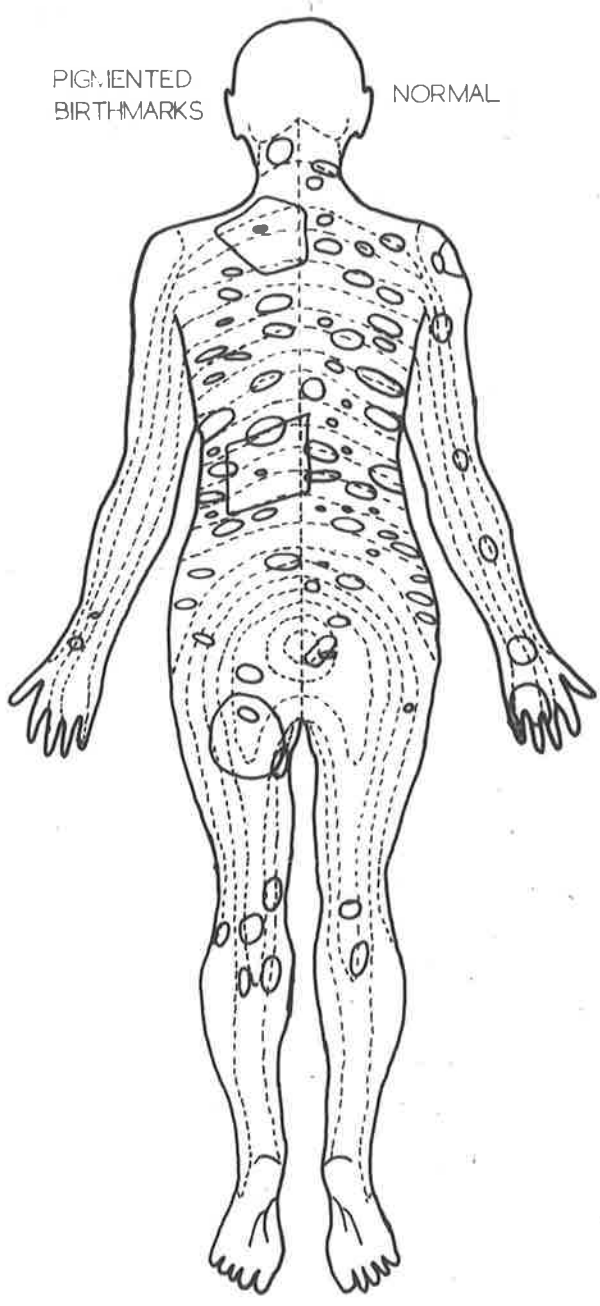


Fig. 10. Outlines of birthmarks seen on normal school children drawn on figure showing segmental nerve supply (dotted lines).

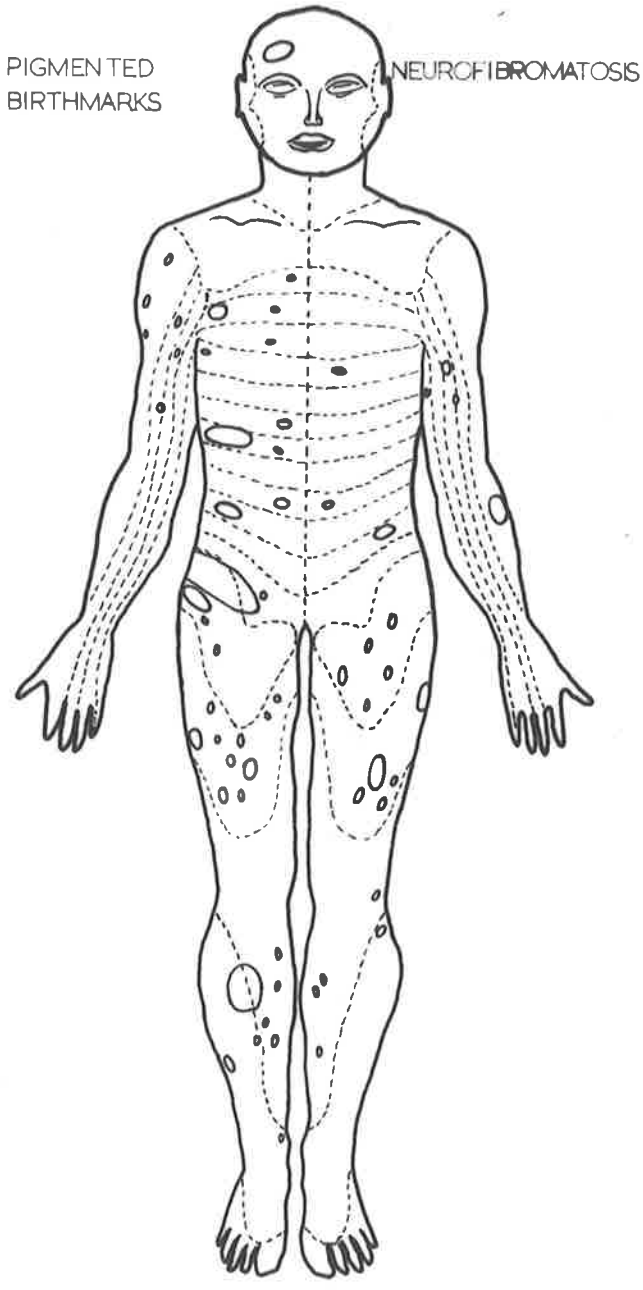


Fig. 11. Outlines of cafe au lait patches of neurofibromatosis cases drawn on figure showing segmental nerve supply (dotted lines).

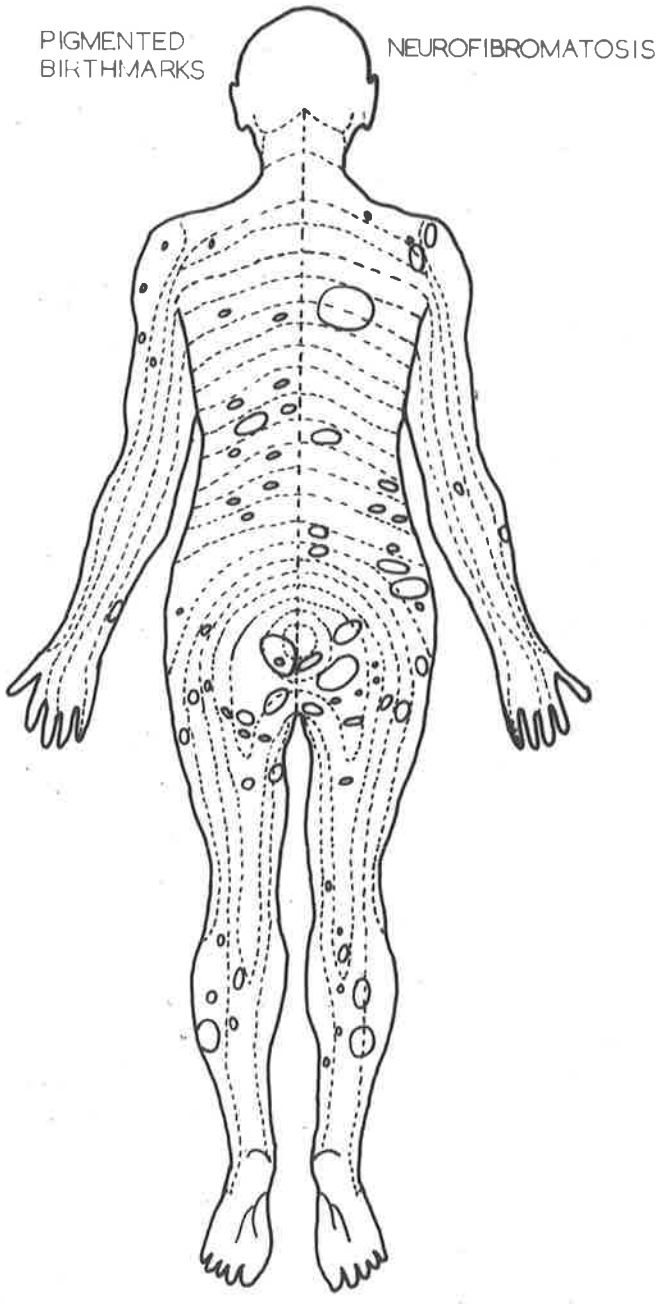


Fig.12. Outlines of cafe au lait patches of neurofibromatosis cases drawn on figure showing segmental nerve supply (dotted lines)

Fig. 13. "Soft naevi", p.206, vol. 11, The British Encyclopaedia of Medical Practice. The obtuse angle ventrally and the acute angle dorsally formed by the pigmented area is characteristic. A number of books on dermatology show pictures of such patients, and a similar case is pictured in "Multiple Neurofibromatosis" by Crowe Schull & Neel (1956). This latter case is diagnosed as a case of neurofibromatosis, and is identical in appearance with the one pictured here, including evidence of plexiform neuroma about the lower lumbar region.

C. Pigmented Birthmarks.

One learns in clinical medicine that people with von Recklinghausen's Disease or Multiple Neurofibromatosis are characterised by large numbers of "café au lait" patches (milk coffee coloured pigmented birthmarks). It is not so clearly stated that many other individuals also have identical café au lait patches which they generally refer to as birthmarks, and which usually only differ in number from those of neurofibromatosis. Histologically the freckling of neurofibromatosis, the café au lait patches, the freckling of normal individuals and their birthmarks are all identical - monolayers of melanocytes with greater melanic activity than those of surrounding skin.

In surveying large numbers of school children the term "birthmark" for the large pigmented patch is in most cases a satisfactory designation - due to personal curiosity, and/or discussions between parent and child, many school children are able to point out without hesitation those lesions which they regard as birthmarks. Sometimes the "birthmarks" prove to be vascular types or what might be better described as moles. Rarely other types of birthmarks are seen. Not every birthmark is identifiable as such by the child and the chief reasons for this are position on the body, faintness of the lesion, or general lack of interest. There is no reason to believe that these latter pigmented patches are different in any way from those which are clearly stated to have been present since birth, so for the purposes of this thesis all such lesions have been included as birthmarks. There is one aspect which deserves comment - birthmarks appear to darken somewhat in the early childhood years, as most of the epidermal pigment does, and faint birthmarks may have been

genuinely invisible at birth. One feels that this would constitute a fairly small percentage of the total number observed and since these lesions have the same characters as the darker ones no misgivings have been felt about recording all patches regardless of possible arguments as to whether they are truly "birthmarks".

The orientation of pigmented birthmarks on the body is interesting. One becomes impressed in examining the birthmarks on normal and neurofibromatosis patients that on every area of the body there is a characteristic orientation of these lesions. The Figures (9-12) show dorsal and ventral views of normal and neurofibromatosis birthmarks superimposed over body outlines showing the segmental distribution of cutaneous nerves. The normals were taken from school children seen in the survey, the neurofibromatosis cases were personal cases and derived from photographs found in dermatological and other text books.

The characteristic orientation of these lesions could be taken as evidence for their occurrence at an early embryological stage. The earlier the change in the individual cell which brought about the development of the lesion, the more nearly the lesion follows the orientation of the skin segments supplied by particular spinal nerves. Where whole sections of the trunk are affected there is also a characteristic distribution of the pigmented area dorsally and ventrally (Fig. 13).

It seems likely that from the study of the orientation of larger and smaller patches in a particular area, and with a full knowledge of the embryological history of different areas, one might be able to make a good estimate of the time at which the particular cellular change seen as

Fig. 14. "Divided nevus" of the eyelids showing large upper segment and small lower segment, quite separated except when the eye is closed.

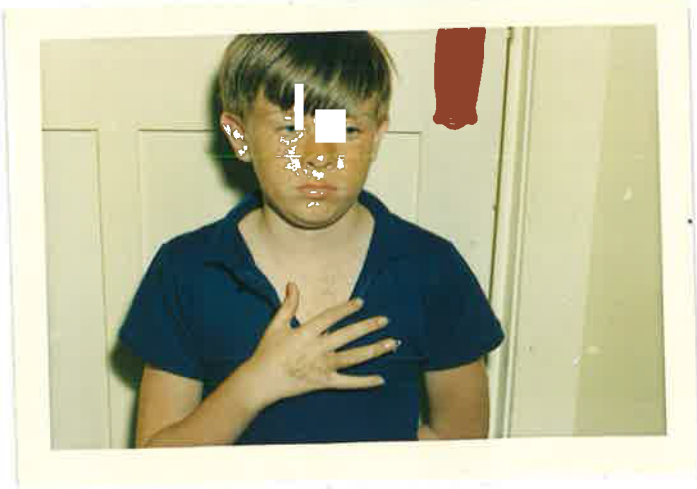


Fig. 15. Birthmark on back of R hand of irregular shape when fingers are separated, but becoming a regular oval shape with fingers together. The margins are marked in ink.

a developed birthmark may have occurred. Two observations made during the survey, one of which has been reported a number of times previously, were of some interest in relation to estimates of the time of occurrence of these lesions. The first observation was of a "divided naevus" of the eyelids (Fig. 14). The photograph shows the large segment of the naevus on the upper lid and the much smaller segment on the lower lid. With the eye open these appear quite unrelated in position and orientation, but when the eye is closed the two segments assume a perfect oval shape showing the origin of this lesion from a single developmental event. The second lesion of interest in this regard was a birthmark on the dorsum of the right hand of a schoolboy (Fig. 15), and this birthmark extended a little way onto the third and fifth fingers, but nearly the whole length of the fourth finger. With the fingers spread out it appeared to have a very irregular shape, but when the hand was closed it assumed a neat oval shape, partly on the hand and partly on the fingers.

Embryologically the eyelids are united between the eighth and sixteenth weeks, and the hand is forming and the fingers beginning to differentiate between the fifth and sixth week. This gives some indication of the probable time of development of the lesions.

The relation of pigmented birthmarks to freckling is interesting and important. Histologically a birthmark and a freckle are identical and one aim of this study is to demonstrate other relationships between the two, the concept being that a pigmented birthmark is in effect a freckle that occurred spontaneously before birth, and in many cases at an early embryological stage. There is a close correlation between the occurrence

Fig. 16. A small birthmark surrounded by freckles showing the clinical similarity.

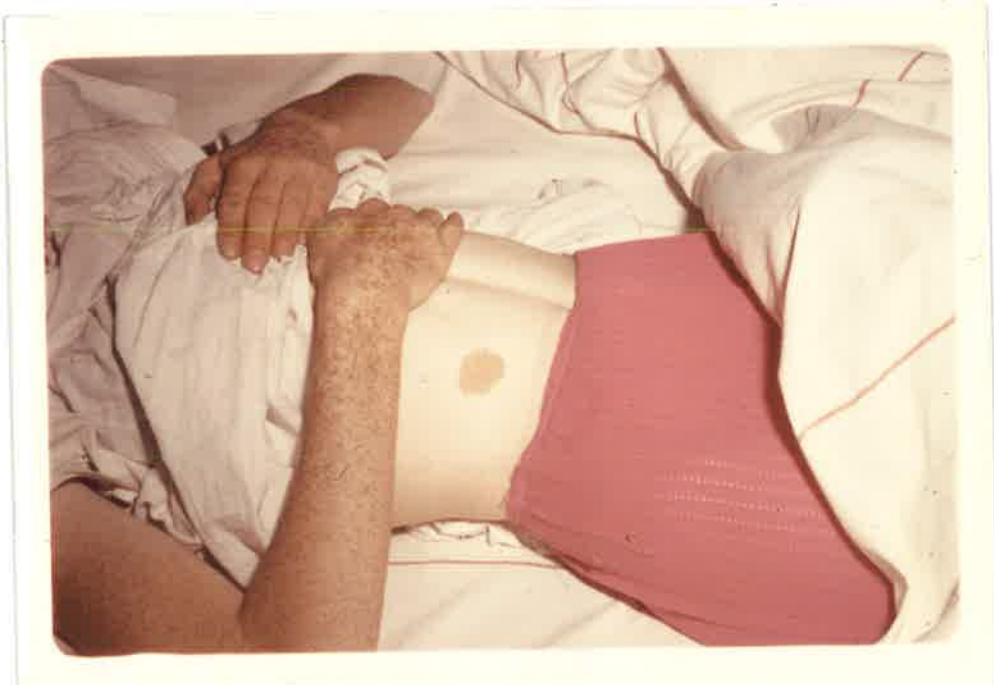


Fig. 17. Birthmark and freckles showing the contrast in colour between these and unfreckled skin. Freckles "fade" with age on exposed parts because the skin assumes a uniform and darker colour, with individual freckles gradually losing their identity.

Fig. 18. Freckles (marked in in ink) surrounding a completely unfreckled birthmark.

Fig. 19. A birthmark about to disappear. Situated three inches from the midline in the L mid-lumbar area, and extending laterally about 3 inches, this birthmark was at one time clearly visible, but due to subsequent freckling has become practically undistinguishable.

of freckles and birthmarks on different individuals and this will be shown later in this chapter.

The clinical similarity can be demonstrated in the photographs (Figs. 16-19). Fig. 16 shows the similarity between a small birthmark and freckles, and also gives an idea of the way in which a birthmark is usually distinguishable by its size and orientation even when surrounded by freckles. Fig. 17 demonstrates the similarity in colour between a birthmark and freckles on the same person, and the marked contrast between these and the unfreckled skin. This subject was a mental hospital patient and much of the pigmentation of the arm is due to freckling rather than general ultra-violet stimulation. The suggestion from this and many other observations is that the skin of a "freckler", when sufficiently freckled, assumes a new and darker pigmentation, and that if one accepts genetic determination for freckles, genes for freckling represent some of the "polygenes" responsible for skin colour.

Fig. 18 shows a birthmark surrounded by a dense aggregation of freckles which were marked in ink in a band around the birthmark. There were no freckles on the birthmark, showing that in this respect there is a basic difference between the skin of the birthmark and the surrounding skin - the ability to freckle. On the hypothesis of somatic mutation this is explained by saying that the birthmark is a large "freckle" of mutant skin, and that a specific gene of the genome had made the change within itself and the same change could not occur again in the same group of cells.

Fig. 19 is necessarily a difficult one to visualise - it is of a

Fig. 20. Freckling on and around a birthmark on the R leg of a red haired boy. Approximately half the number of freckles on the birthmark as on the surrounding skin.

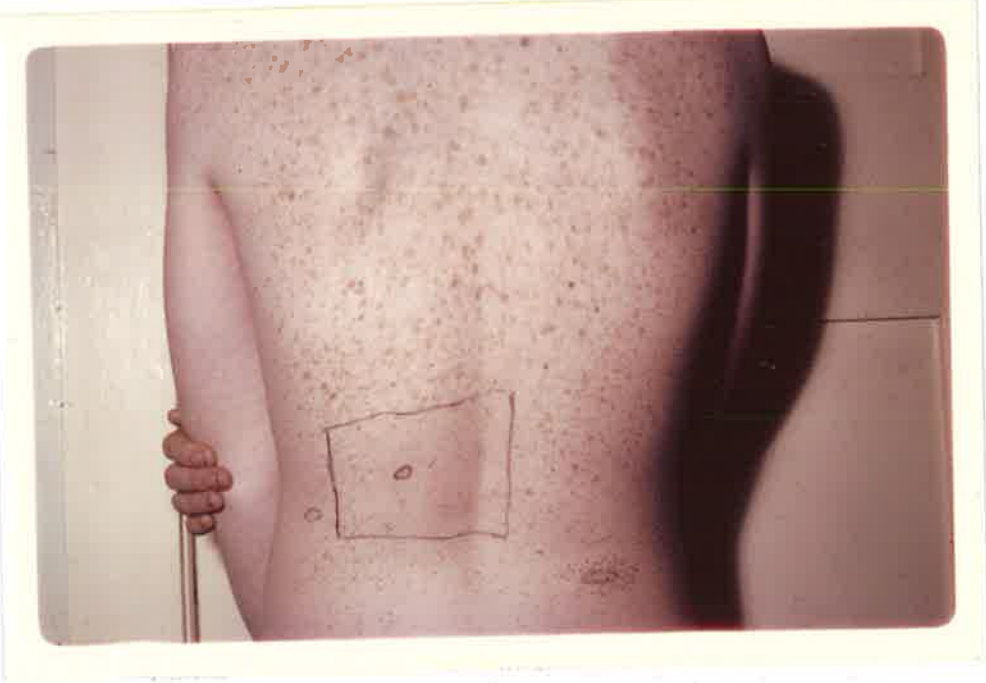


Fig. 21. Large birthmark on red haired boy's back. Fewer freckles on the birthmark than on surrounding skin. Unfreckled small birthmark on the large birthmark. Freckled birthmarks elsewhere on this subject.

birthmark about to vanish because the freckling around it has become so dense that the skin is largely occupied by changed pigment producing cells. This man stated that at one time he had a very distinct birthmark on his back and no freckles. The conclusion to be drawn is that birthmarks in exposed areas disappear at a time when the surrounding areas become completely freckled over and the skin has become uniformly darker in colour. Crowe Schull and Neel (1956) commented on the fewer café au lait patches in older people with neurofibromatosis, and one would suggest that the freckling which occurs in this disease accounts for the disappearance of many café au lait patches in the first few decades of life.

A further phenomenon of freckling in relation to birthmarks has been observed in a number of cases, two of which are pictured. This phenomenon has only been seen in red haired persons, and this brings one to the hypothesis which has been formulated in relation to red hair. This hypothesis is that D and E type frecklers with brown hair represent heterozygotes for the gene which in homozygotes produces red hair.

Fig. 20 shows the birthmark on the leg of a red haired boy. The freckles on the birthmark, which is outlined in ink, and on the surrounding skin, have been inked in and it is seen that there are many fewer, approximately half the number of freckles on the birthmark as there are on the surrounding skin. If the normal skin of this boy was homozygous for a freckling gene and the birthmark was heterozygous this result could come about when freckling occurred. A similar result has been observed in the next case (Fig. 21) where the boy has a large pale birthmark

Fig. 22. Mole like birthmark and numerous pigmented moles.

Fig. 23.
Multiple halo nevi.

Fig. 24.
Eyespots.

on the L lumbar region which is freckled to a lesser degree than the surrounding skin, and has a small unfreckled birthmark outlined near the centre of it. Other freckled birthmarks, for example one on the R lumbar region, occurred in this boy, who was red haired.

Finally mention should be made of the fairly frequent occurrence of thickened "mole like" birthmarks. A photograph of this type is shown (Fig. 22), and in the same way as there are various clinically distinguishable types of moles, so there are also the same clinically distinguishable types of birthmarks. It is a common observation that where a certain type of birthmark occurs on a person, there will also be moles of similar type, as there were in this individual.

D. Moles

The study of freckles, birthmarks and moles was based on a clinical survey of schoolchildren in conjunction with the N.S.W. School Medical Service and permission to examine children was based on the general permission which parents give to the Medical Officers of this service. Taking of biopsies, samples of hair, or even photographs of lesions was not possible under this arrangement, hence any form of microscopical correlation with clinical situations was minimal and was in fact based on the examination of post mortem material at the Royal Alexandra Hospital for Children and Sydney Hospital. As with the general phenomena of freckling and birthmarks, so to a lesser extent with moles, it is felt that there is much to be learned by a purely clinical approach. Many clinical types of moles have been observed and there is a strong clinical impression that certain people have a tendency to produce their "own kind" of mole.

Most have the small flat dark brown mole of 1 - 2 mm in diameter, but others have a number of the same type of lesions up to 4 - 5 mm in diameter. Some have small elevated moles, others have numbers of large elevated dark moles. In some cases there is a tendency to produce hair in the mole, and in yet other cases the moles are large, fleshy, but not deeply pigmented. Some moles approximate in type to neurofibromata clinically. At some future time these clinical impressions may be definable more specifically than is at present possible, and if this should be the case, then useful information pointing to genetic predisposition to this or that type of mole may be forthcoming. A simple clinical classification, to be described shortly, was followed in this study, but no analysis has been attempted so far.

From the point of view of neoplasia in general one can say that a mole is the commonest form of tumour and the most accessible; the fact that it is generally benign should be no deterrent in the study of neoplasia, and there is at least one phenomenon which may well be relevant to the therapy of benign and malignant tumours. This is the phenomenon of the "halo naevus" in which a mole becomes reddened, and appears to be undergoing an inflammatory reaction; it becomes depigmented, and so does a "halo" up to 5 mm in width around the mole. The mole then gradually disappears, and this whole process may take two or three years. Some individuals will develop as many as 5 - 10 such halo naevi but it is seldom that all the moles on a particular person will be affected. It occurs most frequently in the 10 - 15 age range, and is relatively infrequent (10 cases in 1500 school children). In one case the sister of an affected boy also had one

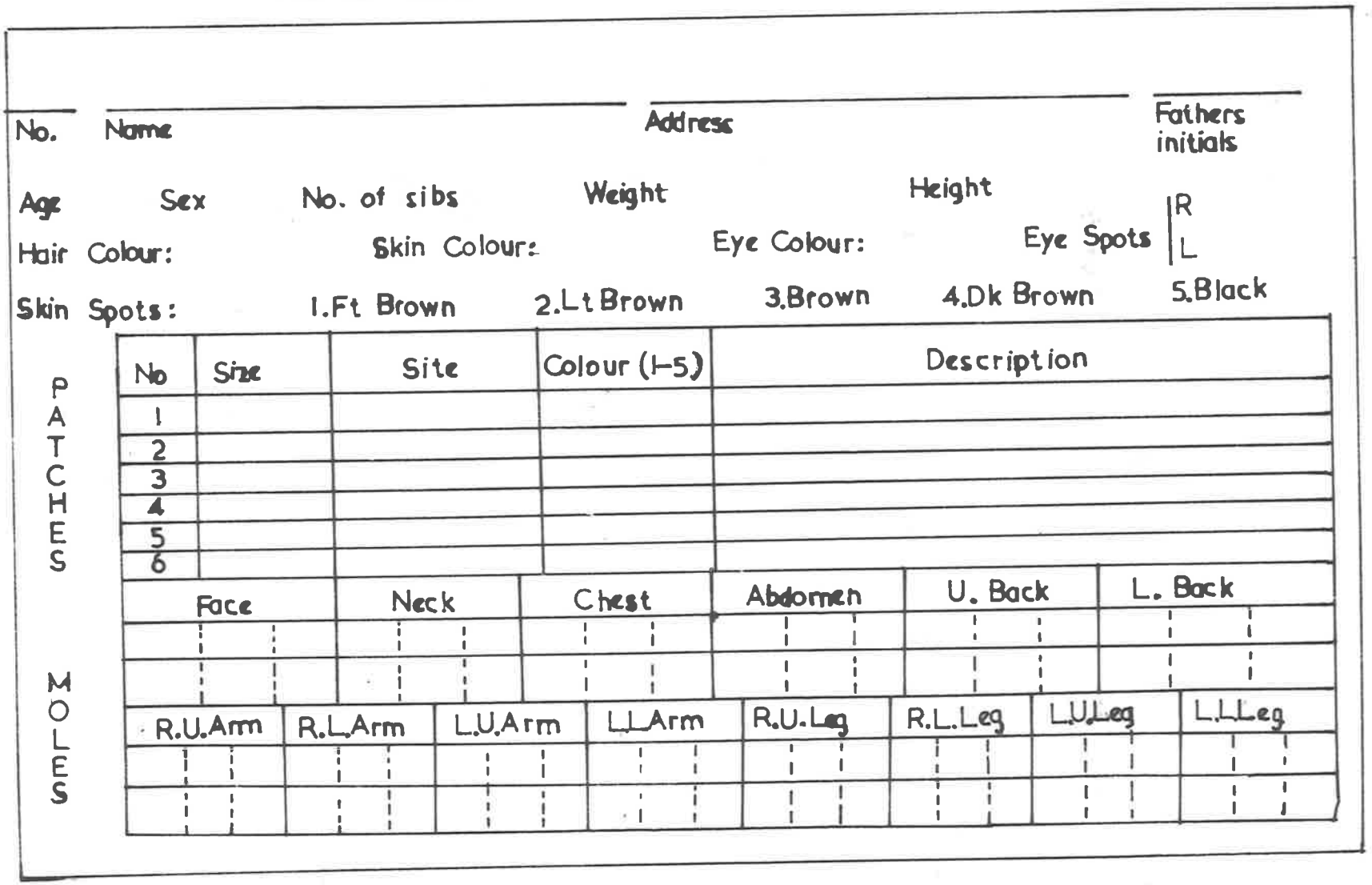
halo naevus and numerous depigmented spots particularly on the legs. Since halo naevi appear to occur most frequently on chest and back, the occurrence of depigmented spots on the legs in this case could not be convincingly linked with the occurrence of the naevus. It has been suggested that the halo naevus may result from an antibody type attack by the body on the "new growth", and the thought that the body may in fact "recognize" mutant gene products will be discussed later.

In the study of school children all moles were counted, excluding those less than two millimeters in diameter. This removed the ambiguity between the mole and the freckle (even though many freckles are greater in diameter than 2 mm), and allowed one to ignore many very doubtful small lesions. The clinical classifications of moles already referred to were five in number. Class I was the flat plaque like mole 2 - 4 mm in diameter; class II was the same type but in excess of 4 mm diameter; class III were the conspicuously elevated moles between 2 and 4 mm diameter; class IV were the same type in excess of 4 mm diameter; class V were those which were unclassifiable into the first four classes and usually merited some separate description. The colours ranged from pale brown to black, although the most common colour was dark brown.

E. Eye Spots.

The occurrence of brown pigment spots in the iris is analogous in many ways to freckling. The spots develop during childhood, becoming more obvious in adolescence. A simple classification of iris colour was used; blue and brown which are both determinable with very little ambiguity, and grey which includes most of the intermediate shades between blue and brown,

Fig. 25. Replica of card used in survey of schoolchildren.



including hazel and green which seem to be favourites with some people. For each of the three classes blue, grey and brown the occurrence of spots of darker pigment was recorded - it is by far the most common in grey eyes. It is possible to count the number of such spots in each eye but the correlation between number of spots and number of moles has not been worked out, although other data have been presented.

The colours of the spots are in the vast majority of cases some shade of brown, although rarely black spots have been seen.

Other observations on eye colour recorded were the occurrence of segments of brown in grey or blue eyes and this occurred in six of approximately 1500 schoolchildren. This usually occurs in grey eyes with brown spots, but as suggested can also occur in blue eyes. The occurrence of one "blue" and one brown eye has been seen two or three times by this observer, but not in the series of schoolchildren. The "blue" eye has always been classifiable as a grey eye with brown spots and whether this is the more common occurrence of this condition is not known.

F. Interrelationships.

Interrelationships between various pigmentary characters were studied in the survey of school children. The form used is shown in the accompanying reproduction (Fig. 25). In all 1456 children were seen at various schools. In 1964, 721 children mainly from High Schools were seen, and in 1965 and 1966 735 from Primary Schools were seen. These groups constitute Series I and Series II, the differences between the groups being average age, and the change in classification of freckling already discussed, which was made at the end of Series I. The ages and numbers are set out

in the Table.

TABLE I: SCHOOLCHILDREN SEEN IN SURVEY -- AVERAGE AGES.

	Male	Female	Total
Series I	13.9 years (531)	14.7 years (190)	14.1 years (721)
Series II	10.1 years (467)	10.5 years (268)	10.3 years (735)

The name, address, age, sex and number of sibs of each child was recorded, and it was possible to visit some families subsequently and get family data. The families selected for this purpose were those with a large number of children, or where the propositus had some interesting feature, e.g. red hair.

Weight and height were recorded, and from this, using the nomogram in Documenta Geigy, surface area of each child was estimated. Hair colour was graded as Blonde, Light Brown, Brown, Dark Brown, Black and Red. The author's discrimination of red appears to be sufficient for practical purposes as the spectrophotometric analysis of 76 hair samples in Fig. 56 shows no overlap between reds and browns.

Skin colour was graded as Pale Brown, Light Brown, Brown and Dark Brown. Eye colour was graded as already discussed (Blue, Grey, Brown, Grey with spots, Brown with spots). Freckles ("skin spots") were allotted a letter according to the clinical assessment, and the various colours of spots were recorded. Birthmarks ("patches") were recorded as shown. Size was measured in millimetres and was recorded for longest and shortest diameters. Areas of birthmarks were later roughly calculated as the product of length times breadth, and it is thought that this was sufficiently accurate for the purpose for which it was to be used. The colours of

birthmarks were recorded in the same categories as the colours of freckles (Faint Brown, Light Brown, Brown, Dark Brown and Black). The description included such features as hairiness, thickening, freckling, and any other observable feature.

All moles in excess of 2 mm diameter were counted using the criteria of a mole already laid down. The distribution on the body was recorded in the spaces provided. From this eventually one might obtain data showing that the more exposed parts of the body have more moles than the covered parts, but this has not been done. The clinical division of moles into five categories was followed, and the category number and number in the category were recorded on the cards.

The analysis of the results comprises various frequency distribution tables. It is to be noted that Standard Deviations are high in all classes because overall the variation in numbers of moles per person was from 0 - 241, and no class was exempt from rather wide variations in numbers.

Many differences in mean values have been found. Some of these are rather less relevant to the purpose of this thesis than others, but are recorded for completeness. Comments as to the statistical significance of some results is made after presentation of the results in the tables. Rather than obscure the more important points with actual data on significance, an appendix (Appendix I) giving these results is included at the end of the Thesis.

(a) <u>Freckling in relation to age</u>.

As the classifications and the mean ages differed between the two

series, and the assessment of "frecklers" and non frecklers" clinically made allowance for differences in progression of the freckling phenomenon at different ages, these data do not add anything further to the relation of freckling to age.

(b) Moles in relation to age.

In the age range studied moles increase rapidly with age, and males have more moles than females. The only big divergence from the general trend is in 15 year old boys. This is hard to explain, as 14 and 16 year olds examined at the two schools where the greatest number of 15 year olds were seen conformed closely to the general pattern. One feels sure that there was no form of bias towards 15 year olds and the result must be attributed to chance and perhaps some genuine peak in frequency of moles at this age. How this could come about is not apparent unless some moles disappear after this age. As regards the sex difference, method of examination might have only a limited effect, and for younger girls none at all; it is in these that the biggest difference proportionally occurred. All males were examined in shorts or underpants; all females were examined in singlets and pants.

TABLE II. NUMBER OF MOLES BY AGE.

	Males					Females				
Age	No.	No. of Moles		S.D.		No.	No. of Moles		S.D.	
7	(18)	7.56	± 1.83	SD	7.75	(3)	2.67	± 0.33	SD	0.57
8	(52)	6.92	± -0.89	SD	6.41	(13)	1.46	± 0.39	SD	1.39
9	(85)	8.19	± 1.15	SD	10.56	(20)	2.60	± 0.91	SD	4.08
10	(120)	9.30	± 0.99	SD	10.82	(84)	7.52	± 1.06	SD	9.75
11	(151)	12.93	± 1.54	SD	18.91	(95)	6.49	± 0.76	SD	7.42
12	(99)	11.66	± 1.53	SD	15.22	(53)	7.40	± 1.44	SD	10.45

Age	Males No.	Males No. of Moles	Males S.D.	Females No.	Females No. of Moles	Females S.D.
13	(139)	19.15 ± 1.69	SD 19.87	(12)	13.83 ± 4.23	SD 14.67
14	(130)	23.97 ± 2.25	SD 25.71	(60)	17.28 ± 2.32	SD 18.00
15	(128)	37.14 ± 3.37	SD 38.12	(90)	22.84 ± 2.29	SD 21.77
16	(65)	22.72 ± 2.87	SD 23.17	(24)	24.17 ± 3.23	SD 15.80
17	(7)	23.43 ± 7.15	SD 18.93	(1)	3	

Inspection of these data shows a roughly linear increase with age. A regression equation of the form $y = a + bx$ has been calculated for males and females, where y = number of males and x = age. "a" for males is -19.61 and for females is - 24.47, while "b" for males is 3.08 and for females is 3.01. These equations give marked negative values for the number of moles at zero age but this is the result of unwise extrapolation from the data rather than incorrect mathematics. The regression coefficients of 3.08 and 3.01 have standard errors of 0.29 and 0.15 respectively which indicates that the positive values obtained differ significantly from zero i.e. the data show a genuine increase of numbers of moles with age.

The average numbers of moles in each series are shown in Table III.

TABLE III. Moles in Two Series of Schoolchildren.

Series I	24.38 ± 1.00	S.D. 26.77	(531 males, 190 females) (average age 14.1 years)
Series II	7.61 ± 0.35	S.D. 9.53	(467 males, 268 females) (average age 10.3 years)

(c) Birthmarks in relation to age.

The two series (average ages 14.1 and 10.3 years) gave different values for the number of birthmarks, 0.67 per child, and 0.51 per child respectively. Two reasons probably account for this difference. Firstly,

the size of the child and the child's birthmark. A lesion considered to be too small to count in a 10 year old might be counted after four years of additional growth in a 14 year old. Secondly, degree of pigmentation. The body and hair pigment of 14 year olds is on the average darker than that of 10 year olds and many birthmarks are very pale in the younger age groups, becoming more obvious later. Hence in an examination some are undoubtedly missed, and the younger the child the more likely is this to happen. It is felt that the result obtained does not indicate a serious discrepancy.

Comparing the effect of age on moles, and age on birthmarks, one sees that the number of moles in Series I is roughly three times that in Series II, whereas there is an increase of only 32% in the number of countable birthmarks. Further data on birthmarks will be given in subsequent sections.

(d) Moles and racial type.

The population of schoolchildren in the area visited comprised individuals of Australian birth usually with English, Scottish or Irish surnames, migrants from the British Isles and Germany, migrants from Italy and Greece, and Chinese. The groups were divided into Northern European, Southern European, and Oriental. There were marked differences in freckling, hair colour, birthmarks and moles.

TABLE IV. Moles according to racial origin.

		Series I				
Northern European	(610)	26.47	±	1.14	SD	28.12
Southern European	(96)	13.19	±	1.35	SD	13.19
Oriental	(15)	8.53	±	2.37	SD	9.17

		Series II				
Northern European	(562)	8.87	±	0.43	SD	10.21
Southern European	(153)	3.48	±	0.42	SD	5.21
Oriental	(14)	2.07	±	0.82	SD	3.05

All the differences in mean value are highly significant except those between Southern Europeans and Orientals which approach significance ("t" tests).

(e) Moles and Hair Colour.

With various shades of brown there was no very pronounced trend. The possession of either black or red hair leads to the possession of fewer moles. The black haired individuals were almost exclusively Southern European or Chinese with much darker than average skin colour. The causes of reduced numbers of moles in red haired individuals will be considered later.

TABLE V. The Number of Moles According to Hair Colour.

	Series I					Series II				
Blonde	(39)	24.13	± 4.32	SD	26.96	(91)	8.46	± 1.10	SD	10.45
Lt.Brown	(161)	25.60	± 2.45	SD	31.14	(222)	9.75	± 0.73	SD	10.84
Brown	(324)	27.61	± 1.67	SD	30.00	(261)	7.15	± 0.54	SD	8.66
Dk. Brown	(100)	23.25	± 2.45	SD	24.51	(97)	4.97	± 0.75	SD	7.40
Black	(61)	14.26	± 2.00	SD	15.62	(26)	3.00	± 0.89	SD	4.53
Red	(36)	8.06	± 1.69	SD	10.14	(38)	4.34	± 1.38	SD	8.51

In general the statistically significant differences in mean values are between the first four sets of values and the last two in Series I, and the first three and the last three in Series II. Many children

classified as having dark brown hair in the second series might have been classified as having black hair four years later, and this probably accounts for the smaller mean number of moles in those classified as having dark brown hair in the second series.

(f) Moles and Skin Colour. The only significant differences were in Series II where children classified as having brown skin had significantly fewer moles than others.

TABLE VI. Number of Moles According to Skin Colour.

	Series I				Series II			
Pale Brown	(96)	23.51 ± 2.75	SD	26.93	(66)	7.94 ± 1.47	SD	11.95
Lt. Brown	(324)	26.01 ± 1.51	SD	27.09	(556)	8.30 ± 0.41	SD	9.64
Brown	(301)	23.33 ± 1.52	SD	26.38	(110)	3.93 ± 0.60	SD	6.29
Dark Brown					(2)	0.5		

(g) Moles and Eye Colour.

Brown eyed children had significantly fewer moles than Blue or Grey. The greatest variations are in distinctions between "Eye Spots" and "No eye spots" but these will not be considered in this table.

TABLE VII. Number of Moles According to Eye Colour.

	Series I				Series II			
Blue	(238)	25.34 ± 1.79	SD	27.69	(278)	9.11 ± 0.61	SD	10.14
Grey	(331)	26.71 ± 1.46	SD	26.65	(230)	8.81 ± 0.70	SD	10.57
Brown	(151)	17.82 ± 2.01	SD	24.65	(225)	4.37 ± 0.43	SD	6.41

(h) Eye Spots

The interest in dark pigmented spots in the iris was the rather close parallel between these and the phenomena of freckling. Seldom present at

birth they appear in the first few years of life and increase in number. Numbers were counted and recorded. In Series I there were 224 of 721 (31.7%) with eye spots; in Series II there were 116 of 735 (15.8%) with eye spots. These differences were highly significant. The mean values did not differ greatly in the two series, comparing only those with at least one spot.

TABLE VIII. Number of Eyespots in Children with at least one Spot.

Series I (224) 3.25 ± 0.22 SD 3.25 Series II (116) 3.01 ± 0.26 SD 2.78

(i) Moles and Eye Spots.

The first comparison is between those with eye spots and those without, and the differences in the means are significant.

TABLE IX. Number of Moles in Those With and Those Without Eyespots.

	Series I	Series II
Eye spots	(224) 30.97 ± 2.11 SD 31.61	(116) 9.91 ± 1.02 SD 10.98
No eyespots	(497) 21.41 ± 1.06 SD 23.71	(613) 7.18 ± 0.37 SD 9.22

Further analysis of the effect of eye spots will be considered after the discussion to follow on freckling.

(j) Freckling and Birthmarks.

Clinical features of birthmarks have already been discussed. A birthmark, one would expect, disregarding Old Wives' Tales, to be very free from environmental influence. On the other hand freckling is manifestly conditioned by environment. An association clinically, histologically and statistically between the two raises interesting possibilities. The concept has been developed that a birthmark is a freckle developing before birth in a genetically predisposed individual. Birthmarks have

been analysed according to degree of freckling.

TABLE X Number of Birthmarks According to Degree of Freckling.

Series I

	Number of Birthmarks		Mean	Area of Birthmark per Child	
A_1	(104)	42	0.404	171	sq. mms.
A_2	(230)	101	0.439	121	sq. mms.
B_1	(345)	301	0.872	264	sq. mms.
B_2	(42)	42	1.000	1480	sq. mms.

Series II

	Number of Birthmarks		Mean	Area of Birthmark per Child	
A	(3)	0	0	0	
B	(232)	78	0.336	128	sq. mms.
C	(201)	78	0.338	71	sq. mms.
D	(277)	198	0.715	223	sq. mms.
E	(16)	22	1,375	480	sq. mms.

Comparing all freckled with all unfreckled or relatively unfreckled people in the two groups one gets 2.08 and 2.00 times as many birthmarks in the former as the latter. These differences are highly significant. If one considers the extreme degrees of freckling, and those with fewest freckles of all, the difference is more marked than this.

One objection to the technique of recording birthmarks used in this study was that the only lower limit to size of birthmark recorded was dictated by uncertainty as to whether small lesions were freckles or birthmarks. Clinically one was satisfied that valid distinctions were

made, but in order to test further this validity all birthmarks smaller than 25 sq. mms. have been omitted in the following table.

TABLE XI. FREQUENCY OF BIRTHMARKS GREATER THAN 25 SQ. MM IN AREA ACCORDING TO DEGREE OF FRECKLING.

Series I	A1	A2	B1	B2
	(104) 0.35	(230) 0.33	(345) 0.73	(42) 0.88

Series II	A	B	C	D	E
	(3) 0	(232) 0.29	(201) 0.36	(277) 0.62	(16) 1.25

This adjustment does not alter the results greatly. Crowe et al (1956) studying neurofibromatosis had a lower limit of 1.5 cm. in the greatest dimension of café au lait patches, and did not count any smaller than this. It is felt that this is a rather too restrictive limitation for this study where the numbers of birthmarks are smaller.

(k) Freckling and moles.

It was apparent early in this study that those with moderate freckles had more moles than the average, whereas those with a very great number of freckles usually had a smaller number. The final results bear this out.

TABLE XII. NUMBER OF MOLES ACCORDING TO DEGREE OF FRECKLING.

		Series I	
A_1	(104)	15.88 $\pm$ 1.85	SD 18.83
A_2	(230)	21.84 $\pm$ 1.29	SD 19.50
B_1	(345)	30.03 $\pm$ 1.73	SD 32.17
B_2	(42)	12.91 $\pm$ 2.50	SD 16.18
		Series II	
A	(3)	1.33 $\pm$ 0.33	SD 0.58

Series II

B	(232)	3.98	± 0.36	SD	5.51
C	(201)	8.93	± 0.65	SD	9.21
D	(277)	9.85	± 0.68	SD	11.31
E	(16)	6.25	± 3.15	SD	12.61

In Series I the only means which do not differ significantly are the means for A1 freckling and B2 freckling. In Series II A and E, B and E, C and D, C and E, and D and E, taken in pairs do not differ significantly. All other values do.

(1) Freckling and eye spots - number of moles.

It has been seen that individuals with freckles have more moles than the average, and those with eyespots similarly have more than the average. The clinical impression was that those who had eyespots and also freckles, excluding the very heavily freckled or red haired, were the ones most likely to have an excessive number of moles. The data have been prepared to show whether this observation has validity. The very heavily freckled classes (B2 in Series I and E in Series II) have been pooled with the unfreckled as these individuals are often deficient in moles.

TABLE XIII. NUMBER OF MOLES IN RELATION TO FRECKLING AND EYESPOTS.

Series I

Freckling (B1)	Eyespots	(102)	38.19	± 3.76	SD	37.99
A_1 A_2 B2,	Eyespots	(122)	24.94	± 2.13	SD	23.57
Freckling B_1	No Eyespots	(243)	26.61	± 1.85	SD	28.78
A_1 A_2 B_2	No Eyespots	(254)	16.43	± 1.01	SD	16.10

Series II

Freckling D, Eyespots	(47)	12.74	± 2.06	SD	14.09
ABC and E, Eyespots	(69)	7.97	± 0.94	SD	7.79
Freckling D, No Eyespots	(230)	9.25	± 0.70	SD	10.59
ABC & E, No Eyespots	(383)	5.93	± 0.41	SD	8.04

In this table the second and third means recorded in Series I are not significantly different. All other differences in mean values are significant. In Series II, comparisons between the first and third, the second and third, and the second and fourth mean values show no significance, whereas other comparisons do.

In studying these data, one can make two comparisons between freckled and unfreckled, and two comparisons between those with eyespots and those without in each Series. Taking these in order of occurrence, freckling accounts for an increase of 53.1%, 62%, 59.8% and 56% in the numbers of moles, and the occurrence of eyespots accounts for increases of 43.8%, 51.8%, 37.7% and 34.4%. These results are consistent, and even though it can be argued that the differences seen may be due to other racial and pigmentary characters with which the freckling and eye spots are associated, one would suggest that the tables are also revealing a very real distinction in the occurrence of moles in children of specific genotype.

m. Somatic mutation rate.

Based on the hypothesis that freckled individuals with brown hair, and freckled individuals with red hair are respectively heterozygotes and homozygotes for a specific gene pair, and that the freckling results from somatic mutation occurring in these genes, somatic mutation rates have been worked out. The result depends on the proportion of total surface

area of groups of individuals affected by the development of birthmarks and uses the same reasoning as that already discussed in the study of presumed somatic mutations by Russell and Major (1957).

The surface area of red haired people, the presumed homozygotes, is counted twice. Since "non-frecklers" can also have birthmarks, an estimate is made of the area of birthmarks in "frecklers" which may not be due to the major freckling genes, based on the occurrence of birthmarks in the "non-frecklers".

Series I

Total areas of all birthmarks in A1 and A2	....	45,564 sq. mms.
Total surface areas of all individuals in A1 and A2		525,480,000 sq. mms.
Total areas of all birthmarks in B1 and B2	...	153,115 sq. mms.
Total surface areas of all individuals in B1 and B2		588,560,000 sq. mms.
Surface area of those with red hair	....	54,040,000 sq. mms.
Estimated area of birthmarks in B1 and B2 not due to major freckling genes	....	51,034 sq. mms.

$$\text{Mutation rate} = 1 \Big/ \frac{588,560,000 + 54,040,000}{153,115 - 51,034} = 1 \Big/ 6,295.$$

Series II

Total areas of all birthmarks in A, B and C	....	44,043 sq. mms.
Total surface area of A, B and C	....	512,600,000 sq. mms.
Total areas of all birthmarks in D and E	....	69,424 sq. mms.
Surface area of those with red hair	....	45,480,000 sq. mms.
Estimated area of birthmarks in D and E not due to major freckling genes	....	29,637 sq. mms.

$$\text{Mutation rate} = 1 \Big/ \frac{344,930,000 + 45,480,000}{69,424 - 29,637} = 1 \Big/ 9,813$$

These results show a degree of consistency despite the differences in criteria between the two series; also the values are of the "right order" based on known mutation rates in human genetic disorders. One feels that the results would tend to be too high due to pigment spread, and it is suggested that the true values may be between one in 10,000 and one in 50,000.

G. Acknowledgment.

Many people have rendered help in the progress of this study. It is not possible to record all of those who have helped in some way, but a few names warrant particular mention. Dr. N. S. Solomons, the Director of the School Health Service of N.S.W., made the survey of schoolchildren possible. A number of the Medical Officers and Sisters of this Service co-operated greatly, and in particular Dr. B. M. Lowe. The analysis of results was facilitated greatly by help with card punching at the Computer Centre at the University of N.S.W., and by programming carried out by Mr. C. J. Clark at that Centre.

Dr. E. Hirst, Senior Pathologist at Sydney Hospital, and Dr. R.D.K. Reye, Director, Institute of Pathology, Royal Alexandra Hospital for Children, Sydney, made available post mortem material and arranged for cutting and staining of sections. Mr. K. Deason of the Department of Medical Illustration, University of N.S.W., prepared some microphotographs and directed the reproduction of all graphs and many photographs, in this and subsequent chapters.

Some of the colour photographs were obtained from Mental Hospital patients, particularly from Callan Park Mental Hospital. Apart from these there were the many subjects of the study, some of whose homes were visited.

Fig. 26. Mother, Father, and three children, all red haired.

Fig. 27. Red haired Indonesian.

Chapter IV - The Genetics of Red Hair

A. Introduction

The calculation of a somatic mutation rate at the end of the last chapter depends in part on being able to show that a single specific gene pair is involved in the phenomena studied. The hypothesis is that the gene for red hair is also the gene which determines the most obvious type of freckling. Effort therefore has been directed towards defining the genetics of red hair.

As stated earlier there is also a need to define the biochemistry of red pigment, partly for the better understanding of the genetics of red hair, partly to provide some basis for the proposed mechanism of formation of a freckle, and partly to provide a theoretical background to the microspectro-photometric study of hair colour variability. In this and subsequent chapters these matters will be considered.

B. Literature

A noteable feature of Europeans with red hair is that they are in most instances also obviously freckled. This is not invariably the case. One Greek migrant girl amongst 66 red haired individuals in the survey of school children had no freckles. Both she and her parents said that the colour was natural. Barnicot (2, 1952) in a survey of Nigerians with red hair found no freckles in the 14 red haired Nigerians of 40,000 examined.

Cockayne (1933) gives data on families in which red hair occurred. When one parent was red haired, and one child red haired, there were 46 red haired children and 48 non-red haired. When neither parent was red haired, but one red haired child was found in the family, there were 59 red and 116 non red. In sibships of 9 or more in which red hair occurred (non-red haired parents) there were 26% of red haired children. In 5 marriages of red haired parents 16 red haired children were born.

Occasionally a non red haired child is born to red haired parents. Neel (1943) discussed the hypotheses suggesting dominant or recessive inheritance. In marriages between two red haired persons there were 111 red haired and 13 non red haired children. These familes were not examined. He suggested that heterozygotes might be sandy red. Reed (1952) concluded that there was no doubt about the inheritance of red hair. He was greatly concerned with finding an objective way of determining the phenotype "red hair" and used reflectance spectrophotometry with a degree of success in defining redness. Reed gives a summary of some older surveys and some of the conclusions that might be drawn from them. He supports a recessive hypothesis as a result of his own investigations and suggests that the individuals whom Michelson (1933) found to have only a few red hairs might be heterozygotes. Singleton (1964) found red hair in six generations of a family and constructed a pedigree which suggested recessive inheritance. Two men had changed

Fig. 28. Reproduction of the form used for family studies. Original size, quarto. The reverse side of the sheet was similarly printed so that a family of mother, father and six children could be recorded.

Name Address Propositus

Age Sex Hair colour Skin colour Eye colour Spots R L

Skin spots 1 Ft Brown 2 Lt Brown 3 Brown 4 Dk Brown 5 Black

PATCHES Size Site Colr. Description

MOLES Other details

Age Sex Hair colour Skin colour Eye colour Spots R L

Skin spots 1 Ft Brown 2 Lt Brown 3 Brown 4 Dk Brown 5 Black

PATCHES Size Site Colr Description

MOLES Other details:

Age Sex Hair colour Skin colour Eye colour Spots R L

Skin spots 1 Ft Brown 2 Lt Brown 3 Brown 4 Dk Brown 5 Black

PATCHES Size Site Colr Description

MOLES Other details

Age Sex Hair colour Skin colour Eye colour Spots R L

Skin spots 1 Ft Brown 2 Lt Brown 3 Brown 4 Dk Brown 5 Black

PATCHES Size Site Colr Description

MOLES Other details

from red hair in childhood to brown hair in adulthood.

C. Family studies

Subsequent to the survey of school children families were visited in order to obtain data on pigmentation. The results of this study are shown in Appendix II. The form used for this purpose was a card (Fig. 28) with four spaces on the front and four on the back, each space prepared for the details of one individual. The actual details determined can be seen from the card, and represent an abbreviated form of the card used in the survey of school children. The same amount of detail was not obtained because it was felt that the average suburban family has a certain amount of patience which would not extend to such interferences as weighing, measuring height, counting moles or searching for birthmarks.

The pedigrees shown are those in which pigmentary details of both parents and at least one child other than the propositus were available. The majority of individuals were actually seen personally and it was only in cases where the mother or some other responsible person could supply details with considerable confidence that people not seen were included. A small number of pedigrees are shown without any recorded propositus; these were certain big families known to the writer who were willing and available to be seen, and were not chosen because of the occurrence of any particular character in any individual. It was not felt necessary to exclude any individual

from calculations in these families, as was done in the families in which a definite propositus was available through the school survey.

The family data support the hypothesis that frecklers with brown hair are heterozygotes and red haired individuals are homozygotes, with a few exceptions. Occasional exceptions might be due to illegitimacy or adoption, but consideration of all the facts lead one to believe that this does not explain all the exceptions.

One problem is change of hair colour during life - this may involve change from one shade of red to another, but one must also admit that some genuinely red haired children grow up later to be brown or red-brown haired adults. Freckling does not express itself on every pigmentary background. The very fair skinned but light red haired individual may have very pale freckles, and it would appear that the development of a freckle represents the unmasking of a pigmentary potential in the skin - if this pigmentary potential is very slight because of other genetic factors, then the freckles will be very small, very faint, or virtually absent. Equally one must refer to the very dark skin which does not freckle noticeably e.g. in the negroes that Barnicot (1952) studied. Another factor in freckling is the relation to sunlight. Many potential frecklers do not freckle because they purposely or by nature do not expose themselves to sufficient strong sunlight to make it possible.

The pedigrees in Appendix II are grouped in seven categories. These are, in order; Neither parent freckled; One parent freckled;

Both parents freckled; One parent freckled, one parent red haired; One parent freckled, one red haired child; One parent red haired, one unfreckled; and Both parents red haired.

The results will be set out according to these divisions.

1. Neither parent freckled.

   51 unfreckled children; 2 freckled.

2. One parent freckled.

   42 unfreckled children; 45 freckled.

3. Both Parents freckled.

   22 unfreckled, 38 freckled, 14 red haired.

4. One parent freckled, one red haired.

   13 freckled children, 8 red haired, 2 children unfreckled.

5. One parent freckled, one unfreckled, at least one red haired child.

   5 unfreckled children, 5 freckled children, one red haired.

6. One parent red haired, one unfreckled.

   3 freckled children.

7. Both parents red haired.

   3 red haired children.

Class I has two exceptions to the hypothesis; otherwise the result does not conflict.

Class 2 in which errors of assessment cannot be checked shows a close approximation to the expected segregation ($\chi^2 = 0.10$, $P = 0.7$).

Class 3 has observed 22, 38 and 14 whereas expected would be 18.5, 37 and 18.5 ($\chi^2 = 1.8$, $0.3 < P < 0.5$).

Class 4 has two exceptions; otherwise 13 freckled and 8 red haired; expected, 10.5 & 10.5. $\chi^2 = 1.09$ $p = 0.3$.
Class 5 as a whole represents an exception to the hypothesis, and the numbers are so small that further analysis is not worth while.
Class 6 and Class 7 do not disagree with the hypothesis.

D. Population Studies

On the basis of a two allele hypothesis for freckling and red hair, homogeneous population groups should obey the Hardy Weinberg Law for the distribution of phenotypes. Since both presumed heterozygotes and homozygotes can be identified, gene frequencies can be worked out, and expected values in each of the three Classes can be determined. χ^2 for one degree of freedom can be calculated, comparing observed with expected. Various groups are available and an alysis of these is presented, including expected numbers on a Hardy Weinberg distribution and χ^2 for the degree of agreement between observed and expected. The groups are considered under the name of the school at which the children were seen. These results have not been pooled as in some areas there were varying numbers of Southern Europeans and Chinese, and estimated gene frequencies vary a good deal between different groups. Also given are the data for 405 patients at the Peat and Milson Island Hospital, and 107 prisoners at Parramatta Gaol. Two large additional groups of school children were seen and assessed purely on the basis of freckling and hair colour.

The criteria of freckling were those of the second series of school children.

The results are as follow:

Series I

Enmore High School

	Non Frecklers	Frecklers	Red Hair	Total
Observed	95	61	5	161
Estimated gene frequencies 0.78, 0.22.				
Expected	98	55	8	161

χ^2 (1DF) = 1.86.

$0.1 < p < 0.2$.

Sydney Girls' High School

	Non Frecklers	Frecklers	Red Hair	Total
Observed	90	93	8	191
Estimated gene frequencies 0.71, 0.29.				
Expected	96	79	16	191

χ^2 (1DF) = 6.88

$0.001 < p < 0.01$

Sydney Boys' High School

	Non Frecklers	Frecklers	Red Hair	Total
Observed	44	55	6	105
Estimated gene frequencies 0.68, 0.32.				
Expected	48	46	11	105

χ^2 (1DF) = 4.37

$0.01 < p < 0.02$.

Dulwich Hill High School

	Non Frecklers	Frecklers	Red Hair	Total
Observed	30	46	3	79
Estimated gene frequencies	0.67, 0.33.			
Expected	35	35	9	79

χ^2 (1DF) = 8.17.

$0.001 < p < 0.01$.

Lewisham High School

	Non Frecklers	Frecklers	Red Hair	Total
Observed	50	67	6	123
Estimated gene frequencies	0.68, 0.32.			
Expected	57	54	12	123

χ^2 (1DF) = 6.99.

$0.001 < p < 0.01$.

Fort Street Boys' High School

	Non Frecklers	Frecklers	Red Hair	Total
Observed	25	30	7	62
Estimated gene frequencies	0.65, 0.35.			
Expected	26	28	8	62

χ^2 (1DF) = 0.31.

$0.50 < p < 0.70$.

Series II

Stanmore Primary School

	Non Frecklers	Frecklers	Red Hair	Total
Observed	134	67	8	209
Estimated gene frequencies 0.8, 0.2.				
Expected	134	67	8	209

χ^2 (1DF) = 0

$p > 0.99$

Redfern Primary School

	Non Frecklers	Frecklers	Red Hair	Total
Observed	110	51	9	170
Estimated gene frequencies 0.8, 0.2.				
Expected	109	54	7	170

χ^2 (1DF) = 0.75.

$0.30 < p < 0.50$.

Newtown Primary School

	Non Frecklers	Frecklers	Red Hair	Total
Observed	90	63	11	164
Estimated gene frequencies 0.74, 0.26.				
Expected	90	63	11	164

χ^2 (1DF) = 0

$p > 0.99$.

Haberfield Primary School

	Non Frecklers	Frecklers	Red Hair	Total
Observed	108	74	10	192
Estimated gene frequencies	0.76,	0.24.		
Expected	111	70	11	192

χ^2 (1DF) = 0.40

$0.5 < p < 0.7$

Peat & Milson Island Hospital

	Non Frecklers	Frecklers	Red Hair	Total
Observed	233	170	2	405
Estimated gene frequencies	0.78,	0.22.		
Expected	246	139	20	405

χ^2 (1DF) = 24.21

$p < 0.001$

Parramatta Gaol

	Non Frecklers	Frecklers	Red Hair	Total
Observed	59	42	6	107
Estimated gene frequencies	0.75,	0.25.		
Expected	60	40	7	107

χ^2 (1DF) = 0.26

$0.5 < p < 0.7$.

Population I (school children).

	Non Frecklers	Frecklers	Red Hair	Total
Observed	358	153	18	529
Estimated gene frequencies	0.82,	0.18.		
Expected	356	156	17	529

χ^2 (1DF) = 0.13

$0.7 < p < 0.8$.

Population II (school children).

	Non Frecklers	Frecklers	Red Hair	Total
Observed	315	175	18	508
Estimated gene frequencies	0.79,	0.21.		
Expected	317	169	22	508

χ^2 (1DF) = 0.95.

$0.3 < p < 0.5$.

It can be seen that there is a strong general trend in the direction of conformity to the Hardy Weinberg distribution. In Series I of school children there are a number of results which deviate from expectation by a greater amount than can be attributed to chance. These were examined using the original criteria of freckling which included many who would later have been classed as unfreckled. Series II conforms closely to expectation on the Hardy Weinberg distribution. These were seen at intervals during the course of two years; some were seen before the next result was obtained, some after. The next result is that for Peat and Milson

Island Hospital which deviates markedly from expectation. The Peat and Milson Island Hospital houses some of the most severely mentally retarded children and young adults in New South Wales. The disorders suffered from are various and unselected and include many undiagnosed conditions. The remarkable thing about this group was the small number of red haired individuals, there being only 2 in 405. Of exactly 2,600 in the other groups listed there were 115 listed as red haired or 4.5 per hundred. If the Peat and Milson Island Hospital group had the same frequency there would have been 18 red haired individuals there. Adjusting the values obtained on this basis one gets 233 non frecklers, 154 frecklers and 18 red haired, a gene frequency of 0.2, and expected numbers unchanged from previous calculation of 246, 139 and 20. χ^2 would be 2.64 (1DF). Thus the discrepancy can be attributed to the very low frequency of red hair at this hospital, which is itself significant. Why there should be such a low frequency of red hair amongst this group of mentally defective males is not apparent.

The last two groups of school children give results which are in agreement with the hypothesis, and the only comment to make is about the gene frequency which shows a small difference between the groups. The first group was taken from inner city suburbs with a high frequency of Greek and Italian migrants, and numerous Chinese, whereas the second group were from outer suburbs which had much smaller numbers of these classes.

E. Acknowledgements

In addition to acknowledgements made in the last Chapter, one must in particular acknowledge the help of Dr. M. M. Ramadan, Superintendant of the Peat and Milson Islands Hospital, whose patients contributed hair samples and were examined, and Mr. Moroney, Comptroller General of Prisons, who made it possible to examine and collect hair samples from prisoners at Parramatta Gaol.

Chapter V - Marsupial Pigments

A. Introduction

It is felt that the phenomena to be discussed in this chapter provide an important key to the biochemistry of epidermal pigments in all mammals.

The concept of freckling, and of hair colour variability to be discussed later, depends among other things on showing biochemical mechanisms for a switch of pigment production by melanocytes of skin or hair. If one can show that by a simple modification of a basic molecule a new and differently coloured pigment is produced, then this may be referrable to the effect of a single enzyme and therefore perhaps a single gene.

Marsupials secrete pigment precursors onto their skin, often in considerable quantity. These are closely related chemically, and have features in common with dopa. With tyrosinase they produce pigments, the most prominent of which is a bright red pigment which appears to be the same as that of mammalian red hair and Rhode Island Red feathers. The present evidence suggests that these precursors may be derived from aromatic amines closely related to tyrosine.

B. Literature

The subject of "chromidrosis" or coloured sweat was first dis-

cussed by James Yonge (1709), when he described a girl who produced dark secretions on her forehead. Similar reports have been made at intervals since. Zlotnikov (1933) discussed a man who had a large Naevus on the L side of his body and secreted brown coloured sweat from the axilla of this side. Shelley & Hurley (1954) report investigations into human cases claiming that individual apocrine glands can secrete yellow, green, blue, brown and black sweat, and that the glands from which the sweat comes might be similarly coloured.

Weber (1888) discussed the red material which he found powdered on the hair of the male red kangaroo, in an area over the upper sternum and lower neck. He decided that it was a red secretion, or a colourless secretion which later became red, from glands in the neck which he showed to be greatly enlarged sebaceous and apocrine sweat glands associated with hairs in the area. He found no coloured secretions in the ducts of these glands and the epithelia of the glands were uncoloured. The epidermis of the area was also free of pigment. He found that the pigment which was originally soluble in water was rendered insoluble by extraction in alcohol. In the female red kangaroo he found much smaller glands in the area studied and does not report having found pigment.

Bolliger & Hardy (1944) discussed the pigmented sternal area of the possum. They state that in a fully grown male there is a "moist and frequently copious secretion with a distinct but not

disagreeable odour". They found that the sudoriferous and sebaceous glands in this area were very much larger and apparently more active than those on the general body surface. In the ducts of the glands there was an eosinophilic secretion, and by giving the animal an injection of pilocarpine they could induce profuse sweating, which sweat if blotted onto filter paper changed from being initially colourless to brown within a few days of exposure to air. In the female they found that a similar chromogen was secreted in the pouch and on the teats, although the amount secreted in the sternal area was much less.

In a later paper Bolliger (1944, 1) showed that castration of the male animal caused a regression of the pigmented area, which could be restored by injections of testosterone. In Bolliger (1944, 2) he found that the skin and hair of the possum had a marked sky blue fluorescence under ultra-violet light, and that the fluorescent material could be scraped off the skin in some areas.

Mackenzie (1934) described a pigmented area over the upper sternum of the male koala and stated that there was present a "peculiar type of alveolar gland which secretes a viscid milky fluid direct on the fur".

Bourne (1934) lists other species - Notomys which has a glandular area on the ventral surface of the neck, the secretions of which are of a "sexually attractive nature"; also a gular pouch in the bat Nyctinomys; modified gular glands in Podanomalus; and a large

Fig. 29. Male red kangaroo (Macropus rufus) showing red pigment in cervical and sternal area.

Fig. 30. The same kangaroo as in Fig. 29 showing orange yellow hair, coloured in part by a soluble free pigment, found on dorsal areas of the body.

compound tubular presternal gland in Myrmecobius. He does not state whether any of the secretions from these glands are pigmented.

In the orangutan Wislocki & Schultz (1925) describe a sternal gland and say that such glands usually have the function of producing a characteristic odour in those species which possess them. Schwarz (1937) describes the paired organ on the sternum of Ateles "composed of long winding glands in the form of loops". He attributes to this an odour producing function "similar to the human axillary organ". He says that the hairs are usually colourless in association with the glands but may be coloured by secretion.

C. General description

In the red kangaroo the male produces a bright red pigment in a roughly triangular area extending in one axis from the level of the mandible to the middle of the sternum and in the other between the forelegs ventrally. There may be streaks of this same pigment around the flanks as well. On the dorsum under the rather tawny orange red hair of this region an orange yellow pigment is found.

Figs. 29 & 30, both of the same animal, show the pigment stained hair and skin. The quantity of pigment on many animals is sufficient to rapidly stain the hands of any person handling these animals. In the female there is also free pigment, but this is very much less in quantity and may be hard to demonstrate.

In the possum there is a red brown pigmented area on the neck and upper sternum which corresponds exactly in location to the bright

Fig. 31. Microphotograph showing hair fibres with red pigment adhering to the hairs, Macropus rufus.

Fig. 32. The orange yellow pigment of the same kangaroo as in Fig. 31.

red area of the kangaroo. It is much more marked in the male although present also in many mature females. The female produces a pigment in the pouch which appears to be identical with that of the male breast, and becomes obvious at the time of sexual maturity. The ventral surface of both male and female is often stained with a yellow brown secretion and there may also be evidence of pigment apart from normal hair pigment over much of the dorsum, particularly in the male.

Other marsupials with free pigment secretion include the grey kangaroo which secretes smaller amounts of pigment than the red kangaroo, the koala which was mentioned by Mackenzie, the Parry's Wallaby or whip tail kangaroo which in certain areas secretes quite large amounts of a yellow brown pigment, the New Guinea tree kangaroo, and other wallabies and wallaroos. Poterous and the ring tailed possum also have free pigment, and in fact so many marsupials do that it may well be a general phenomenon amongst these animals, worthy of much more attention than has till now been devoted to it. The efforts in preparing this thesis have been directed more towards defining the nature and behaviour of the material in two or three species than to a general study at the zoological level.

D. Microscopic appearance

The red kangaroo and the grey possum have provided the greater part of the material for experimental study. In the red kangaroo the gross appearance is closely paralleled by the microscopic - a

Fig. 33. Red brown sternal area of the grey possum, Trichosurus vulpecula. Other ventral areas yellowed by pigment.

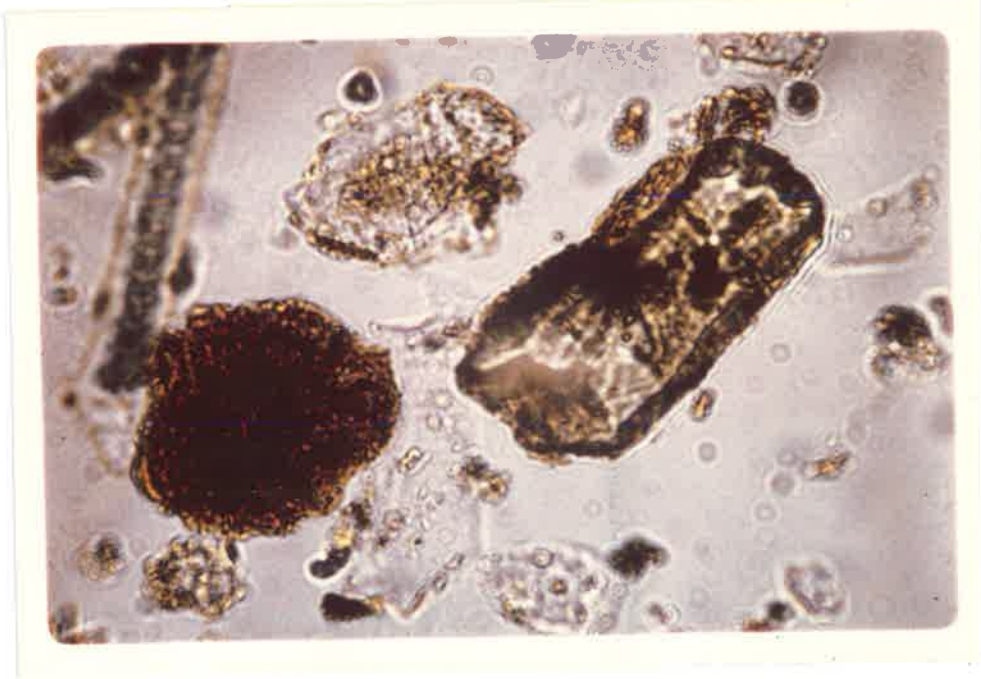


Fig. 34. Brown and colourless material found in possum hair.

Fig. 35.

Yellow pigment found on Parry's wallaby.

Fig. 36.

High power view of small deposit of material in Parry's Wallaby showing partly crystalline structure.

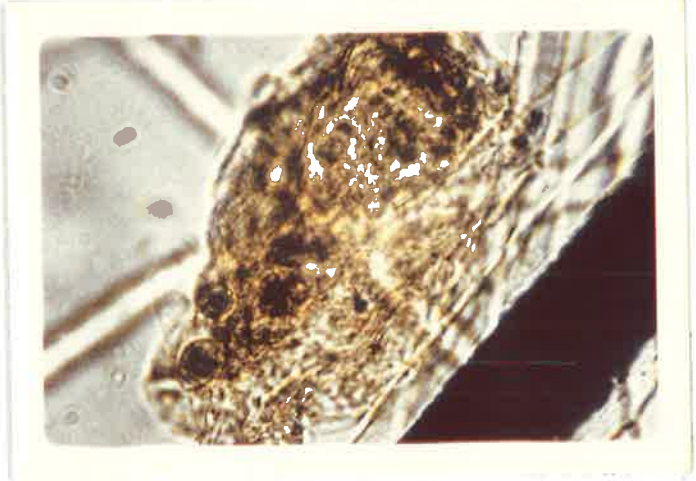


Fig. 37.

Pigmented material found amongst goat hair.

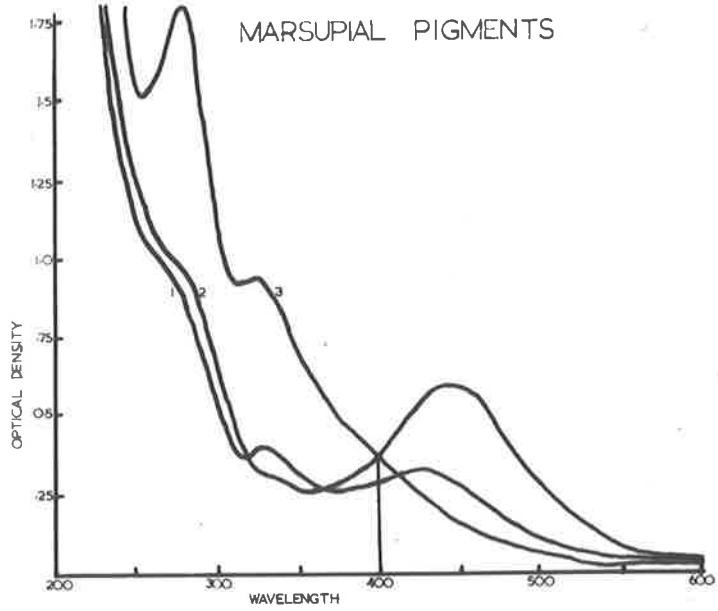
bright red and an orange yellow pigment being found amongst the hair of the particular regions (Figs. 31 & 32) and sticking to the hair as one would expect when a moist liquid containing the pigment dries on the hair. The pigment is uniform in character, although differing in the two areas, and there is no evidence of colourless material. In the possum there may be a variety of colours; red, yellow, brown and black, and frequently small masses of colourless material as well (Fig. 34). The quantity of material is not as great in the possum as it is in the kangaroo, although animals vary in this respect.

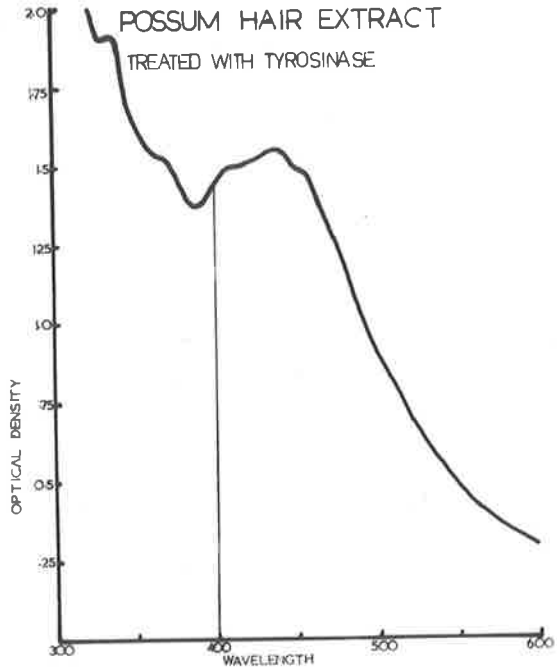
In Parry's wallaby one also sees large quantities of yellow pigment on appropriate parts of the animal (Fig. 35). High power views in wallaby and possum frequently reveal evidence of crystalline structure in the colourless material (Fig. 36). Similar pigment was found in small quantity on goats (Fig. 37) although the situation has not been thoroughly studied.

E. Solubility and relation to tyrosinase

The pigments of the red kangaroo are very soluble is distilled water and a very small amount of hair may provide quite strong solutions for spectro-photometry or other investigations. The pigments are insoluble in organic solvents e.g. choloroform, ether, acetone or alcohol. Alcohol as Weber (1888) pointed out renders the pigments insoluble in water. A suspension of the pigments can

Fig. 38. Aqueous extracts of (1) orange yellow pigment of red kangaroo (2) red pigment of red kangaroo (3) possum hair. Transmission spectrophotometry. Note similarities in





U.V. range, and peaks in visible range of Kangaroo pigments.

Fig. 39. Effect of adding tyrosinase to possum hair extract (Compare Fig. 38, 3).

be prepared in chloroform and this has been useful for spotting in chromatography.

In the possum the brown pigment is quite insoluble in water even on prolonged boiling. However treatment of hair with either hot or cold water produces quite a strong solution of a chromogen which appears to correspond to that studied by Bolliger & Hardy (1944). This, if left standing, becomes reddish in colour and then eventually brown. It was found that by adding tyrosinase to this colourless hair extract the evolution of pigment was speeded up to the point where full red colouration developed in a few hours.

F. Spectrophotometry.

Various methods of spectrophotometry were applied to the study of the pigments, and some useful information was obtained from the use of each technique.

Transmission spectrophotometry. A solution of pigment was prepared by adding 30-50 m.l. of distilled water to 1 gm of hair. In the case of possum hair heating was carried out to 100° C for a few minutes. The solution was drained off and centrifuged, and the spectral absorption was determined on a Unicam spectrophotometer with distilled water as a control. If the solution from kangaroo hair was too concentrated dilutions were made and these were then used for spectrophotometry.

The results are as shown (Fig. 38). The colourless possum hair

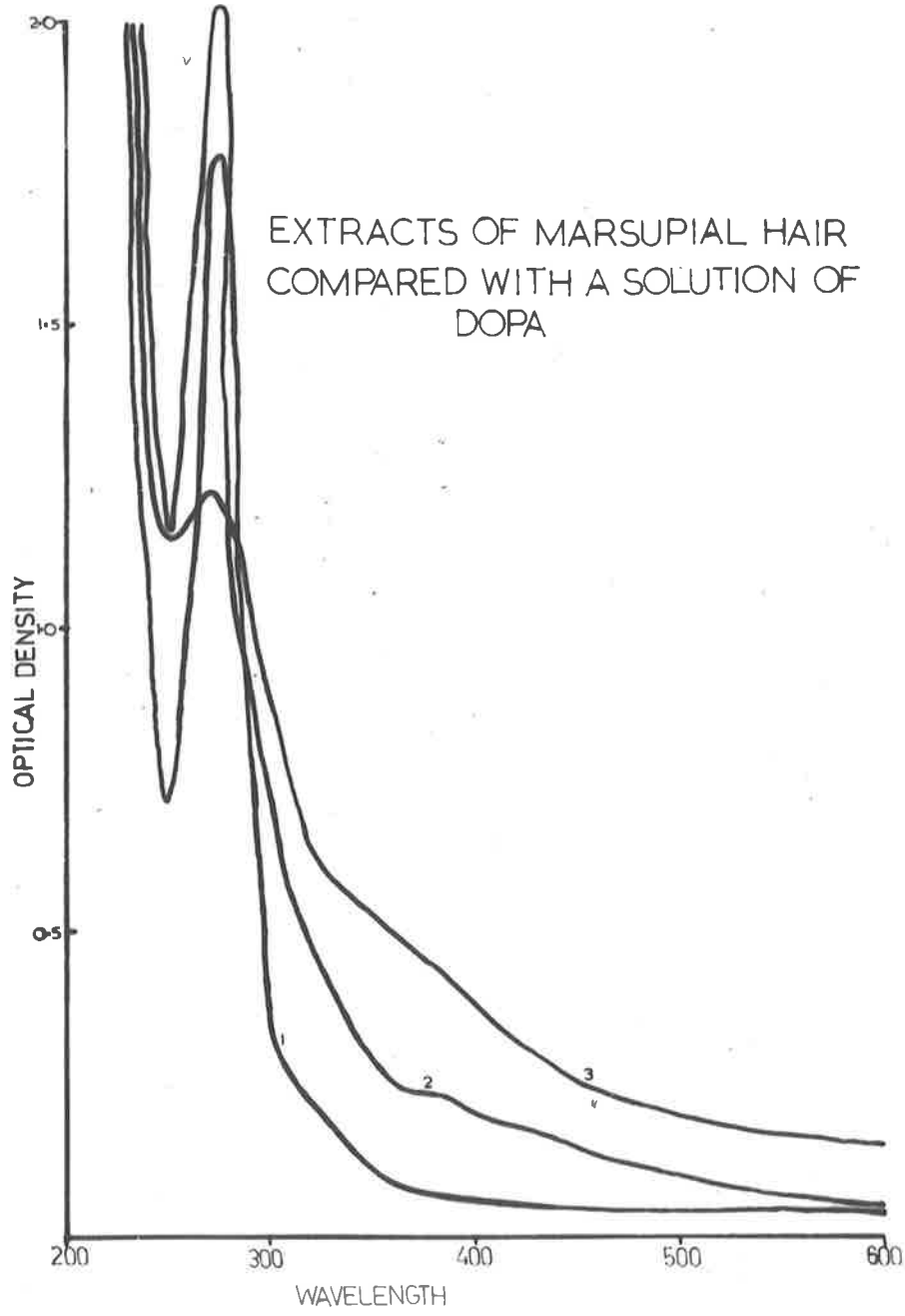


Fig. 40. Results of an experiment in which a solution of dopa (1) was compared with extracts of possum (2) and female red kangaroo (3). (Transmission spectrophotometry).

extract had two peaks of optical density at 290 mμ and 330 mμ. At wavelengths greater than the latter a rapid decline in optical density occurred. In the ultraviolet range the kangaroo pigments showed similarities in shape to the possum curve, while the orange yellow pigment had a low flat peak at 425 mμ, and the red pigment a peak at 450 mμ in the visible range.

Fig. 40 shows the results of another experiment in which possum and female red kangaroo hair extracts were compared with a suitably diluted solution of dopa. There is considerable similarity in the ultraviolet region but subsequent chromatography failed to reveal the presence of dopa.

On adding 1 mgm of tyrosinase to 10 ccs of possum hair extract and waiting one to two hours a change occurred which was read on the spectrophotometer (Fig. 39). The curve became much more like the curves of the two red kangaroo pigments. The colour of this solution had a rather characteristic red similar to that of the kangaroo extracts, but when a much greater dilution of hair extract was treated with tyrosinase a light red purple colour, unlike the colour of the stronger solution, appeared and this was the first indication that there might be more than one chromogen present.

The behaviour of the pigments in acidic and alkaline solutions was investigated. The pH of the kangaroo pigments was altered through a wide range by diluting a strong solution with varying proportions of the buffer of Theorell and Stenhagen (1939). In a

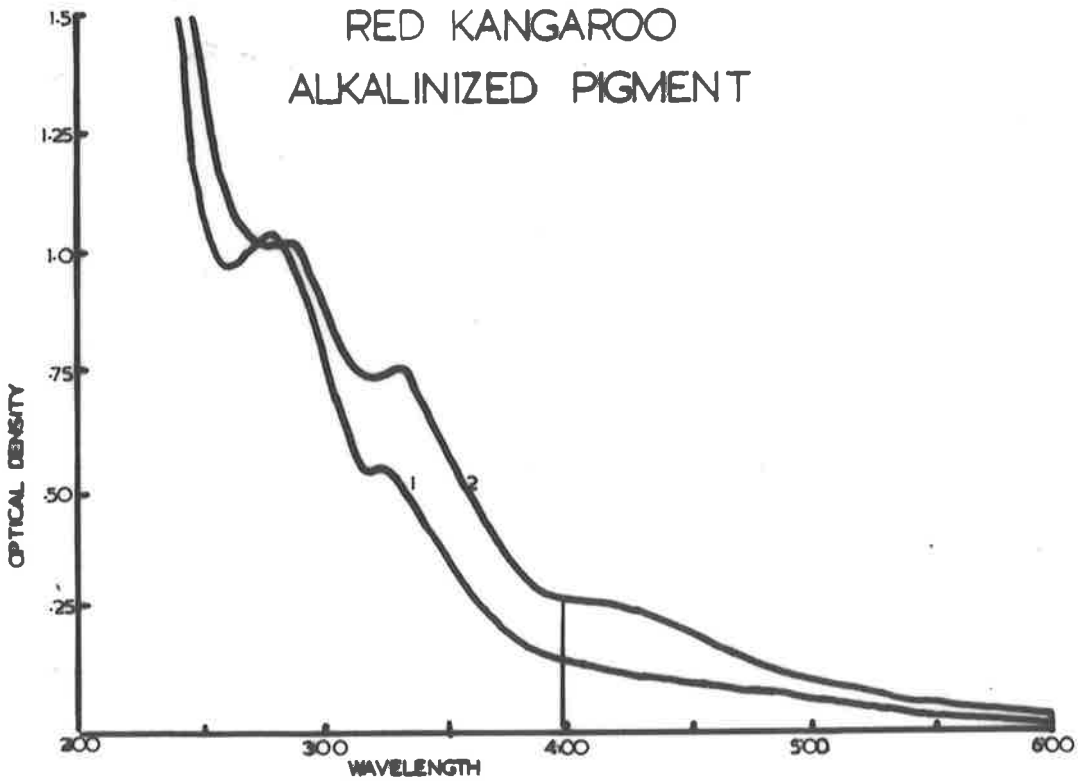


Fig. 41. Flattening of peaks in visible range and greater prominence of peaks in ultraviolet range in alkaline solutions of pigment (1) orange yellow kangaroo pigment (2) red kangaroo pigment.

moderate degree of acidity the pigments remained unchanged, but when alkalinized a progressive loss of colour occurred with an increasing density in the ultraviolet range, which tended to make the curves much more like the possum extract curves (Fig. 41).

Reflectance spectrophotometry.

This technique has been used to study hair and skin pigmentation by several workers. In this case a mass of hair was compressed between two 2" x 2" glass slides which were then taped around the edges. Using a Perkin Elmer reflectance spectrophotometer a continuous recording of the percentage of reflectance was obtained from 350 mμ to 750 mμ, using magnesium oxide as a standard 100% reflectance.

The result from three samples of marsupial hair is shown in Fig. 42. The curves have a general similarity, the most significant being that of the red pigment of the red kangaroo (Curve 1). This shows a plateau between 400 mμ and 550 mμ, with a steep rise after that. This may be compared with the curve of the red pigment in Fig. 43 where as one would expect approximately the inverse situation applies.

Microspectrophotometry.

The technique of microspectrophotometry has been used extensively in this study. As an adjunct to studying pigmentary variability in skin in the form of freckles, birthmarks etc., it was felt that the individual variability in colour of hairs was

Fig. 42.

Reflectance spectrophotometry of marsupial hair.
(1) Red pigment and hair from neck of red kangaroo
(2) Orange yellow pigment and hair of red kangaroo
(3) Hair from grey kangaroo.

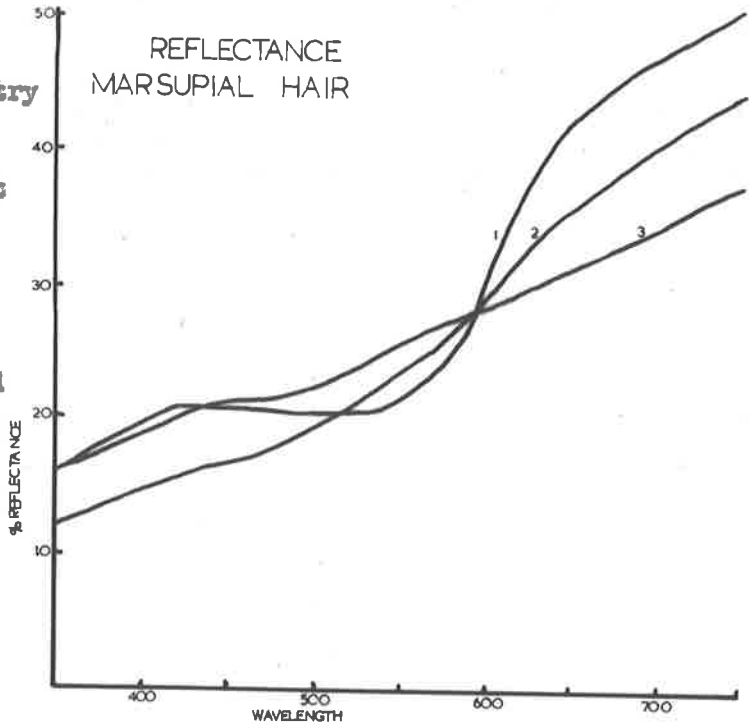
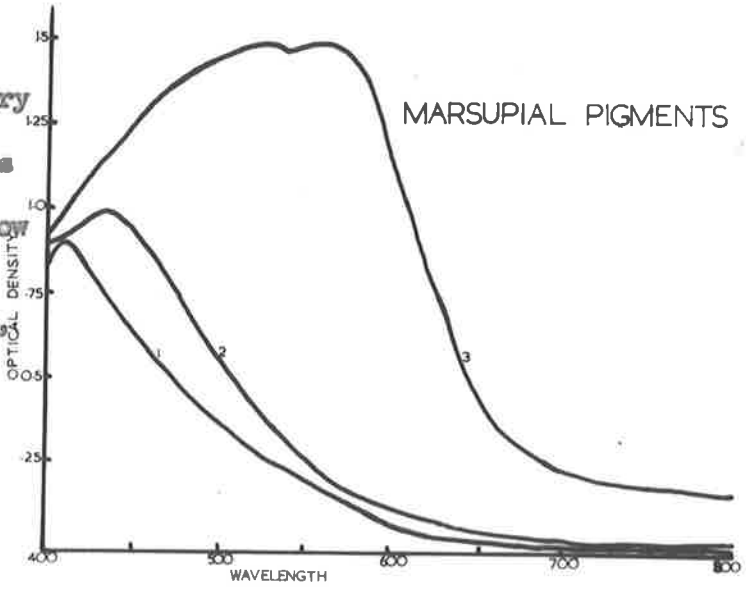


Fig. 43.

Micro-spectrophotometry
(1) Yellow pigment, Parry's Wallaby
(2) Orange yellow pigment, kangaroo
(3) Red pigment, kangaroo.



relevant and important. It was found that microspectrophotometry offered a way of assessing the pigment in individual hairs, and having established a technique for doing this free pigments were also studied by much the same technique.

The technique as applied to hair, and free pigment in association with hair, is as follows. A microscope slide is prepared with a number of short sections (about 1 cm in length is suitable) of the hair to be studied, mounted in a mounting medium of heavy liquid paraffin (4.85cc) and monobromonaphthalene (5.15cc). About 50 hairs on a slide is suitable, and after addition of the mounting medium a 2" x 1" glass coverslip is applied. The coverslip is not sealed onto the slide as with care it will stay in position as long as is necessary for estimation of optical densities. Cleland (1965) describes the microspectrophotometer which was used. By using either a continuous recorder or a series of readings at short wavelength intervals, readings are obtained representing the percentage of transmission against the background value of transmission, this latter being the transmission through the slide at a point free of hair or pigment at the particular wavelength. Optical density is obtained from the calculation of (log background transmission - log sample transmission). In general readings were made using the low power (10X) objective lens on the microscope as this provided an adequate field of hair shaft or pigment particle. It was possible to use a high power objective for smaller particles.

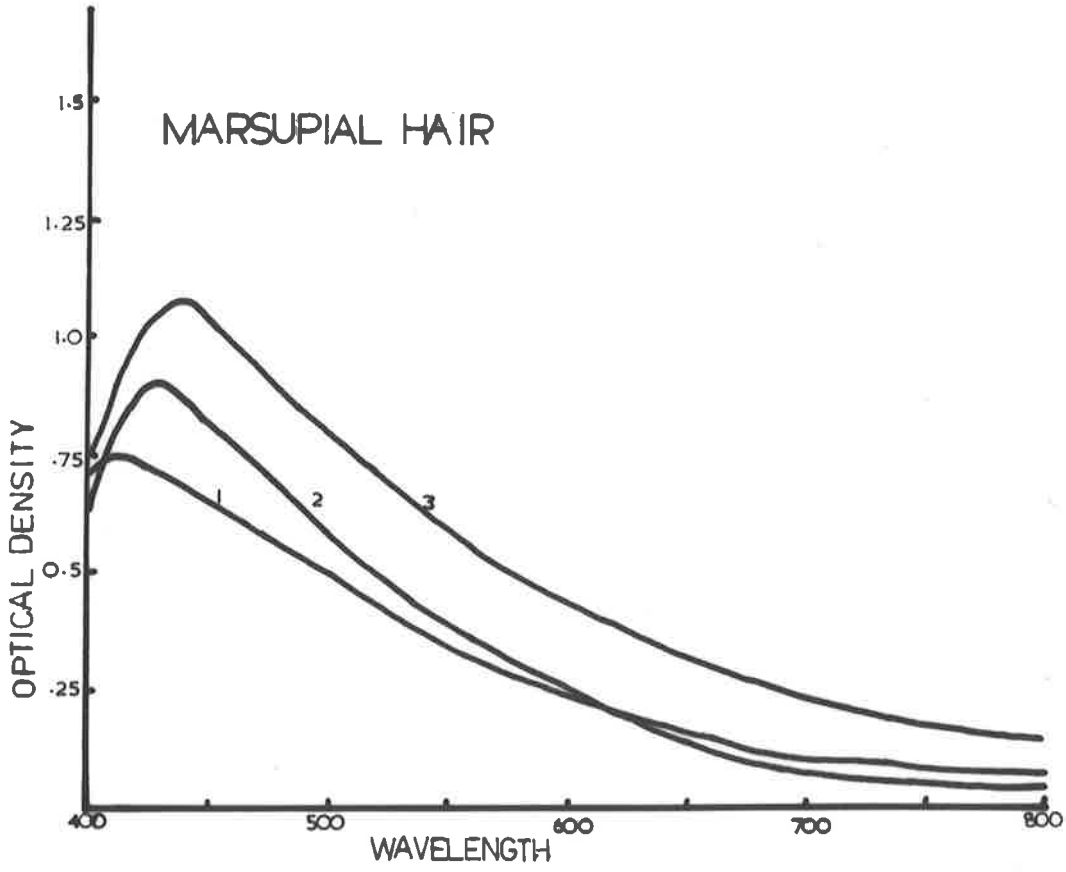


Fig. 44. Microspectrophotometry of marsupial hair.
1. Wallaby - yellow hair
2. Red Kangaroo - orange yellow hair
3. Wallaby - red hair

The results obtained on pigments are shown in Fig. 43. Graph 1 is of yellow pigment from Parry's wallaby and has a peak at 405 mμ; Graph 2 is of orange yellow pigment of the red kangaroo and has a peak at 430 mμ, and Graph 3 is of the red pigment of the red kangaroo. The two flattened peaks shown in this curve were obtained on all samples examined but there was evidence in many curves of several peaks between 450 mμ and 600 mμ, suggesting that in the dry form this pigment undergoes an association of molecules producing a number of polymers with different peaks of optical density. If this is so the process is readily reversible as the solution of the pigment has a single peak of optical density at 450 mμ and is a deep orange red, the change to this colour from a bright red occurring immediately water is added.

The results obtained with marsupial hair on microspectrophotometry are shown in Fig. 44. The pigments in this case are entirely within the hair shafts and one would draw attention to the similarity of curves of free pigment in solution, pigment particles on a slide, and hair pigments as measured on a slide by the microspectrophotometer. The only result which deviates greatly from the general pattern is that for the red kangaroo pigment in the dry state, and there may be a simple explanation for this on the basis of a slightly altered physico-chemical state as already indicated.

G. Chromatography

The evidence suggesting the presence of one or more chromogens

in possum hair led to the preparation of samples for chromatography.

Two grammes of hair is heated with 100 millilitres of water to boiling point in a 250 m.l. beaker. The sample is maintained at boiling point until approximately half of the sample has evaporated. The remaining 50 ml of water is then drained off from the hair and centrifuged. The supernatant is transferred about 20 ml at a time to a long test tube 9" x 1" in a water bath of boiling water, and nitrogen is bubbled through the solution until all water has evaporated leaving a slightly pigmented amorphous residue. This residue is then dissolved in 1 cc. of 10% isopropyl alcohol in water, using a long glass stirring rod to encourage solution at the bottom of the test tube. The resultant solution is drained out of the test tube and centrifuged. The reddish solution obtained is stored in small bottles under refrigeration until required for testing. Nitrogen is used for evaporating the solution in the belief that the pigment precursors are highly oxidizable, and that rapid evaporation in an atmosphere of nitrogen will lessen this possibility.

Another method of preparing samples for chromatography is to treat the hair with chloroform and evaporate this at low temperatures. A suspension of free pigment is obtained by this method and both pigments and pigment precursors are found to correspond to those found in material which has been boiled, indicating that the boiling does not appear to have any appreciable effect on the nature of these materials.

The method of boiling in water was originally used to concentrate extracts of marsupial hair where the pigment precursor was presumed to be external to the hair shaft. The same method was applied to hair of other mammals and to human hair, and to feathers. Boiling of red hair, and in particular of red feathers, and even soaking red feathers in cold distilled water for 24 hours extracts much soluble red pigment from these materials.

The two techniques of chromatography used were paper chromatography and thin layer chromatography. Paper chromatography was carried out with Whatman No. 3 paper using Butanol-Acetic-Water (57:19:30) or Butanol-Pyridine-Water (35:35:35), or Phenol-Water (500:125) solvents. Butanol-Acetic and Phenol or Phenol Acetic (99:1) were used in thin layer chromatography. The thin layer chromatography was carried out by a chromatostrip technique in 9" x 1" test tubes using an 8" x $\frac{3}{4}$" slide cut from window glass and coated with silicagel by dipping into a test tube containing an aqueous suspension of the silica (Kieselgel, Merck) and dried on a rack suspended over a bunsen burner.

About 0.05 ml of the 1 ml preparation was spotted onto the paper or silicagel for chromatography, the amount depending on indications from preliminary experiments of how much active material might be present in a sample.

Methods of Identification.

Several methods were used. Pigments were identified by their natural colours, particularly on thin layer chromatography. By a

technique developed using tyrosinase, pigment precursors were identified and then studied using other reagents. The methods used were those of Ivor Smith (1960).

Ninhydrin was used to identify components in the hair extracts. The ninhydrin showed the presence of many aminoacids but did not immediately identify the chromogens, although there was a delayed reaction at the sites of the chromogens, a purple spot appearing at these sites 2 - 3 days after treatment of the paper when other spots had begun to fade.

Ferric chloride gave a brown spot at the sites of the chromogens - a reaction which one would expect with a phenolic pigment precursor.

Paradimethylaminobenzaldehyde gave a bright orange spot at the site of the chromogens. This is the reaction one would expect with an aromatic amine, and the result obtained with the faster moving of the chromogens in the New Guinea tree kangaroo was in fact identical in type and ultimate behaviour to the result obtained with kynurenin, but at a different site. For the two principal chromogens of the possum the orange spot after some days lost its brightness and became browner, although even months later these spots were still clearly identifiable as orange spots.

Ultraviolet light. The chromogens have blue-purple fluorescence under ultraviolet light and one would believe that this was the source of the fluorescence which Bolliger (1944, 2) found in possum hair. Ultraviolet light identification had the advantage that the spots could be marked in pencil and then other reagents applied.

Tyrosinase. The tyrosinase technique used was as follows. Two mgms of tyrosinase was dissolved in 50 ml of distilled water, and to this was added, immediately before dipping the paper chromatogram, 4 ml of 1/2,500 solution of catechol. The catechol enhances the effect of the tyrosinase greatly, and brings up immediate spots at the sites of the chromogens. The same spots appear more slowly when catechol is not used.

Two way chromatography was performed in many identifications, and by this means it was shown that a regularly occurring bright yellow spot appearing with paradimethylaminobenzaldehyde was urea which was present in all hair and feathers. The identity of spots found in different species was also shown by two way chromatography.

Results:

Paper chromatography

Four chromogens were identifiable in marsupial hair. Three of these were present in quantity and one feels that these three at least have significance in understanding the pigmentation of these animals. The reaction with p-dimethylaminobenzaldehyde was the same with each - the development in the course of 2 - 3 hours of an orange spot. The spots have been lettered A - D in the table which shows Rf values in three solvent systems.

Table XIV. Rf values of marsupial pigment precursors in three solvent systems -

	A	B	C	D
Butanol Acetic	0.89	0.90	0.53	0.42
Butanol Pyridine	0.91	0.80	0.63	0.23
Phenol	0.58	0.88	0.28	0.17

The grey possum frequently had all four substances A, B, C & D present simultaneously although A and C were usually most prominent. A, which is the precursor of the red pigment was occasionally absent from a sample of possum hair. The New Guinea tree kangaroo was notable for the large amount of component B present, with less amounts of the others. The red kangaroo seldom had more than a trace of A or B, the only component regularly present being C.

The component "A" with tyrosinase and catechol immediately develops a bright red spot which remains indefinitely with only a slight dulling of shade. Components, B, C & D parallel closely the reactions of dopa with tyrosinase. The first reaction is the development of a purple colour which after an hour or so fades and is replaced by a light yellow colour. Spot B then gradually becomes a light red brown colour, spot C a brown, and spot D remains a dull yellow.

The red pigment of the red kangaroo, when butanol-acetic solvent is used, adsorbs strongly onto the filter paper in chromatography, and produces a streak starting at the origin and extending a variable distance up the paper according to the original concentration of the pigment present. In very high concentrations it may reach half way up the paper (Rf 0.50). The same pigment is a component of the orange

yellow pigment of the red kangaroo and the pigment produced from precursor "A" of the possum. With butanol-pyridine slight adsorption occurs, but the main body of the pigment moves to a point corresponding to Rf 0.4. In phenol there is no apparent adsorption and the pigment moves to a point corresponding to Rf 0.56

There are a number of other pigment spots produced on the paper - in general these are behind the red pigment spot with butanol pyridine solvent and ahead of it with butanol acetic solvent. These are the pigments which are seen much more clearly in thin layer chromatography and it has not been possible to define them adequately on paper. For the orange yellow pigment of the kangaroo, apart from the red pigment already mentioned there are two main spots running towards the front with the butanol acetic solvent, and there is no apparent adsorption of these on the paper.

Thin Layer Chromatography

As stated this technique had its principal application in the study of pigments. In most preparations a large number of pigment bands were obtained. A principal red pigment (Red 1) was found in kangaroo, (red and orange yellow pigments) and possum. This represented possibly 90% of the total of the red pigment of the red kangaroo, and a lesser percentage of the orange yellow and possum pigments. In the orange yellow pigments of the red kangaroo a second red pigment (red 2) and a yellow and an orange pigment were found. In the possum red 1 and red 2 were found and numerous minor bands. In all three cases there was a purple band in some preparations,

most marked in the possum. Also there were two or three brown bands in most preparations, with low Rf values.

The red pigment precursor (spot A of paper chromatography) ran just behind the red 2 band in the possum extract when Phenol-Acetic was used as solvent. This latter solvent was used principally but Butanol-Acetic and Butanol-Pyridine were also satisfactory.

Rf values obtained are shown in Table XV. A blue band which appeared in one or two orange yellow pigment preparations is also included.

Table XV - Rf values of some pigments on thin layer Chromatography

Phenol-Acetic	Red 1.	0.49	Red 2.	0.65
	Yellow	0.64	Orange	0.47
	Blue	0.35		
Butanol-Acetic	Red 1.	0.64		
Butanol-Pyridine	Red 1.	0.90	Blue	0.93

H. Synthesis

As a result of the conclusion that the pigment precursors were aromatic amines extensive experiments were carried out with all available compounds in this class. These experiments included treatment with tyrosinase, efforts to produce pigment by autoxidation, and chromatography of the pigments produced. Small amounts of catechol or dopa were added in some experiments as these have the effect of increasing the activity of some reactions with tyrosinase. The most interesting substance tested was 3-Aminotyrosine which with

tyrosinase and catechol initially produced an active reaction producing a purple pigment similar to that produced with tyrosine. After a few minutes of this activity the reaction became very much slower and even after many days a weak yellow colour was the best pigmentary result obtained. It was found meanwhile that when dispersed on paper or on silica gel as for chromatography that red pigment was produced. After further experiment it was found that solution in aqueous phenol (4:1 phenol:water), aqueous acetone, or 70% ethanol, led to more active pigment production. The pigments produced had many similarities to the naturally occurring marsupial pigments, and some of the major fractions of these may in fact be represented by minor fractions of the artificial pigments.

It was found that a red pigment with Rf value 0.81 in Butanol-Acetic was present after exposure of 3-Aminotyrosine to sunlight in aqueous phenol, and the same pigment was the principal pigment formed when possum hair extract in aqueous phenol was exposed to sunlight.

The chromatographic features of these two pigments are set out. (Thin Layer Chromatography)

	AMT pigment	Possum pigment
Phenol water	0.50 red purple	0.50 red purple
Butanol Acetic	0.81 pink	0.81 pink
Butanol Pryidine	0.75 red brown	0.75 red brown

As controls for this experiment phenol-water alone, and with kynurenin, tyrosine, dopa, N-Acetyl-tyrosine, phenylalanine and

tryptophane were used. None of these latter produced any pigment in quantity, except dopa which produced a black pigment, and none produced the particular pigment under investigation.

The conclusion is arrived at that 3 Amino-tyrosine is the precursor of a series of yellow, red and purple pigments, and that it may be the precursor of the pigments of marsupials.

I. Interpretation

Why do maraupials produce these pigments? The secretions from both possums and kangaroos have a characteristic odour and are related to the sex hormones and sexual differences between male and female. Possums stain their daytime hideouts with this material and one suggestion is that it enables them to find these hiding places in the darkness, through the sense of smell. It almost certainly has some relevance to the mating and communal activities of these animals. The bright pigment secreted possibly has a function in making the fur more lustrous at various seasons. Finally it could be an adaptation in an animal living in a dry climate as the substances are amino-compounds and represent the excretion of nitrogenous material in the sweat.

Further evidence linking the red pigments studied so far with those of other mammalian species, and of Rhode Island Red poultry, will be presented in the next chapter, and although it is not possible at this stage to present a complete picture of the biochemistry of

these pigments, it is felt that at least some progress in this direction has been made, sufficient at least for the purposes of this thesis.

J. Acknowledgement

Professors R.J. Walsh and G.B. Sharman, of the University of New South Wales suggested the study of kangaroo pigments. Many people have co-operated in the collection of hair samples. Drs. F.J. Moss and P.A.D. Rickard of the University of N.S.W. carried out reflectance spectrophotometry, and Prof. K.W. Cleland of Sydney University made available the microspectrophotometer and helped establish the technique of study of hair pigments. This technique was originally suggested in discussion with Mr. S. Singh of the University of N.S.W.

Chapter VI - Mammalian and Avian Pigments. Human Pigments.

A. Literature

Sorby (1878) treated human red hair with hot acid and obtained a pink solution. Flesch and Rothman (1945) boiled red hair in 0.1 NHCl for two hours and obtained a reddish solution, the colour of which depended on the original colour of the hair. Horse, dog, cat and rabbit hair were all negative to this treatment. The pigment obtained from human hair was purified to a dark red brown solid which was insoluble in water. In acid it had an absorption band at 535 mμ. They concluded that the pigment "contributed little or nothing to the red hair colour". Barnicot (1956) also extracted the red pigment which was called trichosiderin using N/10 NaOH at room temperature for 12 hours. He obtained a yellowish solution which became pink on heating for 5 minutes with acid at pH 0.2, and had the characteristic absorption band of trichosiderin.

Other significant attempts to study the red pigments have been made on feathers of domestic poultry. Smyth, Porter and Bohren (1951) extracted red feathers with first acid and then alkali, and then chromatographed the pigments on a talc celite column. They obtained four pigments; a dark purple one with an absorption band at 567 mμ in acid and 475 mμ in alkali, and a red purple one with peaks at 535 mμ (acid) and 450 mμ (alkali), and two brown pigments which they called brown I and brown II. They found these pigments

present in different breeds of poultry, and also found the red purple pigment, which they considered to be trichosiderin, in human hair but not in the hair of the red setter dog. Brown I was the chief component of both the human and red dog hair.

Red pigments from other sources have created some interest. Kennedy & Nicol (1959) discuss the red head stripe of Chaetopterus variopedatus which is extractable with 0.2% NaOH, is bleached by H_2O_2 and has a broad absorption band between 400 and 450 mu which resembles the human melanoid described by Edwards and Duntley (1939). They suggest that it might be a "natural red melanin". Edwards and Duntley had discovered evidence of the pigment melanoid by spectrophotometry of skin pigments and suggested that it might be a breakdown product of melanin. Evidence in this chapter could suggest that it may be the natural pigment of red hair, and a component of brown hair pigmentation. Zussmann, Lyon & Vicher (1960) studied a pigment produced by T. rubrum, formation of which is dependent on a supply of phenylalanine or tyrosine. This organism produces yellow, orange and red pigments which are insoluble in water and a purple pigment which is soluble and has absorption bands at 425 mμ and 535 mμ.

Speculations as to the origin of red pigments in hair have produced no final solutions. Fuseau-Braesch (1960) obtained a yellow pigment by treating N-acetyl-tyrosine with tyrosinase, and found incorporation of tyrosine into both black and yellow areas of Gryllus

bimaculatus. She suggested that N-acetyl tyramine or N-acetyl tyrosine might be a precursor of a yellow pigment. Fitzpatrick & Kukita (1959) suggested that since numerous pigments can be produced from kynurenin, a derivative of tryptophane, then tryptophane might be the precursor of the red pigment of red hair. Rebell, Lamb, Mahvi & Lee (1957) found that albino rat hair which is faintly yellow in colour contained quite large quantities of kynurenin and a yellow pigment which could be separated by chromatography of an extract obtained by boiling rat hair.

B. Microspectrophotometry

The technique of microspectrophotometry is important in this chapter. In the last chapter the method of preparing a series of readings of optical density is recorded. The only thing to add to this is the technique of "two-wavelength" assessment of pigment character. Brown pigments have a relatively flat curve in the visible range of the spectrum whereas red pigments have high absorption at the blue end of the spectrum and low at the red end. Consequently a distinction between red and brown substances, hair or otherwise, can be made as a numerical index.

The curves for red pigments studied had peaks at 430 mμ or 450 mμ most often; generally then there was rapidly diminishing optical density to about 650 mμ at which there was a point of inflexion and a flattening of the curve. The optical densities at 440 mμ and 650 mμ were chosen to provide a ratio for "two wavelength" assessments,

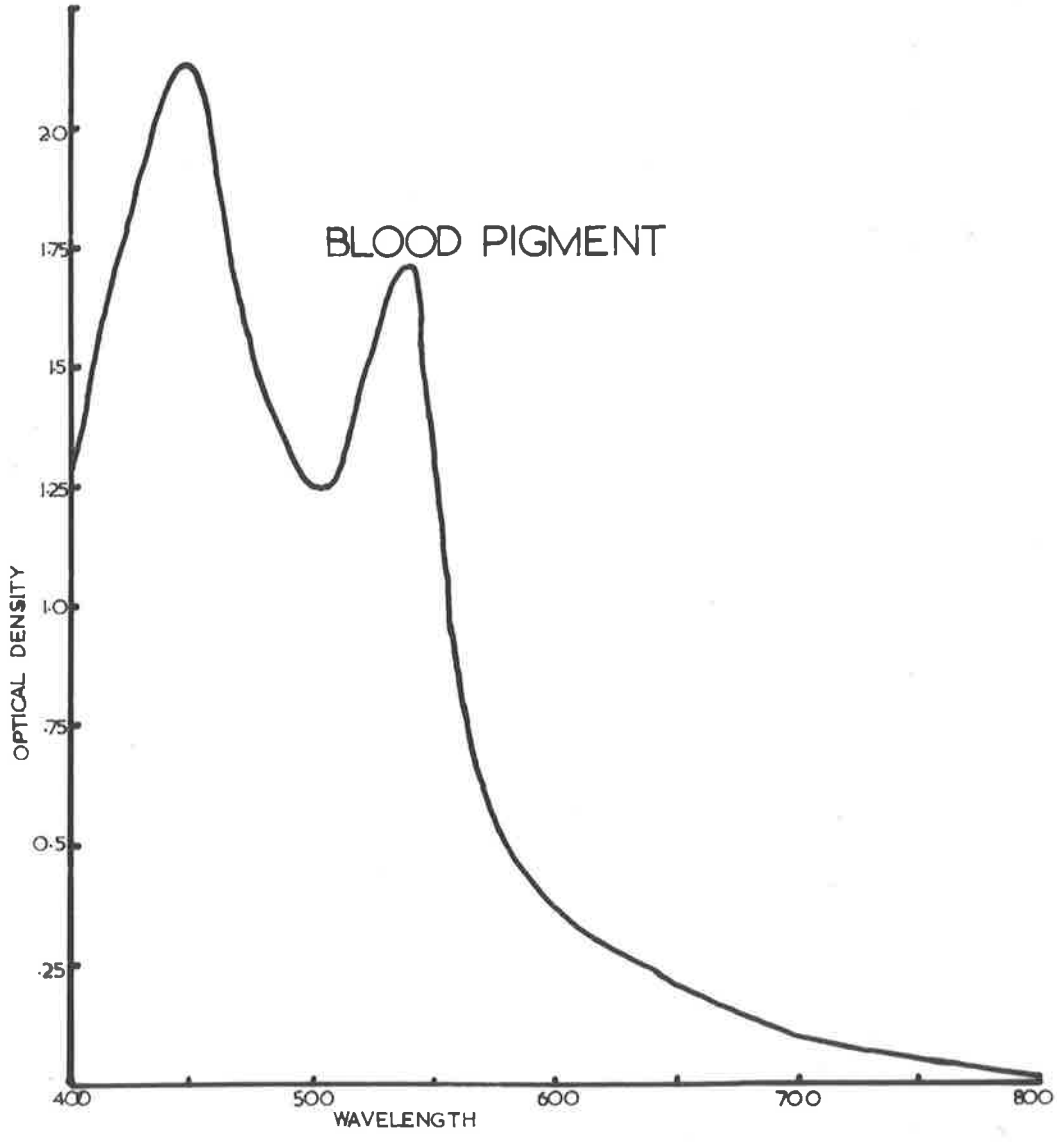


Fig. 45. Spectral absorpiton of blood pigment found in a sample of hair, examined by the same technique of miroo-spectrophotometry as used for hair pigments.

and an index of redness was obtained, being the ratio (optical density at 440 mμ/optical density at 650 mμ). Greater use of this ratio will be made in the latter half of this chapter.

C. Blood pigments

At an early stage when using this technique readings were taken on hair from a skin factory. A bright red pigment seen attached to some of the hair attracted attention and readings were taken of the optical densities of this pigment at different wavelengths. It was realized on further inspection of the sample and on examination of the curve that this was a blood pigment and the curve is shown in Fig. 45.

The inclusion of this result, and the one to follow showing finch carotenoids, in this thesis, is felt to be necessary because the use of microspectrophotometry in the study of hair pigments has not been reported previously, to the knowledge of this author. These curves are so characteristic in their particular areas of relevance, and so well known, that it is felt that they add validity to the extensive use made of this technique.

In passing one would point out that a microscopic amount of blood is identifiable by this method and this might have forensic value in identifying spots of blood on hair and other material.

D. Carotenoids

The Dept. of Genetics, University of Adelaide, is maintaining a

Fig. 46.

Black head feather of Gouldian Finch.

Fig. 47.

Intermediate type of feather giving some idea of the mechanism of the change of structure.

Fig. 48.

Red head feather of Gouldian Finch showing modification of morphology of feather.

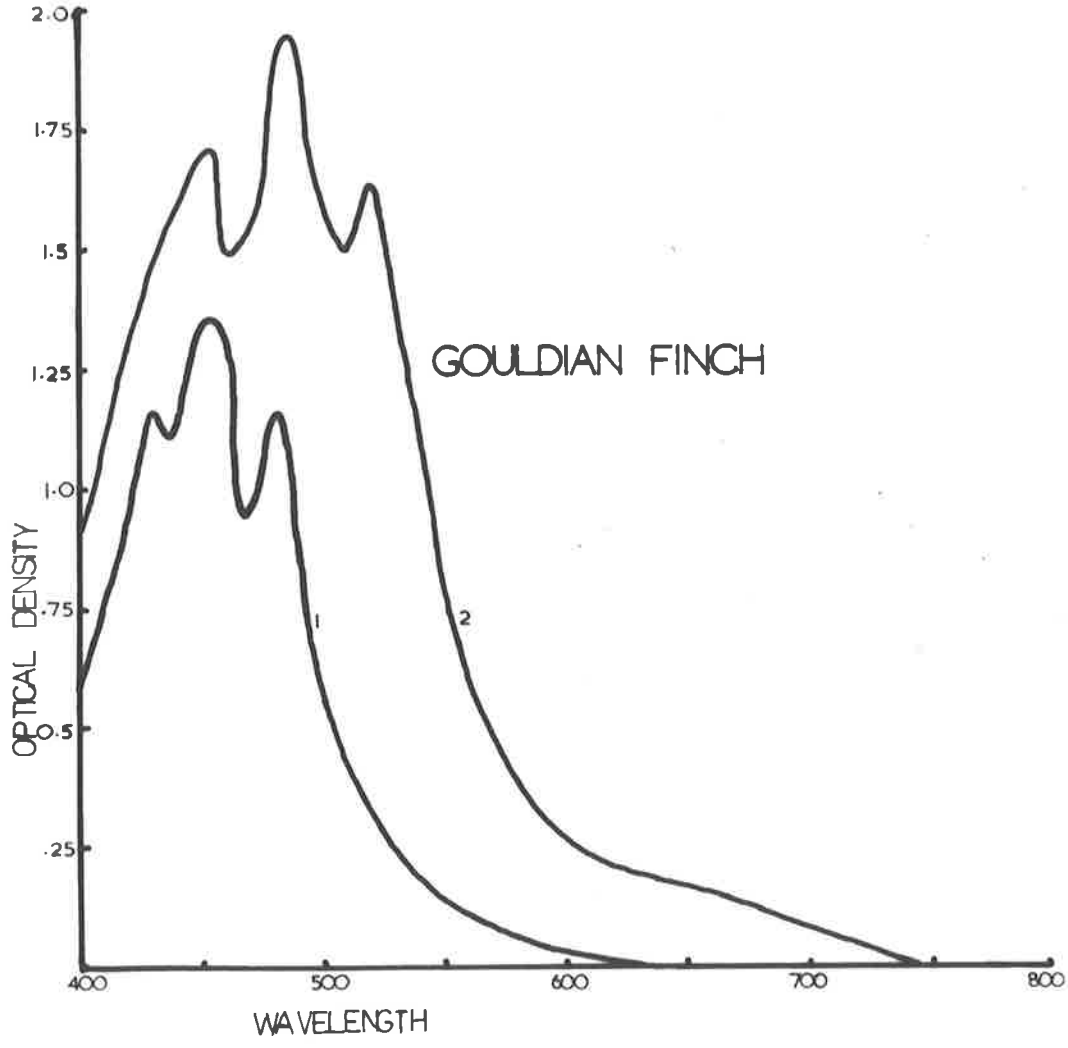


Fig. 49. (1) Yellow feathers of breast of Gouldian finch.
(2) Red head feathers of red headed variant — microspectrophotometry.

colony of Gouldian Finches (Poëphila gouldiae) in which there is a dimorphism of black headed and red headed types. The red headed variants may differ from the black by a single sex linked dominant gene. Since there seemed to be this clear genetic difference between red and black it was felt worthwhile to study this with the facilities available.

It was found on microscopy of feathers that the black feathers had a rather regular structure as seen in the photograph (Fig. 46). In the red headed variant there were red feathers (Fig. 48) and in a rim around the area of red feathers a feather obviously intermediate in type between the red and the black (Fig. 47). The appearance suggests that the laying down of red pigment in the feather has the effect of altering the development of the distal part of the barb so that it takes on an irregular tattered appearance and is completely lost. When a large amount of pigment is laid down in the barb it appears to be partly accommodated in a region where the barbules are clumped together and lose their identity.

Microspectrophotometry (Fig. 49) was carried out on yellow feathers of the breast of the finch and the red feathers shown in the pictures. The pigments were previously considered to be carotenoids, and the curves obtained support this suggestion (Fox and Vevers, 1960).

The curves obtained with these pigments contrast with the curves obtained with presumed "red melanins" of Rhode Island Reds and Mammals

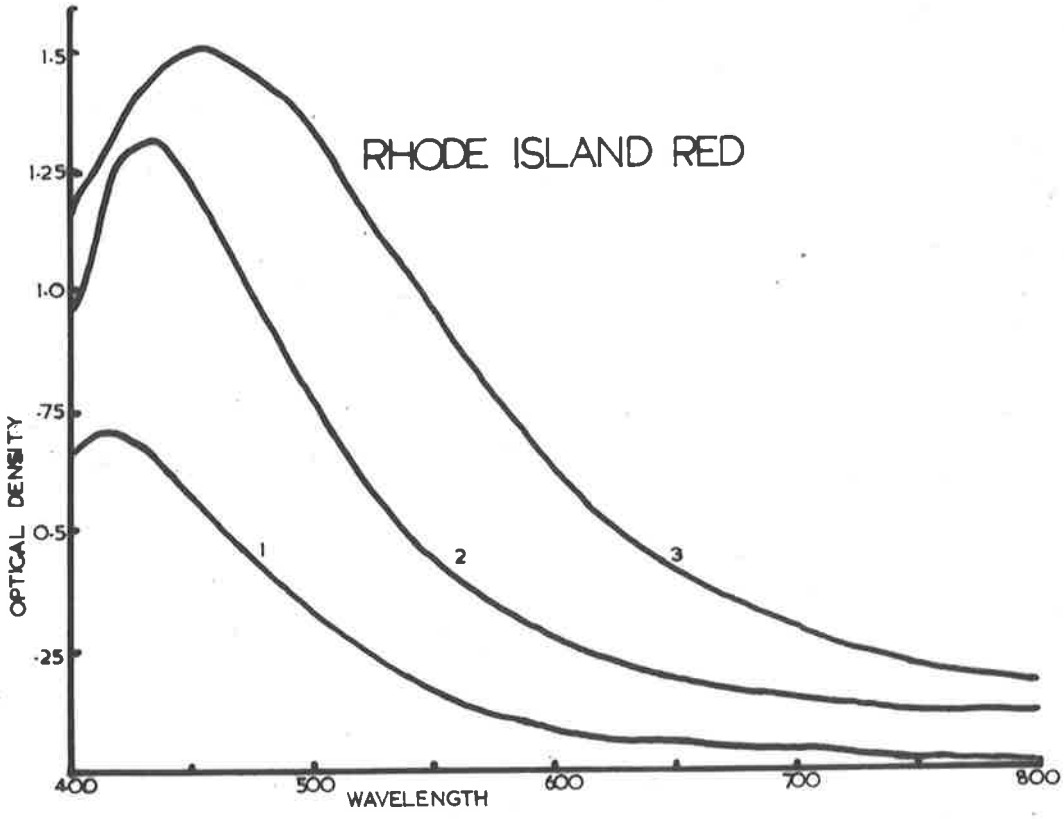


Fig. 50. Rhode Island Red Feathers (1) yellow (2) orange red (3) red. Microspectrophotometry.

about to be described. Other marked differences include insolubility of the finch pigments in water and instability when dissolved in aqueous phenol.

E. Red Melanins of Poultry

Thin layer chromatography of orange yellow pigment of the red kangaroo reveals yellow, red, and orange pigments. In Rhode Island Red feathers the pigments extracted are yellow, orange, red and purple in colour. Microscopically feathers have some parts which are yellow, some orange, and some red in colour. Amongst animal hair the colours, apart from brown and black, range from yellow, to orange red to darker red. Individual hairs are usually classifiable into one or other colour group, although all may occur on the one individual, whereas in feathers the several colours may be seen on different parts of the one feather. In a Rhode Island Red for instance the more exposed parts of barbs and barbules may be uniformly red while the central parts of the feathers may be more orange or yellow. Considerable similarity between yellows of different origin, and between reds of different origin, were found, whether it was in poultry, horse, cow or human. Fig. 50 shows representative results with Rhode Island Red feathers. The darker red feathers may have an even broader absorption band than that shown and this may be due to admixture with brown pigment or to some alteration of the optical properties of the red pigment in high concentration.

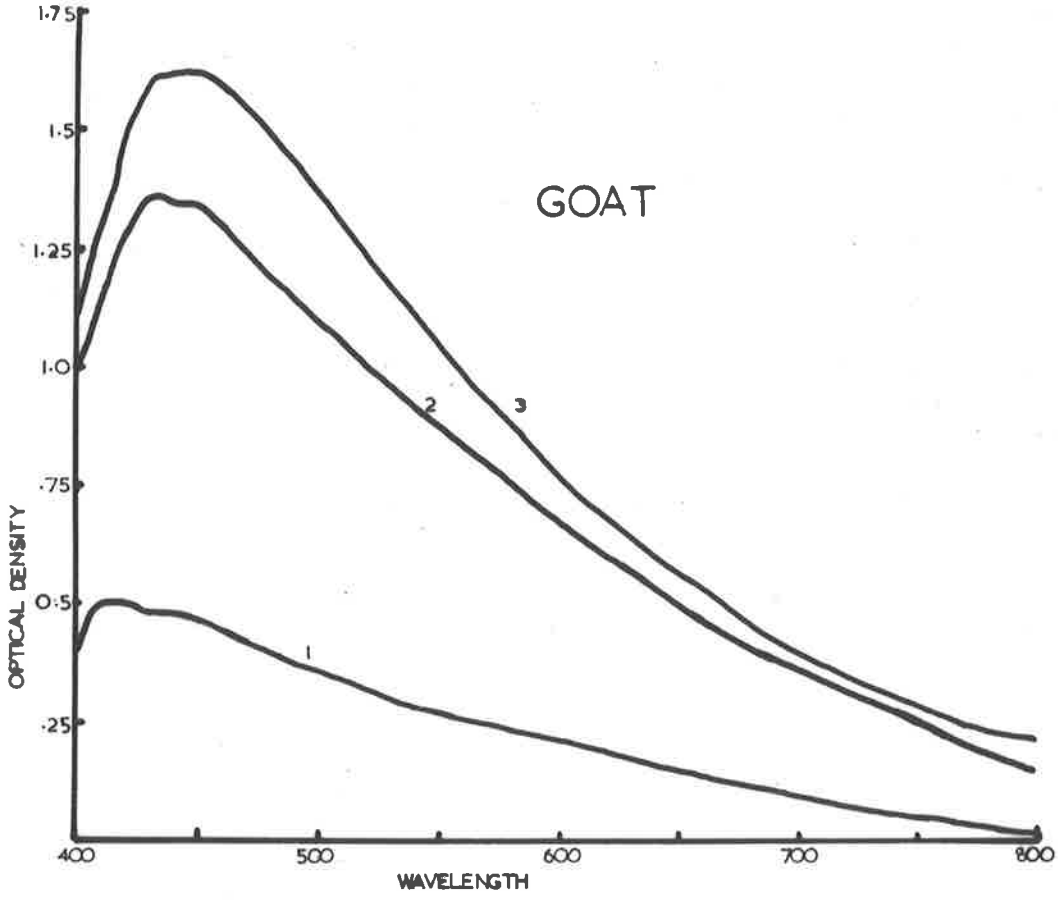


Fig. 51. Goat hair (1) yellow (2) orange yellow (3) red. Microspectrophotometry.

Fig. 52.

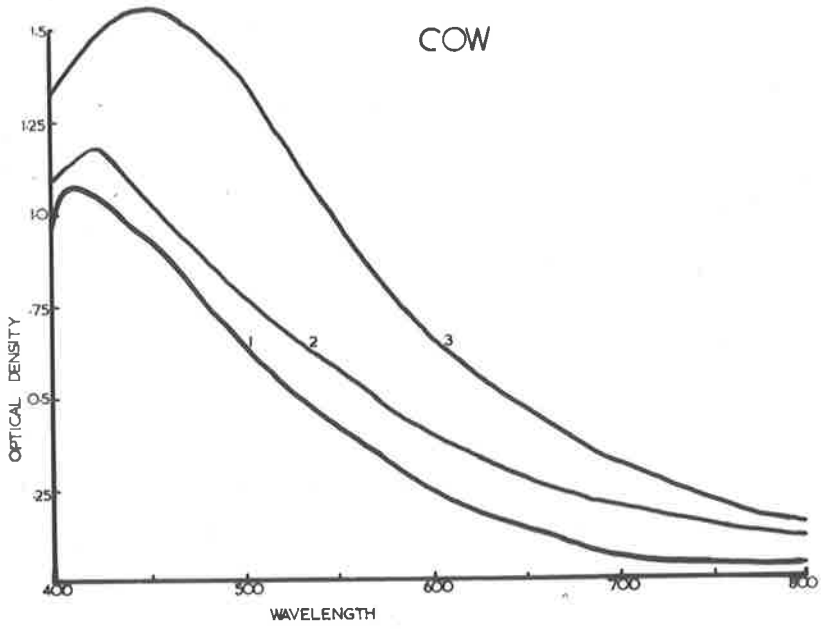
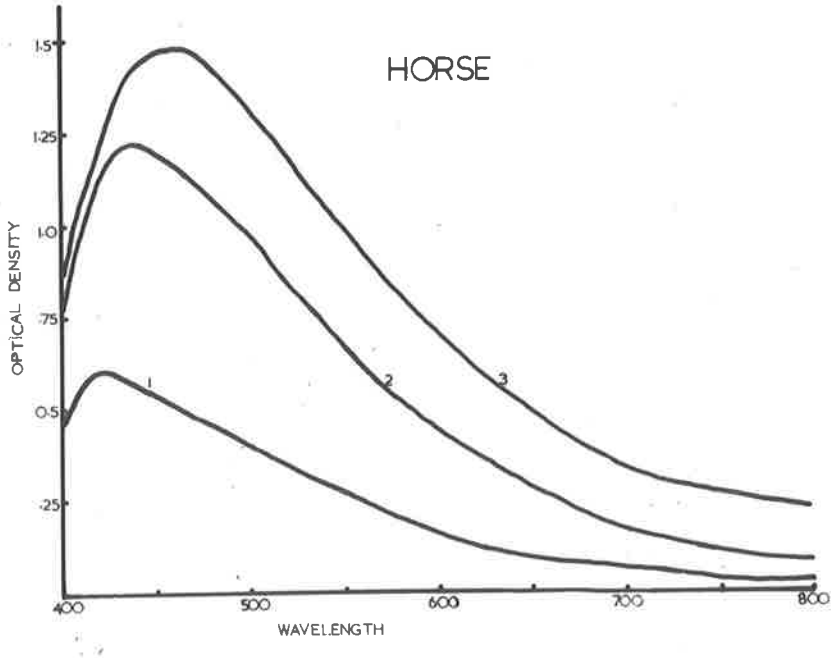


Fig. 53.



Figs. 52 & 53. Cow and horse hair (1) yellow (2) orange yellow (3) red. Microspectrophotometry.

F. Red melanins of mammals.

The curves for goat, cow and horse are shown (Figs. 51, 52 & 53). The general pattern is of peaks of optical density at about 410 mμ, 430 mμ and 450-60 mμ according to the absolute value of the optical density. The hairs recorded are yellow, orange yellow and red.

If one supposed a common precursor for these pigments two possibilities might exist - either a uniform amount of precursor converted to pigment of a particular colour would have a lower optical density if the pigment was yellow than if it was orange yellow or red; or alternatively with graded amounts of precursor the pigment produced from low concentrations of precursor would be yellow; from medium amounts of precursor, orange yellow; and from large amounts of precursor, red.

In the red Hereford cow the band of greatest absorption is even wider than shown here in the reddest hair, and one has the possibility, if there is a single precursor to all these pigments, of higher polymers of a basic molecule being present, with absorption bands at progressively higher wavelengths, as has already been suggested with the free red pigment of the red kangaroo. Difficulties arise in reading very dark red hairs on the microspectrophotometer - the readings of optical density become very high and subject to inaccuracies on this account (Cleland 1965). Hence further understanding of what happens in the very dark red hair may have to wait until a broader

Fig. 54.

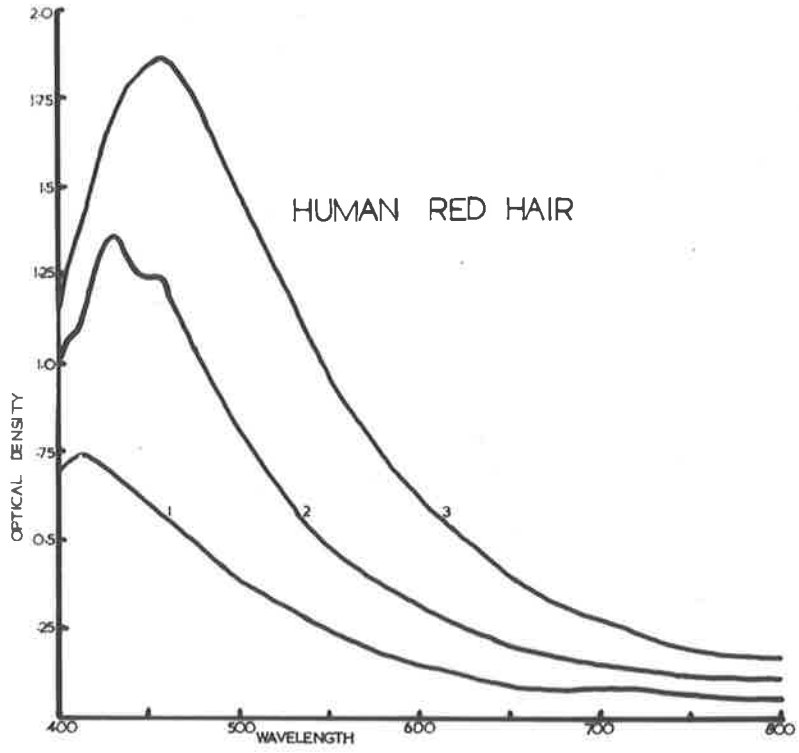


Fig. 55.

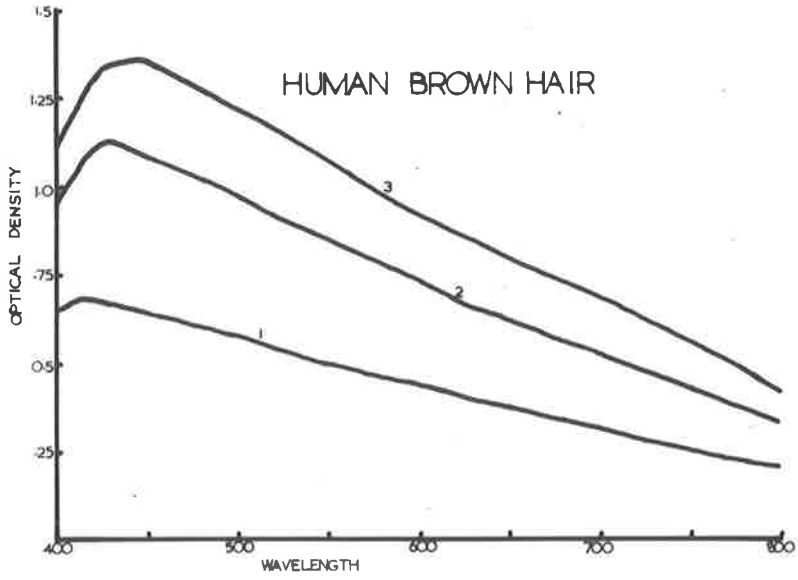


Fig. 54. Human red hair (1) yellow (2) orange yellow (3) red.

Fig. 55. Human brown hair (1) pale yellow brown (2) light brown (3) brown.

basis of knowledge is available.

G. Human Red Hair.

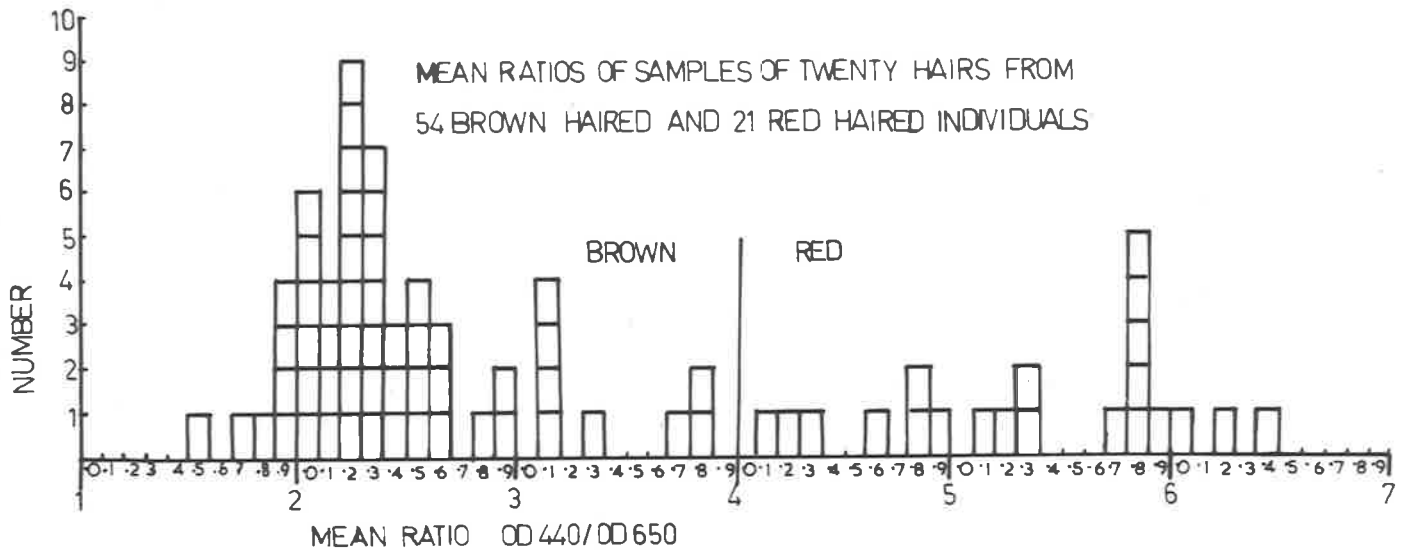
Human red hair has essentially the same features as other mammalian hair. Yellow hairs have a peak of absorption at about 410 mμ, orange yellow at 430 mμ and red at 450 mμ.

It seems significant that both light brown and dark brown hairs have a similarly increased absorption at the same points as yellow and red hairs. This may mean that there is some yellow or red pigment in every brown hair. The curve for brown pigment should be relatively flat, and this was found by Smyth et al (1951) in studying brown pigments of poultry. A combination of such a curve with one of the curves in Fig. 54 would give the shape of curve seen in Fig. 55.

It is worthy of note that the curves in Fig. 55 correspond quite closely to the curves for "melanoid" described by Edwards and Duntley (1939).

Whilst considering the curves for red and for brown hair further comment should be made about the ratio used as an index of redness. Inspection of these curves shows why a ratio of (optical density at 440 mμ/optical density at 650 mμ), gives a clearcut index of redness in the individual hair. Microscopically assessed individual hairs are black when the index is between 1 and about 1.3, brown between 1.3 and 3, red brown between 3 and 4, and red above 4. Values below 1 are not possible with these pigments, and in several thousand

Fig. 56. Seventy five samples of hair assessed by independent observers as being either red or brown show clearcut differentiation when examined by the microspectrophotometer.



readings were not observed. Problems of assessing where red brown ends and red begins are difficult even given the clearcut results obtained in this study (see next section) although one feels that the objective differentiation between red and brown is much nearer solution than when Reed (1952) wrote.

H. Samples of reds and browns.

During the course of this study various people contributed samples of red hair. This collection represented the pooled opinions of many people on what constituted red hair. The School of Hairdressing at Sydney Technical College at one stage collected 54 samples of what they regarded as brown hair. It was felt that the best measure of a person's hair colour was to take unselected hair cuttings, prepare slides without bias to colour of individual hairs, and to estimate the ratio of redness of 20 consecutive hairs as seen under the microscope.

Making readings of this sort is time consuming and 20 readings could be performed in half an hour. Preparation of material and calculation of results, and checking of various aspects of the technique at each session of photometry all take time and for each such result on 20 hairs of an individual approximately 1½ hours of tedious work was expended. In the case of the samples of reds and browns the calculations of ratios were done by computer, which only made the work a little easier. Even though one could outline a scientifically acceptable method of assessing the character "red

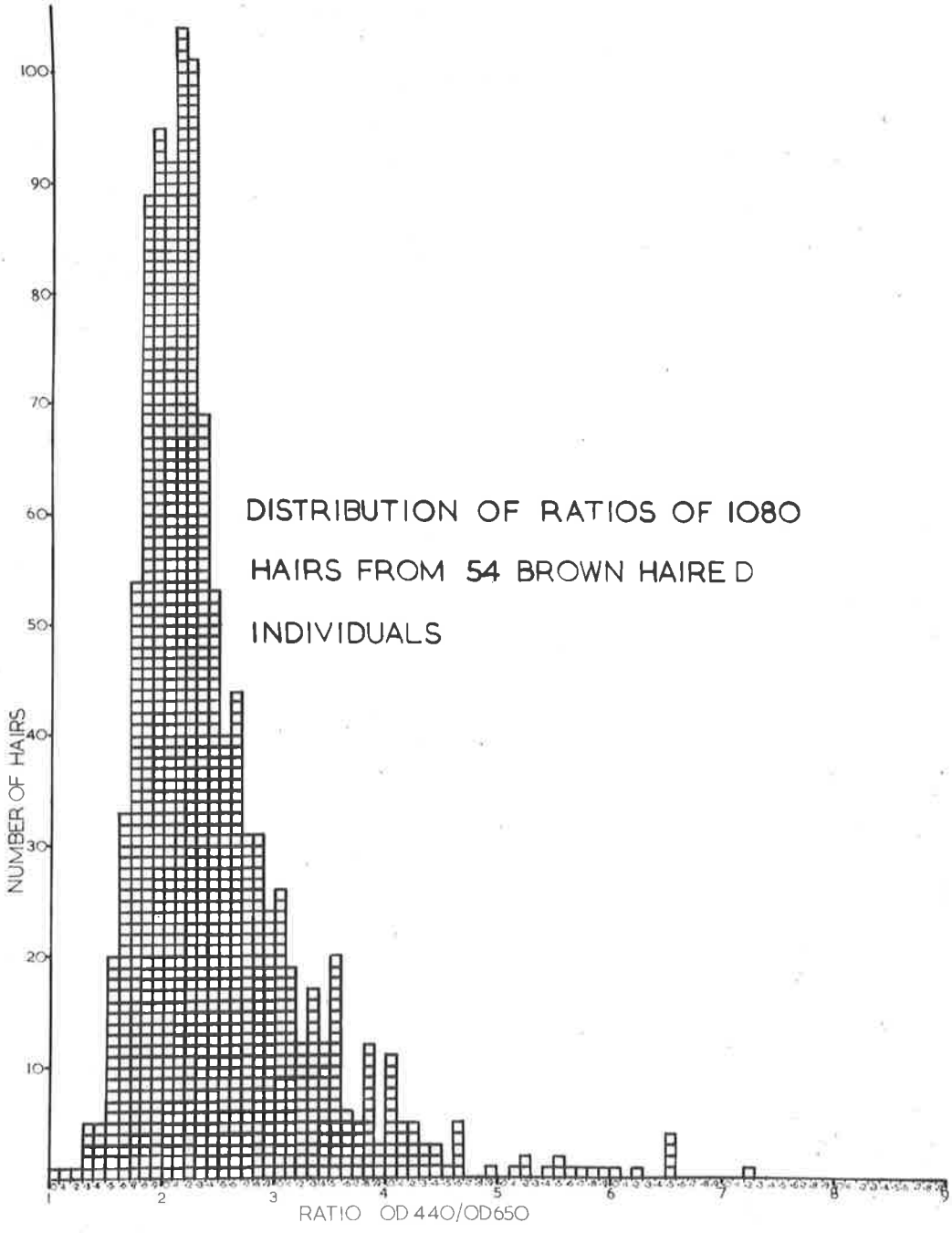


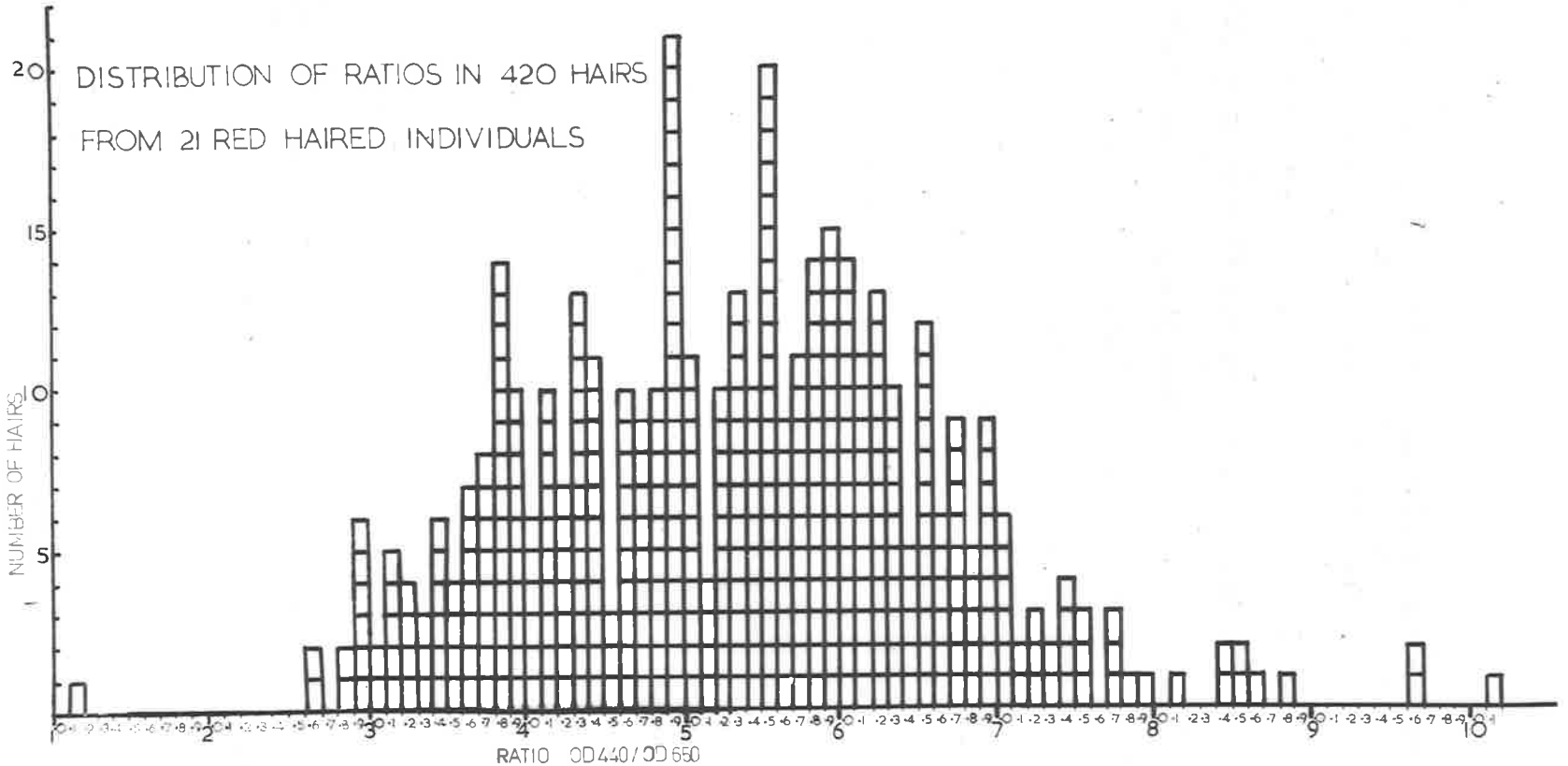
Fig. 57. Positively skewed curve of 1080 readings from 54 brown haired individuals, with a long tail of readings with ratio greater than 4.

hair" in the community by this means, the man hours involved would be prohibitive. One would suggest that what "should" be done is to take 1,000 randomly selected individuals and work out the ratios of 20 hairs randomly selected from the scalp of these 1,000 individuals. Having calculated the results and drawn a rather ungainly graph one might see a bimodality in the graph suggesting that "red hair" was a distinct phenotype. The very large number of samples to be tested would be needed because of the small percentage of red haired people in the community - even at an estimate of 4% this would provide only 40 cases in 1,000. The collection of hair samples from such a large group without bias would be difficult, and even when a clearly bimodal graph is obtained mathematical proof of the distinction into two classes is difficult.

The compromise then was to calculate mean ratios of 54 sets of "brown" hair clippings and 21 sets of "red" hair clippings. The results are shown in Fig. 56 and in Appendices III and IV. The results show that a mean ratio in excess of 4 indicates what is generally believed to be red hair. There is no overlap. It can be seen that the character "brown" has a roughly normal distribution around a mode of 2.2. There is a greater scatter of mean ratios of red hair samples.

Fig. 57 shows the 1,080 readings on 54 brown hair samples, and the marked positive skewness is noteworthy. Later some further data on the occurrence of red hairs on brown haired individuals will be given. Based on inspection of Fig. 57 one would not conclude that

Fig. 58. Ratios of 420 hairs of 21 red haired individuals.
The curve appears nearly normal in distribution and has many readings less than 4. There are several deep troughs particularly at ratios 4.0, 4.5, 5.1, 5.6 and 6.4. These may have no significance.



the occurrence of hairs of ratio greater than 4 (4.8% of the total) was any more significant than as an expression of the skewness of the curve, which is partly caused by hair with a ratio less than 4.

Fig. 58 shows the distribution of ratios amongst the 21 samples of red hair, comprising 420 individual hairs. Of these 17.6% had a ratio of less than 4.

I. Random Hairs - Reddest Hairs

The problem of defining "red hair" as a discrete character need not necessarily only concern heads of hair, but it can also concern individual hairs. Michelson (1933) found that approximately 18% of adult males have some individual red hairs. With the thought that this might be an important aspect of somatic variability an extensive study of this problem was carried out. It has been suggested that these individuals might be heterozygous for a "red hair gene" and on a somatic mutation hypothesis a simple justification for this suggestion can be made out. If as has been suggested red hair is a recessive character then in theory only those hairs with two "red hair genes" will be red. Those with a single "red hair gene" will be brown. In the heterozygote mutation of the normal allele of the "red hair gene", to another of the same sort will produce homozygosity in individual hairs and will account for the appearance of red hairs amongst the brown.

Samples of hair from 552 individuals were collected and slides were prepared. At one end of each slide there were a few randomly

Fig. 59. Ratios of randomly chosen single hairs from each of 552 individuals.

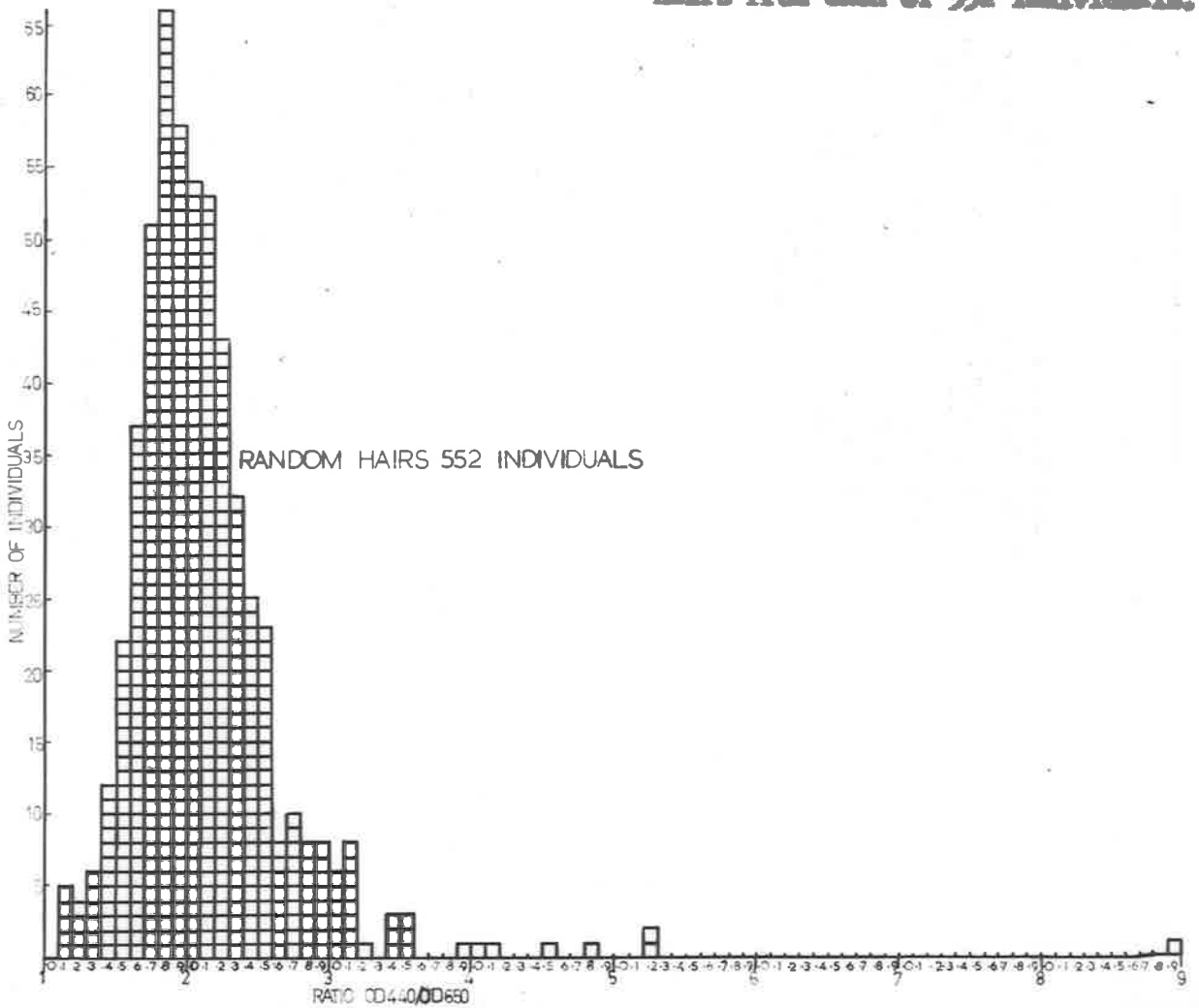
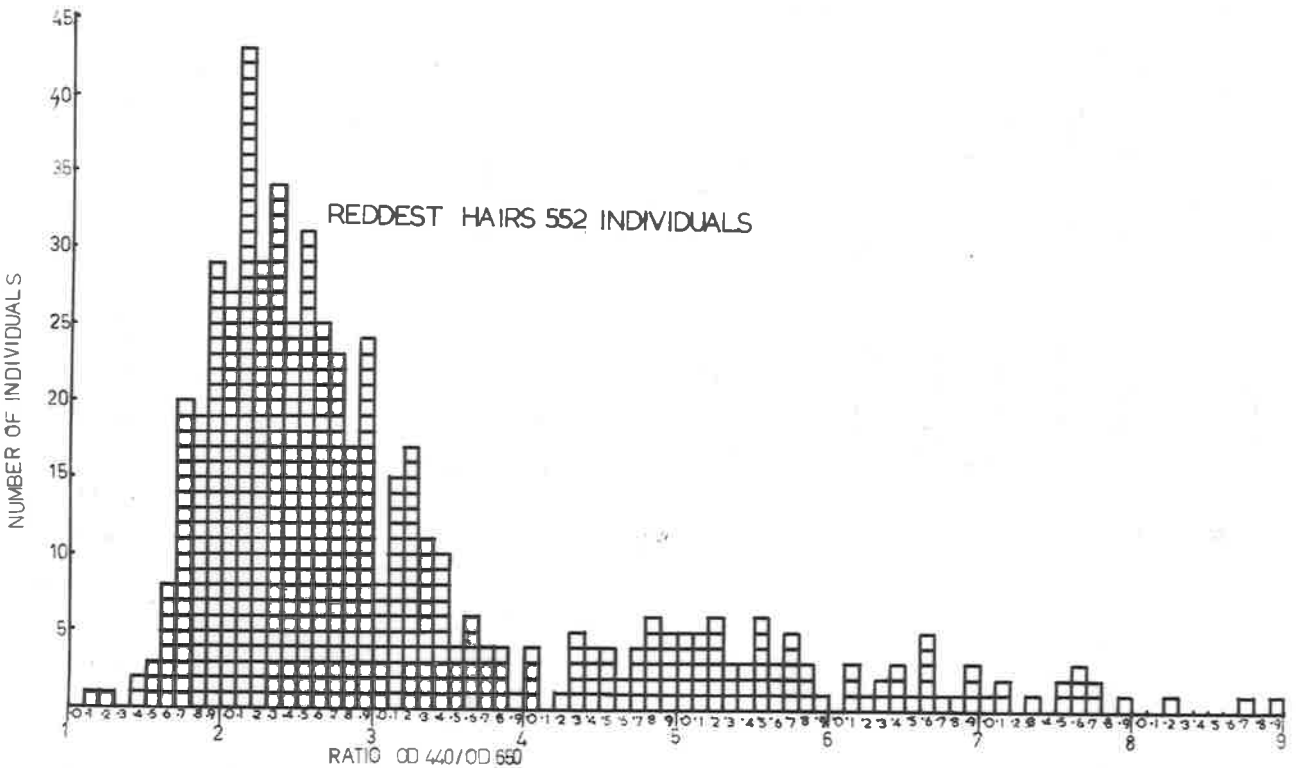
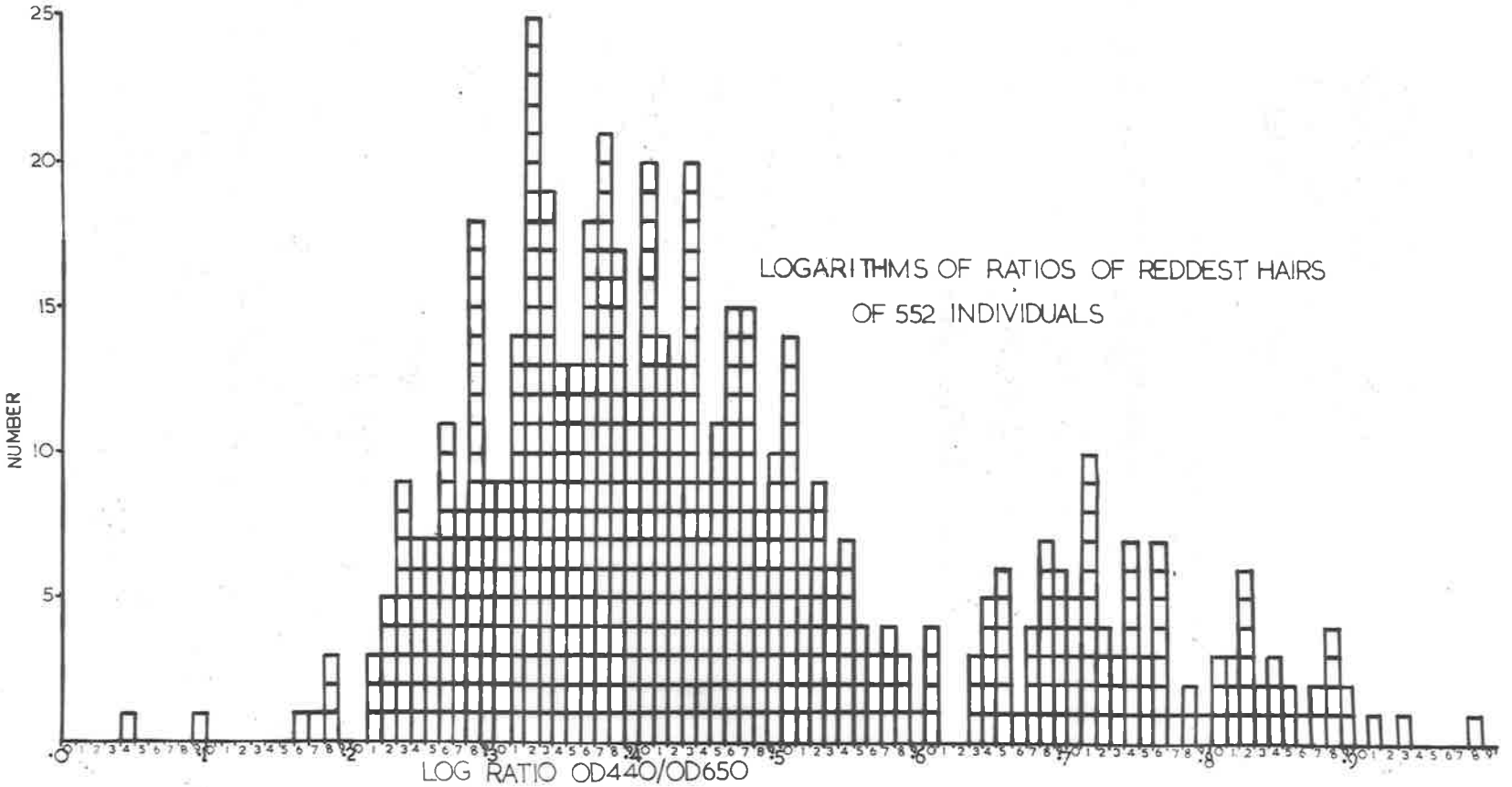


Fig. 60. Ratios of single "reddest" hairs chosen from each of 552 individuals.



selected hairs. At the other end there were one or more of the reddest hairs that could be found in the whole sample under a strong illumination. Spectrophotometer readings were made - one random reading at the brown end of the slide, and a reading of the "reddest" hair at the red end of the slide. In 80% of cases no red hair could be found but the effort of searching for a "red" hair was made on every sample. From this study two graphs have been prepared, one being the graph of readings of random hairs (Fig. 59), the other the graph of readings of reddest hairs (Fig. 60). Surprisingly only 10 individuals in the 552 of the sample were red haired whereas on the basis of other surveys one would have expected about 20; and only 7 of these were recorded as having a ratio greater than 4 in examining random hairs. An individual with a mean ratio above 4 may have quite a large percentage of hairs with a ratio less than 4 and the fact that three individuals were missed as being red by only taking one reading is not too improbable. The reverse situation of accounting as red a brown haired individual did not occur. The graph of ratios of reddest hairs shows 110 with a ratio greater than 4 which is 20% of the total and in close accord with Michelson. The purpose of including these graphs here is particularly to show, in the second graph (Fig. 60) that there is evidence of a separate class of hair in the long "tail" of the curve. Fig. 61 in which a logarithmic transformation of the ratios has been graphed demonstrates even more clearly a bimodality in the data, suggesting that "redness" in hair is truly a discrete character and not part of a continuous

Fig. 61. Logarithms of the readings shown in Fig. 60, demonstrating the bimodality of the data.



distribution. The effect of this transformation in Fig. 61 is to reduce the skewness of both the "brown" and the "red" modes of the bimodal curve.

Appendix V relates the actual readings on brown hairs and "reddest" hairs, and also age, freckling status and observed hair colour. The mean values of random ratios do not differ significantly when divided into those who have at least one red hair and those who do not.

J. Extraction of red pigments from hair and feathers.

Acid and alkali were used in a repetition of the methods of Flesch and Rothman (1945) and Barnicot (1956). In addition hair and feathers were soaked in cold water, and boiled, for both short and long periods.

Microspectrophotometric methods just described suited the purpose of this thesis and were highly reproducible, hence extraction methods were not persisted with, particularly using acid and alkali. The author's belief is that these may produce significant artefacts. Trichosiderin, an iron containing red pigment, could not be found in all samples of red hair from humans, and many red haired animals have none. Microspectrophotometry gives consistent results with all species examined, and with red poultry. The major portion of the pigment from all of these sources is obviously not trichosiderin. Some of the curves obtained with acid and alkaline extracts were similar to those obtained with microspectrophotometry.

The greatest interest was in regard to water extraction. Boiling of human and animal hair extracts red pigment which can be chromatographed although no positive conclusions can as yet be drawn from the results available. A sample of Rhode Island Red feathers soaked in cold water for 24 hours gave, after concentration and thin layer chromatography, distinct purple, red, yellow and brown pigment bands corresponding in position to similar bands obtained with the orange yellow pigment of the red kangaroo. From another sample of feathers a red pigment was obtained by washing the feathers in water at room temperature for half an hour. This was shown in three solvent media to run in identical positions to the "red 2" pigment of possum and red kangaroo, on thin layer chromatography.

A further sample of feathers was boiled for 3 - 4 hours each day for 8 days. The water from each day's boiling was concentrated and chromatographed. Yellow and brown bands were obtained near the origin (phenol acetic solvent, thin layer chromatography) and a distinct red band with Rf 0.50, was obtained with every extraction. This corresponds in character to a red band obtained by heating the "Red 1" band of the red kangaroo, and may be due to the freeing of an altered pigment in the boiling of the feathers.

These results do not paint the whole picture but are facets of what is obviously a complex situation. In summary of the chromatographic results in this and the last chapter one may say that 3 - A minotyrosine is the precursor of a large number, perhaps 10 -

20, yellow, orange, red, purple and brown pigments. In the free pigments of marsupials and in extractable mammalian and poultry pigments there may be a comparable number of components. Evidence is increasing that these different groups may have components in common, but the work involved and the record of final conclusions is beyond the scope of this thesis.

Fig. 62. Giraffes, Taronga Park Zoo.

Fig. 63. Zebra, Taronga Park Zoo.

Chapter VII - Variability of Hair Colour

A. Literature

Naturalists have written on the protective colouring of animals. This colouring has come about through natural selection and is genetically controlled with sometimes environmental modification. In humans there is variability of colour between different parts of the body. Variability of colour of individual hairs in brown haired people has been discussed in part, and this discussion will be extended in this chapter.

Boas & Michelson (1932) studied hair removed from the right temple of a large number of men of various ages. Examination of the hair under a magnifying glass revealed four types of depigmentation (1) depigmentation throughout the length of a hair (2) fading of one end of a hair relative to the other (3) partial loss of pigment throughout the length of a hair (4) hair partially depigmented in one part, completely depigmented in another. Michelson (1933) found that 435 of 2,397 males who contributed hair samples had some red hairs. He had no objective method of determining the redness of these hairs.

Kawamura, Kojima, Ikeda and Kobayashi (1963) discussed a phenomenon with nevi (birthmarks) in 3 young men aged 24, 17 and 20 years respectively. These individuals had some normally pigmented and some white ("grey") hairs on the nevi examined.

Keogh and Walsh (1965) concluded from a study of Australian blood

donors that there is no great difference in the rate of greying between males and females and between dark haired individuals and light haired individuals.

B. Variability of hair colour in animals.

The coat patterns of animals are of interest both with regard to evolution, and to the basic biochemistry which may be operative in production of the pattern. The pigmented areas of giraffe and zebra (Figs. 62 & 63) vary in colour through a wide range - the zebra of browns and blacks, and the giraffe of red and dark red brown - but the patterns remain constant in type in each species. The zebra is adapted to life on an open plain with tall grass or low trees and the pattern serves to make the animal's presence less obvious in these circumstances. The giraffe is adapted to an existence in the mottled light between taller trees. The evolution of these patterns must have entailed the development of a precise and complex genetic mechanism.

C. Variation of hair colour in man - regional.

The regional variations of hair colour in man may be minimal, or they may be striking. A systematic examination of this aspect by spectrophotometry has not been carried out but some observations are in order. Pubic, eyebrow, and eyelash hair are usually darker than other body and scalp hair, although one occasionally sees an individual with red hair who has pale eyebrow hair. Both pubic

hair and beard tend to emphasize red pigment in the red haired person. If more men had beards at the present time there would be some interest in studying the frequency of red beards amongst brown haired men. The reverse situation of red scalp hair and dark, almost brown beard has been observed.

The greying of hair is of interest with regard to regional effects. In particular one is aware of the individual with white scalp hair and black eyebrows.

D. Variability of hair colour in man - local.

Close inspection reveals that apart from the obvious greying of hair, adults have hair of many shades of colour. Some of the hairs can be jet black, and some can be shades of brown, and some shades of red. Further study of these phenomena might be ultimately very instructive both genetically and biochemically. The hair of the scalp itself has regional variations of colour and any comparison of scalp hair between two individuals for instance should be based on hair from a carefully defined part of the scalp.

The question is whether the mosaicism which every adult shows is based on a physiological variability which, in a constant genetic background, shows differences of degree and kind from one hair to the next; or whether the different coloured hairs may not in fact be due to genetic differences in the melanocytes in particular hair follicles. The melanocytes move into the hair follicles at an early stage of development (before the fourth month of embryonic life (Hamilton, Boyd & Mossman 1957)) and from that point on as hairs are

grown and shed from the particular hair germ, the same or descendant melanocytes are responsible for the pigment of the hairs. In the skin, due to pigment spread, one can theorize that where a melanocyte loses capacity for pigment production it is soon replaced, but this does not happen in the hair follicle - melanocytes which have ceased to function or have altered their function reveal their weaknesses by the production of no melanin or melanin of a different colour or quantity.

Hair of all colours has been studied, and these will now be considered; the possible changes based on a somatic mutation theory will be indicated.

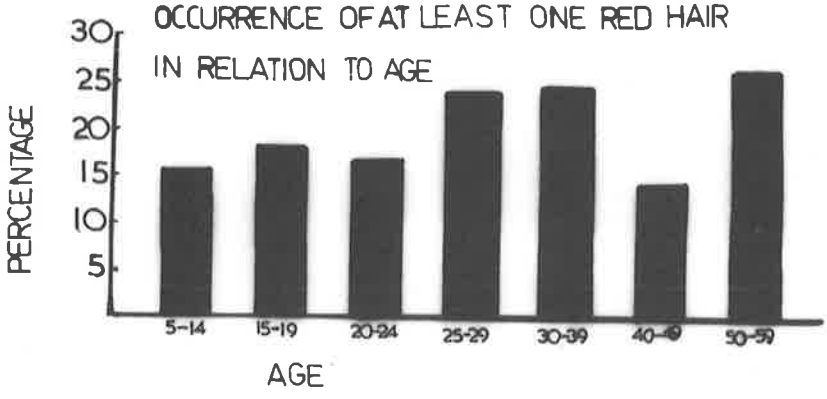
(1) Black haired persons

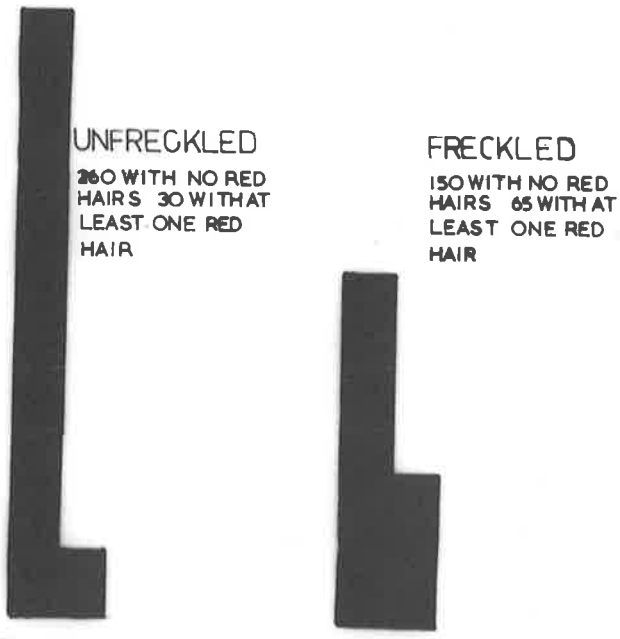
Microscopic examination of hair of a black haired person revealed 13 brown hairs, 106 dark brown and 89 completely black in 208 hairs counted. Genetically, black brown distinctions are not known in man. In the mouse the difference between brown coat colour and black coat colour is that of one dominant gene determining blackness; the recessive alleles determine brown in homozygotes. If a single gene could determine the difference between black and brown hair, then mutation of this gene in some hairs would produce brown hairs amongst the black. Whether the situation is ever as simple as this is not known.

(2) Brown Haired Persons

The most extensive studies of hair colour variability in this thesis apply to brown hair, and some of this has already been recorded. The largest sample of 552 individuals showed 20% with some red hairs.

Fig. 64. Data on 552 subjects, 20% of whom had at least one red hair. The total numbers vary in each group. There is no consistent increase in frequency with increasing age.





	UNFRECKLED	FRECKLED	
NO RED HAIRS	260	150	410
AT LEAST ONE RED HAIR	30	65	95
	290	215	505

$\chi^2 = 10.5$

$0.001 < p < 0.01$

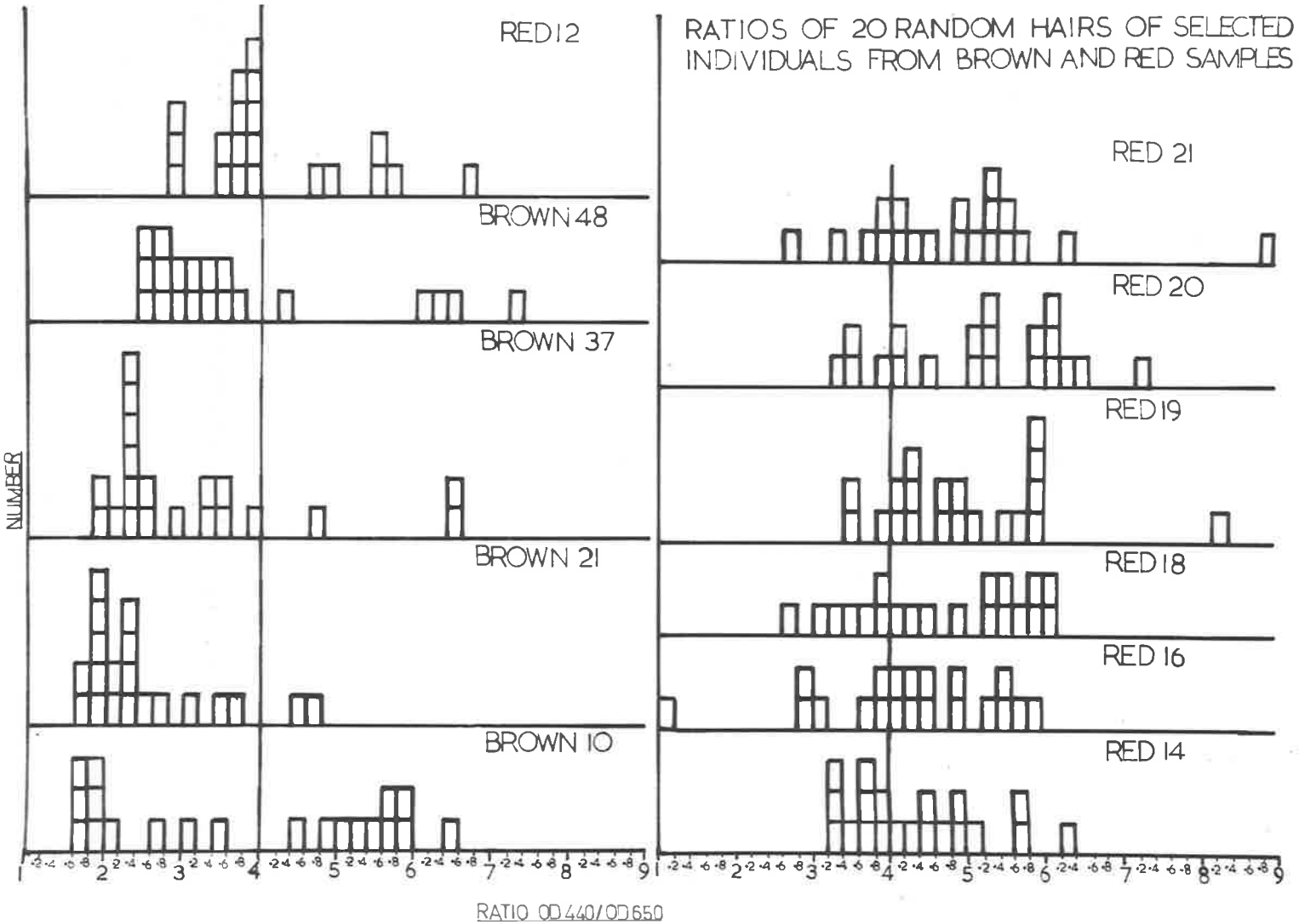
Fig. 65. Relation of freckling to the occurrence of at least one red hair.

The true figure must be higher than this, although possibly not much higher as Michelson (1933) reported 18% in his study. The percentage of red hairs in those samples in which it occurred was not determined. The impression is that most brown haired individuals would have less than 1%, but an occasional subject might have as many as 10% of red hairs.

The occurrence of at least one red hair in brown haired subjects in relation to age is shown in Fig. 64. There may be a small average increase in the frequency of the phenomenon with increasing age but it is not marked. Whether the percentage of red hair increases with age in those who have at least one red hair has not been studied but the impression is that it does. Michelson (1933) claimed that the number of subjects with at least one red hair, and the percentage of red hairs, both increased with increasing age. Fig. 66 shows 4 brown haired subjects who had quite a marked mosaicism, particularly "brown 10". Some of these samples had a high percentage of grey hairs. The ages of the subjects were not known.

Fig. 65 shows the relationship between freckling and the occurrence of at least one red hair. It has been suggested that freckled individuals with brown hair may be heterozygous for a red hair gene, and that people with brown hair and a few red hairs may also be heterozygotes. The two phenomena then should show a high degree of agreement when studied together. There is significant agreement in the data of Fig. 65 but perhaps not of the absolute degree suggested by the hypothesis. If one is not to abandon the

Fig. 66. **Selected individuals from series of 54 brown haired subjects and 21 red haired subjects, showing a high degree of variability of hair colour with some brown hairs and some red hairs.**



hypothesis, then ways will have to be found to explain the discrepancies. It is not intended to try too hard in this respect, but at this point some points might be brought forward. Firstly the clinical assessment of freckling does not deal in absolutes but is only a "clinical assessment" which is necessarily somewhat subjective. The subjects were mental hospital patients and prisoners of all ages, which would be some of the poorest material available for the type of study, and whose greatest merit was their availability. Secondly the microspectrophotometric assessment of hair colour has the same limitations as all biological measurements - experimental variation about the mean. Thirdly is the possibility of variation in colour of individual hairs not mediated by truly genetic causes. Lastly, if one adheres to the hypothesis of somatic mutation causing red hairs in heterozygotes, is the possibility of the double event in a homozygous normal - mutations occurring in both specific genes of a melanocyte as independent events.

The discussion in this section has so far centred on the finding of red hairs in brown haired subjects. This is by no means the only aspect of variability in such subjects. The colours very often range through black, dark brown, brown, pale brown and white (grey), in addition to the occurrence of red in a restricted number of individuals. It was thought that if the variations were due to separate genetic steps one might in a suitable subject find stepwise changes in the graph of hair ratios. With this in mind 176 hairs were examined from one subject (Fig. 67). The result is not conclusive.

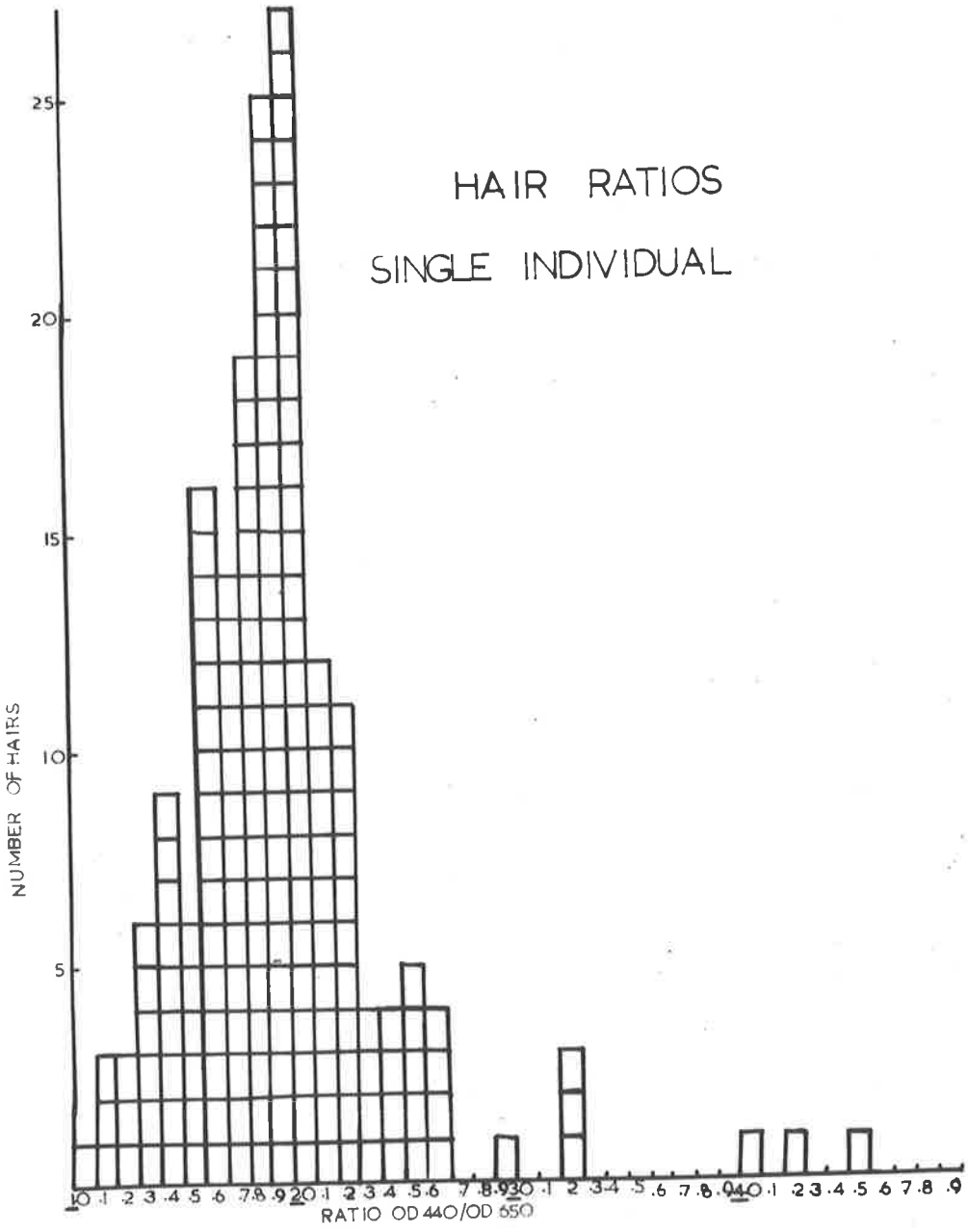


Fig. 67. Hair ratios of a single individual showing black, dark brown, brown and red hairs.

Perhaps several thousand hairs from one subject would show definite peaks of frequency. Alternatively, but perhaps with less validity, a subject such as "Brown 10" in Fig. 66 might be chosen by inspection and assessed more fully.

(3) Red haired persons.

According to hypothesis a mutation of one of the genes for redness in a small clone of melanocytes within a hair follicle of a red haired individual would produce a brown hair. This would render the hair melanocytes effectively heterozygous for the red hair gene. The number of such brown hairs should increase with age. Certainly most red haired individuals have some brown hairs. In the series of 21 red haired subjects used earlier 19 had at least one brown hair in the sample despite the fact that some of the samples were quite small. The two samples without brown hairs came from subjects in their teens. Seven selected samples from this series are shown in Fig 66. The question arises as to whether hairs with ratio less than 4 represent a non genetic biological variation or a true genetic variation. Reference to Fig. 58 which includes these cases shows an approximately normal curve between ratio values of 2.5 and 8.1 for hairs from red haired subjects. The high percentage of brown hairs in red haired subjects therefore may have limited significance, at least in a mathematical sense. To examine this situation further one might work out a mean and standard error value for a series of ratios on a hair sample and then test the significance of a deviation from the mean values in

Fig. 68. Pigmented birthmark (outlined by outer circle) and grey hairs (outlined by inner circle).

Fig. 69. Birthmark (outlined by outer circle) with hairs (outlined by inner circle) which are browner than those on the surrounding skin.

selected brown hairs, or alternatively examine a large enough sample to show that the distribution of ratios has more than one mode.

E. The greying of hair.

This subject is of some general and theoretical interest. Whether willingly or not one is committed in this thesis to suggest that the greying of hair comes about by a process of somatic mutation. It might immediately be said against this idea that this would imply a very high rate of mutation, particularly in people who become grey relatively early. However there may be a number of genes each of which individually might produce greyness if a mutation occurred. Those people who are heterozygous for albinism might develop grey hairs if the remaining functional gene ceased to produce the appropriate enzyme. There are possibilities of dominant genes with an effect on pigment production being produced by mutation. There may be factors, genetically controlled, determining the ability of the melanocyte to function normally within the hair bulb. Silvers (1961) studied the effect of the hair bulb environment on the melanocyte.

No systematic study of greying of hair has been made but one interesting observation is worthy of record. An individual with some red hairs with an otherwise dark brown hair colour was found to have a birthmark. The hairs on a central area of the birthmark were similar to those on surrounding skin, except that many of them were white (grey).

If one supposed this individual to be heterozygous for the red hair character, and that the birthmark represented a somatic mutation of the single gene for red hair, then the melanocytes in the hairs of this central area have lost their capacity to produce melanin much sooner than those on surrounding skin, and a single gene change has in this case been responsible for a greatly increased rate of greying. In this case the gene involved might be responsible for the supply of a pigment precursor, and it may be that a failure of supply of pigment precursors could be responsible for some grey hairs at least.

The fact that only the central area of the birthmark is affected may be evidence of "pigment spread". The central hairs only would have the genetic character of the original birthmark which in the years since the original change occurred has been gradually getting bigger relative to the rest of the body surface. Fig. 69 demonstrates the same possibility of pigment spread, in this case with no grey hairs, in a schoolgirl.

Chapter VIII - Somatic Variability in a Number of Inherited Diseases in Man.

A. Introduction.

The thesis so far has considered somatic variability in the pigmentary system of man and animals. The biochemistry of red hair pigments has been considered because of the relation of freckling to red hair. A technique of microspectrophotometry has been developed for the purpose of studying hair colour variability. It has been shown that freckled people have more moles and more birthmarks than normal, and this has been related to a simple theory of inheritance, and on genetic and biochemical grounds the thesis is advanced that these phenomena are due to somatic mutation.

This chapter concerns twenty or more conditions in which somatic mutation may play a part. The "Statement of the Thesis" suggests that, if tumour formation bears any relation to somatic mutation, then the process of multiple tumorigenesis may be found particularly in people bearing a defective gene expressing itself as a dominant gene. Most of the conditions listed here are dominant conditions in which multiple tumours occur. A few show somatic variability without tumour formation. Two are recessive conditions in which there is evidence of some mechanism greatly speeding up the harmful effects of ultraviolet light on the skin. These are the only two conditions in which supposed mutations are completely generalized as to cell type, and the only cells affected are those near the surface of the skin.

Fig. 70. Presumed neurofibromatosis, showing early facial freckling and a cafe au lait patch on the R forehead near the hairline. This child's mother had neurofibromatosis, and the child had other cafe au lait patches.

B. Neurofibromatosis.

The first and best known of the conditions in which multiple tumours and dominant heredity are combined is multiple neurofibromatosis, which occurs in the community with a frequency of about one case in 2,500 to 3,000 people.

There are two distinct and major groups of changes found in this condition. The first group of changes are pigmentary, the second tumorous in the form of neurofibromata. The association between these two distinctly different phenomena can be explained by the fact that melanocytes and peripheral nerves both owe their origin to the neural crest in embryology.

Pigmentary phenomena.

The pigmentary phenomena include café au lait patches which are indistinguishable clinically and histologically from the normal pigmented birthmark (Crowe, Schull & Neel, 1956), except that whereas very few normal people have more than three pigmented birthmarks, Crowe et al found that 78% of people diagnosed as having neurofibromatosis had more than 6 café au lait patches, in which the broadest diameter equalled or exceeded 1.5 cms. The characteristic orientation of these café au lait patches has already been shown (Figs. 11 & 12). These were derived from personal cases and photographs of the condition which abound in the literature. The general orientation of these lesions are the same as that of the pigmented lesions which occur in normal people.

Freckling also occurs in multiple neurofibromatosis and was seen

Fig. 71.

Multiple neurofibromatosis showing neurofibromata, freckles and cafe au lait patches.

From Ormsby and Montgomery, "Diseases of the Skin."

Fig. 72.

Neurofibromatosis showing multiple neurofibromata.

by Crowe et al (1956) in 30% of their 223 cases. The freckles in adults are usually only found in covered areas - axillae, buttocks, perinea. The same process of blending of freckles into a more uniform darker pigmentation occurs sooner in this disorder than in other types of freckling. The picture of a three year old girl (Fig. 70), who by the rule put forward by Crowe et al (1956) regarding numbers of birthmarks, has inherited neurofibromatosis from her mother, shows a café au lait patch on the right forehead and early freckling.

Cockayne (1933) reported that there may be numerous pigmented naevi, and in Crowe et al (1956) there are pictures of individuals with "bathing trunk naevi" extending over a large area of body surface which are indistinguishable from cases of giant naevi pictured in the older dermatological textbooks. On page 206 of Volume II of the British Encyclopaedia of Medical Practice there is a picture entitled "Soft Naevi" (Fig. 13) and on page 284 of the same volume a picture of a clinically identical condition captioned "Large Mole, bathing drawers type". (Fig. 73). Both of these are clinically identical with cases pictured by Crowe et al (1956). It seems possible that the occurrence of giant naevi is perhaps more often associated with an underlying neurofibromatosis than had been earlier observed.

Neurofibromata and tumours of the nervous system.

The neurofibromata in this condition are usually small sessile or pedunculated bodies which may be present in quite small or very

Fig. 73.

Photograph from The British Encyclopaedia of Medical Practice, Vol 11, p. 284 captioned "Large Mole, bathing drawers type". The evidence of tumorous masses around the buttocks is similar to that found in Neurofibromatosis and it is suggested that this may be the underlying pathogenesis in this case.

Fig. 74.

Large plexiform neuroma in a case of neurofibromatosis

From Kennedy (1959)

large numbers, 9,242 tumours having been counted on one person (Sheklakov (1950)). The tumours are composed of strands of loose wavy fibrils, amongst which are found many pale staining oval or spindle shaped nuclei. Mucoid degeneration of the collagen in these tumours may occur. Sometimes the tumours occur along peripheral nerves in which case they can be felt under the skin. Palmar and plantar neurofibromas are unusual, Crowe et al (1956) having found 6 cases amongst 168 cases, with a suggestion of a familial concentration for this character.

A number of tumour types have been reported in association with neurofibromatosis. Crowe et al reported sarcomatous degeneration of neurofibromata in 4 of 168 patients. One patient developed two separate spindle celled sarcomata. In the same series 10 persons had acoustic nerve tumours, two had retrobulbar neurofibromata, one had a glioma of the optic nerve, three had spongioblastoma polare, two developed meningiomata, one an astrocytoma, and one an unspecified brain tumour. One patient developed a phaeochromocytoma. Another feature of the condition seen by these authors was interosseous cysts but it is not stated whether these can be related to any neoplastic phenomenon.

Crowe et al make other interesting observations on four cases in which they concluded that there was sectorial but not general disease present. They suggest the "possibility of somatic mutation giving rise to a sector of any size differing in genetic potentialities from the rest of the body". This suggestion that a mutant gene

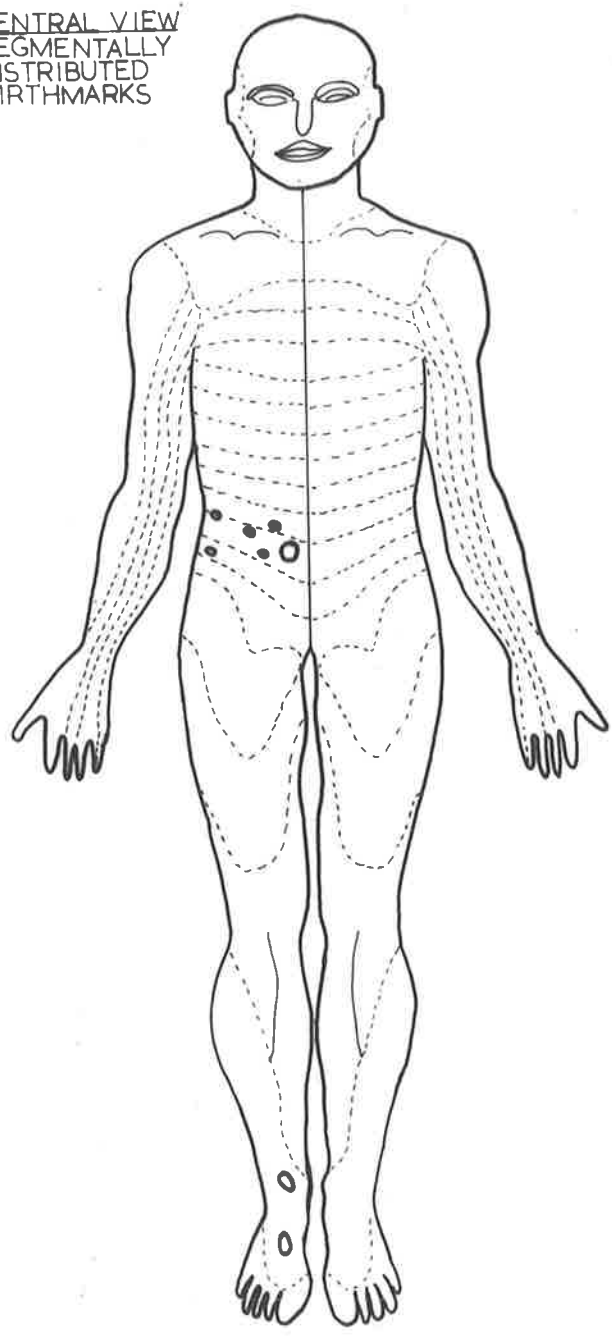


Fig. 75. Segmentally distributed birthmarks on a schoolgirl thought to be due to sectorial neurofibromatosis.

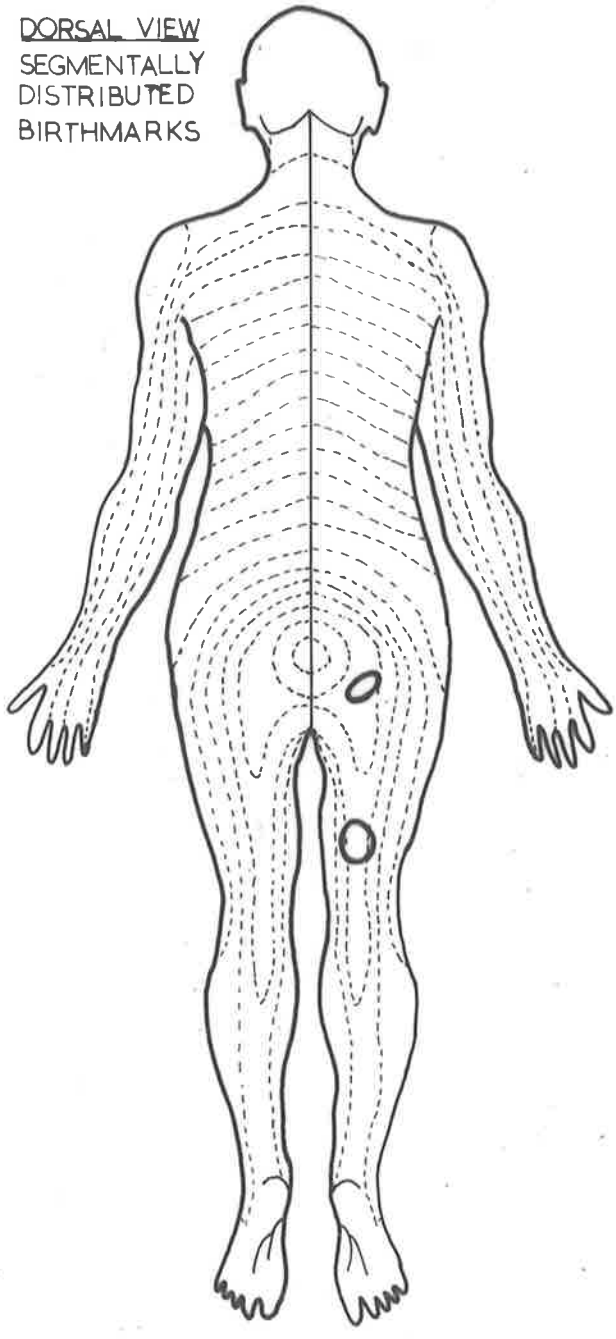


Fig. 76. Segmentally distributed birthmarks on a schoolgirl thought to be due to sectorial neurofibromatosis.

may be acting in a small area is the same in principle as is suggested for this thesis; the difference is that these authors are suggesting a primary mutation occurring in one of two normal alleles in an otherwise normal individual, whereas the suggestion which one would make in the case of the person who inherits one gene for neurofibromatosis, is that the lesions seen are the result of mutation in the remaining normal gene at the same locus. In fact in the case of sectorials one would suggest "two stage" mutations, the first somatic mutation occurring during some embryonic period in one of the normal alleles, and sub-sequently developing café au lait patches, freckles, or neurofibromata being due to second mutations occurring subsequently. During the survey of school children one 9 year old girl was seen considered to be a case of sectorial neurofibromatosis. On an area involving the right side of the abdomen and the right leg (11th thoracic segment to 3rd sacral) there were 5 café au lait patches in excess of 1.5 cm. in one dimension, and none elsewhere on her body (Figs. 75, 76 & 77). There were smaller café au lait patches in the same area and freckling, but none elsewhere. No other member of the family had any evidence of neurofibromatosis. On the basis of the work of Crowe et al the chance of such a number of café au lait patches in the absence of neurofibromatosis is negligible, and from my own data the chance of such a well defined area of altered pigmentation in any "normal" condition would be one in many thousands. If this is a segmental neurofibromatosis it occupies about 10% of the surface area of one out of 1,500 school-

Fig. 77.

Probable segmental neurofibromatosis. The same case as depicted in Figs 75 and 76.

Fig. 78.

Epiloia. Many small papules with characteristic distribution. Note plaque on forehead.

Fig. 79.

Epiloia. Many small, some large, papules with Coalescence of some into warty masses.

children, which would give a mutation rate, assuming that this child was average in size, of one in 30,000 per gene. It would seem likely that the majority of such areas of abnormal pigmentation would be missed unless they were quite large; assuming a number of smaller areas totalling at least as much if not two or three times as much in area as this one, one is left with a possible frequency of such mutations of the order of 1 in 10,000, which approximates reasonably well with suggested rates of germinal mutation. Calculations of this sort of course leave much to be desired but at least one can say that the occurrence of such a case does not deny the validity of a somatic mutation hypothesis, and might at some time provide the basis of a more precise quantitative approach to such problems.

Crowe et al (1956) make one other interesting observation in comparing numbers of café au lait patches between sibs and in parent-child comparisons. The "r" value of sib-sib correlations was 0.642 and of parent-child 0.194. They found the latter value "disconcerting" as coefficients of relationship between sib and sib and parent and child should be the same in genetically determined disorders. One would suggest that there is one genetic situation that these data could fit - that is if the number of café au lait patches was determined by mutations in the allele of the affected gene and if there were a number of such alleles each with different mutation rates. The allele then is inherited from the unaffected parent; there are two such alleles for each normal parent, and a correlation between sibs of the order seen is reasonable, with a lesser correlation between

Fig. 80.

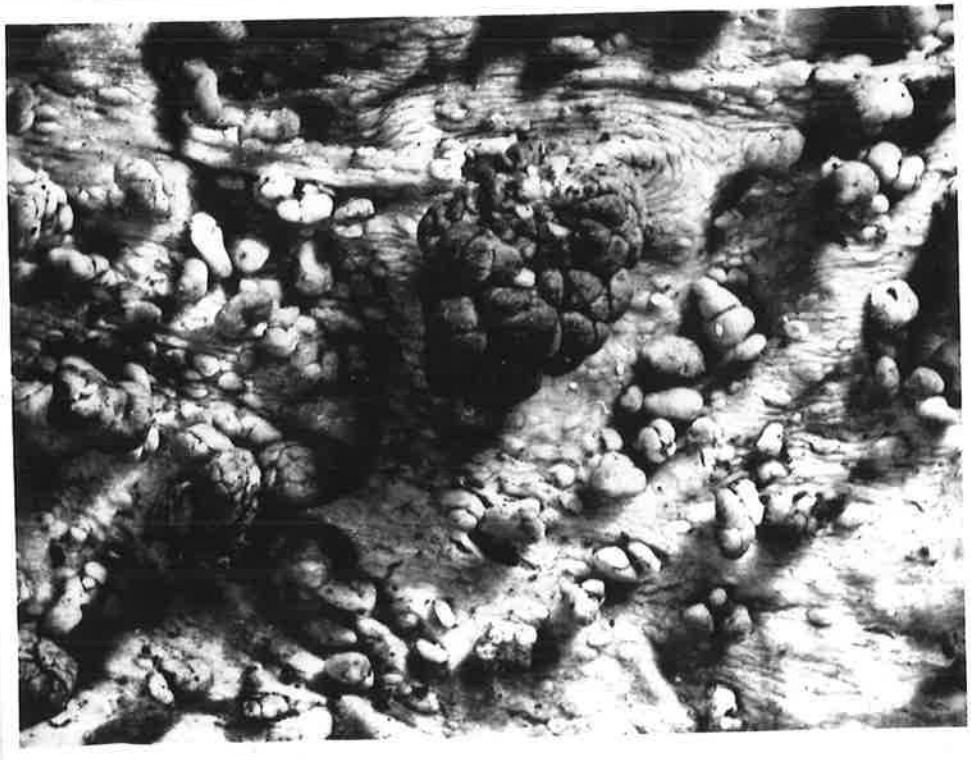
Epiloia, showing the distribution of early lesions in the "butterfly" area of the face.

From Butterworth & Strean (1962)

Fig. 81.

Multiple polyposis coli.

From Dukes (1952)



the affected parent and his or her children.

C. Epiloia. (Tuberous Sclerosis, Pringle's disease).

The most characteristic features of epiloia are the triad of abnormalities - mental deficiency, adenoma sebaceum, and epilepsy. The condition is similar in many ways to neurofibromatosis, has dominant inheritance although there are many sporadic cases, and is a more lethal condition with fewer individuals able to reproduce, and a much lower incidence estimated at 1 in 50,000 in the population.

The number and variety of benign lesions which can be associated with this condition is considerable and one can at best give them only brief mention. Butterworth and Strean (1962) state that the facial lesions of adenoma sebaceum appear during the first decade of life, appearing first as small red papules and later developing a warty or nodular character (Figs. 78, 79). Plaques may occur on various parts of the body but characteristically on the forehead and other parts of the face. The plaques and the nodules are essentially the same histologically. Hyperplasia of hair follicles is stated to be the fundamental change whereas sebaceous hyperplasia is infrequent. Telangiectases may be present. The histology of the lesions varies according to the clinical appearance.

Other lesions which can develop are the gliotic tubera of the brain, phacomata which are flat mulberry like retinal tumours, fibrous hyperplasias of the skin, subungual and periungual fibromas, fibrous nodules of the gingiva, palate and other parts of the mouth,

fibromas of the scalp, large numbers of tiny flesh coloured miliary fibrous tubercles which can occur either individually or as a patch on the trunk, lipomas, fibromyolipomas, angiofibrolipomas, syringocystadenomas, and various pigmented and vascular naevi. Disturbances of pigmentation include café au lait patches, leucodermatous patches, and very numerous "pale" freckles. Renal tumours or cysts can occur in as many as 80% of cases, there is an increased incidence of brain tumours, and tumours of liver, adrenal, thyroid, ovaries, lungs and rarely the gastro-intestinal tract can occur. Willis (1965) mentions rhabdomyomas of the heart, and cystic and other changes in the lungs. Eadie, Tyrer, Tod and Sutherland (1962) discuss a number of features and mention the punctate erythematous macules of the face.

D. Nervous system tumours.

These are included here because they may in individual cases be related to neurofibromatosis. Parkinson & Hall (1962) and Metzel (1964) discuss familial oligodendroglioma, Joynt & Perret (1965) mention meningioma in mother and daughter; Willich & Buschmann (1964) discuss a case where a man had ganglioneuroma and two children by different marriages each had neuroblastoma; Cimino (1965) discusses the case of a man and his sister each of whom had a paraganglioma of the ear - later the man developed the same lesion in the opposite ear.

E. Retinoblastoma

In this condition malignant tumours histologically derived from primitive retinal cells occur in the retina, and where individuals have lived long enough to reproduce dominant inheritance has been shown. Klein (1962) states that the dominant gene has about 80% penetrance; 60% of cases are bilateral and the remainder unilateral; only 4% of cases are familial. It occurs in about 1 in 20,000 to 1 in 30,000 births and estimates of the mutation rate are of the order of 2×10^{-5}. He states that there is a non hereditary form which may be a "phenocopy" or "somatic mutation".

Drews (1962) reports cases in a pedigree where there was transmission of the disorder through unaffected members of the family. Watillon, Weekers, Mairiaux and Joachim (1964) report five members of a family affected in three generations, with one affected member who recovered spontaneously without treatment. Gorer (1958) states that the tendency to regression in retinoblastousa is greater than in any other tumour, and that cases seldom occur beyond the age of 10 years. The tumour leaves a scar on the retina after regression, and these scars may be found in suspected carriers of the gene. He also suggests that sporadic occurrence of the disease may be due to somatic mutation.

F. Familial endocrineadenomatosis (multiple endocrine adenomatosis, M.E.A.).

This syndrome has only been recognized in recent years but there

have been many reports in recent literature. The syndrome in the broadest form can include multiple lesions of any endocrine organ. It appears that the much more specific Zollinger Ellison syndrome can be part of the syndrome. In this latter the features are fulminating peptic ulceration, gastric hyperacidity, and non-insulin secreting islet cell adenomas of the pancreas.

The inheritance is dominant with high penetrance for some feature or other, or perhaps a number simultaneously. Some of the reports will be mentioned to show the general pattern of the syndrome. Cushman (1962) described a patient with phaeochromocytomas, thyroid carcinoma, and parathyroid adenoma. Mieher, Hartsock, Geokas, Ballard & Frame (1962) who described a case of Zollinger Ellison syndrome stated that the patient had a sister with tumours of the pituitary, parathyroids, pancreas and adrenals, and that 8 other members of the family had been effected. Manning, Molnar, Black, Priestly and Woolner (1963) described bilateral phaeochromocytomas, thyroid carcinoma and parathyroid tumours in a patient. Underwood & Jacobs (1963) described a man with hyperinsulinism from islet cell tumours, bronchial carcinoma, and two parathyroid adenomas. He had affected relatives. Ellison & Wilson (1964) reviewing work on the Zollinger Ellison syndrome said that the cause was a gastrin like secretion from a non-B islet cell tumour; 260 cases had been seen of which 8, or 3%, had had familial endocrine adenomatosis. Ballard, Frame & Hartsock (1964) give a general review of some of the lesions possible - chief cell hyperplasia of the parathyroid glands, chromophobe

adenoma of the pituitary, tumours of pancreatic islets which may be benign or malignant, and may affect α or β cells, tumours of the adrenal cortex, phaeochromocytoma, thyroid carcinoma, bronchial carcinoids, and multiple lipomas. They agree that the condition is inherited as a dominant trait.

G. Phaeochromocytoma.

The dominant inheritance of phaeochromocytoma as a separate entity is not so clearly acknowledged. It occurs as a part of the pattern of neurofibromatosis and also of multiple endocrine adenomatosis, both of which are dominant conditions. However there are numerous reports of phaeochromocytomas occurring in various members of a family without evidence of other conditions being present. Von Doepp (1962) found 7 cases amongst 17 family members in three generations. Tisherman, Gregg & Danowski (1962) found it in 7 members of a sibship numbering 199. Von Hagen & Barrows (1963) found familial phaeochromocytoma in association with ependymoma of the spinal cord but in the absence of other signs of neurofibromatosis. Nourok (1964) reported a mother and daughter with the condition, and DeGraeff & Herak (1964) reported a family in which 6 individuals were affected with phaeochromocytoma, with another 10 individuals suspected. They stated that many tumours are unilateral but some may be bilateral.

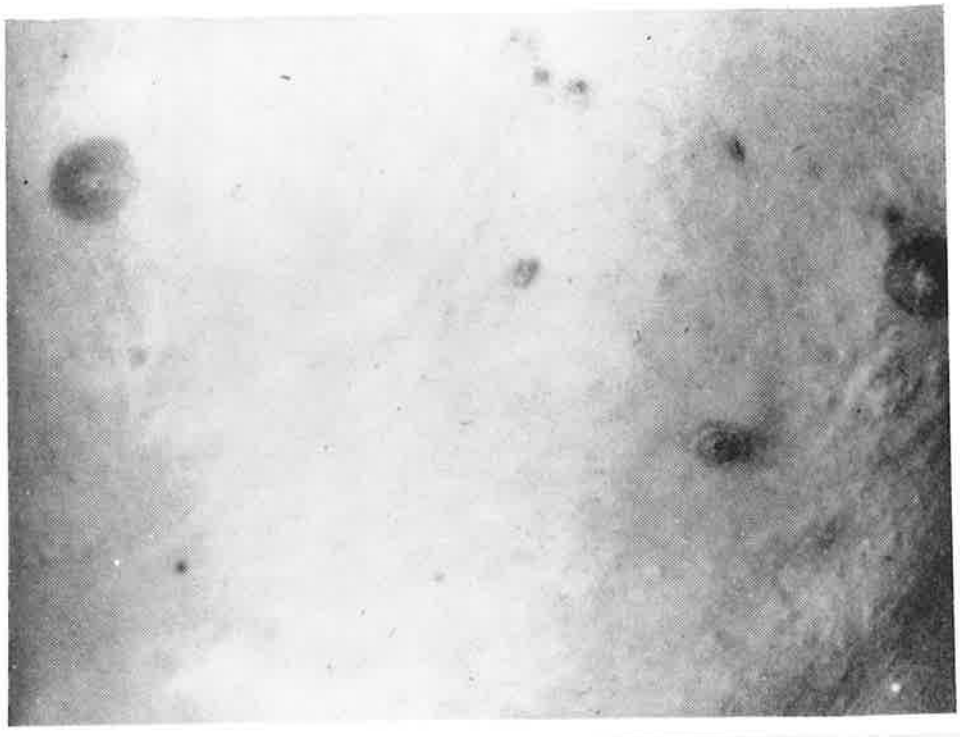


Fig. 82.
Gardner's Syndrome. Multiple Epidermoid Cysts.
Butterworth & Strean (1962)

Fig. 83.
Peutz Jeghers Syndrome, showing distribution of pigmented spots.
From Marshall (1960) "Diseases of the Skin."

H. Familial polyposis coli.

This dominant condition is characterized by gradually increasing numbers of polypi (Fig. 81) developing particularly in the large bowel and manifesting itself in those bearing the gene between the ages of 20 and 40. The tumours are simple pedunculated masses of epithelial hyperplasia and have one serious implication - one or more invariably becomes malignant and typical carcinoma of the bowel results. Colectomy at an early age can prevent this complication. There has been controversy over cytoplasmic particles seen in affected cells, but McKusick (1962) says that these are not thought to be virus particles as at first suggested. A number of families have been reported in recent years eg. Lopez (1963) and Knapp (1965).

I. Gardner's Syndrome.

This syndrome is associated with a number of diverse lesions, some of which are frequent, some infrequent. Cramer (1962) describes polyposis coli which is at first benign and later becomes malignant, osteomatosis occurring in membrane bones and in particular the mandible, mesenchymal cell proliferation giving rise to desmoid tumours, and subcutaneous cysts and masses. Gordon, Rast & Whelan (1962) say that fibrous tissue tumours can include desmoid tumours, fibromas, and leiomyomas, and they describe a case with fibromas of the small bowel mesentery. Gardner in 1962 reported on two cases developing fibrosarcoma. Siguier, Godeau, Levy, Salamon

& Thenot (1963) gave a report of familial incidence and of one individual with a fibrous tumour of the pancreas. Huyghe, Catry & DeGroote (1965) described a case with benign adrenal adenoma. Apart from these there have been other reports stressing familial occurrence and dominant inheritance (Savage (1964), Govaerts & Govaerts, (1964), Verduron, Potet, Nora, Got, Rougeuille & Lambling (1965)).

J. Turcot's Syndrome.

In this dominantly inherited syndrome tumours of the brain are associated with polyposis coli, which does not differ from other types (McKusick, 1962).

K. Peutz Jeghers Syndrome.

This is the last clearly defined syndrome in the polyposis group. There are two distinctive features. The first is the pigmentary spots which can develop particularly circumorally but also on the face with similar distribution to that of normal freckling; pigment spots also occur on the fingers, backs of the hands and on the feet and lower legs. The second is the polyps which can occur throughout the gastrointestinal tract but are usually confined to the small intestine and seldom occur in the colon. The tumours are globular in shape, have an acinar arrangement of the epithelial cells, and cystic dilatation of the glands. There is an abrupt transition from normal epithelium to that of the

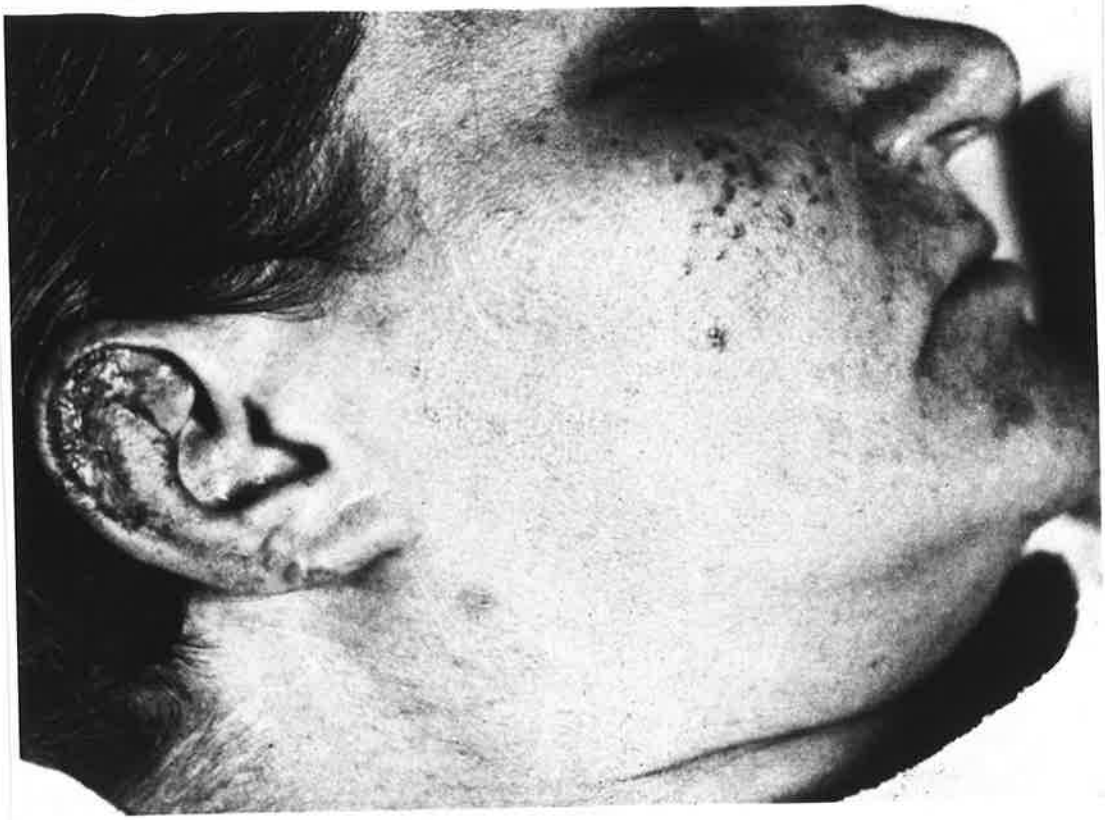


Fig. 84. Osler's Disease - note distribution of lesions on butterfly area of face, and ear. From Bean (1958).

Fig. 85. Large capillary angioma with segmental distribution. Do the three cavernous angiomata represent further steps of somatic mutation ? From Roxburgh (1939).

polyp (Burdick, Prior & Scanlon, 1963). Malignant change is rare although Horn, Payne & Fine (1963) report a case terminating fatally with a carcinoma at the gastroduodenal junction. Willis (1965) states that "the glandular tissue in the polypi of this disease is often so prolific and sometimes so irregular in parts that the pathologist often entertains a suspicion of malignant change". Christian, McLoughlin, Cathcart & Eisenberg (1964) record three cases which had an associated granulosa theca cell tumour of the ovary.

Dominant inheritance has been accepted without question and there are numerous reports of familial occurrence e.g. Loeb (1962), Sheward (1962), Fernando (1964), Lips (1965).

L. Osler's Disease.

This is the most common and perhaps the best understood hereditary condition in which vascular abnormalities occur. Some doubt exists as to whether the angiomas of Osler's disease should be included amongst hereditary tumours. Certain of the lesions might be classed as tumorous, but in the most typical the lesion is a deficiency of tissue rather than an overdevelopment of any cell type.

Baumgarten (1963) describes the two types of lesions seen on the skin - elevated red purple plaques, or alternatively vascular spiders which can appear on the skin of face hands and feet, or in fact almost any part of the body. Histologically the lesions are thin walled sinuses with a marked absence of muscular and elastic tissue. Butterworth and Strean (1962) state that the vascular

lesions begin to appear in the first years of life and become more frequent and more obvious in the third and fourth decades of life.

Other lesions which occur include arteriovenous aneurysm especially of the lung, (Hodgson & Kaye, 1963) gastrointestinal haemorrhages, haemangiona of the tongue, and aneurysm of the aortic arch (Muggia 1964), and arteriovenous malformation in the central nervous system. (Boczko, 1964)

Dominant heredity has been supported by numerous reports including Croizat, Revol, Robert & Manipoud (1963) who reported the condition in 6 generations of a family, and suggested that there was almost 100% penetrance. They say that the homozygote dies in utero or in early infancy. Harrison (1964) reported 3 pedigrees.

Tunte (1963) estimated the mutation rate of the gene and arrived at a figure of the order of 2×10^{-6}.

M. Sturge Weber Disease

This disease along with Lindau's disease has been credited with dominant heredity, and each involves an angiomatosis of specific anatomical areas. The generalized nature of the lesions which is found in many of the other conditions discussed in this chapter is not so obvious although reports suggest that some cases have a greater diversity of lesions than was originally defined in the original descriptions of these disorders. These conditions are comparatively rare.

In Sturge Weber disease the classic picture is one of "homolateral angiomatosis of the skin, choroid and pia mater and

manifested clinically by convulsions, paralysis, mental retardation and visual disturbances" (Butterworth and Strean, 1962). Givner, Roisin, Berman & Gold (1957) however in a post mortem on a case found, as well as the classic features, angiomatous formations in the lungs, thymus, spleen, testes, lymph nodes and pituitary glands. Butterworth and Strean (1962) state that "perhaps the disease is more widespread than formerly thought".

N. Lindau's Disease.

The classic lesions here are angiomas of the retinae and cerebellum, with autosomal dominant inheritance. Melmon & Rosin (1964), Joe & Spencer (1964) and Langsley, Wolton & Goodman (1964) each published extensive investigations of familial occurrences. Regarding a greater generalization of the lesions Butterworth and Strean (1962) state that the haemangiomata may be situated in the medulla oblongata or the spinal cord; hypernephroma, and cysts of the liver, pancreas, kidneys, lungs, and epididymus may be found. Phaeochromocytoma and facial angioma have also been found in a few cases.

O. Glomangioma (multiple glomus tumours)

This condition which is very rare completes the range of more clearly defined vascular abnormalities. A glomus tumour is "composed of convoluted vascular channels resulting from benign hypertrophy of special organs of arteriovenous anastomosis, the

Fig. 86. Keratoacanthoma. From Butterworth & Strean (1962).

Fig. 87. Trichoepithelioma.

Sucquet-Hoyer canals, located in the subpapillary layer of the skin". (Butterworth and Strean, 1962).

The tumours occur in the early years of life and tend to be found on extremities, up to 90 having been reported on one individual. Autosomal dominance has been found in the few familial cases reported. Schnyder (1965) reported 8 cases in 2 generations of a family. Willis (1965) states that most tumours are represented by solitary tumours but many are multiple and familial.

P. Keratoacanthoma

Butterworth and Strean (1962) describe this condition as a "pseudoepitheliomatous neoplasm characterized by rapid and benign growth and spontaneous involution with scarring". It can occur in a generalized form and the lesions have a predilection for exposed parts of the body (Fig. 86). The generalized form has been shown to be familial in many cases. There is also a localized form in which individual or very small numbers of lesions occur in localized areas, and this is never hereditary. Randazzo & Giardina (1964) describe the periodic appearance of lesions on the scalp of an 80 year old woman, and these were later spontaneously reabsorbed. They traced 8 affected members in a sibship of 50 over 5 generations, showing autosomal dominant inheritance. Ereaux & Schopflocher (1965) described a man and his brother both affected.

Q. Trichoepithelioma (multiple benign cystic epithelioma)(Fig. 87).

Butterworth and Strean (1962) state that these are "benign

tumours of the basal cell type originating from the surface epidermis or from the hair follicle". Two distinct types of lesion are seen. The Brookes type or epithelioma adenoides cysticum in which nodules 1 mm to 3 cm or more in size are found primarily on the face neck and chest, and the Spiegler type or cylindroma in which nodules from 5 mm to 5 cm in size or larger are located principally on the scalp. Guggenheim (1962) describes 2 families; nine affected in four generations of one family, and 10 in four generations of another. He states that both types may occur in the one individual and that the condition is dominant with a penetrance of 60-80%.

R. Basal cell naevus syndrome(Syndrome of Gorlin & Goltz).

In this syndrome multiple basal cell epitheliomata, cysts of the jaw, and congenital skeletal deformities occur. These latter include most commonly, bifid or anomalous ribs, dorsal scoliosis, and frontal bosses.

Reports give other lesions which have been found in association with this basic pattern. Clendenning, Herdt & Block (1963) described ovarian fibromas and mesenteric cysts, Howell, Anderson & McLendon (1964) in a study of 7 families with 43 affected members described pits of the hands and feet. Gorlin, Vickers, Kelln & Williamson (1965) include medulloblastoma as a frequent feature, and Kragh & Grotting (1965) in describing a familial occurrence quote also the possibility of developing ameloblastosis.

The dominant autosomal inheritance has been shown in a number

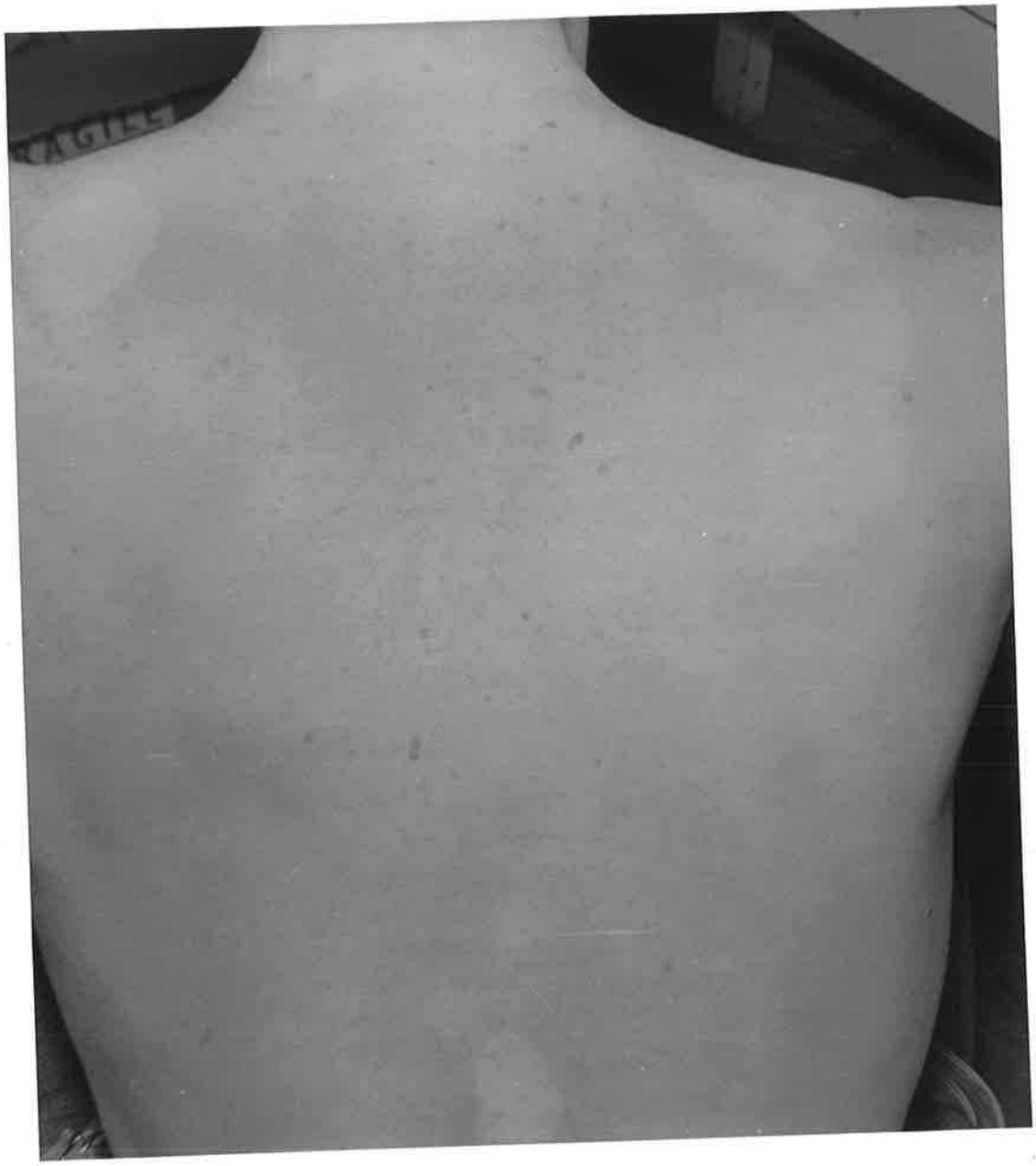


Fig. 88. Leucoderma with pigmented dorsal stripe. Depigmented areas on either shoulder.

of studies eg. Howell et al (1964).

S. Osteomatosis (Hereditary multiple exostoses).

In this dominantly inherited condition multiple exostoses develop particularly at the metaphyses of the long bones. This may be primarily a fault in overproliferating cartilage derived from the metaphysis of the long bone. Bethge (1963) states that the cartilaginous cap of the osteomas may develop chondrosarcoma. Murken (1963) found 15 of 24 patients in families with autosomal dominant transmission in some cases for four generations. He calculated a mutation rate of the order of 7.5×10^{-6}. Schmute & Keim (1964) claim that there are differences between multiple exostoses and multiple enchondromatosis.

T. Leucoderma (Piebaldness).

In this condition a number of depigmentations can occur of different types. There may be a white forelock, white spots liberally occurring over the body surface, large oval depigmented patches, or a white dorsal stripe extending along the back from the sacral region. (Fig. 88). The occurrence of white forelock with depigmented patches represents one type, dorsal stripe with depigmented patches another, and some cases have only the depigmentations. The depigmented patches have a characteristic distribution and orientation similar to that of the pigmented birthmarks already studied. The inheritance in all cases is autosomal dominant. Campbell & Swift (1962) suggest that melanocytes have not been found

Fig. 89. Waardenburg's Syndrome. When first seen this patient had completely confluent eyebrows, part of which he later shaved. He is deaf.

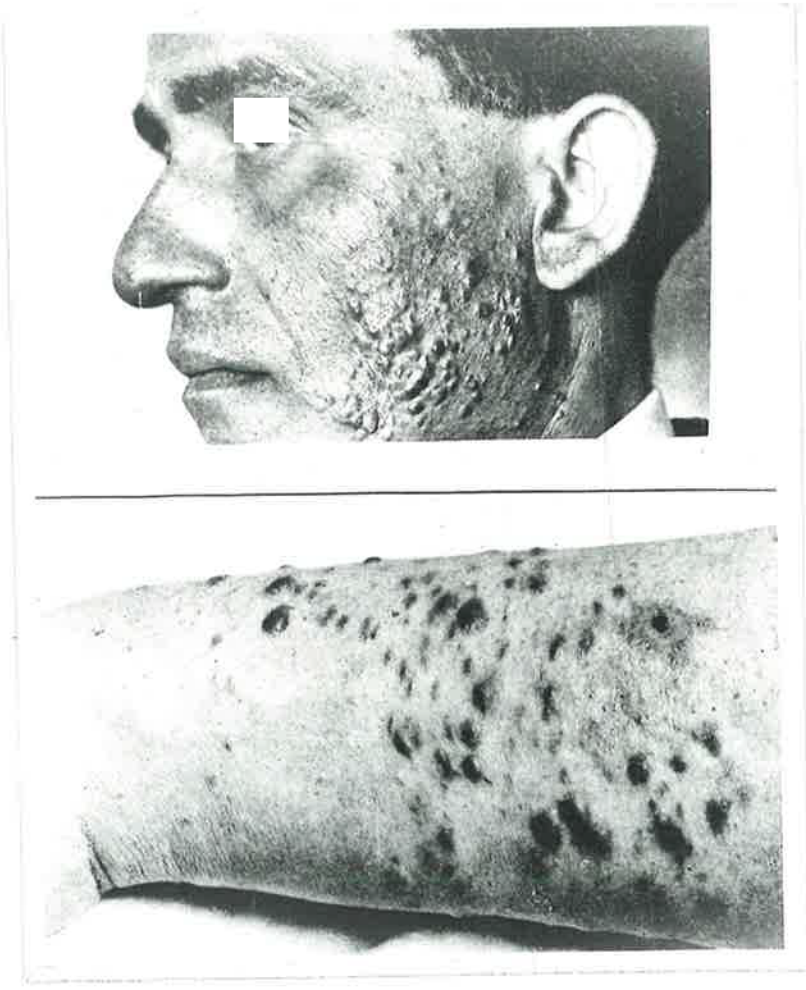


Fig. 90. Multiple leiomyomata.
From Ormsby & Montgomery "Diseases of the Skin".

in the depigmented areas, but Butterworth and Strean (1962) suggest that there is a lack of tyrosinase only. Some inherited depigmentations in animals have absence of melanocytes in the depigmented areas.

This disorder has to be distinguished from an acquired condition of vitiligo in which irregular patches of depigmentation develop during the lifetime of the individual - these patches are often associated with inflammatory redness and hyperpigmentation at the spreading margins.

U. Waardenburg's Syndrome.

An autosomal dominant condition which has been the subject of many reports in recent literature is Waardenburg's syndrome. Ghosh (1963) describes the features of the condition which are present in most cases (1) lateral displacement of the inner canthi (2) high broad nasal root (c) confluence of the eyebrows with hypertrichosis (4) partial or total heterochromia iridis (5) white forelock (6) unilateral or bilateral congenital deafness. All degrees of expression of the various characters are possible and some people with the genes are virtually normal phenotypically. Other interesting features in the present context are that many individuals become prematurely grey (Hansen, Aokaouy & Crump (1965) report on a man who became grey at the age of 20), and there are occasionally depigmented areas on other parts of the body.

Recent family studies have been made by Partsch (1962) Aasved (1962), Dunn (1964) and Rugel & Keates (1965).

Fig. 91. Multiple Lipomatosis. From Sutton (1956).

Fig. 92. Xeroderma Pigmentosum. From Mackey (1955)

Two relatives of the individual pictured (Fig. 89) were reported to have become grey early in life. The mother of this individual (also a bearer of the gene) is firmly convinced that some mental abnormalities express themselves in individuals she knows with the condition. Her son is mentally slow but this could be attributed to his deafness. One individual with the condition was found amongst 500 at the Peat and Milson Island Hospital in which most of the inmates would have IQ less than 50.

V. Multiple Leiomyomata.

This rare condition has been shown to have an autosomal dominant type of inheritance in cases studied. The tumours which are situated in the dermis consist of masses of interlacing spindle shaped smooth muscle fibres, mixed with a stroma of connective tissue. The lesions usually appear in the second decade of life and are distributed over the skin particularly of the face, neck, extensor surfaces of the extremities and the upper parts of the chest and back. Knoth & Knoth-born (1964) report the conditions in two sisters and Mezzadra (1965) reports the occurrence of it in three generations, and states that it may be associated with uterine fibromyomata.

W. Lipomatosis.

Multiple lipomatosis is a dominantly inherited disorder in which many lipomata appear scattered over the body wherever there is fatty tissue. The solitary lipoma which is a reasonably common

occurrence is not hereditary, but in most cases where there are multiple tumours the familial nature of the condition is seen. Rarely a malignant change can occur in lipomata producing liposarcoma.

X. Miscellaneous.

For completeness a few tumorous conditions will be mentioned which have shown familial distribution, but where so few cases have been reported that no final conclusions can be arrived at. It would be wrong just because of the occurrence of such a disorder in parent and child to immediately suggest that this was the result of "dominant inheritance".; it could as well be due to chance, environment or polygenic effects. All the conditions up to this point in this chapter are acceptable as being dominantly inherited, even though many "sporadic" cases occur. The more lethal a dominant condition is of course, the more sporadic cases there must be as so many will be due to new mutation. There comes a point where there are so many sporadic cases that the possible inheritance of the disorder will be obscured.

Recent reports to mention are the following -

Multiple myeloma Thomas (1964), Leongine & Korngold (1964).

Hodgkin's disease Galardeschi & Tortoli (1962), Manigant, Macrez, Chome, Bosson, Delamare & DeParis (1964).

Waldenstrom's Macroglobulinaemia Vannotti (1963).

Reticulum cell Sarcoma Smith (1961), Zachau-Christiansen & Rasmussen (1963).

Sarcoidosis Quinn (1963), Allison (1964), Dahlerup & Dreyer (1964), Jorgenson (1964).

Carotid Body Tumours Katz (1964).

Y. Xeroderma Pigmentosum

This is a most interesting if very unpleasant condition (Fig.92) and is a rare type amongst the group of inherited tumorous conditions - it has clearcut recessive heredity. Butterworth and Strean (1962) state that it is an "inherited disease which appears early in life and is characterized by freckle like pigmentation followed by atrophy of the skin, telangiectases, keratoses, papillomatous growths and malignant tumours". The early signs are an unusual sensitivity to sunlight followed by heavy and progressively increasing freckling. Ocular manifestations include conjunctivitis, extreme photophobia and lacrimation, followed later by blepharitis, symblepharon, ectropion and keratitis. Basal cell epitheliomas, squamous carcinomas, melanomas, sarcomas, endotheliomas, fibromas, peritheliomas, angiomas, angiosarcomas, and keratoacanthoma have all been reported. An increased sensitivity to light and freckling in some normal siblings is explained as being due to partial effect in the heterozygote. The mother and four sibs of two cases in one family seen by the author had none of these features. The mother at least would be heterozygous and probably one or more of the children. The mother and father were second cousins. Other disorders which can be found in sufferers are mental deficiency,

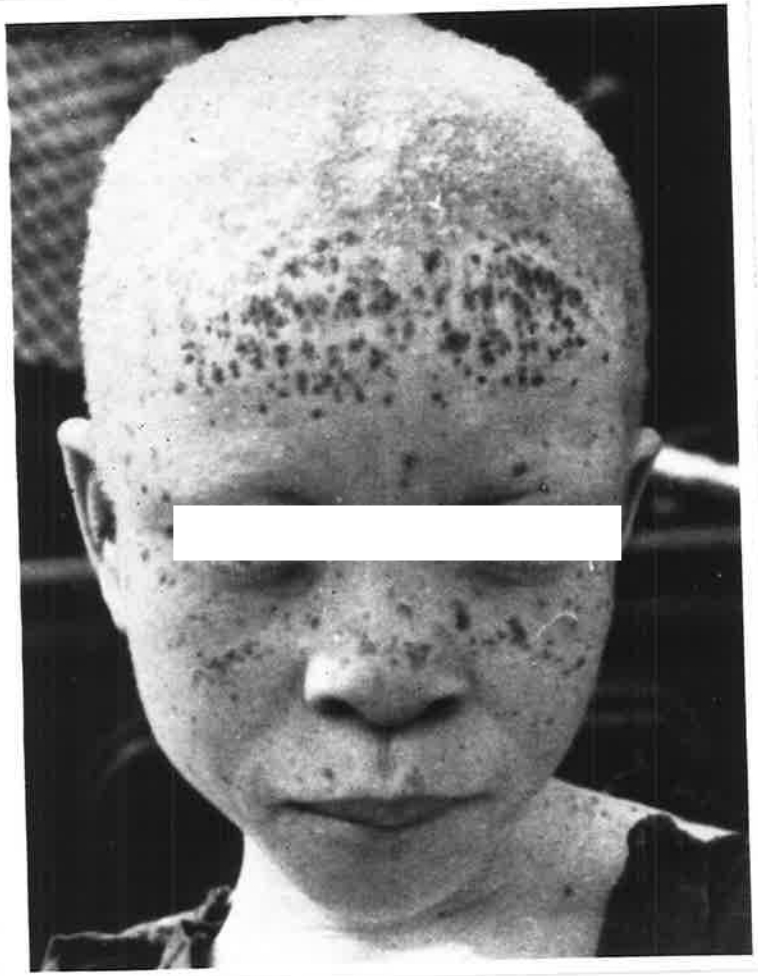


Fig. 93. Freckling in a Nigerian Albino. Note similarity of distribution to that found in normal Freckling. From Barnicot (1952, 1)

Fig. 94. Freckling in Nigerian Albino. Many European children have irregularly star shaped freckles similar to those seen here, and occasionally of the same colour.

speech disturbances, somatic underdevelopment, joint malformation, deafness, epilepsy and neurologic disorders.

Various reports eg. El-Hefnawi & El-Hawary (1963), El-Hefnawi & Rasheed (1964), El-Hefnawi & Mortada (1965), Lemaire & Gaumond (1965) and Reed, May & Nickel (1965) bear out the different aspects of the condition. The pattern of activity is interesting in relation to tumour production generally, and other disorders discussed in this chapter. The lesions include malignancy of every possible cellular type near the surface of the skin. The other conditions discussed, where tumours were produced, had tumours of only one or two types of cells, or cells of a general class such as fibroblast, epidermal cell etc. Whatever the basic force for tumour production might be it was rather specific in each disorder to the particular cell type. In xeroderma pigmentosum there is a force which is completely generalized and one feels that this must be perhaps a rather specific biochemical factor imposing an increased mutability in all cells which may be exposed to sunlight. The protective influence lacking is not a pigmentary one and one can only speculate on what it might be.

Z. Albinism.

The greatest interest in albinos in this thesis is the "freckling" which may develop, but many albinos, particularly in tropical countries, develop malignant tumours with potentially the same diversity of type as in the case of Xeroderma Pigmentosum but we

have a much more specific situation to blame - the lack of pigment to protect the malpighian cells, the melanocytes and the dermal cells. Once again this is a recessive condition, said to be due to absence of tyrosinase, although this may not be invariably the case (Mishima 1965).

The direct access of ultraviolet light to the nuclei of skin cells brings about hyperkeratoses, epitheliomas, and melanotic or amelanotic melanomas. A recent report on amelanotic melanoma in an albino was made by Kennedy & Zelickson (1963).

The most interesting phenomenon in a "somatic mutation" context is the freckling seen in Africans, and other dark skinned races, and which has also been observed much less dramatically in European albinos by this writer. How can a person reputed to be lacking in a gene controlled enzyme, tyrosinase, produce such dramtic pigmented spots as shown in Fig. 93 and 94 (Barnicot 1952,1) unless a mutant change to tyrosinase production has occurred in the skin? Here therefore we have two clearcut situations occurring - the first being a high frequency of somatic change due to the loss of the protection afforded by the skin melanin, and the second a phenomenon best explained in the present state of knowledge as a mutant change in a gene from nonfunctional to a functional condition. The other point worthy of close attention in these photographs is the distribution of the pigmented spots and the irregular star shaped outline of many of them. If one looks back at the photograph, Fig. 5, of the small freckle faced boy, one sees an identical distribution of freckles, and the freckles on the shoulders of the second

case are faithfully reproduced by many Sydney schoolchildren, certainly as to shape and frequency, and occasionally in individual freckles also as to colour.

Chapter IX - Discussion

The concept motivating this thesis is that malignant disease can be explained by sequential somatic mutation. This is not a new idea in principle, and various theories have been put forward, dating initially from the 1920's, using somatic mutation as a basic concept.

The "Statement of the Thesis" in Chapter II outlines in general terms what this writer regards as logical steps in understanding a somatic mutation theory of cancer. The component hypotheses have been made as general and unspecific as possible because the present state of knowledge does not justify anything more dogmatic.

The "Statement of the Thesis" does not indicate what particular pigmentary data are to be discussed, nor what hypotheses specifically relating to pigmentation are to be introduced. Some of these hypotheses may have already had adequate discussion, but some recapitulation is necessary. More important perhaps is to show the relevance of the pigmentary phenomena studied, and of the diseases recorded in Chapter VIII, to the main thesis, and also to extend the ideas to the general phenomena of malignancy.

The occurrence of mutation in somatic cells is, and may remain for a long time, a phenomenon which it is impossible to demonstrate by any direct method. Hence all discussions about it must remain hypothetical; but while this may be the case in a fundamental sense there seems much scope for the study of phenomena which may

be explainable by this concept.

The pigmentary system was chosen for a study of somatic mutation in relation to neoplasia because, as geneticists have found for many years, the major part of this system is available for direct observation. The melanocytes form a monolayer at the dermoepidermal junction, with relatively constant numbers of cells per unit of area, consequently some degree of quantitation of cell populations is possible.

Certain evidence was sought for, based on an analysis of the requirements of the thesis as set out in the "Statement of the Thesis". The list below represents a breakdown of this necessary evidence into definable sections and the headings here will be used to provide the basis for further discussion.

1. Somatic variability. The first requirement in any consideration of somatic mutation is somatic variability.

2. Biochemical basis of pigmentary changes. Some understanding of the biochemical basis of the observed pigmentary changes is necessary.

3. Single gene effects. It must be possible to relate at least some of the phenomena observed to single gene effects.

4. Kind and frequency of changes. The changes must be of such a kind and frequency as reasonably to support the suggestion that they may be due to somatic mutation.

5. Reproductive advantage in supposed mutant cells. There must be evidence of some reproductive advantage in at least some of

the supposed mutant cells to support the suggestion that somatic mutations are both cause and effect of the development of successive clones of cells with increasing rates of multiplication.

6. Progression to malignancy. There must be evidence that in at least a small percentage of cases changes continue to occur, perhaps in step-wise fashion, to the point where frank malignancy arises.

7. Applications. Evidence of the general applicability of the phenomena studied must be brought forward.

8. Ultimate effects and practical significance. The ultimate effects of the application of the principles outlined and the possible practical significance in understanding and therapy must be considered.

1. Somatic variability.

Somatic variability in the human pigmentary system is both regional and local. The regional variation is probably analogous to the marked variations seen in some animal species eg. giraffe and zebra, although not so dramatic as these and with no obvious adaptive significance. Skin and hair both show local variability. In the skin the expression of this variability is in the form of freckles, birthmarks and moles, and in the hair individual hairs can vary greatly in colour from one to another, and this variation appears to increase with age and the phenomenon of greying of hair may be related to this variability.

2. Biochemical basis of pigmentary changes

This aspect has been studied intensively, and much discussion has already been made of the results obtained. There are two reasons why the biochemistry of red pigments is important.

The red hair phenotype in humans is so far the only one which has been related to the effects of a single pair of allelic genes. Somatic variability of human skin pigmentation is closely related to the red hair phenotype, in that most red haired Europeans have more freckles and birthmarks than normal. Breathnach (1964) has shown that the melanosomes of a freckle of a red haired person are like those found in the normal skin of a brown haired person, and quite unlike those found in the unfreckled skin or hair of the red haired subject. He has suggested somatic mutation as the basis of this phenomenon. Other work has suggested the specific chemical identity of the red hair pigment, as being quite distinct from melanin. Thus we have three aspects - genetic identity, somatic variability with specific electromicroscopic changes, and chemical identity which all can be linked to the red hair phenotype.

The second reason for pursuing biochemical studies arose from efforts to give quantitative expression to local variations in hair colour. A technique of microspectrophotometry was developed for studying hair pigments, and distinctions between red and brown pigments were defined by a ratio between optical densities at different wavelengths. The technique however was of general value and blood pigments, caretenoids, free pigments found in marsupial

hair, and the spectral characters of red hair pigments generally were studied by this method, partly to establish the validity of the technique and partly to further the understanding of the red pigments.

The first Chapter on biochemistry of pigments (Chapter V) is devoted to marsupial pigments. Possums and kangaroos secrete colourless pigment precursors onto the skin from enlarged sweat and/or sebaceous glands. Treatment of these secretions with tyrosinase produces red and brown pigments, and the same process occurs on the skin of the animals to produce free pigments amongst and stuck to the hair. The brown pigments found mainly in the possum are insoluble in water and remain firmly adherent to the hair on boiling. The red pigments are very soluble in water and can be studied by spectrophotometry and chromatography.

The bright red pigment found on the breast and neck of the red kangaroo has a principal red band on thin layer chromatography, and several minor bands; an orange yellow pigment on the body of the kangaroo has yellow, orange, red, purple and blue bands, varying in amount between different samples. One of the red bands in this pigment is identical with the principal red band from the neck of the animal. Regularly found on the red kangaroo is also a pigment precursor C which gives rise to a brown pigment with tyrosinase. Precursors A, B and D are also found in small quantities in some samples.

The soluble material from the possum is relatively unpigmented,

but contains variable, often large, amounts of precursors A-D. The precursor A can be separated and found to produce the red pigment as found on the red kangaroo, with tyrosinase.

Chemical reactions of these precursors show that they are aromatic amines, and they resemble dopa in spectral absorption. The reactions with tyrosinase are very similar to that of dopa.

The spectral absorption curves of the free red pigments are the same as those obtained with human and other mammalian red hair and Rhode Island Red feathers, and the presumption that they are in fact identical is furthered by the results of extraction of pigments from hair and feathers, and chromatography of the extracts. These latter pigments are soluble in water although the yield is low from any sample of hair or feathers. Boiling extracts pigment, but with feathers amounts sufficient for chromatography were obtained by soaking in water at room temperature. The chromatographic pattern of pigments obtained with one such extraction was identical to that obtained with the orange yellow pigment of the red kangaroo.

Based on the conclusion that the precursor to the red pigment is an aromatic amine, various appropriate substances have been tested. 3-Amino-tyrosine has been found to be a major source of red pigments with certain types of manipulation, and in fact preliminary evidence shows identity between at least one red marsupial pigment and a pigment produced from 3-Amino-tyrosine.

The biochemical studies have produced other results which should be mentioned here. A series of red and brown hair samples

gave a clear division of mean ratios indicating that brown hair lies in the range 1.3 - 4, and red hair lies in the range 4 - 10, of mean ratios. These were selected brown and red samples - an adequate random sampling of a population would be impossible because of the work involved. A population of 552 individuals was investigated by measuring the ratio of one randomly chosen hair from each sample, and the "reddest" hair from each sample (20% of people have at least one red hair). This latter gave a clearcut bimodality with an antimode at a ratio of 4, and this is taken as an indication of some biological discontinuity in red-brown distinctions, and is supporting evidence for the concept of genetic and biochemical identity of the red hair character.

3. Single gene effects.

Red hair in most past studies has been attributed to recessive inheritance, with often a few cases which would not fit into this pattern. The association of freckling in families where there was red hair has been noted, and a study was carried out, observing red hair, freckling, and other pigmentary details. The conclusion is that red hair arises in an individual who is homozygous for a gene which in heterozygous form produces brown hair and freckling.

Fourteen hundred and fifty six school children were examined, and seventy four family pedigrees prepared. There were several hundred other school children and adults seen under various circumstances and population and family data were prepared. Based on expectations

on the Hardy Weinberg Law the population data in general supported the single locus, two allele hypothesis. Exceptions occurred in two main groups - one group examined at a time when the criteria of "freckling" were not adequately defined and many individuals with quite minor degrees of freckling were being included in the freckled class (practically all individuals have at least a few freckles, and an adequate definition of what is regarded as "freckling" must be made); and a second group of individuals who were nearly all sub-normal mentally and had an exceptionally low frequency of red hair.

The family studies supported the hypothesis if one can ignore a few exceptions. The impression gained was that a few individuals who should have been freckled according to the hypothesis were classifiable as unfreckled, and, if the hypothesis is correct one can attribute this only to illegitimacy, adoption, lack of environmental stimulation, or failure to express freckles because of other genetic attributes. It is possible that some or all of these factors may have been operating.

In the course of the survey of school children observations were made on brown pigmented spots of the iris. These are rarely present at birth and develop in parallel fashion to the development of freckles and moles in the early years of life. Some family pedigrees have suggested dominant inheritance, but while the occurrence of this character is shown in Appendix II in the pedigrees, no analysis has been attempted. Originally a clinical observation was made that people with both freckles and eye spots have more moles

than normal, and the survey of schoolchildren showed that freckling was associated with 60% more moles than the average of the unfreckled, eyespots with 40% more than in those without eyespots, and those with both characters had twice as many moles as those with neither.

As a further study of the effect of genes for red hair a large number of hair samples were investigated for the occurrence of a few red hairs in otherwise non-red specimens. It has been suggested that these individuals may be heterozygous for the red hair character. Many freckled individuals with brown hair (also by hypothesis heterozygotes) had a few red hairs, and the numbers were statistically significant, but there was not an absolute distinction; some with a few red hairs were unfreckled, and some freckled individuals had no detectable red hairs. This discrepancy has already been discussed in Chapter VII.

The relevance of hair colour variability to a somatic mutation hypothesis is that the melanocytes of a hair follicle are a small isolated clone of cells which will ultimately reflect the effects of any somatic mutations which may occur in them. In theory, for example, in a person heterozygous for the red hair character heterozygous melanocytes produce mainly brown pigment (based on the hypothesis for red hair just outlined), but if the normal gene in a melanocyte should mutate to the "red" allele, then the cell becomes homozygous and produces red pigment.

The switch from brown pigment production to red pigment production appears to be a quite well defined step rather than a gradual change from one kind of hair to the other. The percentage

of those people who show this character is reasonably constant, and their hair colour ratios are not significantly different from those who do not show the character. Although the objective evidence has not been obtained in this Thesis (except as related above), there is reason to believe that all changes of colour of individual hairs may be stepwise rather than continuous and that this also includes the phenomenon of greying of hair as a special case.

4. Kind and Frequency of Changes.

The evidence has been presented that red haired individuals are homozygotes for the gene which is heterozygous form causes freckling of brown haired people.

Thus freckling occurs in both heterozygotes and homozygotes by this theory. Some of the pigment in human red hair is soluble in water and red marsupial pigments are completely soluble. It may be that the association of the pigment with keratin in hair is responsible for the difference. The skin of red haired people and frecklers is paler than normal in most cases, and this could be because of formation of red pigment, some of which might not remain in the skin as brown or black melanin does. If a melanocyte switched from red pigment production to brown pigment production and established a clone of a few hundred or more cells the change would be observable as a pigmented spot or freckle. If the change occurred before birth presumably the spot would grow with the individual and a pigmented birthmark would result. If the change was due to mutation one would expect this rate of mutation to be speeded up by exposure

to ultraviolet light.

Two observations are basic to the mutation concept. The hypothesis is that the change in pigment production is due to a mutation in the actual gene under observation, and not due to an increased mutability in other genes of the organism brought about by possession of this gene. A pigmented birthmark in a supposed heterozygote therefore has one originally normal gene and one "freckling" gene which has mutated to normal. This birthmark must differ from surrounding skin in being incapable of freckling. Figure 18 demonstrates this situation. The second observation concerns the type of birthmark in the supposed homozygote, that is in the red haired person. A birthmark in this person according to the theory is due to the mutation of one of two "freckling" or "red hair" genes. The surrounding skin is "homozygous" but the birthmark is "heterozygous". If freckling occurs around this birthmark it will in theory be twice as dense as freckling that can occur on the birthmark. Figures 20 and 21, one believes, demonstrate this situation.

Direct estimates of supposed mutation rates is of no great value on exposed areas as one has a variable environmental factor to contend with. This is not so when freckling occurs on covered parts and an average of two freckles per square centimetre have been observed on the buttocks of a 10 year old freckled child. This would give a mutation rate of the order of 1 per 50,000 for the 10 year period, on the presumption that each freckle resulted from a mutation in one of the approximately 100,000 melanocytes present.

If one was sure that all supposed mutations were detected by this means this would no doubt be the most valid direct method of estimating the mutation rate.

The alternative method of estimating the mutation rate is based on the fraction of body surface covered by pigmented birthmarks in a group of people. Firstly one must say that freckled people have 2 - 3 times as many pigmented birthmarks as the unfreckled and this is in itself a basic fact relating an obviously environmentally modified character, freckling, to one which has no likely environmental modification. Unfreckled people do have a small number of birthmarks and allowance has been made for these in the estimate of the rate of change from normal skin to the skin of the birthmark. The estimates based on two series of schoolchildren give a result of about one mutation in 10,000 cell divisions for one gene. The phenomenon of "pigment spread" would account for the birthmarks being bigger proportionally than they were at the time of formation, so that this estimate is likely to be too high and the true value might be a little smaller or several times smaller than this.

5. Reproductive advantage in supposed mutant cells.

The phenomenon of "pigment spread" gives a theoretical basis for assuming an increased rate of multiplication of a cell producing a darker pigment, relative to a cell producing a lesser kind or amount of pigment. Melanocyte populations can be accounted quite accurately and hence the changes observed can where necessary be given a quantitative expression. Breathnach (1958) observed the

growth of freckles from minute spots, and the suggestion has been made that this growth is the same as the "pigment spread" phenomenon. One sees in any freckled area many freckles of pinpoint size and many which may be quite large, ½ to 1 cm in diameter. The biggest may be irregularly star shaped in outline with evidence of growth from the centre more rapid in some directions than in others. Logically it is hard to believe that a freckle may not have arisen in the first place from a single cell. Birthmarks in which some evidence of pigment spread is visible have also been seen, but the human body hairs do not make possible the clearcut demonstration of the phenomenon as was first observed in animals. There is observational evidence that those who produce very distinctive freckles in colour in relation to the surrounding skin also have the largest freckles, and one would believe that the pigment spread phenomenon is an effect of a pigmentary gradient - if there is not this sharp distinction the freckle grows very slowly and may remain little more than pin point in size. This may account for the often very small size of freckles in the very dark skinned and the very fair skinned.

An observation that deserves note is the multicoloured freckles in some very freckled schoolchildren. The colours clinically belong to well defined classes of pale brown, light brown, brown, dark chocolate brown and black freckles. All five classes may be seen on some very heavily freckled children, but the frequency is in inverse relation to the depth of pigmentation of the freckle. Such

a child may have 10,000 visible pale brown freckles, a few hundred light brown freckles, 100 or so brown freckles, 30-40 dark chocolate brown freckles and 5 or 6 black freckles. This suggests a stepwise unmasking of a basic pigmentary potential, and one feels that genetic changes are intrinsically more likely to provide such discrete steps than other physiological influences.

The cellular changes occurring in these cases obviously happen under strong ultraviolet stimulation, and one can postulate intense activity in cell division of the melanocytes with rapid production of many generations of descendent cells, circumstances which would favour frequent mutations.

These changes, by definition as they occur in freckles, do not produce cells which abandon the monolayer type of colonial organization. However, some freckled people, and some with pigmented spots of the iris have, as already discussed, many more moles than average. A mole has a multilayered organization. They occur frequently in freckled people and are virtually absent in completely unfreckled people, and they may have a range of colours comparable with those of freckles.

One would put forward the suggestion that moles have fundamentally the same causation as freckles, that the early stages of a mole's formation may be based on pigmentary changes of the sort producing freckles, with at some stage a change of character leading to the adoption of the multilayered type of colonial organization. Since some children with many freckles and eye spots have no moles, and

one had more than 240, this suggests that the adoption of the "multilayer" habit in such a colony of cells may in itself be based on a genetic component, perhaps a genetic situation of the sort postulated for the formation of a pigment spot in which a somatic mutation can bring about a change of behaviour of a cell and those cells descendent from it.

Further evidence of the effect of an individual genetic background on the formation of moles is seen in those people who have a large number of a particular clinical type of mole.

Other aspects of mole formation are the increased number of moles on exposed areas, the increase in number of moles with age, and the frequent origin of malignant melanomas in moles.

6. Progression to malignancy.

The fact of occurrence of malignant molanoma in moles has been mentioned. Practically every benign tumour which can develop on or in the body can progress to malignancy in rare instances.

The discussion of the formation of moles in the last section in effect suggests that a limited number, perhaps only three or four, somatic mutations occurring in sequence, could be causative in the development of moles. Moles like other benign tumours in the body eventually reach equilibrium within themselves and with surrounding tissues and cease to get bigger. One must postulate the sort of physiological controls which produce a balance between all cells of the mature organism. At a point where a benign tumour ceases to get bigger one must presume that cell multiplication diminishes

greatly in frequency and perhaps the chance of further somatic mutations is diminished. If the tumour does not reach equilibrium and perhaps, if mutagenic agencies originally causative of the benign tumour are still operative, then further mutations may occur. The selective force suggested in the "Statement of the Thesis" would continue to select for cells with increasing rates of multiplication, and one would submit that a dynamic situation capable of producing a malignant tumour would be in existence.

Let us add four further somatic mutations in sequence to the four postulated for mole formation and the 8 which are now being suggested do not differ greatly from the 7 which Nordling suggested as being likely in many malignant conditions.

As with many theories this argument has become more tenuous the further it has been extended from its original base. There is no evidence in this thesis whether four or forty extra mutations would be necessary. One can but say that there is suggestive evidence of at least some benign tumours arising as a result of somatic mutation, and that malignant tumours very often arise from benign ones, and we have no more reason to postulate a different mechanism for these latter as we have to assume the same mechanism.

7. Applications.

The specific applications of the hypotheses in the "Statement of the Thesis" which one has in mind at this point are the disorders listed in Chapter VIII.

Most of these disorders are dominantly inherited and the reasons for believing that this is more than coincidental have already been discussed. One believes that in homozygous form most of these conditions would be lethal. In heterozygous form the genes are believed to provide a situation in which, in effect, one of the seven steps of Nordling has already been taken and further steps lead much more readily to the initiation of clones of cells in which perhaps the whole sequence of changes to malignancy may occur.

In looking for evidence of somatic mutation certain criteria have emerged. The lesions must be multiple and discrete, usually expressing as multiple benign tumour formation. They must where the appropriate cells are near the surface of the body, show a greater frequency on exposed parts. Some have a characteristic distribution on face and arms. They must in isolated cases progess to malignancy. There must be no continuous change as against discontinuous change eg. in the form of a general malformation of the body or the like. There must occasionally be cases with evidence of antenatal change as against post natal change.

These criteria are not met by all of the cases listed, and even if the lesions in some of these disorders are accepted as being due to somatic mutation there could undoubtedly be controversy over others.

In passing one may say that there is no certain evidence that just a single dominant gene is responsible for each of the disorders listed. These disorders might have different genetic causes, in

volving different dominant genes at different loci, but still be the same diseases clinically. This would be important in estimating mutation rates and in linkage studies.

The conditions will be discussed very briefly in the order in which they appeared in Chapter VIII.

Neurofibromatosis. One would emphasize the pigmentary phenomena, freckles and birthmarks. An analogy may be drawn between freckling and birthmarks in normal individuals, and freckling and birthmarks in neurofibromatosis, and also neurofibromata and plexiform neuroma in the latter. The evidence is that each of these represents a postnatal and an antenatal expression of a similar phenomenon. The occurrences of pigmentary phenomena and nervous tumours in the same condition are not hard to link when one considers the embryological origin of the melanocyte. There is evidence that many of the largest abnormally pigmented areas have an underlying plexiform neuroma - this could arise if a mutation occurred in the stemline of cells before differentiation to melanocyte and nerve cell occurred.

The occurrence of tumours in the central nervous system shows that it is not only the peripheral nervous system which is affected. There is a high rate of malignancy in the tumours arising in this disorder.

Epiloia. The first comment to make is on the facial lesions which vary somewhat between cases. This may be due to differences in the stage of development in different cases, or to different genetic

backgrounds, on which the dominant gene is acting, or to different dominant genes. Another point to emphasize is the very wide variety of tumours which are found in this condition.

Nervous system tumours. These show no clear heredity in most cases. The few cases that do may be due basically to some other gene eg. a gene for neurofibromatosis.

Retinoblastoma. The chief interest, apart from the invariable onset of malignancy, is the evidence that some cases undergo spontaneous regression, or show evidence of this having happened. If mutations produce abnormal products post natally, can these be the subject of immune reactions ?

Familial Endocrine Adenomatosis. This is a recently recognized disease of considerable interest. The variety of lesions is interesting, and the relevance to a class of cells which has long been recognized as responsible for tumour formation.

Phaeochromocytoma. This may be found in neurofibromatosis, familial endocrine adenomatosis, as a familial condition per se, and as a sporadic case either unilaterally or bilaterally. Cases such as these prompt the suggestion that there may be more than one dominant gene responsible for some of those disorders at present regarded as due to a single gene.

The question at this point is whether if one could identify four dominant genes A, B, C & D as being individually causative of a disorder, and if one explained the neoplastic process in terms of sequential somatic mutation, then could not several of these genes

be involved in the progression to neoplasia, even though the specific syndrome may be based on A, or B, or C, or D alone as the initiator of the train of mutations ?

Familial polyposis coli, Gardner's Syndrome, Turcot's Syndrome. The situation with these disorders closely parallels that with phaeochromocytoma - benign polyps progressing to malignancy, but divisible into at least three separate hereditary syndromes. Added to these is the frequent familial occurrence of carcinoma of the colon.

Peutz Jeghers Syndrome. An interesting association of pigmentary spots and polypi of the small bowel is found in this disorder (this constitutes another condition of dominantly inherited pigment spots).

Osler's Disease. There are some variations in clinical type in this disease. This is possibly the only disease in this group of dominant disorders in which a supposed homozygote has been seen.

Sturge Weber Disease and Lindau's Disease. These disorders do not fit the general pattern as well as others recorded if one only considers the classic descriptions, which suggest in each case a rather limited distribution of lesions, rather than the generalization of lesions as occurs in such diseases as neurofibromatosis. Some reports however suggest that these diseases are more generalized than formerly reported.

Glomangioma. A rather rare disorder which fits the general pattern of these diseases well.

Keratoacanthoma, trichoepithelioma and basal cell naevus syndrome. These all affect epidermal cells. The first two fit the common pattern of high frequency of lesions on exposed parts of the body. The third condition deviates in that there are some lesions which depart from the pattern of generalized discrete lesions, and cause developmental anomalies.

Osteomatosis. This disorder fits a pattern of multiple discrete abnormalities well, and there is a high frequency of chondrosarcoma in the lesions.

Leucoderma. This does not produce tumours but the pigmentary changes may be attributable to somatic mutation.

Waardenburg's Syndrome. Several features of this disorder do not fit into a possible somatic mutation pattern of random discrete lesions. The chief interest in the present thesis is the frequency of depigmentations and early greying of the hair.

Multiple Leiomyomata. This rare disorder fits the general pattern of changes well.

Lipomatosis. There is an occurrence of multiple discrete lesions and the rare occurrence of malignant change in a lesion.

Miscellaneous. The miscellaneous group may have no significance at all or they may represent new mutations to highly lethal genes which are kept at a very low level of frequency in the community by selection.

Xeroderma pigmentosum and albinism. The discussion of these recessive conditions has already been adequate, and one should only

emphasize again that among a long list of hereditary conditions these are the only ones with recessive inheritance, and the somatic mutation hypothesis is applicable to these because of the evidence of an undue sensitivity to the effects of ultraviolet light for a reason which is not obvious in xeroderma pigmentosum, and a reason which is obvious in albinism.

8. Ultimate effects and practical significance.

How does this study help in the understanding of malignancy in general? Why should rare disorders be given prominence in trying to understand common disorders? It should hardly be necessary to point out that a dominantly inherited disorder producing tumours would have to be a rather mild condition in its effects to be recognizable in the community, otherwise natural selection would so rapidly eliminate sporadic cases due to new mutation that the inheritance of the gene would remain unknown. One must perhaps distinguish between importance in clinical medicine and possible importance to the understanding of a natural phenomenon.

If the interpretations in this thesis can be accepted in the present form or in some modified form at a future time then the mutation theory provides a logical basis for the understanding of all malignancy. In large populations of people with large populations of cells the random possibility of a number of mutations initiating clones of potentially malignant cells is always present, and this becomes more important when possible mutagenic agents in our environment are considered.

There is one practical consideration which must be recorded at this point. If in fact tumours are clones of mutant cells there must be new antigens present in these cells - new in the sense of not being present in the body in the early stages of life when the body is learning to recognise "self" and "not self". The body may be stimulated to make antibodies to these antigens, and this situation might be used therapeutically if ways were developed to potentiate the effect.

This is not a view entirely unsupported by evidence - quite a long list of spontaneous recoveries from tumours of different sorts have been recorded, some of which may have been due to this effect. Retinoblastoma has already been mentioned in which there is evidence that the malignancy could have been subjected to immune attack. Malignant melanoma sometimes regresses quite dramatically and the phenomenon of the halo naevus is by no means excessively rare. Do these occurrences hold out any hope of therapeutic progress ?

Chapter X - Summary

Germinal mutations in many organisms have been studied, and one must believe that the same sort of mutation can occur in somatic cells. The modern theory of the gene - enzyme relationship makes it possible to generalize to a degree on the possible effects of somatic mutation. Because of numerically vast cell populations somatic mutation must be of frequent occurrence, and generally there would be no harmful effects from such mutations, but because of the large number involved and the probable harmful effects of some of these one must assume firstly that these effects may be detectable clinically and histologically, and secondly that the body must provide mechanisms for the suppression of the more commonly occurring harmful effects.

The theory is put forward that one of the observable effects of somatic mutation is benign and malignant tumour formation. The reasoning is that some somatic mutations must impart an increased relative rate of cell division to the mutant cells and that by a process of selection within large groups of cells an increasing rate of division may occur as a result of sequential mutations. One must postulate that as cells lose certain genetic determinants they tend to revert to a more primitive behaviour, involving a process of de-differentiation, and that their powers of cell division and other characters become more like that of the primitive embryonic cell.

It is further put forward that individuals heterozygous rather than homozygous for certain normal genes will have a much higher

probability of developing multiple benign tumours, and ultimately malignant tumours, because of a much increased probability that clones of mutant cells will be initiated.

The pigmentary system has been studied with a view to finding evidence for this theory. The phenomenon of "pigment spread" provides the necessary theoretical basis for the development of clones of mutant cells. It is suggested that freckles, moles, and birthmarks all provide evidence of somatic mutation, and that each individual freckle, mole or birthmark represents a clone of cells originating from a single mutant cell. The occurrence of the freckle has been related to a single gene effect - a person is either heterozygous or homozygous for this gene; if the latter he will be red haired, and it is suggested that a point mutation occurring in this particular gene in a particular melanocyte causes an alteration in the pigment producing capacity of the melanocyte initiating a clone of cells producing more melanin than the surrounding cells.

A birthmark fits into this picture through the concept that it is a large freckle acquired before birth due to a spontaneous mutation. Children with freckles or red hair have 2 - 3 times as many birthmarks as those without. A mole may occur before birth or be induced spontaneously or by sunlight after birth. It is shown that children with freckles have 60% more moles than those without, and those with pigmented spots of the iris have 40% more than those without. Those who have both characters have twice as many moles as those who have neither, and this could be attributed in part to

an effect resulting from the possession of two genes with dominant effect.

A special application of the sequential mutation concept is the type of birthmark seen in some red haired individuals. Another phenomenon of comparable interest is the stepwise darkening of freckles on very freckled children; the largest number of freckles are the very pale ones, and there may be three darker shades of brown and ultimately black freckles, all progressively less in frequency.

The biochemistry of epidermal pigments in marsupials, birds, higher mammals and humans has been studied. The evidence is that the principal pigments in red animals and birds are separate yellow, orange and red pigments, but that there are also a second red pigment and a purple pigment in appreciable quantities. Minor pigmentary components are numerous and these include brown, yellow, orange and red colours.

Pigment precursors have been found particularly in marsupial hair extracts, and also in minor quantities in some higher mammalian hair extracts. These substances are aromatic amines.

It has been found that 3-Aminotyrosine can be made to produce a range of pigments comparable with those found in marsupials, but this work is as yet unfinished.

However by solution of 3-Aminotyrosine and marsupial hair extracts in aqueous phenol, and exposure to sunlight, a pigment has been produced in quantity which is chromatographically the same from the two sources.

Work has been done on pigmentary variability in human hair, and this variability can be related in at least one aspect to the genetic hypothesis of the inheritance of red hair, and it is suggested that the variability may be due to somatic mutation.

Finally a number of disorders with a specific abnormal gene, and producing multiple tumours, have been reviewed. Also included with these are certain dominant conditions in which no new growths occur but in which there is thought to be some relevance to the general concepts of the thesis. Two recessive disorders of particular interest have been included because it is thought that, unlike the dominant conditions, they represent some generalized increase in sensitivity to the mutation inducing effects of ultraviolet light.

Conclusions.

1. The genetics of red hair. In general the red haired freckled person is a homozygote and the brown haired freckled person is a heterozygote for a single pair of alleles. There are exceptions, particularly the failure of freckling in a supposed heterozygote, but these do not seem to be sufficiently numerous to alter the general applicability of the hypothesis.

2. Some marsupials secrete large amounts of pigments or pigment precursors onto their skin.

3. There is evidence that the precursor to the red hair pigment is a tyrosine derivative, probably 3-Aminotyrosine.

4. The melanocyte may produce pigments the colour of which are

determined by the precursor available. If tyrosine is the precursor of black pigment and substances closely related to tyrosine the precursors to other pigments, then there is the basis for a simple switch mechanism for pigment production. With regard to the pigments of hair there is found in a percentage of brown haired people a switch from brown pigment production to red pigment production in some hairs. In red haired individuals, most have a few hairs which produce brown pigment. In black haired people some hairs are brown or occasionally red. It is suggested that the change from production of one type of pigment to another may be the result of somatic mutation.

5. In the melanocytes of the skin, the phenomenon of pigment spread explains the development of freckles from a single mutant cell capable of producing more intense pigment than its neighbours. The concept is that the freckled person, who has one or two genes producing red pigment, loses the biochemical effect of one of these by somatic mutation and in so doing releases precursor for the production of more of a darker melanin.

6. Study of the size, orientation and histology of birthmarks suggests that they represent the same sort of change antenatally that occurs in a freckle postnatally.

7. Birthmarks are related to freckles and red hair statistically, there being approximately 2 - 3 times as many birthmarks in freckled or red haired children as in those with no freckles.

8. On the basis of a somatic mutation hypothesis it is estimated that the changes which occur in a heterozygous freckler producing birthmarks

occur at a rate of the order of once in 10,000 cell divisions, which is therefore the somatic mutation rate. It is thought that the gene which mutates is the specific gene producing red hair in the homozygous form.

9. The freckles of heavily freckled children may be present in as many as four shades of brown, and also black. As one passes from pale brown to dark brown to black the number of freckles in each class progressively decreases. It has been estimated that a heavy freckler may have as many as 10,000 pale brown freckles, and 5 or 6 black freckles have been seen on some children. This apparently stepwise change in pigment producing capacity may be related to stepwise somatic mutation.

10. Red haired people are occasionally seen with birthmarks having approximately half the number of freckles as the surrounding skin. This can be related to the genetic hypothesis.

11. There is some evidence from family studies that eye spots are dominantly inherited.

12. Freckled children have 60% more moles than unfreckled, and those with eyespots have 40% more than those without. Those with both freckles and eyespots have more than twice as many as those without.

13. It is suggested that as a logical outcome of a somatic mutation hypothesis there should be a class of dominantly inherited disorders in which there occur multiple tumours. Some twenty such disorders are reviewed.

14. Two disorders due to recessive genes are given prominence because it is felt that they represent special cases in which there is a

generalised capacity for somatic mutation rather than a very specific one as in the dominant conditions. In the latter it is thought that the disorder is initiated by mutations of the normal allele of the abnormal gene.

15. It is suggested that the body in some instances treats mutant cells as foreign and that immune responses may be stimulated. If this is so there may be therapeutic possibilities in further stimulating such responses.

Appendix I - Tests of significance of differences in mean values in Tables of Chapter III

The tables I - XIII relate various frequencies of moles or birthmarks to such factors as hair colour, skin colour, freckling type etc. There are significant differences in mean values in many of these tables. The different mean values are compared two at a time, and "t" values are presented in tabular form. The table number and heading to the table is given, and each character in the table is allotted a number. Number of degrees of freedom are given with the "t" values, but no comment as to significance is made. In all of these tables a value of "t" greater than 1.96 is significant at the 5% level. Certain tables have been omitted as inapplicable.

Table III - Moles in two series of schoolchildren

t (1454 DF) = 15.8

Table IV - Moles according to racial origin

Northern European (1) Southern European (2) Oriental (3).

	Series I	Series II
1 - 2	t (704 DF) = 7.38	t (713 DF) = 8.97
1 - 3	t (623 DF) = 6.82	t (574 DF) = 7.31
2 - 3	t (109 DF) = 1.71	t (165 DF) = 1.53

Table V - The number of moles according to hair colour

Blonde (1) Lt. Brown (2) Brown (3) Dk. Brown (4) Black (5) Red (6)

	Series I	Series II
1 - 2	t (198 DF) = 0.30	t (311 DF) = 1.24
1 - 3	t (361 DF) = 0.75	t (350 DF) = 1.07

	Series I	Series II
1 - 4	t (137 DF) = 0.18	t (186 DF) = 2.62
1 - 5	t (98 DF) = 2.07	t (115 DF) = 3.87
1 - 6	t (73 DF) = 3.46	t (127 DF) = 2.34
2 - 3	t (483 DF) = 0.68	t (481 DF) = 2.86
2 - 4	t (259 DF) = 0.68	t (317 DF) = 4.55
2 - 5	t (220 DF) = 3.59	t (246 DF) = 5.87
2 - 6	t (195 DF) = 5.89	t (258 DF) = 3.47
3 - 4	t (422 DF) = 1.47	t (356 DF) = 2.36
3 - 5	t (383 DF) = 5.11	t (285 DF) = 3.99
3 - 6	t (358 DF) = 8.21	t (297 DF) = 1.90
4 - 5	t (159 DF) = 2.84	t (121 DF) = 1.93
4 - 6	t (134 DF) = 5.10	t (133 DF) = 0.40
5 - 6	t (95 DF) = 2.51	t (62 DF) = 0.82

Table VI - Number of moles according to skin colour

Pale Brown (1) Light Brown (2) Brown (3)

	Series I	Series II
1 - 2	t (418 DF) = 0.80	t (620 DF) = 0.24
1 - 3	t (395 DF) = 0.06	t (174 DF) = 2.52
2 - 3	t (623 DF) = 1.25	t (664 DF) = 6.01

Table VII - Number of moles according to eye colour

Blue (1) Grey (2) Brown (3)

	Series I	Series II
1 - 2	t (567 DF) = 0.59	t (506 DF) = 0.32
1 - 3	t (387 DF) = 2.80	t (501 DF) = 6.32
2 - 3	t (480 DF) = 3.58	t (453 DF) = 5.41

Table VIII - Number of eyespots in children with at least one eyespot

No test of significance is applied to the average number of eyespots. In Series I 224 of 721 had eyespots, and in Series II 116 of 735 had eyespots. In a 2 x 2 table this gives $\chi^2 = 46.7$ for 1 DF, a highly significant difference between the two Series.

Table IX - Number of moles in those with, and those without eyespots.

	Series I	Series II
	t (719 DF) = 4.05	t (727 DF) = 2.50

Table X - Number of birthmarks according to degree of freckling

(2 x 2 tables)

Series I χ^2(1 DF) = 34.0 (comparing A1 + A2 with B1 + B2)

Series II χ^2(1 DF) = 32.8 (comparing A + B + C with D + E)

Table XII - Number of moles according to degree of freckling

Series I - A1(1) A2(2) B1(3) B2(4)

Series II - B(1) C(2) D(3) E(4)

	Series I	Series II
1 - 2	t (332 DF) = 2.65	t (431 DF) = 6.69
1 - 3	t (447 DF) = 5.59	t (507 DF) = 7.62
1 - 4	t (144 DF) = 0.96	t (236 DF) = 0.72
2 - 3	t (573 DF) = 3.79	t (476 DF) = 0.98
2 - 4	t (270 DF) = 3.18	t (215 DF) = 0.83
3 - 4	t (385 DF) = 5.63	t (291 DF) = 1.11

Table XIII - Number of moles in relation to freckling and eyespots

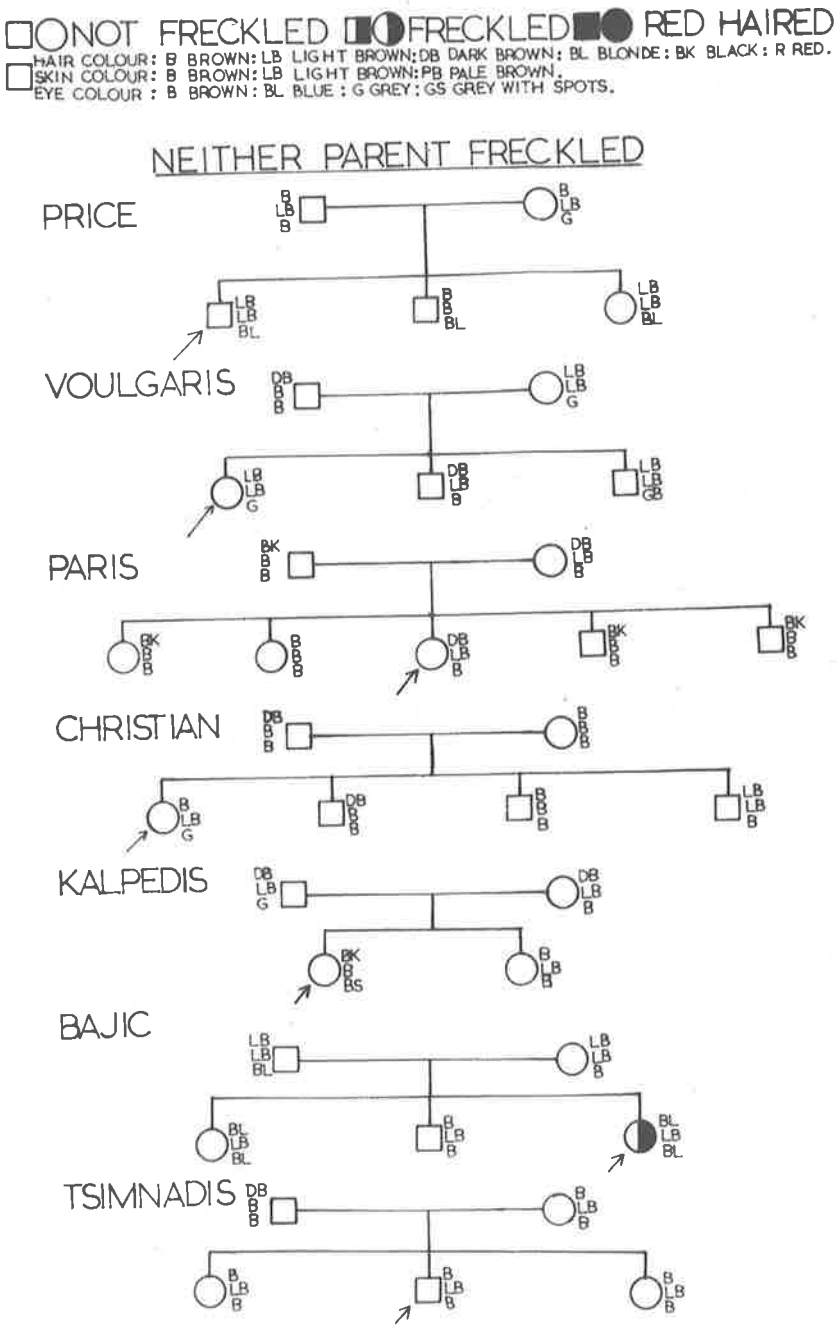
Series I - Freckling B1 + Eyespots (1); A1, A2, B2 + Eyespots (2);

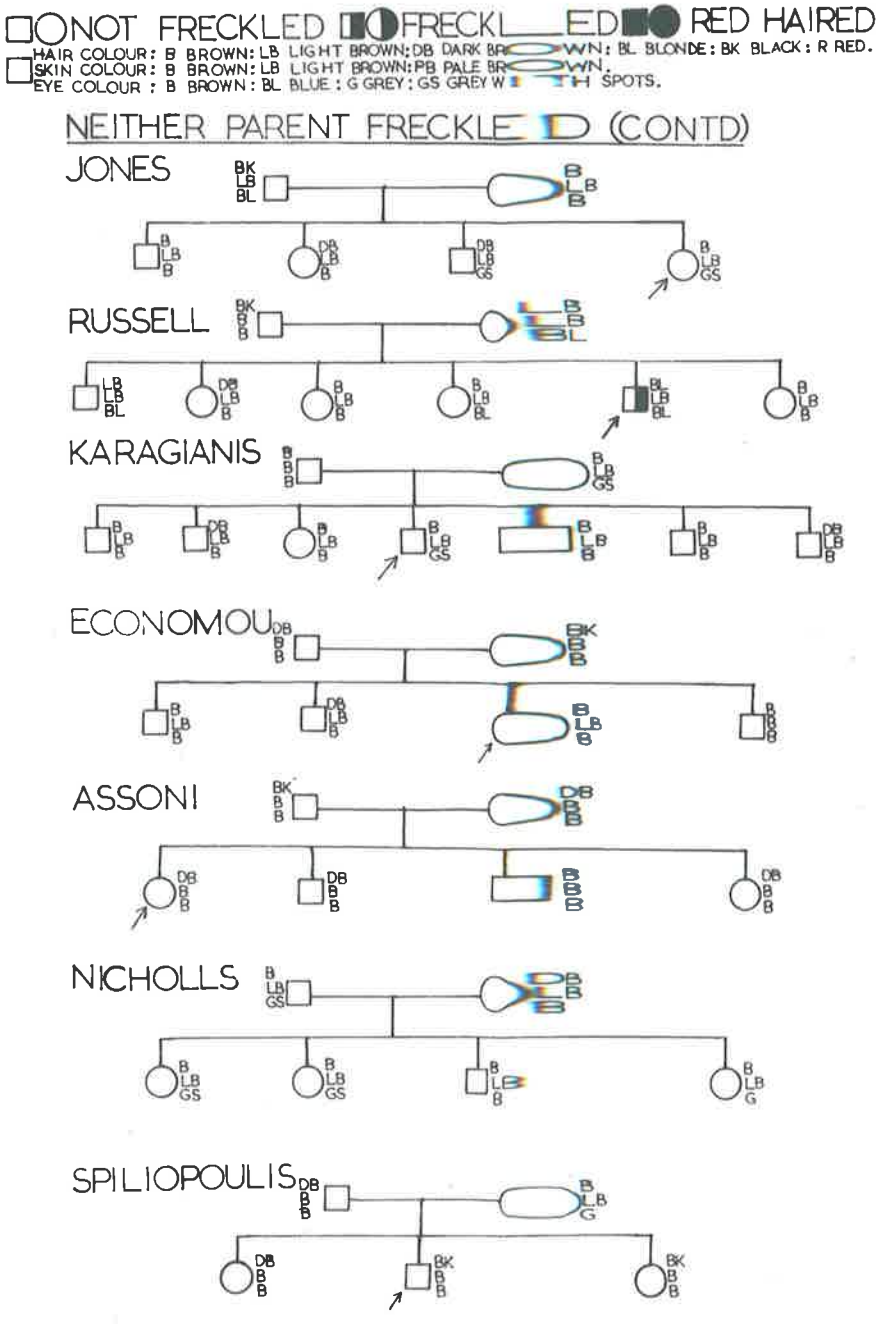
Freckling B1, No eyespots (3); A1, A2, B2, No eyespots (4)

Series II - Freckling D + Eyespots (1); A, B, C, E + Eyespots (2);
Freckling D, No eyespots (3); A, B, C, E, No eyespots (4)

	Series I	Series II
1 - 2	t(220 DF) = 3.07	t(114 DF) = 2.10
1 - 3	t(343 DF) = 2.76	t(275 DF) = 1.60
1 - 4	t(354 DF) = 5.59	t(428 DF) = 3.24
2 - 3	t(363 DF) = 0.59	t(297 DF) = 1.09
2 - 4	t(374 DF) = 3.61	t(450 DF) = 1.98
3 - 4	t(495 DF) = 4.83	t(611 DF) = 4.10

Appendix II - Data on 74 families showing pigmentary details of hair, eyes and skin, including freckling.



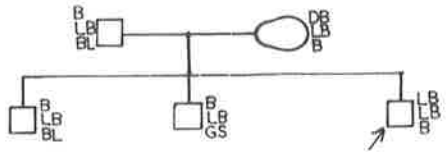

NOT FRECKLED
FRECKLED
RED HAIRED
HAIR COLOUR: B BROWN: LB LIGHT BROWN: DB DARK BROWN: BL BLONDE: BK BLACK: R RED.
SKIN COLOUR: B BROWN: LB LIGHT BROWN: PB PALE BROWN.
EYE COLOUR : B BROWN : BL BLUE : G GREY : GS GREY W SPOTS.
NEITHER PARENT FRECKLED (CONTD)
JONES
RUSSELL
KARAGIANIS
ECONOMOU
ASSONI
NICHOLLS
SPILIOPOULIS

NOT FRECKLED FRECKLED RED HAIRED

HAIR COLOUR: B BROWN: LB LIGHT BROWN: DB DARK BROWN: BL BLONDE: BK BLACK: R RED.
SKIN COLOUR: B BROWN: LB LIGHT BROWN: PB PALE BROWN.
EYE COLOUR: B BROWN: BL BLUE: G GREY: GS GREY WITH SPOTS.

NEITHER PARENT FRECKLED (CONTD)

OLSON



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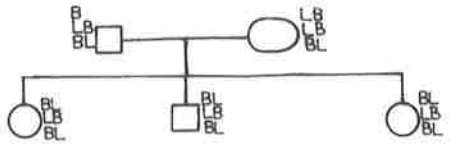

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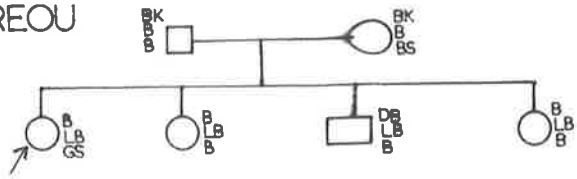
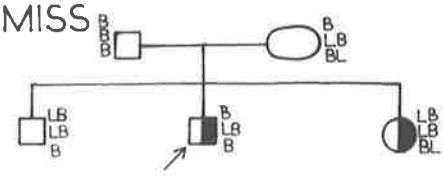


MIDDLEMISS

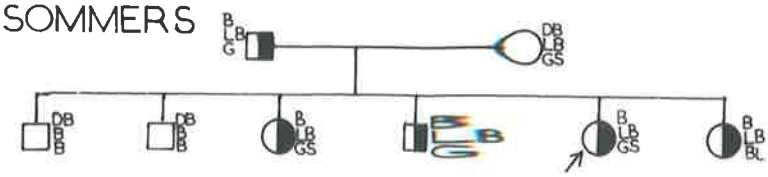


NOT FRECKLED FRECKLED RED HAIRED

HAIR COLOUR: B BROWN: LB LIGHT BROWN: DB DARK BROWN: BL BLONDE: BK BLACK: R RED.
SKIN COLOUR: B BROWN: LB LIGHT BROWN: PB PALE BROWN.
EYE COLOUR : B BROWN : BL BLUE : G GREY : GS GRE WITH SPOTS.

ONE PARENT FRECKLE

SOMMERS



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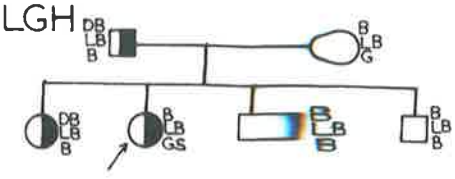
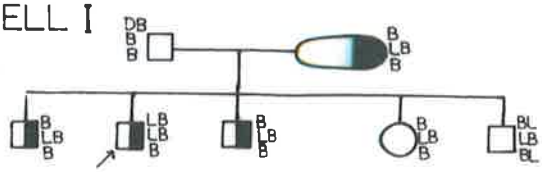
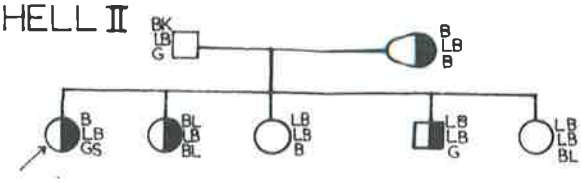


MITCHELL I



MITCHELL II



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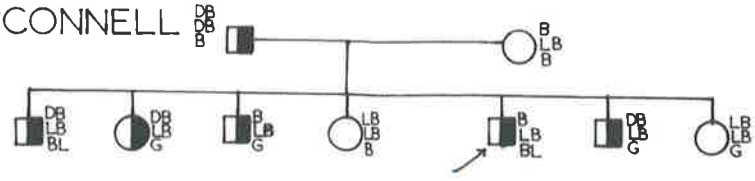

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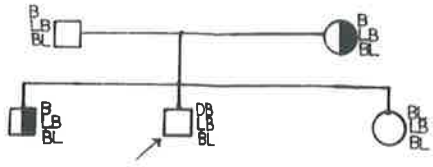
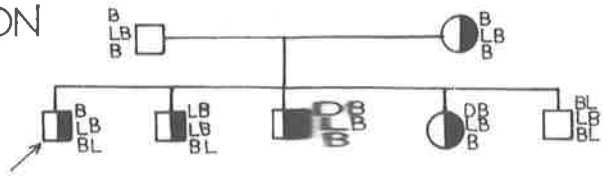


NOT FRECKLED FRECKLED RED HAIRED

HAIR COLOUR: B BROWN: LB LIGHT BROWN: DB DARK BROWN: BL BLONDE: BK BLACK: R RED.
SKIN COLOUR: B BROWN: LB LIGHT BROWN: PB PALE BROWN.
EYE COLOUR: B BROWN: BL BLUE: G GREY: GS GREY WITH SPOTS.

ONE PARENT FRECKLED (CONTD)

JOHNSON



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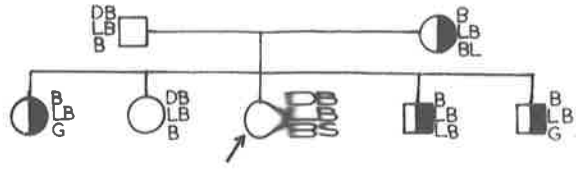

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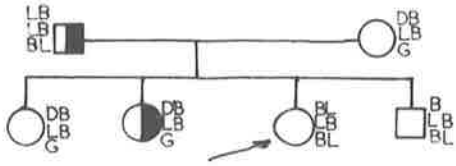

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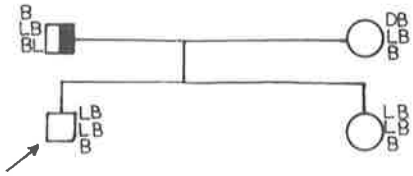

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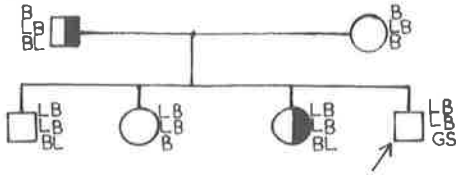

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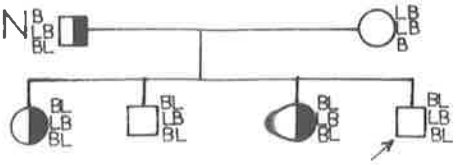
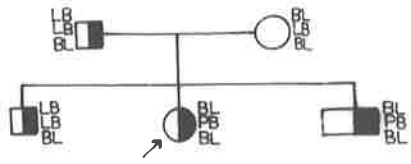


ONE PARENT FRECKLED (CONTD)

WENDT



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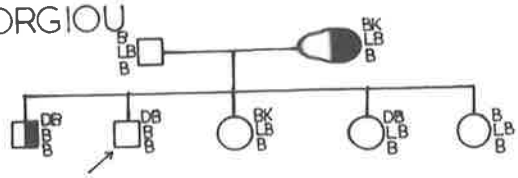

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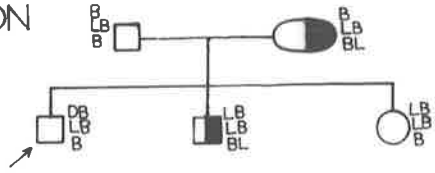

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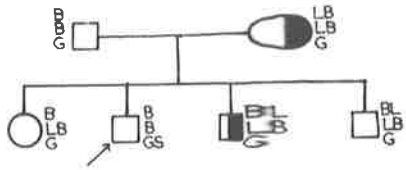

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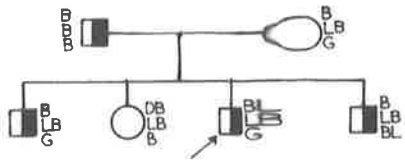

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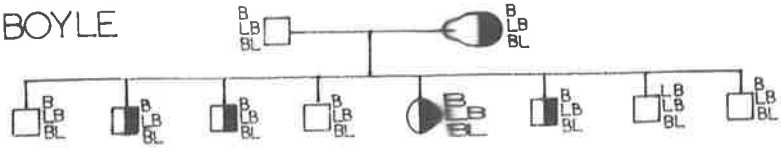

NOT FRECKLED FRECKLED RED HAIRED

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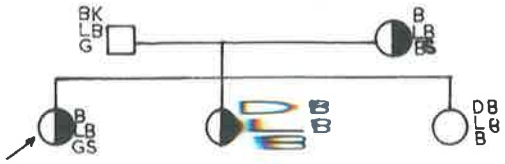
EYE COLOUR : B BROWN : BL BLUE : G GREY : GS GREY WITH SPOTS.

ONE PARENT FRECKLED (CONTD)

KOROBILAS



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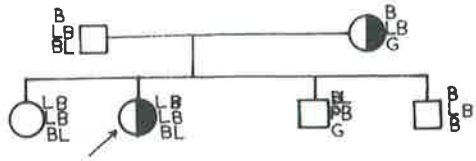

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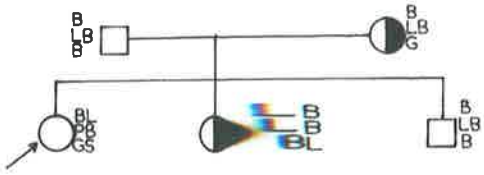

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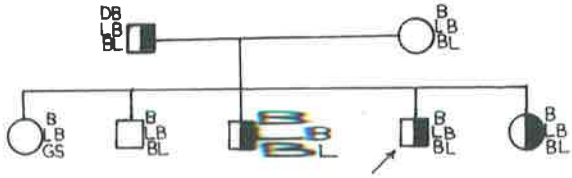

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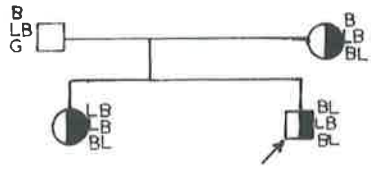

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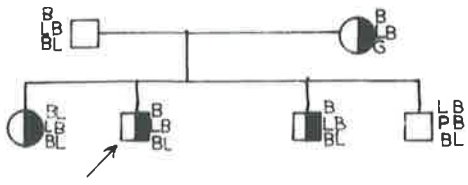

NOT FRECKLED FRECKLED RED HAIRED

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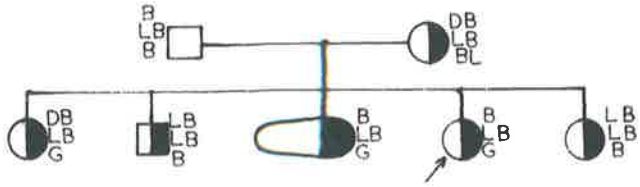
EYE COLOUR : B BROWN : BL BLUE : G GREY : GS GREY WITH SPOTS.

ONE PARENT FRECKLED (CONTD)

KING



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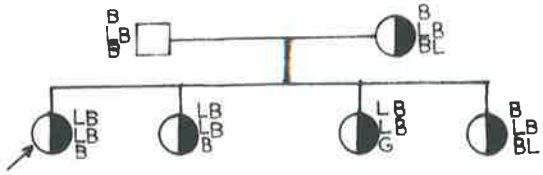

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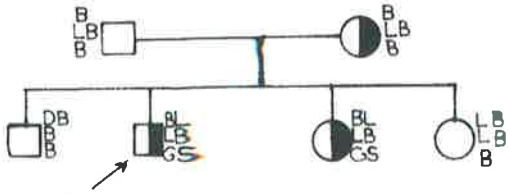

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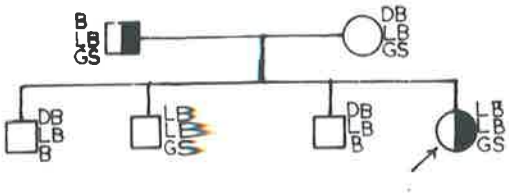

NOT FRECKLED FRECKLED RED HAIRED

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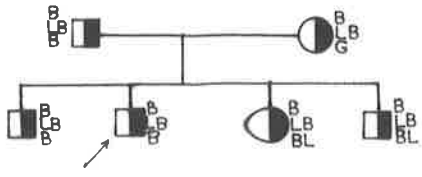
EYE COLOUR : B BROWN : BL BLUE : G GREY : GS GREY WITH SPOTS.

BOTH PARENTS FRECKLED

MAY TOM



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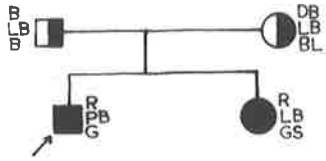

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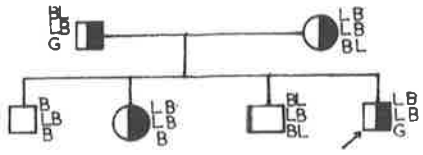

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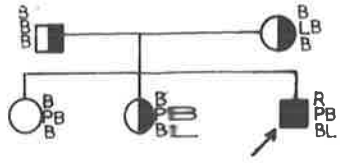

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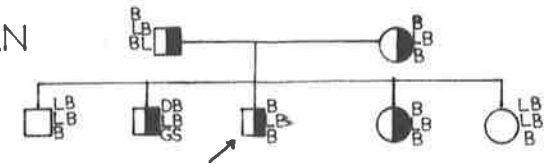

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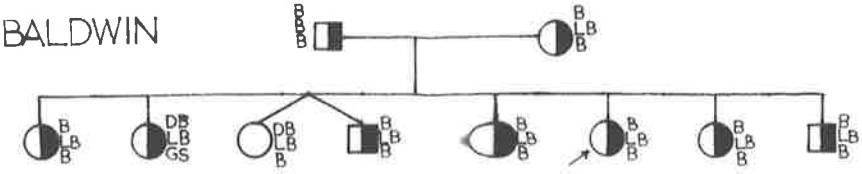

NOT FRECKLED FRECKLED RED HAIRED

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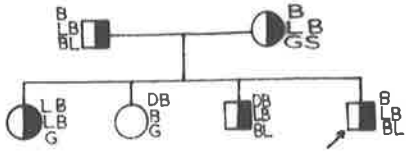
EYE COLOUR : B BROWN : BL BLUE : G GREY : GS GREY WITH SPOTS.

BOTH PARENTS FRECKLED (CONTD)

MILLS



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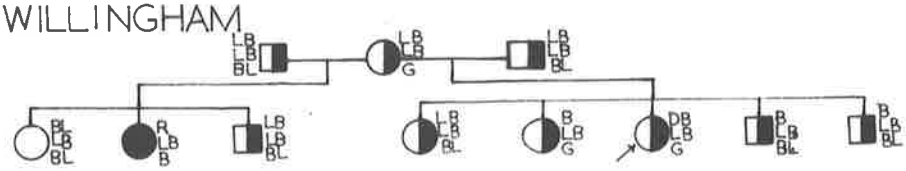

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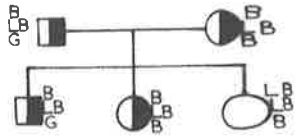
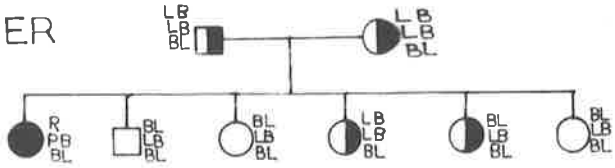
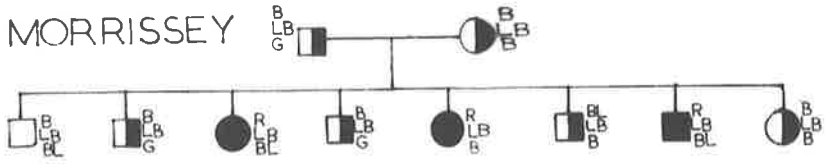


CAYZER



MORRISSEY



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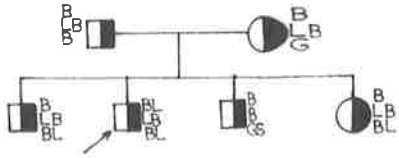

NOT FRECKLED FRECKLED RED HAIRED

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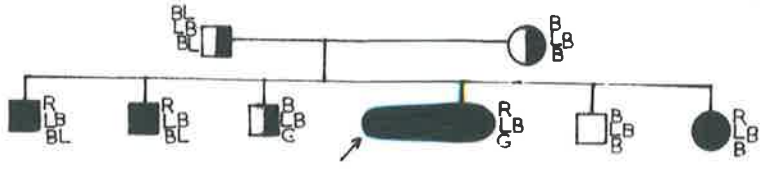
EYE COLOUR : B BROWN : BL BLUE : G GREY : GS GREY WITH SPOTS.

BOTH PARENTS FRECKLED (CONTD)

SMITH



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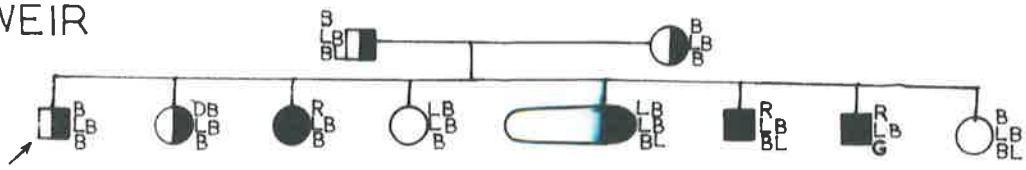
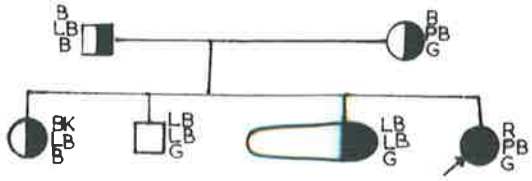
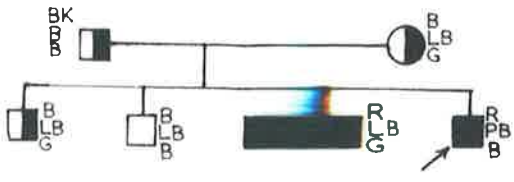


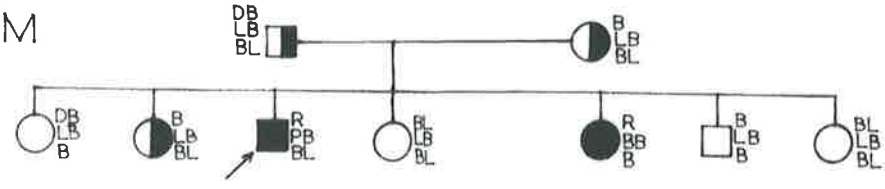
PREH



DONNELLY



THOM



NOT FRECKLED FRECKLED RED HAIRED

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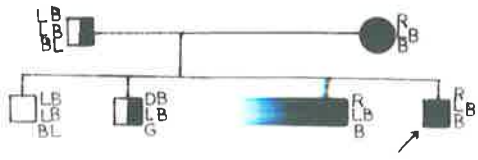
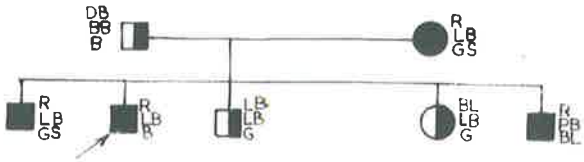
ONE PARENT FRECKLED, ONE RED HAIRED

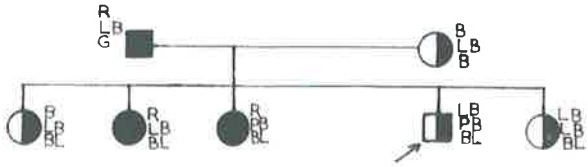
ERICKSON



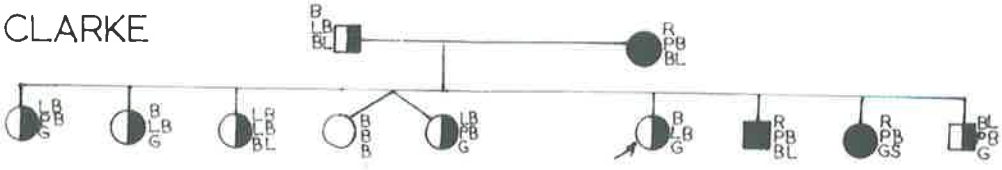
COLEMAN



BYRNE

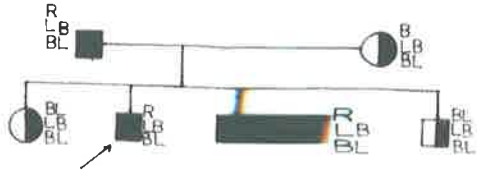


CLARKE



WOOD

BRITTAIN

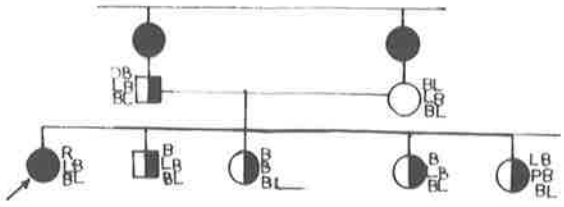


NOT FRECKLED FRECKLED RED HAIRED

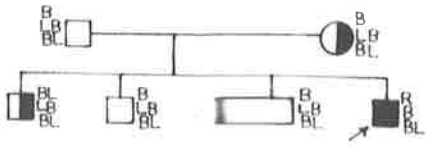
HAIR COLOUR: B BROWN: LB LIGHT BROWN: DB DARK BROWN: BL BLONDE: BK BLACK: R RED.
SKIN COLOUR: B BROWN: LB LIGHT BROWN: PB PALE BROWN.
EYE COLOUR : B BROWN : BL BLUE : G GREY : GS GREY WITH SPOTS.

ONE FRECKLED PARENT, ONE RED HAIRED CHILD

COOPER



GOODWIN



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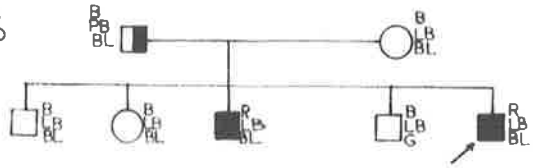
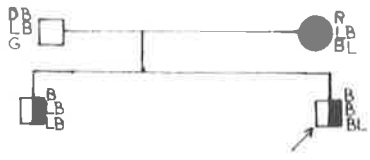


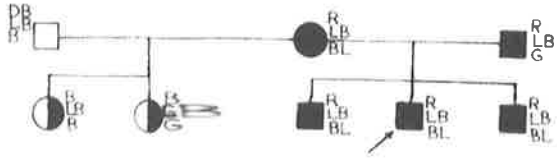
ONE PARENT RED HAIRED

FITZGERALD



BOTH PARENTS RED HAIRED

McDONALD



APPENDIX III

Mean values of 20 random readings on 54 samples of human brown hair.

	Mean	SD	Coeffct of Variation	Reddest Hair
1	2.32 ± 0.11	0.49	21.2	6.56
2	2.47 ± 0.13	0.58	23.4	4.64
3	2.21 ± 0.09	0.41	18.3	3.72
4	1.89 ± 0.06	0.28	14.9	3.64
5	2.03 ± 0.07	0.30	14.8	5.11
6	2.24 ± 0.20	0.89	39.6	5.52
7	1.92 ± 0.05	0.23	11.8	2.54
8	3.84 ± 0.10	0.43	11.1	4.31
9	3.35 ± 0.06	0.29	8.5	5.28
10	3.88 ± 0.04	0.18	46.5	4.80
11	2.42 ± 0.12	0.55	22.7	2.42
12	2.10 ± 0.05	0.23	10.8	3.30
13	2.62 ± 0.07	0.34	13.1	7.54
14	2.20 ± 0.12	0.53	24.3	2.15
15	2.61 ± 0.09	0.42	16.1	3.23
16	2.21 ± 0.06	0.28	12.5	3.07
17	2.31 ± 0.06	0.29	12.6	2.66
18	1.71 ± 0.03	0.13	7.3	1.92
19	2.07 ± 0.04	0.18	8.8	2.01
20	2.31 ± 0.08	0.34	14.6	1.89
21	2.55 ± 0.19	0.87	34.0	4.62
22	2.01 ± 0.07	0.32	15.7	2.61
23	3.11 ± 0.11	0.47	15.2	7.55
24	2.36 ± 0.12	0.56	23.6	2.85
25	1.93 ± 0.05	0.24	12.3	2.35
26	1.55 ± 0.04	0.20	12.8	2.91
27	1.98 ± 0.05	0.21	10.4	2.33

	Mean	SD	Coeffot of Variation	Reddest Hair
28	2.26 ± 0.06	0.28	12.5	2.42
29	2.98 ± 0.12	0.54	18.2	2.98
30	2.18 ± 0.07	0.30	13.6	2.66
31	2.41 ± 0.13	0.58	24.0	4.33
32	2.17 ± 0.06	0.26	12.2	2.88
33	2.26 ± 0.13	0.59	25.9	3.71
34	1.96 ± 0.08	0,35	17.7	3.60
35	2.88 ± 0.18	0.79	27.5	4.47
36	2.08 ± 0.04	0.16	7.5	3.10
37	3.15 ± 0.30	1.36	43.1	6.51
38	2.23 ± 0.07	0.30	13.5	2.59
39	2.07 ± 0.06	0.28	13.5	7.34
40	2.13 ± 0.05	0.23	10.7	3.50
41	2.97 ± 0.18	0.78	26.4	5.25
42	2.06 ± 0.06	0.29	14.0	3.50
43	2.58 ± 0.16	0.73	28.3	2.67
44	2.37 ± 0.12	0.54	22.7	2.24
45	2.34 ± 0.23	0.10	43.6	5.16
46	2.56 ± 0.07	0.33	12.7	2.24
47	2.33 ± 0.08	0.36	15.3	3.48
48	3.77 ± 0.34	1.51	40.2	7.28
49	2.68 ± 0.19	0.84	31.3	4.32
50	2.53 ± 0.06	0.26	10.3	2.90
51	2.27 ± 0.10	0.47	20.6	5.80
52	3.44 ± 0.11	0.48	15.2	3.74
53	2.20 ± 0,07	0.33	15.0	3.79
54	3.16 ± 0.07	0.33	10.5	3.88

APPENDIX IV.

Mean values of 20 random readings on 21 samples of human red hair.

	Mean	Standard Deviation	Coeffot of Variation	Brownest Hair
1	5.88 ± 0.22	0.96	16.4	4.34
2	5.25 ± 0.26	1.14	21.8	3.92
3	5.38 ± 0.32	1.41	26.2	2.33
4	5.91 ± 0.54	2.42	40.9	1.90
5	5.81 ± 0.23	1.04	17.8	2.51
6	6.00 ± 0.19	0.85	14.2	3.84
7	6.43 ± 0.31	1.37	21.3	4.22
8	5.77 ± 0.22	1.00	17.3	1.60
9	5.80 ± 0.33	1.49	25.7	3.14
10	4.87 ± 0.27	1.22	25.0	2.23
11	5.86 ± 0.24	1.07	18.3	2.37
12	4.15 ± 0.23	1.04	25.0	2.89
13	5.30 ± 0.25	1.13	21.3	2.40
14	4.33 ± 0.20	0.89	20.6	2.64
15	5.86 ± 0.21	0.96	16.3	3.06
16	4.27 ± 0.26	1.15	27.0	2.56
17	6.20 ± 0.25	1.13	18.2	2.43
18	4.66 ± 0.24	1.09	23.3	2.68
19	4.99 ± 0.26	1.14	22.9	1.99
20	5.15 ± 0.25	1.14	22.1	2.48
21	4.84 ± 0.30	1.33	22.5	2.68

APPENDIX V.

Results of taking a random reading and a "reddest" reading on 552 samples of hair (microspectrophotometry)

Note: The hair samples in Series I were supplied approximately 1 year before the clinical examination was conducted. In the meantime some individuals had left the Hospital, accounting for gaps in the table.

Series I - PEAT & MILSON ISLAND HOSPITAL.

No.	Age	Hair Colour	Random reading	Red reading	Freckle type
1	39	B(G)	2.38	2.40	C
2	29	B	2.40	6.12	C
3	26	-	2.05	3.66	-
4	30	B	2.67	5.56	B
5	20	B	1.88	1.88	B
6	35	B	2.50	4.91	D
7	16	BL	2.07	2.14	C
8	25	B	2.16	3.11	C
9	18	B	2.23	3.46	C
10	21	LB	1.31	1.86	D
11	30	B	1.79	2.83	B
12	41	B	2.19	2.57	D
13	19	B	2.28	2.69	C
14	23	B	1.64	2.31	C
15	32	BL	3.42	2.61	D
16	26	BL	1.83	1.97	A
17	22	LB	1.75	3.26	C
18	54	B	2.92	3.24	C
19	20	B	2.13	4.90	D
20	18	BL	2.64	3.70	D
21	22	B	2.11	3.61	D
22	22	B	2.24	1.62	B
23	22	B	2.02	3.32	D
24	16	B	2.15	5.08	D
25	19	B	2.13	2.52	D

B - Brown; LB - Light Brown; DB - Dark Brown; BL - Blonde;
B(G) - Brown becoming grey; G(B) Grey, originally brown;
BK - Black; R - Red.

No.	Age	Hair Colour	Random reading	Red reading	Freckle type
26	31	B	1.88	2.73	C
27	28	B	2.13	2.96	D
28	46	B	1.74	3.39	B
29	40	B	1.56	1.78	D
30	46	B	2.39	3.33	C
31	24	-	2.12	2.54	-
32	19	B	3.13	2.55	C
33	19	B	1.68	2.73	D
34	20	B	2.34	5.83	D
35	32	B	1.87	3.07	C
36	23	B	1.89	1.77	C
37	51	B	1.91	1.85	B
38	13	LB	2.36	2.78	C
39	23	LB	2.20	1.73	C
40	15	B	2.41	5.21	D
41	8	LB	2.10	2.39	D
42	20	B	2.28	1.92	D
43	16	B	2.22	2.05	D
44	14	B	2.25	2.94	B
45	13	BL	2.25	3.50	D
46	11	BL	2.40	3.00	C
47	15	B	3.55	2.52	B
48	18	DB	1.46	1.64	D
49	16	B	3.15	5.30	C
50	16	B	2.17	2.43	D
51	30	B	1.84	2.26	C
52	16	B	2.02	1.94	C
53	15	B	2.60	7.79	B
54	23	B	1.87	2.44	C
55	18	B	2.05	2.18	C
56	40	B	1.94	2.41	D
57	15	BL	2.50	4.40	D
58	19	LB	1.77	1.90	D
59	25	DB	1.80	1.90	B
60	40	B(G)	2.34	2.40	C
61	45	B	2.08	1.92	B
62	56	G	2.02	2.00	D
63	54	DB(G)	1.56	2.19	B
64	56	G	1.92	1.75	D
65	28	B	1.96	2.08	B

No.	Age	Hair Colour	Random reading	Red reading	Freckle type
66	13	B	2.24	2.36	D
67	15	B	1.75	2.17	C
68	56	G	1.61	2.35	C
69	9	-	2.28	2.41	-
70	40	-	1.86	2.08	-
71	44	B	1.97	2.23	C
72	41	B	1.94	1.94	C
73	44	B	1.75	2.39	D
74	36	B	2.30	2.63	D
75	25	B	1.66	2.15	D
76	47	B	2.34	2.90	C
77	45	B(G)	1.62	2.20	B
78	20	-	2.11	2.04	-
79	26	BL	2.34	3.51	B
80	21	B	1.88	2.26	C
81	15	B	2.17	2.99	D
82	29	B	1.60	2.24	B
83	25	B	2.81	2.37	D
84	20	B	1.82	4.05	D
85	31	DB(G)	1.71	2.19	C
86	32	-	2.14	2.85	-
87	18	B	2.17	2.13	C
88	34	B	2.14	2.27	C
89	41	B	2.21	3.60	D
90	24	-	1.96	2.18	-
91	25	-	2.31	7.52	-
92	23	R	9.01	6.94	D
93	22	B	2.37	1.84	D
94	23	-	4.88	8.71	-
95	19	BL	3.47	3.11	C
96	42	B	2.00	2.54	D
97	17	B	2.29	2.75	B
98	15	B	2.53	2.54	D
99	18	B	1.53	2.14	B
100	20	B	1.97	2.64	C
101	17	B	2.14	6.40	D
102	21	B	2.51	2.07	C
103	15	B	1.76	2.10	D
104	20	B	2.04	2.93	D
105	24	B	2.48	7.58	D

No.	Age	Hair Colour	Random reading	Red reading	Freckle type
106	33	B	2.14	3.28	C
107	24	B	1.67	2.43	B
108	17	B	2.54	2.37	B
109	17	LB	2.91	2.41	C
110	15	LB	2.10	2.05	C
111	16	BL	2.32	2.30	D
112	15	B	1.72	2.10	D
113	13	R	2.15	4.37	D
114	13	LB	2.05	2.50	C
115	17	LB	2.60	3.60	C
116	19	B	2.18	5.73	C
117	19	-	2.07	3.38	-
118	20	B	1.81	7.62	D
119	22	B	2.11	2.29	D
120	16	B	2.05	2.43	C
121	16	B	1.83	2.18	D
122	23	-	2.29	1.85	-
123	45	B	1.86	2.86	C
124	55	B	1.63	1.73	B
125	22	B(G)	2.12	2.55	D
126	59	G	1.66	1.80	C
127	45	B(G)	2.26	5.16	C
128	23	-	1.87	2.82	-
129	20	B	2.44	2.31	B
130	21	BL	3.02	3.22	B
131	26	B	2.03	3.17	D
132	15	B	2.04	2.33	D
133	19	LB	3.10	3.21	C
134	20	DB	1.79	1.79	C
135	42	B	2.41	2.84	C
136	18	B	2.62	2.39	C
137	22	B	2.05	7.60	D
138	21	DB	1.82	5.80	B
139	23	B	2.20	2.43	D
140	20	B	1.79	1.90	B
141	19	BK	1.14	1.24	B
142	41	B	1.91	2.26	C
143	48	G	2.00	2.57	B
144	44	G	1.62	2.01	B
145	21	BK	1.69	2.63	B

No.	Age	Hair Colour	Random Reading	Red Reading	Freckle type
146	23	B(G)	1.88	2.48	B
147	21	B	2.83	3.21	D
148	20	B	2.71	2.70	C
149	28	B	1.87	6.13	D
150	37	-	3.02	7.93	-
151	39	B(G)	1.67	2.07	C
152	39	LB	2.38	6.49	D
153	22	B	2.57	2.70	D
154	40	B(G)	2.44	2.73	B
155	21	B	2.12	2.31	D
156	33	B	1.95	2.98	B
157	40	G(B)	1.95	6.60	B
158	21	-	1.48	2.79	-
159	34	B	1.74	2.40	B
160	34	DB(G)	1.64	2.96	C
161	35	B	2.60	2.29	B
162	36	B	1.75	2.24	C
163	25	B	1.77	2.11	D
164	41	B	1.59	4.80	C
165	39	DB	1.89	5.80	C
166	24	-	1.81	3.10	-
167	32	B	2.26	2.12	C
168	17	B	2.21	4.00	C
169	19	-	1.95	5.45	-
170	15	B	1.54	2.97	B
171	21	B	2.29	2.10	D
172	23	LB	1.83	2.96	C
173	18	B	2.15	4.61	D
174	30	DB	1.66	1.98	C
175	35	-	1.83	2.42	-
176	20	B	1.90	4.74	D
177	16	B	1.96	1.94	D
178	39	LB	1.97	2.67	D
179	35	LB	2.20	3.27	C
180	21	B	1.93	2.39	C
181	20	B	2.21	2.39	D
182	19	DB	2.12	3.42	B
183	48	BK(G)	1.73	1.80	C
184	40	-	1.78	2.38	-
185	32	LB	3.28	2.51	C

No.	Age	Hair Colour	Random Reading	Red Reading	Freckle type
186	36	B	2.74	4.98	D
187	26	B	1.45	2.36	B
188	23	B	2.60	2.11	D
189	26	LB	2.44	2.26	D
190	26	-	2.00	3.16	-
191	39	B(G)	1.55	2.12	B
192	31	DB	1.34	1.71	C
193	25	BK	1.63	2.77	B
194	25	B	1.84	6.71	D
195	25	B	2.21	8.23	C
196	26	-	2.08	5.56	-
197	16	DB	2.56	2.57	D
198	22	B	1.97	3.13	C
199	24	BK	1.85	1.10	B
200	48	LB	2.69	2.70	C
201	57	B	1.74	6.40	D
202	36	LB	2.25	6.94	D
203	22	-	1.64	1.66	-
204	25	-	1.95	2.62	-
205	28	B	1.87	2.65	D
206	21	B	2.43	1.84	C
207	20	B	2.50	2.21	D
208	24	DB	1.80	2.72	D
209	15	-	3.13	3.33	-
210	22	B	1.80	2.74	B
211	35	B	2.26	2.19	B
212	46	B	1.75	2.57	D
213	40	B	2.24	3.22	D
214	24	B	2.24	2.94	C
215	27	B	2.17	2.30	C
216	28	B	2.19	6.68	D
217	40	B	2.36	5.02	D
218	37	B(G)	1.88	4.88	D
219	34	BL	2.20	2.83	C
220	54	G	1.24	5.79	D
221	19	LB	1.97	3.35	C
222	21	B	1.85	2.28	C
223	25	B	1.64	2.14	D
224	19	B	2.21	2.98	D
225	38	DB	2.19	3.42	C

No.	Age	Hair Colour	Random Reading	Red Reading	Freckle type
226	24	B	2.43	2.50	C
227	27	BL	2.71	3.80	D
228	24	BL	2.87	2.82	D
229	37	B	1.51	3.41	D
230	20	B	2.31	3.08	D
231	26	LB	2.94	6.42	D
232	54	G(B)	1.59	5.79	D
233	28	-	1.82	1.93	-
234	28	B	2.04	3.49	C
235	46	B	2.04	3.12	B
236	16	B	1.96	1.96	B
237	24	-	2.41	5.58	-
238	34	B	2.01	2.55	B
239	25	-	1.81	2.52	-
240	19	LB	1.73	2.44	C
241	21	B	1.80	2.90	C
242	19	-	3.14	3.50	-
243	38	-	1.81	2.30	-
244	20	B	2.77	2.10	C
245	31	B	1.63	6.20	B
246	27	DB	2.11	5.58	D
247	35	B	3.01	6.62	C
248	33	B	2.05	5.18	D
249	37	DB	2.42	1.84	B
250	26	B	1.92	2.51	D
251	42	DB(G)	1.65	2.13	D
252	55	B	1.47	3.28	D
253	48	B	1.87	3.51	D
254	61	DB	1.94	1.98	C
255	41	B(G)	2.12	2.50	B
256	67	DB(G)	1.86	2.93	C
257	34	B	1.92	3.38	C
258	34	B	1.90	1.86	D
259	29	B	1.86	2.48	B
260	68	G	1.70	4.30	C
261	37	BK(G)	2.04	2.06	C
262	28	DB	1.87	1.98	C
263	13	LB	1.93	2.75	D
264	30	DB(G)	1.64	1.85	D
265	15	LB	1.77	1.66	D

No.	Age	Hair Colour	Random Reading	Red Reading	Freckle type
266	15	B	2.00	2.66	B
267	15	LB	2.02	2.79	B
268	29	B	2.24	2.39	D
269	26	-	2.23	3.00	-
270	29	DB	2.49	2.45	C
271	42	B	2.30	2.09	B
272	13	B	2.25	2.91	C
273	13	LB	3.16	2.85	C
274	12	LB	2.90	5.14	D
275	18	B	1.86	2.10	D
276	19	DB	2.03	2.31	B
277	37	-	2.13	2.42	-
278	14	B	1.73	5.51	D
279	15	B	1.84	4.98	D
280	17	BL	2.56	3.00	B
281	16	B	1.91	3.96	D
282	45	DB(G)	1.61	2.98	D
283	20	-	2.02	3.06	-
284	47	B(G)	1.50	2.65	D
285	28	B	2.02	2.71	B
286	24	B	3.53	2.73	D
287	31	G(B)	1,49	1.79	D
288	20	B	1.96	3.47	C
289	30	B	2.94	2.42	C
290	15	LB	1.69	1.90	C
291	20	B	1.66	6.50	D
292	14	-	1.60	4.06	-
293	16	BL	2.73	2.64	D
294	15	DB	1.51	1.73	C
295	16	B	2.21	2.72	C
296	16	B	2.25	2.57	D
297	17	LB	2.83	4.85	C
298	46	B(G)	1.93	3.04	D
299	29	LB	1.80	2.12	C
300	39	B	1.97	7.11	D
301	30	BL	3.74	3.66	C
302	46	B	1.46	1.94	D
303	26	RB	2.87	5.97	D
304	31	DB	1.76	2.86	C
305	19	BL	2.08	4.07	D

No.	Age	Hair Colour	Random Reading	Red Reading	Freckle type
306	35	B(G)	1.10	5.68	C
307	22	B	2.09	2.76	C
308	41	B	2.93	2.61	C
309	34	B	1.87	2.58	B
310	43	DB	1.64	2.47	C
311	60	G	1.81	2.54	D
312	14	BL	1.83	2.47	D
313	14	B	1.60	2.17	D
314	13	LB	1.34	4.46	D
315	15	LB	2.28	2.68	C
316	12	LB	1.78	1.96	B
317	17	BK	1.41	1.70	B
318	14	B	1.81	2.38	D
319	15	LB	1.80	4.50	C
320	13	B	2.72	2.91	C
321	14	DB	1.94	2.55	B
322	11	DB	1.88	3.35	D
323	14	DB	2.44	9.62	D
324	10	B	2.36	2.40	D
325	22	LB	2.96	5.24	C
326	16	-	2.97	2.19	-
327	12	DB	1.73	2.31	D
328	16	B	1.70	5.06	D
329	18	LB	1.37	5.47	B
330	13	B	1.65	4.34	D
331	15	B	2.17	2.94	D
332	14	B	2.28	2.15	C
333	20	B	1.95	1.82	D
334	12	BL	1.95	2.61	C
335	55	B	1.99	3.23	D
336	23	BK(G)	1.51	2.30	D
337	19	B	1.67	3.77	D
338	21	B	2.60	2.12	B
339	19	B	2.33	2.75	B
340	17	B	1.98	2.10	D
341	28	DB	2.17	4.42	D
342	21	B	2.11	2.24	B
343	44	-	2.02	3.26	-
344	21	B	1.79	2.86	C
345	22	-	1.85	2.03	-

No.	Age	Hair Colour	Random Reading	Red Reading	Freckle type
346	13	B	1.89	2.57	D
347	20	B	2.64	3.72	D
348	14	B	2.51	2.52	C
349	30	B	1.87	1.84	C
350	15	B	3.43	2.91	D
351	19	B	1.82	2.40	D
352	21	B	1.61	2.54	C
353	50	B(G)	1.90	2.13	D
354	20	LB	2.43	2.49	C
355	33	-	1.40	5.87	-
356	19	B	2.25	6.86	C
357	15	B	2.49	2.49	C
358	31	B(G)	1.86	2.89	D
359	19	B	2.37	2.42	D
360	37	LB	2.90	2.96	D
361	66	B	2.19	2.07	D
362	35	B	2.47	2.44	D
363	17	B	2.33	2.30	C
364	20	DB	1.71	2.37	D
365	15	B	2.22	2.02	B
366	25	B	2.36	4.69	C
367	16	LB	1.93	2.54	D
368	12	BK	1.73	1.53	B
369	14	LB	2.46	3.12	D
370	13	LB	2.15	2.58	C
371	14	LB	2.09	3.35	C
372	17	B	2.40	3.88	C
373	16	B	1.92	3.22	D
374	15	LB	3.26	3.23	C
375	13	BL	3.04	2.29	D
376	16	B	2.78	2.40	C
377	19	LB	2.15	2.94	B
378	23	B	2.61	2.74	B
379	15	B	1.99	2.64	C
380	40	LB	1.96	2.77	D
381	17	B	2.11	3.07	C
382	18	B	2.21	1.94	C
383	16	BL	2.05	3.13	B
384	12	B	3.07	2.39	D
385	14	B	1.97	2.18	D

No.	Age	Hair Colour	Random Reading	Red Reading	Freckle type
386	38	B	2.09	2.74	D
387	15	B	2.41	2.10	D
388	12	DB	1.68	2.18	C
389	14	B	1.93	3.48	C
390	28	B	2.74	2.97	C
391	25	BK	1.94	2.10	C
392	13	B	2.07	2.06	B
393	15	LB	1.93	2.72	D
394	13	LB	2.36	2.64	B
395	17	LB	2.16	3.18	C
396		-	1.88	2.33	-
397	40	B	2.27	2.93	C
398	26	B	2.57	3.17	B
399	59	G	2.03	2.30	B
400	17	B	2.52	2.57	C
401	16	B	1.92	1.91	C
402	17	B	1.88	2.86	C
403	12	B	2.14	2.16	D
404	17	BL	2.37	2.05	B
405	20	B	2.08	4.80	C
406	15	BL	1.31	3.47	B
407	17	B	2.42	2.99	C
408	12	B	2.39	2.89	B
409	13	B	1.96	2.67	C
410	15	B	1.49	2.79	C
411	19	B	3.13	5.26	D
412	14	DB	1.73	2.56	C
413	13	DB	2.16	2.51	C
414	17	B	2.50	2.06	C
415	53	-	1.78	3.73	-
416	17	B	2.41	2.69	D
417	16	DB	1.71	2.13	B
418	19	-	2.00	2.23	-
419	16	B	1.89	2.27	B
420	22	B	2.42	2.13	B
421	16	B	1.86	1.88	B
422	17	-	2.34	3.53	-
423	17	DB	2.35	3.06	C
424	17	B	1.85	2.97	D
425	19	BL	2.78	3.35	D

No.	Age	Hair Colour	Random Reading	Red Reading	Freckle type
426	18	B	2.14	2.82	D
427	22	LB	2.01	5.62	D
428	22	B	2.02	2.11	C
429	16	B	2.58	2.59	C
430	16	BL	2.88	2.62	D
431	18	B	2.49	3.23	C
432	61	BK(G)	1.58	3.42	C
433	20	B	2.15	2.67	C
434	20	B	1.89	2.16	C
435	22	BK	1.90	2.83	C
436	24	B	1.85	2.82	B
437	9	-	2.58	2.74	-
438	11	B	2.00	5.23	D
439	13	B	1.69	3.10	C
440	17	R	4.50	4.49	D
441	65	G	2.18	4.74	D
442	16	B	2.94	7.13	C
443	22	B(G)	1.67	7.62	D
444	17	-	2.56	2.70	-
445	19	-	2.18	7.05	-

SERIES II - PARRAMATTA GAOL

1	29	B	2.03	2.05	B
2	18	LB	2.95	3.22	C
3	19	B	1.82	2.28	B
4	19	B	2.50	3.16	C
5	45	BK	1.12	7.71	D
6	18	B	2.05	4.80	D
7	18	B	1.21	2.69	C
8	19	R	4.17	5.33	D
9	30	B	2.15	5.34	C
10	18	B	2.05	2.22	D
11	29	B	1.71	2.37	B
12	28	B	1.88	1.96	B
13	28	LB	2.01	1.46	D
14	19	B	3.12	2.28	D
15	26	B	1.83	2.16	D

No.	Age	Hair Colour	Random Reading	Red Reading	Freckle type
16	19	B	2.54	2.10	C
17	22	BL	2.21	2.95	D
18	67	LB(G)	2.05	6.91	D
19	18	B	2.47	5.85	C
20	18	B	2.04	2.14	D
21	31	B	1.87	2.18	C
22	20	B	1.98	2.02	B
23	19	B	3.51	5.57	C
24	24	B	1.14	2.36	B
25	46	B	1.75	1.66	C
26	18	LB	2.37	2.54	D
27	34	B	1.59	1.93	C
28	19	B	1.65	1.89	D
29	22	B	1.79	2.09	C
30	18	B	2.08	2.03	C
31	28	B	1.86	5.68	C
32	52	B(G)	1.69	1.76	D
33	34	B	2.16	3.89	C
34	37	B	1.16	4.54	D
35	47	LB	1.63	1.81	D
36	46	B(G)	1.45	2.24	C
37	46	B(G)	1.58	2.66	C
38	22	B	1.71	1.94	B
39	34	B	1.57	1.94	D
40	19	LB	2.32	4.49	D
41	35	LB	1.80	2.01	C
42	24	B	1.50	1.73	D
43	39	DB	1.76	6.67	D
44	20	B	1.86	1.86	C
45	41	R(G)	1.90	5.48	D
46	29	B	1.23	1.68	C
47	30	LB	2.71	3.18	C
48	21	B	1.55	2.24	B
49	50	B	1.72	2.13	C
50	26	B	2.10	2.14	C
51	44	RB	2.24	4.31	D
52	23	B	1.85	3.73	C
53	40	B	2.08	2.12	C
54	18	B	2.26	3.25	D
55	21	LB	1.69	2.03	C

No.	Age	Hair Colour	Random Reading	Red Reading	Freckle type
56	33	DB	1.50	1.70	D
57	49	B(G)	1.60	2.60	D
58	28	B	1.29	4.88	C
59	33	B	1.96	1.53	C
60	35	B	1.43	2.37	D
61	24	DB	1.60	1.77	C
62	45	B	1.56	4.97	D
63	39	B	1.98	2.16	B
64	20	B	1.20	2.70	C
65	24	B	1.86	5.25	D
66	26	DB	1.99	1.91	C
67	19	B	1.97	1.76	D
68	36	R(G)	2.12	5.10	D
69	51	B(G)	1.89	3.86	C
70	60	G	1.78	5.20	D
71	34	B	2.55	2.29	C
72	41	LB	2.10	2.17	D
73	19	LB	1.76	1.92	D
74	29	B	2.18	4.51	D
75	24	B(G)	1.71	1.97	C
76	47	B	3.16	2.33	C
77	25	B	1.80	1.94	D
78	23	B	1.74	1.67	C
79	21	R	4.00	5.36	D
80	30	B	2.09	1.88	D
81	31	BK	1.58	1.52	C
82	36	B	1.96	1.73	D
83	24	B	1.97	2.11	C
84	24	B	2.04	1.94	B
85	51	R(G)	2.21	6.63	D
86	23	LB	1.83	1.85	B
87	27	LB	2.20	2.10	D
88	19	DB	1.76	1.95	C
89	20	B	2.05	5.16	E
90	30	B	1.92	4.57	D
91	19	B	1.91	2.23	C
92	19	LB	2.37	2.20	C
93	19	LB	2.55	3.32	D
94	26	BK	1.72	1.66	C
95	24	B	1.68	4.73	C

No.	Age	Hair Colour	Random Reading	Red Reading	Freckle type
96	38	B	1.70	1.49	D
97	60	LB(G)	2.13	1.77	D
98	23	B	2.33	2.41	C
99	37	B	2.04	2.31	B
100	35	RB	2.16	4.90	D
101	21	B	2.54	2.06	B
102	24	R	5.22	7.35	D
103	20	B	1.63	1.78	D
104	29	B	2.16	1.72	C
105	28	LB	1.78	1.94	D
106	28	LB	2.33	4.38	D
107	18	B	2.00	6.19	C

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