PLATELETS

AND

ASCORBIC ACID

THESIS

SUBMITTED FOR THE DEGREE OF

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BY

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1968

Copy B

Department of Pathology,
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July 7th, 1969.

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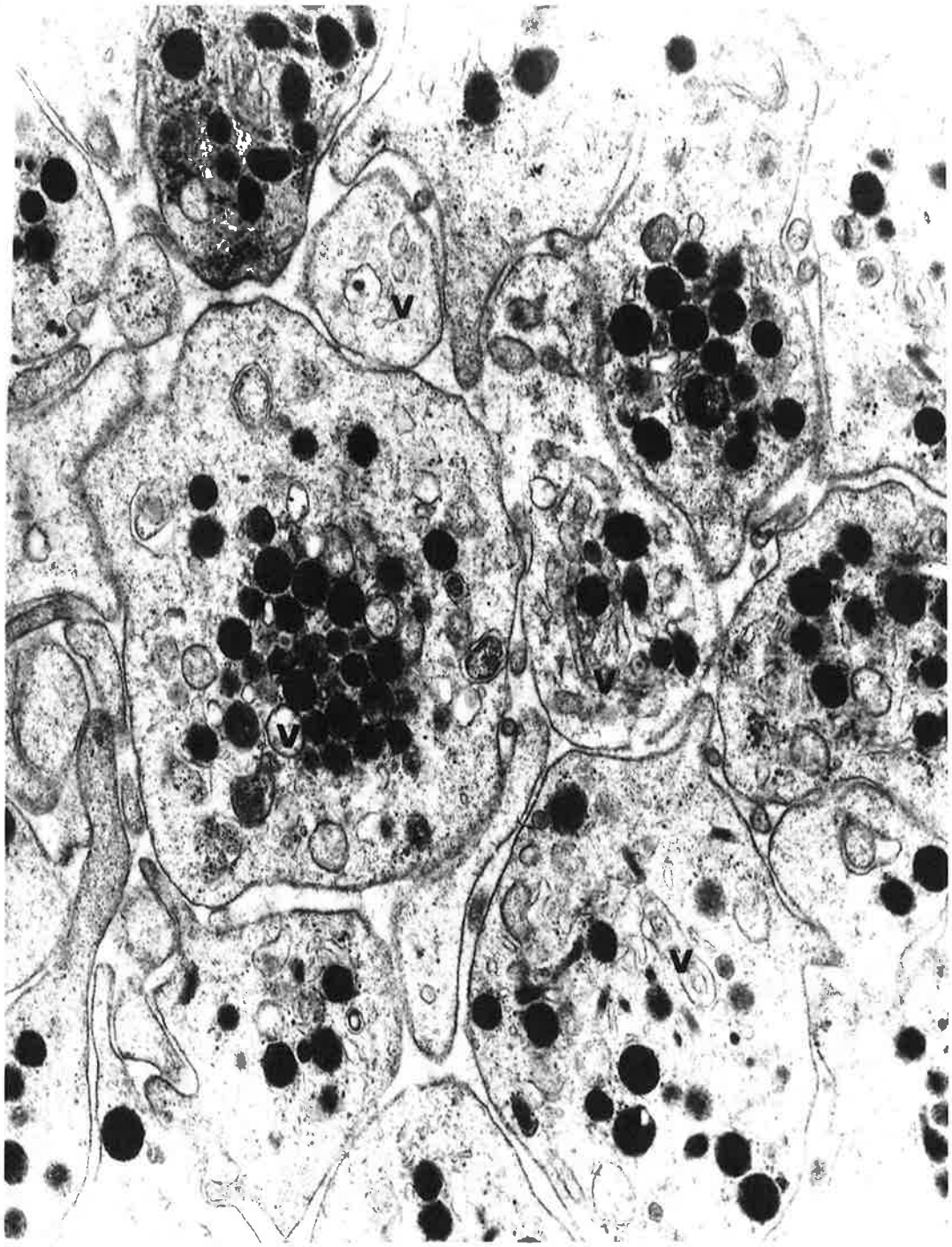

PLATE 1: Platelets viewed by electron microscopy. Specimens fixed and sectioned by the author and subjected to microscopy by Dr. G.E. Rogers, Reader in Biochemistry, University of Adelaide. (V = vesicle.)

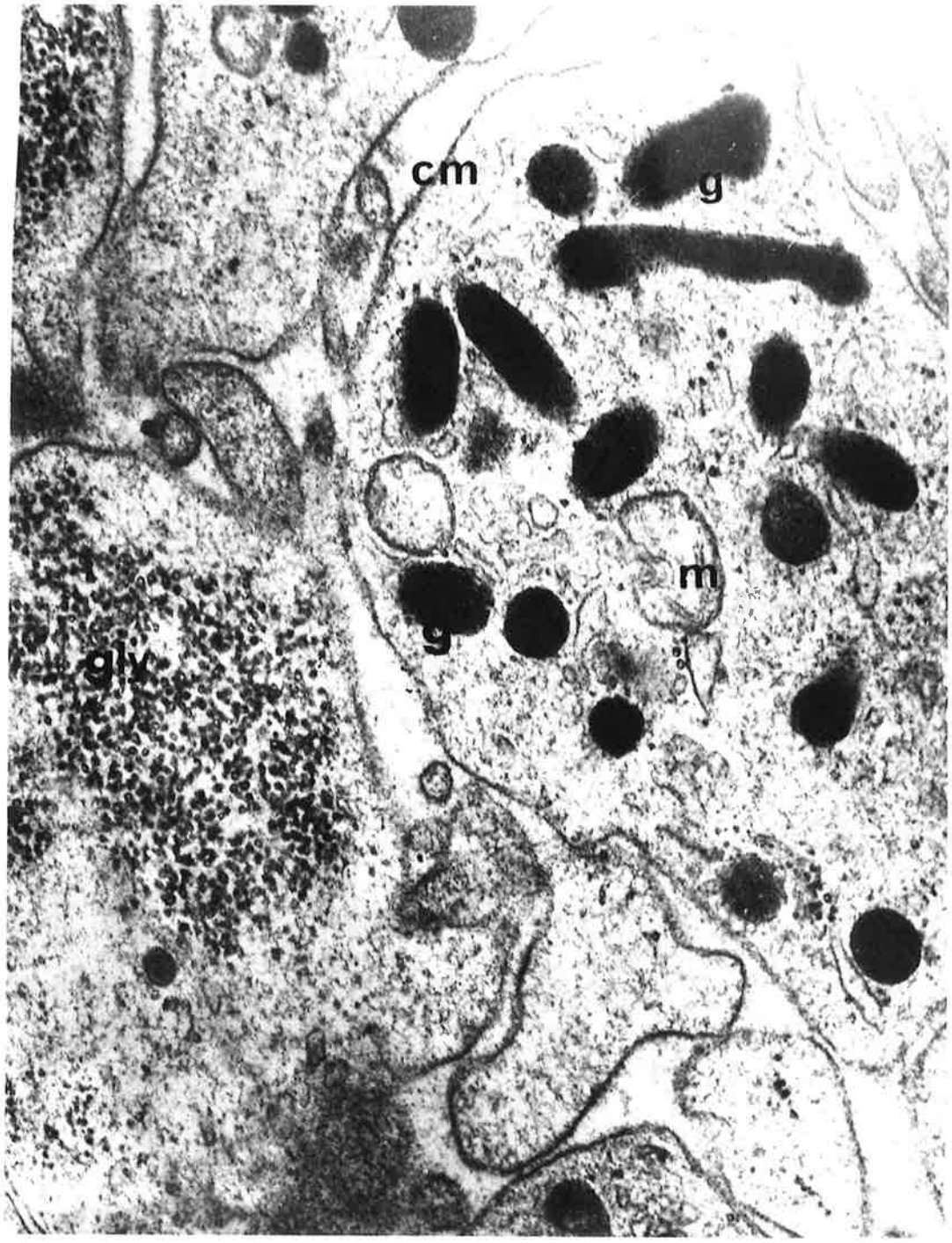

PLATE 2: Platelets viewed by electron microscopy. Specimens fixed and sectioned by the author and subjected to microscopy by Dr. G.E. Rogers, Reader in Biochemistry, University of Adelaide.

(cm = cell membrane; g = granule; m = mitochondrion.)

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DECLARATION OF ORIGINALITY

I DECLARE THAT THIS THESIS IS MY OWN COMPOSITION AND IS A TRUE RECORD OF THE RESEARCH DONE WHILE WORKING IN THE DEPARTMENT OF MEDICINE, UNIVERSITY OF ADELAIDE.

INTRODUCTION

INTRODUCTION

The importance of the role played by platelets in haemostasis and thrombosis has been well established. Intensive investigations are now being carried out by many workers to elucidate further the mechanisms by which platelets take part in these processes. Since it is likely that a complete understanding of the role of platelets will be at the molecular level many of these studies have been devoted to the investigation of the biochemistry of these elements. Since the early 1950's the development of methods for the separation of platelets from whole blood coupled with the development of micro-chemical techniques suitable for application to small amounts of biological sample has allowed the estimation of a large number of platelet constituents.

One substance which exists in a high concentration in platelets is ascorbic acid. Until recently the only report of the ascorbic acid content of platelets was by Barkhan and Howard (1958) who estimated that the ascorbic acid concentration in platelets was some twenty times as high as that in the plasma. Wilson et alii (1967) have now reported values which are some three hundred times those stated by Barkhan and Howard.

Taken with the finding of a high concentration of ascorbic acid in platelets the severe purpura which occurs in scurvy

suggests that ascorbic acid may be important in platelet metabolism. That platelet function is in fact abnormal in scurvy has been shown recently by studies demonstrating an impairment in the ability of platelets from humans and guinea pigs suffering from scurvy to stick to a glass surface (Born and Wright, 1967; Wilson et alii, 1967).

The essential role which ascorbic acid plays in tissue metabolism is emphasised by the fact that animals which cannot synthesise their own ascorbic acid will die of scurvy if deprived of vitamin C in the diet. Yet, despite an enormous amount of work devoted to this subject, the nature of this role remains unknown. Ascorbic acid does not appear to act as a specific cofactor in enzyme reactions as do the other vitamins, and though its function may be related to its action as a redox potential buffer, this remains to be proven. Since it is likely that the function of ascorbic acid in platelets is either related to or the same as its function in other cells, studies of its function in platelets could eventually lead to a better understanding of its function in other tissues. Because platelets are readily isolated from whole blood and are easily handled in vitro they provide a convenient model for the study of ascorbic acid metabolism.

An essential tool in the study of ascorbic acid is a method for its estimation in biological samples. A review of the

literature revealed that methods commonly used have the disadvantage that they are not specific for ascorbic acid. Those methods which depend on a measurement of the reducing power of the sample also measure other reducing substances present; methods which depend on the formation of the 2,4-dinitrophenylhydrazone derivative of dehydroascorbic acid are subject to interference from other substances which form hydrazone derivatives. It is surprising that, in view of the obvious importance of ascorbic acid in metabolic processes, better methods have not been devised.

A property of ascorbic acid which lends itself to further investigation is its ability to enhance the absorption of iron from the intestine (Moore et alii, 1939). It is usually claimed that this is due to the reduction of ferric ions to ferrous ions (Hahn et alii, 1945); ferric ions, unlike ferrous ions, are insoluble at the alkaline pH in the duodenal lumen from which site iron is absorbed. This does not explain, however, the fact that ascorbic acid also enhances the absorption of ferrous ions (Greenberg et alii, 1957). It has been shown that iron can also be maintained in solution in the duodenum by the formation of metal-chelate complexes between iron and ascorbic acid (Charley et alii, 1963; Davis and Deller, 1967), and that substances which can complex iron in this way can alter the rate of iron absorption (Stitt et alii, 1962; Davis and Deller, 1967). If complex

formation occurs when ascorbic acid is added to iron, this could explain its effect on iron absorption.

Because of the importance of platelets in haemostasis and thrombosis and the importance of ascorbic acid in metabolic processes, the investigations described in this thesis were devoted to the study of some aspects of the ascorbic acid content of platelets. The main objects of this work were:

1. To develop a more specific method for the estimation of ascorbic acid.

2. To determine the ascorbic acid content of normal platelets and to compare these results with the values previously reported.

3. To study the effect of variation in the dietary intake of ascorbic acid on the platelet ascorbic acid content.

4. To determine whether the ascorbic acid content of platelets differs from normal in diseases in which the platelets are thought to be affected.

5. To study the mechanism by which platelets maintain a higher concentration of ascorbic acid than in the surrounding plasma.

6. To explore the possibility of a relationship between ascorbic acid and adenosine diphosphate induced platelet aggregation.

7. To decide whether ascorbic acid or dehydroascorbic acid will form a stable complex with iron.

CHAPTER 1

HISTORICAL REVIEW

PART A. THE NATURE AND CHEMICAL COMPOSITION OF PLATELETS

I. EARLY HISTORY

The first person to recognise the platelet as a formed element of the blood was Donné who, in 1842, reported that in addition to red globules and white globules, there were little globules "les globulins du chyle". Similar small bodies which tended to form clumps were described in 1846 by Zimmerman and in 1865 by Schultze.

It was soon realised that platelets might have an important function. In 1878, Hayem suggested that platelets may accelerate blood coagulation and Bizzozero (1882) showed that the white portion of a thrombus consisted almost entirely of platelets. Hayem (1882) observed that platelet aggregation was responsible for haemostasis in the jugular veins of dogs and both he and Bizzozero found that platelets would collect at a site of injury to a vessel wall to form a thrombus. In 1883 Krauss described a reduced platelet count in a case of haemorrhagic purpura showing that platelets are important in maintaining vascular integrity.

At this time many workers still believed that platelets were merely artefacts produced during collection of blood. Bizzozero (1882) removed many of these doubts by observations on platelets actually circulating in living animals. He noticed that platelets

which had aggregated were more sticky and had changed in appearance, and he called these changes "viscous metamorphosis". Even after this publication many workers still claimed that the platelets were merely either fibrin deposits or bacteria. The majority of these workers were basing their conclusions on dried smears of blood. Later the introduction of new staining techniques enabled Dominici (1900) to describe platelets as formed elements without a nucleus.

Early workers considered that the platelets were derived from the red cells or the normoblasts (Tocantins, 1948). Dominici (1900) thought they were derived from mononuclear cells. In 1906 and again in 1910 Wright published work which demonstrated clearly that platelets arose from megakaryocytes. He was able to show granules, with staining characteristics identical to platelet granules, in the pseudopods of megakaryocytes. Clusters of these granules could be seen to be separated from the rest of the cell by a zone of hyaline cytoplasm and platelets could be identified in the vicinity of the pseudopods. The process of platelet formation from megakaryocytes has now been followed by electron microscopy (Yamada, 1957) and by microcinematography (Kinosita and Ohno, 1961).

II. STRUCTURE

Platelets are very small cells with a diameter of only about 2 μ. Using light microscopy Wright (1910) recognised that platelets

contained granules, and some workers, for example, Deetjen (1901) and Perroncito (1920) claimed that platelets possessed nuclei. Deetjen also observed that platelets have pseudopodia, and this has since been confirmed by many workers.

Early in its development electron microscopy was applied to platelets, for the ability of platelets to spread out on a membrane to form a flattened disc, which was more transparent to an electron beam, facilitated the use of this technique (David-Ferreira, 1964). Wolpas and Ruska (1939) were the first to describe the electron microscopic appearances of platelets. Bessis (1950) and Rebuck (1949) showed that platelets fixed as soon as possible after collection were disc shaped, but that as the time between collection and fixation increased protoplasmic processes or pseudopods appeared. The pseudopods "by progressive enlargement are transformed into thin sheets (transitional form), which coalesce to form a thin veil of hyaloplasm (spread form)". The final spread form has a "round, ovoidal, or polygonal shape, with clear-cut edges devoid of processes or having only a few tips corresponding to rests of pseudopodia" (De Robertis et alii, 1953).

The full elucidation of the ultrastructure of the platelet was not possible prior to the introduction of ultrathin sections. The first observations using this technique were by Bernhard and

Leplus (1955), by De Marsh et alii (1955), and by Rinehart (1955). Since then many other publications have appeared describing the ultrastructure of platelets (reviewed by David-Ferreira, 1964).

Electron microscopy of thin sections (Plates I and II) has shown that the platelet does not possess a nucleus. A limiting membrane which consists of two dense layers separated by a less dense layer surrounds the ground cytoplasm which is homogeneous and finely granular. Suspended in the cytoplasm are the dense granules which correspond to the azurophilic granules seen by light microscopy (Rinehart, 1955). These granules are oval in shape, 120-300 mμ long, finely granular in appearance, and are surrounded by a limiting membrane.

Also in the platelet thin section are one or two small mitochondria. Some authors, for example Rinehart (1955), believe that the dense granules are derived from the mitochondria, but this is denied by Jones (1960) who has followed the process of formation of the dense granules in megakaryocytes.

Clear elements or vacuoles are seen in the cytoplasm and, depending on the method of fixation, microtubules are visible (Sandborn et alii, 1966). David-Ferreira (1964) concluded that some of the tubules and vacuoles result from pinocytosis and that the possibility that others arise from the Golgi apparatus cannot be excluded.

In some platelets very dense granules less than 200 Å in diameter are seen in the ground cytoplasm. The work of Jean and Gautier (1961) and David-Ferreira and David-Ferreira (1962) supports the contention that these are glycogen granules and not ribosomes.

III. FUNCTION

The main physiological function of the platelet is its role in haemostasis and the maintenance of vascular integrity, and its main pathological function is its role in thrombus formation.

1. Haemostasis is largely attained by the formation of a plug of aggregated platelets at the site of the break in the vessel wall where the platelets are bound together by a fibrin meshwork (Marcus and Zucker, 1965). This was originally demonstrated in the mesentery of an experimental animal by Bizzozero (1882) and in the jugular veins of dogs by Hayem (1882). It has since been confirmed in similar experimental models by Zucker (1947), by Berman and Fulton (1961), and by Roskam (1961). Jorgensen and Borchgrevink (1963) showed that platelet plugs were responsible for haemostasis after small cuts in human skin. Recently Johnson (1966) reported experiments on haemostasis in the small vessels of guinea pig mesentery. Her technique was more physiological than that of other workers for she did not wash the

effluent blood away from the site of injury by continuous irrigation. She showed that"in arterioles 80 to 160 μ in diameter bleeding was arrested not by a platelet plug but by a red cell capsule." In this capsule, however, were "large platelet aggregates from which a fibrin network emanated entrapping individual red blood cells."

After platelets have aggregated at a site where haemostasis or thrombosis is in progress they undergo viscous metamorphosis. During this process the contents of the dense granules are liberated and the platelet membrane is ruptured. This process is probably responsible for the liberation of substances required for further platelet aggregation and for the initiation of blood coagulation (Adelson et alii, 1961).

(a) Platelet stickiness and platelet aggregation. A breakthrough in the understanding of the mechanism by which platelets adhere to a foreign surface and to each other was made in 1960 by Hellem. He showed that a substance extracted from red cells caused an increase in the tendency of platelets to adhere to glass. This substance was subsequently identified as adenosine diphosphate (A.D.P.) by Gaarder et alii, (1961). These studies have stimulated a large amount of work in many different laboratories to find out more about the reaction between platelets and adenosine diphosphate. Some workers measure platelet

stickiness to glass by counting platelets before and after passage of blood or platelet rich plasma through a column of glass beads (Hellem, 1960). Others use the technique of Wright (1941) in which blood is rotated in a glass bulb with measurements of platelet counts before and after a period of rotation. A widely used technique for measuring the adherence of platelet to platelet is the turbidometric method developed independently by Born (1962) and O'Brien (1962). In this simple and reproducible technique the absorbance of stirred platelet rich plasma is measured at about 500 mμ in a spectrophotometer. After A.D.P. is added the platelets aggregate into many small clumps and the absorbance decreases.

Using these techniques it has been shown that there is sufficient adenosine triphosphate (A.T.P.) in normal circulating platelets to account for the amount of A.D.P. which is required to cause platelet aggregation (Kaser-Glanzmann and Luscher, 1962). Thus it is thought by some people that platelet aggregation is initiated by hydrolysis of A.T.P. in the platelet to A.D.P., and that this liberated A.D.P. triggers platelet aggregation. Castaldi and Firkin (1962) found, however, that little or no change occurred in platelet A.T.P. levels for three minutes after recalcification of platelet rich plasma at which time platelet aggregation had already occurred.

Johnson (1966) estimated the total A.T.P. and A.D.P. content of shed blood and found that in whole blood 90% of the adenine nucleotides were in the red cells and only 10% in the platelets. About one half of the total blood A.T.P. was converted to A.D.P. fifteen seconds after bleeding began and before much platelet aggregation had occurred. It appeared therefore that much of the A.D.P. in normal haemostasis could be supplied by red cells rather than platelets.

Substances which will inhibit A.D.P.-induced platelet aggregation if added to platelet rich plasma some minutes prior to adding A.D.P. are adenosine and many adenosine analogues such as adenosine monophosphate (A.M.P.), A.T.P., and 2-chloroadenosine (O'Brien, 1963). Born et alii (1965) have compared the effectiveness of 30 analogues of adenosine as inhibitors of A.D.P.-induced platelet aggregation.

Other substances which cause platelet aggregation in vitro are 5-hydroxytryptamine (serotonin), norepinephrine, epinephrine and thrombin (O'Brien, 1964a). Serotonin is an important constituent of platelets (Born, 1963) and thrombin is generated by the coagulation mechanism at sites of haemostasis. Zucker and Borrelli (1962) found that collagen fibres induced platelet aggregation, and that the aggregated platelets adhered to the collagen particles.

The effect of A.D.P. on platelets in vivo has been investigated. Using rabbits it was shown that when A.D.P. was applied to a blood vessel of the cerebral cortex after a minor injury to the vessel the formation of platelet emboli was induced (Honour and Mitchell, 1963). During an adenosine infusion into the carotid artery a slightly higher concentration of A.D.P. was required to produce the same effect (Born et alii, 1964). The intravenous administration of A.D.P. caused a transient thrombocytopenia in cats (Born and Cross, 1963), rabbits (Regoli and Clark, 1963), humans (Davey and Lander, 1964), and sheep (Lloyd et alii, 1965). This effect is usually considered to be due to a transient adhesion of a proportion of the platelets to vascular endothelium, probably in the splanchnic and pulmonary areas (Davey and Lander, 1964).

Attempts have been made to find a non-toxic drug which will inhibit platelet aggregation in vivo. It is hoped that such a drug could act as a prophylactic against thrombosis. Drugs investigated so far are persantin with promising initial results in rabbits (Emmons et alii, 1965a), and in man (Emmons et alii, 1965b), and glyceryl guiacolate which produced decreased platelet adhesiveness in 22 healthy volunteers (Eastham and Griffiths, 1966).

(b) Structural changes during clotting. The changes in platelets during haemostasis (Kjaerheim and Hovig, 1962), and

during in vivo thrombosis (Vassalli et alii, 1964) are essentially those of platelets in recalcified platelet rich plasma as described by Rodman et alii (1962) and Castaldi et alii (1962). Four main phases have been recognised (Marcus and Zucker, 1965).

(i) Preagglutination: When calcium is added to citrated platelet rich plasma no change is observed until just prior to the onset of aggregation when the dense granules congregate near the centre of the platelet and pseudopods appear.

(ii) *Agglutination:* More pseudopods appear and the platelets adhere to one another. The concentration of granules at the centre of the platelets becomes more marked and fibrin can be seen at the periphery of the aggregates but not between the platelets. The platelet limiting membranes mostly remain intact.

(iii) Thrombocytorrhexis: The platelets lose their granules and the external membranes of platelets at the centre of the aggregate disintegrate. At the periphery of the aggregate the external membranes remain intact and fibrin becomes more prominent.

(iv) Thrombocytolysis: The platelets disintegrate to leave granular debris, sac-like protrusions at the periphery, and fibrin.

When A.D.P. is added to platelet rich plasma the platelets

aggregate but remain intact, the platelet granules do not disintegrate, and fibrin is not formed (Hovig, 1962; Rodman et alii, 1963). Thus the role of A.D.P. is probably restricted to aggregation. Other substances, possibly thrombin, must be responsible for thrombocytorrhexis.

There is some confusion in the literature about the meaning of the term "viscous metamorphosis". Eberth and Schimmelbusch used it to describe aggregation (Sharpe, 1965) but Wright and Minot (1917) applied it to the process whereby platelets which have aggregated proceed to "fuse with one another" so that "their separate identity becomes lost or obscure". Most workers apply the term to the stages of thrombocytorrhexis onwards (Sharpe, 1965) but Marcus and Zucker (1965) recommend its use for the whole process including the initial aggregation. In this thesis the term "viscous metamorphosis" will be used to apply to the stages of thrombocytorrhexis and thrombocytolysis.

2. *Thrombosis.* There is considerable evidence that thrombosis is initiated by the aggregation of blood platelets at a site of endothelial damage (Honour and Ross-Russell, 1962). The platelet aggregate forms the white head of the thrombus and the coagulation mechanism becomes activated resulting in the red tail which consists mainly of red cells trapped in fibrin (Egeberg, 1966; O'Brien, 1964b). Johnson (1966) considers that

in experimental thrombosis the same reactions are involved as in haemostasis: the formation of fibrin, the generation of A.D.P. from A.T.P., platelet aggregation, red cell lysis, and the adherence of red cells to each other. In thrombosis, however, there is (i) more damage to the vessel wall than in haemostasis, (ii) much less fibrin is formed, (iii) there is a much greater tendency for platelet aggregates to adhere to the vessel wall, and (iv) the importance of red cells in the genesis of platelet aggregation is minimal (Johnson, 1966).

3. *Atherogenesis.* An increasing amount of evidence is being gathered to support the hypothesis that deposition of platelets on the walls of blood vessels is responsible for atherogenesis. Duguid (1948) found fibrinous encrustations on normal intima but did not mention platelets. Movat et alii (1959) have demonstrated small platelet-fibrin thrombi on normal intima and have suggested that these deposits become incorporated into the intima to form atheromatous plaques. This encrustation hypothesis which was originally proposed by Rokitansky (1842-1846) had been discarded in favour of the lipid imbibition hypothesis until it was revived by Duguid (1948).

Though the lipid imbibition hypothesis is still generally favoured considerable evidence has now been accumulated to support the encrustation hypothesis and this has been reviewed by Mustard

et alii (1964). Chandler and Hand (1961) have observed the phagocytosis by monocytes of platelets which are rich in lipid. The monocytes were transformed into lipophages similar to those seen in human platelet thrombi or in atherosclerotic plaques. These workers have also produced atheromatous lesions in the pulmonary vessels by the intravenous injection of artificial thrombi consisting largely of platelets (Hand and Chandler, 1962).

Platelets have been shown to come more readily into contact with arterial walls at sites of turbulence (Hadfield, 1950) which occur near vessel origins and bifurcations. These are also the sites of maximum atheroma formation (Mustard et alii, 1961). Similar findings have been obtained in studies of patterns of turbulence and of platelet deposition in silicone coated plastic tubes inserted into the circulations of pigs (Mustard et alii, 1961).

Thus evidence is accumulating to suggest that not only does the platelet play a primary role in the formation of the thrombus which blocks an atheromatous artery, but that it may also play an important role in the formation of the atheromatous deposits themselves.

4, Transport. The platelet has been regarded by some as a method of transportation for certain substances in the bloodstream

(Humphrey and Jacques, 1954; Maupin, 1961). It may be important for a substance to be carried in the bloodstream rather than just free in the plasma for one of several reasons.

(a) The substance may produce unwanted effects if allowed to circulate free in the plasma. Serotonin, for instance, is carried in the bloodstream inside platelets. Arguing teleologically, this prevents it from coming in contact with receptors in the vessel walls and causing vasoconstriction.

(b) The platelet may be required to deliver substances to particular sites. (i) Platelets carry plasma coagulation factors, possibly adsorbed on the platelet surface (Adelson et alii, 1961; Barkhan and Silver, 1962), and platelet factor III (Barkhan and Silver, 1962) which is probably lipoprotein (Marcus et alii, 1966). Since platelets are attracted in large numbers to sites of haemorrhage or thrombosis, they may be responsible for providing a concentration of coagulation factors at this site. (ii) It is probable that platelets contribute to the integrity of the vessel wall. Platelets are antigenically similar to the endothelium of blood vessels (Baldini, 1966) and electron micrographs have shown platelets within endothelial cells (Johnson et alii, 1964). When Cronkite et alii (1961) labelled rat platelets with S^{35} and injected them into thrombocytopenic rats, the S^{35} was

later detected in the blood vessel walls. Mustard et alii (1965) came to similar conclusions after injecting S^{35} into normal pigs.

5. *Phagocytosis.* David-Ferreira (1964) noted the ingestion of thorium oxide by platelets. The colloidal material was contained "in tubules which rounded up into typical phagocytic vacuoles". By a process of phagocytosis platelets can engulf silicon dioxide (Schulz, 1961), ferritin (Schulz, 1961; Hagenau et alii, 1963) and latex particles (Glynn et alii, 1965). After fat infusion and in patients with hyperlipaemia, phagocytosis of fat particles by platelets occurs (Schulz and Wedell, 1962).

IV. THE SURVIVAL OF PLATELETS IN CIRCULATION

This subject has been recently reviewed (Mustard et alii, 1966; Baldini, 1966; Gardner and Cohen, 1966). In modern techniques for the measurement of platelet survival the platelets are labelled with a radioactive isotope. Blood samples are taken at daily intervals thereafter and the radioactivity of the platelets determined. The most commonly used techniques are (i) labelling in vitro by $chromium^{51}$ followed by reinfusion of the labelled platelets into their donor (Aas and Gardner, 1958; Davey and Lander, 1963), and (ii) labelling in vivo with $D.F.P.^{32}$ (Leeksma and Cohen, 1956; Barkhan, 1966a). Using these techniques platelet

lifespan in humans is estimated to be 9-11 days. The survival of platelets has been thought by some workers using the chromium^{51} technique to be linear with respect to time (Aas and Gardner, 1958; Baldini, 1966). Other workers using D.F.P.^{32} have found an exponential relationship (Murphy et alii, 1962; Adelson et alii, 1963). A linear curve is consistent with removal of each platelet from the circulation at the end of a lifespan of about 10 days and an exponential curve is said to suggest removal by a process of random destruction (Davey, 1966). Mustard et alii (1966) have proposed a multiple hit model to explain the variable results found.

V. CHEMICAL COMPOSITION

The first chemical analyses of platelets were by Abderhalden and Doetjen (1907) who investigated the proteases of platelets. Aynaud (1914) analysed platelets from the horse and donkey and found phosphorus, iron, sulphur and calcium. Calorimetric determination by Haurowitz and Sladek (1928) showed that platelets contain 71% protein, 12% lipid, 1-7% cholesterol, and 5.5% of ash. Other early workers are mentioned by Tocantins (1938).

Most of the work on the chemical composition of platelets has been done since the early 1950's when differential centrifugation techniques for the separation of platelets from whole blood were developed (Dillard et alii, 1951; Hirsch et alii, 1952; Minor and

Burnett, 1952; Tullis, 1952). One of the main reasons that this subject received little attention in the first half of this century was that large volumes of blood were required to obtain sufficient platelets on which to perform a chemical estimation. In the experience of the author 30 ml of blood provides only about 0.06 ml of platelets. When Bettex-Galland and Luscher (1961) extracted the contractile protein, thrombosthenin, they used the platelets from 50 litres of human blood which yielded 20-30 ml of packed platelets! Now, with the development of microchemical methods, the analytical chemistry of platelets has received more attention. The published work on the chemical composition of platelets has been reviewed by Maupin (1961), Bettex-Galland and Maupin (1961), Barkhan and Silver (1962), and Marcus and Zucker (1965).

1. Ascorbic acid. In the 1940's several publications appeared on the ascorbic acid content of the buffy layer of centrifuged blood (Butler and Cushman, 1940; Bessey et alii, 1947). This layer contains platelets and white cells and it was not known at this time whether ascorbic acid was predominantly in platelets, predominantly in white cells, or whether it was equally distributed between the two. Crandon et alii (1940) found that when a volunteer was given a diet free of ascorbic acid the plasma concentration of ascorbic acid decreased to very low levels over a period of 3-4 weeks. The buffy layer content of ascorbic acid

fell more slowly and did not reach zero levels for some months, just prior to the onset of scurvy. These findings have been confirmed by the Vitamin C Subcommittee (1953). Most workers now regard the buffy layer ascorbic acid content as a reasonable index of tissue stores and the plasma level as an index of recent intake (Pearson, 1966).

Jordanov et alii (1956) noted blackening of both platelets and white cells after treatment of these cells with silver nitrate. This blackening appeared to increase after large doses of ascorbic acid suggesting that both platelets and white cells contain ascorbic acid. Barkhan and Howard (1958) were the first to publish on the concentration of ascorbic acid in platelets which had been separated from white cells. Using the 2,4-dinitrophenylhydrazine method they found that the level in platelets from six normal subjects was 18.3 mg/100 G which was similar to that in white cells (16.5 mg/100 G). Expressed as a proportion of cell count these results are 2.91 mg/10^{12} cells for platelets and 148 mg/10^{12} cells for white cells. The apparent discrepancy in these figures is due to the fact that a platelet is very much smaller than a white cell. They also estimated the ascorbic acid concentrations in plasma (0.69 mg/100 ml) and in red cells (0.69 mg/100 ml), and were then able to calculate that of the total blood ascorbic acid about 10% was in white cells, 10% was in platelets, and 80% was equally distributed

between red cells and plasma. By saturating their subjects with ascorbic acid they were able to raise the ascorbic acid content of the platelets by about 30% while the concentration in the white cells was almost doubled. They also studied three cases of chronic myeloid leukemia and two cases of chronic lymphatic leukemia. Though the ascorbic acid content of leukocytes was higher than normal in chronic lymphatic leukemia, the platelet concentration of ascorbic acid did not differ significantly from normal in either type of leukemia.

The only other reported value for the ascorbic acid content of platelets is that given by Wilson et alii (1967). Using the dinitrophenylhydrazine technique they found a mean of 858 $\mu g/10^9$ platelets in eight normal subjects. The mean concentration of ascorbic acid in the leukocytes was only 43 $\mu g/10^9$ cells and the mean plasma level was 0 µg/100 ml. It is generally accepted that the normal plasma level is well over 200 µg/100 ml (Pearson, 1966). These authors do not comment on the fact that their results differ so greatly from those published by other workers.

The function of ascorbic acid in platelets was unknown until Born and Wright (1967) found that platelet adhesiveness to glass measured by the rotating bulb method was significantly decreased in scorbutic guinea pigs. This report was soon followed by the paper of Wilson et alii (1967) describing reduced platelet

adhesiveness as measured by the glass bead column technique in two patients with scurvy. These results suggest that the purpura of scurvy may be due to a defect in platelet adhesiveness.

Other possible reasons for the high ascorbic acid content of platelets have been proposed. Maupin (1961) suggested that ascorbic acid is merely using platelets as a passive vehicle for transport. It is conceivable that platelets are required to deliver a high concentration of ascorbic acid to a site where haemostasis is occurring, or that they carry ascorbic acid to the vascular endothelium. Barkhan (1966b) has pointed out that the concentration in ascorbic acid in platelets is similar to that in white cells and has suggested that, since both platelets and white cells are capable of phagocytosis, ascorbic acid may be necessary for phagocytosis. This suggestion is supported by the finding of a reduced phagocytic activity in the leukocytes of scorbutic guinea pigs (Lawrynowicz, 1931; Cottingham and Mills, 1943).

Two other items in the literature relate to the ascorbic acid content of platelets. Denson and Richards (1962) published on the uptake of ascorbic acid by blood cells and included the results of one experiment on the uptake of ascorbic acid by platelets. Gibson et alii (1966) pointed out that the buffy layer ascorbic acid content was likely to be low in patients with thrombocytopenia.

2. Other substances "transported" by platelets. Under the heading of "substances probablement non constitutives, véhiculées par les plaquettes" Maupin (1961) included the three amines adrenaline, histamine and serotonin, the proteins of the "atmosphère plasmatique", and ascorbic acid. This classification may be more convenient than accurate, as many of these substances have since been shown to have important effects on platelet behaviour.

(a) Serotonin (5-hydroxytryptamine). Almost all the serotonin in the blood is contained in the platelets (Humphrey and Jacques, 1954). When platelets were separated from blood with minimal damage to the platelets, the amount of serotonin in the plasma was only about 0.002 µg/ml whereas the total blood serotonin varied from 0.19 µg/ml in man to 5.2 µg/ml in rabbits (Humphrey Jacques, 1954).

Platelets concentrate serotonin from the surrounding medium by an active transport mechanism which is inhibited by enzyme inhibitors such as sodium mono-iodoacetate, potassium cyanide, dinitrophenol (Born and Gillson, 1958, 1959; Sano et alii, 1958), fluoride, and cardiac glycosides (Weissbach and Redfield, 1961). There is evidence that platelets do not contain serotonin when they are formed from megakaryocytes, and that they take up this

substance while they are passing through the blood vessels in the walls of the intestine (Toh, 1954; Erpsamer and Testini, 1959).

It is possible that serotonin in the platelet is bound to A.T.P. (Born, 1963). Davis and Kay (1965) tried to determine the location of serotonin in the platelet by electron microscope autoradiography using tritium-labelled serotonin. Firm conclusions were not possible because of possible movement of serotonin during fixation. Wurzel et alii (1965) found that the platelet granules obtained by separation on a continuous sucrose gradient contained tightly bound serotonin. The platelet membrane fraction did not contain bound serotonin, but free serotonin was probably present. This free serotonin may have originated from disrupted platelet granules.

It has been shown that serotonin is released from platelets by thrombin (Zucker and Borrelli, 1955) suggesting that it may aid haemostasis by inducing vasoconstriction, but Berman and Fulton (1961) failed to obtain visible vasoconstriction during the experimental production of thrombi in arterioles and venules.

Under certain conditions serotonin has a direct effect on platelets: it causes platelet aggregation both in vitro (O'Brien, 1964a) and in vivo (Honour and Mitchell, 1963) and in vitro this effect is inhibited by specific antiserotonins, by adenosine and by A.M.P. (O'Brien, 1964a).

(b) Epinephrine and norepinephrine. Whether epinephrine and norepinephrine are present in higher concentrations in platelets than in plasma is controversial. Weil-Malherbe and Bone (1954) found that 70-80% of both epinephrine and norepinephrine in human platelet rich plasma could be removed by centrifugation. Born et alii (1958) estimated that pig platelets contained 0.2-2.6 µg epinephrine per 10^8 platelets but no detectable norepinephrine. Unfortunately they did not state the concentrations in platelet poor plasma. They found that pig platelets would concentrate epinephrine and norepinephrine which had been added to platelet rich plasma. On the other hand Valk and Price (1956) detected no catechol amines in human platelets and Sano et alii (1959) reported that the concentration of epinephrine in rabbit platelets was very low (about 0.01 µg/mg dry weight of platelets). Sano et alii (1959) found that platelets would concentrate added epinephrine by an active transport mechanism. Dog platelets contained negligible amounts of epinephrine and norepinephrine (Weissbach et alii, 1958), but binding of these substances to platelets occurred when large amounts were infused into the bloodstream.

All the experiments showing that platelets concentrate epinephrine and norepinephrine have been performed in the presence of high concentrations of added epinephrine or norepinephrine.

The only work showing a higher concentration in platelets than in plasma in the absence of added amounts of these substances is that of Weil-Malherbe and Bone (1954). It is only by comparing the concentration of a substance in carefully isolated fresh platelets with that in platelet poor plasma that one can determine whether platelets normally contain a higher concentration of this substance than would be expected to occur due to passive diffusion from plasma.

(c) Histamine. The histamine content of rabbit platelets is high, 2-6.7 µg/10^9 cells whereas only trace amounts (0.002 µg/ml) are found in plasma (Humphrey and Jacques, 1954). Schayer and Kobayashi (1956) have shown that histamine is produced in rabbit platelets by decarboxylation of histidine. Significant but lesser amounts were also found in platelets of other species including man (Humphrey and Jacques, 1954) and these levels were all much higher than those found in plasma. Humphrey and Jacques (1954) concluded that the histamine found in the blood of most species is almost all contributed by platelets. These findings have been challenged, however, by Graherm et alii (1955) who found that the histamine concentration in platelets was the same as that in whole blood and that most of the histamine in the blood was in the basophils.

(d) "Atmosphère plasmatique". The platelet is also important as a carrier of various clotting factors. Roskam (1922) thought that plasma proteins including clotting factors were adsorbed on the platelet surface. This view has been supported by many other workers and the evidence in favour of the "platelet as a sponge" which "adsorbs and concentrates coagulation and other factors and carries them through the circulation" is reviewed by Adelson et alii (1961). Clotting factors present in (or on) platelets include calcium, fibrinogen, prothrombin, and factors V, VII, VIII and IX. It is envisaged that when platelets are attracted to a site where haemostasis is required, the adsorbed coagulation factors are important in initiating fibrin formation.

The evidence that these factors are adsorbed on to the platelet surface rather than being inside the platelet is tenuous and rests mostly on experiments showing that these factors can be easily removed from platelets by washing. More sophisticated techniques, such as those involving the separation of cell fractions by centrifugation on a sucrose density gradient, will be required to settle this point.

3. Lipids. In 1936 Chargaff et alii separated cholesterol and phosphatides from blood platelets and investigated the effect

of these substances on blood clotting. Their discovery that platelet phospholipids are important in blood coagulation has led to the detailed analysis of these phospholipids.

Lipid comprises 15-17% of the dry weight of platelets (Marcus and Zucker, 1965; Barkhan and Silver, 1962). Of this lipid 70-80% is phospholipid (Barkhan and Silver, 1962) and the remainder is neutral lipid. Of the neutral lipid over a half is cholesterol, mostly non-esterified (Barkhan and Silver, 1962). 16% of the phospholipid is in the plasmalogen form (Zilversmitt et alii, 1961).

Many workers, including Barkhan et alii (1961) and Marcus et alii (1962) have attempted to discover which particular phospholipid is important in the initiation of coagulation by determining the activity in the thromboplastin generation test of each type of phospholipid found in platelets. The activity has been found to reside in either or both of the phosphatidyl ethanolamine fraction and the phosphatidyl serine fraction (Barkhan et alii, 1961). The activity in the thromboplastin generation test, however, depends as much on the size and shape of the phospholipid micelles as on the particular phospholipid present (Barkhan and Silver, 1962).

Marcus et alii (1966) have examined the activities of platelet membranes and platelet granules isolated on a sucrose density gradient. Platelet membranes were more effective than granules in

in vitro coagulation systems but lipids extracted from granules were as effective as platelet membranes. They suggested that lipoprotein in platelet membranes may be more available than that in granules and therefore more important in the coagulation mechanism.

4. Inorganic constituents. The inorganic cations in platelets have been studied by Maupin (1953) and by other workers. A resume of the literature on this topic is given by Maupin (1961). Metals found in platelets include sodium, potassium, calcium, magnesium, copper, iron and manganese. The values for the sodium content given by different authors show a wide variation which may be due to extensive washing procedures used by some of these authors (Maupin, 1961).

The only non-metals which have been precisely determined are nitrogen and phosphorus. 80-90% of the nitrogen is protein bound Fantl and Ward, 1956). Of the phosphorus present, 50% is acid soluble and probably comes from A.T.P.

5. Adenine nucleotides. Born (1956) was the first to demonstrate the presence of large amounts of A.T.P. (4.4 µM/G wet weight) in platelets. Schmitz et alii (1962) who recently reported a value of 23.6 µM/G wet weight have also found small amounts of uridine and guanidine nucleotides.

The function of A.T.P. in platelets is controversial. It is hydrolysed during blood coagulation (Born, 1958; Kaser-Glanzmann and Luscher, 1962) and the A.D.P. so formed may have a role in platelet aggregation (see above). A.T.P. may be necessary for clot retraction to occur (Bettex-Galland and Luscher, 1960) and its presence is said to be required for the contraction of thrombosthenin, the contractile protein found in platelets (Bettex-Galland and Luscher,1961). More recently doubt has been thrown on this concept by the finding that platelets depleted of 60-90% of their A.T.P. by thrombin still produce normal clot retraction (Zieve et alii, 1964).

6. Nucleic acids. It is now generally agreed that platelets do not contain detectable amounts of D.N.A. (Maupin, 1961). The traces found by some workers (Fantl and Ward, 1956; Morita and Asada, 1956) were probably due to leukocyte contamination (Maupin, 1961). These findings concur with the fact that electron microscopy has disclosed neither nucleus nor nuclear fragments in platelets.

Some workers have failed to detect R.N.A. in platelets (Mizuno et alii, 1958; Maupin, 1961). Others claim to have found significant amounts of this substance (Morita and Asada, 1956; Bestetti et alii, 1956; Wagner et alii, 1956). These findings must be considered in the light of the information that ribosomes

have not yet been definitely identified in platelets by electron microscopy.

7. Amino acids and proteins.

(a) Amino acids. The free amino acid content of platelets has been studied by McMenamy and Lund (1960) and by Gross and Gerok (1961). Besides all the amino acids normally found in proteins, there are unusually large quantities of taurine (Maupin et alii, 1962) which is probably derived from cysteine (Frendo et alii, 1959).

(b) Proteins. Bezkorovainy and Doherty (1962) have investigated the structural properties of platelet proteins and have found a full complement of amino acids. A contractile protein, thrombosthenin, has been isolated from platelets (Bettex-Galland and Luscher, 1961). This represented 15% of the total platelet protein and was very similar to actomyosin of skeletal muscle. It had two components, one similar to actin and the other to myosin, and contraction occurred in the presence of A.T.P. and magnesium. Fibrinogen has also been identified in platelets by many workers (Marcus and Zucker, 1965). Cooper and Firkin (1967) have described protein synthesis by platelets.

(c) Enzymes. A detailed account of the work done on platelet enzymes prior to 1961 is available (Bettex-Galland and Maupin,

1961). The platelet contains all the enzymes necessary for respiration, those of the glycolytic cycle being quantitatively more important than those of the tricarboxylic acid cycle. Also present are the hydrolytic lysomal enzymes acid phosphatase and beta-glucuronidase which are liberated into the serum during clotting. It has been suggested that these enzymes are liberated from the platelet granules some or all of which may be lysosomes (Marcus and Zucker, 1965).

8. Carbohydrates. Woodside and Kocholaty (1960) who analysed the carbohydrates of human blood platelets found a total carbohydrate content of 8.47% (dry weight). Many monosaccharides including glucose, galactose, mannose, and ribose were detected. The acid mucopolysaccharide fraction contained galactosamine, glucuronic acid, galactose, glucose and ribose. They also found glycogen and sialic acid. Madoff et alii (1964) found a large amount of sialic acid in platelets compared to erythrocytes. Odell and Anderson (1957) labelled human platelets with $S^{35}O_4$ in vivo and subjected the mucopolysaccharides to chemical analysis. They found two fractions: chondroitin sulphate and a uronic acid free mucopolysaccharide.

9. Platelet metabolism. Gross (1961) has compared the platelet to the jump muscle of the African grasshopper which works

in short bursts with long intervals of rest in between. The platelet engages in a large expenditure of energy only once, that is during viscous metamorphosis and clot retraction. The jump muscle contains very few mitochondria as does the platelet, and in both the enzymes of glycolysis are predominant over those of the tricarboxylic acid cycle (Campbell, 1956; Waller, 1959). Cells which metabolise at a high continuous level, such as the flight muscle of the grasshopper and heart muscle, have many mitochondria, and the tricarboxylic acid cycle predominates.

VI. COMMENT

Platelets are small cells which differ from most other cells in that they do not possess a nucleus. Their main roles appear to be in haemostasis, thrombosis, and the maintenance of vascular integrity. Over recent years much progress has been made in the determination of the structure and chemical composition of these elements. The work done so far is only a starting point in the unravelling of the way in which platelets perform their functions.

PART B. THE BIOCHEMISTRY OF ASCORBIC ACID

I. EARLY HISTORY

Scurvy was first adequately described at the time of the renaissance when many sailors contracted the disease during the long sea voyages which were being attempted at that time (Lind,

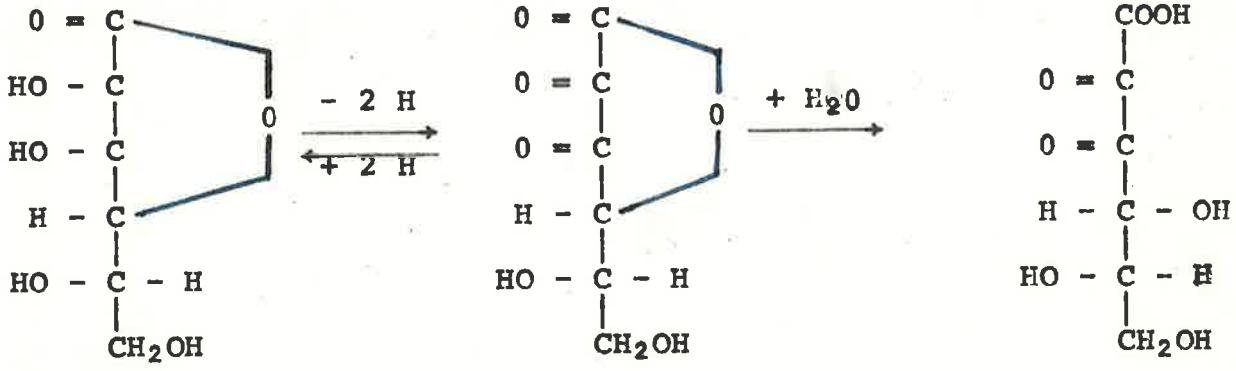
1753). James Lind, a medical officer in the Royal Navy, was the first to prove that the juice of citrus fruit causes a dramatic cure in cases of scurvy. The result of his classical clinical trial on a group of twelve sailors suffering from scurvy is described in his "Treatise on Scurvy" first published in 1753. It was many years before Lind's ideas were fully accepted, and even as late as 1912 Scott left for his fateful expedition to the South Pole with rations containing insufficient vitamin C (Stewart, 1953). During the early part of the twentieth century the concept of deficiency diseases developed. In 1907 Holst and Frolich accidentally produced scurvy in guinea pigs while attempting to induce ricketts. It was fortunate that they chose the guinea pig and not some other experimental animal. Their observations made possible the development of biological assay techniques which eventually led to the isolation of the vitamin by Waugh and King (1932). They found it to be identical to a substance called hexuronic acid previously isolated by Szent-Gyorgy (1928) from adrenal glands. In 1933 the structure of ascorbic acid was finally determined (Farmer, 1933; Hirst and Peat, 1934; Karrer et alii, 1933).

II. CHEMICAL PROPERTIES

L-ascorbic acid is a six carbon atom sugar-like compound ($C_6H_8O_6$) with a γ-lactone ring and an enediol linkage between the

second and third carbon atoms (see below). It forms a white crystalline solid which is very soluble in water and almost insoluble in organic solvents. In aqueous solutions it behaves as a dibasic acid, the hydrogen atoms on the second and third carbon atoms dissociating with pKa of 11.57 and 4.17 respectively.

The most striking chemical property is its strong reducing power which it owes to the ease with which it is reversibly oxidised to dehydroascorbic acid by loss of the hydrogen atoms on the second and third carbon atoms (see below). For this reason ascorbic acid is unstable in aqueous solution being readily broken down by reaction with atmospheric oxygen to dehydroascorbic acid, which is also unstable because it is readily hydrolysed irreversibly to 2,3-diketogulonic acid (Knox and Goswami, 1961).



Ascorbic acid | Dehydroascorbic acid | 2,3-diketogulonic acid

2,3-diketogulonic acid undergoes further degradation leading to a wide variety of products which differ according to the experimental conditions (Hirst, 1953). The nature of these

products has not been properly elucidated (Baker, 1967), but under some conditions, such as in the presence of sodium hypoiodite, oxalic acid is formed (Hirst, 1953).

Ascorbic acid is most stable at pH 3 becoming increasingly unstable as the pH is raised above this value (Uprety and Revis, 1964). At pH 7-8 in aqueous solution it is extremely unstable (Uprety and Revis, 1964). It is more unstable in the presence of traces of ionic copper or iron. These ions probably aid the transfer of electrons from ascorbic acid to oxygen (Roe, 1954). High temperatures and light also enhance the instability of ascorbic acid (Prochazka, 1963).

III. FUNCTION

If primates and guinea pigs had not lost the ability to synthesise ascorbic acid, the importance of this substance in metabolism may not have been realised today. These animals die of scurvy if l-ascorbic acid (or dehydroascorbic acid) is not supplied in the diet. Other animals which have lost the ability to synthesise ascorbic acid are the red-vented bulbul (Roy and Guha, 1958) and the Indian fruit bat (Chattergee et alii, 1961).

Despite its great importance in metabolism and despite the large number of publications which continue to appear on the subject of ascorbic acid, its function in metabolic processes is still unknown. Ascorbic acid is excreted in the urine as ascorbic acid and its breakdown products dehydroascorbic acid, diketogulonic acid and oxalic acid (Baker et alii, 1966). It is not known whether ascorbic acid is metabolised to form these breakdown products or whether they are produced by spontaneous breakdown as occurs in aqueous solution (Griffith, 1967).

1. Redox potential and ascorbic acid. The biological function of ascorbic acid may be related to its strong reducing power. It enhances the activity of many in vitro enzyme systems (Knox and Goswami, 1961), but it can be replaced in these systems by one or more reducing agents. Thus it does not function as a specific coenzyme. For instance, in the oxidation of para-hydroxyphenylpyruvic acid in vitro, ascorbic acid could be replaced by hydroquinone, 2,6-dichlorophenolindophenol, D-ascorbic acid or D-isoascorbic acid, but not by glutathione or cysteine (La Du and Greenberg, 1953). This suggested that some reducing agents provide an optimum redox potential not provided by others.

It has been shown that many enzymes operate most effectively at an optimum redox potential (Reiss, 1942-43 and 1943-44). The theory of redox potential is described in most textbooks of general biochemistry, and a detailed treatment is presented in Ziegler's monograph (1965). Prediction of the redox potential of a system, in which both reduced and oxidised forms of a molecule or ion are present, can be made by applying the Nernst-Peters equation:

$$E = E_o + \frac{RT}{nF} \log_e \frac{[Ox]}{[Red]}$$

where R = the gas constant
T = absolute temperature
n = valency
F = the Faraday
[Ox] = concentration of oxidised form
[Red] = concentration of reduced form
Eo = the potential relative to the hydrogen electrode when equal amounts of the reduced and oxidised forms are present.

This equation is of similar form to that relating pH to concentration of base and acid, $pH = pKa + \log_e \frac{[base]}{[acid]}$. The maximum buffering action of a pH system occurs when [base] = [acid]. Similarly the maximum buffering effect in a redox potential system occurs when [Ox] = [Red].

Until recently it has been impossible to measure with any accuracy the redox potential of an ascorbic acid/dehydroascorbic acid system (Hewitt, 1950). Past measurements were inadequate because of a film of suboxide which formed "on the surface of a platinum electrode, impairing or totally annulling its inertness towards badly poised solutions" (Ziegler, 1965). This effect has also interfered with the measurement of the redox potential of the blood.

Ziegler (1965) has made a major advance in instrumental design by gently polarising the electrode. This prevents deposition of oxygen on the platinum which then remains inert. Using this improved method he has been able to measure accurately the redox potential of the blood in vivo in animals and in humans. He claims that the redox potential of human blood can be accounted for by the ascorbic acid/dehydroascorbic acid system.

"The metabolic pool is maintained in a constant state of flux between the force of reduction, generated by the metabolic processes, and the oxidising force of the ubiquitous oxygen. The intensity of

these opposing influences determines the redox potential of the inner environment. The reversible redox system of the blood and tissues which, under aerobic conditions, guarantees the individual organism a definite redox potential is the ascorbic acid/dehydro-ascorbic acid system" (Ziegler, 1965).

2. *Specific defects in metabolism in scurvy.* A requirement for ascorbic acid has been claimed in several different metabolic pathways: tyrosine metabolism, folic acid metabolism, and collagen synthesis.

(a) Tyrosine metabolism. Tyrosine is metabolised via p-hydroxyphenylpyruvic acid to homogentisic acid. Scorbutic guinea pigs fed tyrosine excreted large amounts of p-hydroxyphenyl-pyruvic acid in the urine (Sealock and Silberstein, 1940). In vitro studies by La Du and Zannoni (1961) have shown that in the absence of ascorbic acid the enzyme p-hydroxyphenylpyruvic acid oxidase is inhibited by its substrate. This inhibition was prevented not only by ascorbic acid but also by other reducing substances such as hydroquinone, D-isoascorbic acid, and D-ascorbic acid in vitro, and by 2,6-dichlorophenolindophenol both in vitro and in vivo. The defect in this enzyme has also been reported in infants and adults suffering from scurvy (Woodruff, 1964). Painter and Zilva (1947) have shown that the defect is demonstrable only when very large doses of tyrosine are given, and that a larger

amount of ascorbic acid is necessary to reverse the lesion than is required to cure scurvy. For these reasons a defect in this enzyme pathway is probably not responsible for the picture of scurvy.

(b) Folic acid metabolism. There is evidence that ascorbic acid is required for the efficient conversion of folic acid to its active form tetrahydrofolic acid in slices of rat liver (Nichol and Welch, 1950) and in vivo in man (Gabuzda et alii, 1952; Jandl and Gabuzda, 1953). This has been thought to explain the megaloblastic anaemia which occurs in many cases of scurvy. In experimental human scurvy where normal amounts of folic acid are supplied in the diet, anaemia does not occur (Crandon et alii, 1940; Vitamin C Subcommittee, 1953). Similarly, monkeys on a diet devoid of ascorbic acid but containing normal amounts of folic acid do not become anaemic (Proehl and May, 1952). When guinea pigs eating an ascorbic acid free diet were fed folic acid in small amounts (amounts just sufficient to prevent anaemia in guinea pigs on a normal diet) megaloblastic anaemia developed (May et alii, 1952). This anaemia responded to either folic acid or to ascorbic acid (May et alii, 1952). It appears therefore that the defect in folic acid metabolism in scurvy does not become manifest unless folic acid intake is also restricted. The clinical situation is more complex as was shown in a study of six patients with anaemia associated with scurvy (Cox et alii, 1967). In these patients

the anaemia did not respond to tetrahydrofolic acid, but did respond to large doses of ascorbic acid. Asquith et alii (1967) have also reported a case of megaloblastic anaemia which responded to ascorbic acid alone.

3. Collagen synthesis. Ascorbic acid is required for the formation of collagen (Wolbach, 1933) but the exact nature of the defect is unknown. The collagen molecule contains a large proportion of hydroxyproline which is not derived from dietary hydroxyproline (Stetten, 1949) but is formed at the site of collagen synthesis by hydroxylation of proline (Green and Lowther, 1959; Robertson, 1961). It has been suggested that ascorbic acid may be necessary for the conversion of proline to hydroxyproline (Gould, 1960; Robertson, 1961). This suggestion is based on experiments which show that the amount of bound hydroxyproline in granulation tissue does not increase in the absence of ascorbic acid. These experiments are really measuring the lack of collagen synthesis in the absence of ascorbic acid, and they do not prove that it is the hydroxylation of proline which is specifically inhibited.

In summary, it appears that very little good evidence has yet accumulated to support the contention that a defect in one or more enzyme systems is responsible for the clinical picture of scurvy. Further work on the effect of ascorbic acid on redox potential may lead to the demonstration that it is this effect which is partly or

wholly responsible for the importance of ascorbic acid in metabolism. A further possible role for ascorbic acid (in the transport of iron) is discussed in chapter 7.

IV. DISTRIBUTION

1. In the tissues. The concentration of ascorbic acid in all tissues is higher than in plasma. It is difficult to compare the concentrations found in one organ with those found in other organs because of differences in methodology between laboratories and because of a scarcity of reports on estimations by the same laboratory on different tissues. Yavorsky et alii (1934) stated the concentration found in nine different human tissues from various age groups. They found a very high content of ascorbic acid in adrenal glands and brain, with lesser concentrations in pancreas, liver, spleen, kidney and lung. Kuether et alii (1944) compared the content of a number of tissues in guinea pigs fed varying doses of ascorbic acid. Further literature on this subject can be found in reviews by Ralli and Sherry (1941) and by Knox and Goswami (1961). In general, very high concentrations of ascorbic acid are present in hypophysis, adrenal cortex, thymus, and corpus luteum (0.5-1.5 mg/G). Intermediate concentrations are found in white cells, platelets, brain, pancreas, liver, and spleen (0.1-0.5 mg/G), and lesser amounts in kidney, lung, and skeletal, smooth, and cardiac muscle (0.02-0.1 mg/G).

2. In the cell. The distribution of ascorbic acid within the cell has been studied by histochemical techniques and by estimations performed on cell fractions after isolation on sucrose density gradients. Staining tissues with silver nitrate caused blackening in the mitochondria, the Golgi apparatus, and the large granules of the adrenal and pituitary macrophages, but there is little evidence that this technique is specific for ascorbic acid (Pearse, 1953). Ascorbic acid estimations on separate cell fractions showed that most of the ascorbic acid was in the microsome fraction, and that some was present in the mitochondria, and that little if any was in the nucleus (Gero and Roux, 1958).

V. NUTRITIONAL REQUIREMENTS IN MAN

Ascorbic acid is absorbed from the gastrointestinal tract and equilibrated in the tissues in less than 4 hours (Woodruff, 1964). The plasma levels which can be attained are limited by the pattern of renal excretion. Urinary excretion of carbon^{14} accounted for 97% of an oral dose of C^{14}-labelled ascorbic acid in a normal subject (Baker et alii, 1966). Ascorbic acid passes into the glomerular filtrate and is then reabsorbed in the proximal tubule (Friedman et alii, 1940). Reabsorption is incomplete at all plasma levels, but when the plasma level exceeds 1.4 mg/100 ml the reabsorption mechanism is working at its maximum rate, and any further ascorbic acid to reach the plasma is very rapidly excreted

in the urine (Friedman et alii, 1940).

In adults an intake of 60-100 mg of ascorbic acid per day is required to produce tissue saturation (Goldsmith, 1961). The plasma concentration of ascorbic acid in saturated subjects is 1.0-1.4 mg/100 ml, and the content of the buffy layer of centrifuged blood has been variously estimated as 0.34 mg/G (Morse et alii, 1956) and 0.18 mg/G (Woodruff, 1964). These differences are probably methodological.

It is generally agreed that the buffy layer content of ascorbic acid provides a reasonable index of tissue stores, whereas the plasma level is a reflection of recent intake (Woodruff, 1964). Experimental depletion of human subjects (Crandon et alii, 1940; Vitamin C Subcommittee, 1953) showed that plasma levels approach zero after about 6 weeks on an ascorbic acid free diet, whereas the content of the leukocyte-platelet layer reaches minimal values after 15-20 weeks, just prior to the onset of scurvy.

The minimal amount of ascorbic acid required to prevent scurvy is 8 mg/day (Johnstone et alii, 1946) and 10 mg/day will produce healing of the disease in 10-14 weeks (Vitamin C Subcommittee, 1953). British and Canadian authorities recommend a daily intake of ascorbic acid of 30 mg, but the Unites States National Research Council recommends 75 mg (Woodruff, 1964). The latter recommendation is based on the belief that near saturation

levels in plasma and tissues are desirable. This partly stems from the teleological view that saturation may be desirable in man because the tissues of animals which synthesise their own ascorbic acid are saturated with this substance (Goldsmith, 1961).

There have been reports that saturation of the body with ascorbic acid confers some protection against colds (Ritzel, 1961) but a recent study by Walker et alii (1967) does not substantiate this. Some Russian authors have suggested that high concentrations of tissue ascorbic acid may help prevent atheroma formation, but their evidence is questionable (see below). In summary, there is no good evidence that saturation of the tissues with ascorbic acid is more or less desirable than a somewhat lower level.

VI. ASCORBIC ACID IN HAEMOSTASIS

1. The purpura of scurvy. One of the main clinical features of scurvy in adults is a severe purpura (Lind, 1753; Vilter, 1962). Early in the course of the disease petechial haemorrhages appear in the hair follicles of the lower limbs, around joints, and in those areas which are exposed to trauma such as pressure from belts and brassieres. The petechiae later coalesce to form large ecchymoses, and haemorrhages also occur into muscles causing painful lumps, and into joints causing haemarthroses. Swelling of and haemorrhage from the gums may occur, but these changes are usually minimal or

absent in patients with good dental hygiene. Petechial haemorrhages of the brain and intestine are common but a severe intracranial haemorrhage is rare, even in fatal cases. Haematuria is common and a haemorrhagic pericardial effusion occasionally occurs. The tourniquet test does not usually demonstrate increased capillary fragility, though new haemorrhages around hair follicles may occur about half an hour after the test.

2. *Cause of the bleeding tendency in scurvy.* The bleeding tendency in scurvy may theoretically be due to a defect in the capillary wall, the platelets, the coagulation mechanism, or a combination of two or more of these factors.

(a) Role of the capillary wall. Prior to the demonstration of a defect in platelet adhesiveness in scurvy (Born and Wright, 1967; Wilson et alii, 1967) it was thought that the purpura in scurvy was due entirely to a defect in the capillary wall (de Gruchy, 1964). The capillary wall has three important components (Chambers and Zweifach, 1947): the endothelial cells which are bound by intercellular cement, the endocapillary layer which is a coating of blood proteins on the luminal surfaces of the endothelial cells, and the connective tissue pericapillary sheath which surrounds the vessel. The pericapillary sheath of which collagen is a major component seems to be the most likely layer to be defective in scurvy. Penney and Balfour (1949) have shown that ascorbic acid is

required for the formation of the pericapillary sheath in new granulation tissue. Ascorbic acid is not necessary for the maintenance of collagen which has been formed for some time (Robertson, 1950, 1952) but in the absence of ascorbic acid collagen is not formed in new granulation tissue and hyaluronic acid accumulates (Robertson, 1961). New capillaries have been observed around hair follicles in experimental scurvy (Vitamin C Subcommittee, 1953) and it therefore seems likely that the haemorrhage occurs from these capillaries.

Reppert et alii (1951) produced evidence from experiments with rabbits which suggested that the increased capillary permeability in scurvy could be due to the inhibitory effect which ascorbic acid exerts on the hyaluronidase-hyaluronic acid reaction. It is difficult to justify their use of an experimental animal in which the tissues are normally saturated with ascorbic acid.

(b) Role of the platelets. "There has been a regrettable tendency to implicate a 'vascular factor' whenever a bleeding diathesis is unexplained" (Marcus and Zucker, 1965). This is not entirely true in scurvy where there is some evidence that the capillary wall may be defective (Penney and Balfour, 1949), but it is true that a platelet defect has not been excluded as a possible cause for the bleeding. The first indication that the platelet may be implicated in scurvy came from the finding of a high ascorbic acid

content in platelets by Barkhan and Howard (1958). If this substance is concentrated by platelets it is probable that it is required in platelet metabolism. Cetingil et alii (1958) found a defective platelet thromboplastic function in one case of scurvy, but this result could not be confirmed by Hart et alii (1964) in three cases of scurvy. Recently, however, it has been shown that platelet adhesiveness is decreased in scurvy (Born and Wright, 1967; Wilson et alii, 1967) and it now seems probable that platelets are responsible at least in part for the purpura of scurvy.

(c) Role of the coagulation mechanism. Barkhan and Howard (1959) studied the blood coagulation mechanism in scorbutic guinea pigs. These animals had an increased platelet count, a long plasma one stage prothrombin time, and impaired blood thromboplastic activity. The clot retraction time and the thromboplastic activity of platelets were normal. The defects in the coagulation mechanism appeared to be multifactorial, but their exact nature was not established. Extensive clotting studies by Hart et alii (1964) in three patients with scurvy revealed no marked anomalies. According to recent reviews (Nutrition Reviews, 1960; Dayton and Weiner, 1961), although deficiencies of various coagulation factors have been demonstrated in scurvy, the degree of magnitude of this alteration and its significance remains controversial. The purpura of scurvy resembles that of platelet or capillary origin, rather

than a deficiency in the coagulation mechanism.

In summary, the blood coagulation mechanism can probably be excluded as a major factor in the aetiology of the purpura of scurvy. The evidence in favour of a defect in the connective tissue sheath of the capillary wall is based mainly on the failure of fibroblasts to form this sheath in new granulation tissue in the absence of ascorbic acid (Penney and Balfour, 1949), and on the appearance of new capillaries around hair follicles in scurvy (Vitamin C Subcommittee, 1953). A defect in platelet adhesiveness in scurvy (Born and Wright, 1967) suggests that a platelet defect may well be responsible at least in part for the purpura which occurs in this condition.

VII. ASCORBIC ACID AND ATHEROMA

It is generally accepted in Russia that ascorbic acid administration acts as a prophylactic against coronary artery disease (Simonson and Keys, 1961). Since deposition of platelets on arterial walls is considered a possible mechanism for atherogenesis (Mustard et alii, 1964) it is appropriate to review the literature on the relation of ascorbic acid to atheroma. The Russian literature was reviewed in 1961 by Simonson and Keys. Experiments in Russia (Anitchkov, 1959; Myasnikov, 1958; Zaitsev et alii, 1964) have shown that the administration of large doses

of ascorbic acid to rabbits fed atherogenic diets lowers the serum cholesterol content and retards the development of atherosclerosis. A report in the western literature (Flexner et alii, 1941) indicated, however, that large intravenous doses of ascorbic acid failed to affect either the serum cholesterol or the development of atherosclerosis in the aorta.

Sedov (1956) in Russia reported the results of a clinical trial in which 106 patients received a course of 0.5-1.0 G of ascorbic acid daily for 20-30 days. This course was repeated after an interval of 1-3 months. In those patients with a serum cholesterol concentration greater than 250 mg/100 ml before treatment a significant fall in serum cholesterol occurred ($P < 0.001$) and clinical improvement was claimed. Samuel and Shalchi (1964) in the United States of America found that 1-6 G of ascorbic acid per day for 5-16 weeks failed to alter the serum cholesterol concentration in 14 patients.

The clinical trials conducted in Russia are criticised by Simonson and Keys (1961) because double blind studies with placebos were not used and statistical evaluation of the results was inadequate.

Some support for a role for ascorbic acid in atherogenesis can be found in the western literature. Willis (1953) reported

hypercholesterolaemia and an increase in the degree of atherosclerosis in scorbutic guinea pigs. King et alii (1953) found that guinea pigs fed C^{14}-labelled acetate showed a progressive increment in the fixation of acetate in cholesterol with the onset of scurvy.

Whether or not ascorbic acid administration will lower the serum cholesterol in some patients with atherosclerosis, the fact remains that there are easier and more certain methods of producing this effect. Most western workers remain sceptical of the value of ascorbic acid in the prophylaxis of atherosclerosis.

VIII. COMMENT

Ascorbic acid is essential for tissue metabolism. Although a large amount of work has been devoted to this substance its role in metabolic processes has not yet been elucidated. Of particular importance in this thesis is the requirement for ascorbic acid in the maintenance of vascular integrity where it may be required in the formation of capillary walls, in platelet metabolism, or both of these.

PART C. METHODS FOR THE ESTIMATION OF ASCORBIC ACID IN BIOLOGICAL SAMPLES

I. BIOLOGICAL ASSAYS

The first estimations of ascorbic acid were carried out by

biological assay (Bliss and Gyorgy, 1951). These methods were of two types, based on the ability of this substance either to prevent the appearance of scurvy in guinea pigs on an otherwise scorbutic diet, or to effect a degree of cure of scurvy already induced in the test animals. Such methods were tedious, difficult to make quantitatively accurate, and comsuming of both time and guinea pigs (Bliss and Gyorgy, 1951).

II. CHEMICAL METHODS

Chemical methods are based on one of two main principles. Some measure the reducing power of the sample; others measure the coloured 2,4-dinitrophenylhydrazone derivative of dehydro-ascorbic acid (Gyorgy and Rubin, 1950; Roe, 1954). Methods based on the reducing power of the sample frequently make use of a coloured dye which can be reduced by ascorbic acid to its leuko form. The amount of reducing substance present in the sample can be determined either by titration or by colorimetry. The dye most commonly used is 2,6-dichlorophenolindophenol (D.C.P.I. procedure, Gyorgy and Rubin, 1950). Other substances which are sometimes used are methylene blue (Lund and Lieck, 1936), iodine (Gyorgy and Rubin, 1950), and mercuric chloride (Lee and Leong, 1964). In the 2,4-dinitrophenylhydrazine (D.N.P.H.) procedure (Roe and Hall, 1939; Roe and Kuether, 1943) the ascorbic acid is oxidised to dehydroascorbic acid which is then coupled to D.N.P.H. to form a hydrazone. On treatment with sulphuric acid this yields a deep

reddish-orange colour which can be estimated by colorimetry (Roe, 1954).

Unfortunately, neither methods based on reduction nor methods based on hydrazone formation are specific. The D.C.P.I. method also measures other reducing substances such as cysteine, glutathione, cuprous and ferrous ions (Gyorgy and Rubin, 1950; Roe, 1954). The D.N.P.H. method may be interfered with by substances such as 2,3-diketogulonic acid (Penney and Zilva, 1943; Pijoan and Gerjovick, 1946), pyruvic acid (Sealock and Scherp, 1941; Lu, 1939) and phenylpyruvic acid (Penrose and Quastel, 1937) as these substances also have carboxyl groups capable of forming hydrazone derivatives (Gyorgy and Rubin, 1950). The lack of specificity and unsatisfactory nature of these methods is evinced by the many modifications which have been published since 1940.

1. 2,6-dichlorophenolindophenol (D.C.P.I.) method. Many of the modifications which are claimed to render the D.C.P.I. method more specific are based on the assumption that ascorbic acid reduces the dye almost instantaneously whereas other reducing substances do so more slowly. In one such modification titration readings are taken at intervals and extrapolated back to zero time to determine the true ascorbic acid concentration (Bessey, 1938). In another modification the unreduced dye is rapidly extracted with xylene to remove it from contact with other reducing substances

(Robinson and Stotz, 1945). These modifications do not completely eliminate interference because the assumption that other reducing substances reduce more slowly than ascorbic acid is not always correct (Lugg, 1942).

In 1943, Levy published a modification in which the extract was allowed to react with hydrogen peroxide before adding the dye. This reduced interference due to sulphur dioxide by converting it to sulphuric acid. The method was not valid, however, in the presence of iron and copper salts. Others (Kirkpatrick, 1941; Mapson, 1941), used acetone or certain sugars to prevent the dye reacting with sulphite ions. Lugg (1942) carried out the reaction in the presence of formaldehyde claiming that this reduced interference from substances such as cysteine, sulphite, hydrogen sulphide, and H_2S-treated pyruvic acid.

Although all these modifications reduce the degree of interference, none is highly specific for ascorbic acid. Campbell et alii (1951) found serious errors in the presence of ferrous, cuprous, or cupric ions when analysing pharmaceutical preparations by seven different modifications of the D.C.P.I. method.

2. 2,4-dinitrophenylhydrazine (D.N.P.H.) method. Roe (1961) claims to have answered objections that the D.N.P.H. method is not specific. He showed that incubation of samples and controls at

15°C for 18 hours gave similar results to incubation at 37°C for 3 hours. He says "If interfering substances that couple slowly with dinitrophenylhydrazine are present in the tissue extracts they would be expected to yield more chromogen at 37°C than at 15°C." This experiment does not seem to be an adequate method for the determination of the presence or absence of specificity. His contention is correct only if the rate of hydrazone formation with interfering substances was affected by temperature changes in a different manner from hydrazone formation with dehydroascorbic acid. Roe (1961) has shown that fructose, glucose and glucuronic acid do not interfere with the D.N.P.H. method, but there are other substances in biological samples capable of forming coloured derivatives with D.N.P.H. (see above).

Another disadvantage of the D.N.P.H. procedure is the 4 hour incubation period. Schaffert and Kingsley (1955) shortened this time to 10 minutes by incubating at 100°C instead of 37°C. This was subsequently shown by Roe (1961) to increase the likelihood of interference from other substances.

3. Use of the ultraviolet absorption of ascorbic acid. Ascorbic acid absorbs strongly in the ultraviolet range. The wavelength of maximum absorbance varies with pH and occurs at 224 mμ when the pH is 3 (Figure 1). Dehydroascorbic acid at this concentration does not absorb in the ultraviolet range (Figure 1).

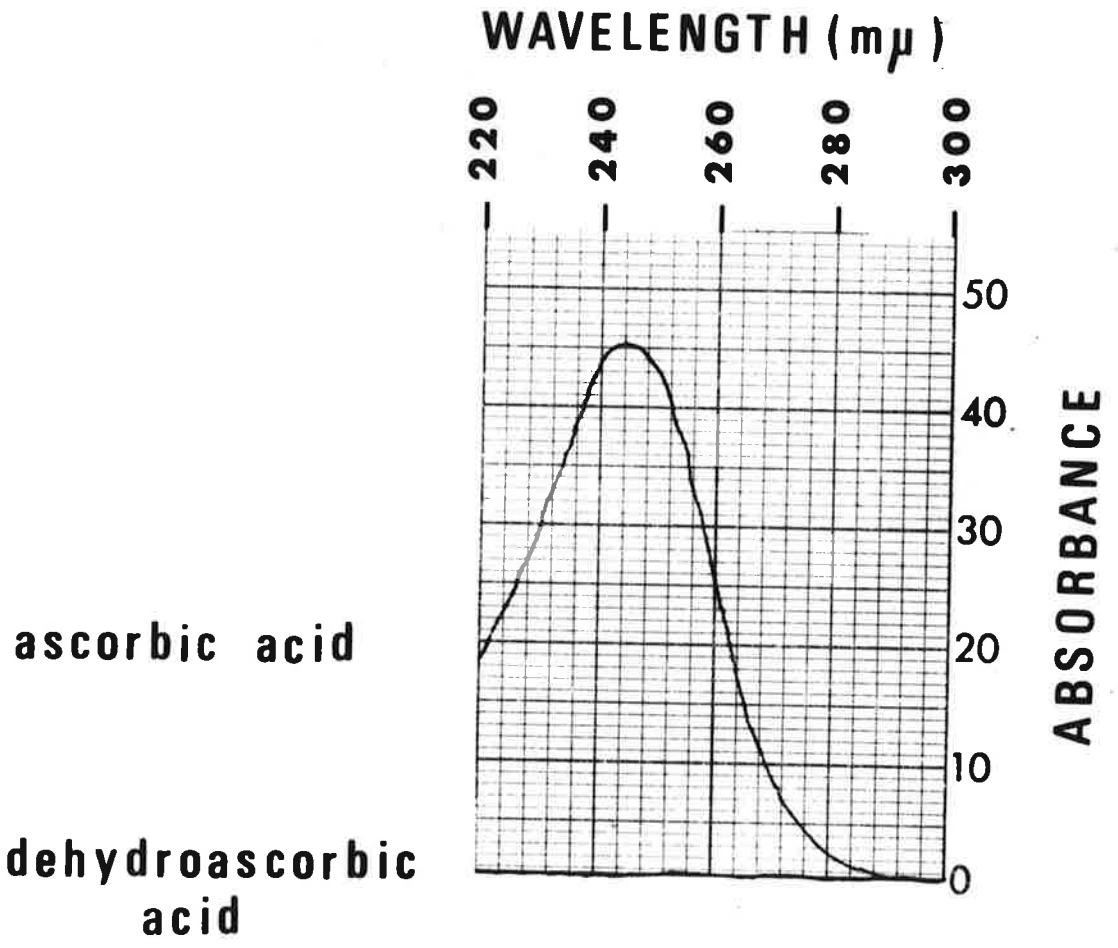


FIGURE 1: Absorbance (x 100) in the ultraviolet of ascorbic acid and dehydroascorbic acid (8.0 µg/ml) in a cell of 1.0 cm light path.

The difference between the absorbance of a solution of the extract in 1:50,000 potassium cyanide and the absorbance of the same solution after destruction of the ascorbic acid by ultraviolet irradiation gave a measure of the ascorbic acid concentration (Robertson, 1934; Chevallier and Choron, 1943). This method has not gained acceptance because of the unpredictable effect of ultraviolet irradiation on a complex biological sample.

4. Chromatographic methods. It is interesting that chromatographic methods give lower values than volumetric procedures, presumably because of the greater specificity obtained by chromatography (Prochazka, 1963). The principal method used has been paper chromatography. A list of references is obtainable (Macek et alii, 1957-1960) and the subject has been thoroughly reviewed (Prochazka, 1963). Using paper chromatography Mapson and Partridge (1949) separated ascorbic acid from reductone, reductic acid, hydroxytetronic acid and dihydroxymaleic acid. They suggested that since these substances can interfere with ascorbic acid estimations, paper chromatography might increase the specificity of these procedures. Heimann et alii (1953) published a paper chromatographic method by which all the substances which usually interfere with the estimation of ascorbic acid such as cysteine, glutathione, and ferrous and stannous ions can be separated from ascorbic acid. Estimation of ascorbic acid by paper chromatography has been performed (Chen et alii,

1953; Herrmann and Zobel, 1962). The ascorbic acid was eluted and subjected to estimation with D.C.P.I. Paper chromatographic techniques have the disadvantage that because of the lengthy nature of the procedure (about 24 hours for development of the chromatogram) it is difficult to prevent a considerable loss of ascorbic acid by oxidation (Prochazka, 1963).

The possibilities of thin layer chromatography have not been adequately explored (Bolliger, 1965). Strohecker and Pies (1962) allowed the biological extract to react with D.N.P.H. and separated the hydrazones so formed by thin layer chromatography on silica gel. The spots were eluted with sulphuric acid and estimated spectrophotometrically. A search of the literature has revealed no other publications on the use of thin layer chromatography in the estimation of ascorbic acid.

III. COMMENT

Ascorbic acid is of vital importance in tissue metabolism, yet its metabolic function remains unknown. An essential tool in the search for a solution to this problem is an accurate method for its estimation. It is surprising therefore that none of the methods at present in use are satisfactory. Procedures commonly used lack specificity, for no attempt is made to separate ascorbic acid from interfering substances. Though methods for the separation of ascorbic acid from interfering substances by paper

chromatography have been published, these have not gained acceptance. This is probably because of the lengthy nature of the procedure and because of the difficulty in preventing the loss of ascorbic acid by oxidation during development of the chromatogram.

CHAPTER 2

DEVELOPMENT OF A NEW METHOD FOR THE ESTIMATION OF ASCORBIC ACID IN PLATELETS

I. INTRODUCTION

The first requirement in the project described in this thesis was for a method for the estimation of ascorbic acid in platelets. A review of the literature revealed that methods presently available for its estimation in biological samples lack specificity (Chapter 1, part C). Those which measure the reducing power of the sample are interfered with by other reducing substances present (Roe, 1954; Gyorgy and Rubin, 1950) and those which depend on the formation of the dinitrophenylhydrazone derivative of dehydroascorbic acid are interfered with by substances which also form coloured derivatives with D.N.P.H. (Gyorgy and Rubin, 1950).

In the search for a more specific method some authors have proposed the separation of ascorbic acid from interfering substances by paper chromatography (Prochazka, 1963). The ascorbic acid can then be eluted and estimated by one of the standard methods (Herrmann and Zobel, 1962). Chromatographic methods give lower values than volumetric procedures because the former are more specific (Prochazka, 1963).

In the method proposed in this chapter thin layer chromatography was used. The disadvantage of paper chromatography is that 24 hours are required for development of the chromatogram. This time factor is of particular importance in the case of an

unstable substance such as ascorbic acid, for the more lengthy the procedure the greater the likelihood of considerable loss of ascorbic acid by oxidation.

Thin layer chromatography, on the other hand, takes only one hour and therefore offers a possible means of quickly separating ascorbic acid from interfering substances. The only previous attempt to use thin layer chromatography for the estimation of ascorbic acid was by Strohecker and Pies (1962) who treated the biological extract with D.N.P.H. and separated the hydrazones so formed by thin layer chromatography on silica gel. The spots were eluted with sulphuric acid and estimated spectrophotometrically.

The method described in this chapter made use of two main principles:

1. *Thin layer chromatography.* Increased specificity was obtained by thin layer chromatography of the platelet extract by which means ascorbic acid was separated from other reducing substances present in the sample. The separated ascorbic acid was eluted from the chromatogram and the amount of ascorbic acid in the eluate estimated by a sensitive colorimetric method.

2. *Estimation by a sensitive colorimetric method.* A very sensitive method for the estimation of ascorbic acid was devised. This was necessary because thin layer chromatography involves loss

of sensitivity; it was possible to apply only a small volume of sample (such as 20 µl) to the origin of the chromatogram, and after development of the chromatogram the ascorbic acid was eluted in a much larger volume of solvent (such as 2 ml) effectively diluting the ascorbic acid by a factor of about 100.

II. DEVELOPMENT OF A SENSITIVE METHOD FOR ASCORBIC ACID ESTIMATION

1. General principle. Before attempting the resolution of ascorbic acid by thin layer chromatography a sensitive and rapid method was required for the estimation of ascorbic acid. It can be predicted from redox potential theory that ascorbic acid should reduce ferric ions to the ferrous form (Hewitt, 1950). If ascorbic acid were added to a molar excess of ferric chloride solution, the amount of ferrous ions liberated should theoretically provide a measure of the amount of ascorbic acid present. For the estimation of the liberated ferrous ions two main principles offering the possibilities of rapidity and great sensitivity were explored. (i) If radioactive iron (Fe^{59} Cl_3) were used the ferrous ions could be estimated by their radioactivity if a method could be found for the separation of ferrous from ferric ions. (ii) A number of chromogens are available which form intensely coloured derivatives with ferrous ions but not with ferric ions.

2. Use of radioactive iron. Two possible methods for the separation of ferrous from ferric ions were considered.

TABLE 1. THE ESTIMATION OF ASCORBIC ACID USING RADIOACTIVE IRON (Fe^{59}

REAGENTS	SAMPLE CONTAINING ASCORBIC ACID	BLANK	CONTROL
1. 0.40 mM $FeCl_3$	10 ml	10 ml	10 ml
2. 0.04 µC Fe^{59}/ml (as $Fe^{59}Cl_3$)	10 ml	10 ml	10 ml
3. Sample solution	10 ml	-	-
4. Distilled water	-	10 ml	10 ml
5. HCl, NH_4OH, H_2O as needed	10 ml	10 ml	10 ml
6. pH adjustment	pH 2 →	pH 8	pH 2

(a) It is possible to separate ferrous ions from ferric ions by thin layer chromatography (personal observations). This possibility has the disadvantage that, in addition to the separation of ascorbic acid from other reducing substances, another chromatographic procedure is required which would involve further loss of sensitivity.

(b) The solubility product of ferrous hydroxide is 1.64×10^{-14} whereas that of ferric hydroxide is 1.1×10^{-36} (Kolthoff and Elving, 1962). Thus while at pH 7 ferrous hydroxide is appreciably soluble ferric hydroxide is extremely insoluble.

Because of the loss of sensitivity involved the first possibility was discarded and the separation of ferrous from ferric ions was attempted on the basis of their differing solubilities. The test was set up as in Table 1. Ferric chloride, radioactive iron, and sample solution containing varying amounts of ascorbic acid were pooled in a 50 ml beaker. Blank and control samples (0 and 100% values) containing no ascorbic acid were included. The pH of the contents of each beaker was adjusted to 2 with hydrochloric acid and then all except the control (which was left at pH 2) were brought to pH 8 with ammonium hydroxide. The final volume was brought to 40 ml and 10 ml aliquots were centrifuged at 1300 g for 30 minutes. The gamma activity of the supernatant was determined by scintillation

counting. Results were expressed as

Percentage radioactivity of supernatant =

$$\frac{\text{Sample activity - blank activity}}{\text{Control activity - blank activity}} \times 100\%$$

As expected ascorbic acid, by maintaining radioactive iron in solution, increased the amount of radioactivity in the supernatant (fig. 2). The relationship between the ascorbic acid concentration and the radioactivity of the supernatant was non-linear however, and an adequate measure of ascorbic acid concentration was possible only over the range 0.06 - 0.1 mM (concentration of ascorbic acid in the 10 ml sample). It was interesting that 100% iron solubility was attained when the molar ratio of iron to ascorbic acid was 4:1. The significance of this finding in relation to possible complex formation between dehydroascorbic acid and ferric ions will be discussed in Chapter 8.

Though this method could probably be made very sensitive by the use of more dilute ferric chloride of a higher specific activity, it had the great disadvantage that it was sensitive over only a small concentration range. It was therefore discarded in favour of the following method.

3. Use of an iron chromogen. (Fe-T.P.T.Z. method). Ferrous ions react with a number of iron chromogens to form

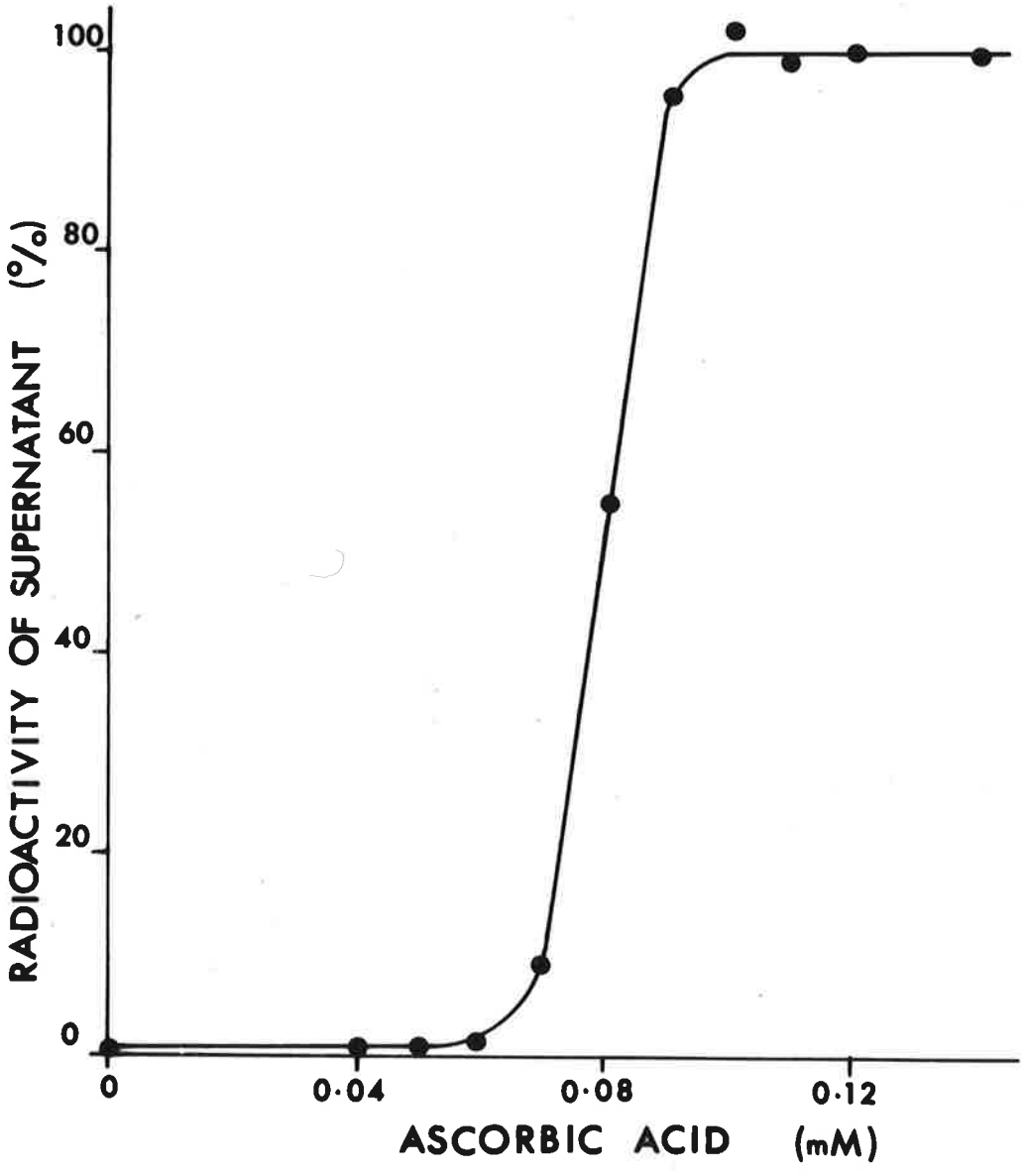


FIGURE 2: Relationship of radioactivity of supernatant to known ascorbic acid concentration in 10 ml sample.

intensely coloured products well suited to spectrophotometric estimation. Some of these chromogens are specific for ferrous ions and do not react with ferric ions. The most sensitive of these is tripyridyl-S-triazine (T.P.T.Z.)(Caraway, 1961; Fischer and Price, 1964) which forms a violet ferrous derivative, $Fe(T.P.T.Z.)_2$, with a molar extinction coefficient of 22,600 in aqueous solution at 593 mμ (fig. 3). At pH 3.4-5.8 the colour formation is complete, stable, and follows Beer's law (Fischer and Price, 1964).

5.0 ml of ferric chloride (0.16 mM in 0.01 M HCl), 5.0 ml of T.P.T.Z. (4.0 mM) and 5.0 ml of ascorbic acid (of known concentration in 1% acetic acid) were pooled in a beaker. The pH was adjusted to less than 2 with hydrochloric acid and then to 4.0-4.5 with ammonium acetate. The final volume was made up to 20 ml with distilled water and the absorbance of the resulting solution measured in a spectrophotometer at 593 mμ using a glass cell of 1.0 cm light path. A range of concentrations of ascorbic acid from 0-0.12 mM were tested.

Beer's law was followed over the concentration range 0-14.7 μg/ml of the 5 ml sample (0-0.021 mM in the 20 ml final volume) (fig. 4). In those samples in which the ascorbic acid concentration exceeded this range no further colour was generated. At the upper limit of this range the concentration of ascorbic

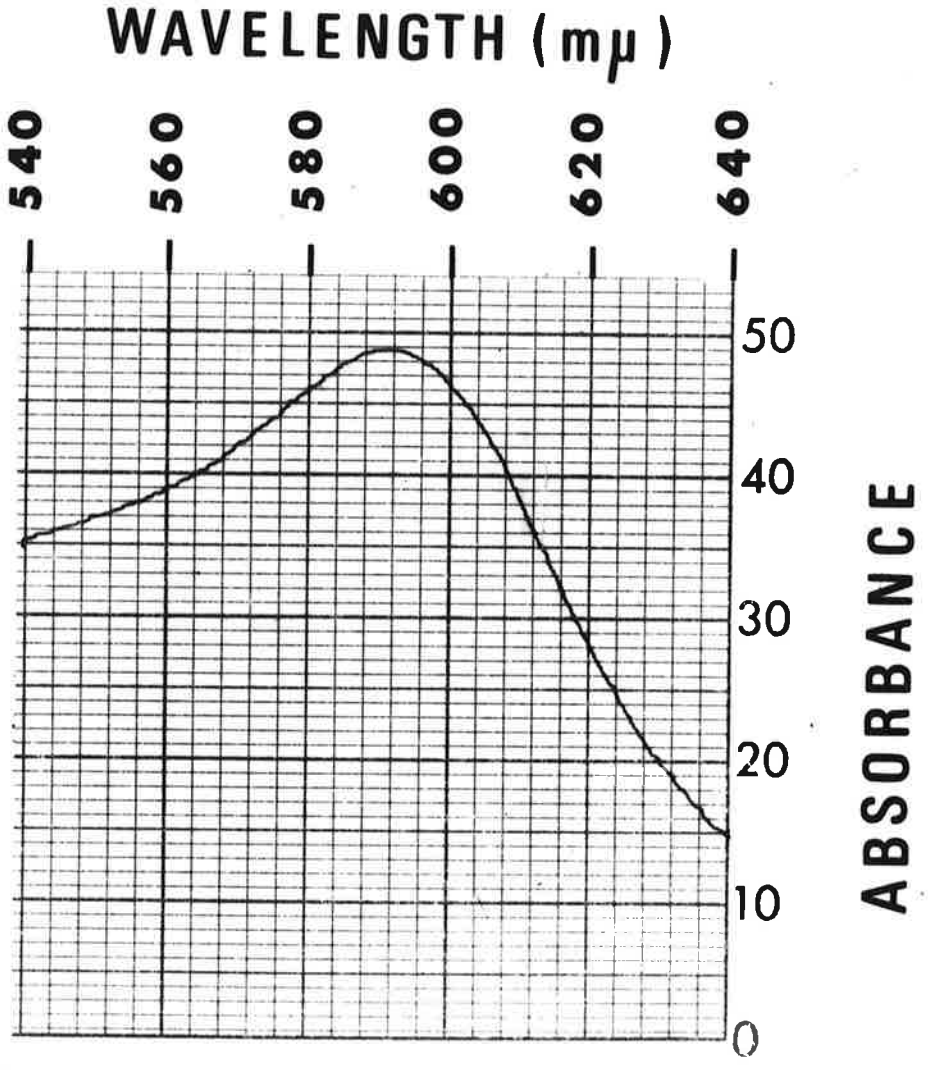


FIGURE 3: Absorbance (x 100) of $Fe(T.P.T.Z.)^2$ complex in a cell of 1.0 cm light path.

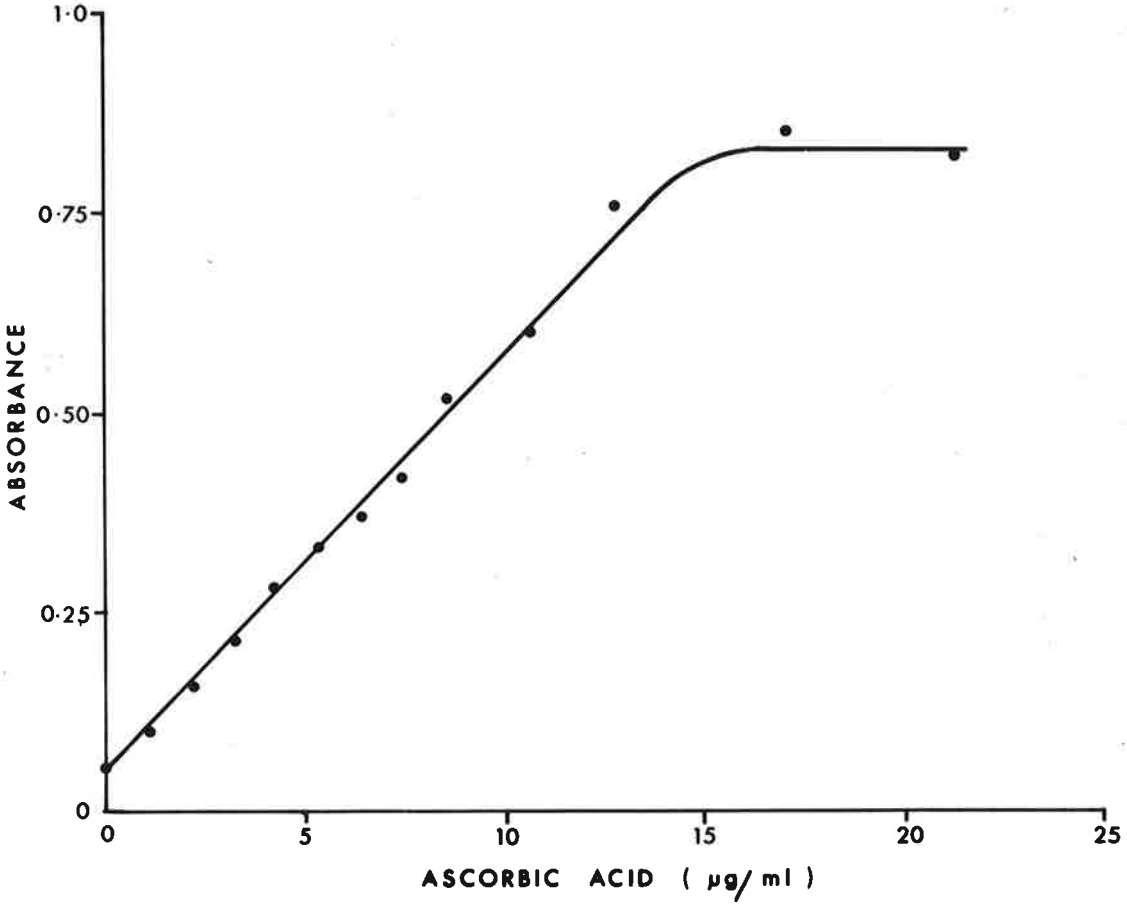


FIGURE 4: Relationship of absorbance to the known ascorbic acid concentration in 5.0 ml samples. Each point represents the mean from two samples.

acid was half the concentration of iron (0.40 mM in final volume). When 5.0 ml samples of dehydroascorbic acid containing 10 µg/ml were tested the absorbance was equivalent to that in blank samples.

When ascorbic acid is oxidised it liberates 2 hydrogen atoms each capable of donating an electron. It would therefore be expected that 1 molecule of ascorbic acid would reduce 2 ferric ions to ferrous ions. This hypothesis is consistent with the fact that concentrations of ascorbic acid greater than 0.021 mM (final volume), which is half the concentration of iron (0.040mM), failed to generate more colour. The reaction can therefore probably be represented as follows:

$$\text{ascorbic acid} + 2\ Fe^{+++} \rightarrow \text{dehydroascorbic acid} + 2\ Fe^{++} + 2\ H^{+}$$

$$2\ Fe^{++} + 4\ \text{T.P.T.Z.} \rightarrow 2\ Fe\ (\text{T.P.T.Z.})_2$$

4. Advantages of the Fe-T.P.T.Z. method over other methods.

(a) The Fe-T.P.T.Z. method is more sensitive than other methods. This can best be shown by comparing the molar extinction coefficients. The molar extinction coefficient can be calculated from the formula

$$a = \frac{A}{bc}$$

where a = extinction coefficient

A = absorbance generated

c = molar concentration

b = length of light path

In the experiment illustrated in Figure 4 the extinction coefficient for ascorbic acid was 36,900. In later experiments under slightly different conditions 42,800 was obtained. This can be compared with 23,200 for the D.N.P.H. procedure and 9,770 for the ultraviolet absorption of ascorbic acid at pH 3 (both values determined in this laboratory.

It is more difficult to compare the sensitivity of other methods for ascorbic acid estimation as extinction coefficients for these are not quoted in the literature. The Fe-T.P.T.Z. procedure when modified for use in a 4.0 cm cell (see below) requires a sample concentration of less than 1.25 µg/ml of ascorbic acid. The D.C.P.I. method, which requires a sample concentration of 2-10 µg/ml appears to have about the same sensitivity as the D.N.P.H. procedure (Roe, 1954). The lower limit of sensitivity of the method of Lee and Leong (1964), which utilises the reduction of mercuric to mercurous ions, is 10 µg/ml. The Fe-T.P.T.Z. method is therefore the most sensitive yet described for the estimation of ascorbic acid.

(b) The Fe-T.P.T.Z. method takes only 2-3 minutes per sample and is therefore more rapid than the D.N.P.H. procedure which includes a 4 hour incubation step.

(c) A disadvantage of the D.N.P.H. method is that all reactions are carried out in a high concentration of sulphuric

acid. The oily highly acid solution is difficult to handle and unpleasant to work with.

(d) An advantage of the Fe-T.P.T.Z. method over the D.C.P.I. procedure is that in the Fe-T.P.T.Z. method the blank has minimum absorbance and colour intensity is directly proportional to ascorbic acid concentration. In the D.C.P.I. procedure the blank has maximum absorbance and colour formation is inversely proportional to ascorbic acid concentration. This increases the lower limit of sensitivity of the D.C.P.I. method, for a small difference between two large values cannot be measured as accurately as the same absolute difference between two small values.

5. Further development of the Fe-T.P.T.Z. method. Because of the advantages of the Fe-T.P.T.Z. method over existing methods it was chosen for the estimation of ascorbic acid which had been eluted from the chromatogram. Further development of the method was therefore undertaken.

(a) Control of pH. Full colour development did not occur and the intensity of colour was not reproducible unless the pH of the mixture of ascorbic acid, ferric chloride, and T.P.T.Z. was reduced to less than 2.0 during the procedure. This is because ferric ions at a pH above 2 aggregate to form multi nuclear complexes which are chemically less reactive than free ferric ions

which exist in acid solution (Eichorn, 1964).

Fischer and Price (1964) stated that maximum colour formation was obtained at pH 3.4-5.8. This was substantiated in this laboratory. Furthermore it was found that if the pH was raised to above 6 loss of colour occurred, and this could not be recovered by further manipulation of the pH. Thus it was important to avoid overtitration. Ammonium acetate (10-30%) proved to be a suitable buffer for raising the pH from 2.0 up to 4.0-4.5.

(b) Stability of the blue complex. Two samples of absorbance 0.495 were left on the bench and tested in the spectrophotometer at 15 minute intervals. A gradual increase in absorbance to 0.52 occurred over an hour, after which it remained constant. No error resulted if all samples were tested at the same period of time after titration. It was customary to determine the absorbance immediately after titration.

(c) Stability of ascorbic acid solutions. At first considerable difficulty was experienced in the preparation of solutions containing a known amount of ascorbic acid because of loss of ascorbic acid due to breakdown by oxidation. It is known that ascorbic acid is unstable in solution if the pH is not near 3 (Uprety and Revis, 1964) or if trace metal ions such as those of iron or copper are present (Roe, 1954). When

ascorbic acid (10 µg/ml) was prepared in distilled water 8% was lost over a period of two and a half hours, irrespective of whether it was kept in the refrigerator or left on the bench. Bubbling oxygen free nitrogen through the solution did not increase the stability of the ascorbic acid.

The problem of ascorbic acid breakdown was partially overcome when it was discovered that the stability was greatly enhanced if the ascorbic acid was prepared in a solution of 1% acetic acid which has a pH of 3; no deterioration occurred over half an hour at room temperature, though 5% was lost over 24 hours at 0-4^{o}C in the refrigerator.

Another cause of ascorbic acid breakdown at this stage of the project was the impurity of the distilled water which was significantly contaminated with iron to the extent of 0.2-0.3 µg/ml by the method of Fischer and Price (1964). When ascorbic acid solutions in the concentration range 0-1.25 µg/ml were used (see below) this degree of contamination was sufficient to considerably increase the rate of breakdown of ascorbic acid. Later in the project distilled water which had been subsequently deionised in a mixed-bed ion exchange resin demineraliser became available. Ascorbic acid solutions prepared in 1% acetic acid using this water did not deteriorate when left at 0-4^{o}C for 24 hours.

(d) Attainment of maximum sensitivity. The sensitivity of a spectrophotometric method can be enhanced by increasing the length of the light path through the sample, and by decreasing the final volume of the reaction mixture. In the spectrophotometer used (Beckman DB) the maximum light path available was 4.0 cm. The minimum volume which could be accommodated in such a cell was 3.6 ml. Spacers 9.0 mm high were then required to raise the lower part of the cell into the path of the light beam. Reproducible results were obtained if a volume of 4.0 ml. was used. Preliminary tests with silica gel showed that 2.0 ml. of 1% acetic acid was sufficient for elution of ascorbic acid from a silica gel thin layer chromatogram. If 1.0 ml of Fe-T.P.T.Z. reagent was added to this 2.0 ml sample the adjustment of pH could easily be carried out without exceeding a total volume of 4.0 ml.

6. Procedure finally used for Fe-T.P.T.Z. reaction.

(a) Reagents. (i) Ferric chloride stock solution (0.01 M $FeCl_3$ in 0.2 M HCl). (ii) 2,4,6-tripyridyl-S-triazine, $C_3N_3(C_5H_4N)_3$,* (T.P.T.Z.), 0.004 M stock solution. This was prepared by dissolving 124.8 mg of T.P.T.Z. in 1 ml of 1.0 M HCl and diluting to 100 ml with deionised water. The solution was stable indefinitely if kept in a well-stoppered bottle in the dark (Fischer and Price, 1964). (iii) Fe Cl_3-T.P.T.Z. reagent.

*G. Frederick Smith Chemical Co. Ohio, U.S.A.

5.0 ml of Fe Cl_3 stock solution was added to 25 ml of T.P.T.Z. stock solution and the volume made up to 500 ml with 0.05 M HCl. The solution then contained 0.1 mM Fe Cl_3 and 0.2 mM T.P.T.Z. in 0.05 M HCl. This solution was stored in the dark in the refrigerator at 0-4°C. (iv) Ascorbic acid standard solution (1.25 µg/ml in 1% acetic acid). (v) Hydrochloric acid 3.0 M. (vi) Ammonium acetate A.R. 30% w/v.

It was essential that all reagents should have a minimal degree of iron contamination. Acid cleaned glassware was used both for preparation of reagents and for carrying out the Fe-T.P.T.Z. reaction.

(b) Procedure. 2.0 ml of the unknown sample and 1.0 ml of the Fe-T.P.T.Z. reagent were pooled in a 10 ml beaker. The pH was momentarily adjusted to 1.7-1.9 with HCl and then brought to 4.0-4.5 with ammonium acetate. The final volume was adjusted to 4.0 ml by the addition of distilled water and the absorbance was then measured in a spectrophotometer at 593 mµ using a glass cell with a 4.0 cm light path. 2.0 ml of 1.25 µg/ml ascorbic acid in 1% acetic acid and 2.0 ml of 1% acetic acid were treated in the same manner as the unknown sample to determine the control (100%) and blank values.

(c) Results. When known concentrations of ascorbic acid were tested by the above procedure there was a linear relationship

between absorbance and concentration of ascorbic acid in the 2 ml sample over the range 0-1.25 µg/ml (Figure 5). For concentrations of ascorbic acid greater than this value the proportionality no longer occurred despite an excess of the reagents (Fe Cl_3 and T.P.T.Z.).

In the experiment illustrated in Figure 4 the failure of the relationship between absorbance and ascorbic acid concentration to remain linear above a certain concentration of ascorbic acid was probably due to exhaustion of the ferric chloride and T.P.T.Z. In this instance, however, the reagents were in excess and the loss of linearity was probably due to a failure of the Fe- T.P.T.Z. complex to continue to adhere to Beer's law above this concentration.

7. Reproducibility of the Fe-T.P.T.Z. method. The method was reproducible only if strict precautions were taken to ensure the absence of iron contamination in all reagents. This was because the reaction detected not only ascorbic acid but also any contaminating ferrous ions; a ferrous ion concentration of only 3 parts per 100 million in the final volume produced an absorbance of 0.05. For this reason all glassware was acid cleaned and beakers were capped with parafilm until ready for use to guard against contamination from atmospheric dust particles. Reproducibility was enhanced by rinsing the reaction beakers in 1.0 M HCl just prior to use.

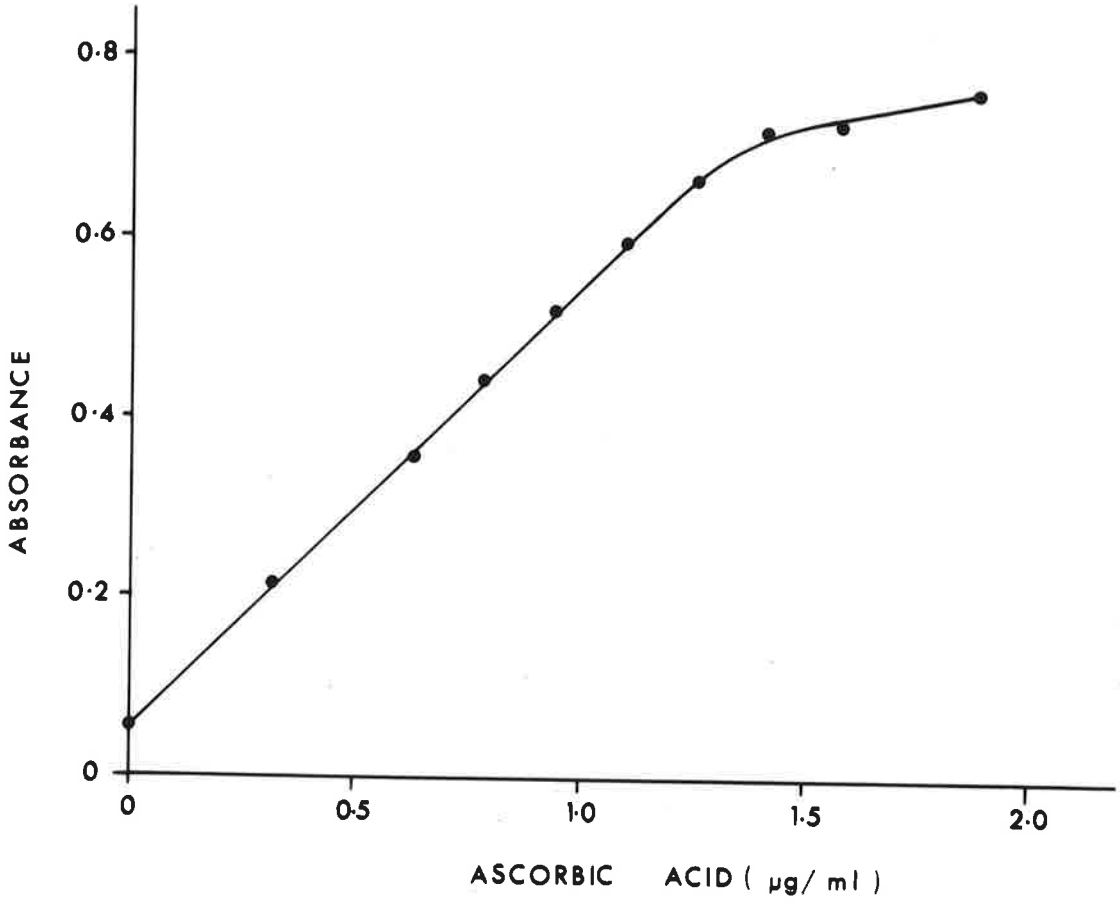


FIGURE 5: Relationship of absorbance to the known concentration of ascorbic acid in 2.0 ml samples. Each point represents the mean from 2 samples.

The reproducibility of the technique was shown on 10 2.0 ml samples from a solution containing 0.625 µg/ml of ascorbic acid. The mean increase in absorbance above the blank was 0.323 ± 0.008, a standard deviation of 2.5%. In 11 blank determinations a mean absorbance of 0.043 ± 0.008 was found.

III. DEVELOPMENT OF A THIN LAYER CHROMATOGRAPHIC METHOD FOR THE SEPARATION OF ASCORBIC ACID FROM OTHER REDUCING SUBSTANCES

1. General principle. Thin layer chromatography provides a simple, versatile, and rapid technique for the resolution of biological samples. Separation takes about 1 hour which is much more rapid than the 24 hours required for paper chromatography. Resolving power is greater than with paper chromatography, partly because increased speed leaves less time for diffusion to occur. Rapidity is also important when an unstable substance such as ascorbic acid is used.[1]

The versatility of the method is greater than for paper chromatography. A wide range of adsorbent materials is available for use as a thin layer and the separating characteristics of the adsorbent can be modified by varying either the solvent used to make the slurry or the degree of heat induced activation of the resultant thin layer. Larger volumes of sample can be accommodated simply by increasing the thickness of the adsorbent layer.

An advantage over column chromatography is that it is possible to run 11 samples simultaneously on a 20 cm x 20 cm plate, whereas only one sample at a time can be resolved on a single column. Another distinct advantage which thin layer chromatography shares with paper chromatography is that most substances can be readily detected by ultraviolet fluorescence or by specific spray reagents.

The main disadvantage of thin layer chromatography compared to column chromatography is that it is possible to apply only a small volume of sample (such as 2-20 µl) in a single spot. This is a drawback when elution of a substance followed by its volumetric estimation is required. The amount of sample which can be applied can be increased by using a thicker adsorbent layer, by prior concentration of the sample, by repeated application of the sample to the same spot with drying between each application, or by application of the sample as a long streak.

Details on the methodology of thin layer chromatography are available (Bobbitt, 1963; Stahl, 1965).

2. Apparatus.

(a) Glass plates 20 cm x 20 cm and 20 cm x 10 cm were used, though in the thin layer chromatogram scanner 20 cm x 2.5 cm were required. Prior to spreading the plates were cleaned by

scrubbing with a mild abrasive, dipping briefly in acid, and rinsing in distilled water. Any traces of grease which still remained were removed just before use with ethyl alcohol followed by n-hexane.

(b) Thin layer chromatogram spreader (Camag).

(c) Application of the sample was by micropipettes (Camag) capable of delivering 1-10 µl or by a micrometer syringe (Agla) which delivered volumes as low as 0.2 µl.

(d) Development was carried out in glass chambers (DeSaga) which had been filled with solvent to a depth of about 1.5 cm. A glass spacer 20 cm x 6 cm x 1 cm reduced the amount of solvent required. Saturation of the atmosphere inside the chamber with solvent vapour was facilitated by lining the walls with filter paper which was wetted with solvent by tilting the tank.

(e) Visualisation of substances separated by chromatography was aided by an ultraviolet lamp with principal wavelengths 254 mµ and 350 mµ and by spray reagents applied with a pressure pack spray apparatus (Camag).

(f) An Actigraph III thin layer chromatogram scanner (Nuclear Chicago) was used when the detection of beta emitting radioactive isotopes was required. The apparatus was operated with a 2π mylar

thin window arrangement and P10 (10% methane, 90% argon) was used as counting gas. The counting efficiency for C^{14} was 8%.

3. Choice of an adsorbent medium. The first requirement was for a combination of adsorbent and developing solvent which would produce a separation of ascorbic acid from other reducing substances without excessive breakdown of ascorbic acid by oxidation. Adsorbent materials commonly used in thin layer chromatography are silica gel, cellulose and Keiselguhr (Diatomaceous earth). Silica gel is the most extensively used. It produces a highly active layer which is capable of separating both hydrophilic and hydrophobic substances depending on the solvents used (Waldi, 1965a). Cellulose and Keiselguhr are both inactive media (Waldi, 1965a) which in combination with a polar solvent can be used to separate a mixture of hydrophilic substances (Stahl, 1965). Silica gel was chosen for assessment as a possible medium for the separation of ascorbic acid from other reducing substances.

4. Assessment of silica gel as a chromatographic medium. Silica gel (Merck), HF254 (with added fluorescent indicator) or H (without indicator), was used. 80 G of gel were thoroughly mixed with about 160 ml of water to form a thin slurry. A layer 0.2 mm thick was applied to the glass plates, allowed to dry overnight, and activated by heating for half an hour in an oven at 110°C.

TABLE 2. SEPARATION OF ASCORBIC ACID FROM OTHER REDUCING SUBSTANCES ON SILICA GEL

Solutions applied	Rf = $\frac{\text{distance from origin x 100}}{\text{length of run (10 cm)}}$			Detection of spots
CHROMATOGRAM 1	Ascorbic acid	cysteine	glutathione	(a) Ultraviolet light 254 mμ. Ascorbic acid dark on green background. (b) Ninhydrin spray (Waldi, 1965). Ascorbic acid, dehydroascorbic acid, cysteine and glutathione pink on white background.
Ascorbic acid 0.02 M	53			
Cysteine 5 mg/ml		15		
Glutathione 10 mg/ml			13	
A:C = 1:1	50	13		
A:G = 1:1	54		13	
CHROMATOGRAM 2	Ascorbic acid		Dehydroascorbic acid	Silver nitrate-ammonia-spray (Waldi, 1965). Ascorbic acid black, dehydroascorbic acid brown after heat (110° for 5 mins). Background white.
Ascorbic acid 0.02 M	54			
Dehydroascorbic acid 0.02 M			65	
A:D = 1:1	55		68	
CHROMATOGRAM 3	Ascorbic acid	$FeSO_4$	$SnCl_2$	(a) Ultraviolet light 254 mμ. Fe^{+++} dark spot. Fe^{++} dark spot after drying. (b) Silver nitrate-ammonia-spray. Sn^{++} brown spot.
Ascorbic acid 0.02 M	52			
$FeSO_4$ 0.02 M		23		
$SnCl_2$ 0.1 M			10	
A:Fe = 1:1	55*	25		
A;Sn = 1:1	58		10	

*Some dehydroascorbic acid appeared at Rf 68.
Fe^{++} catalyses the oxidation of ascorbic acid.

(a) Separation of ascorbic acid from other reducing substances on silica gel. In the search for a solvent which would separate ascorbic acid from other reducing substances on silica gel, 5 µl aliquots of the solutions listed in Table 2 were applied as separate spots to the origins of thin layer chromatograms (silica gel HF254) which were then developed in the test solvents. The solvent n-propanol:acetic acid:water: ethyl acetate = 80:8:10:20 produced a suitable separation of ascorbic acid from other reducing substances and from dehydro-ascorbic acid (Table 2). In addition it was found that sulphite ions, rendered visible as white spots on a blue background by spraying firstly with 0.002 N iodine in methanol and then with 0.5% starch solution, remained at the origin in this system.

(b) Method of elution from the chromatogram. Two different techniques were tried for the elution of ascorbic acid from the chromatogram. In the first technique the adsorbent containing the ascorbic acid was scraped into a beaker and mixed with 1% acetic acid. After centrifugation of the resultant suspension the supernatant contained the eluted ascorbic acid. The disadvantage of this method was that it was difficult to transfer the finely powdered scrapings quantitatively into the beaker. This difficulty was solved by adopting the following method (Malinek, 1965).

Eluting pipettes were constructed from 10 cm lengths of 7 mm internal diameter glass tubing which were constricted at one end and plugged with glass wool (Figure 6). The area of cellulose containing the ascorbic acid was drawn into the tube by tap suction and allowed to form a layer over the glass wool plug. Elution of the ascorbic acid was attained by passing the eluting fluid through the cellulose layer, a process which was hastened with the aid of compressed air.

Using this apparatus 10 mg of ascorbic acid was eluted from a silica gel thin layer with 0.75 ml of 1% acetic acid. This was well within the limit of 2 ml required by the Fe-T.P.T.Z. method.

(c) *Difficulties encountered with silica gel.* 10 µl samples from solutions containing known amounts of ascorbic acid were applied to a thin layer chromatogram which was then developed in the solvent n-propanol:acetic acid:water:ethyl acetate = 80:8:10:20. After drying the chromatogram the ascorbic acid spots were located (see below) and then eluted. The ascorbic acid concentration in the eluate was estimated by the Fe-T.P.T.Z. reaction.

This experiment was repeated many times but no reproducible relationship was obtained between the known amount of ascorbic

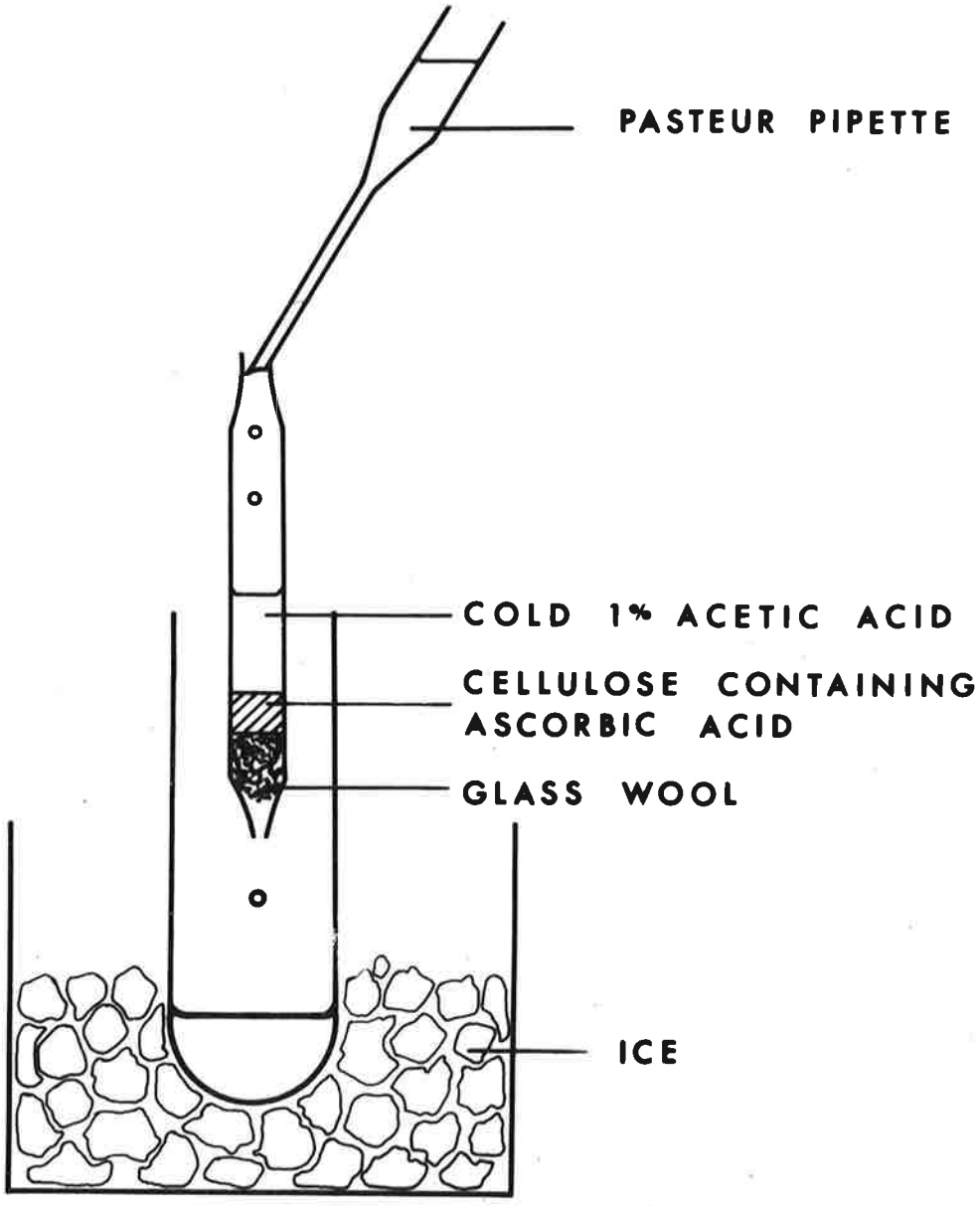


FIGURE 6: The elution of ascorbic acid from cellulose.

acid in each 10 µl sample and the amount detected in the eluate by the Fe-T.P.T.Z. reaction.

Analysis of the results suggested that the lack of reproducibility was due to the following causes:

i. Contamination of silica gel with iron. As stated previously the Fe-T.P.T.Z. reaction detects 3 parts per 100 million of ferrous ions. According to the manufacturers (Merck) the silica gel used contained 0.03% of iron. This relatively large amount of iron impaired the estimation of ascorbic acid for two reasons. Firstly it caused a high and variable blank value in the Fe-T.P.T.Z. reaction. For instance, when two areas of silica gel 1.5 cm x 1.5 cm containing no ascorbic acid were eluted and the eluates estimated, absorbances of 0.15 and 0.30 were obtained. Secondly, at the concentrations of ascorbic acid used (0-2.5 µg/10 µl sample) it considerably enhanced the breakdown of ascorbic acid during chromatography so that little or no ascorbic acid could be detected in the eluate.

ii. Fluorescent indicator. When 2.5 µg of ascorbic acid was eluted from silica gel (Merck) to which fluorescent indicator had been added an absorbance of 0.30 was obtained on estimation of the ascorbic acid in the eluate by the Fe-T.P.T.Z. reaction (mean of 4 estimations). When silica gel (Merck) without added

fluorescent indicator was used the mean absorbance was 0.57 suggesting that the fluorescent indicator either enhanced the breakdown of ascorbic acid or interfered with the reaction between ascorbic acid, ferric ions, and T.P.T.Z.

iii. Location of ascorbic acid spots. Some of the inaccuracy obtained when ascorbic acid was estimated after chromatography on silica gel was due to error in the location of the ascorbic acid spots after development of the chromatogram. When silica gel without fluorescent indicator was used there was insufficient ascorbic acid in the spots to allow their visualisation by ultraviolet light. The position of the sample spots was therefore deduced from the location of control spots containing a larger amount of ascorbic acid. Though this method was successful in most instances, slight distortion of the solvent front sometimes occurred and altered the Rf values of the sample spots in unpredictable fashion.

(d) Purification of silica gel - removal of iron. The purification of silica gel was carried out in the hope that the lack of reproducibility was due mainly to iron contamination of the thin layer. Iron can be removed from silica gel by washing with hydrochloric acid (Bobbitt, 1963). A major disadvantage of the procedure as described by Bobbitt is that the sticky sludge obtained is difficult to wash by filtration and it forms a cake

on oven drying which must be ground and regraded for particle size. For these reasons an alternative process was devised.

The silica gel was washed by suspending in an equal volume of 5 N HCl. The suspension was centrifuged and the supernatant, which was dark yellow due to iron contamination, discarded. This washing procedure was repeated 5 times. The silica gel was then washed with distilled deionised water 8-10 times after which washing once in 0.05 M potassium phthalate brought the pH to 3.2. The sludge was then resuspended in a small amount of 0.05 M potassium phthalate and applied immediately to glass plates, thus obviating the problems which occur on drying in the procedure described by Bobbitt. This process was still tedious, however, and took about 3 hours.

Using this purified thin layer reproducible and low blank determinations were obtained on a series of 2.0 ml eluates estimated by the Fe-T.P.T.Z. procedure (0.077 ± 0.023 on 7 2.0 ml eluates compared with 0.055 ± 0.008 for 8 2.0 ml samples of 1% acetic acid). Thus a considerable degree of purification had been obtained.

(e) Estimation of ascorbic acid after chromatography on purified silica gel. To assess the suitability of purified silica gel for the thin layer chromatographic estimation of

ascorbic acid the following experiment was performed. Known amounts of ascorbic acid (from 0-2.5 µg) in 1% acetic acid were applied as 10 µl spots to a purified silica gel thin layer chromatogram. Marker spots containing a larger amount of ascorbic acid were also applied. The chromatogram was developed in the solvent n-propanol:acetic acid:water:ethyl acetate = 80:8:10:20 and then partially dried in a cold air stream. The location of the ascorbic acid spots was determined from the marker spots which were rendered visible by spraying with silver nitrate while screening the rest of the chromatogram to prevent contamination of the sample spots. Elution of each ascorbic acid sample was carried out with 2.0 ml of 1% acetic acid and the absorbance generated by each eluate in the Fe-T.P.T.Z. reaction was measured.

A linear relationship was found between the amount of ascorbic acid originally applied to the origin of the chromatogram and the absorbance of the blue Fe $(T.P.T.Z.)_2$ complex (Figure 7). The eluate corresponding to the sample containing no ascorbic acid yielded an absorbance of 0.10 and that corresponding to the sample containing 2.5 µg an absorbance of 0.55. 2.0 ml of 1% acetic acid and 2.0 ml of 1% acetic acid containing 2.5 µg of ascorbic acid yielded an absorbance of 0.04 and 0.68 respectively. The recovery of ascorbic acid after chromatography was therefore

$$\frac{0.55 - 0.10}{0.68 - 0.04} \times 100\% = 70\%.$$

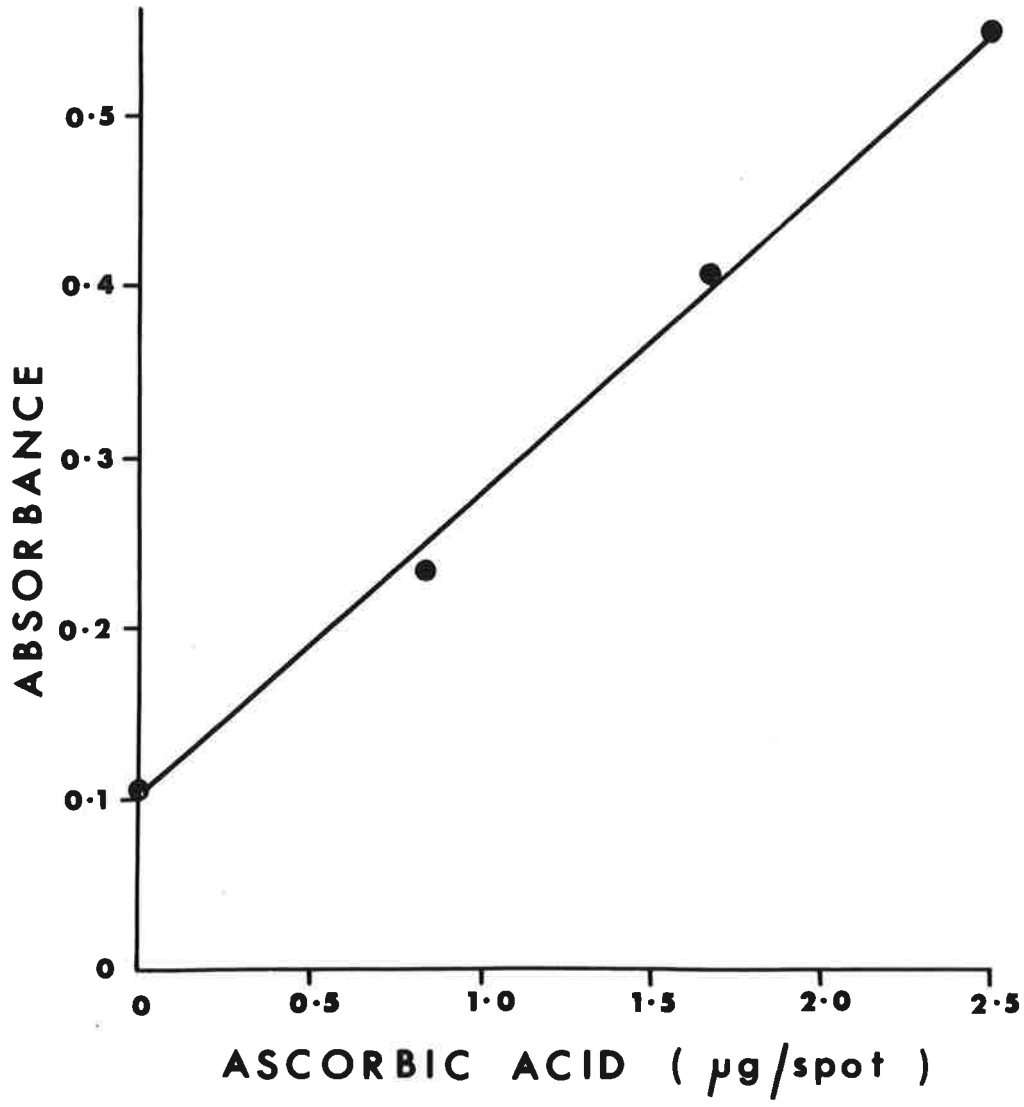


FIGURE 7: Relationship between absorbance and known amounts of ascorbic acid in samples applied to the origin of the chromatogram. Each point represents the mean from two samples.

These results showed that the lack of reproducibility of the results obtained previously was due largely to contamination of silica gel with iron. Furthermore they demonstrated the suitability of purified silica gel for the thin layer chromatographic estimation of ascorbic acid.

5. Assessment of cellulose as a chromatographic medium. The disadvantage of silica gel was that a time consuming procedure was required for the removal of iron contamination. Keiselguhr (Merck) also has a high iron content (0.05%* as compared to 0.03%* for silica gel) but cellulose (Whatman CC41) has an iron content of less than 0.0005%* and should not require purification.

(a) Preparation of plates. In accordance with the recommendations of Bobbitt (1963) for quantitative thin layer chromatography, the cellulose was prewashed once in n-propanol and once in 1% acetic acid. It was then resuspended in sufficient 1% acetic acid to form a thin suspension which was applied to the glass plates as a layer 0.65 mm thick. A 10 µl sample of water applied to a cellulose layer of this thickness produced a spot diameter of 1.0-1.1 cm which was small enough to allow 10 such samples to be accommodated on one 20 cm x 20 cm plate.

(b) Preliminary experiment. Before expending time on the

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*According to the manufacturers.

development of cellulose as a thin layer chromatographic medium for ascorbic acid estimation, an experiment was performed to ensure that ascorbic acid could be estimated by the Fe-T.P.T.Z. reaction after chromatography on cellulose. Known amounts of ascorbic acid (from 0-2.5 µg) were subjected to thin layer chromatography on a cellulose layer using the solvent n-propanol:water = 60:30 with sufficient 1% acetic acid added to bring the pH to 3. The ascorbic acid spots were located at Rf 75, eluted, and estimated by the Fe-T.P.T.Z. reaction. A linear relationship was obtained between the amount of ascorbic acid originally applied and the absorbance generated with the Fe-T.P.T.Z. reagent (Figure 8). The mean blank value, obtained from the 2 samples containing no ascorbic acid, was 0.044 which did not differ greatly from that of 0.034 obtained from 2 samples of 1% acetic acid. This signified a minimal degree of iron (ferrous) contamination. Recovery of ascorbic acid was 78% which compared favourably with the value of 70% obtained when purified silica gel was used (see above).

The above experiment demonstrated that cellulose was suitable for the estimation of pure ascorbic acid samples. By using this medium the lengthy purification procedure required for silica gel would be avoided. For this reason the development of a solvent which would allow the separation of ascorbic acid from other reducing substances on a cellulose thin layer was undertaken.

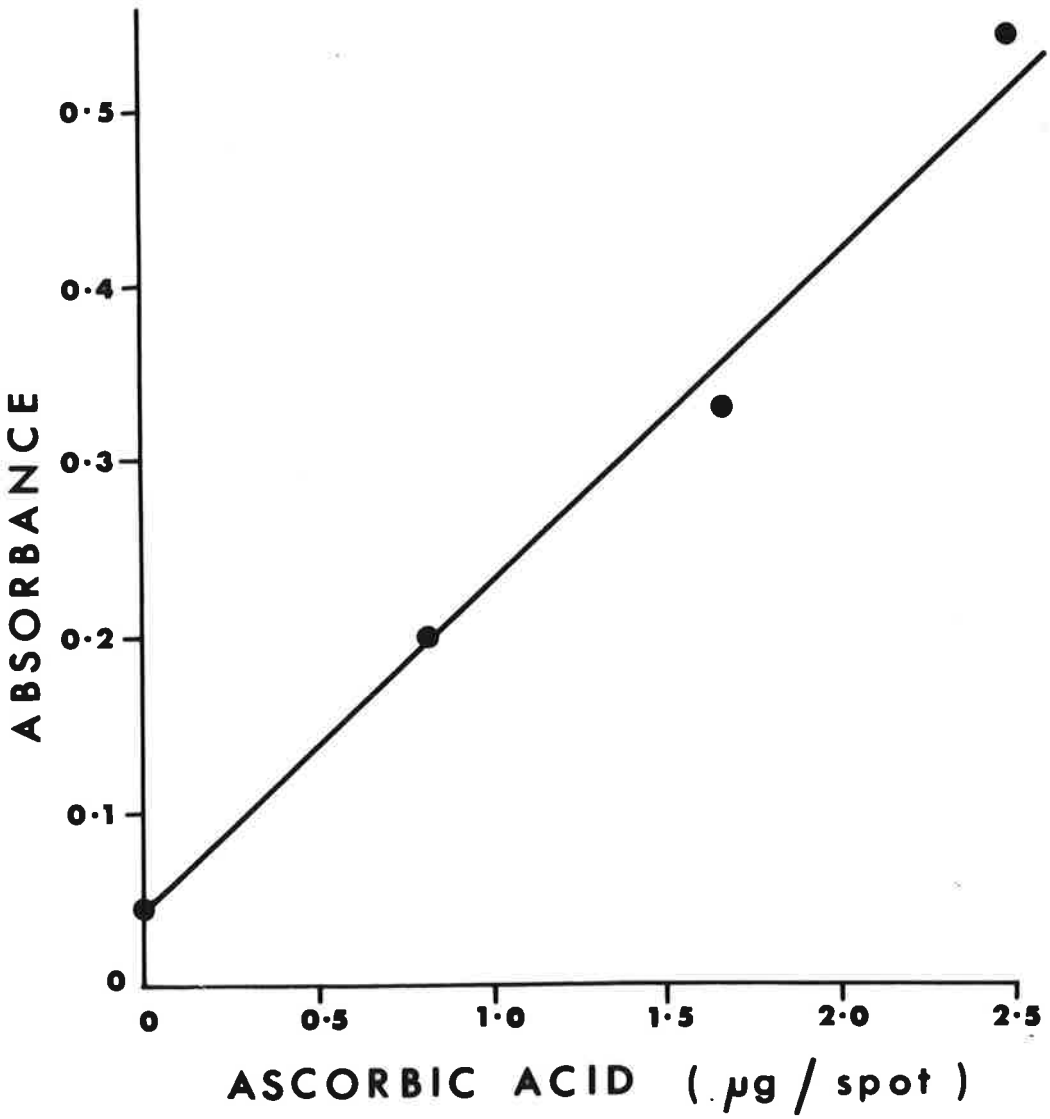

FIGURE 8: The relationship between absorbance and known amounts of ascorbic acid in samples applied to the origin of the chromatogram. Each point represents the mean from two samples.

TABLE 3. SEPARATION OF ASCORBIC ACID FROM OTHER REDUCING SUBSTANCES ON CELLULOSE

Solutions applied	Rf = distance from origin x 100 / length of run (10 cm)			Spray reagents (Waldi, 1965)
CHROMATOGRAM 1	Ascorbic acid	cysteine	glutathione	
Ascorbic acid 0.35 mg/ml	54			
Cysteine 0.3 mg/ml		33		Phosphomolybdic acid followed by heat (80-90°C for 5 minutes)
Glutathione 0.6 mg/ml			33	
A:C = 1:1	53	33		
A:G = 1:1	49		33	
CHROMATOGRAM 2	Ascorbic acid	$FeSO_4$	$SnCl_2$	
Ascorbic acid 0.3 mg/ml	53			
$FeSO_4$ 7 H_2O 10 mg/ml		23		Phosphomolybdic acid followed by heat (80-90° C for 5 minutes)
$SnCl_2$ 5 mg/ml			1	
A:Fe = 1:1	50	23		
A;Sn = 1:1	47		47	
CHROMATOGRAM 3	Ascorbic acid		Dehydroascorbic acid	
Ascorbic acid 0.3 mg/ml	65			Silver nitrate spray followed by heat (110°C for 5 minutes)
Dehydroascorbic acid 0.6 mg/ml			58	
A:D = 1:1	65		59	

(c) Separation of ascorbic acid from other reducing substances on cellulose. 10 µl of the solutions listed in Table 3 were applied to the origin of a cellulose thin layer chromatogram. The solvent propanol:water = 85:15 buffered to pH 3 with 1% oxalic acid produced a separation of ascorbic acid from cysteine, glutathione and ferrous ions. These substances were rendered visible as blue spots on a yellow background by spraying with 5% phosphomolybdic acid in ethyl alcohol and heating for 5 minutes at 80-90°C (Waldi, 1965). Separation was also obtained from sulphite ions which, in high concentration (50 mg/ml anhydrous sodium sulphite) were detected by phosphomolybdic acid spray at Rf 25.

A separation of ascorbic acid from stannous ions was not obtained. Stannous ions subjected to chromatography in the above solvent remained at the origin, but when a mixture of ascorbic acid and stannous chloride was tried, a single spot was formed at Rf 37 (Table 3). This suggests that a complex with Rf value different from either substance was formed between stannous ions and ascorbic acid. This difficulty is not serious as tin has not been found in platelets (Maupin, 1961).

Dehydroascorbic acid had a slightly lower Rf value than ascorbic acid, but a complete separation was not obtained (Table 3).

To test this solvent C^{14} labelled ascorbic acid was added to an extract of platelets (prepared as described below). 20 µl of this extract was resolved by chromatography on cellulose using the solvent system described above. The chromatogram was then sprayed with 5% phosphomolybdic acid and scanned for beta activity in the thin layer chromatogram scanner.

Ascorbic acid appeared at Rf 65 as a discrete oval blue spot. The radioactive scan showed that the radioactivity occurred as a single peak (Figure 9) which coincided with the position of the blue spot. The tail of this peak, however, had a slightly lower Rf value than the tail of the blue spot. This tail of radioactivity was probably dehydroascorbic acid which could have been produced by breakdown of ascorbic acid. We have shown that dehydroascorbic acid has an Rf value slightly lower than that of ascorbic acid (Table 3) and that the recovery of ascorbic acid after chromatography is only 80% suggesting some loss by oxidation. In addition 9% of the ascorbic acid activity was lost during manipulation of the radioactive ascorbic acid prior to its addition to the platelet extract (see Chapter 6).

Other reducing substances in platelets were not present in high enough concentration to be rendered visible by the phosphomolybdic acid spray. They could, however, be detected by eluting the chromatogram and estimating the eluates for reducing activity

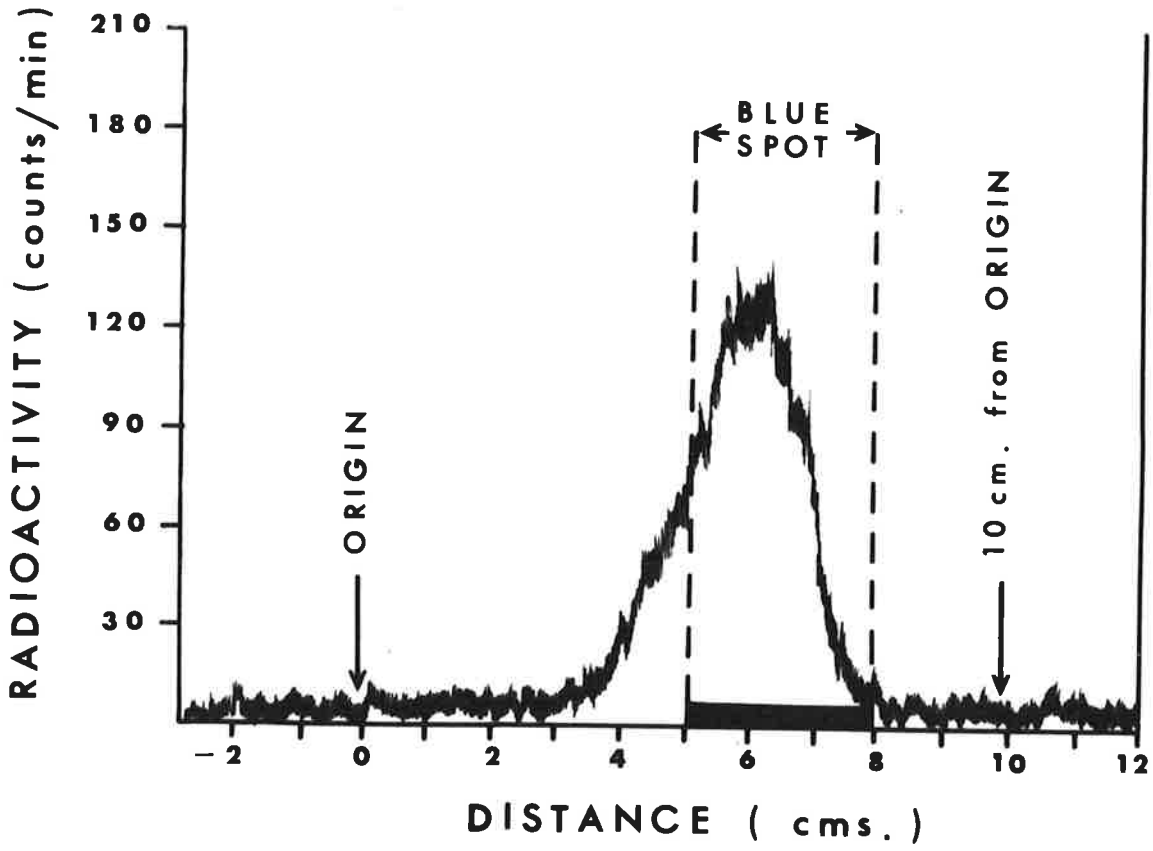

FIGURE 9: Radioactive scan of a thin layer chromatogram after resolution of a platelet extract containing C^{14}-ascorbic acid.

in the Fe-T.P.T.Z. reaction. For instance, when the area (Rf 33) which would be expected to contain cysteine or glutathione was eluted, the eluate generated an absorbance of 0.30 and 0.33 (in 2 separate experiments), values well above blank values of 0.07 and 0.06.

Thus it has been shown that using cellulose and the above solvent system ascorbic acid is separated from reducing substances likely to be present in platelets. The possibility that some other unknown reducing substance happens to migrate to the same position on the chromatogram as ascorbic acid cannot, of course, be completely excluded.

6. Reasons for choosing cellulose. It has now been shown that both cellulose and silica gel could be used for the separation of ascorbic acid from other reducing substances and that both would allow the estimation of eluted ascorbic acid by the Fe-T.P.T.Z. reaction. Cellulose had the advantage that a lengthy purification for iron was not required. A disadvantage of cellulose was that the layer was less stable, being more inclined to crack or to become detached from the glass. This could be avoided, however, if more solvent was added to the slurry to render it less viscid than a silica gel slurry, and if more attention was paid to de-greasing the glass prior to application of the thin layer. Mainly because of its low degree of iron contamination cellulose was chosen

as the adsorbent to be used in the estimation of platelet ascorbic acid.

7. Method for locating ascorbic acid spots. Up to this time the location of ascorbic acid spots prior to elution was deduced from the position of marker spots containing a higher concentration of ascorbic acid. These were detected by spraying with phosphomolybdic acid while the remainder of the chromatogram was screened to prevent contamination of the sample spots with spray reagent. This method has the following disadvantages:

(a) Distortion of the solvent front sometimes occurred due to an uneven thickness of the cellulose layer or to inadequate saturation of the atmosphere of the chromatography tank. When this occurred the method did not allow the accurate prediction of the location of the unknown ascorbic acid spots, and occasionally adjacent samples would run together and contaminate one another.

(b) The marker spots occupied two or more sites on the chromatogram which could otherwise have been used for samples.

(c) The screening procedure was clumsy and did not always prevent the phosphomolybdic acid spray from contaminating one of the sample spots.

These difficulties were overcome when it was realised that

cellulose has a slight natural fluorescence under ultraviolet light (254 mμ). This is sufficient to render ascorbic acid samples of 1-2.5 μg clearly visible as dark spots. It was also found that adjacent samples could be prevented from contaminating one another by ruling the thin layer into a series of vertical columns (one for each sample) with a sharp lead pencil.

8. Elution of ascorbic acid from cellulose. An experiment was performed to ensure that with cellulose, as with silica gel, all the ascorbic acid was eluted in 2.0 ml of 1% acetic acid. 3.0 ml of 1% acetic acid were used to elute 5 different spots each containing 2.5 μg of ascorbic acid. The eluate from each was collected in 3 1.0 ml aliquots. Of the ascorbic acid recovered a mean of 89% was in the first ml and 11% in the second ml of eluate. No additional ascorbic acid was eluted in the third ml.

9. Procedure finally used for thin layer chromatography.

(a) Reagents. (i) Solvent for chromatography: n-propanol: water = 85:15 brought to pH 3 with about 10 ml of 1% oxalic acid. The n-propanol had been redistilled in a glass still to ensure freedom from iron contamination, and distilled water which had been subsequently deionised was used. (ii) Known concentrations of ascorbic acid dissolved in 1% acetic acid.

(b) Procedure. Known amounts of ascorbic acid (from 0-2.5 μg) were subjected to thin layer chromatography. The chromatography

tank was surrounded by a black cardboard screen to minimise the amount of breakdown due to light. When the solvent had travelled for a distance of 10 cm from the origin the chromatogram was removed from the tank and partially dried in a cold air stream. The position of each ascorbic acid spot was determined with the aid of ultraviolet light and the ascorbic acid was eluted by the technique of Malinek (described above) using 2.0 ml of 1% acetic acid. The samples were centrifuged at 1300 g for 5 minutes to remove any cellulose which had passed through the fibreglass plugs of the eluting apparatus. The amount of ascorbic acid in each eluate was then determined by the Fe-T.P.T.Z. reaction.

(c) Results. A linear relationship was obtained between the amount of ascorbic acid originally applied to the chromatogram and the absorbance generated by the eluate with the Fe-T.P.T.Z. reagent (Figure 10). The mean recovery was 83%.

(d) Reproducibility of chromatographic procedure. Nine 10 µl samples of ascorbic acid each containing 2.5 µg were subjected to chromatography on the same plate, eluted, and the ascorbic acid content of the eluate determined by the Fe-T.P.T.Z. reaction. The mean increase in absorbance above the blank value was 0.429 ± 0.024, a standard deviation of 5.6%. 9 blank spots treated in the same manner yielded a mean absorbance of 0.051 ± 0.013. These results represented a recovery rate of 71%.

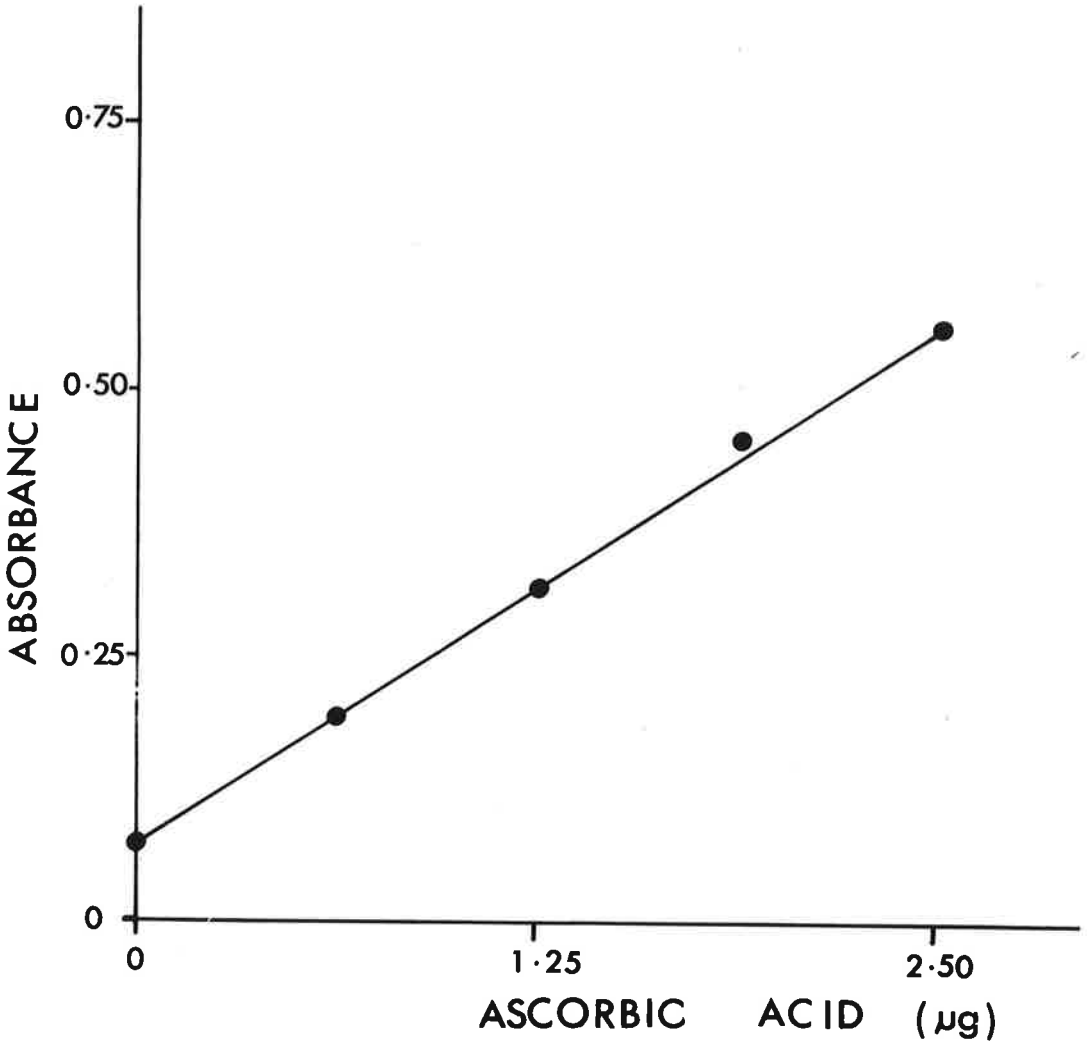

FIGURE 10: The relationship between absorbance and known amounts of ascorbic acid in samples applied to the origin of the chromatogram. Each point represents the mean from two samples.

(e) Conclusions. Thin layer chromatography on cellulose with subsequent estimation of the resolved ascorbic acid should provide a suitable method for the estimation of ascorbic acid in biological samples.

IV. APPLICATION OF THE METHOD TO BLOOD PLATELETS

1. Production of an extract suitable for application to the chromatogram.

(a) Separation of platelets from whole blood. Using siliconised syringes and needles blood was taken by venepuncture into one-tenth its volume of 3% disodium ethylenediaminetetra-acetic acid (E.D.T.A.) in a plastic centrifuge tube. The tube was kept immersed in melting ice during transport to the laboratory. Platelet rich plasma was obtained by centrifugation at 300 g for 20 minutes at 0-4°C, was transferred to another tube using a siliconised Pasteur pipette and centrifuged at 1300 g for 30 minutes at 0-4°C to obtain a platelet button. The platelets from 10 ml of blood were resuspended in 0.5 ml of normal saline and from this suspension films were prepared on microscope slides and stained with Jenner-Geimser stain. Examination by light microscopy revealed no contamination by red cells and only one white cell in over 1000 platelets.

(b) Decision not to wash the platelets. A difficulty encountered in chemical estimations carried out on platelets is

that it is difficult to obtain platelets free from contamination by plasma. A platelet button must contain plasma in the interstices between the platelets and, in addition, platelets are said to adsorb plasma proteins onto their surface (Adelson et alii, 1961). Maupin (1961) stated that "l'éxperience montre qu'un grand nombre de lavages sont nécessaires à l'élimination des protéines adsorbées" and he accordingly washed platelets three times prior to carrying out a chemical estimation. It is probable, however, that repeated washing would damage the platelet membrane sufficiently to allow the release of certain substances.

It is probably best to avoid washing platelets prior to chemical analysis for the following reasons.

(i) In many cases the substance being estimated is in much higher concentration in the platelets than in the plasma, for example, serotonin (Humphrey and Jaques, 1954) and ascorbic acid (Barkhan and Howard, 1958), so that the contribution to the estimation by contaminating plasma is negligible.

(ii) For substances present in a similar concentration in plasma to platelets a technique such as the Evans blue dilution method which Chaplin and Mollison (1952) applied to red cells should enable the amount of contaminating plasma to be estimated and an appropriate correction applied.

(iii) The less the platelets are damaged prior to analysis the greater the probability that the results are representative of the situation in the platelet in vivo.

For these reasons, particularly because of the much higher concentration of ascorbic acid in platelets than in plasma, it was decided not to attempt to wash the platelets.

(c) *Homogenisation of platelets.* There are 3 principal methods for the homogenisation of cells: ultrasound, freeze-thawing, and tissue grinding. Ultrasound was not possible in this instance because suitable equipment was not available. Freeze-thawing proved unsuccessful, for after freezing and thawing 12 times in a dry ice alcohol mixture no visible breakdown of platelets occurred (by phase contrast microscopy). A teflon tissue grinder was therefore tried (Figure 11).

The platelets from 20 ml of blood were resuspended in 0.2 ml of normal saline and transferred to the glass chamber of a teflon tissue grinder (capacity of lower chamber = 1 ml, Figure 11). The teflon rod while being rotated at 4400 revolutions per minute by an electric motor was moved up and down inside the lower chamber about once every 5 seconds. During this procedure the lower chamber was immersed in melting ice. This was effective in dissipating most of the heat produced, for after 2 minutes of this procedure the teflon rod still felt cold to the touch. At 1

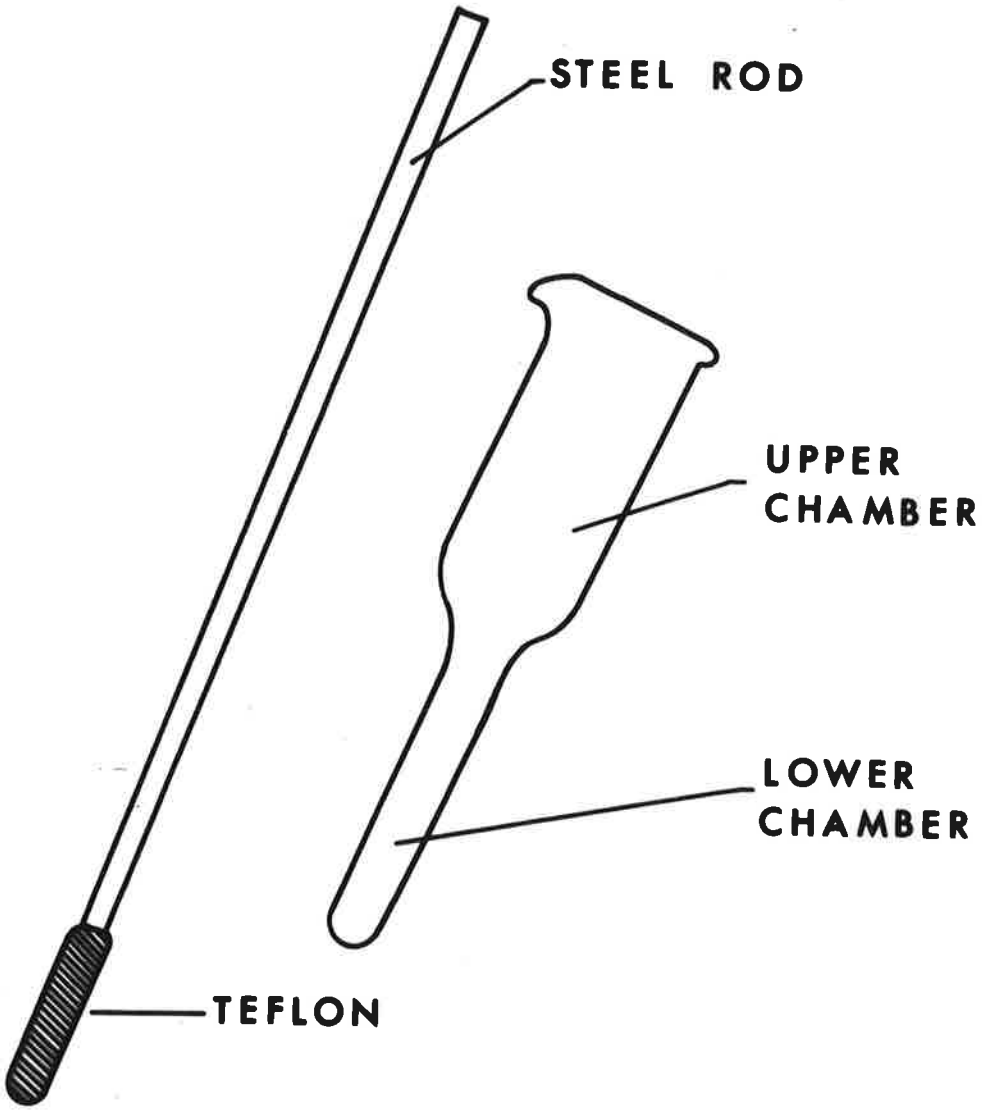

FIGURE 11: Teflon tissue grinder.

minute intervals samples were removed from the grinding chamber and viewed by phase contrast microscopy. After 1 minute only one platelet in 10 remained intact, and after 2 minutes all platelets appeared very ragged, swollen, and granular and much extracellular debris was present.

Since tissue grinding produced the greatest amount of visible cellular disruption this method was adopted. It was shown later that this method released more ascorbic acid from platelets than simply stirring them with a glass rod (Table 6).

A disadvantage of this method was that platelets were lost by becoming adherent to the walls of the centrifuge tube and the Pasteur pipette while being transferred from the centrifuge tube to the grinding chamber. This was overcome by centrifuging the platelet rich plasma in the grinding tubes to obtain the platelet button in the bottom of the grinding chamber, thus avoiding the transfer procedure.

(d) Quantitation of platelets. The number of platelets in each button was determined by performing platelet counts (Brecher and Cronkite, 1950) on the platelet rich and platelet poor plasma, and by recording the volume of the platelet poor plasma.

$$N = 10^3 V (n_1 - n_2)$$

where N = number of platelets in button

V = volume of platelet poor plasma (ml)

n_1 = platelet count of platelet rich plasma (per c mm)

n_2 = platelet count of platelet poor plasma (per c mm)

The weight of the platelet button was found by taring the tissue grinder chamber prior to use and reweighing when all plasma had been removed from the grinding chamber. Contaminating plasma was removed by inverting the chamber for 2 minutes and then wiping the inside of the upper chamber with adsorbent tissue. The small amount of plasma which remained in the lower chamber on reinverting was easily removed with a Pasteur pipette.

(e) Separation of protein from the extract. Homogenisation of platelets in normal saline produced a thick suspension which after a few minutes formed a gel unsuitable for application to the chromatogram. Metaphosphoric acid and trichloracetic acid have been used by previous workers to precipitate protein in animal tissue extracts which were to be submitted to ascorbic acid analysis (Musulin and King, 1936). Metaphosphoric acid is preferred because it inhibits the catalytic oxidation of ascorbic acid by metals such as iron and copper (Roe, 1954). Trichloracetic acid is less often used because it is an oxidising agent and therefore renders ascorbic acid less stable (Musulin and King, 1936).

Metaphosphoric acid proved unsuitable for use in the present method for ascorbic acid dissolved in 3.4% metaphosphoric acid failed to react with the Fe-T.P.T.Z. reagent both before and after chromatography. Presumably either metaphosphoric acid itself, or a contaminant of this reagent, interferes with the Fe-T.P.T.Z. reaction.

Although it is an oxidising agent, the use of trichloracetic acid was assessed as a means of inducing protein precipitation. The stability of ascorbic acid in a solution of trichloracetic acid was determined by preparing a solution of 150 µg/ml ascorbic acid in 8% trichloracetic acid and estimating the ascorbic acid concentration at intervals while maintaining the solution at 0-4°C in the refrigerator. No loss of ascorbic acid occurred over the first hour but after 2 hours a loss of 5% had occurred. This rate of breakdown was negligible because the extract was resolved by thin layer chromatography within an hour of adding the trichloracetic acid and any breakdown which did occur was allowed for by the use of internal standards (see below). The protein from the platelets obtained from 15 ml of blood was effectively precipitated by 0.1 ml of 8% trichloracetic acid and the precipitate was removed by centrifugation at 1300 g for 5 minutes to leave a clear supernatant.

TABLE 4. CHROMATOGRAPHY AND ESTIMATION OF ASCORBIC ACID IN PLATELET EXTRACTS

Sample applied (20 µl)		Absorbance (to nearest 0.05)	Mean absorbance	Absorbance less blank value	Calculated amount (µg) of ascorbic acid in each sample
1. Platelet extract	(i)	0.17	0.17	0.122	1.31
	(ii)	0.17			
2. Platelet extract + 1-2/3 µg ascorbic acid	(i)	0.300	0.315	0.267	2.98
	(ii)	0.330			
3. Platelet extract + 3-1/3 µg ascorbic acid	(i)	0.525	0.505	0.457	4.64
	(ii)	0.485			
4. 8% trichoracetic acid (blank)	(i)	0.055	0.048	0	0
	(ii)	0.040			
5. 2.5 µg ascorbic acid in 8% trichloracetic acid (control)	(i)	0.455	0.455	0.407	2.5
	(ii)	0.455			
6. 2.5 µg ascorbic acid in 2 ml 1% acetic acid	(i)	0.59	0.605	0.570	2.5
	(ii)	0.620			
7. 2 ml. 1% acetic acid (blank)	(i)	0.035	0.035	0	0
	(ii)	0.035			

Samples 1 to 5 were subjected to thin layer chromatography followed by estimation with Fe-T.P.T.Z. Samples 6 and 7 were estimated with Fe-T.P.T.Z. without preceding chromatography.

2. Thin layer chromatography and estimation of ascorbic acid in a platelet extract.

(a) Method. Equal amounts of platelet rich plasma from 45 ml of blood were centrifuged in 3 grinding tubes to obtain 3 separate platelet buttons. To each tube was added 150 µl of 8% trichloracetic acid containing 0, 12.5 and 25 µg of ascorbic acid respectively. The platelet buttons were homogenised and the protein precipitate removed by centrifugation. From each of the 3 tubes 2 samples of supernatant were applied to the chromatogram. It was found that with platelet extracts the samples spread less on the thin layer allowing 20 µl to be applied in each spot, whereas with the pure ascorbic acid samples previously used only 10 µl could be used if it was desired to accommodate 10 samples on one 20 cm x 20 cm plate. Of the remaining 4 places on the chromatogram 2 were occupied by blank spots (20 µl of 8% trichloracetic acid) and 2 by control spots (20 µl samples of 8% trichloracetic acid each containing 2.5 µg of ascorbic acid). The chromatogram was developed, the ascorbic acid was located and eluted, and the eluate was subjected to ascorbic acid estimation by the Fe-T.P.T.Z. method.

(b) Results. From the absorbance generated by the ascorbic acid from the 3 platelet samples the amount of ascorbic acid in each 20 µl sample of the extract was calculated (Table 4). There

was a linear relationship between the calculated amount of ascorbic acid in the extracts and the colour generated in the Fe-T.P.T.Z. reaction (Figure 12). The proportion of ascorbic acid which was recovered in the eluate after addition to platelet extracts and subsequent chromatography was determined. This was calculated from the absorbance generated in the Fe-T.P.T.Z. reaction by the eluates from samples 1, 2 and 3 (Table 4) and the absorbance generated by a solution containing a known amount of ascorbic acid (sample 6, Table 4). The mean recovery of ascorbic acid from platelet extracts was 42%. The mean recovery of ascorbic acid from 20 µl of 8% trichloracetic acid (sample 5, Table 4) was 71%. Thus the recovery after chromatography of ascorbid acid in trichloracetic acid which was added to platelet extracts was lower than that in trichloracetic acid which had not been added to a platelet extract. An experiment which helped to elucidate the reason for this is described later.

(c) Conclusions. These results showed that thin layer chromatography of platelet extracts followed by estimation with the Fe-T.P.T.Z. reagent was a suitable method for the estimation of ascorbic acid in platelets. They also showed that it would not be possible to use external standards because the recovery rate of ascorbic acid from platelets was lower than from a standard solution. It would have been possible to use external standards if the recovery rate was constant from one run to another, but further experiments

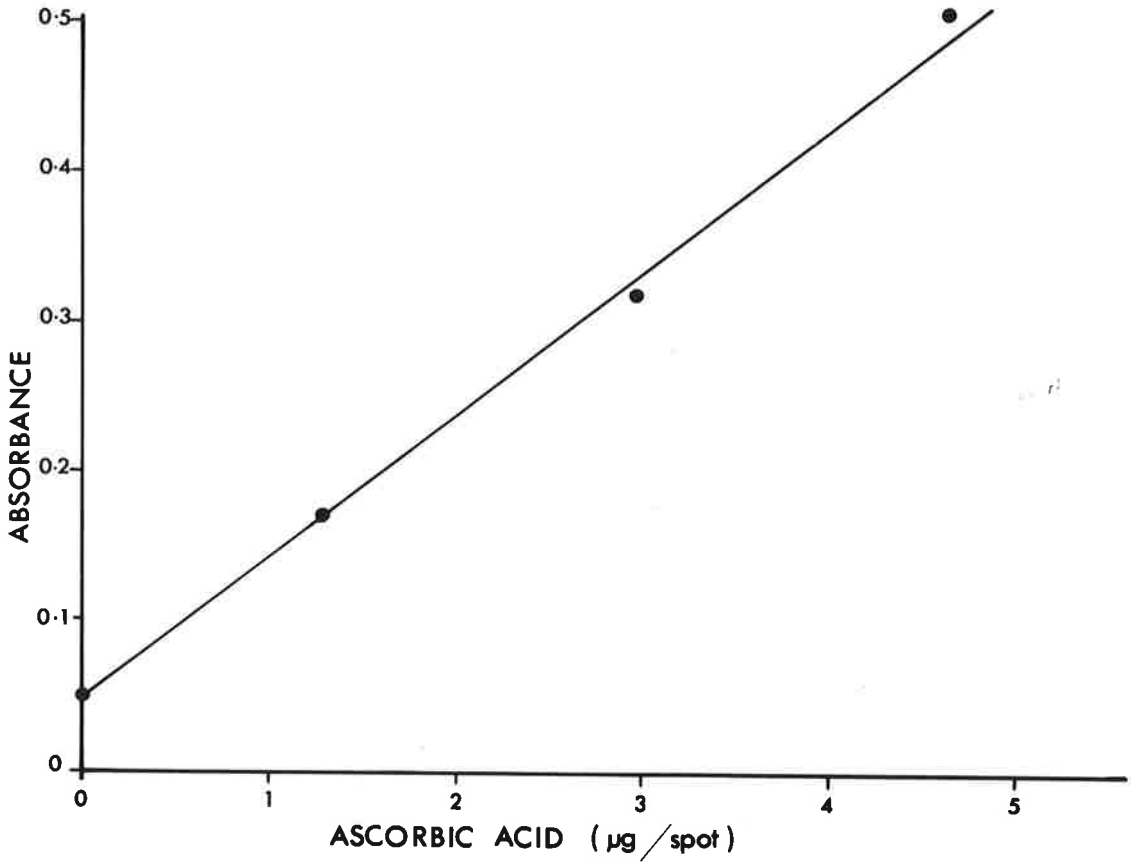

FIGURE 12: The relationship between absorbance and amount of ascorbic acid in samples of platelet extracts applied to a thin layer chromatogram. Each point represents the mean from two samples.

showed that this was not so.

3. Calculations and the use of internal standards. Because of the low and variable recovery of ascorbic acid the use of internal standards was mandatory. Each sample of platelet rich plasma was divided equally between 2 tissue grinder chambers and centrifuged to obtain the 2 platelet buttons which were then weighed. To one platelet button was added 0.1 ml of 8% trichloracetic acid and to the other 0.1 ml of 8% trichloracetic acid containing 15 µg of ascorbic acid (freshly prepared) to form an internal standard. From each of the 2 extracts two 20 µl aliquots were subjected to thin layer chromatography and ascorbic acid estimation with Fe-T.P.T.Z. Two 20 µl samples of 8% trichloracetic acid were included as blanks. The amount of ascorbic acid in the platelet button was calculated from the formula:

$$W = \frac{15\ A_S}{A_R - A_S}$$

where W = weight of ascorbic acid in platelet button

A_S = absorbance generated (less the blank value) by ascorbic acid from the sample

A_R = absorbance generated (less the blank value) by ascorbic acid from the internal standard

In practice the weight of platelets in the 2 platelet buttons often differed and allowance was made for this by modifying the

above formula as follows:

$$W = \frac{15\ A_S}{A_R - \frac{W_R}{W_S} \cdot A_S}$$

where W_R = weight of platelets in internal standard

W_S = weight of platelets in sample

4. Expression of results

Although the results of each estimation were expressed both as a proportion of platelet number (µg of ascorbic acid per 10^9 platelets) and as a proportion of wet weight of platelets (µg of ascorbic acid per mg of platelets) it was unnecessary to repeat all statistical analyses on both sets of results. Expression of values as a proportion of wet weight of platelets does not allow an easy comparison of results from different authors for the number of platelets in a given weight of platelet button depends on the conditions of centrifugation. Barkhan and Howard (1958) obtained 6.3×10^7 platelets/mg of platelet sample whereas Marcus and Zucker (1965) quote a value of $4\text{-}5 \times 10^7$ platelets/mg. In the present study $(3.6 \pm 0.65) \times 10^7$ platelets/mg was obtained in 13 samples from normal subjects. The differences in results from different workers are probably due to variation in the amount of plasma trapped in the platelet buttons at varying centrifuge speeds.

Because it allowed a more valid comparison with the results of previous authors statistical analyses were performed on the platelet ascorbic acid content expressed as a proportion of platelet count.

Some authors recommend that the results of chemical estimations on platelets should be expressed as a proportion of dry weight (Maupin, 1961). The dry weight of platelets in two platelet buttons prepared as for ascorbic acid analysis was 3.7 and 3.1 mg/10^9 platelets corresponding respectively to 18% and 19% of the wet weight. This compares with 2.8 mg/10^9 platelets found by Maupin (1961), 2.1 mg/10^9 platelets by Barkhan and Howard (1958) and 2.9-5.0 mg/10^9 platelets by Gerok and Gross (1959).

5. Reproducibility of method

The error of the method is compounded by two main factors. These are the error in the determination of the amount of ascorbic acid in the platelet button and the error in the determination of the number of platelets in the button.

(a) Standard deviation of ascorbic acid determination. Equal aliquots of a sample of platelet rich plasma were centrifuged to obtain 8 identical platelet buttons. These were paired and one member of each pair was homogenised in 8% trichloracetic acid. The other member was ground in 8% trichloracetic acid to which ascorbic acid had been added to form an internal standard. Thus

4 identical sample extracts were obtained, each with its corresponding internal standard. After homogenisation one 20 µl aliquot from each extract along with 20 µl from the internal standard were subjected to ascorbic acid determination by thin layer chromatography. Using the corresponding internal standard the ascorbic acid content of each of the 4 samples in turn was calculated. The mean ascorbic acid content of the 4 sample buttons in one such experiment was 4.40 ± 0.68 µg, and in another 5.50 ± 0.73 µg, standard deviations of 15% and 13% respectively (a mean of 14%). In routine estimations of platelet ascorbic acid content 2 20 µl aliquots from the sample and 2 from the internal standard were estimated and the means determined. An estimate of the standard deviation for the routine determination of the amount of ascorbic acid in the platelet button is therefore $\frac{14}{\sqrt{2}}\% = 10\%$.

(b) Standard deviation of platelet count. 10 consecutive platelet counts on the same sample of blood yielded a standard deviation of 12%.

(c) Standard deviation of the method. The value for the platelet ascorbic acid content expressed as µg/10^9 cells is therefore subjected to a degree of variation which can be estimated by combining the standard deviations of the ascorbic acid determination and the platelet count, namely, standard deviation of

TABLE 5. EFFECT OF STORING THE FROZEN PLATELET EXTRACT ON THE ESTIMATION OF PLATELET ASCORBIC ACID CONTENT

SUBJECT	VALUE FOR PLATELET ASCORBIC ACID CONTENT ($\mu g/10^9$ platelets)		A MINUS B
	ESTIMATED IMMEDIATELY (A)	ESTIMATED AFTER 4 to 5 DAYS (B)	
1	3.5	3.8	- 0.3
2	3.0	3.5	- 0.5
3	5.5	4.1	+ 1.4
4	2.4	2.4	0
5	6.4	6.8	- 0.4
Mean	4.16	4.12	+ 0.2

$t = 0.1$

$N = 4$

$0.95 > P > 0.90$

TABLE 6. EFFECT OF HOMOGENISATION ON VALUES OBTAINED FOR PLATELET ASCORBIC ACID CONTENT

SUBJECT	VALUE FOR PLATELET ASCORBIC ACID CONTENT ($\mu g/10^9$ platelets)		H MINUS S
	EXTRACTION BY HOMOGENISATION (H)	EXTRACTION BY STIRRING (S)	
1	6.4	5.0	+ 1.4
2	3.8	3.3	+ 0.5
3	3.3	2.1	+ 1.2
4	3.8	3.3	+ 0.5
Mean	4.3	3.4	+ 0.9

$t = 3.8$

$N = 3$

$0.05 > P > 0.025$

method = $\sqrt{10^2 + 12^2}$% = 16%.

6. Storage of the platelet extracts

Though some loss of ascorbic acid occurred (20-30%) if the extracts (samples and internal standards) were stored by deep freezing for 4-5 days prior to estimation by thin layer chromatography, reproducible results were obtained because the internal standards allowed for breakdown of ascorbic acid. There was no significant difference between the values obtained from platelet extracts estimated immediately and those obtained from the same extracts after storage in a deep freeze (Table 5). In the routine estimation of platelet ascorbic acid the extracts were sometimes stored for some days prior to ascorbic acid estimation.

7. Release of ascorbic acid by homogenisation

An evaluation of the effectiveness of the homogenisation procedure was made by treating duplicate platelet suspensions (in 8% trichloracetic acid) for 2 minutes by either homogenisation in a tissue grinder or by stirring with a glass rod. The extracts obtained were centrifuged and the supernatants were subjected to estimation by thin layer chromatography. The amount of ascorbic acid liberated by homogenisation was significantly larger than the amount released by stirring (Table 6).

8. Experiments elucidating the cause of the low recovery of ascorbic acid from platelets

The recovery after chromatography of ascorbic acid added to trichloracetic acid solution was 71% whereas for ascorbic acid added to platelet extracts it was only 42% (Table 4). Attempts were made to increase these recovery rates by applying the samples to the thin layer in an atmosphere of nitrogen or hydrogen sulphide and by developing the chromatograms in a nitrogen atmosphere. With both aqueous solutions of ascorbic acid and with ascorbic acid added to platelet extracts no increase in recovery was obtained by these means.

The loss of ascorbic acid during chromatography of aqueous solutions of ascorbic acid was probably due to breakdown by oxidation (see above). It seemed that the lower recovery obtained when platelet extracts were used could have been due either to an increased rate of ascorbic acid breakdown when in contact with platelet extract or to loss of ascorbic acid either by adsorption on the protein precipitate or by dilution with intracellular fluid from the disrupted platelets.

To further elucidate this problem C^{14}-labelled ascorbic acid was added to 8% trichloracetic acid. A platelet button was homogenised in 0.1 ml of this solution and the protein precipitate removed by centrifugation. The beta activity of 20 µl of the

supernatant, as measured by planchette counting in a proportional counter was only 61% of that of 20 µl of the original trichloracetic acid solution. The effects of sample quenching were eliminated by adding 20 µl volumes of non-radioactive platelet extract to the trichloracetic acid sample and non-radioactive trichloracetic acid to the labelled platelet sample.

This result shows that a large proportion of the platelet ascorbic acid was lost during homogenisation either by adsorption on the protein precipitate or by dilution with intracellular fluid from the disrupted platelets.

9. Comparison of values obtained by the thin layer chromatography (T.L.C.) method with those obtained by the dinitrophenylhydrazine (D.N.P.H.) procedure

1. Method. In 6 normal subjects the platelet content of ascorbic acid was estimated by both the T.L.C. technique and by the D.N.P.H. method (Roe, 1954). Descriptions of the D.N.P.H. method (Rubin, 1950; Roe, 1954) mention only external standards. In the experiment described here, however, internal standards were also used as in the T.L.C. method.

2. Results and conclusions

(i) Use of internal standards in the D.N.P.H. method. The values obtained in the D.N.P.H. method using internal standards

TABLE 7. COMPARISON OF PLATELET ASCORBIC ACID CONTENT MEASURED BY TWO DIFFERENT METHODS

SUBJECT	THIN LAYER CHROMATOGRAPHY METHOD (A)	DINITROPHENYLHYDRAZINE METHOD		B MINUS C	B MINUS A
		USING INTERNAL STANDARDS (B)	USING EXTERNAL STANDARDS (C)		
1	1.7	7.2	3.1	+ 4.1	+ 5.5
2	2.7	11.5	6.3	+ 5.2	+ 8.8
3	6.0	12.5	4.7	+ 7.8	+ 6.5
4	2.2	3.0	2.1	+ 0.9	+ 0.8
5*	6.4	7.3	3.5	+ 3.8	+ 0.9
6	6.5	14.7	5.3	+ 9.4	+ 8.2
Mean	4.3	9.4	4.2	+ 5.2	+ 5.1
			t	3.8	3.3
			N	5	5
			P	0.02>P>0.01	0.025>P>0.02

*This subject had been taking high doses of ascorbic acid.

were much higher than those obtained using external standards (Table 7). Some ascorbic acid had therefore been lost during the procedure, perhaps by adsorption on the protein precipitate as may have occurred in the T.L.C. method (see above). These results show that when the D.N.P.H. method is applied to platelets internal standards must be used to allow for the low recovery. Previous authors (Barkhan and Howard, 1958; Wilson et alii, 1967) make no mention of internal standards and do not quote recovery rates.

In 54 estimations of plasma ascorbic acid concentration by the D.N.P.H. method, no significant difference was detected between the values obtained by external standards and those obtained by internal standards ($t = 1.3$; $N = 53$; $P = 0.2$). Thus external standards are adequate when the D.N.P.H. method is applied to plasma.

(ii) Comparison of T.L.C. method with D.N.P.H. method. The mean values for the platelet ascorbic acid content obtained by the T.L.C. method were significantly lower than those obtained by the D.N.P.H. method using internal standards (Table 7). This is consistent with the hypothesis that the T.L.C. method is more specific for ascorbic acid than the D.N.P.H. procedure.

V. COMMENT

In this chapter the development of a new and more specific method for the estimation of ascorbic acid has been described.

The increased specificity has been attained by thin layer chromatography and sensitivity has been acquired by a new reaction which is the most sensitive yet described for the estimation of ascorbic acid.

The main difficulty encountered in the development of the method was the instability of ascorbic acid. This resulted at first in a low and non-reproducible recovery of ascorbic acid after thin layer chromatography. This problem was overcome and reproducible recoveries of about 80% were obtained.

The increased specificity of the method is shown by the separation which was attained from other reducing substances such as cysteine, glutathione, ferrous ions and sulphite ions, and by the apparently uncontaminated ascorbic acid spot which is produced by chromatography of platelet extracts. The much lower results obtained by the new method compared with the D.N.P.H. technique are further evidence that the method is more specific than previous methods.

CHAPTER 3

PLATELET ASCORBIC ACID CONCENTRATION IN NORMAL SUBJECTS AND ITS VARIATION WITH DIETARY INTAKE

TABLE 8. DISTRIBUTION OF BLOOD ASCORBIC ACID

AUTHOR	METHOD	INTERNAL (I) OR EXTERNAL (E) STANDARD	NO. OF SUB-JECTS	PLATELETS mg/100G	PLATELETS µg/10^9 cells*	WHITE CELLS mg/100 G	WHITE CELLS µg/10^9 cells	PLASMA mg/100 ml
Barkhan & Howard, 1958	DNPH	-[x]	6	18.3	2.91 ± 0.22	16.5	148	0.69
Wilson et alii, 1967	DNPH	-[x]	8	-	858 ± 142	-	43	0.00
Present study	DNPH	I	6	38	9.4 ± 1.8	-	-	0.79†
Present study	DNPH	E	6	18	4.2 ± 0.63	-	-	-
Present study	TLC	I	26	15	3.5 ± 0.27	-	-	-

*Mean ± standard error. All other figures are mean values.

[x]These authors did not state whether or not internal standards were used.

†This plasma level is the mean from 7 normal subjects.

I. PLATELET ASCORBIC ACID IN NORMAL SUBJECTS

1. *Introduction*

A value for the ascorbic acid content of blood platelets was first reported by Barkhan and Howard (1958). Until that time it was not known whether the ascorbic acid in the buffy layer (approximately equal parts by weight of platelets and white cells) of the blood was mainly in platelets, mainly in white cells, or distributed equally between these two. Using the D.N.P.H. method they estimated that the mean value for 6 normal subjects was 18.3 mg/100 G (wet weight) which was similar to the concentration in white cells and much greater than the concentration of 0.69 mg/100 ml in plasma (Table 8). Expressed in terms of cell count these results became 2.91 $\mu g/10^9$ platelets and 148 $\mu g/10^9$ white cells (Table 8). This apparent discrepancy was due to the great difference in size between platelets and white cells.

The only other reported value for the ascorbic acid content of platelets was published by Wilson et alii (1967). They found, again using the D.N.P.H. method, the very much higher value of 858 $\mu g/10^9$ platelets in 8 normal subjects. This was much higher than the concentration of 43 $\mu g/10^9$ cells which they found in white cells (Table 8). If they had expressed their results in relation to cell weight the difference between the concentration in platelets and that in white cells would have been even larger. In the same subjects they found a plasma level

TABLE 9. PLATELET ASCORBIC ACID CONTENT IN NORMAL MALES

SUBJECT	AGE (YEARS)	ASCORBIC ACID CONTENT ($\mu g/10^9$ cells)	($\mu g/mg$)	PLATELET COUNT (x 10^3/cmm	SMOKERS (+) OR NON-SMOKERS (-)
G.D.	19	1.9	0.05	234	-
P.A.	19	4.0	0.12	143	-
R.P.	22	3.0	0.09	313	-
R.C.	28	4.2	0.24	280	+
I.H.	19	2.7	0.12	303	-
G.T.	20	2.7	0.08	158	-
A.W.	19	3.3	0.13	155	+
P.S.	22	1.9	0.07	223	-
D.C.	20	4.2	0.19	275	-
C.L.	24	3.6	0.17	326	+
J.L.	27	2.3	0.18	269	-
N.A.	28	3.3	0.14	200	+
MEAN	22	3.1	0.13	240	
STANDARD DEVIATION		0.8	0.06		
STANDARD ERROR		0.2	0.02		

TABLE 10. PLATELET ASCORBIC ACID CONTENT IN NORMAL FEMALES

SUBJECT	AGE (YEARS)	ASCORBIC ACID CONTENT ($\mu g/10^9$ cells)	(μg/mg)	PLATELET COUNT (x 10^3/cmm)	SMOKERS (+) OR NON-SMOKERS (-)
S.O.	18	6.3	0.19	185	-
S.L.	19	1.7	0.07	310	+
D.V.	19	2.7	0.10	223	+
K.L.	18	3.5	0.15	188	-
D.P.	27	6.0	0.27	285	+
H.E.	18	6.5	0.30	300	-
J.D.	30	4.0	0.15	368	+
R.K.	30	5.3	0.20	298	-
M.H.	26	3.2	0.15	245	-
H.S.	19	1.4	0.13	360	-
P.W.	22	2.2	0.18	228	-
H.B.	20	4.6	0.19	264	+
S.B.	19	2.6	0.12	344	-
B.L.	25	4.2	0.21	304	-
MEAN	20	3.9	0.17	279	
STANDARD DEVIATION		1.7	0.06		
STANDARD ERROR		0.5	0.02		

of 0 µg/100 ml. It is generally accepted that the plasma concentration is far above this value, and that any subject with a level of less than 100 µg/100 ml is having insufficient dietary ascorbic acid (Goldsmith, 1961). Wilson et alii made no comment on the large discrepancies between their results and those of other workers.

The aim of the work presented in this section was to determine the mean and degree of variation of the platelet ascorbic acid content in normal subjects.

2. *Methods*

(a) Subjects studied. A total of 26 normal subjects was studied. Of these 12 were males aged 19-28 (Table 9) and 14 were females aged 18-30 (Table 10). All subjects were questioned to exclude any bleeding tendency in themselves or their families. A dietary history with respect to foods containing ascorbic acid was taken and they were also asked about smoking habits.

(b) Estimations performed. (i) The platelet ascorbic acid content was estimated in all subjects by the T.L.C. method (see Chapter 2). (ii) The plasma ascorbic acid concentration was estimated on platelet poor plasma in 7 of the subjects by the D.N.P.H. technique (Roe, 1954). (iii) Platelet counts were performed by the method of Brecher and Cronkite (1950).

(c) Statistics. An account of statistical methods used in this thesis is in Appendix A.

Results

(a) Platelet ascorbic acid content. The mean platelet ascorbic acid concentration in 26 normal subjects as determined by the T.L.C. method was 3.5 ± 1.4 µg/10^9 cells (S.E. = 0.3) or 0.15 ± 0.06 µg/mg (S.E. = 0.01). That the distribution was approximately normal was confirmed by carrying out probit transformation and plotting on probability paper. Comparison of these results with those of other authors (Table 8) is difficult because they stated neither recovery rates for ascorbic acid in their methods nor whether internal or external standards were used. Nevertheless it appears that the values obtained in the present study by the T.L.C. method do not differ substantially from those obtained by Barkhan and Howard by the D.N.P.H. method (Table 8).

(b) Values in males and females. The platelet ascorbic acid content in males (Table 9) was slightly less than that in females (Table 10) but the difference was not significant [$t = 1.8$; $N = 24$; $0.10 > P > 0.05$ (µg/10^9 cells): $t = 0.52$; $N = 24$; $0.7 > P > 0.6$ (µg/mg)].

(c) Correlation of platelet ascorbic acid with platelet count. There was no correlation between the platelet count and the

TABLE 11. PLATELET ASCORBIC ACID CONTENT IN THE SAME SUBJECT ON FOUR SUCCESSIVE DAYS

DAY		ASCORBIC ACID CONTENT ($\mu g/10^9$ platelets) Each estimation	Mean
1		2.3	2.3
2	(1)	2.6	2.3
	(2)	2.0	
3	(1)	2.5	2.35
	(2)	2.2	
4		3.0	3.0
		MEAN	2.5

TABLE 12. RELATION BETWEEN PLATELET AND PLASMA ASCORBIC ACID CONCENTRATIONS IN NORMAL SUBJECTS

SUBJECT	PLATELET CONCENTRATION (Y) ($\mu g/10^9$ cells)	PLASMA CONCENTRATION (X) (mg/100 ml)
R.K.	5.3	1.05
P.S.	1.9	0.40
H.B.	4.6	0.90
S.B.	2.6	0.55
D.C.	4.2	1.10
B.L.	4.2	1.10
C.L.	3.6	0.45
MEAN	3.8	0.79
STANDARD DEVIATION	1.2	0.32

Regression coefficient of Y on X = + 3.1

N = 5; t = 3.5; 0.02 > P > 0.01

concentration of ascorbic acid in normal platelets ($r = -0.002$; $t = 0.12$; $N = 26$; $P > 0.9$).

(d) Comparison of smokers with non-smokers. In this series there was no demonstrable difference between the platelet ascorbic acid content in smokers (3.7 $\mu g/10^9$ cells) and non-smokers (3.4 $\mu g/10^9$ cells). This differs from the findings of Calder et alii (1963) who showed that both the buffy layer and plasma concentrations of ascorbic acid were lower in smokers than in non-smokers. Their study differs from the present study both in that they studied a larger number (205) of subjects, and that their estimations were performed on a mixture of white cells and platelets.

(e) Day to day variation in a normal subject. In one subject (the author) the platelet ascorbic acid content was measured by the T.L.C. method on 4 successive days. Consistent results were obtained (Table 11). A considerable amount of the variation found can be accounted for by variation in the method which has an estimated standard deviation of 15% (see Chapter 2).

(f) Plasma ascorbic acid concentration. The mean plasma concentration of ascorbic acid measured in 7 of the normal subjects was 0.79 ± 0.32 mg/100 ml which is consistent with previous reports in the literature (Goldsmith, 1961). There was a significant positive relationship between platelet ascorbic acid content and plasma ascorbic acid concentration (Figure 13, Table 12).

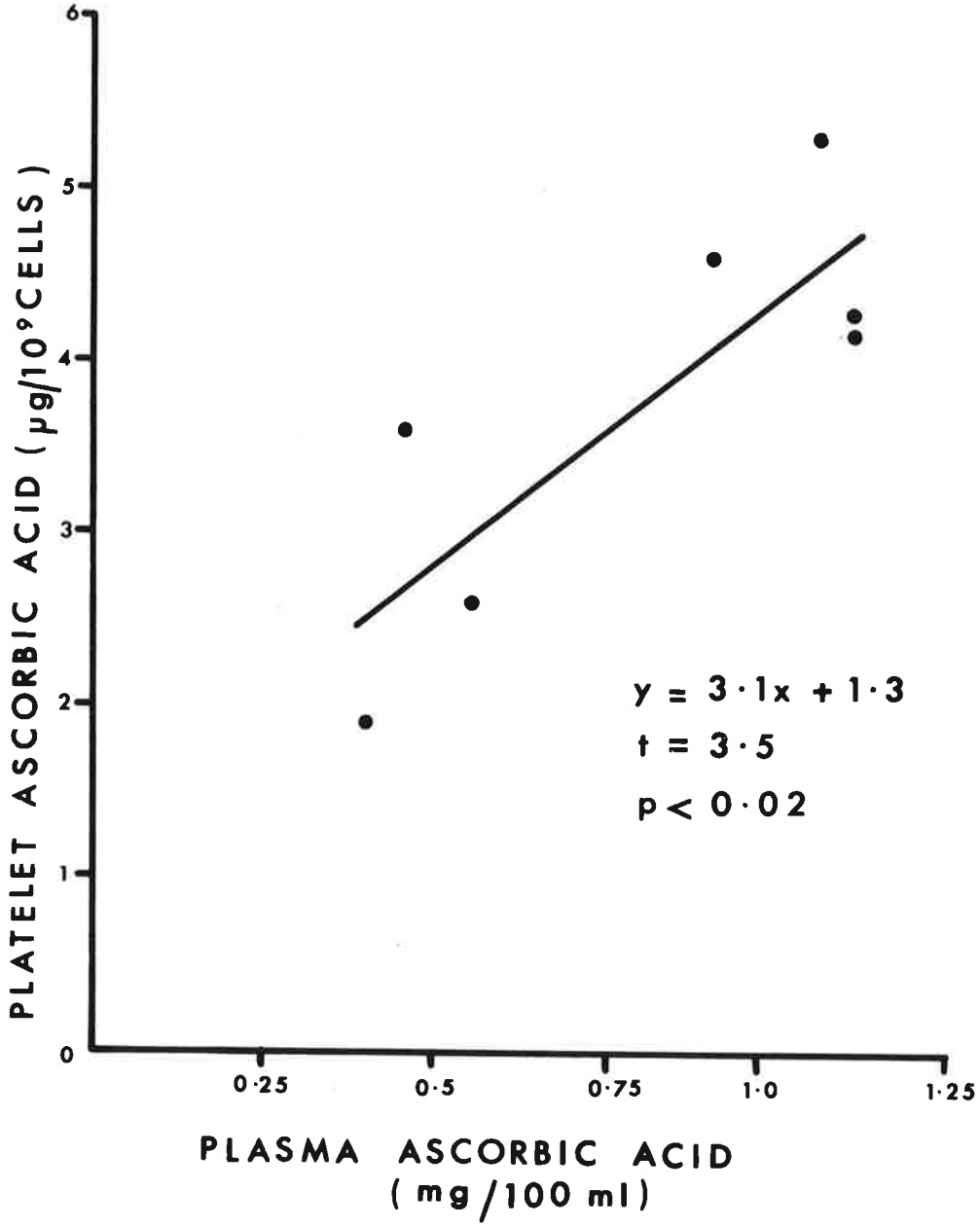

FIGURE 13: Regression relationship between platelet ascorbic acid content and plasma ascorbic acid concentration.

II. VARIATION OF THE NORMAL PLATELET ASCORBIC ACID CONTENT WITH DIETARY INTAKE OF VITAMIN C

1. Introduction

The only reported study on the variation in platelet ascorbic acid with dietary intake of vitamin C in which platelets free of white cell contamination were used, is that of Barkhan and Howard (1958). They found that saturation of 6 normal subjects with ascorbic acid increased the mean platelet ascorbic acid content by about 30%.

In contrast, the variation of the ascorbic acid content of the buffy layer (platelets plus white cells) with dietary intake of this vitamin has been the subject of many investigations (Goldsmith, 1961). Saturation of the body with ascorbic acid is associated with a platelet-white-cell content of 0.18 - 0.34 mg/G and a plasma concentration of 1.0 - 1.4 mg/100 ml (Woodruff, 1964). Dietary deprivation of ascorbic acid causes the ascorbic acid content of the platelet-white-cell layer to fall gradually reaching zero levels after about 17 weeks, just prior to the onset of clinical scurvy (Crandon et alii, 1940; Vitamin C Subcommittee, 1953); the plasma concentration drops more rapidly and reaches zero levels after only 6 weeks. The buffy layer ascorbic acid content is now regarded as a reasonable index of tissue stores, and the plasma concentration is looked upon as an index of recent

intake (Pearson, 1966).

In the previous section it was shown that there is a definite relationship between platelet ascorbic acid content and the plasma concentration of ascorbic acid (Figure 13). The aims of the studies presented in this section are (i) to determine the effect of saturation of normal subjects with ascorbic acid on the platelet ascorbic acid content, and (ii) to determine the effect of dietary deprivation of ascorbic acid on the platelet ascorbic acid content.

2. Methods

(a) Saturation of subjects with ascorbic acid.

(i) Method of saturation. It has been shown (Cuttle, 1938; Barkhan and Howard, 1958) that 1 G of ascorbic acid per day, taken orally as a single dose or in divided doses, will saturate the tissues of human subjects with ascorbic acid in 4 days. This method was therefore used in the following studies.

(ii) Procedure. 4 normal subjects were studied for a period of 14 days. Blood was taken for platelet (T.L.C. method) and plasma (D.N.P.H. method) estimations of ascorbic acid on days 0, 7 and 14. For 4 days prior to day 14 (days 10-13 inclusive) the subjects took 1 G of ascorbic acid per day in tablet form.

4 subjects from a geriatric hospital were also studied. In this group only one estimation was performed prior to the

TABLE 13. EFFECT OF SATURATION OF NORMAL SUBJECTS WITH ASCORBIC ACID

	PLATELET ASCORBIC ACID ($\mu g/10^9$ cells)				PLASMA ASCORBIC ACID (mg/100 ml)			
SUBJECT	C.L.	S.B.	D.C.	B.L.	C.L.	S.B.	D.C.	B.L.
Day 0	3.6	2.6	4.2	4.2	0.45	0.7	1.2	0.8
Day 7	-	3.2	4.7	2.4	-	0.4	1.0	1.4
Mean level prior to saturation (A)	3.6	2.9	4.45	3.3	0.45	0.55	1.1	1.1
Day 14 (post saturation) (B)	4.6	2.8	4.7	2.6	1.0	1.05	0.95	1.2
B - A	+ 1.0	- 0.1	+ 0.25	- 0.7	+ 0.55	+ 0.50	- 0.15	+ 0.1
Mean increase		+ 0.11				+ 0.25		
t (one tail test)		0.28				1.3		
N		3				3		
Probability		P = 0.4				P = 0.15		

administration of ascorbic acid and the ascorbic acid dosage was commenced on that day (day 0). The second estimation was performed on day 4, after the subjects had been saturated. Tourniquet tests and Ivy bleeding times were normal in all 4 subjects.

(b) Ascorbic acid depletion study. One subject (the author) partook of an ascorbic acid free diet for a period of 48 days. The concentrations of ascorbic acid in platelets and in plasma were estimated before, during and after this period. On the 43rd and 45th days 1 G of ascorbic acid was taken orally.

The diet consisted of powdered milk, cereals, eggs, bread, margarine, biscuits, cakes, occasional dried fruit and nuts, well cooked meats (but no organ meats), junkets prepared from powdered milk, and jellies. Excluded from the diet were fresh fruit, vegetables (cooked or uncooked), potatoes, fresh milk, butter and cream.

3. Results

(a) Saturation of 4 normal subjects (Figure 14, Table 13).

(i) Effect on platelet ascorbic acid concentration. There was no significant increase in the mean platelet ascorbic acid content after saturation with this substance. This differs from the increase of 30% observed by Barkhan and Howard, 1958). It

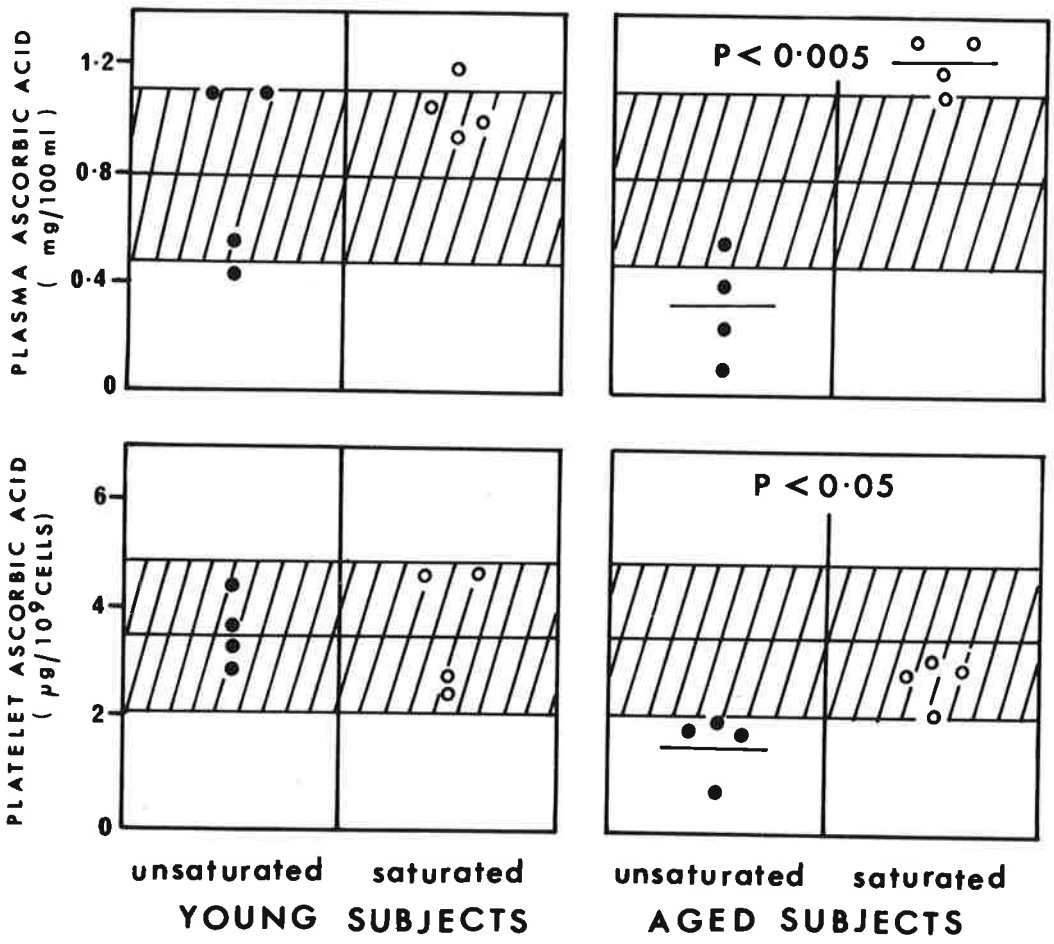

FIGURE 14: Effect of tissue saturation with ascorbic acid on concentrations of ascorbic acid in platelets and in plasma.

TABLE 14. EFFECT OF SATURATION OF GERIATRIC SUBJECTS WITH ASCORBIC ACID

	PLATELET ASCORBIC ACID ($\mu g/10^9$ cells)				PLASMA ASCORBIC ACID (mg/100 ml)			
SUBJECT	G.B.	S.L.	N.L.	T.W.	G.B.	S.L.	N.L.	T.W.
SEX	M	F	F	M	M	F	F	M
AGE (YEARS)	69	58	58	72	69	58	58	72
PRIOR TO SATURATION (A)	2.0	1.9	1.8	0.8	0.55	0.40	0.25	0.10
POST SATURATION (B)	3.2	2.2	2.9	3.0	1.20	1.10	1.30	1.30
B - A	+ 1.2	+ 0.3	+ 1.1	+ 2.2	+ 0.65	+ 0.70	+ 1.05	+ 1.20
Mean increase	+ 1.2				+ 0.25			
t (one tail test)	2.7				5.9			
N	3				3			
Probability	0.05 > P > 0.025				0.005 > P > 0.0025			

would appear therefore that the platelets of the subjects in the present study were either saturated or almost saturated with ascorbic acid prior to the experiment which is consistent with the fact that these subjects had a high citrus fruit intake.

(ii) Effect on plasma ascorbic acid concentration. In 2 subjects (C.L. and S.B.) saturation with ascorbic acid caused the plasma concentrations to increase from 0.45 and 0.55 mg/100 ml respectively to levels which were consistent with saturation (1.0-1.4 mg/100 ml). The plasma concentrations of ascorbic acid in the other 2 subjects (D.C. and B.L.) were already at levels consistent with saturation prior to the experiment. Large doses of ascorbic acid did not significantly affect the plasma concentrations in these 2 subjects.

(b) Saturation of 4 geriatric subjects (Figure 14, Table 14).

(i) Effect on platelet ascorbic acid concentration. The platelet concentrations of ascorbic acid in these subjects prior to saturation were much lower than in the 4 young subjects and the mean level was significantly lower than the normal range ($\bar{x} = 1.6$; $t = 2.6$; $N = 28$; $0.02 > P > 0.01$). This can be related to the low dietary intake of ascorbic acid. They had all resided in the geriatric hospital for some months at least, and had been given only one piece of fruit per week. Though they could buy their own fruit, only one subject (G.B.) did so. After saturation with

ascorbic acid, the mean platelet ascorbic acid content in these 4 subjects was significantly increased to well within the normal range.

(ii) Effect on plasma ascorbic acid concentration. The mean plasma concentration of ascorbic acid in these subjects prior to saturation was also significantly lower than the normal range ($\bar{x} = 0.33$; $N = 9$; $t = 2.4$; $0.05 > P > 0.025$). After saturation the plasma concentrations in all 4 subjects rose to well within the range of 1.0-1.4 mg/100 ml which is consistent with saturation.

(c) Ascorbic acid depletion study (Figure 15). A few days prior to the onset of this study the subject had taken large doses of ascorbic acid for another experiment. The concentrations of ascorbic acid in platelets and in plasma prior to commencement of the diet was therefore high (7 $\mu g/10^9$ platelets and 1 mg/100 ml of plasma). This value for the platelet content of ascorbic acid is unusually high, even for a saturated subject.

As soon as the diet was commenced both platelet and plasma concentrations of ascorbic acid fell concomitantly to reach minimum values after about 3 weeks (1.5 $\mu g/10^9$ platelets and 0.3 mg/100 ml of plasma). The values then remained more or less constant until ascorbic acid supplements were given on the 43rd and 45th days.

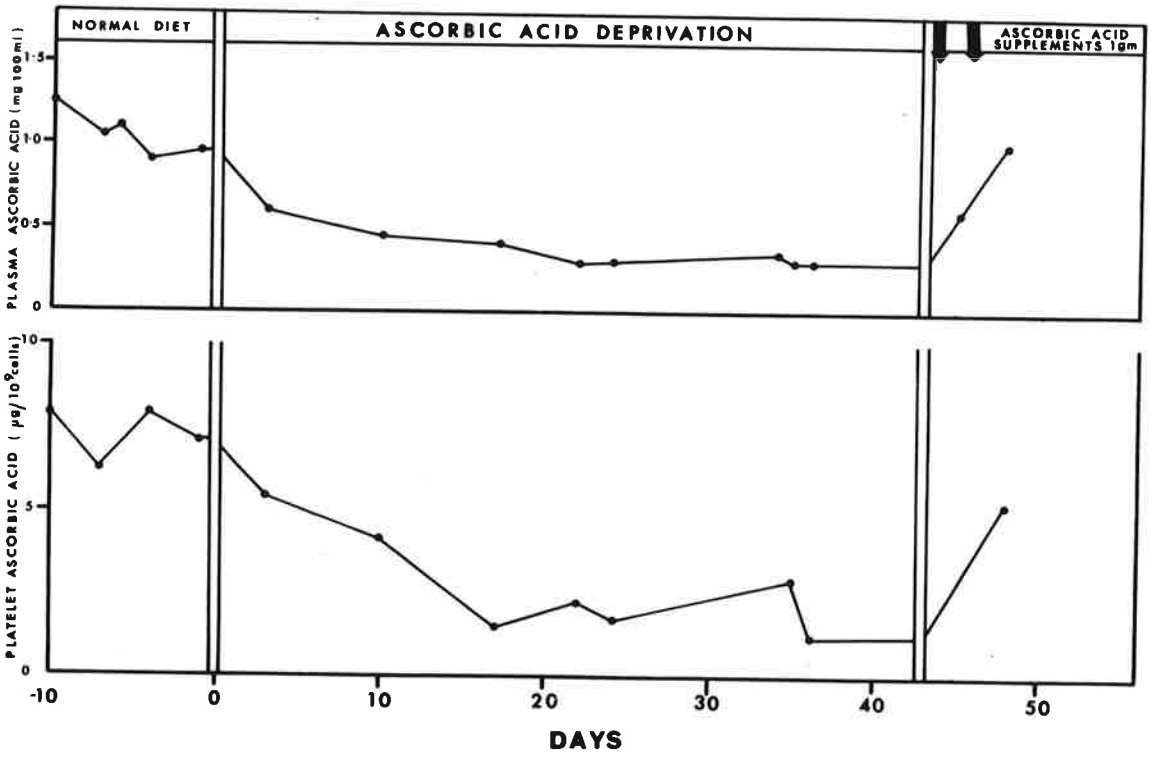

FIGURE 15: Effect of an ascorbic acid free diet on concentration of ascorbic acid in platelets and in plasma in a normal volunteer.

III. CONCLUSIONS

Work presented in this chapter confirmed the high concentration of ascorbic acid found in platelets by Barkhan and Howard. Estimations by the T.L.C. method on 26 normal subjects yielded 3.5 ± 1.4 $\mu g/10^9$ platelets. No significant difference existed between males and females or between smokers and non-smokers, and there was no relation between the ascorbic acid content of platelets and the platelet count. The platelet ascorbic acid content was related to the plasma ascorbic acid concentration, and experiments in which the ascorbic acid intake of normal subjects was varied suggested that the platelet ascorbic acid content depends to some extent on the plasma level which depends in turn on the dietary intake.

CHAPTER 4

PLATELET ASCORBIC ACID CONTENT IN PATIENTS WITH VARIOUS DISEASES

I. INTRODUCTION

The only report in the literature on the variation of the ascorbic acid content of platelets in disease is that of Barkhan and Howard (1958). In 3 cases of chronic myeloid leukemia and 2 cases of chronic lymphatic leukemia they found no marked differences from normal in platelet ascorbic acid content.

Platelets are involved in many other disease processes besides leukemia. The bleeding tendency of thrombocytopenic purpura is caused by insufficient platelets, whereas in other diseases such as uraemia and hereditary haemorrhagic thromboasthenia a defect in platelet function is held responsible. Patients who are receiving steroid therapy often develop a purpuric rash. It has been claimed that this haemorrhagic tendency is abolished by the administration of ascorbic acid (Stefanini and Rosenthall, 1950; Holley and McLester, 1951).

It is now well established that platelets play an important part in the initiation of thrombosis (see Chapter 1). In patients with thrombocythaemia an increased tendency to thrombosis is associated with an increase in the platelet count. Another disease in which platelets may be involved is atherosclerosis: an hypothesis that atheromatous lesions are formed by the gradual deposition of platelets on the endothelium of blood vessels has been proposed (Duguid, 1948; Movat et alii, 1959).

TABLE 15. SUBJECTS WHOSE PLATELETS WERE SUBJECTED TO ASCORBIC ACID ESTIMATION

GROUP		NUMBER IN SUBGROUPS	NUMBER IN MAIN GROUPS	TOTAL
NORMAL SUBJECTS				
(1)	Young		26	
(2)	Elderly		4	30
ABNORMAL SUBJECTS				
(1)	Uraemia		13	
(2)	Leukemia			
	(a) acute	8		
	(b) chronic lymphatic	4		
	(c) chronic myeloid	4	16	
(3)	Atheroma			
	(a) myocardial infarction	10		
	(b) other	8	18	
(4)	Polycythaemia vera and thrombocythaemia	4	9	
(5)	Steroid therapy		6	
(6)	Megaloblastic anaemia		5	
(7)	"Young" platelets		6	
(8)	Thrombocytopenia		4	
(9)	Miscellaneous		8	85
			TOTAL	115

In megaloblastic anaemia the platelet count is often low and abnormalities in ascorbic acid metabolism have been described. In pernicious anaemia ascorbic acid is cleared from the plasma more rapidly than normal (Cox et alii, 1958) and in folic acid deficiency there may be concurrent ascorbic acid deficiency (Cox et alii, 1967).

In the studies described in this chapter the platelet ascorbic acid content was estimated in a series of patients with a variety of diseases. Changes in these levels might help elucidate either the function of ascorbic acid in platelets or serve to shed some light on the pathogenesis of the disease processes.

II. METHODS

1. Subjects studied. A total of 85 patients was studied. The number of subjects in each disease category is listed in Table 15. Details of each patient and the results of estimations performed are recorded in Appendix B.

2. Estimations performed. (a) Platelet ascorbic acid content was estimated in all subjects by the T.L.C. method. (b) Plasma ascorbic acid concentration was estimated in 46 of the subjects by the D.N.P.H. procedure. (c) Platelet counts were performed by the method of Brecher and Cronkite (1950). (d) Haemoglobin concentration and white cell count were determined on all subjects. (e) Platelet thromboplastic function

TABLE 16. COMPARISON OF ASCORBIC ACID CONCENTRATIONS IN CASES OF URAEMIA WITH THE NORMAL RANGE

	NO. OF SUBJECTS	CONCENTRATION OF ASCORBIC ACID	DEGREES OF FREEDOM	DEVIATION (t)	PROBABILITY (P)
PLATELETS ($\mu g/10^9$ cells)	13	2.0 ± 2.0	37	2.7	P = 0.01
PLASMA (mg/ 100 ml)	9	1.04 ± 0.51	14	1.1	P = 0.3

NORMAL RANGE = Platelets: 3.5 ± 1.4 $\mu g/10^9$ cells (n = 26)

Plasma: 0.79 ± 0.32 mg/100 ml (n = 7)

was estimated in some subjects by the method of Bonnin and Cheney (1961).

3. Statistical methods. The procedures used for the statistical evaluation of results are set out in Appendix A.

III. RESULTS

1. Uraemia (Table 16, Figure 16). 13 patients with uraemia were studied. The blood urea nitrogen concentration ranged from 30 to 132 mg/100 ml and the cause of renal failure was chronic pyelonephritis in 6 cases, chronic glomerulonephritis in 3 cases, and uncertain in 4 cases. 4 patients were being treated by haemodialysis twice weekly, and in these subjects blood was taken for estimation just prior to dialysis.

The mean value for the platelet ascorbic acid content was 2.0 ± 2.0 $\mu g/10^9$ cells which was significantly lower than normal ($P = 0.01$). Since the distribution appeared to be somewhat skew (confirmed by carrying out probit transformation and plotting on log probability paper) the statistical significance was checked by the Wilcoxon test for 2 samples ($P < 0.01$). The plasma ascorbic acid concentration, however, did not differ significantly from normal being slightly higher than that in normal subjects. There was no relationship between the platelet ascorbic acid content and either blood urea nitrogen concentration ($b = + 0.02$, $t = 1.3$, $N = 11$,

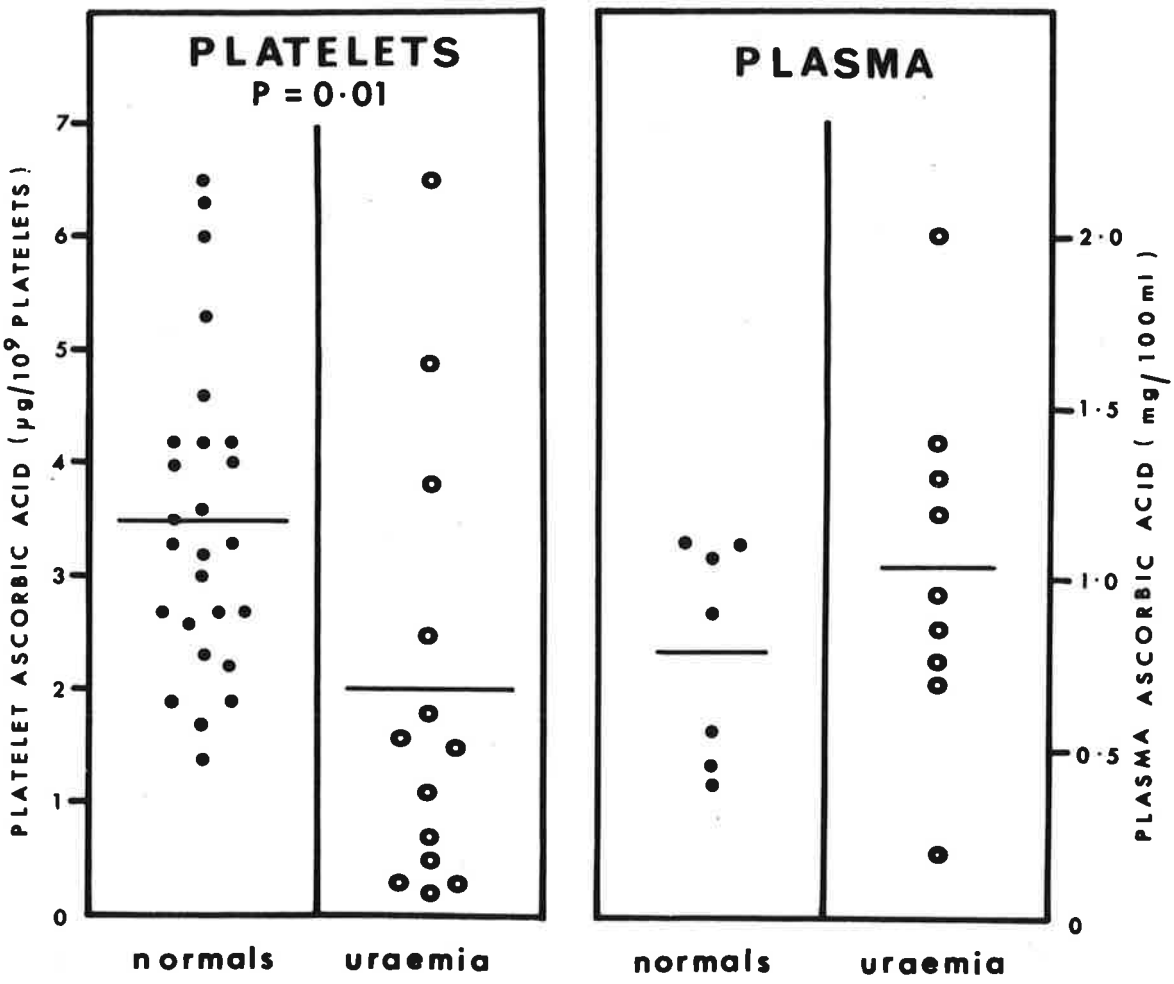

FIGURE 16: Platelet and plasma ascorbic acid concentrations in patients suffering from uraemia and comparison with normal range.

TABLE 17. COMPARISON OF ASCORBIC ACID CONCENTRATIONS IN CASES OF LEUKEMIA WITH THE NORMAL RANGE

TYPE OF LEUKEMIA	PLATELETS OR PLASMA	NO. OF SUB-JECTS	CONCENTRATION OF ASCORBIC ACID platelets, $\mu g/10^9$ cells plasma, mg/100 ml	DEGREES OF FREEDOM	DEVIATION (t)	PROBABILITY (P)
Acute	Platelets	8	1.2 ± 0.7	24*	6.3*	$P < 0.001$
	Plasma	3	0.30 ± 0.05	8	2.4	$0.05 > P > 0.025$
Chronic lymphatic	Platelets	4	4.4 ± 2.1	28	1.1	$P = 0.3$
	Plasma	3	0.58 ± 0.16	8	0.98	$0.4 > P > 0.3$
Chronic myeloid	Platelets	4	1.7 ± 2.2	28	2.1	$0.05 > P > 0.025$
	Plasma	3	0.62 ± 0.60	8	0.54	$0.7 > P > 0.6$

NORMAL RANGE = Platelets: 3.5 ± 1.4 $\mu g/10^9$ cells (n = 26)
Plasma: 0.79 ± 0.32 mg/100 ml (n = 7)

*F test was significant and the t test was modified as in Appendix A.

$0.3 > P > 0.2$) or platelet thromboplastic function ($b = -0.06$, $t = 1.4$, $N = 6$, $P = 0.2$).

2. Leukemia (Table 17, Figure 17). 16 patients were studied (Appendix B2), 8 with acute leukemia, 4 with chronic lymphatic leukemia and 4 with chronic myeloid leukemia. Of the 8 cases of acute leukemia 4 were aleukemic and one other was in haematological remission. One of the 4 cases of chronic lymphatic leukemia was in haematological remission while all 4 cases of chronic myeloid leukemia were in an active phase of the disease.

The mean platelet ascorbic acid content in the patients with acute leukemia was 1.2 ± 0.7 $\mu g/10^9$ cells which was significantly lower than normal ($P < 0.001$). The plasma ascorbic acid concentration, estimated in 3 of the subjects, was also significantly reduced ($P < 0.05$).

In the patients with chronic lymphatic leukemia neither the platelet content nor the plasma concentration of ascorbic acid differed significantly from the normal range.

The platelet ascorbic acid content in chronic myeloid leukemia was significantly lower than normal ($P < 0.05$). The mean plasma concentration, estimated in 3 of the 4 subjects, did not differ significantly from normal, but appeared to be unduly affected by

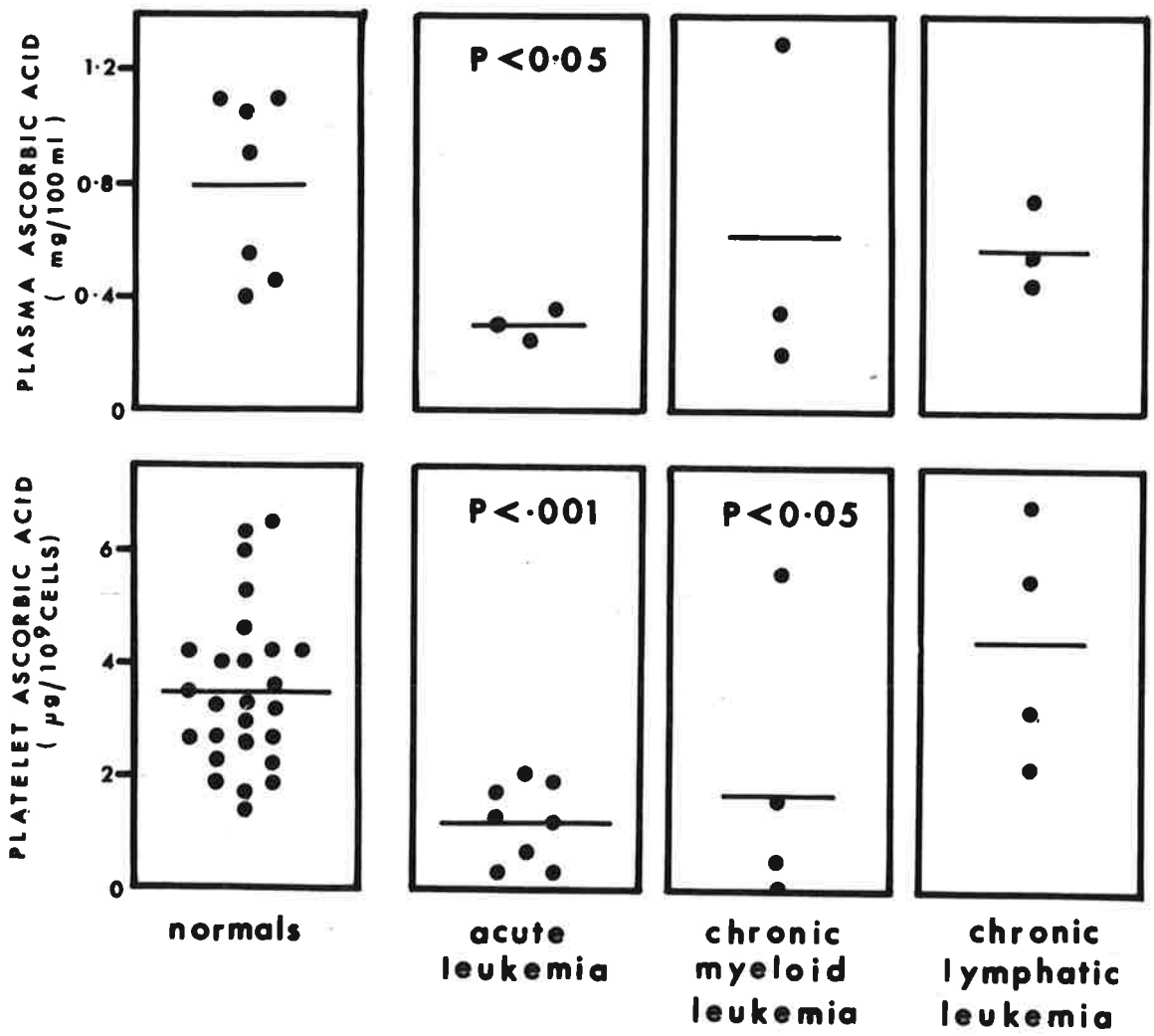

FIGURE 17: Platelet and plasma ascorbic acid concentrations in patients suffering from leukemia and comparison with the normal range.

TABLE 18. COMPARISON OF ASCORBIC ACID CONCENTRATIONS IN CASES OF ATHERSCLEROSIS WITH THE NORMAL RANGE

DIAGNOSIS	PLATELETS OR PLASMA	NO. OF SUB-JECTS	CONCENTRATION OF ASCORBIC ACID platelets, $\mu g/10^9$ cells plasma, mg/100 ml	DEGREES OF FREEDOM	DEVIATION (t)	PROBABILITY (P)
Atheroma with recent myocardial infarct (A)	Platelets	10	2.3 ± 1.4	34	2.2	0.05 > P > 0.025
	Plasma	6	0.61 ± 0.19	11	1.2	0.3 > P > 0.2
Atheroma without recent infarction (B)	Platelets	8	2.4 ± 1.6	32	1.9	0.1 > P > 0.05
	Plasma	3	0.67 ± 0.38	8	0.49	0.7 > P > 0.6
Total (A + B)	Platelets	18	2.3 ± 1.4	42	2.7	P = 0.01
	Plasma	9	0.62 ± 0.24	14	1.2	0.3 > P > 0.2

NORMAL RANGE = Platelets: 3.5 ± 1.4 $\mu g/10^9$ cells (n = 26)

Plasma: 0.79 ± 0.32 mg/100 ml (n = 7)

the high value obtained in one subject (J.S.) who had a high intake of fruit juice.

8. Atheroma (Table 18, Figure 18). 18 patients with evidence of atherosclerosis were studied (Appendix B3). 10 were in hospital for the treatment of myocardial infarction diagnosed on the basis of a history of chest pain coupled with one or both of electrocardiographic changes, and raised serum levels of glutamic oxaloacetic transaminase and lactic acid dehydrogenase. 2 (H.A. and B.W.) had been admitted to hospital because of severe chest pain thought to be of cardiac origin though there was no electrocardiographic or enzyme evidence of infarction, and 6 were outpatients suffering from symptoms of peripheral vascular insufficiency principally affecting the lower limbs.

When the patients with evidence of occlusive vascular disease were considered as a single group, the mean platelet ascorbic acid content was 2.3 ± 1.4 $\mu g/10^9$ platelets which was significantly lower than normal ($P = 0.01$). If the 10 patients with myocardial infarction were considered separately the mean value was also significantly lower than the normal range ($P < 0.05$). The mean value in the remaining 8 patients was lower than normal but the difference was not quite significant.

The values for the mean plasma ascorbic acid concentration were low but not significantly so. It is possible that estimations on a

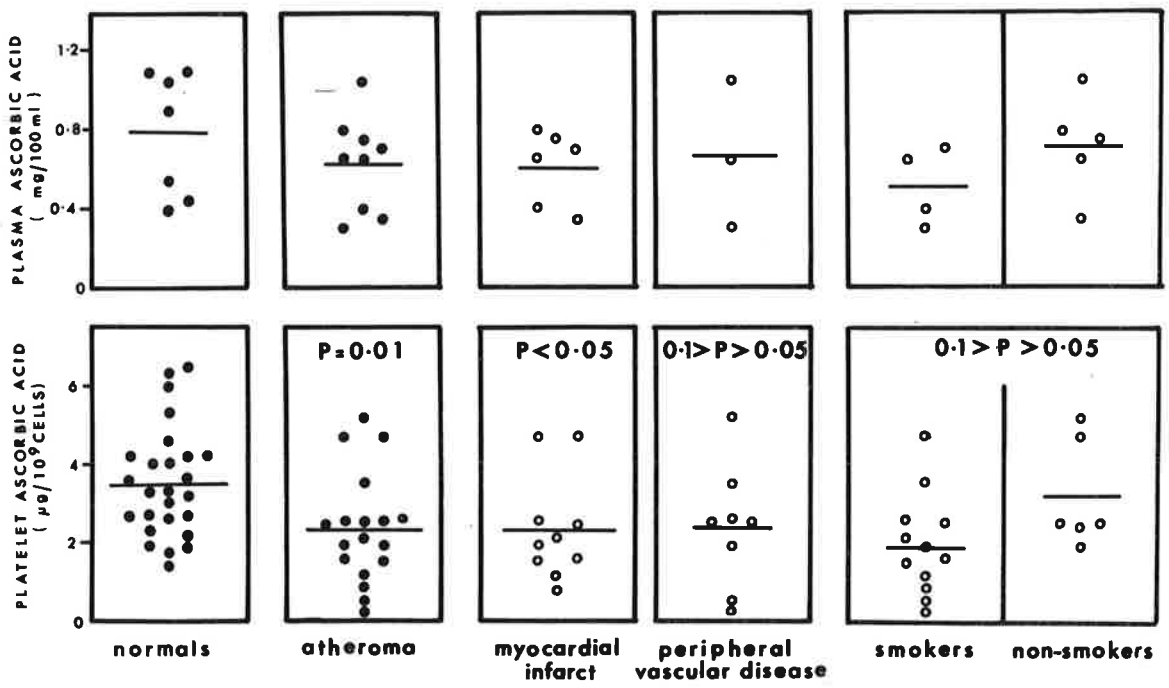

FIGURE 18: Platelet and plasma ascorbic acid concentrations in patients suffering from atherosclerosis and comparison with the normal range.

larger proportion of the subjects would have revealed this difference to be significant. Dietary history revealed no differences from normal, but it was difficult to assess ascorbic acid intake accurately by this means and the data available were not sufficient to exclude dietary causes for the low platelet ascorbic acid content.

The possibility arose that smoking habits were having some effect on the results obtained. Only one-third of the normal subjects smoked whereas two-thirds of the atheroma group did so. There was no difference between the platelet ascorbic acid content in smokers and in non-smokers in the normal group (see Chapter 3). In the atheroma group, however, the platelet ascorbic acid content was lower in smokers than in non-smokers though the difference was not quite significant ($0.1 > P > 0.05$). Calder et alii (1963), using much larger numbers, have shown that smoking does cause a significant reduction of both buffy layer and plasma ascorbic acid concentrations.

The test and control groups were not matched for age, but as there was no relation between platelet ascorbic acid content and age in the atheroma group ($b = + 0.01$, $t = 0.003$, $n = 16$, $99.7 > p > 99.5$), it is unlikely that age had a significant effect on the platelet ascorbic acid content.

TABLE 19. COMPARISON OF ASCORBIC ACID CONTENT OF PLATELETS IN CASES OF POLYCYTHAEMIA VERA AND THROMBOCYTHAEMIA

DIAGNOSIS	NO. OF SUB-JECTS	CONCENTRATION OF ASCORBIC ACID ($\mu g/10^9$ cells)	DEGREES OF FREEDOM	DEVIATION (t)	PROBABILITY (P)
Polycythaemia vera	4	2.0 ± 1.0	28	2.0	P = 0.05
Thrombocythaemia due to other causes	5	3.3 ± 1.6	29	0.26	P = 0.8

NORMAL RANGE = 3.5 ± 1.4 $\mu g/10^9$ cells (n = 26)

TABLE 20. COMPARISON OF ASCORBIC ACID CONCENTRATIONS IN PATIENTS RECEIVING PREDNISOLONE

DIAGNOSIS	PLATELETS OR PLASMA	NO. OF SUBJECTS	CONCENTRATION OF ASCORBIC ACID	DEGREES OF FREEDOM	DEVIATION (t)	PROBABILITY (P)
Steroid therapy	Platelets ($\mu g/10^9$ cells)	6	2.5 ± 1.4	30	1.5	0.3 > P > 0.2
	Plasma (mg/100 ml)	6	0.63 ± 0.36	11	0.25	0.9 > P > 0.08
Steroid therapy with haemorrhagic diathesis	Platelets ($\mu g/10^9$ cells)	4	2.3 ± 1.4	28	1.6	0.2 > P > 0.1
	Plasma (mg/100 ml)	4	0.66 ± 0.37	9	0.56	0.6 > P > 0.5

NORMAL RANGE = Platelets: 3.5 ± 1.4 $\mu g/10^9$ cells (n = 26)

Plasma: 0.79 ± 0.32 mg/100 ml (n = 7)

4. Polycythaemia and thrombocythaemia (Table 19, Figure 19). 4 patients suffering from polycythaemia vera and 5 patients suffering from thrombocythaemia due to other causes were studied (Appendix B4). The mean value of the platelet ascorbic acid content in patients with polycythaemia vera was 2.4 ± 1.0 $\mu g/10^9$ platelets which was lower than normal but the difference was not quite significant ($P = 0.05$). In the 5 patients with thrombocythaemia the mean value was well within normal limits.

5. Patients on steroids (Table 20, Figure 20). 6 patients, 5 receiving prednisolone and one (A.M.) betamethazone were investigated (Appendix B5). 3 of these had a haemorrhagic rash which was thought to be due to the steroid therapy; these 3 patients were being treated with steroids for malabsorption syndrome, rheumatoid arthritis and acute nephritis respectively. Another patient (R.N.) who was receiving prednisolone for asthma had a positive tourniquet test. Whether the 6 patients receiving steroids were considered as a single group or whether the 4 patients with a haemorrhagic diathesis were considered separately, neither platelet nor plasma ascorbic acid concentrations differed significantly from normal.

One patient (A.M.) experienced severe intracutaneous haemorrhages in response to such slight trauma as removal of elastoplast. This haemorrhagic tendency had developed since

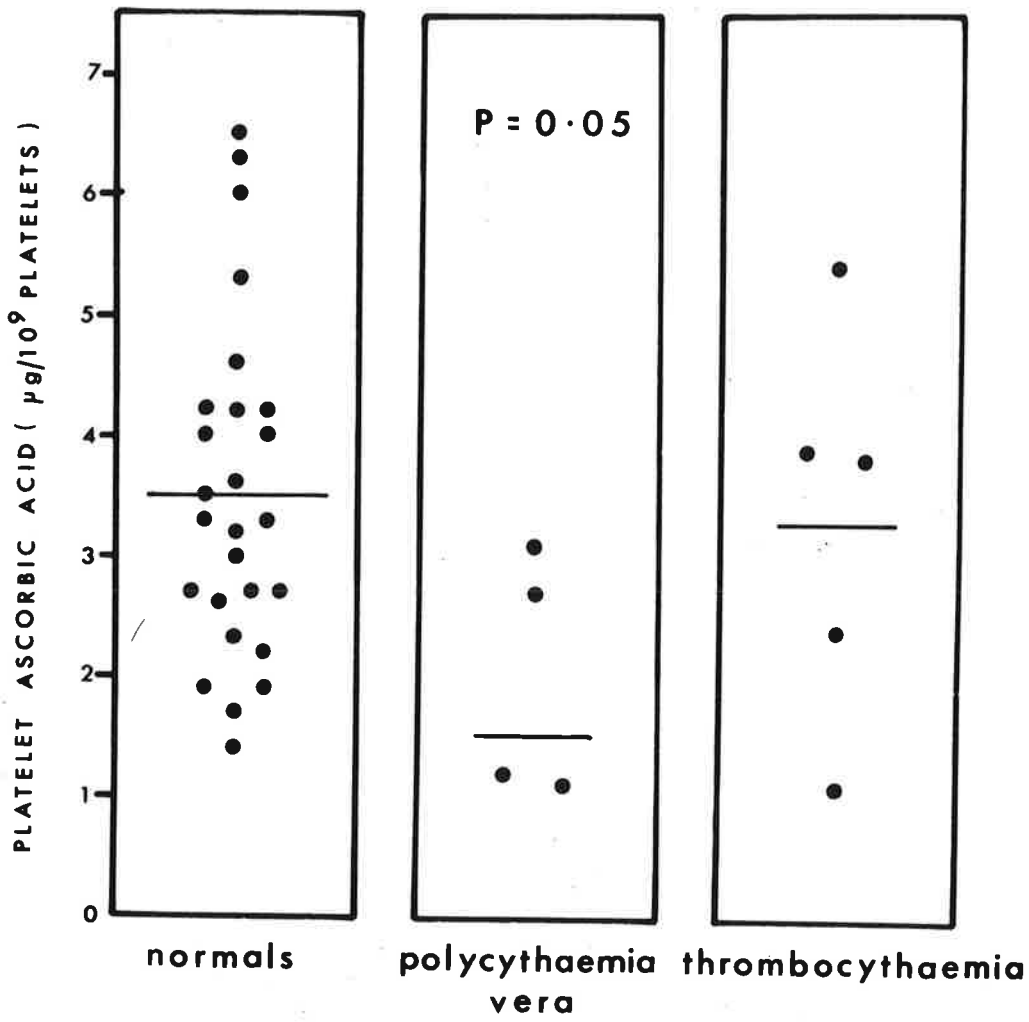

FIGURE 19: Platelet and plasma ascorbic acid concentrations in patients suffering from polycythaemia and thrombocythaemia and comparison with the normal range.

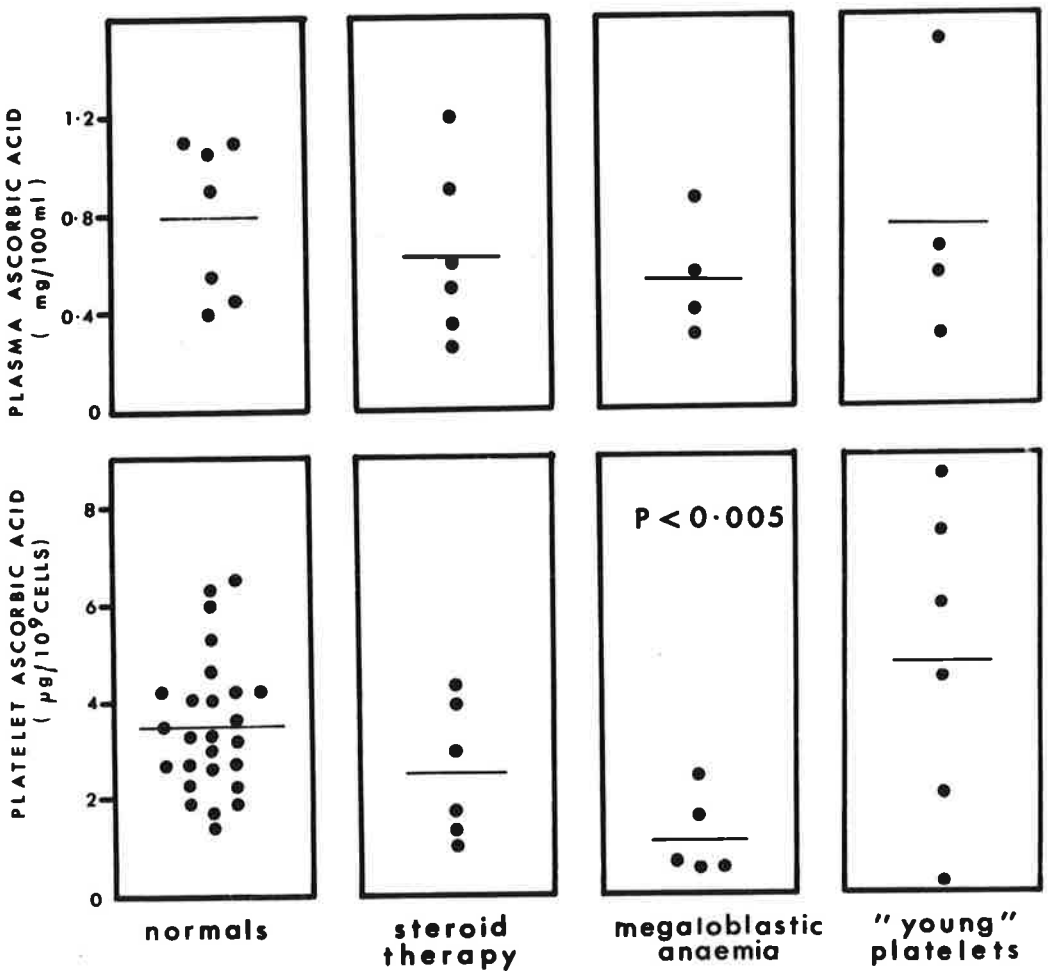

FIGURE 20: Platelet and plasma ascorbic acid concentrations in patients receiving steroid therapy, in patients with megaloblastic anaemia, and in patients thought to have "young" platelets.

TABLE 21. COMPARISON OF ASCORBIC ACID CONCENTRATIONS IN CASES OF MEGALOBLASTIC ANAEMIA

PLATELETS OR PLASMA	NO. OF SUBJECTS	CONCENTRATION OF ASCORBIC ACID	DEGREES OF FREEDOM	DEVIATION (t)	PROBABILITY (P)
Platelets ($\mu g/10^9$ cells)	5	1.1 ± 0.9	29	3.6	0.005>P>0.001
Plasma (mg/100 ml)	4	0.53 ± 0.24	9	1.3	0.3 > P > 0.2

NORMAL RANGE = Platelets: 3.5 ± 1.4 $\mu g/10^9$ cells (n = 26)
Plasma: 0.79 ± 0.32 mg/100 ml (n = 7)

she had been taking steroids. Bleeding time, tourniquet test, and platelet thromboplastic function were all normal. The administration of 1 G of ascorbic acid per day for 4 days produced a marked rise in both platelet and plasma ascorbic acid concentrations (Appendix B5) but there was no obvious decrease in her haemorrhagic tendency: on the day after the completion of the course of ascorbic acid she developed a crop of petechial haemorrhages on the forearm at the site of slight trauma.

6. Megaloblastic anaemia (Table 21, Figure 20). 5 patients with megaloblastic anaemia were studied (Appendix B6). All had megaloblastic changes in the bone marrow. Two patients suffered from pernicious anaemia and one from folic acid deficiency. One patient had had a partial gastrectomy, was being treated with phenytoin for epilepsy, and had low normal serum concentrations of both vitamin B_{12} and folic acid. The fifth patient, who was thought on clinical and biochemical grounds to be suffering from scurvy, had a normal serum vitamin B_{12} level, and a low normal serum folic acid concentration. Unfortunately ascorbic acid therapy had been commenced before the patient could be examined by the author.

The mean platelet ascorbic acid content was 1.1 ± 0.9 $\mu g/10^9$ cells which was significantly lower than normal ($0.005 \geq P > 0.001$). On the other hand the plasma concentration, measured in 4 patients, was not significantly reduced. One patient (A.W.) was observed

at intervals during treatment with vitamin B_{12}. Although the only ascorbic acid received was that supplied in the ward diet, the platelet ascorbic acid content increased from 0.5 $\mu g/10^9$ cells to 5.2 $\mu g/10^9$ cells over a period of 2 weeks (Appendix B6).

7. "Young" platelets (Figure 20). A group of 6 patients in whom it was presumed that the mean platelet age would be less than normal was studied (Appendix B7). This group was made up of 3 different categories:

(a) Two patients had lost a considerable proportion of their blood volume by acute haemorrhage 6 days previously. The lost blood had been replaced by transfusion of 6 pints of stored blood which does not contribute platelets to the circulation. As the platelet lifespan is about 10 days the bone marrow must normally replace about one-tenth of the circulating platelets daily. After severe haemorrhage, however, the marrow must produce a larger number of "young" platelets to replace those lost. In addition the bone marrow often over-reacts after haemorrhage to cause a thrombocytosis.

(b) Two patients had a postoperative thrombocytosis; one of these had had a splenectomy performed for congenital spherocytosis.

(c) Two were cases of thrombocytopenia in whom a remission had just occurred.

The values obtained for the platelet ascorbic acid content showed a wide scatter, and no conclusions could be drawn.

8. Thrombocytopenia (Figure 21). Besides those patients with leukemia and megaloblastic anaemia who had low platelet counts, 4 other patients with thrombocytopenia were studied (Appendix B8). They suffered respectively from Felty's syndrome, disseminated lupus erythematosus, hepatic cirrhosis, and aplastic anaemia. It was not practicable to study a larger number of subjects with thrombocytopenia because of the difficulty of obtaining enough platelets to perform an estimation. The mean platelet ascorbic acid content was 1.95 ± 0.42 $\mu g/10^9$ cells which was significantly lower than normal ($t = 4.5$, $N = 5$*, $0.01 > P > 0.005$).

In the patient (F.D.) with Felty's syndrome the platelet ascorbic acid content was lower than the normal range (mean ± 2 x standard deviation) on the first 2 occasions on which it was measured, and her plasma concentration was near the lower limit of normal (Appendix B8). After large doses of ascorbic acid (1 G per day orally for 4 days) the platelet ascorbic acid content was within the normal range and her plasma concentration was at a level consistent with saturation. Further large doses of ascorbic acid did not change these levels (Appendix B8) indicating that

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*F test was significant and the t test was modified as specified in Appendix A.

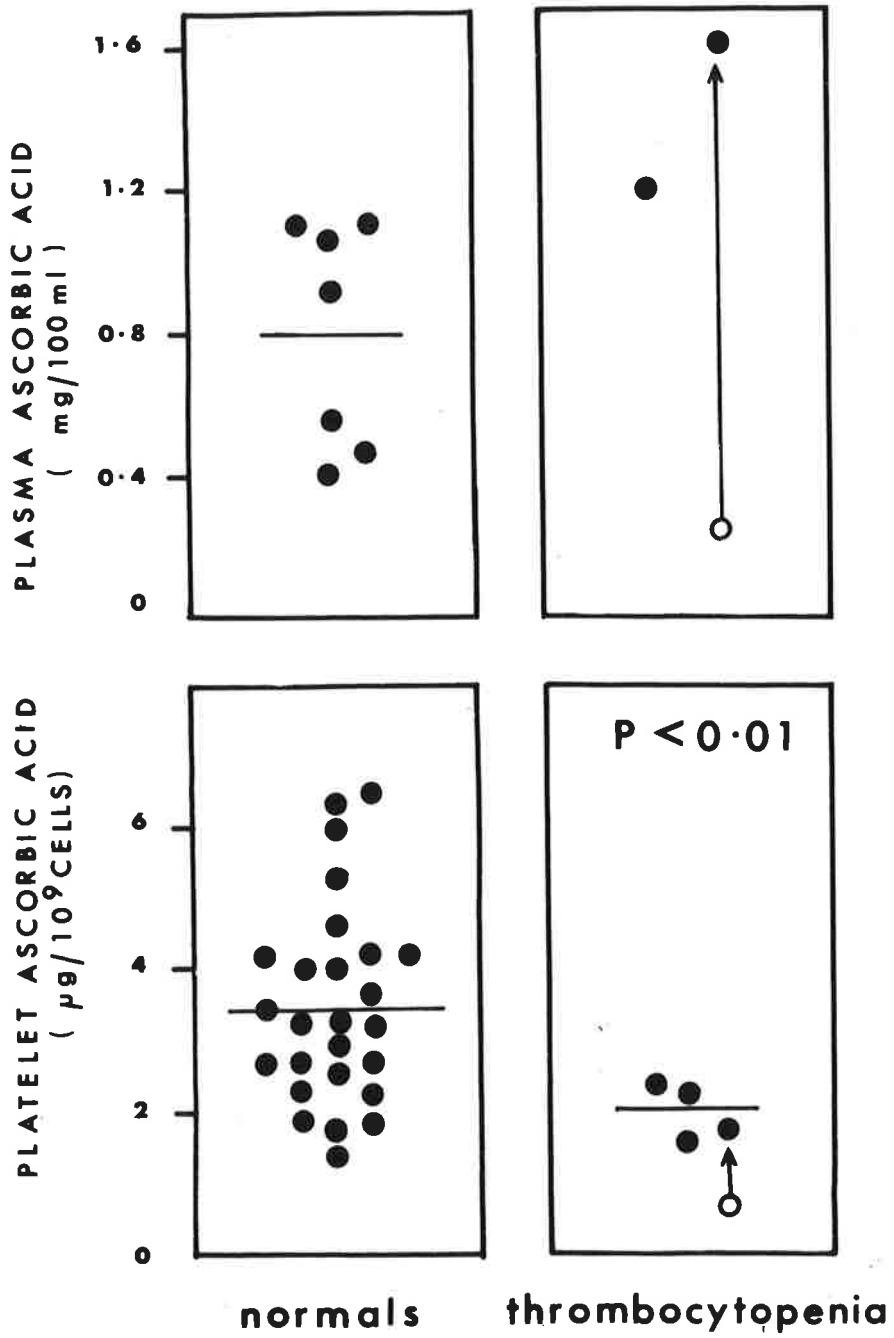

FIGURE 21: Platelet and plasma ascorbic acid concentrations in patients with thrombocytopenia and comparison with the normal range.

saturation had been attained.

IV. COMMENT

The platelet ascorbic acid content was significantly lower than normal in patients suffering from uraemia, acute leukemia, chronic myeloid leukemia, atherosclerosis, megaloblastic anaemia, and in a heterogeneous group of cases of thrombocytopenia.

In uraemia the low platelet ascorbic acid content was associated with a normal plasma concentration. This suggests that there is a defect in the ability of platelets to concentrate ascorbic acid in this condition. On the other hand, the low platelet ascorbic acid content in acute leukemia was associated with a marked depression of the plasma ascorbic acid concentration suggesting that the low platelet content in this condition is secondary to a low plasma concentration.

In atheroma it may appear at first sight that the results suggest an abnormality in the mechanism by which platelets concentrate ascorbic acid. Such a defect has not, however, been conclusively established for an analysis of the plasma concentration in a larger number of subjects may have shown this level to be significantly lower than normal. Another uncertain factor in this investigation was the unknown effect of smoking on the platelet ascorbic acid content. A larger number of subjects must be studied before a conclusion can be reached. Smoking has been shown by

Calder et alii (1963) to depress the buffy layer and plasma ascorbic acid concentrations.

In polycythaemia vera and in thrombocythaemia no marked differences from normal were found. A larger number of patients in each group would be required to exclude any slight differences from the normal range.

The ascorbic acid concentrations in patients receiving steroids were within normal limits. This tends to suggest that the purpura of steroid therapy is not due to ascorbic acid deficiency.

A marked and highly statistically significant depression of the platelet ascorbic acid content occurred in megaloblastic anaemia. In the present study the platelet concentration of ascorbic acid had fallen out of all proportion to the slight fall in plasma concentration. This suggests that in megaloblastic anaemia, as in uraemia, there is a defect in the mechanism by which platelets concentrate ascorbic acid.

An attempt to estimate the ascorbic acid content of platelets which were younger than normal did not demonstrate any abnormalities. A wide scatter of results was obtained.

In the four patients with thrombocytopenia an insufficient number of plasma ascorbic acid estimations precludes the possibility of determining the reason for the low platelet ascorbic acid content.

In such a heterogeneous group of disorders in which the only common factor was a low platelet count, it is surprising that the platelet ascorbic acid content should be lower than normal.

CHAPTER 5

ADMINISTRATION OF HIGH DOSES OF ASCORBIC ACID TO PATIENTS SUFFERING FROM URAEMIA AND LEUKEMIA

I. INTRODUCTION

One important observation in Chapter 4 was that the platelet ascorbic acid concentration in uraemia was significantly lower than normal despite a normal plasma concentration. Another important result was that low platelet ascorbic acid concentration was found in acute leukemia and chronic myeloid leukemia and that this was associated with a low plasma ascorbic acid concentration in all but one of the cases of chronic myeloid leukemia. In chronic lymphatic leukemia, however, both platelet and plasma values were normal.

In this chapter experiments are described which measure the effect of a high oral intake of ascorbic acid on the platelet ascorbic acid content and the plasma ascorbic acid concentration in patients suffering from uraemia and leukemia. The doses given were large enough to saturate the tissues of normal subjects but may not have produced saturation in the patients with leukemia for it has been reported that, whereas saturation in normal subjects is attained by administration of 1 G of ascorbic acid daily for 1-4 days, a longer period is required in leukemia (Cuttle, 1938; Barkhan and Howard, 1958).

II. METHOD

1.0 G of ascorbic acid was taken orally daily for 4 days. Ascorbic acid estimations were performed on the morning of the first

TABLE 22. EFFECT OF ASCORBIC ACID ADMINISTRATION IN URAEMIA

PATIENT	BLOOD UREA NITROGEN (mg/100 ml)	PLATELET ASCORBIC ACID ($\mu g/10^9$ cells)			PLASMA ASCORBIC ACID (mg/100 ml)		
		B	A	C	B	A	C
J.T.	30	2.5	3.4	0.9	0.75	4.2	3.45
D.W.*	73	1.5	4.8	3.3	0.70	5.5	4.80
S.M.*	72	0.7	5.0	4.3	0.95	6.5	5.55
C.D.	54	0.2	4.1	3.9	0.20	2.1	1.90
C.H.	67	1.1	3.0	1.9	1.20	2.8	1.60
	MEAN	1.2	4.1	2.9	0.76	4.22	3.5
	t		4.0			4.0	
	N		4			4	
	P		$0.01 > P > 0.005$ †			$0.01 > P > 0.005$ †	

B = before ascorbic acid administration

A = after ascorbic acid administration

C = change = A-B

*on haemodialysis

†one tailed t test

day of the experiment; ascorbic acid administration was then commenced. 4 days later on the morning after the last dose of ascorbic acid the ascorbic acid estimations were repeated. Also performed on each patient before and after large doses of ascorbic acid were platelet thromboplastic function (Bonnin and Cheney, 1961), Ivy bleeding time (Wintrobe, 1961), and tourniquet test (Wintrobe, 1961).

III. RESULTS

1. *Uraemia*

(a) Subjects studied. 5 cases of uraemia were studied (Table 22). Two of these patients were being treated by twice weekly haemodialysis. None had an abnormal bleeding tendency. Further details of these subjects are contained in Appendix B1.

(b) Before ascorbic acid administration. The platelet ascorbic acid content in the 5 uraemic patients before large doses of ascorbic acid had been administered was significantly lower than the normal range ($t = 3.5$, $N = 29$, $0.005 > P > 0.001$), but the plasma concentration was well within normal limits (Table 22, Figure 22). The platelet thromboplastic function was reduced to 60% in one subject (C.H.) and the bleeding time and tourniquet tests were normal in all 5 subjects.

(c) After ascorbic acid administration. After the administration of large doses of ascorbic acid the mean platelet

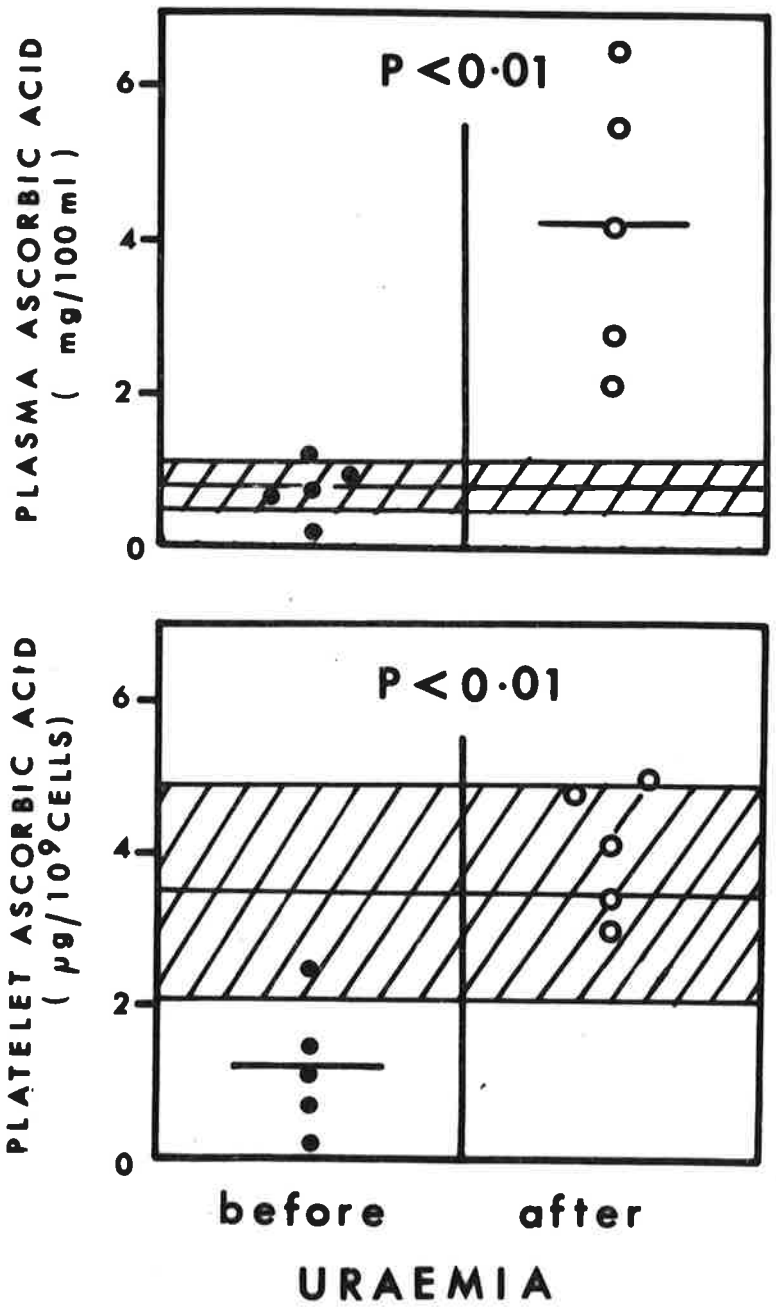

FIGURE 22: The concentration of ascorbic acid in platelets and plasma in patients suffering from uraemia before and after the administration of ascorbic acid. A one tailed t test was used.

TABLE 23. EFFECT OF ASCORBIC ACID ADMINISTRATION IN LEUKEMIA (GROUP I)

PATIENT	TYPE OF LEUKEMIA	WHITE CELL COUNT (c mm)	PLATELET ASCORBIC ACID ($\mu g/10^9$ cells)			PLASMA ASCORBIC ACID (mg/100 ml)		
			B	A	C	B	A	C
A.S.	Acute	3,000	0.3	4.1	3.8	0.30	1.60	1.30
J.D.	Chronic myeloid	49,000	0	2.4	2.4	0.35	1.15	0.80
A.M.	Chronic myeloid	13,400	1.6	5.8	4.2	0.20	1.20	1.0
		MEAN	0.6	4.1	3.5	0.28	1.3	1.03
		t		5.2			5.8	
		N		2			2	
		P	0.025 > P > 0.0125*			0.025 > P > 0.0125*		

B = before ascorbic acid administration

A = after ascorbic acid administration

C = change = A-B

*one tailed t test

TABLE 24. EFFECT OF ASCORBIC ACID ADMINISTRATION IN CHRONIC LYMPHATIC LEUKEMIA (GROUP II)

PATIENT	WHITE CELL COUNT (per c mm)	PLATELET ASCORBIC ACID ($\mu g/10^9$ cells)			PLASMA ASCORBIC ACID (mg/100 ml)		
		B	A	C	B	A	C
Beat. T.	63,000	6.8	7.2	0.4	0.45	2.50	2.05
J.G.	83,000	5.5	6.0	0.5	0.55	2.15	1.60
J.F.	19,000	3.2	2.4	- 0.8	0.75	1.30	0.55
	MEAN	5.2	5.2	0	0.58	1.98	1.4
					t	2.6	
					N	2	
					P	0.1 > P > 0.05*	

B = before ascorbic acid administration

A = after ascorbic acid administration

C = change = A-B

*one tailed t test

ascorbic acid content had risen to well within the normal range and the plasma concentration had risen to a very high level (mean 4.2 mg/100 ml), (Figure 22). This plasma level is very much higher than that attained in normal subjects where the same dose produced a plasma concentration of 1.0-1.4 mg/100 ml (see Chapter 3). The plasma ascorbic acid concentrations attained were particularly high in 2 patients on chronic haemodialysis (D.W. and S.M., Table 22).

There was no significant change in platelet thromboplastic function, bleeding time, or tourniquet test.

2. Leukemia.

(a) Subjects studied. One case of acute lymphatic leukemia, 2 cases of chronic myeloid leukemia, and 3 cases of chronic lymphatic leukemia were studied. For the purposes of discussion these subjects were divided into 2 groups: the case of acute leukemia and the 2 cases of chronic myeloid leukemia in the first group (Table 23), and the 3 cases of chronic lymphatic leukemia in the second group (Table 24).

Though none of the patients was in haematological remission, none had a marked bleeding tendency. Details of these patients are contained in Appendix B2.

(b) Before ascorbic acid administration. Prior to the administration of ascorbic acid the mean platelet and plasma

ascorbic acid concentrations were both lower than normal in the first group and within the normal range in the second group (figure 23). The platelet thromboplastic function was reduced to 30% in one subject (A.S.), and the tourniquet test was positive in another subject (J.G.).

(c) After ascorbic acid administration. After the administration of ascorbic acid the platelet content of ascorbic acid in the first group rose significantly to well within the normal range but did not change significantly in the second group (Figure 23, Tables 23 and 24). The plasma ascorbic acid concentration in both groups rose to levels consistent with saturation. The platelet thromboplastic function in A.S. was still 30% and the tourniquet test in J.G. was still positive.

IV. COMMENT

Prior to the administration of ascorbic acid to patients suffering from uraemia the ascorbic acid content of platelets was lower than would be expected but the plasma concentration was normal. This suggested that there was a defect in the ability of the platelets to concentrate ascorbic acid. After large doses of ascorbic acid, the platelet content of ascorbic acid rose to within normal limits probably because the very high plasma levels attained enabled the platelets to take up more ascorbic acid. The high plasma ascorbic acid concentrations after ascorbic acid administration were probably a manifestation of the impairment

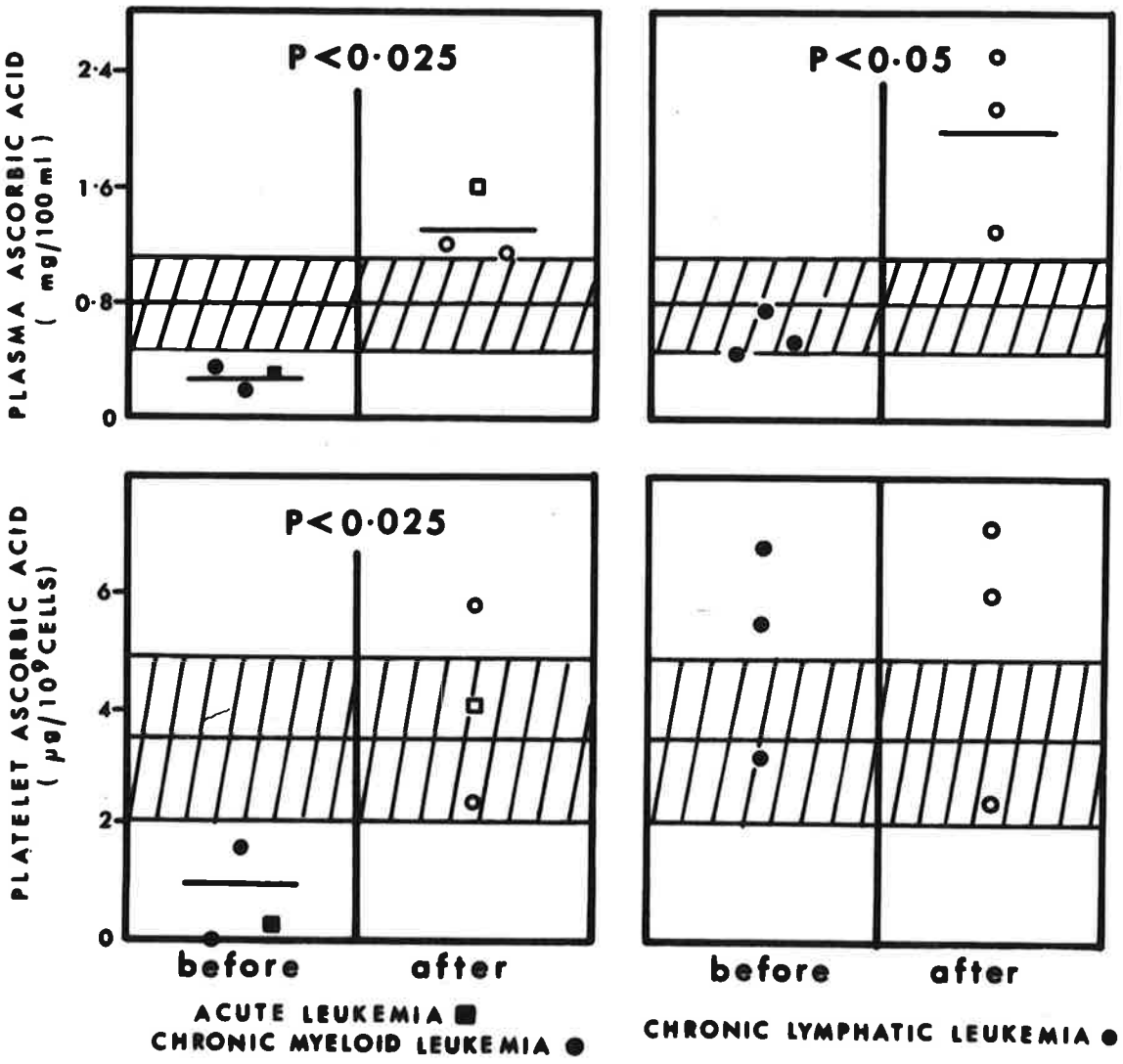

FIGURE 23: The concentration of ascorbic acid in platelets and plasma in patients suffering from leukemia before and after the administration of ascorbic acid. A one tailed t test was used.

in renal excretion of ascorbic acid which has been previously described in uraemia (Sendroy and Miller, 1939).

In the three cases of leukemia in the first group (Table 23) a low platelet ascorbic acid concentration was observed and the plasma concentration was also low. After the administration of ascorbic acid the platelet ascorbic acid content was well within the normal range and the plasma concentration was at levels consistent with those obtained by saturation of normal subjects. These results suggest that the low platelet ascorbic acid concentrations observed in acute leukemia and in chronic myeloid leukemia are due to a decreased amount of ascorbic acid in the body rather than a defect in the platelet mechanism for the concentration of ascorbic acid.

In chronic lymphatic leukemia the platelet ascorbic acid concentration was normal prior to the administration of ascorbic acid and had not increased significantly after large doses of ascorbic acid suggesting that the level was at or near saturation prior to the experiment.

CHAPTER 6

UPTAKE OF ASCORBIC ACID BY PLATELETS

I. INTRODUCTION

The high concentration of ascorbic acid in platelets compared to that in the surrounding plasma implies either that platelets contain a large amount of ascorbic acid when released from megakaryocytes or that they concentrate ascorbic acid from the surrounding plasma. One might expect that both mechanisms would be operative to some extent. Denson and Richards (1962) reporting that white cells will absorb ascorbic acid from plasma against a concentration gradient, included the results of 2 experiments on platelets; when platelet ascorbic acid content was estimated before and after incubation with ascorbic acid an uptake of this substance by platelets was demonstrated.

The experiments described in this chapter were designed to study in vitro the mechanism by which platelets concentrate ascorbic acid from the surrounding medium.

II. METHOD

1. *C^{14}-labelled ascorbic acid*

The contents of an ampoule of L-ascorbic acid-1-C^{14}* (New England Nuclear Corporation) containing 50 µCi (2.4 mg) of labelled ascorbic acid was reconstituted in 50 ml of distilled water acidified to pH 4 with oxalic acid. One ml aliquots (1 µCi,

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*Paper chromatography by the manufacturer indicated 100% purity.

48 µg per aliquot) were lyophilised, sealed in ampoules under vacuum, and stored at 0-4°C. The recovery of ascorbic acid from this procedure was 91% by the Fe-T.P.T.Z. reaction.

2. Procedure

5-10 ml of fresh platelet rich plasma was incubated in a water bath at 37°C in a plastic test tube. C^{14}-ascorbic acid was added along with sufficient carrier ascorbic acid to raise the total ascorbic acid concentration of the platelet rich plasma by 1.0 mg/ 100 ml. 0.5 ml samples of platelet rich plasma were removed immediately and at intervals thereafter. Each sample was centrifuged for 5 minutes at 1300 g at 0-4°C to form a platelet button and the supernatant plasma was then decanted. Any excess plasma was removed by wiping the inside of the tube with adsorbent tissue. In this way the platelets were separated from the platelet rich plasma within 6-7 minutes of taking the sample.

The platelets in each sample were resuspended in 0.2 ml of 10% E.D.T.A. and transferred to a disposable planchette using a siliconised Pasteur pipette. Spreading of the platelets on the planchette to form an even film was aided by a trace of detergent which had been added to the E.D.T.A. solution. The contents of the planchettes were dried by evaporation under an infrared lamp and the beta activity was measured in a proportional counter with an ultrathin window using P 10 (argon-methane counting gas).

Because each sample from a given suspension contained an identical amount of platelets, no allowance was required for self absorption.

III. RESULTS

1. Normal uptake

40 ml of platelet rich plasma was divided into 4 equal aliquots to each of which was added 0.5 μCi of C^{14}-ascorbic acid with carrier ascorbic acid. One aliquot was subjected immediately to platelet ascorbic acid estimation by the T.L.C. method. From the second aliquot 0.5 ml samples were removed at intervals and the platelet radioactivity determined. After 80 minutes the platelets from the third aliquot were examined for ascorbic acid content by the T.L.C. method and the platelets from the fourth aliquot were separated, homogenised in a tissue grinder and the platelet extract subjected to thin layer chromatography on cellulose in the usual solvent used in the T.L.C. method. The developed chromatogram (from the fourth aliquot) was scanned for beta activity in a thin layer chromatogram scanner (Nuclear Chicago).

The uptake of radioactivity (second aliquot) was linear with time for 50 minutes after which no further uptake occurred (Figure 24). A high initial level of radioactivity was present in the first sample. This was partly because 6-7 minutes elapsed between addition of the radioactive ascorbic acid and the separation

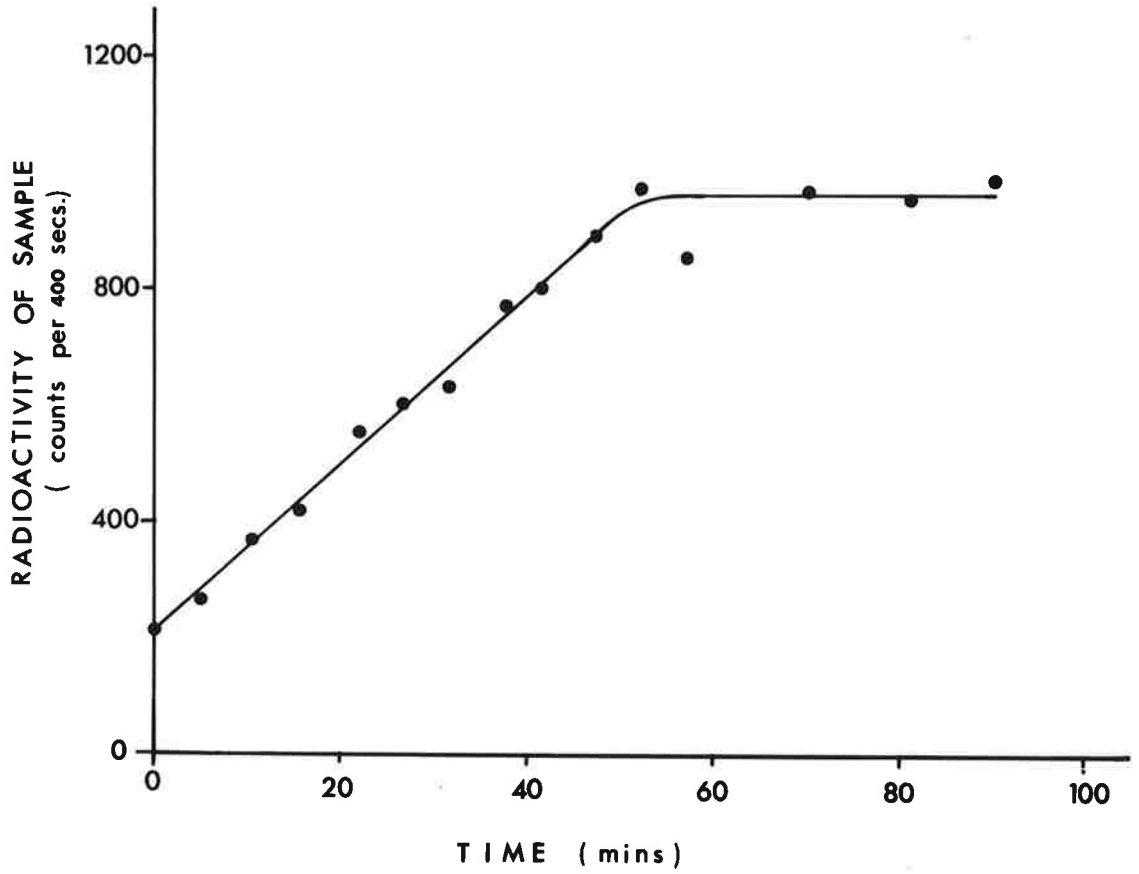

FIGURE 24: Uptake of radioactivity by platelets which have been incubated with C^{14}-ascorbic acid.

of the platelets in the first sample from plasma. Another factor would probably be plasma trapped in the interstices between the platelets.

The uptake of radioactivity was not associated with a large increase in the total amount of ascorbic acid within the platelet. Platelet ascorbic acid concentration was 1.2 $\mu g/10^9$ cells just after the addition of the labelled ascorbic acid (first aliquot) and 1.5 $\mu g/10^9$ cells after incubation for 80 minutes (second aliquot).

From the increase in radioactivity the amount of ascorbic acid which entered the platelet can be estimated as follows:

(a) Self absorption = $\frac{I}{Io} = e^{-nd}$ (Broda, 1960)

where I = intensity of radiation before self absorption

Io = intensity of radiation after self absorption

n = self absorption coefficient (120 for C^{14}; Broda, 1960)

d = layer thickness = 0.0016 G/cm^2

$\frac{I}{Io} = e^{-120 \times 0.0016} = 0.83$

(b) Counting efficiency of beta counter = 4.6%

(c) Assuming that the uptake of activity actually began 6 minutes prior to time 0 (Figure 24) the increase in platelet radioactivity per 0.5 ml sample was 830 counts per 400 seconds.

Therefore increase in counts emitted by samples

$$= \frac{830}{0.046 \times 0.83 \times 400} \text{ per second}$$

(d) specific activity of ascorbic acid in platelet rich plasma = 0.0078 μCi/μg.

(e) Number of platelets in 0.5 ml sample = 1.48×10^8

(f) Amount of ascorbic acid entering the platelet = 1.3 μg/10^9 cells.

Thus, though the increase in platelet ascorbic acid content was shown to be only 0.3 μg/10^9 cells by chemical means, the change in radioactivity showed that about 1.3 μg/10^9 cells had entered the platelets. Two possible explanations for this are:

(a) The ascorbic acid has been broken down or metabolised to another substance. This is unlikely for the radioactive scan (fourth aliquot) was identical to that shown in Figure 9 (Chapter 2). A single peak was obtained and the radioactivity coincided with the position of a reducing spot demonstrated by phosphomolybdic acid spray. It is possible, but unlikely, that the ascorbic acid had broken down before or after entering the platelets to some unknown substance which by chance had the same Rf as ascorbic acid.

(b) While ascorbic acid molecules from the plasma are diffusing into the platelet, ascorbic acid already in the platelet is diffusing out at the same rate, maintaining an equilibrium in which the greater concentration of ascorbic acid is inside the platelet. The specific activity of ascorbic acid inside the platelet would then rise until it

was the same as that of ascorbic acid outside the platelet. The specific activity of ascorbic acid in the platelet when the maximum uptake had occurred was estimated to be 0.0066 μCi/μg. This is of the same order as the specific activity in the plasma which was 0.0078 μCi/μg. The present results are therefore consistent with but do not prove this second hypothesis.

2. *Effect of incubation of platelet rich plasma*

There are two possible reasons for the cessation of uptake of radioactive ascorbic acid after about 50 minutes. Firstly it is possible that there is a failure of the uptake mechanism consequent on a decline in platelet metabolism; this may occur because of a lack of oxygen and other nutrients which the platelet consumes in the normal processes of respiration. Secondly it may be because an equilibrium has been reached between the specific activity of the ascorbic acid inside the platelet and the specific activity of the ascorbic acid in the plasma: thus at equilibrium, the rate at which ascorbic acid is leaving the platelet equals the rate at which it is entering. The following experiment was performed to explore the first possibility.

15 ml of platelet rich plasma were divided into 2 equal aliquots. To one aliquot C^{14}-ascorbic acid was added and the platelet radioactivity determined at 8 minute intervals. The other aliquot was incubated for 32 minutes at 37°C prior to the addition of C^{14}-ascorbic acid and the measurement of uptake.

Uptake of radioactivity occurred to the same extent and for the same length of time after the addition of C^{14}-ascorbic acid in the second aliquot as in the first (Figure 25). If the first hypothesis were correct the uptake in the second tube would be expected to cease after about 20 minutes.

The effect of more prolonged incubation on the ability of the platelet to maintain a high concentration of ascorbic acid in its interior was studied in the next experiment. After addition of C^{14}-ascorbic acid to platelet rich plasma incubated at 37°C, the platelet radioactivity was followed for 4½ hours. In this experiment maximum uptake occurred at about 80 minutes (Figure 26) and after 2 hours there was a gradual decrease in the amount of platelet radioactivity. This suggests that over a prolonged period of incubation platelets gradually lose their ability to maintain a gradient of ascorbic acid concentration between the plasma and the cell cytoplasm.

3. Effects of enzyme inhibitors

Substances tested for their effect on platelet ascorbic acid uptake were potassium cyanide, parachloromercuribenzoate, and dinitrophenol. The substance being tested was added in a volume of 0.5 ml to 10 ml of platelet rich plasma. In each experiment a control was set up by adding 0.5 ml of normal saline to 10 ml of platelet rich plasma. 5 minutes after adding the test substance C^{14}-ascorbic acid was added and the platelet radioactivity

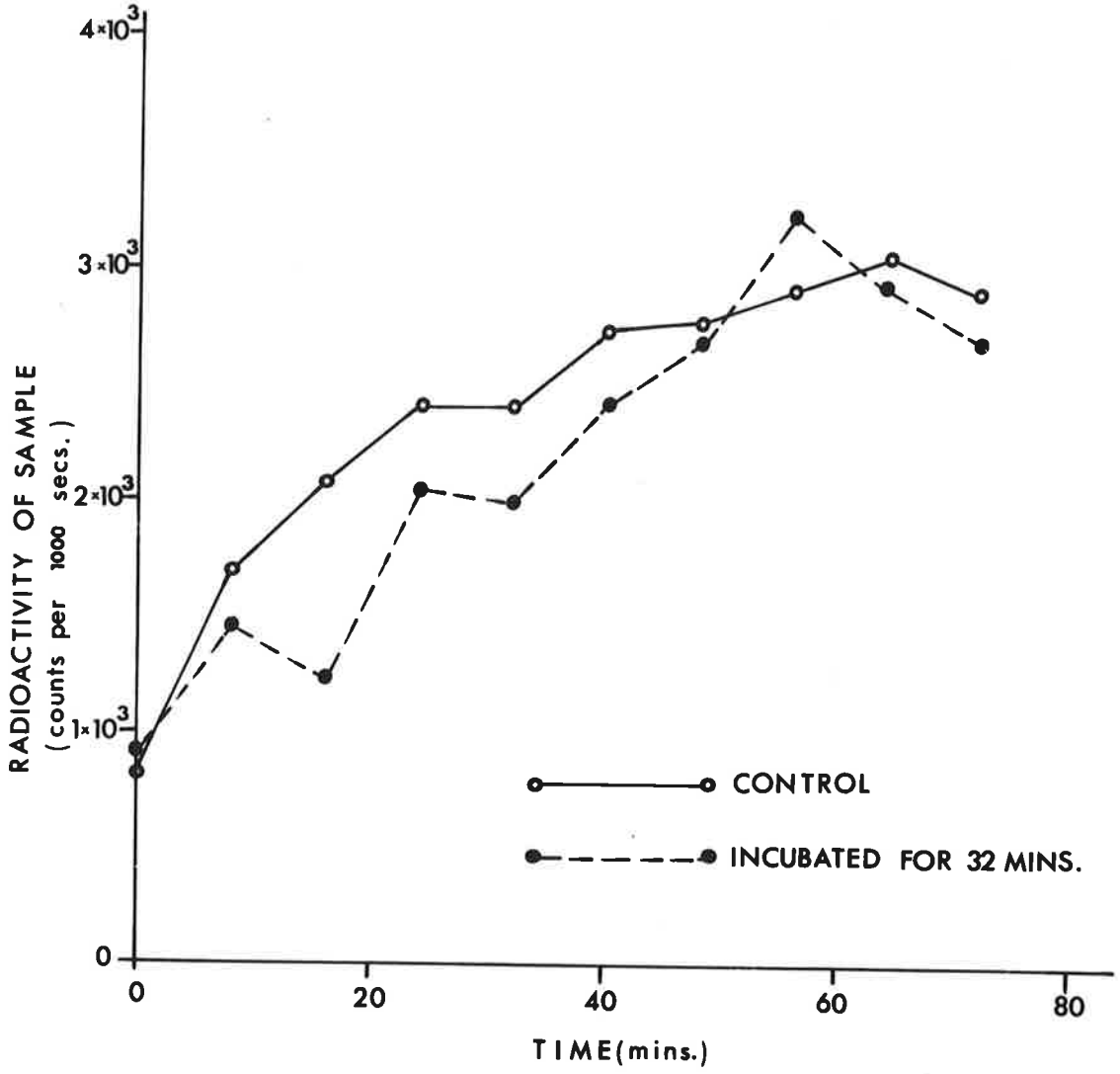

FIGURE 25: Uptake of radioactivity by platelets plotted against time since adding C^{14}-ascorbic acid to platelet rich plasma. In the control tube C^{14}-ascorbic acid was added as soon as the platelet rich plasma was isolated. In the other tube C^{14}-ascorbic acid was added after a period of incubation.

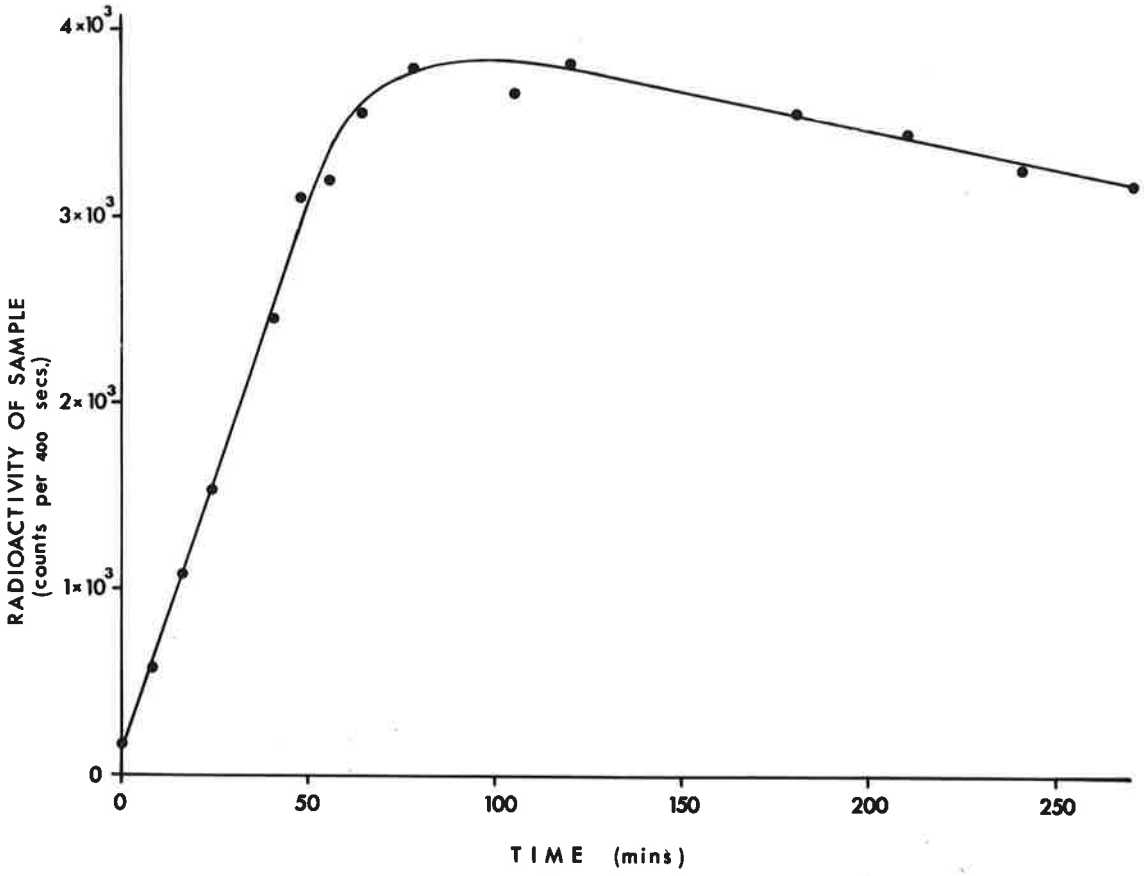

FIGURE 26: In this experiment platelet radioactivity was followed for a total of 4½ hours after the addition of C^{14}-ascorbic acid to platelet rich plasma.

determined at intervals.

Potassium cyanide in a final concentration of 10^{-3} M inhibited the uptake of C^{14}-ascorbic acid, the total uptake being only about one-fifth of that in the control tube (Figure 27).

Parachloromercuribenzoate in a final concentration of 2×10^{-5} M caused no inhibition, but in a concentration of 2×10^{-4} M there was a marked decrease in the initial rate of uptake (Figure 28). The total uptake, however, was similar in all three samples.

Dinitrophenol in final concentrations of 2×10^{-5} M and 2×10^{-3} M did not inhibit the uptake of ascorbic acid.

IV. COMMENT

The experiments described in this chapter show that C^{14}-ascorbic acid added to platelet rich plasma was taken up by platelets. This was not accompanied by the expected rise in the total platelet content of ascorbic acid suggesting that either the ascorbic acid has been broken down before or after entering the cells, or that the diffusion of ascorbic acid into the platelets was accompanied by the diffusion of ascorbic acid out of the platelets. Evidence obtained by subjecting the platelet extracts to thin layer chromatography followed by scanning for radioactivity suggested that the former possibility is unlikely.

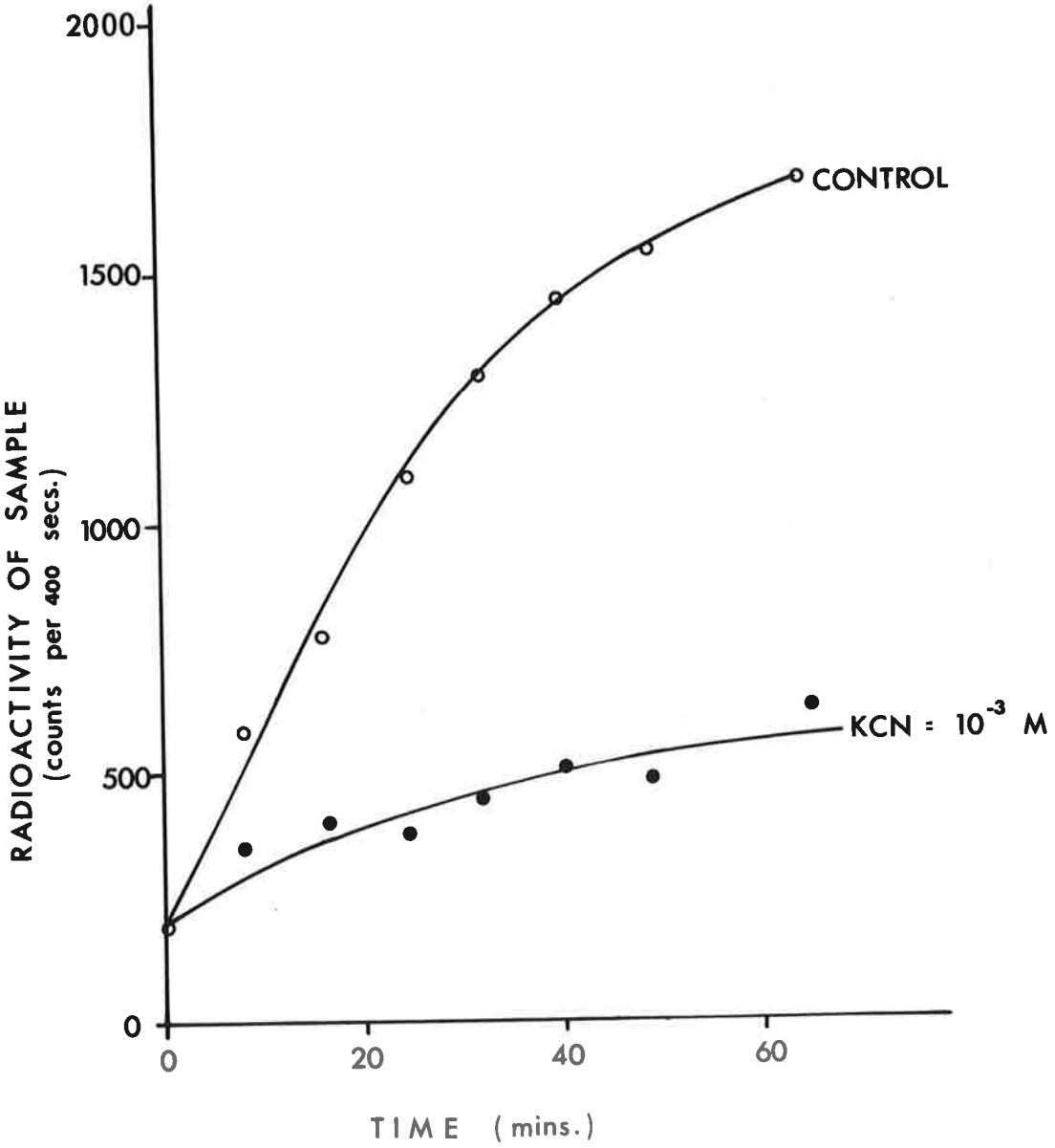

FIGURE 27: Effect of potassium cyanide on the uptake of radioactivity by platelets.

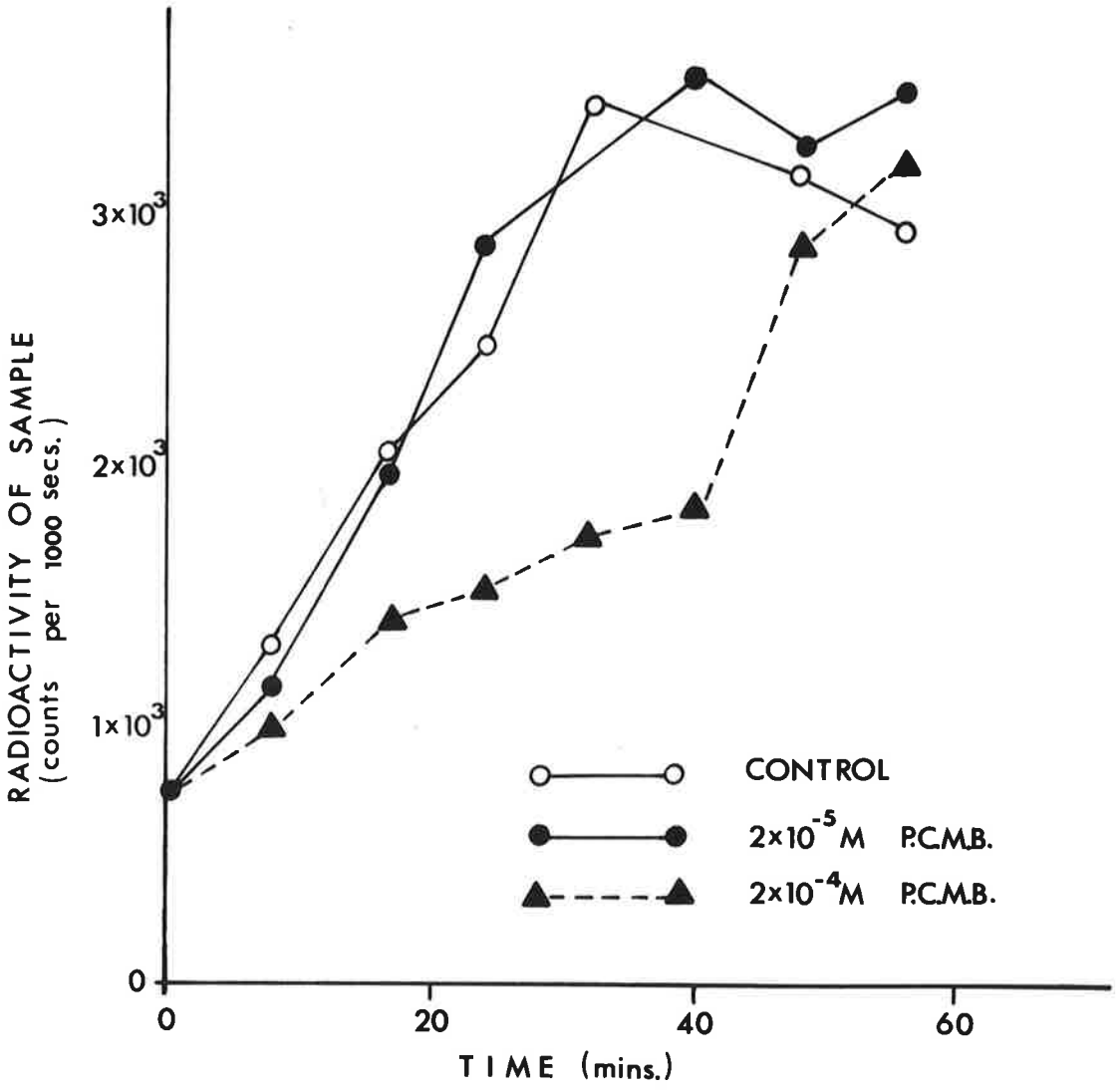

FIGURE 28: Effect of parachloromercuribenzoate on the uptake of radioactivity by platelets.

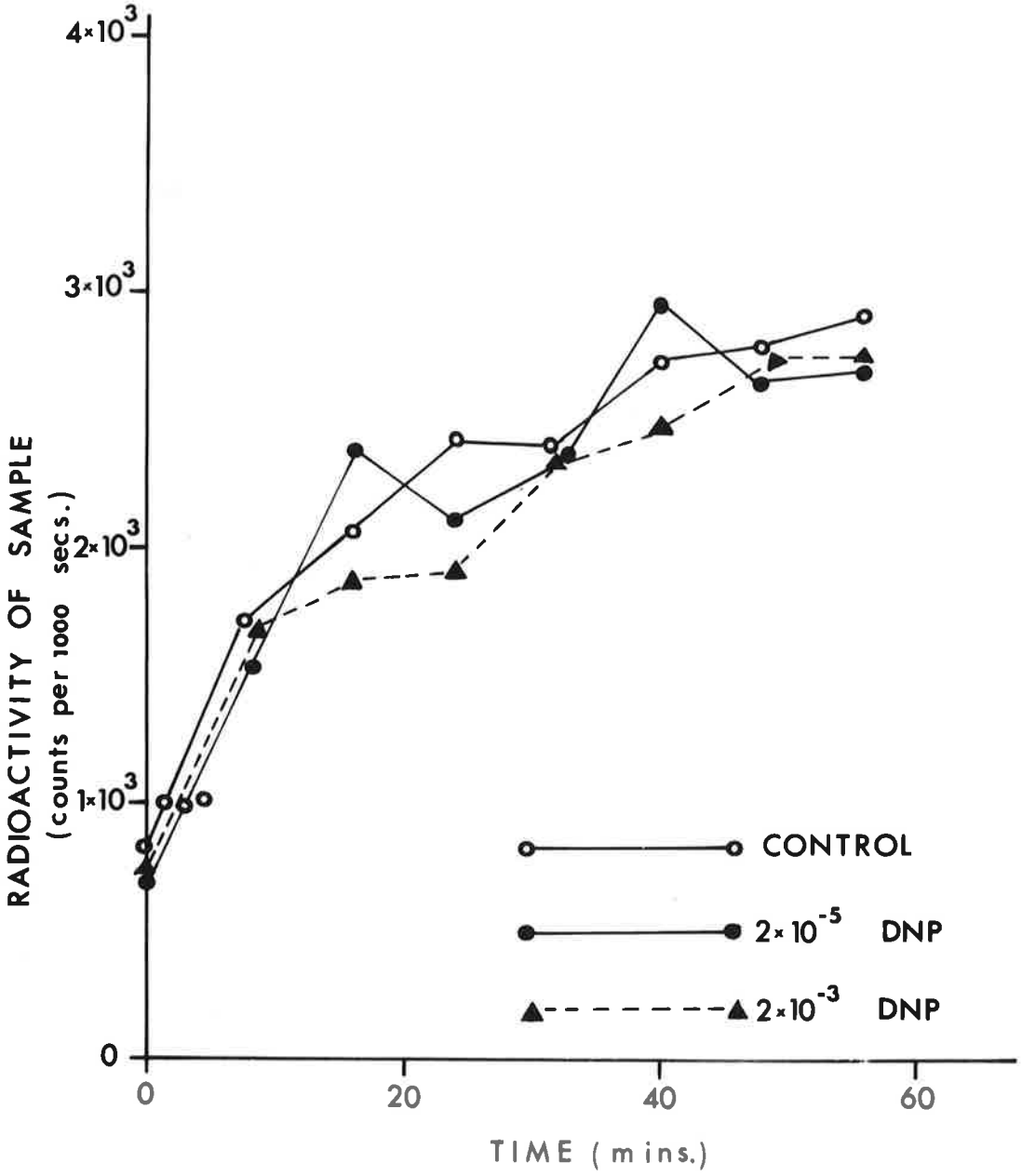

FIGURE 29: Effect of dinitrophenol (D.N.P.) on the uptake of radioactivity by platelets.

The uptake of C^{14}-ascorbic acid by platelets was not impaired by incubation of the platelet rich plasma at 37°C for 30 minutes prior to adding the radioactive ascorbic acid. When platelets which had taken up C^{14}-ascorbic acid were incubated for 4½ hours at 37°C there was a gradual decrease in the amount of radioactive ascorbic acid in the platelets. The uptake of C^{14}-ascorbic acid was blocked by potassium cyanide which forms complexes with metal ions and therefore probably has a non-specific inhibitory effect on cellular metabolism. Parachloromercuribenzoate which inhibits enzymes whose activity depends on sulphydryl groups had a similar effect which, however, did not persist. Dinitrophenol which uncouples oxidative phosphorylation of A.D.P. (White et alii, 1964) had no effect in the concentrations used suggesting that the mechanism does not require a ready supply of adenosine triphosphate.

Though these results do not prove that the mechanism in platelets for maintaining a high concentration of ascorbic acid within the cell is mediated enzymatically, they do show that it is dependent on an intact cellular metabolism.

CHAPTER 7

ASCORBIC ACID AND A.D.P.-INDUCED AGGREGATION

I. INTRODUCTION

An important property of platelets is their ability to respond to certain stimuli by adhering to each other to form platelet aggregates. Platelet aggregation can be followed by recording the transmission of light through a suspension of platelets on to a photoelectric cell (Born, 1962; O'Brien, 1962). This system has been used extensively in the study of the kinetics of platelet aggregation (see Chapter 1). Adenosine diphosphate is one substance which initiates platelet aggregation, and since it is released from red cells and platelets during haemostasis it is likely that A.D.P.-induced platelet aggregation is an important component of the complex mechanism of haemostasis.

The studies described in this chapter were undertaken to assess the role of ascorbic acid in platelet aggregation. Experiments were designed to determine (1) whether ascorbic acid added to platelet rich plasma had any effect on A.D.P.-induced platelet aggregation, and (2) whether ascorbic acid was released from platelets during A.D.P.-induced platelet aggregation.

II. METHODS

1. Measurement of platelet aggregation

Platelet aggregation was measured spectrophotometrically as a decrease in the absorbance of a sample of platelet rich plasma.

3.0 ml of platelet rich plasma contained in a glass cell (length of light path = 1.0 cm) of a Beckman DB spectrophotometer was stirred with a small plastic coated steel rod rotated by a magnetic stirrer. At 30 second intervals the cell was placed in the spectrophotometer and the absorbance measured at 600 mμ. When a series of 5-10 baseline readings had been obtained, 0.1 ml of 1% acetic acid containing enough ascorbic acid to increase the plasma concentration by 1.0 mg/100 ml was added. For control runs 0.1 ml of 1% acetic acid containing no ascorbic acid was used. After the addition of ascorbic acid or 1% acetic acid the absorbance was recorded at half minute intervals for 4 minutes. A solution of A.D.P. in normal saline was then added to produce a concentration of A.D.P. in the plasma of 2.6×10^{-6} - 2.5×10^{-5} M and the absorbance was then recorded at half minute intervals for at least 10 minutes.

2. *The liberation of ascorbic acid from platelets*

10 ml of platelet rich plasma was incubated with C^{14}-ascorbic acid along with sufficient carrier ascorbic acid to raise the plasma ascorbic acid concentration by 1.0 mg/100 ml. After 70 minutes which allowed time for the concentration of C^{14}-ascorbic acid in the platelets to attain a stable level (see Chapter 6), 0.5 ml samples of the platelet rich plasma were removed and subjected to determination of platelet radioactivity as described in Chapter 6. The 8 ml of platelet rich plasma remaining was divided into 2

equal aliquots each of which was placed in a spectrophotometer cell of 1.0 cm light path and stirred on the magnetic stirrer. The absorbance at 600 mμ was measured at intervals in the spectrophotometer. After about 4 minutes the platelets in one of the cells were aggregated with A.D.P. and normal saline was added to the other cell to form a control. When aggregation had occurred samples were withdrawn from both cells for the determination of platelet radioactivity.

III. RESULTS

1. Effect of added ascorbic acid on A.D.P.-induced platelet aggregation

Following the addition of the ascorbic acid (dissolved in 1% acetic acid) to the platelet rich plasma there was a slight decrease in absorbance. This occurred to the same extent when 1% acetic acid alone was added and could be accounted for by the diluting effect of the added fluid. Thus ascorbic acid did not induce measurable platelet aggregation.

The effect of adenosine diphosphate in 2 typical experiments is illustrated in Figure 30. On day 1 platelets from a normal subject were used, and on day 2 platelets were taken from the author who was at that time on an ascorbic acid deficient diet (platelet ascorbic acid content = 1.2 μg/10^9 cells; plasma

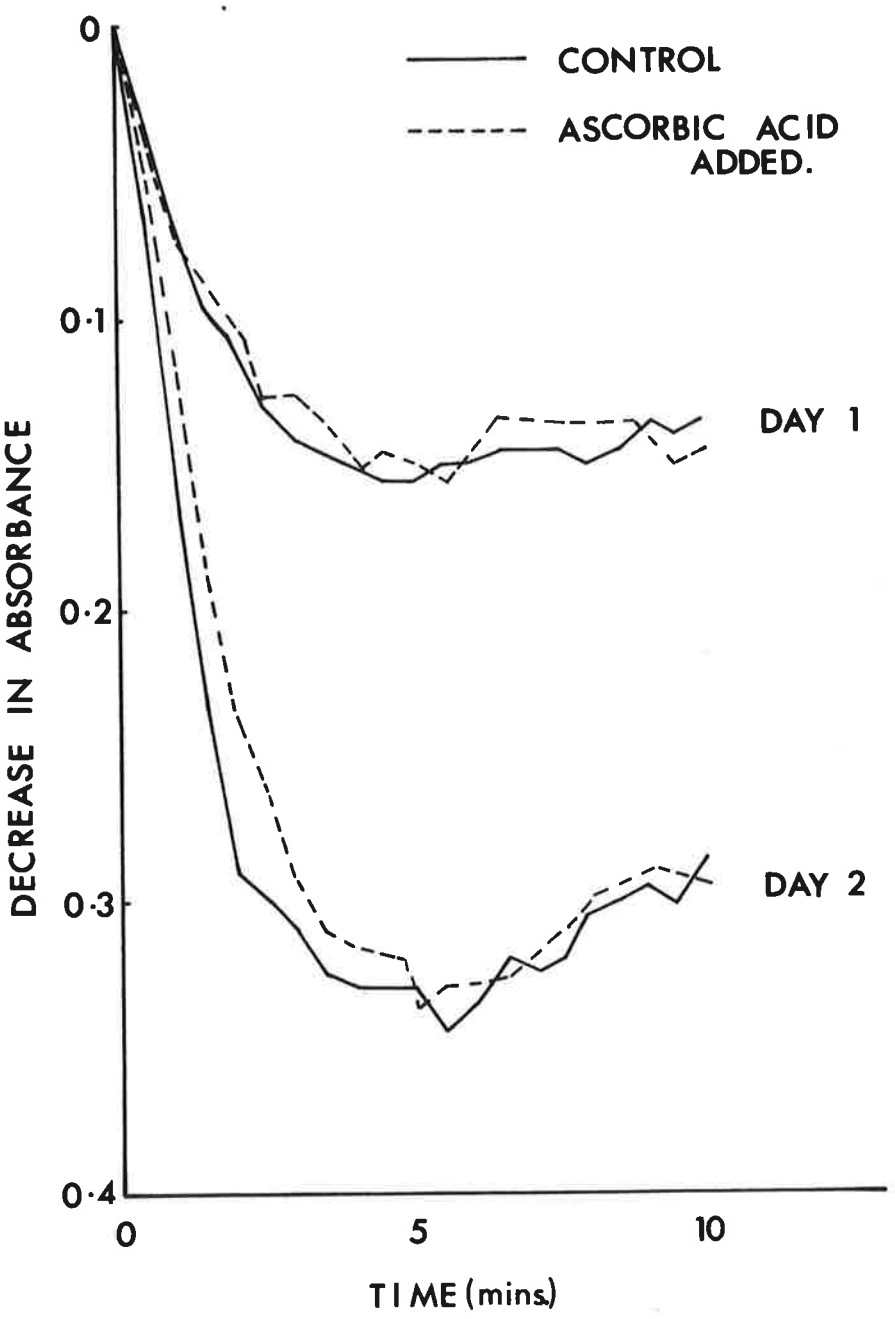

FIGURE 30: Effect of ascorbic acid on A.D.P.-induced platelet aggregation (measured as a decrease in absorbance).

concentration = 0.3 mg/100 ml). There was no significant difference between the pattern of aggregation recorded in the presence of added ascorbic acid and that recorded in the control. The concentration of added A.D.P. in the plasma was 2.5×10^{-6}M on day 2 and 1.25×10^{-6} M on day 1.

2. *The liberation of ascorbic acid from platelets*

The results of one experiment are illustrated in Figure 31. At point A 0.1 ml of A.D.P. in normal saline was added to one of the cells to produce a plasma concentration of 2.5×10^{-6}M. Because this produced only minimal aggregation a further 0.2 ml of the A.D.P. solution was added to increase the A.D.P. concentration to 7.3×10^{-6} M (point B). Once again the degree of aggregation was not marked and at point C a further 0.3 ml of a more concentrated solution of A.D.P. was added to produce a total plasma concentration of 6.7×10^{-5} M. With each addition of A.D.P. equal amounts of normal saline were added to the control tube.

The platelet radioactivity (counts per 400 seconds) in samples taken at points S_1, S_2, S_3 was as follows.

	Control	A.D.P.
S_1 (4 samples)	206; 233; 220; 220 (mean = 220)	
S_2 (1 sample each)	190	214
S_3 (4 samples each)	230; 251; 243; 253 (mean = 246)	243; 273; 245; 231 (mean = 248)

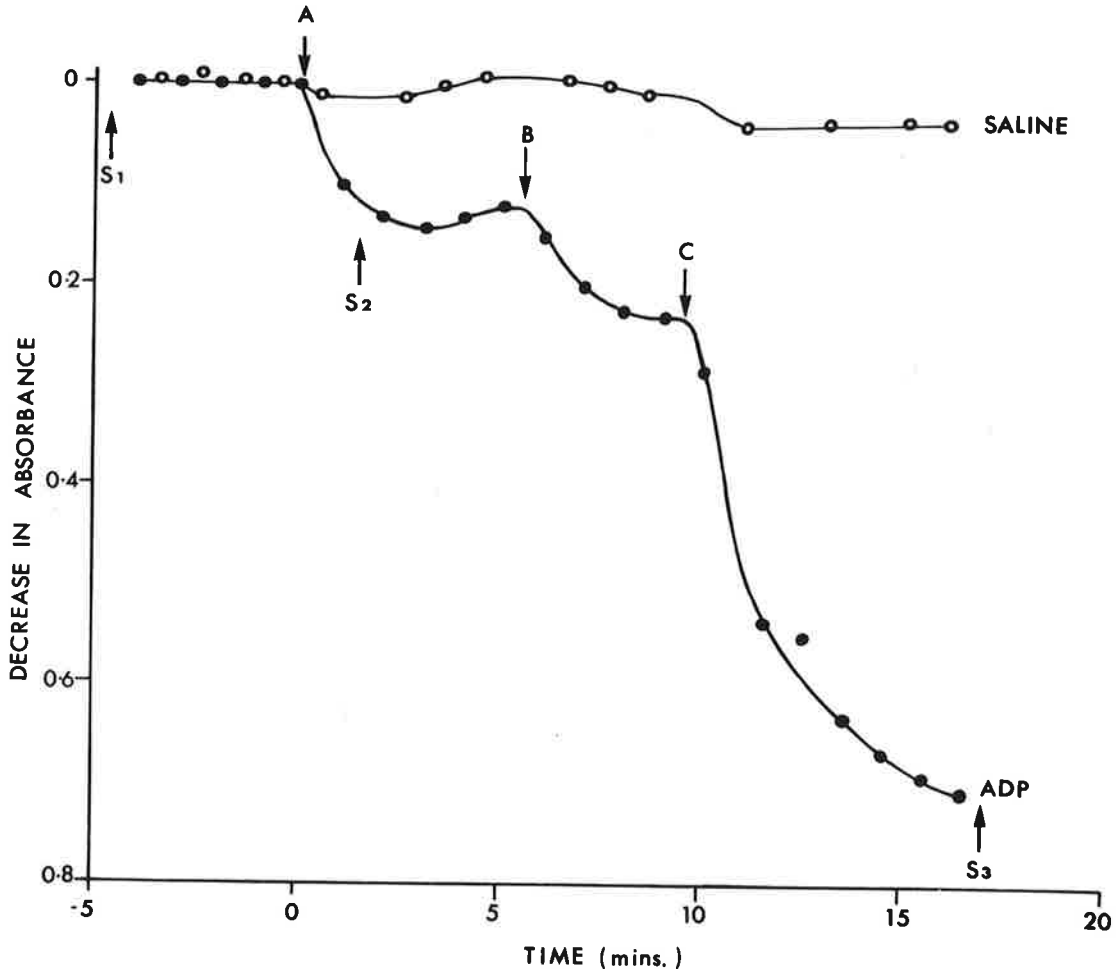

FIGURE 31: Experiment to determine whether A.D.P.-induced aggregation is associated with liberation of ascorbic acid from platelets. A.D.P. was added at points A, B, C. Samples were taken for measurement of platelet radioactivity at S_1, S_2, S_3.

There was no significant difference in radioactivity between the aggregated and the control platelets. In 2 similar experiments there was also no difference between aggregated and control platelets. Thus A.D.P.-induced aggregation was not associated with a release of ascorbic acid from the platelets.

IV. COMMENT

Ascorbic acid had no effect on A.D.P.-induced platelet aggregation even when platelet rich plasma from a subject on an ascorbic acid deficient diet was used. The platelets from this subject still contained a considerable amount of ascorbic acid, however. To completely exclude the possibility that ascorbic acid is required for A.D.P.-induced platelet aggregation, platelet rich plasma from a patient with clinical scurvy should be used.

Ascorbic acid was not released from the platelets during A.D.P.-induced aggregation. This suggests that if ascorbic acid is required for platelet aggregation it is required within the platelet rather than at the outer surface of the platelet external membrane.

CHAPTER 8

INVESTIGATION OF THE IRON BINDING ABILITY OF ASCORBIC ACID AND DEHYDROASCORBIC ACID

I. INTRODUCTION

A property of ascorbic acid which lends itself to further investigation is its ability to enhance the absorption of iron from the intestine. It is usually claimed that this is due to the formation of ferrous ions which, in the alkaline conditions of the duodenum where iron is absorbed, are very much more soluble than ferric ions (Moore et alii, 1939; Hahn et alii, 1945). This has not been proven, however, and another possible explanation for the effect of ascorbic acid is that a chelate complex with iron is formed.

It has been shown that chelates with iron are formed by fructose and by other reducing sugars, and that these chelates are soluble in the alkaline conditions of the duodenum (Charley et alii, 1963). The ferric-fructose chelate is more rapidly absorbed than ferrous sulphate in rats (Stitt et alii, 1962) and it has been recently shown that fructose also enhances iron absorption in humans (Davis and Deller, 1967). Since ascorbic acid has a structure somewhat similar to hexose sugars, the possibility that this substance also chelates iron must be considered.

Besides the effect of ascorbic acid on iron absorption there are other links between ascorbic acid and iron metabolism. It has been shown that ascorbic acid is required for the transfer of iron from plasma transferrin to liver ferritin (Mazur et alii, 1960)

TABLE 25. SOLUBILITY TEST FOR DETECTION OF CHELATING AGENTS (DAVIS, 1965)

Reagents	"Sample"	"Blank"	"Control"
1. 1.0 mM Fe^{+++}	2 ml	2 ml	2 ml
2. 0.01 μCi Fe^{59}/ml	2 ml	2 ml	2 ml
3. Sample solution	2 ml	-	-
4. Distilled water	-	2 ml	2 ml
5. HCl, NH_4OH as needed + water to	2 ml	2 ml	2 ml
6. pH adjustment	pH2 → pH 8		pH 2

and to the site of haem synthesis in liver mitochondria (Lockhead and Goldberg, 1959).

The aim of the experiments described in this chapter was to determine whether an iron-chelate complex is formed when ascorbic acid is added to solutions of iron. These experiments were based on techniques available in the department for the investigation of iron chelates in biological samples (Davis, 1965).

II. METHODS

1. Radioiron solubility test

This test developed by Davis (1965) is based on the fact that iron in its ferric form is extremely insoluble at pH 7-8, whereas most ferric chelates are soluble at this pH. The principle involved is identical to that used in a previous experiment (Chapter 2, Figure 2) for the estimation of ascorbic acid.

The test was set up in 20 ml beakers as shown in Table 25. Radioactive iron (Fe^{59}), carrier iron, and the sample (containing ascorbic acid or dehydroascorbic acid) were pooled in a beaker. Two other beakers, the blank and the control, contained distilled water in place of sample. The pH of the contents of each beaker was brought to 2 with hydrochloric acid. The contents of the sample and blank beakers were then adjusted to pH 8 with ammonium hydroxide, but the control mixture was allowed to remain at pH 2.

After adjusting the total volume of each mixture to 8 ml a suitable aliquot was centrifuged at 1300 g for 30 minutes to remove any precipitated ferric hydroxide. The gamma activity of 2.0 ml of supernatant from each aliquot was measured by scintillation counting.

The radioactivity of the control sample was high because all the iron remained in solution, whereas in the blank tube the radioactivity of the supernatant was almost background for the iron had been removed as precipitated ferric hydroxide. The results were expressed as

Iron solubility index of sample =

$$\frac{\text{Sample activity - blank activity}}{\text{Control activity - blank activity}} \times 100\%$$

In the presence of chelating agents iron solubility in this test is considerably enhanced (Davis, 1965). A false positive may be obtained in the presence of a reducing agent for ferrous ions are soluble at this pH and concentration.

2. *Ion exchange test (Davis, 1965)*

A property of metal chelates is that they are usually negatively charged or neutral, whereas free metal ions are positively charged. Thus chelated iron will not be bound by a cation exchange resin provided the chelating agent binds iron

more strongly than the resin. Free ferric or ferrous ions will, however, be bound strongly by a cation exchange resin.

10 ml of ferric chloride (10 µg iron/ml containing 0.1 µCi per ml of Fe^{59}) was added to 10 ml of sample solution containing a known amount of ascorbic acid or dehydroascorbic acid. After adjustment of pH (see results) the total volume was brought to 25 ml and 2 ml was placed on an ion exchange column. Dowex AG-50W-X8 in the sodium form was used in a glass column of 1.0 cm internal diameter. The bed volume was 5 ml. The column was then eluted with 50 ml of solvent. For the first 10 ml a flow rate of 1 ml per minute was used. For the last 40 ml a rate of 5 ml per minute was used.

The eluate was collected in 5 ml aliquots which were subjected to determination of gamma activity by scintillation counting. A significant amount of gamma activity indicates the formation of a negatively charged or neutral complex with iron. Free ferric ions or a positively charged complex will be bound strongly by the resin.

III. RESULTS

1. Colour formation

10 ml of 0.004 M ferric chloride solution were added to 10 ml of 1% acetic acid and the pH adjusted to 2 with HCl and then to 8 with NH_4OH. As the pH was raised from 2 to 8 a dense red precipitate formed. When 10 ml of 0.1 M ascorbic acid (in 1%

TABLE 26. SOLUBILITY TEST ON ASCORBIC ACID AND DEHYDROASCORBIC ACID

Concentration of 2 ml sample	Ratio of ascorbic or dehydro-ascorbic acid to iron	SOLUBILITY INDEX (%)	
		Ascorbic acid	Dehydro-ascorbic acid
Control (pH < 2)	0:1	100	100
Blank (pH = 8)	0:1	0	0
2.0 mM	2:1	100	98.9
1.0 mM	1:1	96.6	98.9
0.75 mM	3:4	100	100
0.5 mM	2:4	100	100
0.25 mM	1:4	99.7	58.1
0.10 mM	1:10	0.95	0.2

2.0 ml of sample was added to 2.0 ml of 1.0 mM Fe^{+++} Cl_3 and the solubility test performed.

acetic acid) was used in place of 1% acetic acid a visible precipitate did not form on raising the pH and a clear greenish coloured solution resulted. When 0.004 M ascorbic acid solution was used there was again no visible precipitate and the solution was a faint yellow colour.

Thus ascorbic acid appears to keep iron in solution at an alkaline pH. This could theoretically be due either to reduction of the ferric ions to ferrous ions or to the formation of a complex between iron and either ascorbic acid or one of its breakdown products.

2. *Solubility test*

(a) ascorbic acid. A variety of concentrations of ascorbic was tested. When the concentration of ascorbic acid was greater than or equal to 0.25 mM (molar ratio of ascorbic acid to iron = 1:4) all the iron remained in solution (Table 26). When 0.10 mM ascorbic acid was used only 1% of the iron remained in solution. This result concurs with the results of an experiment described in Chapter 2 (Figure 2); in this experiment, which differed from the present procedure only in that a different concentration of iron (0.18 mM) was used, iron was also precipitated when the molar ratio of ascorbic acid to iron was less than 1:4. Thus one molecule of ascorbic acid keeps 4 iron ions in solution.

When one molecule of ascorbic acid is oxidised to dehydro-

ascorbic acid it loses 2 hydrogen atoms. One molecule of ascorbic acid should therefore theoretically reduce 2 ferric ions to ferrous ions:

ascorbic acid + 2 Fe^{+++} → dehydroascorbic acid + 2 Fe^{++} + 2 H^{+}

That this is so is borne out by an experiment previously described in Chapter 2 (Figure 4). In this experiment varying amounts of ascorbic acid were added to a reagent containing a constant amount of ferric chloride and excess T.P.T.Z. The amount of ferrous ions liberated was measured by the blue colour produced. Colour formation was proportional to the amount of ascorbic acid added when the molar ratio of ascorbic acid to iron was less than 1:2. When higher concentrations of ascorbic acid were used such that the ratio was greater than 1:2 no further colour formation occurred.

Accordingly, in the solubility test, one gram molecule of ascorbic acid should maintain two gram ions of ferric iron in solution by reduction to the more soluble ferrous form. In fact one gram molecule of ascorbic acid maintained four gram ions of ferric iron in solution. This suggests that one molecule of ascorbic acid maintains two gram ions of ferric iron in solution by reduction and two more by some means other than reduction, perhaps by complex formation.

When ascorbic acid is added to ferric chloride such that the

molar ratio of ascorbic acid to iron is 1:4 all the ascorbic acid present must be oxidised to dehydroascorbic acid. Thus if complex formation does occur we would expect two ferric ions to combine with one molecule of dehydroascorbic acid, not ascorbic acid. On this hypothesis the reactions which take place could be represented as follows:

i. ascorbic acid + 2 Fe^{+++} → dehydroascorbic acid + 2 Fe^{++} (soluble) + 2 H^{+}

ii. dehydroascorbic acid + 2 Fe^{+++} → dehydroascorbic-acid-$(Fe^{+++})_2$ (soluble complex)

(b) dehydroascorbic acid. According to the above hypothesis, if dehydroascorbic acid is tested by the solubility test, the iron should remain in solution provided the molar ratio of dehydroascorbic acid to iron is greater than 1:2 and precipitation of iron should occur when the molar ratio is less than this value. This is in fact what happened (Table 26) for 100% solubility was attained only when the ratio of dehydroascorbic acid to iron was greater than 1:2.

These results are consistent with the hypothesis that dehydroascorbic acid forms a complex with ferric ions, but they could also be due to reduction of ferric ions by dehydroascorbic acid. That this does not occur was shown by testing dehydroascorbic acid with

the Fe-T.P.T.Z. reagent. Two ml of 1.25 mg/ml dehydroascorbic acid tested in the Fe-T.P.T.Z. reaction yielded absorbances of 0.050 and 0.065 in two separate tests. Two blank determinations using 2 ml of 1% acetic acid gave 0.055 and 0.065, and two determinations using 2 ml of 1.25 µg/ml ascorbic acid yielded 0.59 and 0.58. Thus dehydroascorbic acid does not reduce ferric ions to the ferrous form.

3. Ion exchange test

(a) At pH 3. In this experiment 1.0 mM ascorbic acid was added to the iron solution (0.18 mM) and the pH adjusted to 3.0 with ammonium hydroxide. The column was eluted with 1% acetic acid which has a pH of 3.

All of the radioactivity remained on the column suggesting either that complex formation had not occurred or that the complex was positively charged.

(b) At pH 8. Since the metal chelate complexes are more stable at higher pH the following experiments were performed. After adding the sample solution to the radioactive iron the pH was reduced to 2 with HCl and then brought to 8 with NH_4OH. The 2.0 ml sample was eluted from the column with 50 ml of 0.1 M tris buffered to pH 8 with HCl.

When either ascorbic acid or dehydroascorbic acid was used a

large amount of radioactivity was found in the eluate (Figure 32). If the sample contained no ascorbic acid or dehydroascorbic acid the amount of radioactivity in the eluate was very small (Figure 32).

In an attempt to confirm on the ion exchange column the ratios found in the solubility test various concentrations of dehydroascorbic acid were tested. The results obtained were not sufficiently reproducible to enable any definite conclusions to be drawn as to the combining ratios of ascorbic acid and iron (Figure 33).

IV. COMMENT

In both the solubility tests and the ion exchange tests good evidence has been obtained that when ascorbic acid is added to solutions of ferric ions and the pH adjusted to 8 a soluble complex with iron is formed. This complex is unlikely to be with ascorbic acid for in these experiments ferric chloride was in molar excess with respect to ascorbic acid and the ascorbic acid should therefore have all been oxidised to dehydroascorbic acid. Evidence from the solubility test suggests that one molecule of dehydroascorbic acid may bind two ferric ions. In none of the experiments described in this chapter is it possible to completely exclude the possibility that iron is binding to some breakdown

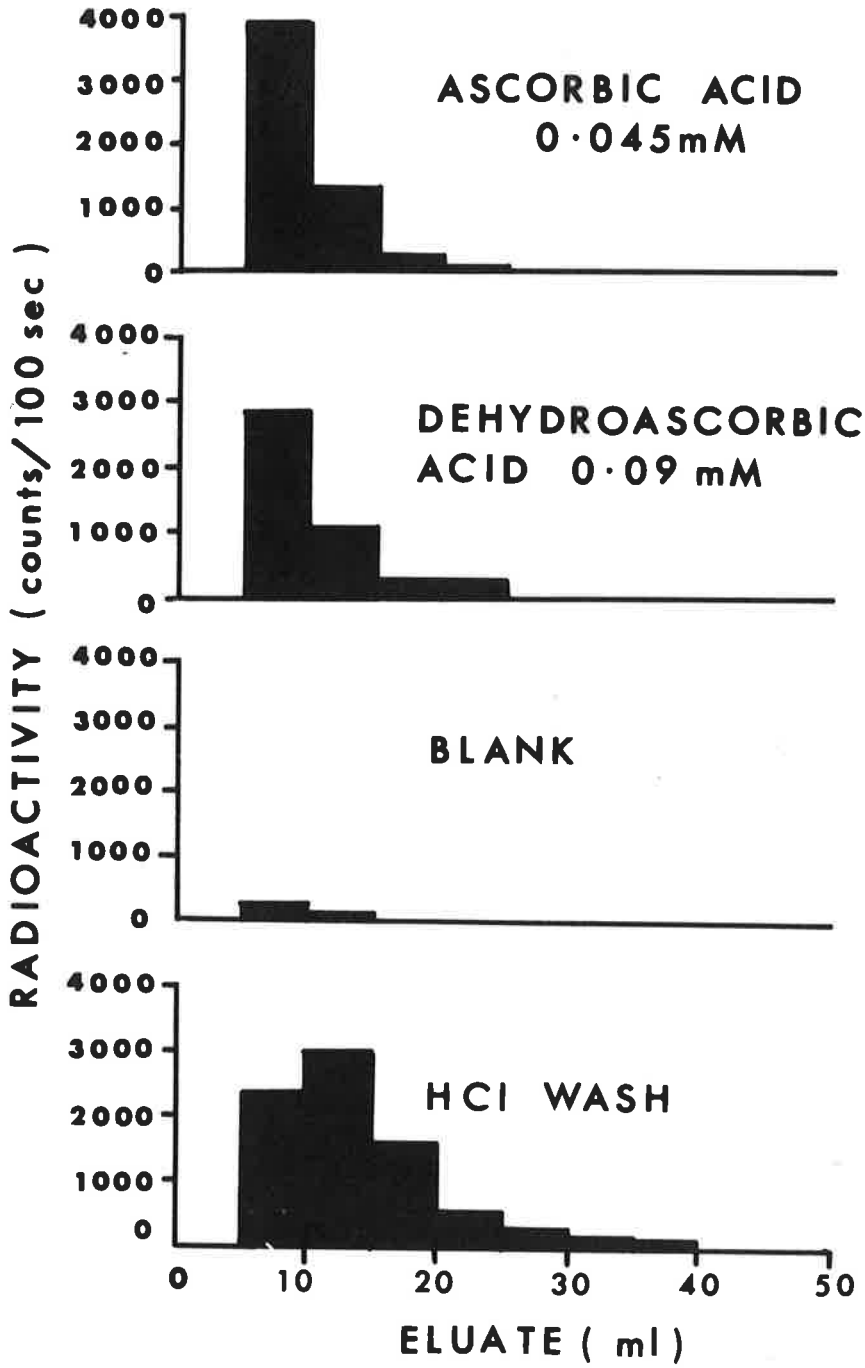

FIGURE 32: Radioactivity of the eluate in the ion exchange test. The test mixture was eluted from the column with buffer of pH 8 and collected in 5 ml aliquots.

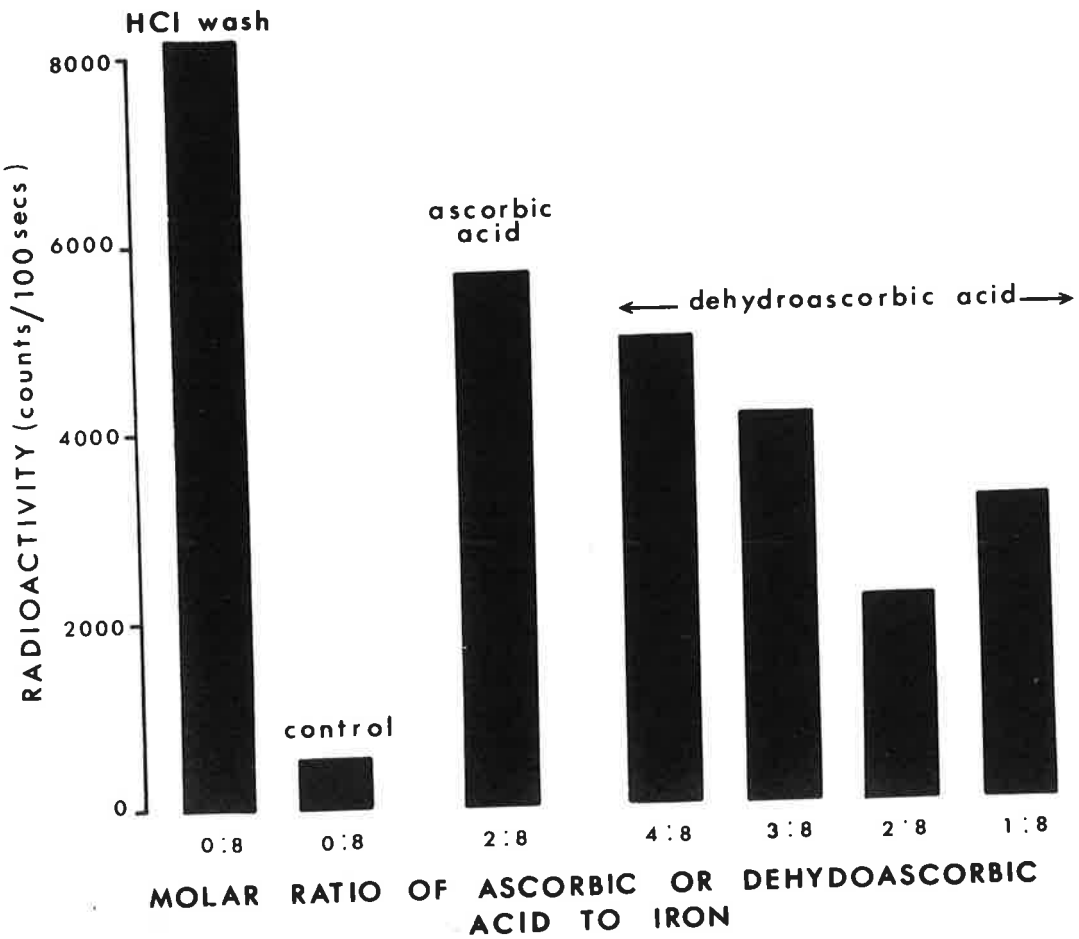

FIGURE 33: Radioactivity of the eluate in the ion exchange test for varying concentrations of dehydroascorbic acid. Each histogram represents the sum of the radioactivity of ten 50 ml aliquots.

product of dehydroascorbic acid such as oxalic acid which also chelates iron. To help exclude this possibility all dehydroascorbic acid solutions used in these experiments were freshly prepared a few minutes prior to adding them to the iron solutions. The final proof that dehydroascorbic acid does chelate iron rests with the isolation and determination of structure of the soluble complex which forms when iron is added to solutions of dehydroascorbic acid.

CHAPTER 9

DISCUSSION

The first requirement in this project was for a method for the estimation of ascorbic acid in biological samples. A review of the literature revealed that although "it is usually supposed that any technician can measure it simply and accurately" (Meiklejohn, 1953) none of the methods commonly in use is specific for ascorbic acid. Though such non-specific methods are adequate for the clinical investigation of a case of scurvy, the progress of research into the role of ascorbic acid in metabolic processes requires higher standards of accuracy.

Accordingly the experiments described in Chapter 2 were designed to develop a new method for ascorbic acid estimation in biological samples. Specificity was obtained by resolution of the sample by thin layer chromatography on cellulose. This procedure was used to separate ascorbic acid from other reducing substances. Sensitivity was obtained by a new reaction which was based on the ability of ascorbic acid to reduce ferric ions to ferrous ions. The ferrous ions were then estimated colorimetrically using the intense blue derivative which they form with the iron chromogen, T.P.T.Z. This reaction is the most sensitive yet described for the estimation of ascorbic acid.

The specificity of the method was demonstrated by dhowing that the chosen solvent was effective in separating ascorbic acid from other known reducing substances likely to be present in biological samples such as platelets. Though the possibility that some

other unknown reducing substance migrates to the same place as ascorbic acid during chromatography cannot be completely excluded by these experiments, this is unlikely. In any case the much lower values obtained for the ascorbic acid concentration in platelets by the T.L.C. method compared with those obtained on duplicate samples by the D.N.P.H. technique demonstrated the increased specificity obtained by the new method.

Analysis of the results obtained by the D.N.P.H. technique revealed that in addition to the lack of specificity there were other grounds on which the platelet ascorbic acid determinations by other authors (Barkhan and Howard, 1958; Wilson et alii, 1967) could be criticised. These authors both used the D.N.P.H. technique; in this method external standards are normally used and it is assumed that recovery of ascorbic acid is complete (Roe, 1954). In fact, as revealed by the present study, though external standards are adequate when the method is applied to plasma, this is not so when it is applied to platelets. The values for platelet ascorbic acid content when determined by the D.N.P.H. method were much higher when internal standards were used than when external standards were used. This indicates that when this method is applied to platelets there is a loss of ascorbic acid, perhaps by adsorption on to the precipitate as may also occur in the T.L.C. method.

When the T.L.C. method was applied to the platelets from 26 normal subjects previous reports of the high concentration of ascorbic acid found in platelets were confirmed. The values obtained were in much closer agreement with the results of Barkhan and Howard (1958) than with the extremely high values obtained by Wilson et alii (1967). Since a more specific method and internal standards were used, and because larger numbers of subjects were studied, the results obtained probably represent a better estimate of the ascorbic acid content of platelets than the two previous reports.

Experiments were carried out to investigate the relationship between the platelet ascorbic acid content, the plasma ascorbic acid concentration, and the dietary intake of this substance. By estimating the plasma ascorbic acid concentration in 7 of the normal subjects it was shown that there was a positive relationship between the platelet ascorbic acid content and the plasma ascorbic acid concentration. The tissues of 4 young subjects and 4 elderly subjects were saturated with ascorbic acid by giving high doses of ascorbic acid (1 G per day for 4 days). By saturation is meant that any further ascorbic acid administered will not cause any increase in the tissue levels of ascorbic acid and will be rapidly excreted in the urine. Once attained, saturation can be maintained by giving about 100 mg of ascorbic acid a day (Goldsmith, 1961). Whereas in the 4 young subjects the platelet content of ascorbic

acid was at or near saturation level prior to the experiment, in the 4 elderly subjects both platelet and plasma concentrations of ascorbic acid were low and were significantly increased by saturation. The differences between young and elderly subjects can be related to the dietary intake of ascorbic acid prior to the experiment. The normal subjects consumed 1-2 pieces of fruit per day whereas the elderly subjects were given only 1 piece of fruit per week.

One normal subject was fed a low ascorbic acid content diet for a period of 6 weeks. The concentration of ascorbic acid in both platelets and plasma fell concomitantly to reach minimun values after 3 weeks. In previously reported depletion experiments the ascorbic acid content of the white-cell-platelet layer of the blood has fallen more slowly than the concentration in the plasma: whereas the buffy layer concentration reached low values only after 4 or 5 months just prior to the onset of scurvy, the plasma concentration reached minimal levels after only about 4 weeks (Crandon et alii, 1940; Vitamin C Subcommittee, 1953). The present study was carried out in only 1 subject and studies are required in a larger number of subjects for a longer period of time before it can be decided whether the pattern of depletion is different in platelets from that in white cells.

From this series of experiments in normal subjects it can be

concluded that the platelet ascorbic acid content depends to some extent on the plasma ascorbic acid concentration which, in turn, depends on the dietary intake of this vitamin. These relationships are important when comparing the platelet ascorbic acid content in diseased subjects with the normal range.

Apart from the 5 patients with leukemia studied by Barkhan and Howard (1958) there are no reports of the ascorbic acid content of platelets from abnormal subjects. Ascorbic acid estimations were therefore carried out on platelets from patients with a variety of disorders in which platelets are affected or thought to be affected.

Changes in the platelet ascorbic acid concentration in disease could be due either to an abnormality in the platelets themselves or to a change in concentration of ascorbic acid in the plasma in which they are suspended. To help differentiate between these two possibilities the plasma concentration of ascorbic acid was estimated in a proportion of the subjects. If, for instance, the platelet concentration of ascorbic acid was low in the presence of a normal plasma concentration, this would point to a defect in the platelets themselves. If, however, the plasma concentration was also low this would indicate that the platelets were not necessarily abnormal in their ability to retain ascorbic acid. In normal subjects a low plasma ascorbic acid concentration is said to point to a low dietary intake of ascorbic acid; it is conceivable,

however, that other mechanisms could operate, particularly in abnormal subjects. Possible such mechanisms are an increased rate of breakdown of ascorbic acid, an increase in the rate of renal excretion of ascorbic acid, or a decreased abosprtion from the intestinal tract.

Ideally it would have been preferable to estimate the plasma ascorbic acid concentration by the T.L.C. method, but this proved impractical. The concentration of ascorbic acid in plasma is so low that the T.L.C. method is not sufficiently sensitive unless prior concentration of the extract by freeze-drying is carried out. Since a freeze-drying apparatus was not readily available it was necessary to use the D.N.P.H. method.

In uraemia the mean platelet ascorbic acid content was significantly lower than normal despite a plasma concentration of ascorbic acid which was well within normal limits. A search of the literature has revealed no previous reports on the ascorbic acid content of platelets or white cells in uraemia. The results obtained here suggest that there is a defect in the ability of platelets to maintain a high concentration of ascorbic acid in this condition, which is consistent with the fact that other defects in platelets have been observed in uraemia; the bleeding tendency which develops in many patients with uraemia is now thought to be largely due to a defect in platelet function as measured by a

decrease in platelet thromboplastic function (Cahalane et alii, 1958; Cheney and Bonnin, 1962) and a decreased tendency to aggregate in response to A.D.P. (Castaldi et alii, 1966) which is improved by dialysis (Stewart and Castaldi, 1967). Thus the change in platelet ascorbic acid concentration is yet another abnormality to be found in platelets in uraemia. It would be interesting to determine whether the abnormality in platelet ascorbic acid content was improved after haemodialysis.

When large doses of ascorbic acid were administered to patients suffering from uraemia the plasma ascorbic acid concentration rose to very high levels. This was probably due to the decreased rate of renal excretion of ascorbic acid previously described in uraemia by Sendroy and Miller (1939). In the presence of this very high concentration of ascorbic acid in the plasma the platelets were able to retain sufficient ascorbic acid to bring their ascorbic acid content within the normal range.

In acute leukemia the platelet content of ascorbic acid was also decreased but the decrease was associated with a low plasma ascorbic acid concentration. Similar results were obtained in chronic myeloid leukemia though the numbers were small and one patient who had a high ascorbic acid intake had a high plasma ascorbic acid concentration. These results suggest that the low ascorbic acid content of the platelets in leukemia was secondary

to a low plasma concentration rather than to a defect in the platelets themselves. In a small number of cases of chronic lymphatic leukemia the concentration of ascorbic acid in both platelets and plasma was normal. These differences between acute leukemia and chronic myeloid leukemia on the one hand and chronic lymphatic leukemia on the other did not appear to be due to differences in the severity of the disease at the time of study.

It is interesting to compare the results of this study with the concentrations of ascorbic acid found in the buffy layer of centrifuged blood by Waldo and Zipf (1955); they have measured the ascorbic acid content of the buffy layer in a group of 42 cases of leukemia including acute and chronic lymphatic leukemia, acute monocytic leukemia, and chronic myeloid leukemia. The mean buffy layer and plasma concentrations of ascorbic acid were less than a quarter of the normal value in each type of leukemia, and the levels were lowered to a similar extent in other diseases of the blood forming organs such as multiple myeloma and malignant lymphoma. Barkhan and Howard (1958) studied the ascorbic acid content of platelets which had been separated from white cells in 3 cases of chronic myeloid leukemia and 2 cases of chronic lymphatic leukemia and found no marked differences from normal, though the plasma concentration was reduced in all 5 patients. Stephens and Hawley (1936) found no marked changes in either the buffy layer or the plasma in a small number of cases of leukemia.

When large doses of ascorbic acid were administered to 1 patient suffering from chronic myeloid leukemia the platelet ascorbic acid content rose to well within the normal range and the plasma concentration of ascorbic acid rose to the high levels that are encountered in saturated normal subjects. Thus when sufficient plasma ascorbic acid was available the platelets were able to concentrate this substance to a normal extent. In leukemia we have not therefore been able to demonstrate any defect in the ability of the platelets to maintain a normal concentration of ascorbic acid. The reason for the low blood ascorbic acid levels and the increased amount of ascorbic acid required for tissue saturation in some patients suffering from leukemia remains unknown.

Low concentrations of ascorbic acid were also found in the platelets of patients with clinical evidence of atherosclerosis. Patients with myocardial infarction or intermittent claudication of the lower limbs were chosen assuming that they would constitute a group with significantly more atherosclerotic disease than the general population. This concept is supported by the findings of Mitchell and Schwartz (1965) that there is "an association between the degree and number of stenosing lesions in the coronary arteries...and the degree and number of stenosing lesions in the carotid and iliac arteries" and "patients with cardiac infarction have more aortic disease than patients from an unselected necropsy group."

The low platelet ascorbic acid content found in atheroma is interesting in view of 2 hypotheses which have been proposed by other workers. One of these, the hypothesis that atheromatous plaques are formed by the gradual deposition of platelets on the walls of blood vessels (see Chapter 1) has still to be proved or disproved. The other, that a low dietary intake of ascorbic acid is an important factor in atherogenesis, is widely held in Russia (see Chapter 1) but has not gained acceptance in the western hemisphere.

The results obtained in the present study did not show whether the low platelet ascorbic acid content in atheroma was due to a defect in the platelets or to a low plasma ascorbic acid concentration. Also, it is possible that the results were affected by the higher proportion of smokers in the atheroma group compared with the control group. The subjects in the atheroma and control group were not matched for age. The ages of the patients in the atheroma group spanned a wide range (41-82 years) and there was no relationship demonstrable between the platelet ascorbic concentration and age. This does not, however, exclude a slight effect due to aging.

In a group of patients receiving steroid therapy neither the platelet content of ascorbic acid nor the plasma concentration was significantly lower than normal. Other authors have described a

haemorrhagic diathesis, which responded to ascorbic acid administration, in patients receiving adrenocorticotrophic hormone (A.C.T.H.) (Stefanini and Rosenthal, 1950; Holly and McLester, 1951). In the present study the ascorbic acid concentrations were much too high to be consistent with scurvy, and the administration of ascorbic acid to one patient did not improve the bleeding tendency. As the patients in this study were receiving prednisolone or betamethazone, the results do not necessarily conflict with the two previously reported studies in which A.C.T.H. was used.

The mean platelet ascorbic acid content in patients with megaloblastic anaemia was significantly depressed to less than a third of the normal value, whereas the mean plasma concentration, though slightly diminished, was well within normal limits. This suggests that in this condition as in uraemia, the platelets are unable to maintain a high gradient of concentration of ascorbic acid between their interior and the surrounding plasma. In one patient (A.W.) the abnormality in platelet ascorbic acid returned to normal during treatment for 2 weeks with vitamin B_{12}.

Cox et alii (1958) have studied the plasma ascorbic acid concentration but not the buffy layer ascorbic acid content. They found, in a larger number of patients, that the slight fall in plasma ascorbic acid concentration in vitamin B_{12} deficiency is

statistically significant. They also found that the rate of disappearance from the plasma of ascorbic acid injected intravenously was increased in vitamin B_{12} deficiency.

No report has yet appeared comparing the chemical composition of young with that of old platelets. McDonald et alii (1964) have shown in rats that platelets become smaller during the aging process, and it seems reasonable to attempt to determine whether changes in chemical composition also occur. In the small series of patients studied in this project no abnormality was demonstrated though an unusually wide scatter of results was obtained. A much larger number of patients must be studied before any conclusion can be drawn.

In all those disease processes in which the platelet ascorbic acid content is low it must be considered whether this abnormality is just a relatively unimportant manifestation of the disease process of whether the low level is in fact of significance in the pathogenesis of the disorder. In uraemia and leukemia, for instance, could the impairment in platelet function be due to the low ascorbic acid content, and is platelet function improved by large doses of ascorbic acid. This seems unlikely because the low ascorbic acid levels found are not as low as the zero levels found in scurvy (Vitamin C Subcommittee, 1953). To help exclude this possibility it would be necessary to study platelet function in

patients with these disorders in whom the bleeding tendency was marked. Tests of platelet function used should include those based on platelet aggregation with A.D.P. and platelet stickiness to glass.

A series of in vitro experiments was carried out to investigate the mechanism by which platelets maintain a high concentration of ascorbic acid in their interior compared to the plasma. C^{14}-ascorbic acid proved a useful tool in this study. When C^{14}-ascorbic acid was added to platelet rich plasma an uptake of ascorbic acid by the platelets occurred over a period of about 1 hour. There was a marked discrepancy between the relatively small difference in the total platelet ascorbic acid content before and after incubation (estimated by the T.L.C. method), and the larger amount of ascorbic acid which had entered the platelets (estimated from the increase in radioactivity). This could have been because the increase in radioactivity was due to a breakdown product of ascorbic acid and not ascorbic acid itself. That this was unlikely was shown by liberating the radioactivity from the platelets by tissue grinding and subjecting it to thin layer chromatography; when the chromatogram was scanned for beta activity a single peak was obtained which was identical to the peak obtained for pure ascorbic acid, and which coincided with the ascorbic acid spot rendered visible with phosphomolybdic acid spray. The results can

best be explained by assuming that there is a continual exchange between ascorbic acid in the platelets and ascorbic acid in the plasma. The uptake of radioactivity then represented the attainment of equilibrium between the specific activity of ascorbic acid in the platelets and that of ascorbic acid in the plasma, without a marked increase in the total amount of ascorbic acid within the platelets.

When platelet rich plasma is incubated in a plastic test tube the supply of nutrients such as oxygen and glucose must eventually become too small for the processes of respiration within the platelet to continue. If the high concentration of ascorbic acid within the platelets is maintained by an active transport mechanism it would be expected that this would fail on prolonged incubation when energy producing processes could no longer be maintained. Incubation of platelet rich plasma for half an hour prior to adding radioactive ascorbic acid did not impair the ability of the platelets to take up radioactivity. On incubation of platelets which had already taken up C^{14}-ascorbic acid for over 4 hours the platelet radioactivity gradually decreased. This suggested some impairment of the ability of the platelets to maintain a high concentration of ascorbic acid within the cell. Alternatively the effect may have been due to gradual breakdown of ascorbic acid, the breakdown products not being retained by the

platelets. If there is a continuous exchange between ascorbic acid in the platelets and ascorbic acid in the plasma there must be a mechanism, perhaps enzymatic, for the maintenance of a higher concentration of ascorbic acid inside the platelet. Theoretically this may act at the level of the cell membrane as does the active transport mechanism proposed for sodium and potassium (Tostesan, 1963), or it may act by causing ascorbic acid to bind to some non-diffusible substance. A possible clue to the nature of this mechanism has been provided by the experiments described by Martin (1961). He has shown that ascorbic acid does not cross cell barriers as rapidly as the non-ionic and more lipid soluble dehydroascorbic acid. Perhaps ascorbic acid crosses cell membranes in the dehydro form and is then actively reduced to ascorbic acid which is then less able to leave the cell.

The reports that the ability of platelets to adhere to glass is impaired in scurvy (Born and Wright, 1967; Wilson et alii, 1967) suggested that the role of ascorbic acid in the adherence of platelet to platelet after the addition of A.D.P. should also be studied. The experiments on A.D.P.-induced platelet aggregation described in this thesis did not demonstrate any effect of ascorbic acid on this mechanism even when the platelets from a subject on an ascorbic acid deficient diet were used. It would be necessary, however, to use the platelets from a subject with untreated

clinical scurvy to completely exclude this. Unfortunately such patients are rare and facilities for rendering a colony of guinea pigs scorbutic were not readily available.

It was also of interest to determine whether ascorbic acid was released from platelets during A.D.P. induced aggregation. Teleologically speaking, this might occur if, for instance, ascorbic acid was required to ensure an optimum redox potential at the outer surface of the external platelet membrane, or if it was required for some other part of the mechanism of haemostasis such as the formation of fibrin. In fact, no release of ascorbic acid from the platelets occurred during A.D.P.-induced aggregation. It would be interesting to determine whether other agents, such as thrombin, which induces a more advanced degree of platelet change (Hovig, 1962; Rodman et alii, 1963) will cause a release of ascorbic acid from the platelets.

The final series of experiments reported in this thesis was designed to determine whether an iron-chelate complex forms when ascorbic acid is added to solutions containing iron. These experiments were prompted by the well known fact that when ascorbic acid is added to a preparation of iron in the ferric form prior to ingestion, the absorption of this iron from the intestine is enhanced (Moore et alii, 1939; Moore and Dubach, 1955). It has been thought that this enhancement is due to the formation of

ferrous ions which are better absorbed than ferric ions (Moore et alii, 1939; Hahn et alii, 1945) but this seems unlikely for it has been shown that ascorbic acid also enhances the absorption of ferrous ions (Greenberg et alii, 1957; Brise and Hallberg, 1962). It has been argued that this may be because, in the absence of a reducing agent such as ascorbic acid, ferrous ions are oxidized to the less soluble ferric ions (Brise and Hallberg, 1962).

Iron absorption occurs predominantly from the duodenum (Wheby and Crosby, 1963). It is well known that though iron in the ferric form is soluble in the acid conditions of the stomach it is virtually insoluble at the alkaline pH which occurs in the duodenum; iron in the ferrous form, however, is relatively soluble at this alkaline pH. It is this difference in solubilities which has been thought to explain both the greater absorption of ferrous ions compared to ferric ions and the effect of ascorbic acid on the absorption of iron.

Another possible explanation for the effect of ascorbic acid is that a chelate complex with iron is formed. It has been shown that chelates of iron are formed with fructose and with other reducing sugars, and that these chelates are soluble in the alkaline conditions of the duodenum (Charley et alii, 1963). The ferric-fructose chelate is more rapidly absorbed than ferrous sulphate in rats (Stitt et alii, 1962) and it has been recently

shown that fructose also enhances iron absorption in humans (Davis and Deller, 1967). Since ascorbic acid is a six carbon atom compound with a structure somewhat similar to hexose sugars, the possibility that this substance also forms a chelate with iron must be considered.

In the series of experiments described in Chapter 8 evidence was obtained that dehydroascorbic acid (or perhaps one of its breakdown products) forms a negatively charged or neutral complex with ferric ions and that this complex confers solubility on ferric ions at an alkaline pH. The pH adjustments used in these experiments mimic the changes which occur when a solution is ingested and mixes firstly with the acid juices of the stomach and then the alkaline juices of the duodenum.

Further work is suggested to determine whether dehydroascorbic acid is as effective as ascorbic acid in enhancing iron absorption. If this proves to be so, strong evidence will have been obtained that it is the formation of a soluble complex which is important, and not the reducing properties of ascorbic acid.

Besides the intestinal lumen the formation of a soluble complex between iron and dehydroascorbic acid has possible implications in iron metabolism elsewhere in the body. It has been shown in experiments with liver slices that ascorbic acid is

necessary for the transfer of iron from plasma transferrin to liver ferritin (Mazur et alii, 1960); ascorbic acid could not be replaced by the usual reducing agents. It seems possible that ascorbic acid may be required, not for its reducing action, but for its ability to form an iron-dehydroascorbic acid complex which can more easily cross the cell membrane into the liver cell. Similarly Lockhead and Goldberg (1959) have shown that ascorbic acid enhances the transfer of iron from plasma transferrin to the site of haem synthesis in liver mitochondria. It would be interesting to determine the effect of other chelating agents which are not reducing agents in the experimental models described by these two groups.

The contributions to knowledge made by the work described in this thesis are the development of a new method for the estimation of ascorbic acid which has the advantage over previous methods of being more specific; the development of a spectrophotometric method for the estimation of ascorbic acid which is more sensitive than previously described methods; the demonstration that when the D.N.P.H. method is applied to ascorbic acid estimations in platelets internal standards are required, not external standards as are usually used in this method; the determination of the normal range for the platelet ascorbic acid content as estimated by the T.L.C. method; the demonstration of the way in which the platelet ascorbic acid content varies with changes in the dietary intake of

this vitamin; the determination of the variation from normal of the platelet ascorbic acid content in a variety of diseases with the finding of abnormally low levels in uraemia, acute leukemia, chronic myeloid leukemia, megaloblastic anaemia, and atherosclerosis; the demonstration that the low platelet ascorbic acid content in uraemia and in megaloblastic anaemia was probably due to a defect intrinsic in the platelets, whereas in leukemia it was probably due to a low plasma ascorbic acid concentration; the finding that the administration of large doses of ascorbic acid to patients suffering from leukemia and uraemia allowed the platelet ascorbic acid content to return to normal values, and that in uraemia the plasma concentration rose to very high levels; the discovery that C^{14}-ascorbic acid added to platelet rich plasma was taken up by the platelets and that this effect was blocked by cyanide ions; the finding that ascorbic acid added to platelet rich plasma did not affect the pattern of A.D.P.-induced platelet aggregation; the finding that ascorbic acid was not released from platelets during A.D.P.-induced platelet aggregation; the demonstration that when ascorbic acid or dehydroascorbic acid was added to iron solutions and the pH raised to 8 a soluble complex was formed, probably between iron and dehydroascorbic acid.

Further work is suggested along the following lines:

(1) When ascorbic acid oxidase becomes available commercially it will be possible to develop a rapid, very sensitive, and highly specific method for the estimation of ascorbic acid in biological samples. Such a method would not suffer from the loss of sensitivity involved in chromatographic procedures.

(2) It would be of interest to know whether the low platelet ascorbic acid content in uraemia and leukemia is correlated with other aspects of platelet function such as platelet stickiness and platelet aggregation. In addition it would be of interest to measure the platelet ascorbic acid content in patients suffering from uraemia before and again immediately after haemodialysis.

(3) A larger series of patients suffering from atherosclerosis should be studied by the estimation of the ascorbic acid concentrations in platelets and in plasma. The values obtained should be compared with a control series matched for age, sex, and smoking habits.

(4) It would be interesting to determine whether agents other than A.D.P., such as thrombin, will cause a release of ascorbic acid from platelets.

(5) Experiments to determine the location of ascorbic acid within the platelet could be performed. This could be done by analysis of platelet fractions separated on a sucrose density gradient. Alternatively, electron microscope autoradiography using tritiated ascorbic acid could be used, but this requires highly specialised techniques to ensure that ascorbic acid does not migrate by diffusion during fixation.

(6) In vivo experiments are suggested to further elucidate the role of ascorbic acid in iron absorption.

(7) It should be possible to isolate and determine the chemical structure of the complex which forms when iron is added to ascorbic acid or dehydroascorbic acid.

(8) The affinity of ascorbic acid for other metals such as copper, zinc and tin should be determined. It is possible that ascorbic acid may have a general role in metal transport.

Ascorbic acid is present in high concentrations in other tissues besides platelets. A question which has not been answered is whether its function in platelets is the same as or different from its function in other tissues. Is it important in platelet stickiness just because it is required to maintain the general metabolism of the platelet, or has it some more specific

function in the mechanism of platelet stickiness? Thus platelet suspensions may constitute an experimental model suitable for the further elucidation of the function of ascorbic acid not only in platelets but in other body tissues. Platelets are easily isolated from blood which is more readily sampled than other tissues. It is possible that further studies of ascorbic acid in platelets could lead to a greater understanding not only of the biochemistry of the blood platelets but also of the role of ascorbic acid in metabolic processes.

SUMMARY

SUMMARY

The complete understanding of the role of platelets in the body must await the elucidation of the biochemical changes which occur in platelets during haemostasis and thrombosis. A first step in the understanding of platelet function at the molecular level is the study of the chemical composition of the platelet. In the work presented in this thesis the substance chosen for study was ascorbic acid. This substance is in high concentration in platelets compared to plasma and the severe purpura which occurs in scurvy suggests that it may play an important role in platelet metabolism.

The first part of the project was devoted to the development of a new and more specific method for the estimation of ascorbic acid. Specificity was obtained by the use of thin layer chromatography to separate ascorbic acid from other reducing substances. Sensitivity was obtained by the use of a new method for the spectrophotometric estimation of ascorbic acid. The ascorbic acid was eluted from the chromatogram and allowed to react with excess ferric ions. The ferrous ions so formed combined with tripyridyl-S-triazine to form an intensely coloured derivative.

To test the specificity of the new method (T.L.C. method)

estimations of platelet ascorbic acid content were carried out on 6 normal subjects by both the T.L.C. method and the 2,4-dinitrophenylhydrazine method (D.N.P.H. method). Significantly lower results were obtained by the T.L.C. method than by the D.N.P.H. method. This investigation also revealed that when the D.N.P.H. method is applied to platelets the recovery of ascorbic acid is low; external standards which are usually used in this method are therefore inadequate and should be replaced by internal standards.

To determine the normal range for the platelet ascorbic acid content the T.L.C. method was applied to the platelets from 26 normal subjects. A normal range of 3.5 ± 1.4 $\mu g/10^9$ platelets was obtained. No relationship could be shown between platelet ascorbic acid content and sex, smoking habits, or platelet count, but there was a significant positive relationship between the platelet ascorbic acid content and the plasma ascorbic acid concentration.

The relationship of the platelet ascorbic acid content to the dietary intake of vitamin C was studied by administering to 4 young subjects and 4 elderly subjects doses of ascorbic acid large enough to saturate their tissues with ascorbic acid; in addition one normal subject was given a diet deficient in ascorbic acid. In the 4 young subjects saturation produced no demonstrable rise in the platelet ascorbic acid content indicating

that the platelets were at or near saturation levels prior to the experiment. In the elderly subjects, however, a significant rise in both the platelet ascorbic acid content and the plasma ascorbic acid concentration occurred. The differences between these 2 groups of subjects can be related to differences in their intake of ascorbic acid prior to the experiment. In the subject on an ascorbic acid deficient diet the ascorbic acid concentrations in both the platelets and the plasma fell to low levels over a period of about 3 weeks and rose again on the introduction of ascorbic acid supplements.

The platelets from 85 subjects with a variety of diseases affecting platelets were subjected to ascorbic acid estimation. The platelet ascorbic acid content was low in uraemia (13 patients), acute leukemia (8 patients), chronic myeloid leukemia (4 patients), atherosclerosis (18 patients), and megaloblastic anaemia (5 patients). In uraemia and megaloblastic anaemia the plasma concentration of ascorbic acid was normal indicating that a platelet defect was probably responsible for the low values. In leukemia, however, the plasma concentration of ascorbic acid was significantly lower than normal suggesting that the low platelet ascorbic acid content was secondary to the low plasma level. No significant difference from the normal range was found in chronic lymphatic leukemia (4 patients), polycythaemia vera (4 patients), thrombocythaemia (5 patients), patients receiving steroid therapy

(6 cases), and patients in whom it was thought the platelet population would be younger than normal (6 cases).

Large doses of ascorbic acid were administered to 5 patients suffering from uraemia and 6 patients suffering from leukemia. In uraemia the platelet concentration of ascorbic acid rose to normal values in the face of a plasma concentration which rose to very high levels. In 2 cases of chronic myeloid leukemia and 1 case of acute leukemia the platelet ascorbic acid concentrations rose from low levels to well within the normal range, and the plasma concentration rose to values similar to those obtained on saturation of normal subjects. In 3 patients with chronic lymphatic leukemia no significant change occurred in the platelet ascorbic acid concentration though the plasma levels rose to values consistent with saturation.

The uptake of ascorbic acid by platelets was studied using C^{14}-labelled ascorbic acid. When this substance was added to platelet rich plasma it was taken up by the platelets over a period of about 1 hour. This uptake was blocked by cyanide ions, temporarily blocked by parachloromercuribenzoate, and not blocked by 2,4-dinitrophenol.

The relationship of ascorbic acid to A.D.P.-induced platelet aggregation was studied. Ascorbic acid added to platelet rich

plasma had no effect on the aggregation which occurred on the subsequent addition of A.D.P. Neither did A.D.P. cause any liberation of ascorbic acid from platelets.

A possible clue to the function of ascorbic acid was provided, not by experiments on platelets, but by in vitro experiments prompted by the knowledge that ascorbic acid enhances the absorption of iron from the intestine. When ascorbic acid or dehydroascorbic acid was added to solutions containing ferric ions a soluble complex was formed which did not precipitate on raising the pH of the mixture to 8. This complex was negatively charged or neutral for it was not bound by a cation exchange resin. The results suggested that the complex consisted of 2 iron atoms bound to 1 molecule of dehydroascorbic acid. The formation of this complex may explain the effect of ascorbic acid on the absorption of iron from the intestine and on the transfer of iron across cell membranes elsewhere in the body.

APPENDIX A

STATISTICAL METHODS USED IN THIS THESIS

Results were analysed according to statistical methods outlined by Moroney (1956) and Bailey (1959).

1. LIST OF ABBREVIATIONS USED

All normal ranges are stated as mean ± one standard deviation ($\bar{x} \pm S$) unless stated otherwise.

- n = Number of observations in sample
- x = observed measurements in a sample, independent variables in a regression
- $\bar{x}$ = Mean of sample of measurements x
- y = Observed measurements in a sample, dependent variables in a regression
- $\bar{y}$ = Mean of sample of measurements y
- Σ = Summation symbol
- S = Estimated standard deviation
- S.E. = Standard error
- N = Number of degrees of freedom
- P = Probability that observed deviation is due to chance alone
- t = "Student's" t
- r = Correlation coefficient
- b = Regression coefficient

II. STATISTICAL FORMULAE

1. Calculation of mean and standard deviation

mean $(\bar{x}) = {}^{1}/_{n} \Sigma x$

estimated standard deviation $(S) = \sqrt{\frac{\Sigma (x - \bar{x})^2}{n - 1}}$

2. *Comparison of mean of two samples*

(a) If normally distributed:

(i) For difference ($\bar{x}$) between means of 2 paired samples.

$$t = \frac{\bar{x}\ \sqrt{n-1}}{s}$$

(ii) For comparing means ($\bar{x}$ and $\bar{y}$) of 2 samples.

$$t = \frac{\bar{x} - \bar{y}}{s_{xy}\sqrt{\frac{1}{n_x} + \frac{1}{n_y}}}$$

where $s_{xy} = \sqrt{\frac{n_x s_x^2 + n_y s_y^2}{n_1 + n_2 - 2}}$

This test was used only if F was not significant at P = 0.05 level.

$$F = \frac{s_1^2}{s_2^2}$$

where $s_1 = s_x$ or s_y whichever is the larger

$s_2 = s_x$ or s_y whichever is the smaller

(iii) For comparing means ($\bar{x}$ and $\bar{y}$) of 2 samples when F test was significant.

$$t = \frac{\bar{x} - \bar{y}}{\sqrt{\frac{s_x^2 + s_y^2}{n_x + n_y}}}$$

where degree of freedom = N =

$$\frac{1}{\frac{u^2}{n_x - 2} + \frac{(1 - u)^2}{n_y - 2}}$$

where $u = \frac{S_x^2/n_x}{S_x^2/n_x + S_y^2/n_y}$

(b) If not normally distributed.

The Wilcoxon ranking test was used (Documenta Geigy, 1962)

8. *Correlation coefficient*

$$r = \frac{\Sigma (x - \bar{x})(y - \bar{y})}{\sqrt{\Sigma (x - \bar{x})^2 \Sigma (y - \bar{y})^2}}$$

$$t = \sqrt{\frac{n - 2}{(1 - r^2)}}$$

4. *Regression coefficient*

$$b = \frac{\Sigma (x - \bar{x})(y - \bar{y})}{\Sigma (x - \bar{x})^2}$$

$$S^2 = \frac{1}{n - 2} \{\Sigma (y - \bar{y})^2 - [\frac{\Sigma (x - \bar{x})(y - \bar{y})}{\Sigma (x - \bar{x})^2}]^2\}$$

$$t = \frac{b - \beta}{S/\sqrt{\Sigma (x - \bar{x})^2}}$$

where β = expected value for regression coefficient on a null hypothesis.

III. SIGNIFICANCE LEVELS

$P \geq 0.05$ was taken as not significant.

$P < 0.05$ was taken as significant.

APPENDIX B

CLINICAL DETAILS AND RESULTS OF ASCORBIC ACID ESTIMATIONS IN ABNORMAL SUBJECTS

ABBREVIATIONS USED IN APPENDIX B

b.d.	twice daily
Bet.	betamethazone
B.U.N.	blood urea nitrogen
Chlor.	chlorambucil
I.M.	intramuscular
6 M.P.	6-mercaptopurine
Myl.	myleran
n.d.	not determined
Pred.	prednisolone
Rad.	radiotherapy
t.d.s.	three times daily
V.A.M.P.	vincristine, amethopterin, 6-mercaptopurine, prednisolone

NORMAL RANGES

Platelet count: 150,000 - 350,000 per c.mm.

Blood urea nitrogen: less than 20 mg/100 ml.

Red cell mass: 25 - 32 ml/kg.

Mean corpuscular volume = 80 - 96 cμ.

APPENDIX B1 : URAEMIA

PATIENT	AGE (YEARS)	SEX	DIAGNOSIS	B.U.N. (mg/ 100 ml)	PLATELET ASCORBIC ACID ($\mu g/10^9$ CELLS)	PLASMA ASCORBIC ACID (mg/100 ml)	PLATELET COUNT (x 10^3/ c.mm.)	PLATELET THROMBO-PLASTIC FUNCTION	HAEMORRHAGIC MANIFESTATIONS
H.B.	65	F	Chronic pyelo-nephritis. Hypertension	61	0.3	n.d.	353	n.d.	Spontaneous bruising for one week
W.G.	80	M	Chronic renal failure. Multiple tel-angiectasia	42	0.3	n.d.	243	n.d.	Chronic anaemia due to tel-angiectasia
R.R.	39	M	Chronic pyelo-nephritis. Quadriplegia. Prerenal element. B.U.N. = 25 after treat-ment.	110	3.8	1.3	123	n.d.	Nil
B.L.	23	M	Chronic renal failure, on haemodialysis. ?Congenital renal hypo-plasia	88	1.6	2.0*	248	n.d.	Nil

*On 45 mg of ascorbic acid daily.

APPENDIX B1 (CONTINUED) : URAEMIA

M.R.	56	F	Chronic pyelo-nephritis. Prerenal element. B.U.N. = 30 after treat-ment.	90	6.5	n.d.	485	50	Had always bruised easily
C.H.	49	F	Chronic renal failure. Hypertension	67	1.1	1.20	388	60	Nil
C.D.	61	M	Chronic pyelo-nephritis. Renal calculi	54	0.2	0.20	200	100	Nil
S.M.	28	F	Chronic pyelo-nephritis. On haemodialysis	72	0.7	0.95	190	100	Had always bruised easily.
D.W.	41	F	Chronic glomerulo-nephritis. On haemodialysis.	73	1.5	0.70	228	70	Had always bruised easily.
J.T.	65	M	Chronic renal failure. Hypertension, congestive cardiac failure	30	2.5	0.75	158	100	Nil
H.R.	57	F	Chronic glomerulo-nephritis. On haemodialysis.	132	4.9	1.40	433	80	Severe melaena 2 days previously Bleeding time > 18 mins.

APPENDIX B1 (CONTINUED) : URAEMIA

S.P.	43	M	Chronic glomerulo-nephritis.	126	0.5	0.85	313	n.d.	Recent severe nose bleed
L.V.	42	M	Chronic renal failure. Hypertension	135	1.8	n.d.	315	85	Nil

APPENDIX B2 : LEUKEMIA

PATIENT	AGE (YEARS)	SEX	DIAGNOSIS	ASCORBIC ACID CONCENTRATION PLATELETS µg/ 10^9 cells	PLATELETS µg/ mg	PLASMA mg/ ml	PLATELET COUNT (x 10^3/ c.mm.)	WHITE CELL COUNT (c.mm.)	PLATELET THROMBO-PLASTIC FUNCTION (%)	SPECIFIC THERAPY	HAEMORRHAGIC MANIFEST-ATIONS AT TIME OF ESTIMATION
J.B.	47	M	Acute myeloid leukemia in haematological remission	1.7	0.11	n.d.	423	6,500	n.d.	V.A.M.P.	Nil
K.B.	23	M	Acute myeloid leukemia	1.9	0.06	n.d.	58	14,000	n.d.	V.A.M.P.	Bleeding tooth socket
I.L.	19	M	Acute myeloid leukemia	1.3	0.06	0.25	216	22,900	n.d.	6 M.P.	Nil
H.C.	65	M	Acute myeloid leukemia	2.1	0.04	0.35	64	28,500	n.d.	Pred. 6 M.P.	Nil
Bett. T.	37	F	Acute aleukemic myeloid leukemia	0.7	0.02	n.d.	60	4,900	n.d.	V.A.M.P.	Bruised easily
K.O.	14	F	Acute aleukemic myeloid leukemia	1.2	0.10	n.d.	84	7,800	n.d.	6 M.P. Pred.	Nil
A.S.	79	M	Acute aleukemic lymphatic leukemia	0.3	0.02	0.3	83	3,000	33	Chlor.	Bruised easily for several years

APPENDIX B2 (CONTINUED) : LEUKEMIA

M.S.	37	F	Acute aleukemic myeloid leukemia	0.3	0.02	n.d.	110	5,500	n.d.	V.A.M.P.	Nil
Beat. T.	64	F	Chronic lymphatic leukemia	6.8	0.22	0.45	90	63,000	100	Nil	Nil
J.G.	70	F	Chronic lymphatic leukemia	5.5	0.15	0.55	213	83,000	80	Nil	Nil
J.F.	61	M	Chronic lymphatic leukemia	3.2	0.11	0.75	120	19,600	80	Chlor.	Nil
F.D.	63	F	Chronic lymphatic leukemia in haematological remission	2.2	0.06	n.d.	176	5,700	n.d.	Pred.	Nil
J.D.	50	M	Chronic myeloid leukemia	0	0	0.35	65	49,000	100	Myl. Pred.	Nil
A.M.	69	M	Chronic myeloid leukemia	1.6	0.10	0.20	118	13,400	100	Nil	Nil
A.W.	37	F	Chronic myeloid leukemia	0.5	0.09	n.d.	2,710	79,900	n.d.	Rad.	Occasional bruises
J.S.	60	F	Chronic myeloid leukemia	4.8	0.29	1.3	542	256,000	100	Myl.	Nil

APPENDIX B3 : ATHEROMA

PATIENT	AGE (YEARS)	SEX	DIAGNOSIS	ASCORBIC ACID CONCENTRATION: PLATELETS µg/10^9 cells	ASCORBIC ACID CONCENTRATION: PLATELETS µg/mg	ASCORBIC ACID CONCENTRATION: PLASMA mg/100 ml	PLATELET COUNT (x 10^3/ c.mm.)	SMOKER (S) OR NON-SMOKER (N)
E.P.	46	M	Myocardial infarct 5 days previously	1.9	0.08	0.70	343	S
J.J.	53	F	Myocardial infarct 7 days previously	4.7	0.28	0.75	323	N
A.G.	66	M	Myocardial infarct 6 days previously	0.8	0.03	0.65	213	S
A.W.	76	F	Myocardial infarct 7 days previously	2.5	0.12	0.35	180	N
L.K.	82	M	Myocardial infarct 3 days previously Intermittent claudication of lower limbs	2.1	0.10	0.40	183	S
C.P.	56	M	Myocardial infarct 10 days previously	2.4	0.15	0.80	200	N
A.B.	60	M	Myocardial infarct 9 days previously	4.7	0.08	n.d.	188	S
R.E.	42	M	Myocardial infarct 11 days previously. Infarct 3 years before	1.1	0.06	n.d.	513	S
L.G.	56	M	Myocardial infarct 15 days previously	1.5	0.14	n.d.	343	S

APPENDIX B3 (CONTINUED) : ATHEROMA

A.F.	78	M	Myocardial infarct 10 days previously. Past history of cerebrovascular accident.	1.6	0.12	n.d.	302	S
G.L.	50	M	Peripheral vascular insufficiency lower limbs	2.6	0.10	n.d.	125	S
J.K.	54	M	Peripheral vascular insufficiency upper and lower limbs	2.5	0.10	n.d.	198	S
A.B.	71	F	Peripheral vascular insufficiency lower limbs	5.2	0.18	n.d.	288	N
I.N.	72	M	Peripheral vascular insufficiency lower limbs	2.5	0.18	1.05	210	N
I.M.	70	F	Peripheral vascular insufficiency lower limbs	1.9	0.11	0.65	155	N
B.D.	47	M	Peripheral vascular insufficiency lower limbs	0.2	0.02	0.30	288	S
H.A.	55	M	Myocardial insufficiency	0.5	0.05	n.d.	218	S
B.W.	41	M	Myocardial insufficiency. Previous infarcts 4 months and 3 months previously	3.5	0.15	n.d.	125	S

APPENDIX B4 : POLYCYTHAEMIA AND THROMBOCYTHAEMIA

PATIENT	AGE (YEARS)	SEX	DIAGNOSIS	ASCORBIC ACID CONCENTRATION IN PLATELETS $\mu g/10^9$ cells	ASCORBIC ACID CONCENTRATION IN PLATELETS $\mu g/mg$	PLATELET COUNT ($\times 10^3$ / c.mm)	WHITE CELL COUNT (c.mm)	HAEMOGLOBIN (G/100 ml)	SPECIFIC THERAPY
J.F.	50	M	Polycythaemia vera (red cell mass = 39.4 mg/kg)	3.1	0.20	198	9,900	16.2	Venesection
F.V.	51	M	Polycythaemia vera	2.7	0.07	258	5,300	13.4	Venesection P^{32} 18 months ago
W.F.	55	M	Polycythaemia vera	1.2	0.05	220	7,400	17.4	Venesection
R.R.	58	M	Polycythaemia vera with thrombocythaemia (red cell mass = 29.4 mg/kg)	1.1	0.06	586	11,900	20.6	Nil
W.B.	82	M	Post haemorrhagic thrombocytosis, haematemesis (P.T.F.= 100%)	2.4	0.06	440	10,300	17.3	Nil
P.C.	56	M	Thrombocythaemia, lymphadenitis, exfoliative dermatitis, cause uncertain (P.T.F. = 50%)	1.1	0.13	608	15,600	17.8	Prednisolone 10 mg q.i.d.
W.M.	59	M	Thrombocythaemia, anaemia, myeloproliferative disorder (P.T.F.= 70%). Large platelets	5.4	0.30	790	8,400	7.8	Nil

APPENDIX B4 (CONTINUED) : POLYCYTHAEMIA AND THROMBOCYTHAEMIA

E.R.	65	F	Thrombocythaemia, cause uncertain	3.9	0.20	788	8,600	13.2	Nil
F.S.	33	M	Thrombocythaemia, myelofibrosis. Platelets vary in size	3.8	0.37	2,990	26,000	12.1	Nil

APPENDIX B5 : PATIENTS ON STEROIDS

PATIENT	AGE (YEARS)	SEX	DIAGNOSIS	ASCORBIC ACID CONCENTRATION PLATELETS µg/10^9 CELLS	ASCORBIC ACID CONCENTRATION PLATELETS µg/ mg	ASCORBIC ACID CONCENTRATION PLASMA (mg/ 100 ml)	PLATELET COUNT (x 10^3/ c.mm)	STEROID DOSAGE	HAEMORRHAGIC MANIFESTATIONS
M.C.	60	F	Malabsorption syndrome	1.3	0.08	0.35	440	(1) Pred. for 2 years. Present dose 20 mg day. (2) Ascorbic acid 100 mg 3 times a week I.M.	Purpura in response to slight trauma since commencing Pred. Tourniquet test negative.
A.M.	58	F	Rheumatoid arthritis	2.9	0.11	0.50	155	Bet. 0.5 mg b.d. for 5 years	Purpura in response to slight trauma sincecommencing betamethazone. Tourniquet test negative. Bleeding time normal. P.T.F. = 100%.
A.M.	58	F	Repeat estimation after 1G ascorbic acid daily for 4 days	6.0	0.41	1.9	198		Large area of purpura on forearm in response to recent slight trauma.

APPENDIX B5 (CONTINUED) : PATIENTS ON STEROIDS

P.O.	24	M	Acute nephritis	1.0	0.10	0.60	438	Pred. 35-60 mg a day for 114 days, 35 mg a day at present	Haemorrhagic rash developed on day of estimation. Tourniquet test, bleeding time, thromboplastin generation screening test were all normal. P.T.F. = 100%.
P.O.	24	M	13 days later after reduced amounts of steroids	1.9	0.19	0.65	400	Pred. 10 mg a day	Rash fading.
R.N.	64	F	Asthma	3.9	0.26	1.20	343	Pred. 10 mg a day for 10 years; 40 mg a day for the last 11 weeks	Nil except positive tourniquet test.
D.E.	44	F	Disseminated lupus erythematosus	1.7	0.09	0.25	260	Pred. 30 mg b.d. for 70 days	Nil
W.F.	67	M	Chronic bronchitis and emphysema	4.3	0.17	0.90	160	Pred. 15 mg b.d. for 9 days	Nil

APPENDIX B6 : MEGALOBLASTIC ANAEMIA

PATIENT	AGE (YEARS)	SEX	DIAGNOSIS	ASCORBIC ACID CONCENTRATION PLATELETS µg/10^9 cells	ASCORBIC ACID CONCENTRATION PLATELETS µg/mg	ASCORBIC ACID CONCENTRATION PLASMA mg/100 ml	PLATELET COUNT (x 10^3/c.mm.)	HAEMOGLOBIN (G/100 ml)	MEAN CORPUSCULAR VOLUME c.µ	TREATMENT
A.M.	73	M	Scurvy							Since 4th day of admission (1) ascorbic acid 250 mg t.d.s. (2) folic acid 20 mg daily (3) vitamin B_{12} 200 µg I.M. daily
			(1) on admission	n.d.	n.d.	n.d.	64	7.3	112	
			(2) 23 days after admission	0.5	0.01	0.85	68	10.4	103	2 G ascorbic acid daily for 2 days
			(3) 28 days after admission	1.3	0.06	0.70	70	-	-	2 G ascorbic acid daily for 5 days
D.W.	76	F	Pernicious anaemia pretreatment	0.6	0.01	0.55	77	7.4	125	Nil
A.W.	76	M	Pernicious anaemia pretreatment	0.5	0.01	n.d.	53	5.3	128	Nil
			Treated for 7 days	0.5	0.03	n.d.	320	10.6	100	1,000 µg B_{12} daily
			Treated for 14 days	5.2	0.11	n.d.	376	12.3	n.d.	1,000 µg B_{12} daily

APPENDIX B6 (CONTINUED) : MEGALOBLASTIC ANAEMIA

G.H.	56	M	Folic acid deficiency	2.4	0.09	0.30	275	12.6* 14.2†	117* n.d.†	Vitamin B_{12}, folic acid, pyridoxine for 7 days. No ascorbic acid
R.S.	51	M	Postgastrectomy syndrome (also on phenytoin for epilepsy)	1.6	0.10	0.40	590	10.0* 10.8†	118* n.d.†	Vitamin B_{12} 1,000 µg I.M. twice weekly for 1 week. Folic acid 10 mg daily for 1 day

*Prior to treatment.

†On day of estimation.

APPENDIX B7 : YOUNG PLATELETS

PATIENT	AGE	SEX	DIAGNOSIS	ASCORBIC ACID CONCENTRATION: PLATELETS µg/10^9 cells	ASCORBIC ACID CONCENTRATION: PLATELETS µg/mg	ASCORBIC ACID CONCENTRATION: PLASMA (mg/100 ml)	PLATELET COUNT (x 10^3/c.mm.)
L.W.	70	M	Haematemesis, carcinoma of stomach. Transfused 6 pints of stored blood 5-6 days ago.	0.2	0.01	0.05	412
P.L.	57	M	Haematemesis, cirrhosis of the liver, ?peptic ulcer, ?oesophageal varices. Transfused 6 pints of stored blood 6 days ago.	5.9	0.30	1.5	208
F.P.	69	M	Operation 10 days ago for gangrenous toe. Now has postoperative thrombocythaemia	8.6	0.43	n.d.	475
J.M.	11	F	Thrombocytosis after splenectomy for spherocytosis. (1) 2 days postoperatively	3.1	0.11	0.40	666
			(2) 5 days postoperatively	4.4	0.21	0.30	648
R.M.	45	M	Idiopathic thrombocytopenic purpura. (1) 7 days ago platelet count was less than 10,000 (2) 7 days ago 50% survival time of donor platelets was 2 days (chromium^{51} platelet survival) (3) Platelet count rose to 273,000 since platelet transfusion 6 days ago	7.4	0.34	n.d.	273
B.K.	38	M	Thrombocytopenia probably secondary to dexchlorpheniramine (polaramine)	2.0	0.16	0.65	370

APPENDIX B8 : THROMBOCYTOPENIA

PATIENT	AGE	SEX	DIAGNOSIS	ASCORBIC ACID CONCENTRATION: PLATELETS µg/10 cells	PLATELETS µg/mg	PLASMA mg/100 ml)	PLATELET COUNT (x 10^3/c.mm)
F.D.	61	F	Felty's syndrome: rheumatoid arthritis, anaemia, leucopenia. Also diabetes mellitus and aortic incompetence. Haemoglobin = 9.8G/100 ml. White cell count = 32,000/c.mm. P.T.F. = 67%.				
			(1) first estimation (day 0)	0.6	0.02	0.25	93
			(2) second estimation (day 5)	0.5	0.03	0.45	130
			(3) after 1 G ascorbic acid daily for 4 days (day 9)	1.5	0.06	1.80	163
			(4) after 1 G ascorbic acid daily for 3 more days (day 12)	1.7	0.05	1.60	115
M.H.	31	F	Disseminated lupus erythematosus. Cerebral thrombosis 3 days ago. P.T.F. = 100%.	2.4	0.06	n.d.	95
V.M.	24	F	Cirrhosis of the liver, ?hypersplenism	2.2	0.18	1.2	74
J.W.	72	M	Pancytopenia; recent course of butazolidin for polymyalgia rheumatica. Haemoglobin = 9.3 G/100 ml. White cell count = 4,800/c.mm.	1.5	0.02	n.d.	95

APPENDIX B9 : MISCELLANEOUS

PATIENT	AGE	SEX	DIAGNOSIS	ASCORBIC ACID CONCENTRATION			PLATELET COUNT ($\times 10^3$/ c.mm)
				PLATELETS $\mu g/10^9$ cells	μg/ mg	PLASMA mg/ 100 ml)	
M.B.	39	F	Malabsorption syndrome	3.2	0.08	n.d.	185
A.W.	94	F	Hypothermia. Neglected old woman.	1.4	0.09	n.d.	363
M.B.	64	M	Severe duodenal ulcer. Mild iron deficiency anaemia. Haemoglobin 13.1 G/100 ml.	1.6	0.04	n.d.	190
D.E.	72	M	Iron deficiency anaemia. Deep vein thrombosis	2.1	0.16	n.d.	230
R.D.	71	F	Iron deficiency anaemia. Probably nutritional in origin.	1.0	0.06	0.075	450
J.F.	56	F	Haemochromatosis	4.1	0.19	n.d.	198
S.B.	64	M	Haemochromatosis	0.7	0.03	n.d.	413
D.S.	48	M	Hodgkin's disease	2.4	0.09	n.d.	208

REFERENCES

AAS, K.A., GARDNER, F.H. (1958) "Survival of platelets labelled with chromium." J. Clin. Invest. 37, 1257-1268.

ABDERHALDEN, E., DOETJEN, H. (1907) "Weitere Studien über den Abbau einiger Polypeptide durch die roten Blutkorperchen und die Blutplättchen des Pferdeblutes." Z. Physiol. Chem. 53, 280-293.

ADELSON, E., RHEINGOLD, J.J., CROSBY, W.H. (1961) "The Platelet as a sponge: A review." Blood 17, 767-774.

ADELSON, E., KAUFMAN, R.M., LEAR, A.A., KIRBY, J.C., RHEINGOLD, J.J. (1963) "Physiology of platelet destruction as revealed by tagging of cohorts. I. Studies in dog." J. Lab. & Clin. Med. 62, 385-393.

ANITCHKOV, N.N. (1959) "Certain results and perspectives in experimental study of arterial atherosclerosis. Aterosklerоz i Infarkt Miokarda (Moscow, Medgiz) p. 68. Cited by Simonsón and Keys (1961)

ASQUITH, P., OELBAUM, M.H., DAWSON, D.W. (1967) "Scorbutic megaloblastic anaemia responding to ascorbic acid alone." Brit. Med. J. 4, 371-432.

AYNAUD, M. (1914) "Sur la composition chimique des globulins." C.R. Soc. Biol. (Paris) 76, 480-481.

BAILEY, N.T.J. (1959) "Statistical Methods in Biology." The English Universities Press Ltd., London.

BAKER, E.M. (1967) "Vitamin C requirements in stress." Amer. J. Clin. Nutr. 20, 583-590.

BAKER, E.M., SAARI, J.C., TOLBERT, B.M. (1966) "Ascorbic acid metabolism in man." Amer. J. Clin. Nutr. 19, 371-378.

BALDINI, M. (1966) "Platelet circulation and survival." XIth Congress of the International Society of Haematology, Plenary Sessions, 332-346.

BARKHAN, P. (1966a) "Platelet survival studies in man with diisopropylphosphorofluoridate (DFP^{32})." Brit. J. Haemat. 12, 25-36.

BARKHAN, P. (1966b) "Ascorbic acid in leucocytes and platelets." Brit. Med. J. 1, 1357.

BARKHAN, P., HOWARD, A.N. (1958) "Distribution of ascorbic acid in normal and leukaemic human blood." Biochem. J. 70, 163-168.

BARKHAN, P., HOWARD, A.N. (1959) "Some blood coagulation studies in normal and scorbutic guinea-pigs." Brit. J. Nutr. 13, 389-400.

BARKHAN, P., SILVER, M.J. (1962) "Biological Activities of Human Platelets: Biochemical, Physiological and Clinical Aspects." *in* Progress in Haematology, ed. by L.M. Tocantins, Grune and Stratton, N.Y. and Lond. Vol. III, 170-202.

BARKHAN, P., SILVER, M.J., O'KEEFE, L.M. (1961) "The lipids of human erythrocytes and platelets and their effects on thromboplastin formation." *in* Blood Platelets ed. by Johnson, S.A., Monto, R.W., Rebuck, J.W., Horn, R.C. Little, Brown & Co. Boston, pp 303-318.

BERMAN, H.J., FULTON, G.P. (1961) "Platelets in the peripheral circulation." *in* Blood platelets ed. by Johnson, S.A., Monto, R.W., Rebuck, J.W., Horn, R.C. Little, Brown & Co. Boston, 7-20.

BERNHARD, W., LEPLUS, R. (1955) "La méthode des coupes ultrafines et son application à l'étude de l'ultrastructure des cellules sanguines." J. Suisse Med. 85, 897-900.

BESSEY, O.A. (1938) "A method for the determination of small quantities of ascorbic acid and dehydroascorbic acid in turbid and coloured solutions in the presence of other reducing substances." J. Biol. Chem. 126, 771-784.

BESSEY, O.A., LOWRY, O.H., BROCK, M.J. (1947) "The quantitative determination of ascorbic acid in small amounts of white blood cells and platelets." J. Biol. Chem. 168, 197-205.

BESSIS, M. (1950) "Studies in electron microscopy of blood cells." Blood, 5, 1083-1098.

BESTETTI, A., CROSTI, P.F., LUCCHELLI, P. (1956) "Chemical investigations on platelets." Blood 11, 1162.

BETTEX-GALLAND, M., LUSCHER, E.F. (1961) "Thrombosthenin - a contractile protein from thrombocytes. Its extraction from blood platelets and some of its properties." Biochim. Biophys. Acta 49, 536-547.

BETTEX-GALLAND, M., LUSCHER, E.F., BERNE, T.R.I. (1960) "Studies on the metabolism of human blood platelets in relation to clot retraction." Thrombos. Diathes. Haemorrh. 4, 178-195.

BETTEX-GALLAND, M., MAUPIN, B. (1961) "Chimie des plaquettes sanguines: II. Equipment enzymatique et metabolisme." Hemostase 1, 375-400.

BEZKOROVAINY, A., DOHERTY, D.G. (1962) "Properties of structural proteins of bovine platelets." Arch. Biochem. Biophys. 86, 412-418.

BIZZOZERO, J. (1882) "Ueber einen neven Formbestandtheil des Blutes Und dessen Rollen bei der Thrombose und der Blutgerinnung." Virchows Arch. Path. Anat., 90, 261-332.

BLISS, C.I., GYORGY, P. (1951) "The Animal Vitamin Assays" *in* Vitamin Methods" ed. P. Gyorgy, Vol. II, 244-261, Academic Press Inc. N.Y.

BOBBITT, JAMES, M. (1963) "Thin-layer chromatography." Reinhold Publishing Corporation, N.Y.

BOLLIGER, H.R. (1965) "Vitamins" *in* Thin Layer Chromatography by Stahl, E. ed. Springer-Verlag, Berlin, Academic Press, N.Y. pp 210-248.

BONNIN, J.A., CHENEY, K. (1961) "The P.T.F. test: an improved method for the estimation of platelet thromboplastic function." Brit. J. Haemat. 7, 512-522.

BORN, G.V.R. (1956) "Adenosinetriphosphate (ATP) in blood platelets." Biochem J. 62, 33P.

BORN, G.V.R. (1958) "Changes in the distribution of phosphorus in platelet-rich plasma during clotting." Biochem. J. 68, 695-704.

BORN, G.V.R. (1962) "Quantitative investigations into the aggregation of blood platelets." J. Physiol. 162, 67P.

BORN, G.V.R. (1963) "Functions of the adenine nucleotides of blood platelets." Scientific Basis of Medicine : Annual Reviews, 249-265.

BORN, G.V.R., CROSS, M.J. (1963) "Effect of adenosine diphosphate on the concentration of platelets in circulating blood." Nature 197, 974-976.

BORN, G.V.R., GILLSON, R.E. (1958) "The effect of 2,4-dinitrophenol and of potassium on the uptake of 5-hydroxytryptamine in platelets." J. Physiol. 141, 39P.

BORN, G.V.R., GILLSON, R.E. (1959) "Studies on the uptake of 5-hydroxytryptamine by blood platelets." J. Physiol. 146, 472-491.

BORN, G.V.R., HASLAM, R.J., GOLDMAN, M. (1965) "Comparitive effectiveness of adenosine analogues as inhibitors of blood-platelet aggregation and as vasodilators in man." Nature 205, 678-680.

BORN, G.V.R., HONOUR, A.J., MITCHELL, J.R.A. (1964) "Inhibition by adenosine and by 2-chloroadenosine of the formation and embolization of platelet thrombi." Nature 202, 761-765.

BORN, G.V.R., HORNYKIEWICZ, O., STAFFORD, A. (1958) "The uptake of adrenaline and nor-adrenaline by blood platelets of the pig." Brit. J. Pharmacol. 13, 411-414.

BORN, G.V.R., WRIGHT, H. PAYLING (1967) "Platelet adhesiveness in experimental scurvy." Lancet 1, 477-478.

BRECHER, G., CRONKITE, E.P. (1950) "Morphology and enumeration of human blood platelets." J. Appl. Physiol. 3, 365-377.

BRISE, H., HALLBERG, L. (1962) "Iron absorption studies II." Acta med. scand. 171 (supp. 376), 7-73.

BRODA, E. (1960) "Radioactive isotopes in biochemistry." Elsevier Publishing Company, Amsterdam, London, New York, Princeton.

BUTLER, A.M., CUSHMAN, M. (1940) "Distribution of ascorbic acid in the blood and its nutritional significance." J. Clin. Invest. 19, 459-467.

CAHALANE, S.F., JOHNSON, S.A., MONTO, R.W., CALDWELL, M.J. (1958) "Acquired thrombocytopathy. Observations on the coagulation defect in uraemia." Amer. J. Clin. Path. 30, 507-513.

CALDER, J.H., CURTIS, R.C., FORE, H. (1963) "Comparison of vitamin C in plasma and leukocytes of smokers and non-smokers." Lancet 1, 556.

CAMPBELL, J.A., CHAPMAN, D.G., ROCHON, O. (1951) "Estimation of ascorbic acid in pharmaceuticals." Anal. Chemistry, 23, 1113-1115.

CAMPBELL, E.W., SMALL, W.J., DAMESHEK, W. (1956) "Metabolic activity of human blood platelets, I." J. Lab. Clin. Med. 47, 835-843.

CARAWAY, W.T. (1961) "Macro and micro methods for the determination of serum iron and iron-binding capacity." Clin. Chem. 7, 572.

CASTALDI, P.A., FIRKIN, B.G. (1962) "Platelet changes in coagulation: Chemical studies." J. Aust. Soc. Med. Res. 1, 35.

CASTALDI, P.A., FIRKIN, B.G., BLACKWELL, P.M., CLIFFORD, K.I. (1962) "An electron microscopic study of the changes in platelets during viscous metamorphosis." Blood 20, 566-579.

CASTALDI, P.A., ROZENBERG, M.C., STEWART, J.H. (1966) "The bleeding disorder of uraemia." Lancet 2, 66-69.

CETINGIL, A.I., ULUTIN, O.N., KARACA, M. (1958) "A platelet defect in a case of scurvy." Brit. J. Haemat. 4, 350-354.

CHAMBERS, R., ZWEIFACH, B.W. (1947) "Intercellular cement and capillary permeability." Physiol. Reviews 27, 436-463.

CHANDLER, A.B., HAND, R.A. (1961) "Phagocytized platelets: a source of lipids in human thrombi and atherosclerotic plaques." Science 134, 946-947.

CHAPLIN, H., MOLLISON, P.L. (1952) "Correction for plasma trapped in the red cell column of the hematocrit." Blood 7, 1227-1238.

CHARGAFF, E., BANCROFT, F.W., STANLEY-BROWN, M. (1936) "Studies on the chemistry of blood coagulation. III. The chemical constituents of blood platelets and their role in blood clotting, with remarks on the activation of clotting by lipids." J. Biol. Chem. 116, 237-251.

CHARLEY, P.J., SARKAR, B., STITT, C., SALTMAN, P. (1963) "The chelation of iron by sugars." Biochim et Biophys Acta 69, 313-321.

CHATTERJEE, I.B., KAR, N.C., GHOSH, N.C., GUHA, B.C. (1961) "Aspects of ascorbic acid biosynthesis in animals." New York Academy of Sciences, Annals, 92, 36-56.

CHEN, Y.T., ISHERWOOD, F.A., MAPSON, L.W. (1953) "Quantitative estimation of ascorbic acid and related substances in biological extracts by separation on a paper chromatogram." Biochem. J. 55, 821-823.

CHENEY, K., BONNIN, J.A. (1962) "Haemorrhage, platelet dysfunction and other coagulation defects in uraemia." Brit. J. Haemat. 8, 215-222.

CHEVALLIER, A., CHORON, Y. (1943) "Sur une méthode spectrophotométrique de dosage de l'acide ascorbique dans les tissus." Bull. Soc. Chim. Biol. 19, 511-526.

COOPER, I.A., FIRKIN, B.G. (1967) "The transport of aminoacids into human platelets and subsequent protein synthesis." Proc. Aust. Soc. Med. Res. 2, 106.

COTTINGHAM, E., MILLS, C.A. (1943) "Influence of environmental temperature and vitamin deficiency upon phagocytic functions." J. Immunol. 47, 493-502.

COX, E.V., GADDIE, R., MATTHEWS, D., COOKE, W.T., MEYNELL, M.J. (1958) "An inter-relationship between ascorbic acid and cyanocobalamin." Clin. Sci. 17, 681-692.

COX, E.V., MEYNELL, M.J., NORTHAM, B.E., COOKE, W.T. (1967) "The anaemia of scurvy." Amer. J. Med. 42, 220-227.

CRANDON, J.H., LUND, C.C., DILL, D.B. (1940) "Experimental human scurvy." New Engl. J. Med. 223, 353-369.

CRONKITE, E.P., BOND, V.P., FLIEDNER, T.M., PAGLIA, D.A., ADAMIK, E.R. (1961) "Studies on the origin, production and destruction of platelets." *in* Blood Platelets ed. by Johnson, S.A., Monto, R.W., Rebuck, J.W., Horn, R.C. Little, Brown & Co. Boston (1961) 595-609.

CUTTLE, T.D. (1938) "Observations on the relation of leukocytosis to ascorbic acid requirements." Quart. J. Med. 7, 575-584.

DAVEY, M.G. (1966) "The survival and destruction of human platelets." Bibl. Haematologica, Fasc.22.

DAVEY, M.G., LANDER, H. (1963) "The labelling of human platelets with radiochromate." Aust. J. exp. Biol. med. Sci. XLI, 581-593.

DAVEY, M.G., LANDER, H. (1964) "Effect of adenosine diphosphate on circulating platelets in man." Nature 201, 1037-1039.

DAVID-FERREIRA, J.F. (1964) "The blood platelet: Electron microscopic studies." Internat. Rev. Cytology 17, 99-148.

DAVID-FERREIRA, J.F., DAVID-FERREIRA, K. (1962) "L'ultrastructure des plaquettes sanguines mise en evidence du glycogène." Z. Zellforsch. Mikroskop. Anat. 56, 789-802.

DAVIS, P.S., (1965) "Formation of metal chelates in biological systems: Methods of investigation." Proc. Aust. Assoc. Clin. Biochem. 1, No. 6, 190-192.

DAVIS, P.S., DELLER, D.J. (1967) "Effect of orally administered chelating agents EDTA, DTPA and fructose on radioiron absorption in man." Aust. Ann. Med. 16, 70-74.

DAVIS, R.B., KAY, D. (1965) "Demonstration of 5-hydroxytryptamine in blood platelets by electron microscope autoradiography." Nature 207, 651.

DAYTON, P.G., WEINER, M. (1961) "Ascorbic acid and blood coagulation." Ann. N.Y. Acad. Sci. 92, 302-306.

DEETJEN, H. (1901) "Untersuchungen uber die Blutplattchen." Virchows Arch. Path. Anat. 164, 239-263.

DE GRUCHY, G.C. (1964) "Clinical Haematology in Medical Practice." Blackwell Scientific Publications, Oxford, second edition, 1964, p. 532.

DE MARSH, Q.B., KAUTZ, J., MOTULSKY, A.G. (1955) "An electron microscope study of sectioned platelets and megakaryocytes." J. Clin. Invest. 34, 929-930.

DENSON, K.W., RICHARDS, J.D.M. (1962) "Levels and uptake of ascorbic acid from plasma by white cells, platelets and leukaemic cells." Nature 196, 1218.

DE ROBERTIS, E., PASEYRO, P., REISSIG, M. (1953) "Electron microscopic studies of the action of thrombin on blood platelets." Blood 8, 587-597.

DILLARD, G.H.L., BRECHER, G., CRONKITE, E.P. (1951) "Separation, concentration and transfusion of platelets. Proc. Soc. Exp. Biol. N.Y. 78, 796.

DOCUMENTA GEIGY (1962) "Scientific Tables." Geigy Pharmaceuticals, ed. by Diem, J., sixth edition, 1962.

DOMINICI, M. (1900) "Sur l'histologie de la rate au cours des états infectieux." Arch. Med. Exper. 12, 733-768.

DONNÉ, A. (1842) "De l'origine des globules du sang, de leur mode de formation, et de leur fin." C.R. Acad. Sci. 14, 366-368.

DUGUID, J.B. (1948) "Thrombosis as a factor in the pathogenesis of aortic atherosclerosis." J. Path. Bact. 60, 57-61.

EASTHAM, R.D., GRIFFITHS, E.P. (1966) "Reduction of platelet adhesiveness and prolongation of coagulation time of activated plasma by glyceryl guiacolate." Lancet 1, 795-796.

EGEBERG, O. (1966) "The role of coagulation in thrombosis." XIth Congress of the International Society of Haematology, 1966, Plenary Sessions, 172-182.

EICHORN, G.L. (1964) *in* "Iron Metabolism" ed. by Gross, F. Berlin, Springer-Verlag, 1964, p 87.

EMMONS, R.R., HARRISON, M.J.G., HONOUR, A.J., MITCHELL, J.R.A. (1965a) "Effect of a pyrimidopyrimidine derivative on thrombus formation in the rabbit." Nature 208, 255-257.

EMMONS, P.R., HARRISON, M.J.G., HONOUR, A.J., MITCHELL, J.R.A. (1965b) "Effect of dipyridamole on human platelet behaviour." Lancet 2, 603-606.

ERPSAMER, V., TESTINI, A. (1959) "Observations on the release and turnover rate of 5-hydroxytryptamine in the gastrointestinal tract." J. Pharmacol. (Lond.) 11, 618-623.

FANTL, P., WARD, H.A. (1956) "Nucleotides of human blood platelets." Biochem. J. 64, 747-754.

FARMER, E.H. (1933) "Ascorbic acid." Chemical Society Annual Reports, 167-175.

FIRKIN, B.G. (1963) "The platelet." Aust. Ann. Med. 12, 261-274.

FISCHER, D.S., PRICE, D.C. (1964) "A simple serum iron method using the new sensitive chromogen tripyridyl-S-triazine." Clin. Chem. 10, 21-31.

FLEXNER, J., BRUGER, M., WRIGHT, I.S. (1941) "Experimental atherosclerosis. II. Effect of thiamine hydrochloride and ascorbic acid on experimental atherosclerosis in rabbits." Arch. Path. 31, 82-84.

FRENDO, J., KOJ, A., ZGLICZYNSKI, J.M. "Taurine in human blood platelets." Nature 183, 685-686. (1959).

FRIEDMAN, G.J., SHERRY, S., RALLI, E.P. (1940) "The mechanism of the excretion of vitamin C by the human kidney at low and normal plasma levels of ascorbic acid." J. Clin. Invest. 19, 685-689.

GAARDER, A., JONSEN, J., LALAND, S., HELLEM, A. (1961) "Adenosine diphosphate in red cells as a factor in the adhesiveness of human blood platelets." Nature (Lond.) 192, 531.

GABUZDA, G.J., PHILLIPS, G.B., SCHILLING, R.F., DAVIDSON, C.S. (1952) "Metabolism of pteroylglutamic acid and the citrovorum factor in patients with scurvy." J. Clin. Invest. 31, 756-761.

GARDNER, F.H., COHEN, P. (1966) "Platelet life span." Transfusion 6, 23-31.

GÉRO, E., ROUX, J.M. (1958) "L'acide ascorbique dans la cellule." C. Rend. Soc. Biol. (Paris) 152, 329-331.

GEROK, W., GROSS, R. (1959) "Aminosaüren in normalen menschlichen Thrombozyten." Thromb. Diath. Haemorr. 3, 654-657.

GIBSON, S.L.M., MOORE, F.M.L., GOLDBERG, A. (1966) "Measurement of leukocyte ascorbic acid." Brit. Med. J. 1, 1152-1153.

GLYNN, M.F., MOVAT, H.Z., MURPHY, E.A., MUSTARD, J.F. (1965) "Study of platelet adhesiveness and aggregation with latex particles." J. Lab. & Clin. Med. 65, 179-200.

GOLDSMITH, G.A., (1961) "Human requirements for vitamin C and its use in clinical medicine." New York Academy of Sciences, Annals, 92, 230-245.

GOULD, B.S. (1960) "Ascorbic acid and collagen fiber formation." Vitamins and Hormones 18, 89-120.

GRAHERM, H.T., LOWRY, O.H., WHEELWRIGHT, T.F., LENZ, M.A., PARISH, H.H. (1955) "Distribution of histamine among leukocytes and platelets." Blood 10, 467-481.

GREEN, N.M., LOWTHER, D.A. (1959) "Formation of collagen hydroxyproline in vitro." Biochem J. 71, 55-66.

GREENBERG, S.M., TUCKER, R.G., HEMING, A.E., MATHUES, J.K. (1957) "Iron absorption and metabolism. I. Interrelationship of ascorbic acid and vitamin E." J. Nutr. 63, 19-31.

GRIFFITH, W.H. (1967) Amer. J. Clin. Nutr. 20, 591-593.

GROSS, R. (1961) "Metabolic aspects of normal and pathological platelets." *in* Blood Platelets ed. by Johnson, S.A., Monto, R.W., Rebuck, J.W., Horn, R.C. Little, Brown and Co. Boston, 1961. p. 407-421.

GROSS, R., GEROK, W. (1961) "Quantitative determination of the amino acids and other ninhydrin positive substances in normal human platelets." Thrombos. Diathes. et Haemorrh. 6, 462-469.

GYORGY, P., RUBIN, S.H. (1950) "Chemical methods of vitamin assay." *in* Vitamin Methods, ed. by P. Gyorgy, Acad. Press Inc., N.Y., 1950. p. 260-276.

HADFIELD, G. (1950) "Thrombosis." Ann. Roy. Coll. Surgeons England, 6, 219-234.

HAGENAU, F., HOLLMANN, K.H., LEVY, J.P., BOIRON, M. (1963) "Etude au microscope électronique des plaquettes sanguines dans les leucemies humaines." J. de Microscopie 2, 529-537.

HAHN, P.F., JONES, E., LOWE, R.C., MENEELY, G-R., PEACOCK, W. "The relative absorption and utilization of ferrous and ferric iron in anemia as determined with the radioactive isotope." Amer. J. Physiol. 143, 191-197. (1945)

HAND, R.A., CHANDLER, A.B. (1962) "Atherosclerotic metamorphosis of autologous pulmonary thromboemboli in the rabbit." Amer. J. Path. 40, 469-486.

HART, H.C., PLOEM, J.E., PANDERS, J.T., VERLOOP, M.C. (1964) "Haemorrhagic diathesis and anaemia in scurvy." Acta Medica Scand. 176, 497-508.

HAUROWITZ, F., SLADEK, J. (1928) "Über die chemische Zusammensetzung der Blütplattchen." Z. Physiol. Chemie 173, 233-234.

HAYEM, G., (1878) "Recherches sur l'évolution des hématies dans le sang de l'homme et des vertebrates." Arch. de Phys. norm. et Path. 5, 692-734.

HAYEM, G. (1882) "Sur le mecanisme de l'arrêt des hémorrhagies." C.R. Acad. Sci. (Paris) 95, 18-21.

HEIMANN, W., STROHECKER, R., MATT, F. (1953) "Zur Bestimmung der Ascorbinsäure auf papierchromatographischem Weg." Z. Lebensm. Unters. Forsch. 97, 263-270.

HELLEM, A.J. (1960) "The adhesiveness of human platelets in vitro." Scand. J. Clin. Lab. Invest. 12 (supp. 51).

HERRMANN, J., ZOBEL, M. (1962) "Paper-chromatographic determination of ascorbic acid, dehydroascorbic acid and ascorbigen in heated biological material. I. Paper chromatographic determination of ascorbic acid." Anal. Abstracts 9, 4951.

HEWITT, L.F. (1950) "Oxidation-reduction potentials in bacteriology and biochemistry." Sixth edition (1950), E. & S. Livingstone Ltd., Edinburgh, p 60-61.

HIRSCH, E.O., GARDNER, F.H., THOMAS, E.D. (1952) "Isolation and concentration of human blood platelets. Their properties in vitro and in vivo." J. Clin. Invest. 31, 638-639.

HIRST, E.L. (1953) "The Chemistry of Vitamin C" *in* Lind's Treatize on Scurvy" reprinted in 1953 Edinburgh, University Press, ed. by Stewart, C.P. and Guthrie, D., p 413-424.

HIRST, E.L., PEAT, S. (1934) "Ascorbic acid and its analogues." Chemical Society, Annual Reports, p 177-187.

HOLLEY, H.L., McLESTER, J.S. (1951) "Manifestations of ascorbic acid deficiency after prolonged corticotrophin administration." Arch. Intern. Med. 88, 760-761.

HOLST, A., FROLICH, T. (1907) "Experimental studies relating to ship-beri-beri and scurvy." J. Hyg. (Camb.) 7, 634-671.

HONOUR, A.J., MITCHELL, J.R.A. (1963) "Platelet clumping in vivo." Nature (Lond.) 197,1019-1020.

HONOUR, A.J., ROSS-RUSSELL, R.W. (1962) "Experimental platelet embolism." Brit. J. Exper. Path. 43, 350-362.

HOVIG, T. (1962) "The ultrastructure of rabbit blood platelet aggregates." Thromb. Diath. Haemorrh. 8, 455-471.

HUMPHREY, J.H., JAQUES, R. (1954) "The histamine and serotonin content of the platelets and polymorphonuclear leukocytes of various species." J. Physiol. 124, 305-310.

HUMPHREY, J.H., TOH, C.C. (1954) "Absorption of serotonin (5-hydroxytryptamine) and histamine by dog platelets." J. Physiol. 124, 300-304.

JANDL, J., GABUZDA, G. (1953) "Potentiation of pteroylglutamic acid by ascorbic acid in the anemia of scurvy. Proc. Soc. Exper. Biol. and Med. 84, 452-455.

JEAN, G., GAUTIER, A. (1961) "Essais de cytochimie ultrastructurale. Sur la mise en évidence du glycogène dans les thrombocytes humains normaux et pathologiques." C.R. Acad. Sci. (Paris) 253, 2274-2276.

JOHNSON, S.A. (1966) "Role of blood vessel in thrombosis." XIth Congress of the International Society of Haematology, Plenary Sessions, 1966, p 133-149.

JOHNSON, S.A., BALBOA, R.S., DESSELL, B.H., MONTO, R.W., SIEGESMUND, K.A., GREENWALT, T.J. (1964) "The mechanism of the endothelial supporting function of intact platelets." Exp. Molec. Path. 3, 115-127.

JOHNSTONE, W.M., DRAKE, T.G.H., TISDALL, F.F., HARVIE, F.H. (1946) "A study of the ascorbic acid metabolism of healthy young Canadians." Canad. Med. Ass. J. 55, 581-585.

JONES, O.P. (1960) "Origin of megakaryocyte granules from Golgi vesicles." Anat. Record 138, 105-113.

JORDANOV, J., TARALOV, S., GUEORGUIEV, I., BÉLÉVA-STAKOVA, R. (1956) "Recherches sur l'histochimie de la vitamin C du sang." C.R. Assoc. Anat. 42, 671-676.

JORGENSEN, L., BORCHGREVINK, C.F. (1963) "The platelet plug in normal persons." Acta Path. Microbiol. Scand. 57, 40-56.

KARRER, P., SALOMON, H., SCHOPP, K., MORF, R. (1933) "Zur Kenntnis des antiskorbutischen Vitamins (Vitamin C)." Helv. Chim. Acta 16, 181-183.

KASER-GLANZMANN, LUSCHER, E.F. (1962) "The mechanism of platelet aggregation in relation to thrombosis." Thrombos. Diathes. et Haemorrh. 7, 480-490.

KING, C.G., BURCH, H.B., BECKER, R.R., SALOMON, L. (1953) "New functional role of ascorbic acid." Fed. Proc. 12, 470.

KINOSITA, R., OHNO, S. (1961) "Biodynamics of Thrombopoiesis" *in* Blood Platelets, ed. by Johnson, S.A., Monto, R.W., Rebuck, J.W., and Horn, R.C. Little, Brown & Co., Boston, 1961, p 611-616.

KIRKPATRICK, H.F.W. (1941) "Further notes on the determination of ascorbic acid and sulphur dioxide in fruit juices." J. Soc. Chem. Ind. 60, 298-299.

KJAERHEIM, A., HOVIG, T. (1962) "The ultrastructure of haemostatic blood platelet plugs in rabbit mesenterium." Thrombos. Diathes. Haemorrh. 7, 1-15.

KNOX, W.E., GOSWAMI, M.N.D. (1961) "Ascorbic acid in man and animals." Adv. Clin. Chem. 4, 122-205.

KOLTHOFF, I.M., ELVING, P.J. (1962) "Treatise on Analytical Chemistry - Part II. Analytical Chemistry of the Elements Vol. 12." New York, Interscience.

KRAUSS, (1883) Inaugural Dissert. Heidelberg, 1883. Cited by Tocantins, L.M. "Historical notes on blood platelets." Blood 3 (1948) 1073-1082.

KUETHER, C.A., TELFORD, I.R., ROE, J.H. (1944) "Tissue distribution of ascorbic acid." J. Nutr. 28, 347-358.

LA DU, B.N., GREENBERG, D.M. (1953) "Ascorbic acid and the oxidation of tyrosine." Science 117, 111-112.

LA DU, B.N., ZANNONI, V.G. (1961) "The role of ascorbic acid in tyrosine metabolism." Ann. N.Y. Acad. Sci. 92, 175-191.

LAWRYNOWICZ, M.A. (1931) "Observations relatives à l'influence des vitamines sur les propriétés des microbes et le cours de l'infection." J. Physiol. Path. gén. 29, 270-276.

LEE, K., LEONG, P.C. (1964) "A new method for the determination of ascorbic acid in urine." Clin. Chem. 10, 575-580.

LEEKSMA, C.H.W., COHEN, J.A. (1956) "Determination of the life span of human blood platelets using labelled diisopropylfluorophosphate." J. Clin. Invest. 35, 964-969.

LEVY, L.F. (1943) "The determination of ascorbic acid in the presence of sulphur dioxide." Biochem. J. 37, 713-716.

LIND, J. (1753) "A treatise on the scurvy" reprinted in "Lind's treatise on scurvy" ed. by C.P. Stewart and D. Guthrie. University Press, Edinburgh (1953) p 5-355.

LLOYD, J.V., LANDER, H., KINLOUGH, R.L. (1965) "The effects of the adenosine nucleotides on the circulating platelet level in sheep." Proc. Aust. Soc. Med. Res. 1, 154.

LOCKHEAD, A.C., GOLDBERG, A. (1959) "Transfer of iron to protoporphyrin for haem biosynthesis. Role of ascorbic acid and glutathione." Lancet 2, 271-272.

LU, G.D. (1939) "Studies on the metabolism of pyruvic acid in normal and vitamin B_1-deficient states. I. A rapid, specific and sensitive method for the estimation of blood pyruvate." Biochem. J. 33, 249-254.

LUND, H., LIECK, H. (1936) "A specific reaction for the qualitative and quantitative determination of ascorbic acid in serum." Nature 137, 784.

LUGG, J.W.H. (1942) "Use of formaldehyde in the estimation of ascorbic acid and dehydro-ascorbic acid. Nature (Lond.) 150, 577.

McDONALD, T.P., ODELL, T.T., GOSSLEE, D.G. (1964) "Platelet size in relation to platelet age." Proc. Soc. Exp. Biol. Med. 115, 684.

McMENAMY, R.H., LUND, C.C. (1960) "Concentration of unbound amino acids in human platelets. Biochim. et Biophys. Acta 45, 603-605.

MACEK, K., HAIS, I.M., GASPARIC, J., KOPECKY, J., ROBEK, V. (1962) "Bibliography of paper chromatography." Publishing House of the Czechoslovak Academy of Sciences, Prague (1962) p 440-442.

MADOFF, M.A., EBBE, S., BALDINI, M. (1964) "Sialic acid of human blood platelets." J. Clin. Invest. 43, 870-877.

MALINEK, M. (1965) "Quantitative recovery of lipids from thin-layer chromatography by a simple micro technique." Aust. J. Exp. Biol. Med. Sci. 570.

MAPSON, L.W. (1941) "Vitamin Methods IV. The estimation of ascorbic acid in the presence of reductones and allied substances." J. Soc. Chem. Ind. 62, 223-232.

MAPSON, L.W., PARTRIDGE, S.M. (1949) "Separation of substances related to ascorbic acid." Nature 164, 479-480.

MARCUS, A.J., ULLMAN, H.L., SAFIER, L.B., BALLARD, H.S. (1962) "Platelet phosphatides. Their fatty acid and aldehyde composition and activity in different clotting systems." J. Clin. Invest. 41, 2198.

MARCUS, A.J., ZUCKER, M.B. (1965) "The Physiology of Blood Platelets." Grune and Stratton, N.Y. and London, 1965.

MARCUS, A.J., ZUCKER-FRANKLIN, D., SAFIER, L.B., ULLMAN, H.J. (1966) "Studies on human platelet granules and membranes." J. Clin. Invest. 45, 14-28.

MARTIN, G.R. (1961) "Studies on the tissue distribution of ascorbic acid." Ann. N.Y. Acad. Sci. 92, 141-147.

MAUPIN, B. (1953) "Acquisitions récentes sur l'histochimie et la biochimie des plaquettes sanguines de l'homme." Revue d'hématologie 8, 302-315.

MAUPIN, B. (1961) "Chimie des plaquettes sanguines: I. Chimie analytique et histochimie." Hémostase I, 29-54.

MAUPIN, B., SAINT-BLANCARD, J., STORCK, J. (1962) "Soufre, taurine, protéines et A.T.P. plaquettaires." Rev. Franc. Études Clin. Biol. 7, 169-173.

MAY, C.D., HAMILTON, A., STEWART, C.T. (1952) "Experimental megaloblastic anaemia and scurvy in the monkey." Blood 7, 978-991.

MAZUR, A., GREEN, S., CARLETON, A. (1960) "Mechanism of plasma iron incorporation into hepatic ferritin.: J. Biol. Chem. 235, 595-603.

MEIKLEJOHN, A.P. (1953) "The physiology and biochemistry of ascorbic acid" *in* Vitamins and Hormones, ed. by Harris, R.S., Marrian, G.F. and Thimann, K.V. Acad. Press Inc., N.Y. Vol. 11. p 61-96.

MINOR, A.H., BURNETT, L. (1952) "A method for separating and concentrating platelets from normal human blood." Blood 7, 693-699.

MIZUNO, N.S., SAUTTER, J.H., SCHULTZE, M.O. (1958) "Enzyme and nucleic acid content of thrombocytes from normal and thrombocytopenic calves." Proc. Soc. Exp. Biol. Med. 98, 42-46.

MOORE, C.V., ARROWSMITH, W.R., WELCH, J., MINNICH, V. (1939) "Studies in iron transportation and metabolism. IV. Observations on the absorption of iron from the gastrointestinal tract." J. Clin. Invest. 18, 553-580.

MOORE, C.V., DUBACH, R., (1955) "Observations on the absorption of iron from foods tagged with radioiron." Trans. Assoc. Amer. Phys. 64, 245-256.

MORITA, H., ASADA, T. (1956) "Morphological and biochemical studies of the human blood platelets." Acta Haematol. Japon. 19, 426-449.

MORRISON, F.S., BALDINI, M. (1966) "Antigenic relationship between vascular endothelium and blood platelets." XIth Congress of the International Society of Haematology (1966), Abstracts of Papers, CA6.

MORSE, E.H.M., POTGIETER, M., WALKER, G.R. (1956) "Ascorbic acid utilization by women." J. Nutr. 58, 291-298.

MOVAT, H.Z., HAUST, M.D., MORE, R.H. (1959) "The morphological elements in the early lesions of arteriosclerosis. Amer. J. Path. 35, 93-101.

MURPHY, E.A., MUSTARD, J.F., ROBINSON, G.A. (1962) "The pattern of platelet destruction." Blood 20, 765.

MUSTARD, J.F., DOWNIE, H.G., MURPHY, E.A., ROWSELL, H.C. (1961) "Lipids, platelets and atherosclerosis." *in* Blood Platelets ed. by Johnson, S.A., Monto, R.W., Rebuck, J.W., Horn, R.C. Little, Brown & Co., Boston (1961) 191-204.

MUSTARD, J.F., MURPHY, E.A., ROWSELL, H.C., DOWNIE, H.G. (1964) "Platelets and atherosclerosis." J. Atherosclerosis Res. 4,1-28,

MUSTARD, J.F., ROWSELL, H.C., MURPHY, E.A. (1966) "Platelet economy (platelet survival and turnover)." Brit. J. Haemat. 12, 1-24.

MUSTARD, J.F., ROWSELL, H.C., MURPHY, E.A., GLYNN, M.F. (1965) "The platelet and endothelium." Blood 25, 613.

MUSULIN, R.R., KING, C.G. (1936) "Metaphosphoric acid in the extraction and titration of vitamin C." J. Biol. Chem. 116, 409.

MYASNIKOV, A.L. (1958) "Influence of some factors on development of experimental cholesterol atherosclerosis." Circulation 17, 99-113.

NICHOL, C.A., WELCH, A.D. (1950) "Synthesis of citrovorum factor from folic acid by liver slices; augmentation by ascorbic acid." Proc. Soc. Exper. Biol. Med. 74, 52-55.

NUTRITION REVIEWS (1960) "Scurvy and blood coagulation." Vol. 18, 242-244.

O'BRIEN, J.R. (1962) "Platelet aggregation. Part II. Some results of a new method of study." J. Clin. Path. 15, 452-456.

O'BRIEN, J.R. (1963) "Further observations on platelet aggregation induced by adenosine diphosphate, thrombin, and triethyltin." J. Clin. Path. 16, 223-226.

O'BRIEN, J.R. (1964a) "A comparison of platelet aggregation produced by seven compounds and a comparison of their inhibitors." J. Clin. Path. 17, 275-281.

O'BRIEN, J.R. (1964b) "The mechanism and prevention of platelet adhesion and aggregation considered in relation to arterial thrombosis." Blood 24, 309-314.

ODELL, T.T., ANDERSON, B. (1957) "Isolation of a sulfated mucopolysaccharide from blood platelets of rats." J. Exp. Biol. Med. 94, 151-156.

PAINTER, H.A., ZILVA, S.S. (1947) "The influence of l-ascorbic acid on the rupture of the benzene ring of L-tyrosine consumed in high doses by guinea-pigs." Biochem. J. 41, 511-519.

PEARSE, A.G. (1953). "Histochemistry, theoretical and applied. J. & A. Churchill Ltd., London (1953) p 159-162.

PEARSON, W.N. (1966) "Assessment of nutritional status: Biochemical methods." *in* Nutrition 3, 1966. Ed. by G.H. Beaton, Adademic Press, N.Y. and London,, 265-315.

PENNEY, J.R., BALFOUR, B.M. (1949) "The effect of vitamin C on mucopolysaccharide production in wound healing." J. Path. Bact. 61, 171-178.

PENNEY, J.R., ZILVA, S.S. (1943) "The determination of 2:3-diketo-L-gulonic acid." Biochem. J. 37, 39-44.

PENROSE, L., QUASTEL, J.H. (1937) "Metabolic studies in phenylketonuria." Biochem. J. 31, 266-274.

PERRONCITO, A. (1920) "The mammalian blood platelet in health and disease." Haematologica 7, 111. Cited by Tocantins, L.M. (1938) Medicine 17, 155-258.

PIJOAN, M., GERJOVICH, H.J. (1946) "The use of 2,4-dinitrophenylhydrazine for the determination of ascorbic acid." Science 103, 202-203.

PROCHÁZKA, Z. (1963) "Ascorbic acid and related compounds" *in* Paper Chromatography ed. by I.M. Hais and K. Macek, Academic Press, New York and London, 1963. p 679.

PROEHL, E., MAY, C.P. (1952) "Experimental nutritional megaloblastic anemia and scurvy in the monkey. III. Protoporphyrin, coproporphyrin, urobilinogen and iron in blood and excreta." Blood 7, 671-682.

RALLI, E.P., SHERRY, S. (1941) "Adult scurvy and the metabolism of vitamin C." Medicine 20, 251-340.

REBUCK, J.W. (1949) "A simple method for the electronmicroscopy of peripheral blood cells." Amer. J. Clin. Path. 19, 217-228.

REGOLI, D., CLARK, V. (1963) "Prevention by adenosine of the effect of adenosine diphosphate on the concentration of circulating platelets." Nature (Lond.) 200, 546-548.

REISS, P. (1942-43) "Actions du potential d'oxydation-réduction du milieu sur l'activite de différentes protéinases: hydrolyse et condensation." Arch. Phys. Biol. 16, Suppl. (1942-43). Cited by Ziegler E. (1965).

REISS, P. (1943-44) "Les mesures de potentiels d'oxydation-réduction dans le sang. Revue bibliographique." Arch. Phys. Biol. 17, Suppl. (1943-44).

REPPERT, E., DONEGAN, J., HINES, L.F. "Ascorbic acid and the hyaluronidase hyaluronic acid reaction." Proc. Soc. Exp. Biol. Med. 77, 318-320.

RINEHART, J.F. (1955) "Electron microscopic studies of sectioned white blood cells and platelets." Amer. J. Clin. Path. 25, 605-619.

RITZEL, VON G. (1961) "Critical evaluation of vitamin C as a prophylactic and therapeutic agent in colds." Helv. Med. Acta 28, 63-68.

ROBERTSON, E.B. (1934) "Spectrophotometric estimation of ascorbic acid." J. Soc. Chem. Ind. 53, 277.

ROBERTSON, W. VAN B. (1950) "Concentration of collagen in guinea pig tissues in acute and prolonged scurvy." J. Biol. Chem. 187, 673-677.

ROBERTSON, W. VAN B. (1952) "The effect of ascorbic acid deficiency on the collagen concentration of newly induced fibrous tissue." J. Biol. Chem. 196, 403-408.

ROBERTSON, W. VAN B. (1961) "The biochemical role of ascorbic acid in connective tissue." Ann. N.Y. Acad. Sci. 92, 159-167.

ROBINSON, W.B., STOTZ, E. (1945) "The indophenol-xylene extraction method for ascorbic acid and modifications for interfering substances." J. Biol. Chem. 160, 217-225.

RODMAN, N.F., MASON, R.G., BRINKHOUS, K.M. (1963) "Some pathogenetic mechanisms of white thrombus formation: agglutination and self-destruction of the platelet." Fed. Proc. 22, 1356-1365.

RODMAN, N.F., MASON, R.G., McDEVITT, N.B., BRINKHOUS, K.M. (1962) "Morphologic alterations of human blood platelets during early phases of clotting." Amer. J. Path. 40, 271-282.

ROE, J.H. (1954) "Chemical determination of ascorbic, dehydro-ascorbic and diketogulonic acids" *in* Methods of Biochemical Analysis ed. by D. Glick, 1, 115-139 (1954).

ROE, J.H. (1961) "Appraisal of methods for the determination of L ascorbic acid." Ann. N.Y. Acad. Sci. 92, 277-283.

ROE, J.H., HALL, J.M. (1939) "The vitamin C content of human urine and its determination through the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid." J. Biol. Chem. 128, 329-337.

ROE, J.H., KUETHER, C.A. (1943) "The determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid." J. Biol.Chem. 147, 399-407.

ROKITANSKY, C. (1842-1846) "Handbuck der Pathologischen Anatomie." Braunmüller u. Seidel : Vienna (1842-1846). Cited by Mustard et alii (1961).

ROSKAM, J. (1922) "Contribution à l'étude de la physiologie normale et pathologique du globulin (plaquette de Bizzozero)." Arch. Internat. Physiol. 20, 241-330.

ROSKAM, J. (1961) "Role of platelets in the formation of a hemostatic plug." *in* Blood Platelets ed. by Johnson, S.A., Monto, R.W., Rebuck, J.W., Horn, R.C. Little, Brown & Co. Boston, 153-162 (1961).

ROY, R.N., GUHA, B.C. (1958) "Production of experimental scurvy in a bird species." Nature (Lond.) 182, 1689-1690.

SAMUEL, P., SHALCHI, O.B. (1964) "Effect of vitamin C on serum cholesterol in patients with hypercholesterolemia and arteriosclerosis." Circulation 29, 24-25.

SANDBORN, E.B., LEBUIS, J., BOIS, P. (1966) "Cytoplasmic microtubules in blood platelets." Blood 27, 247-252.

SANO, I., KAKIMOTO, Y., TANIGUCHI, K. (1958) "Binding and transport of serotonin in rabbit blood platelets and action of reserpine. Amer. J. Physiol. 195, 495-498.

SANO, I., KAKIMOTO, Y., TANIGUCHI, K., TAKESADA, M. (1959) "Active transport of epinephrine into blood platelets." Amer. J. Physiol. 197, 81-84.

SCHAFFERT, R.R., KINGSLEY, G.R. (1955) "A rapid simple method for the determination of reduced, dehydro, and total ascorbic acid in biological material." J. Biol. Chem. 212, 59-68.

SCHAYER, R.W., KOBAYASHI, Y. (1956) "Histidine decarboxylase and histamine binding in rabbit platelets." Proc. Soc. Exp. Biol. Med. 92, 653-655.

SCHMITZ, H., SCHLEIPEN, T., GROSS, R. (1962) "Freie nucleotide in normalen menschlichen blutplattchen.: Klin. Wschr. 40, 13-15.

SCHULTZE, M. (1865) Arch. f. Mikr. Anat. I, 36 (cited by Tocantins, 1948).

SCHULZ, H. (1961) "Uber die phagozytose von kolloidalem siliziumdioxyd durch thrombozyten mit bemerkungen zur submikroskopischen struktur der thrombozytenmembran." Folia Haemat. (N.F.) 5, 195. Cited by Marcus and Zucker (1965).

SCHULZ, H., WEDELL, J. (1962) Elektronenmikroskopische Untersuchungen zur Frage der Fettphagocytose und des Fetttransportes durch Thrombocyten.: Klin. Wschr. 40, 1114-1120.

SEALOCK, R.R., SCHERP, H.W. (1941) "The determination of keto acids and d-amino acids in biological materials." J. Biol. Chem. 140, CXIV.

SEALOCK, R.R., SILBERSTEIN, H.E. (1940) "The excretion of homogentisic acid and other tyrosine metabolites by the vitamin C-deficient guinea pig." J. Biol. Chem. 135, 251-258.

SEDOV, K.R. (1956) "Prevention and therapy of atherosclerosis with ascorbic acid." Terap. Arkh. 28, 58. Cited by Simonson and Keys (1961).

SENDROY, J., MILLER, B-F. (1939) "Renal function as a factor in the urinary excretion of ascorbic acid." J. Clin. Invest. 18, 135-140.

SHARP, A.A. (1965) "Present status of platelet aggregation." New Engl. J. Med. 272, 89-92.

SIMONSON, E., KEYS, A. (1961) "Research in Russia on vitamins and atherosclerosis." Circulation 24, 1239-1248.

STAHL, E. (1965) "Thin Layer Chromatography." Academic Press Inc. N.Y., London. (Editor).

STEFANINI, M., ROSENTHAL, M.C. (1950) "Hemorrhagic diathesis with ascorbic acid deficiency during administration of anterior pituitary corticotrophic hormone (ACTH)." Proc. Soc. Exp. Biol. Med. 75, 806-808.

STEPHENS, D.J., HAWLEY, E.E. (1936) "The partition of reduced ascorbic acid in blood." J. Biol. Chem. 115, 653-658.

STETTEN, M.R. (1949) "Some aspects of the metabolism of hydroxy-proline studied with the aid of isotopic nitrogen." J. Biol. Chem. 181, 31-37.

STEWART, C.P. (1953) "Scurvy in the nineteenth century and after" *in* Lind's Treatise on Scurvy, ed. by C.P. Stewart and D. Guthrie, University Press Edinburgh, 1953, p 404-412.

STEWART, J.H., CASTALDI, P.A. (1967) "Uraemic bleeding: a reversible platelet defect corrected by dialysis." Quart. J. Med. 36, 409-424.

STITT, C., CHARLEY, P.J., BUTT, E.M., SALTMAN, P. (1962) "Rapid induction of iron deposition in spleen and liver with an iron-fructose chelate." Proc. Soc. Exp. Biol. Med. 110, 70-71.

STROHECKER, R., PIES, H. (1962) "Improved photometric determination of vitamin C in the presence of carbohydrates by the dinitrophenylhydrazine method." Z. Lebensmitt Untersuch. 118, 394-397.

SZENT-GYÖRGY, A. (1928) "Observations on the function of peroxidase systems and the chemistry of the adrenal cortex. Description of a new carbohydrate derivative." Biochem. J. 22, 1387-1409.

TOCANTINS, L.M. (1938) "The mammalian blood platelet in health and disease." Medicine 17, 155-260.

TOCANTINS, L.M. (1948) "Historical notes on blood platelets." Blood, 3, 1073-1089.

TOH, C.C. (1954) "Release of 5-hydroxytryptamine (serotonin) from the dog's gastro-intestinal tract." J. Physiol. 126, 248-254.

TOSTESON, D.C. (1963) "Active transport, genetics, and cellular evolution." Fed. Proc. 22, 19-26.

TULLIS, J.L. (1952) "Separation and purification of leukocytes and platelets." Blood 7, 891-896.

UPRETY, M.C., REVIS, B. (1964) "Elevated temperature studies on stability of ascorbic acid in certain fruit juice and aqueous vehicles." J. Pharmaceutical Sci. 53, 1248-1254.

VALK, A.T., PRICE, H.L. (1956) "The chemical estimation of epinephrine and nor-epinephrine in human and canine plasma. I. A critique of the ethylenediamine condensation method." J. Clin. Invest. 35, 837-841.

VASSALLI, P., SIMON, G., ROUILLER, C. (1964) "Ultrastructural study of platelet changes initiated in vivo by thrombin." J. Ultrastructure Res. 11, 374-387.

VILTER, R.W. (1962) "Principles of Internal Medicine." Ed. by T.R. Harrison. McGraw Hill Book Co. Inc. N.Y., Toronto. Kogakusha Co. Ltd., Tokyo, p 539-541 (1962).

VITAMIN C SUBCOMMITTEE (1953) "Vitamin C requirement of human adults." Med. Res. Council Special Report, Serial No. 280 (1953).

WAGNER, R., MEYERRIECKS, N., SPARACO, R. (1956) "Enzyme studies on white blood cells and blood platelets. V. Dehydrogenase activity." Arch. Biochem & Biophys. 61, 278-290.

WALDI, D. (1965a) "Coating materials for thin-layer chromatography." *in* Thin layer chromatography, ed. by E. Stahl, Academic Press Inc., N.Y. and London, p 29-34.

WALDI, D. (1965b) "Spray reagents for thin layer chromatography." *in* Thin Layer Chromatography, ed. by E. Stahl, Academic Press, N.Y. and London, p 483-502.

WALDO, A.L., ZIPF, R.E. (1955) "Ascorbic acid level in leukemic patients." Cancer 8, 187-190.

WALKER, G.H., BYNOE, M.L., TYRRELL, D.A.J. (1967) "Trial of ascorbic acid in prevention of colds." Brit. Med. J. 1, 603-606.

WALLER, H.D., LOHR, G.W., GRIGNANI, F., GROSS, R. (1959) "Uber den energiestoffwechsel menslicher thrombozyten." Thromb. Diath. Haemorrh. 3, 520-547.

WAUGH, W.A., KING, C.G. (1932) "Isolation and identification of vitamin C." J. Biol. Chem. 97, 325-331.

WEIL-MALHERBE, H., BONE, A.D. (1954) "Blood platelets as carriers of adrenaline and nor adrenaline." Nature 174, 557-558.

WEISSBACH, H., BOGDANSKI, D.F., UDENFRIEND, S., (1958) "Binding of serotonin and other amines by blood platelets." Arch. Biochem. 73, 492-499.

WEISSBACH, H., REDFIELD, B.G. (1961) "Studies on the uptake of serotonin by platelets" *in* Blood Platelets, ed. by Johnson, S.A., Monto, R.W., Rebuck, J.W., Horn, R.C. Little, Brown & Co. (1961) p 393-405.

WHEBY, M.S., CROSBY, W.H. (1963) "The gastrointestinal tract and iron absorption." Blood 22, 416-428.

WHITE, A., HANDLER, P., SMITH, E.L. Editors "Principles of Biochemistry, third edition (1964), McGraw-Hill Book Co. N.Y., Toronto, London. Kogakusha Co. Ltd., Tokyo. p 293.

WILLIS, G.C. (1953) "An experimental study of the intimal ground substance in atherosclerosis.: Canad. Med. Ass. J. 69, 17-22.

WILSON, P.A., McNICOL, G.P., DOUGLAS, A.S. (1967) "Platelet abnormality in human scurvy." Lancet 1, 975-978.

WINTROBE, M.M. (1961) "Clinical Haematology." Fifth edition, Lea and Febiger, Philadelphia, U.S.A.

WOLBACH, S.B. (1933) Amer. J. Path. 9, supplment page 689-700.

WOLPERS, C., RUSKA, H. (1939) "Strukturunter suchungen zur blutgerinnung." Klin. Wschr. 18, 1077-1081.

WOODRUFF, C.W. (1964) "Ascorbic acid" *in* Nutrition, Volume II, 1964. Academic Press, N.Y. and London. p. 265-298.

WOODSIDE, E.E., KOCHOLATY, W. (1960) "Carbohydrates of human and bovine platelets." Blood 16, 1173-1183.

WRIGHT, J.H. (1906) "The origin and nature of the blood plates." Boston Med. Surg. J. 154, 643-645.

WRIGHT, J.H. (1910) "The histogenesis of blood platelets." J. Morphol. 21, 263-278.

WRIGHT, J.H., MINOT, G.R. (1917) "The viscous metamorphosis of the blood platelets." J. Exper. Med. 26, 395-409.

WRIGHT, H.P. (1941) "The adhesiveness of blood platelets in normal subjects with varying concentrations of anti-coagulants." J. Path. Bact. 53, 255-262.

WURZEL, M., MARCUS, A.J., ZWEIFACH, B.W. (1965) "Subcellular distribution of serotonin in rabbit platelets." Proc. Soc. Exp. Biol. (N.Y.) 118, 468-472.

YAMADA, E. (1957) "The fine structure of the megakaryocyte in the mouse spleen." Acta Anat. 29, 267-290.

YAVORSKY, M., ALMADEN, P., KING, C.G. (1934) "The vitamin C content of human tissues." J. Biol. Chem. 106, 525-529.

ZAITSEV, V.F., MYASNIKOV, L.A., KASATKINA, L.V., LOBOVA, N.M., SUKASOVA, T.I. (1964) "The effect of ascorbic acid on experimental atherosclerosis." Cor Vasa 6, 19-25.

ZIEGLER, E. (1965) "The redox potential of the blood in vivo and in vitro." Charles C. Thomas, Springfield, Illinois, U.S.A. (1965).

ZIEVE, P.D., GAMBLE, J.L., JACKSON, D.P. (1964) "Effects of thrombin on the potassium and ATP content of platelets." J. Clin. Invest. 43, 2063-2069.

ZILVERSMIT, R.D., MARCUS, A.J., ULLMAN, H.L. (1961) "Plasmalogen in human blood platelets." J. Biol. Chem. 236, 47-49.

ZIMMERMANN, R. (1846) "Uber die Formgebilde des menschlichen Blutes in ihrem naheren Verhaltris zum prozesse der Entzundung und Eiterung." Rust's Mag. Fur die Ges. Heilk. 65, 410. Cited by Davey, M.G. *in* "The survival and destruction of human platelets." Bibliotheca Haematologica, Fasc. 22 (1966).

ZUCKER, M.B. (1947) "Platelet agglutination and vasoconstriction as factors in spontaneous hemostasis in normal, thrombocytopenic, heparinized and hypoprothrombinemic rats." Amer. J. Physiol. 148, 275-288.

ZUCKER, M.B., BORRELLI, J. (1955) "Relationship of some blood clotting factors to serotonin release from washed platelets." J. Appl. Physiol. 7, 432-442.

ZUCKER, M.B., BORRELLI, J. (1962) "Platelet clumping produced by connective tissue suspensions and by collagen." Proc. Soc. Exp. Biol. 109, 779-787.

PUBLICATION OF PAPERS

LANDER, H., KINLOUGH, R.L., DAVEY, M.G., ROGERS, G.B., LLOYD, J.V. (1965) "Studies of the sites of segregation and sequestration of normal and altered human platelets." Comm. VIII Amer. Soc. Hemat. Philadelphia, 1965.

LANDER, H., LLOYD, J.V., SCHULTZ, B.G. (1965) "Studies of platelet survival and behaviour in sheep." Proc. Soc. Clin. Res. 38, 44.

LANDER, H., LLOYD, J.V., KINLOUGH, R.L. (1965) "The effects of catecholamines and of chlorpromazine on the circulating platelet level in sheep." Proc. Aust. Soc. Med. Res. 1, 153.

LLOYD, J.V., LANDER, H., KINLOUGH, R.L. (1965) "The effects of the adenosine nucleotides on the circulating platelet level in sheep." Proc. Aust. Soc. Med. Res. 1, 154.

LLOYD, J.V., DAVIS, P.S., LANDER, H. (1966) "A new method for the estimation of ascorbic acid in blood platelets." XIth Congress of the International Society of Haematology, Sydney, 1966, Abstract of Papers AG12.

LANDER, H., LLOYD, J.V., KINLOUGH, R.L., DAVEY, M.G., ROGERS, G.E. (1966) "Studies of the effects of heat and of N-ethyl maleimide on the sequestration and survival of platelets." XIth Congress of the International Society of Haematology, Sydney, 1966, BE9.

LLOYD, J.V., DAVIS, P.S., EMERY, H.M., LANDER, H. (1966) "The application of a new method for the determination of ascorbic acid to human blood platelets." Proc. Aust. Soc. Med. Res. 2, 36.

LLOYD, J.V., DAVIS, P.S., LANDER, H. (1967) "Platelet ascorbic acid." Proc. Aust. Soc. Med. Res. 2, 110-111.