A CLINICAL STUDY OF TESTS FOR VITAMIN C DEFICIENCY.

A Thesis submitted for the degree of M.D. Edinburgh

by

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C O N T E N T S.

INTRODUCTION.

Although the vitamins are commonly thought to be a comparatively recent discovery, it is of interest to note that as far back as the early part of the 18th century the suggestion was made that some of the diseases affecting human beings might be due to a deficiency of certain food stuffs in the diet. Such excellent accounts of the history of the vitamins, however, appear in two recent publications - "The Vitamins" by Sherman and Smith (1) and the Medical Research Council Report on Vitamins (2) - that no further historical references need be given here. It is sufficient to state that Vitamin C as such was first mentioned in 1915, although an "antiscorbutic vitamine" had been postulated some three years earlier. At that time, however, the vitamin was only known to be some substance present in orange and lemon juice and certain vegetables, which was necessary for the prevention and cure of scurvy: nothing was known of its nature or chemical properties.

During the next fifteen years a tremendous amount of experimental work on Vitamin C was carried out by investigators in several centres, in this country notably by Zilva and his colleagues. This work culminated in the successful isolation of crystalline

vitamin C in 1932, an accomplishment of such merit that a short account of some of the stages through which the work passed, is worth recording. (For fuller details the reader is referred to an excellent account by Zilva (3) and to the two publications already mentioned.)

ISOLATION and IDENTIFICATION of VITAMIN C.

After the discovery by Holst and Frolich in 1912 that experimental scurvy could be produced in guinea pigs, great strides were made in the estimation of the antiscorbutic power of many fruits and vegetables and their juices: this was followed by attempts to concentrate some of these known antiscorbutic substances with a view to isolating Vitamin C. Most workers attempted to isolate vitamin C from lemon juice, which was thought at that time to be the most potent source of the vitamin; although very concentrated preparations were made they were none of them the pure vitamin and most were very unstable. Knowledge of the chemistry of the vitamin was gradually accumulating - for instance most of the early preparations were found to be strongly reducing and it was widely recognised that the vitamin was rapidly destroyed under conditions

of mild oxidation.

An important stage was reached in 1928, when Szent Gyorgi (4) isolated from the adrenal cortex hexuronic acid - a highly reducing compound which he was also able to isolate from oranges and cabbages. He suggested that this substance was "possibly identical with the reducing substance present in active vitamin C concentrates".

No further advance was made for four years, until in 1932 confirmation of Szent Gyorgi's suggestion was provided by three different groups of workers and the isolation of vitamin C became an accomplished fact.

First came the report of Waugh and King (5) that they had isolated from lemon juice a crystalline compound which was active in preventing scurvy in guinea pigs. They found that the properties of this active crystalline substance corresponded with those given for the hexuromic acid studied by Szent Gyorgi and they believed the two substances to be identical.

Almost simultaneously with the publication of Waugh and King's results came a report from Svirbely and Szent Gyorgi (6) that they had found that hexuromic acid prepared from animal adrenal glands was active as an antiscorbutic: they concluded that "vitamin C is a

single substance and identical with hexuronic acid".

Then Tillmans and Hirsch and their colleagues (7) who, with the aid of an indicator 2:6 dichlorophenol indo-phenol, had been working out the reducing capacity of lemon juice and other fruit and vegetable juices, stated that "from the parallelism between the vitamin C content of the juices and their reducing capacity, it is concluded that the substance responding to the indo-phenol titration was vitamin C".

It might have been thought that no further proof of the identity of vitamin C was needed, but it was still necessary to prove that the hexuronic acid itself and not some adhering impurity was the antiscorbutic agent. Experimental work with pure hexuronic acid had so far been difficult on account of the scarcity of animal adrenals from which most of it was prepared, so that when Szent Gyorgi discovered that large amounts of hexuronic acid could be obtained from paprika (the Hungarian red pepper plant), further research was greatly facilitated.

In a short while the identity of pure hexuronic acid as the antiscorbutic agency was proved by Szent Gyorgi (8) and (9), whose work was confirmed at his own request by Harris (10) and Zilva (11), to whom he

supplied some of his own hexuronic acid, and by others.

The final confirmation was supplied by the synthesis of vitamin C, the first account of which was published in August 1933 by Haworth and Hirst and their colleagues at Birmingham (12) and (13), followed almost immediately by a report from Rechstein and his colleagues in Zürich (14) of a slightly different method of synthesis.

In the following year Haworth, Hirst and Zilva (15) demonstrated "that the synthetic vitamin showed the same degree of antiscorbutic potency as the natural vitamin", thus completing the chain of events.

In 1933 Szent Gyorgi and Haworth (16) suggested that the name hexuronic acid be changed to ascorbic acid as the vitamin was found to be not properly a hexuronic acid, and because the name hexuronic acid is the name usually applied to a certain group of substances.

In 1935 the Council on Pharmacy and Chemistry of the American Medical Association (17) adopted the name Cevitamic acid as a non-proprietary name for crystalline vitamin C.

CHEMISTRY and PHYSIOLOGY of VITAMIN C.

Knowledge of the chemistry of vitamin C has been greatly increased since the isolation of the vitamin in 1932. Vitamin C is now known to be l-ascorbic acid, an odourless, white or yellowish white crystalline powder, with a melting point of from 189-192°C. It is freely soluble in water, methyl alcohol, ethyl alcohol and acetone; it oxidises on exposure to air and light and is decomposed when heated above 185°C. (This list of chemical properties is derived largely from Wright and Lilienfield (18), whose article gives a full list of references.)

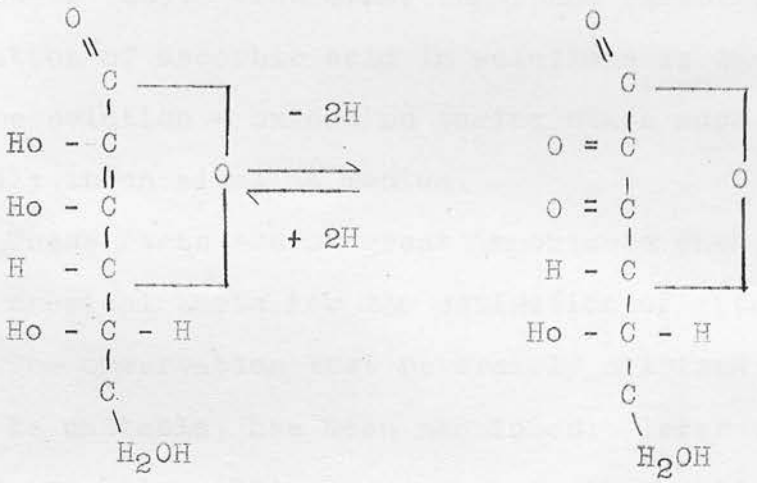
The high reducing power of the vitamin was shown by Szent Gyorgi (8). The acid titration of the vitamin corresponds with the formula $C_6 H_8 O_6$ (Birch and Harris (19)).

Various structural formulae were originally suggested for ascorbic acid but the one now commonly accepted (20) is that shown below.

Tillmans and Hirsch (21) showed that the vitamin could be oxidised by iodine or H_2O_2 to a first oxidation product which was unstable and which could be reconverted to ascorbic acid by treatment with H_2S. This has been confirmed by several subsequent workers and

the first oxidation product is now known as dehydro-ascorbic acid; it has been shown to be active anti-scorbutically to almost the same extent as ascorbic acid (Hirst and Zilva (22)) but to have no reducing power (Berry and King (23)).

The relation between the two forms may be shown thus:-



l-ascorbic acid (reduced form)

l-dehydro ascorbic acid (reversibly oxidised form)

It is often stated that pure crystalline vitamin C is practically stable even in the presence of atmospheric oxygen (e.g. Zilva (3)), a statement which does not seem compatible with the well known fact that vitamin C is readily oxidised by atmospheric oxygen when in solution. The explanation, as pointed out by the same writer, is that the oxidation of ascorbic acid in solution is considerably hastened by the presence of minute quantities of metallic impurities, particularly

copper, which act as catalysts (see also Barron et al (24)). These may exist in the water with which a solution of pure vitamin C is made up, or in the vessel in which the solution is placed (Musulin and King (25)) or they may exist in minute quantities in biological fluids such as urine, a point emphasised by Pijoan and Klemperer (26). The other important factor in the oxidation of ascorbic acid in solutions is the reaction of the solution - oxidation taking place much more rapidly in an alkaline medium.

These facts are of great importance when dealing with chemical tests for the estimation of vitamin C.

The observation that reversibly oxidised ascorbic acid is unstable, has been mentioned: later work by Hirst and Zilva (22) showed that further oxidation may take place beyond dehydro ascorbic acid to a stage which is irreversible and at which antiscorbutic activity is lost. Recent work by Borsook and his colleagues (27) has confirmed these results and goes on to suggest that at this irreversible stage the power of reduction is greater even than that of ascorbic acid itself.

Although this is perhaps not the proper place for such a discussion, a few remarks on vitamin C as it exists in the body will be interpolated here. According to present evidence, it would seem that most of the vitamin C exists in the body tissues and biological fluids as the reduced form (Kellie and Zilva (28)), and that even if dehydro ascorbic acid is given to an individual it will be reduced in the tissues (Johnson and Zilva (29) and Borsook et al (27)). Although the presence of dehydro ascorbic acid has been demonstrated in biological fluids outside the body, e.g. in urine and blood, it is most probable that this has resulted from oxidation of ascorbic acid before the estimation was carried out.

Numerous writers have shown that there exist in various vegetables enzymes capable of oxidising ascorbic acid, e.g. Szent Gyorgi (30), Hirst and Zilva (22), Tauber, Kleiner and Mishkind (31), Kertesz, Dearborn and Mack (32) Hopkins and Morgan (33); and it was shown by van Eckelen (34) that these enzymes were liberated during the process of extraction of vitamin C, being then able to act on the vitamin and cause formation of dehydro ascorbic acid. No definite evidence has yet been offered that similar enzymes exist in animal

tissues or fluids although Roe and Barnum (35) have suggested that a reducing enzyme is present in blood. On the other hand, Barron, Barron and Klemperer (36) have shown that inhibitory mechanisms protecting ascorbic acid from oxidation exist in many biological fluids, including urine: they suggest that the protecting mechanism in the case of the blood serum is glutathione.

PHYSIOLOGICAL FUNCTIONS.

Several functions in animal physiology have been credited to vitamin C but none can be said to be definitely proved: the following short list includes those for which there seems to be some suggestive evidence.

1. Tissue Oxidation. The work of Szent Gyorgi (4) and (30) has suggested that ascorbic acid is of vital importance in tissue respiration, playing the part of a hydrogen transport agent by way of two or more oxidase enzyme systems. This suggestion is supported by later studies which showed that animals with scurvy had a diminished oxygen consumption (Harrison (37)) and that slices of tissue from scorbutic animals had a decreased oxygen content which was increased

when ascorbic acid was added in vitro (Söderström and Törnblom (38)).

2. Regulation of the colloidal condition of intercellular substance: a function suggested by the work of Wolbach and his associates (39) and (40). According to these workers, this function includes maintenance of the health of capillary walls: other writers have suggested that the maintenance of adequate nutrition of the capillary walls is a separate function of vitamin C, e.g. Findlay (41) and Samson Wright (42).

3. Haemopoietic Function. There is now considerable evidence to support the suggestion that vitamin C is necessary throughout all stages of development of the red blood corpuscles, more particularly perhaps at that stage of erythropoiesis at which Hb. is being rapidly assimilated by the ripening corpuscle. The anaemia which may arise in scurvy responds specifically to vitamin C, no other treatment being necessary. (See Parsons and Smallwood (43), Mettier, Minot and Townsend (44), Whitby and Britton (45), and Dunlop and Scarborough (46).)

4. Blood Coagulation. Several writers have suggested that the presence of vitamin C is necessary in normal blood clotting, and that its absence results

in a prolonged clotting time, e.g. Presnell (47) showed that the blood of scorbutic guinea-pigs had a longer clotting time than had the blood of normal guinea pigs, but was unable to state whether this also applied to human beings. Other writers have found no such change in the blood in human scurvy (e.g. Dunlop and Scarborough (46)).

Several reports have been published which claim that the giving of vitamin C may cause a decrease of blood coagulation time in certain pathological blood conditions, such as purpura - for example Kuhnau (48), Böger and Schröder (49), Engelkes (50) - but others have been unable to obtain any similar good results, e.g. Wright and Lilienfield (18).

The existence of a definite relationship between vitamin C and blood coagulation, therefore, still awaits proof.

5. Teeth. The work of Fish and Harris (51) and of Hanke (52) suggests that the vitamin has a specific function in assisting the normal development of the enamel and dentine of the teeth. On the other hand, Wolbach and Howe believe that the development of the teeth is influenced by the function stated under 2. Here again further work will be necessary

before a definite statement can be made.

ORIGIN and DISTRIBUTION of VITAMIN C.

The vitamin is very widespread in plants, practically all fresh fruits and vegetables contain definite amounts of it; it is formed rapidly in sprouting seeds and in all rapidly growing stem and root tips, in green leaves, seeds and pods. The precursor of the vitamin in plant tissues has, however, not been definitely established.

A number of animals, including man, guinea pigs and monkeys, are entirely dependent on their diet for a supply of vitamin C. Other animals, e.g. dogs and rats, are apparently able to synthesise their own vitamin, although no one has yet succeeded in localising the site of the synthesis.

Evidence at present available, including estimations on human post mortem material (53), indicates that vitamin C is present in all tissues of the higher animals, being most abundant in glandular tissues such as the adrenals, pituitary, corpus luteum, pancreas, liver, spleen, etc., but is also found in high concentration in the lens and aqueous humour of the eye.

Both human and cows' milk are known to contain definite but variable amounts of the vitamin.

EFFECTS of DEFICIENCY of VITAMIN C.

I. SCURVY.

The clinical condition resulting from complete absence of vitamin C from the diet for a sufficient period is scurvy - a rare disease nowadays.

In adults the main clinical features of scurvy are weakness, lassitude, a tendency to haemorrhage (into skin, or muscles, or mucous membrane), swelling and bleeding of the gums and looseness of the teeth, often oedema of the legs, and frequently anaemia.

In infants under 2 years of age the condition is often called Barlow's disease and is characterised by irritability, swelling and great tenderness of one or both legs caused by subperiosteal haemorrhage which is most frequent in the femur, occasionally affection of the gums if teeth are present; loss of weight and anaemia are other common findings. The swelling and tenderness may affect the arms instead of, or as well as, the legs; occasionally haemorrhage occurs in or around the orbital cavity and may cause proptosis.

In both adults and infants it is not uncommon for the haemorrhagic tendency to manifest itself as a mild haematuria, in which a few red blood corpuscles are found in the urine on microscopical examination.

The Pathology of Scurvy has mostly been studied in guinea pigs: both in the experimental animal and in man, however, it has been found that the most characteristic changes are those associated with haemorrhage, which is most commonly found in the skin and subcutaneous tissues, and subperiosteally, though it may occur in many other situations: the haemorrhage is always due to bleeding from small vessels and capillaries.

McCallum (54) and also Park (55) have laid stress on the changes in the bones - bone formation becomes almost stagnant everywhere but decalcification continues and the bones become fragile and rarified.

Hanke (52) has stressed the changes in the teeth, which he describes as showing a condition rather like caries.

With regard to the question of haemorrhage, Hess (56) suggested many years ago that "a failure of the integrity of the blood vessels occurred" and that "this was due to a lesion of the endothelial cells and

their cement substance". Wolbach and Howe (39) (40) (57) more recently concluded that the essential pathological change in scurvy is an inability of the supporting tissue to produce and maintain intercellular substances: they believe that the absence of vitamin C prevents the formation of the matrices of white fibrous tissue, bone, cartilage and dentine - thus explaining all the gross lesions of scurvy. They showed in support of this theory that administration of vitamin C by mouth or parenterally was promptly followed by the production of intercellular substance.

II. SUBCLINICAL SCURVY or LATENT SCURVY.

A recognisable clinical condition in infants due to deficiency of vitamin C but not a manifest scurvy, was described by Hess (56) as the result of his observation of what almost amounted to an epidemic of this condition in a home for infants. He called the condition "prescorbutic dystrophy" and gave the typical symptoms as - muddy complexion, stationary weight, lack of appetite, fretful disposition: tachycardia and an increased respiratory rate were also sometimes got: all the symptoms were found to clear up when vitamin C in adequate amounts was given.

Further descriptions of this condition have been given by Frölich (58) and by Rohmer and Bezssonoff (59): Frölich gives a description of one case seen by him and suggests that in addition to the syndrome described by Hess there may be a reduced power of resistance against infection, a tendency to intestinal disturbances and occasionally haemorrhagic symptoms such as haematuria. Rohmer and Bezssonoff draw attention to the anaemia which may exist in this condition. Both these writers found that all the symptoms they described cleared up when vitamin C was given, thus providing support for their diagnoses.

No such definite clinical syndrome has been described in adults, but Öhnell in studying 22 cases of scurvy which he had seen himself, thought that several might have been diagnosed as being in the stage of latent scurvy; he suggested that symptoms associated with latent scurvy are fatigue, mental depression, drowsiness, feeling of oppression, "rheumatic" pains, perhaps anaemia.

Others who have made similar suggestions, for example Mettier, Minot and Townsend (44) and Wright and Lilienfield (18), have also been of the opinion that there may be present certain suggestive symptoms

which if spotted may lead to a suspicion of scurvy before any of the classical signs have appeared.

CIRCUMSTANCES UNDER WHICH SYMPTOMS OF VITAMIN C DEFICIENCY MAY ARISE.

1. Lack of Vitamin C in the diet.

Infantile scurvy usually occurs between the 8th and 12th months, usually in bottle fed babies, very rarely in breast fed infants. It is due to destruction of the vitamins in the milk, either by the process of manufacture if a dried milk, or by boiling or pasteurisation if fresh milk has been used, and also to a failure on the part of the parent or nurse to add any other source of vitamin to the infant's feed. It was shown by Still (61) in 1919 that infants required to have extra vitamin C added to a diet of cows' milk because the vitamin in the milk was mostly destroyed by the process of preparation.

Cases of infantile scurvy occasionally are reported at the age of 15-18 months: these cases are usually due to failure on the part of the parent to provide vitamin C containing foods, either because of ignorance or because of poverty, though sometimes the infant has

been found to dislike the antiscorbutic food (Barlow, (62)).

It is generally accepted that infantile scurvy takes about 4-6 months to develop - this apparently long time being explained by the supposition that the diet is never completely devoid of vitamin C and that the minute quantities of antiscorbutic present delay the full development of scurvy. Occasionally infantile scurvy is reported as coming on in less than four months and in such cases it is usually found that some intercurrent infection has precipitated the disease. (Many cases of infantile scurvy are described by Barlow (62) and Park et al (55).)

Cases of adult scurvy were seen in large numbers during the World War of 1914-18 in areas where troops were by some mischance deprived of their antiscorbutic ration for long periods. Apart from such epidemics, cases are now rare but have been described at all ages and in either sex.

A characteristic type of case is that occurring in elderly males who live alone and do their own housekeeping and who, for reasons of convenience or of poverty, take no antiscorbutic foods: such cases are known as "bachelor scurvy" and have been described

recently by, amongst others, Archer and Graham (63), Mettier, Minot and Townsend (44), Dunlop and Scarborough (46).

Cases have also been described in persons who, by reason of food "faddism" or because they disliked certain kinds of food, had been taking inadequate amounts of antiscorbutics (Schultzer (64)).

A number of cases of scurvy have been recorded in persons who have been for long periods on special therapeutic diets which have contained little or no vitamin C: such diets have been prescribed chiefly in cases of peptic ulcer but also in urticaria, colitis and other conditions, and these patients have often been all the time under the care of their doctors who in most cases failed to diagnose the onset of scurvy. Cases of this sort have been described by Öhnell (60), Barling (65), Platt (66) and many others.

Wood (67) has described a case of scurvy in a woman who for ten years had been on more or less rigid milk food diet because of severe recurrent digestive symptoms.

2. Conditions causing interference with the absorption of ascorbic acid.

These may lead to symptoms of vitamin C deficiency.

It was first suggested by Gothlin (68) that the achylia following alcoholic gastritis might cause deficient absorption of vitamin C, as the vitamin was known to keep only in an acid medium; cases which appear to confirm this suggestion have been described by Schultzer (64) and by Dunlop and Scarborough (46).

Persistent vomiting may similarly result in deficient absorption of vitamin C, a striking example of this being seen in a case of scurvy resulting from the vomiting of pregnancy, which was described by Swanson (69).

Wright and Lilienfield (18) have suggested, on theoretical grounds only, that other possible causes of deficient absorption of the vitamin may be.-

Bacterial destruction of the vitamin in the upper gastro-intestinal tract.

Inflammatory changes of the mucous membranes.

Diarrhoeal states of various sorts.

METHODS FOR ESTIMATING THE STATE OF NUTRITION OF THE BODY WITH REGARD TO VITAMIN C.

The need for such methods.

Before going further it is perhaps well to enquire whether there actually exists any need for such methods as are about to be described. The following remarks should help to provide an answer.

In the preceding sections of this paper dealing with the effect of deficiency of Vitamin C it has been shown that scurvy either in adults or infants is nowadays a comparatively rare condition. For the purpose of this thesis, however, the interest of scurvy lies not so much in its clinical appearances as in the multiplicity of factors which may, so to speak, "condition" it. Fundamentally scurvy is due to lack of vitamin C and the large number of ways in which this lack may arise has already been demonstrated. Although in infants the most common cause of scurvy is the destruction of the vitamin in the course of preparation of the milk food, other contributory factors are - a failure to provide any other source of vitamin in the diet, occasionally the infant does not like the vitamin containing food, occasionally orange juice

cannot be given on account of it causing diarrhoea, and other not fully understood possibilities such as the influence of diarrhoeal and vomiting states. In adults scurvy has been shown resulting from bad planning of the diet due to ignorance of the necessity for antiscorbutics, from dislike of antiscorbutic foods, from poverty, from special therapeutic diets and from persistent vomiting: the influence of achlorhydria has also been pointed out and the possible interference of such conditions as diarrhoea and inflammatory conditions of the intestinal canal has been suggested.

The importance of these facts lies in the realisation that although these factors rarely produce a fully developed case of scurvy, they must in a great many cases produce a condition of vitamin C insufficiency. Another very important factor in the production of vitamin C insufficiency is the instability of vitamin C: it has been shown (1) that the cooking or canning of fruits and vegetables causes the destruction of a large proportion of the vitamin C content (although in the latter case the destruction is now known to be avoidable by the employment of canning under anaerobic conditions.). So that under present conditions the main sources of vitamin C in the adult diet must be

fresh fruits and fresh green uncooked vegetables. But it is just those foods which are usually the most expensive and which very large numbers of people have to do without for the greater part of the year. In support of this statement one may well refer to Sir John Orr's findings (70): of the six grades into which he divided the families under review, two groups (those with the smallest incomes) had a very small inclusion of vitamin C containing foods in their diet and another group had a barely sufficient amount - in other words almost 50% of the population were shown to be getting insufficient vitamin C.

Reference has already been made to the condition of subclinical scurvy or latent scurvy, which some writers maintain is recognisable clinically. A careful scrutiny of the symptomatology of this condition, however, suggests that the clinical picture is a very vague one and at best could only be recognised by someone who had had previous experience of such cases. But that there is a widespread tendency to believe that cases of subclinical scurvy do exist is undoubted; Sir Thomas Barlow in his original article on scurvy wrote that "minor degrees of scurvy are not so rare as might be thought", and the more recent opinions of

Hess, Frölich and others have already been quoted. Youmans (71), as recently as January of this year, stated that "it is becoming better recognised that the mild or latent forms of the vitamin deficiencies are more important in practice at present than the fully developed cases". Many others have expressed similar opinions (e.g. Rinehart (72)).

From these remarks it can be seen, therefore, that states of vitamin C deficiency are probably very common and in most cases are not recognisable clinically - hence the need for special tests.

The methods available for estimation of vitamin C nutrition are three in number, namely -

1. Estimation of the strength of skin capillaries.
2. Estimation of the urinary excretion of vitamin C.
3. Estimation of the vitamin C content of the blood.

These will each be dealt with in some detail.

1. METHODS OF ESTIMATING THE RESISTANCE (OR THE STRENGTH) OF THE SKIN CAPILLARIES.

There are essentially two different groups of such methods - in one group the additional strain on the capillaries is provided by a positive internal pressure and in the other group by a negative external pressure.

The "positive pressure" capillary resistance test.

The development of this test began with the work of Hess and Fish (73) in 1914; they were the first to show that a connection existed between scurvy and the state of the skin capillaries. They utilised the phenomenon of Rumpel and Leede, who had found that in some cases of scarlet fever without a rash, minute haemorrhages could be produced in the skin of the bend of the elbow by the application of a compression band around the upper arm. Using a "blood pressure band or tourniquet" Hess and Fish kept up a pressure such that the radial pulse was almost obliterated for three minutes; they considered the test positive when numerous petechial spots appeared on the forearm. They got positive results in the majority of cases of scurvy but did not consider the test specific for scurvy.

Stephan (74) in 1920 was probably the next to employ this test. He did considerable work with it and found that he got positive results in many conditions besides scurvy.

While investigating possible methods of diagnosing latent scurvy Öhnell (60) employed the "Hess test", as he called it. He used the arm band of a Riva Rocci blood pressure apparatus and kept it applied to the upper arm for three minutes at a pressure just low enough for the radial pulse to be clearly felt: a positive result showed haemorrhages in the skin in front of the elbow. He did not detail his results but stated that, like Stephan, he found positive results in many different conditions: he therefore concluded that when the test was being used in the diagnosis of latent scurvy, "the interpretation of the results must be carried out with great care".

The first to place the test on a more standardised basis was Gothlin (68), who employed it as an indirect means of estimating the "vitamin C standard" of individuals. He elaborated a careful technique for carrying out the test and fixed standards by which results might be compared. His apparatus included a rubber tourniquet (or arm band) as used in a sphygmomanometer,

a special mercury manometer with a thin column for more accurate reading and with red marks on the scale at 35, 50, 65 mm. mercury, and a large rubber Politzer bag. The bag is connected by rubber tubing and metal T-piece to the arm band and manometer and is fitted with a special screw compressor so that the desired pressure may be rapidly attained in the system at the beginning of the experiment, and easily kept constant during the experiment. To carry out a test, the patient is put at ease, sitting or lying down, with the arm supported horizontally on the same level as the heart. A circle of 60 mm. diameter is then stamped on the arm with its centre exactly over the front of the bend of the elbow. The tourniquet is applied to the upper arm, with its lower edge at least one inch from the edge of the circle, and is quickly inflated to the desired pressure. This pressure is maintained for fifteen minutes by slight adjustment of the screw compressor when necessary. At the end of that time the pressure is sharply released and when the normal colour of the skin is restored, the enclosed area is carefully scrutinised in a good light for capillary haemorrhages which are counted with the aid of a magnifying glass. In small children a circle of 40 mm.

diameter is recommended, the number of petechiae in such cases being multiplied by 2.25 to correspond to a reading of the larger circle.

Gothlin argues that the pressure applied should be below the diastolic pressure of the patient's brachial artery on the grounds that if the pressure is supra-diastolic there is slight interference with the passage of arterial blood beyond the tourniquet, and that by using an infradiastolic pressure it is possible to apply the same pressure to a series of individuals without having to make allowances for individual variations in arterial pressure. He suggests starting with a pressure of 50 mm. mercury; if no capillary haemorrhage follows, another test is made on the same arm with a pressure of 65 mm. mercury. "If the 50 mm. mercury pressure has produced between 1-4 petechiae, no further trial is necessary as the limit of strength is then somewhere around 50 mm. mercury. If there are more than 4 petechiae at the pressure of 50 mm. the other arm should be tested at 35 mm., and if at least 2 petechiae are plainly visible at this pressure, then the limit of strength is already passed at 35 mm." He records four grades of results:-

Grade I. Petechiae do not appear below 65 mm. and not even then.

Grade II. Petechiae at 50 mm. but not more than 4.

Grade III. More than 4 petechiae at 50 mm. but none at 35 mm.

Grade IV. Petechiae - at least 2 - at 35 mm.

and states that "in the Nordic race a test resulting in Grade III indicates an undoubted though not pronounced reduction, in Grade IV a pronounced reduction to below the normal strength of the cutaneous capillaries". As the result of further experience with the test, Gothlin (75) was able to establish some further precautions for making the test specific: he found that in order to obtain accurate results it was necessary to ensure that the temperature of the room should be between 16°C. and 21°C., that the subject must not have had a hot bath on the day of examination nor done anything energetic within the preceding three hours, and that in winter subjects must have been indoors long enough not to feel in the least cold. As various acute infections, such as measles and scarlet fever were found to cause a long persisting reduction in capillary resistance, it was found necessary to make sure that no person being tested had had any acute infections disease within the preceding two months. He finally concludes that at a pressure of 50 mm.

mercury cases showing 5-8 petechiae suggest transitional cases, 4-5 petechiae being fairly normal and 7-8 rather subnormal, "but these conclusions cannot be definite owing to the limits of error of the method".

He suggests that it is necessary to allow at least two weeks to elapse before repeating a test on the same arm.

At the time of its inception and during the next three years the method was used by Gothlin and other Scandinavian workers (e.g. Gedda (76), Falk Gedda and Gothlin (77), Nordenmarsh (78)) to examine large groups of school children and of University students. A considerable number of children in a school North of the Arctic Circle were found to show reduced capillary resistance and many of these children had a diet which was deficient in vitamin C. Another investigation revealed a "mutual relationship between the mean capillary strength and the mean vitamin C intake".

In another series of cases a seasonable variation was found, resistance being generally weaker in winter and early spring and being stronger in summer and autumn. In some cases of lowered capillary resistance it was found possible to restore normality by feeding ascorbic acid.

The method of testing capillary resistance just described and henceforth known as the "Gothlin technique" has been employed by numerous workers since it was first introduced: a short note of some of the principal results follows.

Stocking (79) used the method to examine a group of normal children and found that her results fell well within the normal standards given by Gothlin. She believes that the test is practical for determining individual standards for vitamin C.

Schultzer (80) has employed Gothlin's technique in examining several groups of adult hospital patients: he has published his results at considerable length and may be jusitfiably regarded as an authority in the use of the test. He found reduced capillary resistance in about 20% of ordinary hospital medical cases and considered that a large number of those with reduced capillary resistance were suffering from vitamin C deficiency. In another series of cases he was able to produce a lowering of capillary resistance by feeding with a diet low in vitamin C. Some but not all cases of reduced capillary resistance had their resistance increased after administration of vitamin C. He finally concludes that lowered capillary resistance

should only be considered an index of vitamin C deficiency in those cases in which the resistance can be improved by giving vitamin C.

Greene (81) examined groups of healthy children who had been on quite satisfactory diets and also a group of children whose parents were on poor relief and who were getting a diet poor in fresh fruit and vegetables. He found that the percentage of positive results was almost as great in the healthy, well nourished children as in those on inadequate diet: he also tested some of the cases on several occasions and found great variation from time to time, and also great differences in the readings of the two arms in individual cases. He concluded that "a positive reaction to the capillary test does not necessarily denote an insufficient vitamin C intake".

The test was used in cases of infantile scurvy by Goettsch (82), who found the results positive in only four out of eight cases and negative in the other four. During the healing stage the test fluctuated from positive to negative and back to positive although the technique was constant throughout. She therefore concluded that the test was "not reliable as a measure of the scorbutic process in infants".

The results of a study undertaken at the request of the late A. F. Hess were published by Molitch (83), who examined over 400 boys in the New Jersey State Home for Boys, whose diet Hess had estimated to be adequate in all respects, especially in antiscorbutic foods. Molitch found a low incidence of positives, a fact which he thought was due to the adequate diet: retesting without treatment proved the test to be consistent in its results. He concluded that "although we do not consider the test specific, we nevertheless do think it is the best available method for the recognition of subclinical scurvy".

The results which were obtained by Harris and Ray (84), using the Gothlin method, were found to agree with the results obtained by their urine titration method and both sets of results agreed with the dietary intake of vitamin C.

In the course of an investigation into a possible connection between vitamin C and rheumatism, Rinehart (72) made use of the test in the examination of most of his cases. He states that many of the cases were living on a diet which was greatly deficient in vitamin C: capillary resistance tests revealed in general low levels (particularly in cases with clinical evidence of recent rheumatic activity).

A considerably modified Gothlin technique was described by Wright and Lilienfield (18). Their modifications were planned to make the test still more standardised and easier to perform, by dispensing with special apparatus. Instead of a special manometer and special compressor they use an ordinary sphygmomanometer with mercury column and rubber bulb inflator: the arm band is applied as before and inflated to a pressure midway between the systolic and diastolic pressures of the individual. They admit that the pressure they use is open to criticism but they affirm that it gives a definite increase of capillary pressure with no venous escape. With such a pressure they had found that the number of petechiae produced over the front of the elbow was very variable, so they chose an area on the inner aspect of the forearm on which to stamp a circle of 2.5 cm. diameter, the upper edge of the circle being 4 cm. below the crease of the elbow. They claim that the distribution of petechiae in such an area at the pressure used is more regular than over the elbow fold. The pressure is maintained for 15 minutes, or for 7½ minutes when the patient finds it too uncomfortable, and the number of petechiae then counted - without using a magnifying glass. They

state that a normal count rarely exceeds 10, that there is a marginal zone between 10 and 20 and that a count above 20 is definitely abnormal. They investigated cases of vitamin C deficiency, of haemophilia and of purpura, and in some cases showing apparent decrease of capillary resistance they were able to increase the resistance by giving vitamin C. These authors "readily admit that other conditions in addition to scurvy may produce or increase the number of petechiae, and also that other factors..... may cause fluctuation: this test is nevertheless..... a valuable aid, not only in the study of these conditions but also as a guide to therapeutic results".

The "negative pressure" capillary resistance test.

The other method for the estimation of capillary resistance is that known as the negative pressure method, of which there are two or three variations. The method consists of applying suction to a small area of skin, thereby creating a negative pressure on the skin capillaries, and measuring the number of petechiae which are produced after a given pressure has been exerted for a limited time, or measuring the amount of negative pressure required to produce petechiae, after it has been applied for a stated time. The

first person to use a negative pressure apparatus was Hecht in 1907 (85), whose instrument consisted of a suction cup connected with a mercury manometer and a suction pump: he, however, used the test only in some of the infectious fevers and not in scurvy. Hecht's apparatus was very cumbersome and subsequent workers have modified it, for example da Silva Mello (86), who devised a very simple portable apparatus with a small syringe-like suction pump and a spring manometer instead of a mercury one. He used this instrument in the diagnosis of "states of capillary fragility".

One of the first to carry out extensive investigations with the negative pressure method was Dalldorf (87). He employed a simple portable instrument of his own design based upon that of da Silva Mello. The suction cup has an internal diameter of 1 cm. and is applied to successive areas on the skin of the outer surface of the arm: suction is applied for one minute each time and the pressure gradually increased, the observer noting the least pressure necessary to produce macroscopic capillary haemorrhages. Dalldorf examined some 300 cases, many of them several times, and came to the conclusion "that capillary resistance as estimated by this method may be used as a criterion of

subclinical scurvy". In a group of children from poor homes the incidence of subclinical scurvy as estimated by this method was found to be very high.

Dalldorf and Russell (88) collaborated to produce some slight improvements in the instrument used, namely the use of a smaller pump and the use of an automatic valve.

In a series of cases they found that "the results further substantiate our observation that the common condition of capillary fragility represents a mild form of scurvy or clinical scorbutus". In cases of increased capillary fragility they got improvement by feeding ascorbic acid.

The method described by Dalldorf has been used by Weld (89), who found great variations in the responses of normal individuals. He also found that several children suffering from scurvy or known to have had no source of vitamin C for weeks had normal resistances. He concluded that capillary resistance determination is not a useful means of determining the state of nutrition with regard to vitamin C.

A negative pressure method for estimating fragility of capillary walls, in which the results were read by the use of a capillary microscope, was described by

Cutter and Marquardt (90). They found the method unsuitable for clinical application, however, and ultimately were able to produce a better instrument, described by Cutter and Johnson (91). The instrument is compact and portable, the suction is supplied by a small electric motor and the pressure measured on a mercury manometer which is capable of very accurate adjustment. The suction cup has an internal diameter of 5 mm. and has a flange to fit more easily on the skin: suction is applied for 30 seconds, and the number of petechiae recorded at different levels of negative pressure. The small aperture of the suction cut was chosen as being the most comfortable and also the most suitable for use in children. The method was used by Abt, Farmer and Epstein (92) in examining children of all ages: they worked out normal values in some 60 cases and found that in infants and children on low vitamin C diet capillary skin resistances were not consistently lower than normal. An extensive investigation into skin capillary resistance in normal persons was carried out by Anderson Hawley and Stephens (93), using the suction method with an instrument similar to that of Dalldorf but having a mercury manometer instead of the spring type. These workers

examined 100 normal individuals between the ages of 15-50, and found "a high degree of variation in the values for the capillary resistance": a further study of 100 patients, chosen at random in hospital, gave results which were "without significance either individually or when grouped according to disease condition".

Schultz (94), when investigating capillary fragility in a large series of rheumatic children between 4 and 19 years of age found great individual variation in the amount of negative pressure required to produce capillary haemorrhage. The cases were in two groups, one of which received daily doses of ascorbic acid during late winter and early spring, and he found that "as indicated by tests of capillary permeability the development of subclinical scurvy was prevented in the treated group....."

Lindquist (95), using a suction method in examining 240 children, found that capillary resistance was high during the first few months of life and then fell gradually till it reached a constant level between the second and third years. He was also able to show that capillary resistance varied in different parts of the body surface.

Two reports have been published of comparisons

between the positive and negative pressure methods.

Brock and Malcus (96) compared the two methods in 150 children and decided that the negative pressure method gave more accurate results.

More recently O'Hara and Hauk (97) compared the two methods in four adults who were tested repeatedly over a considerable period, using the method of Gothlin for the positive pressure readings and the method of Dalldorf and Russell for the negative pressure. They concluded that "determination of the capillary resistance by either of the methods used, did not give an adequate indication of the state of nutrition with respect to vitamin C". They found marked variations in response to the capillary resistance tests, not only in different individuals but in the same subjects from time to time. They failed to find any correlation between the results obtained by the two methods.

Discussion of the Capillary Resistance Tests.

Some confusion appears to exist with regard to the use of the terms "capillary resistance" and "capillary fragility". Gothlin and the early workers with the positive pressure method referred to the test as a

means of estimating the strength of the capillaries and talked of a "decreased capillary resistance". Since the introduction of the negative pressure methods, however, it would seem that some workers prefer to use the term "capillary fragility" when talking of decreased capillary resistance, and to infer that when decreased resistance is found on testing then the capillaries are "fragile".

Other workers with each of the methods use the term "capillary fragility" where Gothlin uses the term "capillary resistance". They refer to "tests for estimating capillary fragility", and talk of "increased capillary fragility".

On account of this apparent confusion it has been thought best to adhere, in this discussion and other remarks, to Gothlin's original use of the term "capillary resistance" test and to avoid, as far as possible, reference to capillary fragility.

It has long been known that a tendency to haemorrhage is one of the most characteristic features of manifest scurvy in human beings, and early investigators with experimental scurvy in guinea pigs were able to show that the development of capillary haemorrhages is one of the earliest features of scurvy in these animals.

The recognition of these facts led eventually to the employment of the different methods of estimating capillary resistance which have just been described.

At first these tests were applied as a diagnostic aid in cases of developed scurvy but later they came to be used by different workers in an endeavour to establish the existence of latent scurvy or subclinical scurvy, or hypovitaminosis C.

It has been recognised by most of the workers with these tests that many other conditions besides scurvy (manifest or latent) may give rise to a decreased capillary resistance. Some idea of the variety of factors which may influence capillary resistance may be got from a glance at the following table by Weimer (98), quoted by Dalldorf. He divides the possible influencing factors into two groups:

I. Factors which may directly damage the endothelium.

   a. Poisons, e.g. opium neoarsphenamine.

   b. Toxins, e.g. scarlet fever and diphtheria.

   c. Metabolic products, e.g. acetonaemia.

   d. Scurvy.

II. Factors which may indirectly affect the endothelium or capillary tonus.

   a. Physiological variations - e.g. menstruation.

b. Endocrine disorders.

c. Diseases of spleen and reticulo-endothelial system.

The interfering influence of most of the factors in the above list has since been confirmed by other workers, e.g. Wright and Lilienfield.

Gothlin is the only investigator who has endeavoured to eliminate the influence of these interfering factors and it must be admitted that even his precautions are somewhat inadequate: this is almost inevitable, however, as it is impossible clinically to detect the presence of all the above factors.

With regard to the two different methods of performing the test, the positive pressure methods have been criticised on the grounds that they take a long time to perform and that the same arm cannot be used for retesting under 2-3 weeks; neither of these criticisms is very serious if it could be shown that the test gave accurate results. The negative pressure methods offer several points for criticism. In the first place there are so many variations of the method that comparison of the results of one worker with those of another who has used a different modification is almost impossible. Of all the modifications it may be said that on account of the small size of suction

cup used there appears to be considerable possibility of injury to capillaries by trauma at the edges of the cup. It has been shown (98) that undue stretching of the skin as a result of applying suction is enough to cause damage to the capillaries; some workers have used a flanged cup in an endeavour to minimise the marginal trauma. It has also been demonstrated that considerable variations are obtainable in the results of application of the suction cup on different parts of the body and even on different parts of the arm (95) (97) (98).

With regard to the results obtained with the tests, it is significant that most of the claims for the usefulness of the test come from those who have used positive pressure methods and particularly Gothlin's method. Many of these workers - e.g. Gothlin and the other Scandinavians, Schultzer, Molitch, Wright and Lilienfield and others, most of whom have carried out large series of tests - have concluded that the test is of value as a measure of vitamin C nutrition and that the results of the test are in agreement with the previous dietary intake of vitamin C of the subjects being tested. Of those who have used the negative pressure method, Dalldorf is one of the

few who have reported favourably on their results; many others, including Abt, Farmer and Epstein, Anderson Hawley and Stephens, and Weld, have concluded that the method is not of value as a test for vitamin C nutrition.

Two who reported unsatisfactory results with Gothlin's method were Goettsch and Greene, both of whom only tested a few cases: but in addition to these two, others have reported negative results in scurvy and have also found great variation in the same individual at different times, and differences in the two arms of the same individual tested at the same time.

Conclusions.

From this comprehensive review of the subject it is evident that there is no general agreement that any one of the present available methods of estimating capillary resistance constitutes a reliable clinical aid in the diagnosis of states of vitamin C subnutrition. It is difficult to explain satisfactorily the great differences in the results and in some cases the apparently contradictory results obtained by different workers.

Nevertheless there is considerable evidence to suggest that the Gothlin method may be of some value and that it is at least worthy of further clinical trial.

2. THE ESTIMATION OF VITAMIN C IN URINE.

A number of chemical methods of estimation of vitamin C have been described, some of which have been found applicable to the study of the vitamin C content of urine. Practically all such methods depend on the fact that vitamin C has powerful reducing powers which are made use of to produce colour changes in various indicators.

By far the most commonly used indicator is the dye 2:6 dichlorophenolindophenol, the test with which will now be described in considerable detail. Other methods of estimation will be described later.

The Method of Titration with 2:6 dichlorophenol-indophenol.

This dye is a well known oxidation-reduction indicator which was first introduced by Cohen, Gibbs and Clark in 1924 (99). It was Tillman (100) in 1930 who first pointed out that there is a reducing substance present in natural foodstuffs which can be estimated by titration against the 2:6 dichlorophenolindophenol indicator, and which appears to run parallel with their recorded vitamin C content. In 1932 Tillman and his colleagues (7) showed that this reducing power might

be used as a criterion for separation of antiscorbutic fractions. They found that titration values of extracts were the same as the values of the vitamin C content of the extracts when estimated by biological tests. They eventually showed that the substance causing the reduction of the indicator was, in fact, vitamin C.

The method employed by Tillman (by whose name the indicator is frequently referred to) was to titrate the indicator into a solution of the unknown at a neutral or faintly acid reaction, until no further reduction occurred. (The indicator is blue in alkaline solution, red in acid and colourless when reduced.)

When Harris and Ray in 1933 (101) were trying to find a suitable method for chemical estimation of hexuronic acid, they tried "Tillman's indicator" amongst others. They found that the method of using the indicator as originally described was not satisfactory as reduction occurred with various other substances. They therefore carried out the titration in acid medium and titrated the unknown into the indicator instead of vice versa: by these means they claimed to get more accurate results. Reduced glutathione, which had been found to reduce the indicator in alkaline medium,

did so no longer in the acid medium. Pyrogallol and quinol still reduced the indicator very slowly in acid medium, as well as some other substances such as boiled sucrose or fructose solutions. Birch Harris and Ray (102) (103), satisfied with these modifications, introduced a microchemical adaption of this method which they used for the estimation of hexuronic acid in foodstuffs. The vitamin C was liberated by grinding up the foodstuff with sand and 5% trichloracetic acid, and titrating the extract so obtained against a measured amount of the dye. They did not claim that the method was completely specific as they were aware that free cysteine, for example, might reduce the indicator, but they believed that the results were more accurate than could possibly be obtained by biological methods. They stated that by ensuring an acid medium and by carrying out the test rapidly, they could get "perfectly reliable results with fruits and vegetables as ordinarily dealt with".

The first to apply this method to the examination of urine were van Eekelen and his colleagues (104), whose results were published in August 1933. It had never previously been suggested that vitamin C might be present in urine, in fact van der Walle (105) in 1922 had

tested some normal urine biologically and had failed to show that it possessed any antiscorbutic properties. van Ekelen and his fellow workers found a reducing substance in blood and urine which they thought might possibly be vitamin C because, amongst other things, they found that "people taking more fruit and vegetables showed more reducing substance in their blood and urine than those using more cereals", and that "guinea pigs on scurvy producing diet gradually lost the reducing substance in blood and urine". In a second publication a few months later (106) they confirmed the presence of vitamin C in urine by a biological test - they found that "human urine containing relatively large amounts of the reducing substance showed growth promoting activity in guinea pigs". They also found that intake of a large dose of vitamin C (400 mgm.) caused a variable response in urinary output which they thought probably depended on "the state of saturation" of the individual.

As a result of employing the test to estimate "the excretion of vitamin C in the urine and its dependence on the dietary intake", Harris, Ray and Ward (107) were able, in November 1933, to publish some figures for the normal daily excretion of vitamin C; they also

noted a sharp rise in the concentration of vitamin C in the urine after giving a very large dose of vitamin C to a normal person. Their results encouraged them to suggest that the test might have a possible application in the diagnosis of hypovitaminosis C in human beings. At about the same time Hess and Benjamin (108) were also endeavouring to confirm the suggestion of Ekelen that vitamin C might exist in the urine. These two, whose work had been completed before the results of Harris and the later results of Ekelen were known to them, used the microchemical method of Birch et al; they concluded that under ordinary nutritional conditions vitamin C is not excreted in appreciable amounts in human urine. They stated that, in children, ingestion of relatively large amounts of vitamin C led to urinary excretion after the body stores had been completely saturated.

The same technique was used by Johnson and Zilva (29) in a study of the urinary excretion of vitamin C, as a result of which they concluded that "the urinary excretion of ascorbic acid under normal conditions of existence is variable"; and that "the output of ascorbic acid in the urine is conditioned by the amount stored in the body,i.e. the 'state of saturation' and the

amount consumed in the diet". They also conducted a biological test to establish the presence of vitamin C in the urine. By feeding guinea pigs with urine they were able to show that the urine excreted an antiscorbutic effect equivalent to that of the amount of ascorbic acid which they had estimated to be present in it. They therefore felt justified in ascribing the indophenol reducing property of the urine mainly to the ascorbic acid.

The method became much more widely known after it had been fully described in the Lancet in 1935, by Harris and Ray (84). These authors review the development of the test and give details of the procedure, with all the precautions necessary in carrying out the test. They claim that the method has been shown to be suitable for the diagnosis of vitamin C subnutrition. Subsequent to the appearance of this article the method is frequently referred to by other workers as the method of Harris and Ray.

The method may be shortly described as follows:- Estimations should always be carried out on specimens covering 24 hours at least. In order to avoid loss of ascorbic acid by oxidation, the specimens should either be titrated fresh or be preserved by the addition

of 10% glacial acetic acid - the authors claim that this method of preservation will allow specimens to be kept for 10-12 hours without appreciable loss of ascorbic acid. The dye 2:6 dichlorophenolindophenol, being liable to deterioration, should have been recently standardised and be made out in watery solution at a strength of about .1% so that .05 cc. are approximately equal to .025 mgm. ascorbic acid. The urine is placed in a 2 cc. microburette and enough dye solution is used (usually about .05 cc., measured out accurately by a micropipette) to require between 1.5 and 2 cc. of urine to neutralise it. (It may be necessary in some cases to dilute the urine.) The urine is titrated into the dye solution and the end point is shown by complete discharge of the dye colour, determination of the end point being made easier by comparison with another specimen of the same urine diluted to the same extent. If necessary, the urine may be cleared before titration by filtration after acidification. If the specimen is being titrated fresh, then about two drops of glacial acetic acid should be added to the indicator in order to ensure titration in an acid medium. In other cases where the urine has been acidified before titration, allowance

must be made for this added acid when calculating results. The authors stress particularly the importance of the fact that the titration should be completed within two minutes as substances other than vitamin C also reduce the dye but more slowly.

In a further publication almost a year later, Abbasy, Harris, Ray and Marrach (109) give the results of their recent investigations with the test. With regard to the technique, they now point out that owing to the more rapid destruction of vitamin C when exposed to light, it is necessary to preserve the urine specimens in dark coloured bottles - if this precaution is observed then no significant loss of ascorbic acid is stated to occur up to 12-15 hours. They also suggest that 5% of added glacial acetic acid is adequate for the preservation of urine, instead of the 10% used previously.

The use of the method as an aid in the diagnosis of vitamin C subnutrition does not stop at the estimation of the amount of ascorbic acid in the 24 hours urine excretion. The next step, according to the procedure followed by Harris and his colleagues, is to give a "test dose" of ascorbic acid and to estimate the excretion of ascorbic acid in the succeeding 24

hours. Harris and his fellow workers concluded that a suitable size of test dose would be 100 mgm. for an infant of about eight or nine months and, at the other end of the scale, 700 mgm. for an adult of 10 st., with doses for children graded according to their weight.

These workers used this method in large scale investigations on both infants and adults, including a number of infants suffering from manifest scurvy (84) (109). In support of their claim for the usefulness of the method, they found that infants suffering from manifest scurvy or with a history of vitamin C underfeeding excreted less vitamin C in their urine (measured chemically) than did well nourished infants of the same age tested under the same conditions (on controlled diets low in vitamin C). They also found that in adults a low urinary output of vitamin C and a low response to test doses went parallel with a history of vitamin C underfeeding and with a state of vitamin C subnutrition as indicated by a lowered capillary resistance.

The technique just described has been used by Harris and his colleagues (110) (111) in further extensive investigations, and also by many other investigators,

some of whom have accepted the method in its original form while others have criticised it in various ways.

Some of the points on which opinions differ will now be dealt with.

I. The Specificity of the Method.

It has already been noted that Birch Harris and Ray, in their original work, found that when using the indicator to titrate food stuffs and tissue extracts, etc., other substances such as glutathione and certain phenolic compounds would also cause reduction of the dye.

The reaction between ascorbic acid and Tillman's indicator is given by Bessey and King (23), and also by King (112), as follows:-

$C_6H_8O_6$ + $O{:}C_6H_2Cl_2 : N.C_6H_4O\,H$ gives

$C_6H_6O_6$ + $HO.C_6H_2Cl_2.NH.\ C_6H_4O\,H$, a reduction which is theoretically possible by other substances besides ascorbic acid (King (112)).

Substances occurring in urine which might reduce the indicator are glutathione, cysteine, ergothioneine, thiosulphate and possibly others. Glutathione, while it will reduce the indicator in an alkaline medium, has been shown by Birch Harris and Ray (103) to be ineffective in an acid medium - hence the importance of

ensuring titration at an acid reaction. It was Emmerie and van Ekelen (113) (114) (115) who pointed out the interfering action of cysteine, ergothioneine and thiosulphate, and these workers have suggested the following method for removal of these interfering substances before titration:- 20% mercuric acetate is added to the acidified urine, care being taken to avoid excess of the acetate: this precipitates out the interfering substances. The ascorbic acid, however, has now been reversibly oxidised and has to be reconverted to the reduced form by treatment of the urine for some hours with H_2S., which is subsequently removed by bubbling a stream of nitrogen through the urine. This method, however, has not escaped criticism. It was pointed out by Fischer (116), and also by Euler and Malmberg (117) that the heavy precipitate produced by the use of mercuric acetate caused some adsorption of ascorbic acid, and that subsequent readings were too low. Ahmad (118) pointed out that the method required considerable time and he also suggested that the prolonged treatment of the urine with H_2S. upset the final titration: a similar suggestion was made by Gabbe (165). In a later article, van Ekelen and Emmerie (119) pointed out that in using their method it was important to

realise that mercuric acetate precipitation causes oxidation of ascorbic acid and that if the next stage is not carried out rapidly, then irreversible oxidation will take place. The precipitate must therefore be centrifuged off, and they specify that the time taken between adding mercuric acetate and passing H_2S into the subsequent filtrate must not exceed 5-10 minutes.

The possibility that there exists in urine a substance which is not ascorbic acid but which is capable of reducing Tillman's indicator and which cannot be eliminated by mercuric acetate precipitation, is suggested by the following observations. Harris and Ray (101), in some of their earlier work in 1933, allude to the fact that Tillman's indicator was reduced by boiled sugar and fructose solutions. A similar production of unknown, or rather unidentified reducing substance had been suggested by Zilva (120) in 1930. The work of von Euler and Martius (121) in 1933 provided an explanation by describing their investigations on "reductone": this substance was obtained by heating glucose with alkali; it was shown to have very strong reducing properties and to resemble ascorbic acid in many ways but it showed no antiscorbutic activity. Rechstein and Oppenauer (122) in 1933 described the

preparation and properties of "reductic acid", which was also referred to by van Ekelen (115) in 1934. Reductic acid was formed in ordinary urine by a process of hydrolysis at high temperature in an acid medium; it was found to contain the characteristic reducing group of ascorbic acid, it would reduce Tillman's indicator and after being oxidised it could be re-generated with H_2S: it could not be precipitated with mercuric acetate and showed no antiscorbutic activity. It had thus been shown that treatment of ordinary urine by special methods could result in an increase of its reducing power due to formation of reducing compounds from substances normally occurring in the urine.

A similar observation was reported by Stewart and Scarborough (123) in 1937. They found that urine after boiling in an inert atmosphere and after prolonged treatment with H_2S, acquired an increased power of reducing 2:6 dichlorophenolindophenol. As a result of their observations and experiments they concluded that part of this reducing power was due to a substance which was not ascorbic acid but which was neither cysteine, glutathione, ergothioneine, glycuronic acid, galacturonic acid nor glucose.

The importance of these observations lies in the fact that since an increase in the reducing power of the urine may be brought about by the production of reductone or reductic acid as the result of special methods of treatment, and since these substances resemble ascorbic acid closely in many respects and are formed from substances which may occur in the urine, it is impossible at present to be certain that such substances as reductone or reductic acid may not under special circumstances occur in the urine or that they may not be formed at some stage of the titration technique. (Ahmad (118) has suggested that after prolonged treatment of the urine with H_2S, as in the mercuric acetate method for precipitation of interfering substances, there is an increase of reducing substances in the urine. Ekelen (115) has suggested that reductic acid may be formed from glycuronic acid, which may be present in normal urine.)

II. The need for special methods of removing interfering substances from the urine before titration.

There is considerable difference of opinion on this point. Harris and his colleagues have always admitted that interfering substances do exist in the urine but claim that "while the rapid method of analysis described may estimate small traces of other

substances present in urine as well as vitamin C, this in no way affects its utility as a convenient clinical method for investigating the state of vitamin C nutrition of the human subject" (109). Support is given to Harris's view by Youmans and his colleagues (124) and by Sendroy and Schultz (125), who found that other substances which may reduce the indicator do so at such a slow rate that their effect is rendered negligible by rapidity in titration. Harris's technique has been accepted by many other workers as a satisfactory method of investigating "vitamin C nutrition".

On the other hand Emmerie and van Ekelen and their colleagues have from the first stressed the importance of eliminating interfering substances from the urine. van Ekelen (115) was also able to show that even small quantities of thiosulphate (which may occur in normal urine) would rapidly reduce the indicator in acid solution. Archer and Graham (126) are inclined to the view that "the method of estimation (by Harris's technique) is disturbed by the presence of small quantities of other reducing substances in the urine, but we have no evidence as to their nature. When little ascorbic acid is present in the diet the error caused by the reducing substances may be serious."

That there is a definite need for special methods for ensuring a more specific titration is shown by the following facts. Ahmad (127) and Chakraborty and Roy (128) independently announced that high protein diets (with and without excess of meat) produced an increase of the ascorbic acid content of the urine. The method used by each was that of Harris and Ray (84). These results were very soon criticised by Heinemann (129) (130) (131), who referred first to the work of van Ekelen (132) (115), whereby it had been shown that thiosulphate, which might exist in ordinary urine and could be removed by the mercuric acetate precipitation method, would reduce Tillman's indicator. van Ekelen had also stated that an increased excretion of thiosulphate might be expected after the consumption of much meat, or in diseases with an increased protein metabolism. Heinemann repeated the experiments of Ahmad and of Chakraborty and Roy but was unable to confirm their results. He concluded that their different results were due to the fact that "these authors did not remove interfering reducing substances", and that the increased reducing power was due to thiosulphate and not to ascorbic acid.

Estimates of the amount of "non-specific reducing

titre" have been made by a few workers - using the van Ekelen method. Harris (109) estimated that this did not "normally rise above an equivalent of 3-6 mgm. (of substances other than vitamin C) per day in the average case excreting a total of about 20 mgm. of vitamin C per day". On the other hand van Ekelen (133) has stated that "after mercuric acetate precipitation of normal human urine about one half of the amount of reducing substance is found to be vitamin C - the other half must be attributed to other reducing substances", and has given comparative figures of the results obtained before and after mercuric acetate precipitation, in confirmation of this statement. Heinemann (131) also published figures which showed that after removing interfering substances, the total reduction figure had been reduced by about one third. Borsook (27) states that "in urine only a fraction of the reducing material titratable with 2:6 dichlorophenolindophenol is ascorbic acid". It is thus evident that the reducing substances other than ascorbic acid in urine represent a very important factor in these titrations. There is considerable variance in the estimates of the amount of these non-specific reducing agents which are present in normal urine and for this

reason it would seem necessary to have some reliable method either of estimating these substances separately or of eliminating them before carrying out the titration, in order to get an accurate estimate of the amount of ascorbic acid present.

III. The method of preserving the urine.

In their original work Harris and Ray (101)(107) used trichloracetic acid as a preservative of the urine specimens, but they later suggested (84) that 10% glacial acetic acid "gave better results". It has been shown by Fujita and Iwatake (134) that trichloracetic acid is unsatisfactory in these estimations as in its presence the indicator is liable to fade, being apparently reduced: this finding was confirmed by Guha and Gosh (135) and later by Ahmad (118), who noticed that trichloracetic acid was particularly unsatisfactory when the concentration of vitamin C was low.

The necessity for storing the urine specimens in dark bottles owing to the loss of ascorbic acid as the result of the action of light was pointed out by Harris (109) and later confirmed by Wright (136).

Various workers have suggested the use of acids other than glacial acetic as preservatives for the

urine, claiming better preservation of the ascorbic acid content. Fujita and Iwatake (134) suggested the use of 2% metaphosphoric acid in place of trichloracetic acid and Musulin and King (25) followed this up by stating that the addition of 2% metaphosphoric acid to 4-8% of glacial acetic acid was the most effective method as the metaphosphoric acid helped to protect the vitamin C against oxidation in the presence of atmospheric oxygen. These workers also claimed that the metaphosphoric acid would prevent oxidation of the vitamin C even in the presence of small quantities of copper. Johnson and Zilva (29) claimed that when urine was collected under sulphuric acid the ascorbic content was stable for several days; this claim was supported by Youmans and his colleagues (124) and by Emerson and Daniels (137). Chopra and Roy (138) compared several methods of preserving the urine specimens and found that while none was entirely satisfactory, the titration readings after using 10% acetic acid were lower than with any of the other methods, this result suggesting that the acetic acid was the least effective preservative of those tried.

Sendroy and Schultz (125) stated that the most important factors in preservation of ascorbic acid in

urine were oxygen tension, temperature, and pH.; they found that little loss of ascorbic acid occurred if oxygen was excluded from specimens and they were kept on ice. They kept their specimens in small bottles fitted with special stoppers which ensured the exclusion of oxygen: these bottles were then placed in a refrigerator. The slower rate of loss of ascorbic acid from specimens kept on ice was confirmed by Wright (136).

Most of the workers who have used preservatives other than glacial acetic acid have done so because they found that the glacial acetic acid was not satisfactory. No generally accepted alternative has been discovered, however, although sulphuric acid and metaphosphoric acid have strong advocates.

IV. Standardisation of the indicator solution.

It is acknowledged by all that the indicator solution becomes gradually decolorised on standing and that it must therefore be periodically standardised. Harris and Ray state that the indicator solution should be standardised "frequently" against a known solution of ascorbic acid, which in its turn is standardised by iodine titration. Bessey and King (23) were satisfied if their dye solution was standardised every fifth

day; others, for example Archer and Graham (63), Youmans et al (124) suggest that standardisation every third day is necessary, while others such as Hess and Benjamin (108) and Sendroy and Schultz (125) are of the opinion that standardisation every second day is necessary.

It seems reasonable to conclude that it would be unwise to rely on any dye solution which was more than three days old, without standardisation.

V. The "Test Dose".

The use of the test dose method is based on the following findings. Harris and others of the early investigators had noticed that excretion of vitamin C in the urine went on for some time after all vitamin C containing foods had been excluded from the diet: as it was known that human beings could not synthesise the vitamin, it was concluded that they must possess stores or reserves of the vitamin in their tissues. Several of these workers had also noticed that in the normal person on adequate diet administration of a large dose of vitamin C was followed by an increased secretion of the vitamin in the urine: it was supposed that in such cases the body reserves were high or that the tissues were well "saturated" and that the excess

of vitamin had not been needed and hence was excreted into the urine. There thus arose the method of giving a test dose of vitamin C and estimating the effect of this on the output of vitamin C in the urine during the succeeding 24 hours.

Harris (109) suggested giving a test dose on the basis of 700 mgm. for a 10 stone man: if one test dose resulted in no increase in excretion of the vitamin, then a second or even a third or fourth test dose should be given on successive days until a response was obtained.

Archer and Graham (126) have suggested that after a preliminary 24 hours estimation the individual should be given 1000 mgm. of ascorbic acid on each of two successive days, followed by a dose of 400 mgm. on the third day, the percentage excretion of this final dose forming the chief criterion of the state of saturation of the tissues - a satisfactory excretion, according to the authors, consisting of 75% or more of the final dose.

Sendroy and Schultz (125) have recommended the giving of a daily dose of 250 mgm. ascorbic acid for seven days and noting the day on which a response to the test dose was elicited, i.e. when a rise in the excretion occurred. Youmans and his colleagues (124) used a test dose usually of about 600 mgm. in an adult

and suggest that a satisfactory excretion is anything over 30% of this dose in the succeeding 24 hours.

Other Methods of Estimation of Vitamin C in Urine.

1. Grace Medes (139) suggested the use of phospho 18 tungstic acid as the titration reagent. She claimed that this method gave speedier and more accurate results and was more simple to use than the method of Harris and Ray. Her technique consists of taking 5 cc. of urine to which is added 1 cc. of formaldehyde solution, then to the mixture is added 6.5 cc. of a sodium acetate buffer solution and 1 cc. of Folin's phospho 18 tungstic acid reagent. The resulting colour is read in a colorimeter by comparison with standard colour tubes equivalent to known amounts of ascorbic acid. While her method receives occasional mention by other writers, no other worker appears to have made use of it in any published work. In a later article (140) Medes herself admits that estimations by her method are inaccurate at low concentration of ascorbic acid - a fact which had been pointed out by critics of the method.

2. The use of the Bezssonoff colour reaction is

described in an article by Rohmer and Bezssonoff in 1935 (59). This colour reaction had been introduced nearly ten years previously in relation to the vitamin C in fruit juices and was first applied to the estimation of vitamin C in urine in 1934.

Without going into details, it may be stated that the reaction is obtained by adding one drop of a solution of monomolybdophosphotungstic acid to 10 cc. of urine which has been rendered quite clear by treatment with lead acetate, glacial acetic acid and sodium sulphate. The resulting colour reaction is compared with that of a series of tubes containing different quantities of hydroquinone, to each of which one drop of reagent has been added. The "Bezssonoff reagent" gives a violet colouration in the presence of vitamin C, hydroquinone, and other substances, and it is claimed that if the solutions of the hydroquinone standard are prepared according to directions then an estimate may be obtained of the amount of vitamin C in the urine.

Later workers used this method but found it unreliable as other substances also gave the same colour changes (see, e.g., von Euler and Burström (141) and Hess (142).

3. The use of iodine as the titration reagent was tried by Tillmans, Hirsch and their colleagues (7) and later by Harris and his colleagues (101). Both these groups of workers, however, discarded the method as being unsatisfactory on account of the number of other substances which would reduce the indicator. Since then, however, a method of titration using iodine, has been developed by Schroeder (143), who claims good results for it.

4. Robertson (144) has described a method of spectroscopic detection and estimation of vitamin C - a method which is only occasionally referred to in the literature.

There are several other more recent methods of estimating vitamin C, not all of which have as yet been used for testing urine. Amongst these may be mentioned the following:-

5. A ferry-cyanide titration method was described by Tauber and Kleiner (145).

6. Szent Gyorgi (146) suggested the use of a colour reaction with ferrous sulphate exposed to air - a reaction which depends on the formation of a vitamin-Fe complex.

7. Martini and Bonsignore (147) reported that methylene blue was a more specific indicator for ascorbic acid than indophenol.

8. An oxazine dye - "prune" - was used by Melville and Richardson (148) for direct titration of vitamin C.

9. Roe (149)(150) introduced a method of determining ascorbic acid as furfural and claimed a high degree of specificity for his method.

These last five methods have only been mentioned because they show that a considerable number of investigators have been dissatisfied with the present available methods of estimating vitamin C and have sought to produce a better method. While each has made great claims for his own particular method, none of the methods has received much recognition and most have been adversely criticised by others who have used them.

Discussion of the Methods of Estimation of Vitamin C in Urine.

I. Preliminary remarks.

1. Existence of vitamin C. in urine. At this point it is pertinent to enquire whether the existence of vitamin C in the urine has been definitely proved.

It must be admitted that no one has as yet been able to isolate vitamin C from urine. On the other hand two workers were able to demonstrate that urine, when fed to guinea pigs, exerted an antiscorbutic activity which in one case was equal to that of an amount of vitamin C which the urine had been found to contain by biochemical estimation.

On the basis of these findings other workers have accepted the fact as proved, that vitamin C exists in urine. (The failure of van der Walle to demonstrate any antiscorbutic activity in urine has been explained on the ground that the concentration of ascorbic acid in the amount of urine he used was too small to permit of it giving protection against scurvy, and that he did not take sufficient precautions to protect the ascorbic acid in the urine from oxidation.)

2. The excretion of vitamin C. The fact that the knowledge of the factors governing the excretion of vitamin C in the urine is incomplete has already been commented on, but may well be amplified here. It is generally accepted that the amount of excretion of vitamin C in the urine depends on at least two factors - (1) the intake of vitamin C and (2) the state of saturation of the tissues (or the amount of vitamin C

reserves in the tissues). It has been noticed, however, that in none of the reported cases in which large doses of vitamin have been administered to individuals has it been possible subsequently to recover the whole of such a dose from the urine, no matter how well "saturated" the individual had been. No one has offered a satisfactory explanation of what has happened to the rest of the amount of vitamin C taken: some of the suggestions made are that it may not have been absorbed, that it may have been excreted by paths other than through the kidney, that destruction of vitamin may have taken place in the bladder, or that chemical changes may have occurred during metabolism. Failure of absorption is probably more likely to occur if a test dose of the pure vitamin in tablet form has been given. A few records have been made of the vitamin C content of the saliva (151) and of the sweat (152) but so far the amounts excreted by these channels are thought to be very small.

3. The utilisation of vitamin C. While not much is known of the mechanism governing the utilisation or the metabolism of vitamin C, it is important to note that there appears to be an increased utilisation of vitamin C in cases of pyrexia, and of various

infections and intoxications. The importance of this from the point of view of this work lies in the fact that in such cases tests of excretion and saturation may give low readings.

It has been known for a very long time that infection and scurvy are often associated and it has often been noticed that the onset of an infection might precipitate the appearance of symptoms of scurvy. It was Schroeder (143) who was the first to suggest that the need for vitamin C was greater during certain diseases (amongst which were typhoid, cystitis, tuberculosis and others). Shortly afterwards, Harde, Rothstein and Ratish (155) found that the urinary output of vitamin C (especially after test doses) was very much reduced in cases of pneumonia. Later, work by Harris and his colleagues (111) showed a reduced excretion of vitamin C in cases of juvenile rheumatism and of active surgical tuberculosis, while Hasselbach (156) and Heise and Martin (157) have also shown that patients with tuberculosis had low urinary outputs of vitamin C. Harris's work suggested that a febrile illness such as a mild coryza of short duration, could cause a more rapid using up of vitamin C and a fall not only in the urinary excretion of vitamin C but also in the tissue

reserves. A considerable amount of work has also been done in relation to infections in animals, and post-mortem material has been studied. It has been found in animals also that there appears to be an increased metabolic demand for vitamin C during the course of the various infections studied. (A group of articles has just been published by Harris and his colleagues (158)(159)(160). They show an "increased usage" of vitamin C in cases of osteomyelitis, pulmonary tuberculosis and rheumatoid arthritis, and even suggest that determination of vitamin C excretion under controlled conditions might be used as a prognostic guide in some diseases.)

II. The Methods of Estimating Vitamin C in the Urine.

Several methods of estimating vitamin C in urine have been described and most have been already commented on. While this list makes no pretence of including all the known methods, it does at least suggest that no one method has been found to give universal satisfaction. Most of the methods described have received little general support, while others are of too recent date to be yet widely known.

The only method which has been widely used is that which employs the dye 2:6 dichlorophenolindophenol

as the indicator in the titration of the urine. Harris and Ray and their colleagues have been largely responsible for the development of a technique for the use of this dye and their method has been fully described.

Many difficulties and sources of error in the use of the Harris and Ray technique, which have been pointed out by other workers, have been collected from the literature on the subject and these have already been discussed in some detail, (see page 56 et seq.).

Various methods of improving Harris and Ray's technique have been suggested, e.g., better methods of preserving the urine, methods of restoring dehydroascorbic acid to the reduced form before titration, methods of eliminating interfering reducing substances from the urine before titration, and other manoeuvres, but each of these methods has in turn been criticised and none has yet become firmly established as more accurate than the others.

III. The Results.

The results which have been published by different workers of the average vitamin C output in the urine over 24 hours for alleged normal adults (and children) show very great variations and are consequently very puzzling. Some of these results are worth noting here. Harris

and Ray suggested (107) that the average for adults was about 30-33 mgm.; later they reported (84) that the average on a middle class diet was 15-30 mgm. They found that the output in individuals was fairly constant from day to day on controlled diets.

van Eekelen (106) at first suggested an average of 25 mgm., but later (153) gave the figure as 10-15 mgm. (after removal of interfering reducing substances from the urine). Johnson and Zilva (29) and Youmans and his colleagues (124) found very variable figures in apparently normal individuals - the values ranging from 10-80 mgm. in 24 hours - and they did not find that the excretion was at all constant from day to day, even when the intake during the experiment was controlled.

These are only a small number of the results which have been published but they show the difficulty of attempting comparisons of the results of different workers. The results suggest that the 24 hours output is very variable from one individual to another and until more work has been done on the subject, only very approximate figures are at present available. (An apparently very contradictory conclusion by Hess and Benjamin (108), to the effect that under ordinary

circumstances vitamin C is not excreted in appreciable amounts in human urine has been explained by different workers as due (1) to the fact that their subjects had had a deficient intake of vitamin C (124), and (2) to error in the technique (154).)

While several different workers have been able to show that if an individual is kept on a vitamin C free or vitamin C low diet for several days, the daily urinary output of vitamin C will become stabilised, it is more important to know whether the output in any 24 hour period after one or two days dietary control gives any indication of the state of vitamin C nutrition. Harris claimed in his early work that the 24 hours output of vitamin C on a controlled diet gave an index of the state of vitamin C nutrition, that the giving of a test dose brought out more clearly the state of saturation in doubtful cases, and that the response to the test dose was always in proportion to the figure for 24 hours excretion; in his later work he has claimed that the giving of a test dose is unnecessary if the average of two or three days excretion is taken.

These claims have not been confirmed by any other workers, however, and seem scarcely acceptable until more definite limits for "normal values" of excretion

can be fixed. Most other workers appear to lay more stress on the results of administration of test doses, taken in conjunction with the figures of ordinary 24 hours excretion.

On the other hand, Harde and his colleagues (155) found that when they examined some cases of pneumonia they found that excretion figures might give normal results when tests of saturation showed that the tissue reserves were very low. Perry (174) was of the opinion that ordinary 24 hour excretion figures were not reliable and he drew his conclusions from the results of test dose administration only.

The methods of giving test doses vary considerably and make comparison of results difficult. The amounts of the test doses given by different workers have been chosen more or less arbitrarily and until more is known of the process of saturation, it is difficult to form any opinion as to the best method of giving a test dose.

IV. Conclusions.

1. Of the many available methods of estimating vitamin C in the urine, that which uses the dye 2:6 dichlorophenolindophenol for the titration has been most universally used.

2. As a result of this study, the writer has come to the conclusion that there is insufficient evidence to support the claim of Harris and Ray that by using their technique it is possible to make accurate quantitative estimations of the amount of vitamin C in the urine. The following are some of the reasons which have led to this conclusion:-

a. The lack of certain knowledge as to the factors governing the excretion of vitamin C.

b. The lack of certain knowledge as to whether or not any dehydro-ascorbic acid as such is excreted in the urine (dehydro-ascorbic acid does not reduce the indicator and its presence results in low readings).

c. It has been shown (by Harris and also by Wright) that even if urine specimens are acidified with glacial acetic acid and stored in dark glass bottles, at the end of 12-15 hours there may be a loss of 20-30% of the ascorbic acid content; as specimens are usually only titrated twice in 24 hours, it is obvious that there must always be some loss of ascorbic acid from some of the specimens which have been kept for longer periods.

d. The defects of 10% glacial acetic acid as a preservative of the urine have been already pointed out and introduce a factor of uncertainty.

e. The possibility of other reducing substances interfering with the titration is probably the most important point of all. Harris's claim that "if the titration is carried out in acid medium and finished within two minutes, then no other reducing substance interferes" can scarcely be accepted in view of Eekelen's demonstration of the rapid reducing action of thiosulphate (which may

occur in urine), of the error into which Ahmad and Chakraborty and Roy fell when working with high protein diets, and of the possibility that other unidentified reducing substances may exist in urine. The large proportion of the total reducing substances in urine which may consist of substances other than ascorbic acid has been pointed out by several workers.

3. That the method, while not being acceptable for the provision of accurate quantitative results, does give results which are quite acceptable for making qualitative comparisons between individuals or groups of individuals is amply shown by the results not only of Harris and his colleagues but also of many other workers who have used the method in its original, or in a modified form. It has been shown repeatedly, for example, that the titre is very low in cases of scurvy and that tests of saturation show a state of great unsaturation in these cases: the improvement in the figures of excretion and saturation after cure of scurvy has also been demonstrated. The poor figures of excretion and saturation shown by subjects experimentally placed on vitamin C free diets for prolonged periods have also been described. The claims made by Harris and Ray and colleagues for the accuracy of their results are very confident and the figures which they show are very convincing; nevertheless the fundamental

faults of their technique cannot be set aside, although the amount of error may be fairly constant throughout.

4. Despite some evidence to the contrary, it seems advisable in every case to give a test dose or test doses as well as to estimate the vitamin C output during one period of 24 hours.

(Since this work was completed there has appeared an article by T. Meuwissen and E. Moyons in the Acta Brev. Neerl., VII, 92, 1937, which describes the isolation of vitamin C from normal urine. This appears to be the first description of the actual demonstration of vitamin C in urine.)

3. THE ESTIMATION OF THE ASCORBIC ACID CONTENT OF BLOOD.

A number of articles have quite recently been published which deal with the estimation of ascorbic acid in blood. The first to suggest that vitamin C could be detected in human blood were van Ekelen and his colleagues (104)(106) in 1933, whose work has already been referred to (see pages 49 and 50). They concluded that the reducing substance they found in blood and urine was vitamin C. The technique they employed, as described by Emmerie and van Ekelen (114), consisted of treating newly shed blood with 10% trichloracetic acid to precipitate the protein, and then removing interfering reducing substances with mercuric acetate, as in the method for clearing urine, recovering the ascorbic acid with H_2S, removing the H_2S with nitrogen and subsequently titrating with 2:6 dichlorophenolindophenol. van Ekelen at first thought the ascorbic acid in blood was present in the reversibly oxidised form, but he later agreed with Kellie and Zilva's conclusion (28) that it was almost certainly present in the reduced form. van Ekelen (161) also pointed out that ascorbic acid was present not only in the plasma (in the reduced form) but also in the

erythrocytes. He concluded that the quantity of ascorbic acid in the blood and the amount in the urine depend on the intake of vitamin C and the amount stored in the body, and that after a certain level of saturation is passed, surplus excretion occurs in the urine.

Heinemann (162) used van Ekelen's method to confirm his statement that ascorbic acid is present in the erythrocytes as well as the plasma and suggested that the variations in the plasma content tended to follow more closely the variations of ascorbic acid in the whole blood than did the variations of the content in the erythrocytes.

A slight modification of the method of Emmerie and van Ekelen was suggested by Mirsky Swadesh and Soskin (163), and was subsequently used also by Hawley Stephens and Anderson (164). Both these groups of workers were unable to establish any correlation between the vitamin C content of whole blood, and the subject's intake of vitamin C.

The alternative method of estimating vitamin C in blood is to use only the blood serum or plasma - a method which was first used by Gabbe (165), who stated that in most cases he was able to correlate the values so obtained with the previous vitamin C content of the

diet.

The method most commonly used now is that developed by Abt, Farmer and Epstein (166)(167)(92). Their technique, which is based on Harris and Ray's method for urine, is as follows:- 5 cc. of blood are immediately oxalated, centrifuged and the plasma taken off. The plasma is then deproteinised by the tungstic acid method of Folin and the resulting filtrate is titrated against dichlorobensenoneindophenol, thus giving an estimate of the amount of vitamin C in its reduced form. They concluded that "determination of the reduced ascorbic acid content of the blood plasma by the method described indicates the nutritional state of the body relative to vitamin C". They found that in infants and children on low vitamin C diet, blood plasma readings were low, and suggested that the method would prove useful for the detection of subclinical scurvy. (They shortly afterwards described a modification of the method which involved the use of only .3 cc. of blood (168).) This method has been subsequently tried by several workers, amongst whom might be mentioned Taylor, Chase and Faulkener (169), who found a greatly reduced content of ascorbic acid in the blood serum of ten cases of scurvy when compared

with 33 normals. The method was also used by Greenberg, Rinehart and colleagues (170)(171)(172), who found it rapid and reliable; they showed that the reduced ascorbic acid content of blood serum in normal individuals paralleled the intake of vitamin C. These workers specified that the determinations should be made on fasting blood; for this reason they obtained readings which were somewhat lower than those of Farmer and Abt.

The method has been criticised by Pyoan, Townsend and Wilson (173), who found that ascorbic acid was lost from blood which had stood for more than half an hour, and suggested that it was necessary to carry out the estimations at once.

Discussion.

The preceding paragraphs give a short summary of most of the important published work on the estimation of vitamin C in blood. Here again, as in the discussion on urine, it is necessary to enquire if there is any proof of the existence of vitamin C, as such, in blood. No proof has yet been advanced, no biological test has been performed, and the work has all been done in the belief that the reducing substance in blood

which can be measured by certain titration procedures, is ascorbic acid.

The two methods which have been most commonly used in estimating vitamin C in blood are - (1) the estimation of vitamin C in oxalated whole blood as described by van Eekelen and his fellow workers, and (2) the estimation of vitamin C in blood serum or plasma as described by Abt and his colleagues.

van Eekelen claimed that his method gave accurate and significant results but, using the same method, Abt could find no correlation between the vitamin C intake and the vitamin C content of whole blood - a finding which was corroborated by Mirsky and his colleagues and by Hawley and colleagues. van Eekelen's method has latterly been largely given up in favour of Abt's method.

The method of Abt is simple to perform but it has been criticised by van Eekelen on the ground that no attempt is made to exclude from the titration other interfering reducing substances which are known to exist in blood. Nevertheless, using Abt's method, several workers have reported a definite correlation between blood ascorbic acid and the dietary intake of vitamin C, some have found very low values for blood

acid
ascorbic/in scurvy, several have noted an increased ascorbic acid content of blood after a test dose of vitamin C. Figures have been published which are claimed to show the normal ranges of ascorbic acid of blood serum at different ages and it is claimed that this method may be used instead of, or as a confirmation of, other methods of estimating vitamin C nurtition.

There are still many points to be cleared up before such claims can be accepted. For example, the specificity of the method is not yet accepted by all; there is no unanimity of opinion as to the form in which ascorbic acid exists in the blood nor as to how it is distributed amongst the constituents of the blood; nor is there any accurate knowledge of the fluctuations of the ascorbic acid content of whole blood as compared with the fluctuations in the serum and in the corpuscles; mention has already been made of the possible existence in blood of an enzyme capable of reducing reversibly oxidised ascorbic acid.

It is obvious therefore that methods of estimating blood ascorbic acid are not yet sufficiently well developed to yield any accurate information.

THE CLINICAL INVESTIGATION.

The earlier part of this work has been taken up largely with a review of the development of tests for vitamin C deficiency and a discussion of the relative accuracy and usefulness of each test as judged by the results and conclusions of other workers who have applied them clinically. The next part of the work will be taken up with the results of a clinical study of the tests undertaken by the writer. The main purpose of the study was to obtain some idea of the usefulness of the tests clinically, and also to learn more of the factors influencing the state of vitamin C nutrition; it was hoped in addition that it would be possible, by examining a fairly large mixed group of cases, to find out how far their diets had been adequate with regard to vitamin C.

The Methods Used.

Two methods of estimation of the state of vitamin C nutrition have been used - (1) a method of estimating the strength of the skin capillaries, and (2) a method of estimating the vitamin C content of urine.

1. The method of estimating the strength of the skin capillaries.

In the first part of this work the somewhat conflicting reports on the usefulness of the several available methods of measuring capillary resistance were reviewed and the conclusion was reached that the method described by Gothlin was the only one which appeared to merit further clinical trial. As. however, the writer had been impressed with the greater simplicity of the method described by Wright and Lilienfield (see page 35), he determined to try out their method first. Two objections to this method were discovered almost at once and led to its abandonment: the first was that the pressure demanded - a pressure half-way between systolic and diastolic pressures - caused most of the subjects (children) considerable discomfort after a little time and reduced some of them to tears before the 15 minutes of the test had been completed: the second objection was that while the pressure used certainly produced fairly large numbers of petechiae distal to the tourniquet, it was noticed that the distribution of these petechiae on the skin distal to the elbow was much more variable than the distribution of petechiae over the fold of the elbow; it was consequently

felt that comparison of the number of petechiae in circles of 1 inch diameter stamped on a somewhat arbitrary area "on the inner surface of the forearm" was not likely to yield very reliable results. After a few experimental cases had been examined with this method it was given up and the technique of Gothlin was used instead. In order to avoid the necessity for special apparatus, it was resolved to try out several cases using a mercury sphygmomanometer in its ordinary modern form, in place of Gothlin's special manometer and special inflator; after a little practice and with the co-operation of the patients, little difficulty was experienced in keeping the column of mercury steady at the desired pressure or at least within 2 mm. above or below; as the instrument being used was new, there was no leakage of air from any part of the system, the main cause of variations in pressure being movements on the part of the younger patients. It was noticed that many of the children tested in these preliminary trials appeared to have thin and narrow arms; further investigation, however, revealed that they were quite normal in comparison with others in the ward. As it was found difficult to stamp the necessary 60 mm. diameter circle over the fold of the

elbow without overlapping on to the epicondyles even in the older children, it was resolved to use a circle of 40 mm. diameter in all the cases, irrespective of age or size.

When testing the smaller children, specially made smaller arm bands of a suitable breadth were used.

All the other requirements of the Gothlin technique were adhered to and as these have already been described, there is no need to specify them here (see page 27).

2. The method of estimating the vitamin C content of the urine.

The method chosen was that which uses the dye 2:6 dichlorophenolindophenol as the titration agent, according to the technique of Harris and Ray (84)(109) which was fully described on page 52 et seq. The dye solution was made up from tablets of the dye as supplied by Hoffmann-La Roche. According to the instructions in their leaflet, one tablet (the equivalent of 1 mgm. of ascorbic acid) was dissolved in exactly 50 cc. of distilled water and 0.5 cc. of this solution (equivalent to .01 mgm. of ascorbic acid) measured out by a micropipette for each estimation.

The dye solution was made up freshly at least every third day, sometimes more often if it had all been used up; this ensured a constantly fresh dye solution.

All the specimens of urine were collected as soon as voided and at once acidified by the addition of 10% glacial acetic acid. Throughout the duration of the experiment all the specimens except those collected in the middle of the night, were acidified personally by the writer; the night specimens were acidified by a carefully instructed and reliable nursing sister. The specimens were preserved in dark brown bottles which were tightly stoppered and always kept in a cool shaded place, though not in a refrigerator. In almost all cases the urine was titrated in two 12-hour period collections; in a few cases it was titrated three times in the 24 hours. The technique of titration followed was just as described by Harris and Ray and need not be detailed again. The titration of each specimen was repeated at least twice and sometimes three times and an average of the results taken.

Test doses were given in all cases, usually once, sometimes twice, and the amount of the dose required was estimated (to the nearest 50 mgm.) on the basis of 700 mgm. for a 10 st. man (approx. 70 mgm. per stone). The dose was administered

in the form of tablets of "Redoxon", the form in which ascorbic acid is manufactured by Hoffman-La Roche; these tablets were given to the patients to be swallowed whole in most cases, and followed by a drink of water. Few of the patients had any difficulty in swallowing their tablets and only one or two had to have the tablets crushed into powder before being swallowed.

The Material.

The cases investigated were all in-patients in the Children's Hospital, Sheffield, and were all under the writer's care during their stay in hospital. All the patients examined (except for an infant of 7 months) were in the same ward: none from other wards were examined, partly because the supply of material was found to be adequate and partly because this enabled some of the conditions of the tests to be kept more or less constant throughout.

The ward chosen was occupied mostly by older medical cases, male and female, from the age of about 6 years to the age limit of 13 years, with an occasional convalescent surgical case.

With the following exceptions, practically all the

cases which were in the ward at the start of the investigation and which were admitted during the ensuing time were utilised. The cases which were not utilised were:-

(1) Those which were known to have had scarlet fever or measles or other of the acute exanthems within the previous two months, as such might have upset the readings of the tourniquet test.

(2) Cases which showed any urinary abnormality, e.g. albuminuria, pyuria, haematuria, because of the possible interference with the titration and also in the case of albuminuria because of possible interference with the tourniquet test.

(3) Cases which were so seriously ill that the performance of the tests would have been an unkindness to them.

The total number of cases tested in this ward was 36; some of these, however, had the tests repeated once or sometimes twice at later dates.

In addition, one infant in the baby ward was subjected to the tests, and also three adult males - members of the resident medical staff of the hospital - were examined, making a total of 40 subjects.

The routine of the investigation of each case.

Some of the cases tested had been in hospital for some time, while others had only been in for a matter of some hours; in each case, however, before any tests were carried out, an estimate was formed of the clinical condition of the patient at the time and, if possible, the clinical condition during the past few weeks was also reviewed. The past history with regard to the amount of vitamin C containing foods in the diet was then investigated - the parents (or guardians) of each case were interviewed personally and from their statements an estimate was formed of the adequacy or otherwise of the vitamin C content of the child's diet at home. In the case of patients who had been in hospital for some time it was also necessary to find out if they had been in the habit of refusing any of the ordinary diet or if they had (as occasionally happened) been receiving any additional supplies of fruit from outside sources.

A note was also made of the home circumstances of each case with reference to unemployment, the size of the family and the total income being contributed by different members of the family.

The next step was to commence the estimation of

vitamin C in the urine over a 24 hour period, the appropriate test dose was then given and a further 24 hours estimation made; in some cases where the first test dose gave no response, a second one was administered. As far as possible the 24 hour period for urinary collection was from 9 a.m. - 9 a.m., or 9 p.m. - 9 p.m.

The tourniquet test was always performed as soon as possible after the completion of the urine test - usually within three days.

(Control of the diet of the cases prior to testing was carried out in those cases which had been in hospital for the necessary time. For two days these cases were deprived of their morning and afternoon apple or orange and were not allowed any fruit drinks.)

The estimate of the previous diet.

The estimates of the amount of vitamin C containing foods which the patients had consumed at home were formed from the parents' statements. As the most commonly consumed fruits were found to be apples and oranges, it was decided that a "satisfactory" or "good" diet would be one which contained approximately one apple or one orange daily, with some green vegetable in addition, daily. An equally satisfactory diet of course could be one which contained no green vegetable but more

fruit - or vice versa. It was found that tomatoes were only occasionally consumed and then almost always by those whose diet was already adequate in vitamin C. On this basis the diet was graded as poor, fair, good, very good. The ordinary hospital diet which was provided for all the cases under review contained the following supplies of vitamin C containing foods:- In the middle of the morning and again after tea each child got half an orange of half an apple (more usually oranges); for lunch, in addition to a small helping of potato and some green vegetable, there was always some stewed fruit - usually apples. The type of fruit might be changed occasionally, bananas and grapes being the most usual alternative; fresh tomatoes were an infrequent addition to the dietary. During the summer months of July, August and September, when there was a plentiful supply of fruit, extra rations might be handed round once or twice a week. Some patients were allowed to have extra fruit brought in by their parents, others received large amounts of fruit drinks at some period of their illness (usually in the form of orange juice). These additions have been remarked upon in the clinical notes of the cases concerned, and have been indicated in the tables by a "plus" sign after the usual "Full" in the column for hospital diet.

THE RESULTS.

1. A note on the grouping of the cases for presentation of the results.

The results have been fully set out in the pages that follow but a few words of explanation may be helpful at this point.

The cases have been grouped on a clinical basis taken in conjunction with the estimate of their previous dietary history.

Group I contains essentially cases whose previous intake of vitamin C had been adequate for some time and also who showed no signs of active disease. The general health of these cases was good, most were putting on weight regularly and none showed any raised temperature or increased pulse rate from any cause; none showed any evidence of subacute or chronic infection. It will be seen that the list includes some cases of "convalescent rheumatism" and "convalescent chorea"; reference to the clinical notes will show that these had all been mild cases, none had ever shown signs of an acute rheumatic infection and at the time of the test they were in the later stages of convalescence - almost ready for discharge from hospital. The two cases of chorea - Nos. 13 and 33 - which still

showed some choreic movements at the time of examination, were otherwise well and showed no other evidence of rheumatic infection. Case No. 18 was probably not a case of rheumatism.

Group II contains essentially all the cases which were excluded from Group I. These cases have, however, been subdivided into two further groups - II A and II B.

Group II A contains cases whose previous diet of vitamin C was adjudged to have been adequate but who at the time of testing were suffering from some active disease sufficient to cause physical signs. None of these cases were actually at the time suffering from an acute infection such as pneumonia or acute rheumatic fever but they all showed evidence of some subacute or chronic infective process, e.g. in the lungs or pleura, or throat or ears, etc. Some had slightly raised temperatures or gave a history of recent raised temperatures; in many cases the pulse rate was rapid; in addition, several of the cases were in a state of chronic ill health.

Group II B contains cases of mixed types; some were not suffering from any acute or chronic infective disease process but had a history of definite dietary

deficiency, e.g. Case No. 14; other cases not only had a history of deficient vitamin C intake but also showed some evidence of infective disease at work, or gave a history of a very recent infection such as an acute tonsillitis. The numbers of each type were too small to make further subdivision advisable.

GROUP I.

No.	Age in yrs,	Diagnosis	Length of time been in hospital	Previous diet		Home circum-stances	Ascorbic acid excreted in 24 hours before test dose	% of 1st T.D. excreted in 24 hrs.	% of 2nd T.D. excreted in 24 hrs.	No. of petechiae in circle.
				Home	Hospital					
3A	8	Convalescent chorea	11 wks.	Poor	Full	Poor	7.74 mgm.	49%		1
4A	10	Convalescent subacute rheumatism	8 wks.	Fair	Full	Fair	9.47	35%		1
5	8	Concussion - convalescent	2 wks.	Fair	Full	Good	8.9 $\pm$	40% $\pm$		L.arm 3 R.arm 7
6A	9	Mitral stenosis (no active carditis)	4½ mos.	Poor	Full+	Poor	20.36	63%		R.arm 6 L.arm 3
7	8	Mitral stenosis (no active carditis)	14 wks.	Fair	Full	Fair	9.46	64%		0
8	9	Convalescent chorea	7 wks.	Fair	Full	Fair	13.71	46%		1
9A	10	Convalescent subacute rheumatism	10 wks.	Good	Full+	Good	28.25	61%		1
9B	10	do.	1 day	Good	-	Good	7.58	35%		-
10	13	Mental retar-dation	1 day	Good	-	Good	14.31	58%		0
13	13	Mild chorea	1 day	Very good	-	Good	5.67	42%		1
18	10	? subacute rheumatism	1 day	Good	-	Good	7.62	70%		2
20	10	Habit spasm	10 days	Fair	Full	Fair	9.4	25%		1
28	9	Epilepsy	3 days	Very good	Full	Very good	26.12	68%		0
33	8	Hemichorea	1 day	Very good	-	Very good	16.45	86%		0
38	26	Normal	-	Very good			30.82	68%		1
39	25	Normal	-	Fairly good			22.0	41%		0
40	23	Normal	-	Very good			45.9	82%		0

CLINICAL NOTES of the CASES in GROUP I.

Case 3 A. Chapman, age 8. Admitted 11:5:36.
Tested 29:7:36. Convalescent chorea.

On admission he showed some choreic movements, which speedily improved. At time of test he showed no choreic movements, he was getting up for several hours daily, and was taking full diet and putting on weight steadily. He showed no other rheumatic manifestations.

Home circumstances unsatisfactory, both parents being very careless and neglectful of the child: his diet at home contained little fruit or vegetables.

Case 4 A. Heathcote, age 10. Admitted 4:6:36.
Tested 31:7:36. Convalescent subacute rheumatism.

Continued to have rheumatic pains in legs for about a week after admission, associated with increased pulse rate but no temperature: no other rheumatic manifestation. Improved rapidly and for seven weeks prior to test had not complained of pains and pulse rate had been normal.

Home circumstances: house damp (father also a "rheumatic"): only one other child. Income sufficient but patient not fond of vegetables and only gets fruit every other day.

Case 5. Appleyard, age 8. Admitted 18:7:36. Tested 31:7:36. Convalescent concussion.

Knocked down by tram on day of admission: recovery uneventful with rest in bed. No rise of temperature and no wounds.

Home circumstances: quite good, only one other child. Gets fair amount of fruit and regular vegetables.

Case 6 A. Mallinson, age 9. Admitted 17:3:36. Tested 3:8:36. Mitral stenosis (with no active carditis).

Long rheumatic history, on admission had fast pulse and a few rheumatic nodules. At time of test pulse rate been normal for six weeks, rheumatic nodules not been observed for several weeks, patient's general condition very satisfactory - putting on weight steadily.

Home circumstances poor, five other children, father unemployed; child got little vegetables or fruit. In hospital she received extra supplies of oranges and other fruits.

Case 7. Wallace, age 8. Admitted 4:5:36. Tested 10:8:36. Mitral stenosis (with no active carditis).

History of rheumatic pains for one month before admission. Heart enlarged and pulse rapid on

admission but rate slowed with rest in bed and not increased at time of test.
Home circumstances: fairly good, one sister; amount of fruit and vegetables consumed varied.

Case 8. Cook, age 9. Admitted 23:6:36. Tested 12:8:36. Convalescent chorea.

Chorea present for four months before admission; movements marked on admission but improved steadily thereafter. No other rheumatic manifestations.
Home circumstances: father working regularly but has six other children; supply of fruit and vegetables therefore restricted.

Case 9 A. Marsh, age 10. Admitted 8:6:36. Tested 18:8:36. Convalescent subacute rheumatism.

Rheumatic pains for nine weeks before admission but settled quickly after admission: no temperature: pulse rate normal after first week. No other rheumatic manifestations.
Home circumstances: father working regularly, one other child. Diet at home had been well mixed. In ward she was allowed to have extra fruit supplied by parents.

Case 9 B. As above. Readmitted 5:12:36. Tested 5:12:36.

General condition improved, no complaints, apparently no relapse. Diet at home, while well mixed, did not contain so much fruit as she had had in hospital.

Case 10. Impey, age 13. Admitted 21:8:36. Tested 21:8:36. Mental retardation.

Patient was sent in as ? cerebral tumour on account of headaches, which did not trouble her after admission. No further symptoms developed. Patient's mental and physical development was that of a child of 10 years.

Home circumstances: father regularly employed, one other child. Gets some fruit and a lot of green vegetables.

Case 13. Sendall, age 13. Admitted 3:9:36. Tested 3:9:36. Mild chorea.

Has had chorea at intervals during past three years - slightly active movements on admission but no other rheumatic manifestations; no increase of pulse rate. General condition fairly good.

Home circumstances: father out of work but three

older brothers are working and supporting family. Patient gets one orange daily, regularly, plus some green vegetables.

Case 18. Gibson, age 10. Admitted 26:10:36.
Tested 26:10:36. ? Subacute rheumatism.

Said to have had pains in legs for two weeks prior to admission but no other rheumatic history and no evidence of rheumatism found on admission. Home circumstances: two other children, father working regularly. Gets a lot of fruit and fair amount of green vegetables.

Case 20. French, age 10. Admitted 23:10:36.
Tested 2:11:36. Habit spasm.

Movements, in various parts of body, present since age 5 and latterly getting worse. No other abnormalities on admission: general condition quite good. Home circumstances: father dead, one brother aet. 8; one grown up sister contributes her wage to mother's pension of 18/- per week. Patient gets limited amount of fruit and vegetables - not every day.

Case 28. Waller, age 9. Admitted 19:11:36.
Tested 22:11:36. Epilepsy.

Definite history of epilepsy of recent onset. No

other illness recently and otherwise quite well.
Home circumstances: comfortably off, father has his own farm. Patient has always had ample fresh fruit and green vegetables from father's own garden.

Case 33. Fletcher, age 8. Admitted 9:12:36.
Tested 10:12:36. Hemichorea.

Has had right hemichorea for eight months - latterly stationary. No other rheumatic manifestations: rather thin but otherwise well.
Home circumstances: good, father working regularly in good job; three other children. Patient has been getting fruit and vegetables regularly daily.

Case 38. W.H., age 26. Tested 19:7:36.

Healthy in all respects. Diet satisfactory, getting half grape fruit, green vegetables and other fruit daily.

Case 39. E.K.M., age 25. Tested 15:12:36.

Healthy in all respects. Diet good but does not always have fruit every day; has green vegetables daily.

Case 40. H.R., age 23. Tested 18:12:36.

Healthy in all respects. Diet satisfactory: very fond of oranges and gets one or two daily, with green vegetables daily and other fruit occasionally.

GROUP II A.

No.	Age in yrs.	Diagnosis	Length of time been in hospital	Previous diet		Home circumstances	Ascorbic acid excreted in 24 hrs. before test dose.	% Of 1st T.D. excreted in 24 hrs.	% of 2nd T.D. excreted in 24 hrs.	No. of petechiae in circle.
				Home	Hospital					
1	11	Chronic bronchitis Broncho pneumonia	3½ mos.	Fair	Full++	Fair	8.96 mgm.	<10%		2
2A	11	Debility Active chorea	4 days	Poor	Full	Poor	6.33	<10%		0
2B	11	do.	12 wks.	Poor	Full	Poor	6.5 ±	<10%		-
2C	11	do.	19 wks.	Poor	Full	Poor	-	<10%	<10%	-
12A	12	Chorea, Debility: Chronic otitis media	6 wks.	Poor	Full	Fair	8.97	<10%		R.arm 6 L.arm 8
12B	12	do.	11 wks.	Poor	Full	Fair	7.5 ±	<10%		-
22A	9	Influenza and meningismus	2 wks.	Poor	Full++	Poor	5.16	<10%	<10%	0
22B	9	" (convalescent)	3½ wks.	Poor	Full+	Poor	-	-	47%	-
23A	7	Unresolved pneumonia	5½ wks.	Good	Full	Good	12.38	10%		1
23B	7	do.	9 wks.	Good	Full	Good	10.0 ±	10%	26%	-
29	12	Pleural effusion (probably tuberculous)	6 days	Very good	Full+	Very good	10.3	<10%	10%	0
31	6	Subacute rheumatism, septic tonsils, nasal catarrh	2 mos.	Fair	Full	Fair	9.42	<10%	33%	2
32	7	Empyema	7 mos.	Poor	Full+	Poor	7.78	<10%	15%	1
35	6	Spastic diplegia, Tonsils septic, Recurrent tonsillitis	8 mos.	Poor	Full	Very poor	10.15	<10%	<10%	2
36	10	Chorea, Debility	8 wks.	Poor	Full	Fair	6.18	<10%	<10%	2
37	10	Acute chorea Debility	2 days	Good	Full	Good	4.7	<10%	20%	0

CLINICAL NOTES of the CASES in GROUP II A.

Case 1. Thornton, age 11. Admitted 8:4:36. Tested 20:7:36. Broncho-pneumonia, Chronic bronchitis.

Patient was exceedingly ill for first two weeks on account of severe broncho-pneumonia, temperature not settling till after six weeks. At time of test, while temperature and pulse now normal, patient still has lot of cough and widely scattered moist sounds all over chest. Appetite good: beginning to put on weight.

Home circumstances fairly good - father and three brothers working. Getting one orange daily at home and other fruit occasionally. In ward patient had great quantities of orange juice and lemon juice during his illness and subsequently.

Case 2 A. Bentley, age 11. Admitted 23:7:36. Tested 27:7:36. Chorea, Debility.

Been delicate since infancy and often in hospital for "debility". Been ill at home with chorea for nine weeks before admission - movements fairly bad on admission and general condition poor; pale and thin and unhealthy-looking; pulse rate rapid for first week. Home circumstances: unsatisfactory, being looked after

by a foster mother; nevertheless said to have been getting fruit daily and vegetables occasionally.

Case 2 B. As above. Tested 14:10:36.

No improvement of chorea or of general condition; not gaining any weight.

Case 2 C. As above. Tested 23:11:36.

Still very little change: nothing further developed. Been on full diet while in hospital.

Case 12 A. Knight, age 12. Admitted 24:7:36. Tested 1:9:36. Chorea; Debility; Chronic Otitis Media.

Chorea, mostly affecting right side, for six months prior to admission and making very slow progress after admission, interfering with speech and feeding.
General condition not good: pale, under-nourished appearance: has had chorea previously: since age of twelve weeks has had persistent discharge from left ear, present during stay in hospital.
Home circumstances: fair; father chronic rheumatic, working regularly as labourer. Patient only got fruit once or twice weekly and vegetables occasionally.

Case 12 B. As above. Tested 12:10:36.

by a foster mother; nevertheless said to have been getting fruit daily and vegetables occasionally.

Case 2 B. As above. Tested 14:10:36.

No improvement of chorea or of general condition; not gaining any weight.

Case 2 C. As above. Tested 23:11:36.

Still very little change: nothing further developed. Been on full diet while in hospital.

Case 12 A. Knight, age 12. Admitted 24:7:36. Tested 1:9:36. Chorea; Debility; Chronic Otitis Media.

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General condition not good: pale, under-nourished appearance: has had chorea previously: since age of twelve weeks has had persistent discharge from left ear, present during stay in hospital.
Home circumstances: fair; father chronic rheumatic, working regularly as labourer. Patient only got fruit once or twice weekly and vegetables occasionally.

Case 12 B. As above. Tested 12:10:36.

Still much the same: choreic movements marked: still pale and thin: ear still discharging.

Case 22 A. Linton, age 9. Admitted 23:10:36. Tested 6:11:36. Influenza and meningismus.

For two days before and two days after admission had severe febrile illness with some headache, gastro-intestinal upset and slight neck rigidity. Cerebro-spinal fluid normal: no other physical signs developed. Progress for first week slow but thereafter more satisfactory to complete recovery.

Home circumstances: very poor, nine of family living on 42/- weekly. In hospital patient got very large amounts of orange, lemon and other fruit juices for first 7-10 days and thereafter still got extra fruit.

Case 22 B. As above. Tested 16:11:36.

General condition considerably better.

Case 23 A. Wasden, age 7. Admitted 1:10:36. Tested 9:11:36. Unresolved pneumonia.

Pneumonia at home three weeks before admission: failure of chest signs to clear up caused doctor to send him to hospital. On admission, temperature

tending to go up to about 100° at times and chest shows unresolved pneumonia at right base - confirmed by X-ray. Patient felt well at time of test but chest signs were only slightly clearer and temperature had still a tendency to rise occasionally.

Home circumstances: good and been getting fruit and vegetables daily.

Case 23 B. As above. Tested 4:12:36.

Chest not yet clear but temperature seldom rising now. Pulse rate tends to be fast at times. General condition improving slightly.

Case 29. Rumsby, age 12. Admitted 18:11:36. Tested 24:11:36. Pleural effusion - probably T.B.

In-patient five months previously with pleural effusion on right side which cleared up in a few weeks. For three months prior to readmission had not been well - feverish, off his food, getting thinner and paler and having some cough and pain in chest. Now found to have pleural effusion on left side. Fluid clear, thin, containing mostly lymphocytes and no organisms. Pulse rate rather fast. Nil else in chest.

Home circumstances: good, patient had been getting a

lot of fruit and fruit drinks daily, as well as other nourishing foods.

Case 31. Wilson, age 6. Admitted 30:9:36. Tested 1:12:36. Subacute rheumatism, septic tonsils and persistent nasal catarrh.

Been attending O.P.D. for four months with subacute rheumatism, not improving, hence admission to hospital. Some pains in legs, thighs and knees after admission and tonsils seen to be septic. Had acute tonsillitis for two days five weeks after admission, followed by persistent copious nasal and post-nasal discharge.

Home circumstances: fair, father working regularly but has three other children. Patient said to get some fruit daily and also green vegetables fairly often.

Case 32. Ledger, age 7. Admitted 14:5:36. Tested 7:12:36. Empyema.

On admission patient had a right lobar pneumonia followed by an empyema on the same side. Empyema treated by repeated aspiration for next six weeks, after which no pus could be got. Nevertheless the chest remained dull, temperature rose occasionally for a few hours, pulse remained persistently fast (even when asleep) and it was presumed a small collection of

pus still remained between the layers of thickened pleura. General condition improved.
Home circumstances: poor, seven other brothers and sisters, mostly young. In ward - got large amounts of fruit juices at first and later got extra fruit drinks and also extra fruit.

Case 35. Fisher, age 6. Admitted 4:4:36. Tested 15:12:36. Spastic diplegia; septic tonsils; recurrent tonsillitis.

Has been in hospital for the greater part of the last two and a half years for massage and exercise to his legs. Has occasional colds in the head, with sore throat and nasal discharge, temperature being elevated for one or two days. Both tonsils somewhat enlarged and show pus in crypts; otherwise well.
Home circumstances: very poor but takes full diet while in the ward.

Case 36. Connelly, age 10. Admitted 19:10:36. Tested 16:12:36. Chorea; Debility.

Developed chorea following diphtheria five weeks previously; movements still active when admitted. Patient never been very strong: is a T.B. contact and attends dispensary regularly: rather pale and definitely thin: some cough; pulse rate sometimes raised for a few days at a time.

Home circumstances: fair, father works regularly. Patient only got limited amounts of fruit and vegetable at home.

Case 37. Yallop, age 10. Admitted 17:12:36. Tested 19:12:36. Acute Chorea; Debility.

Chorea developed while receiving out-patient treatment for "debility": has never been strong and latterly been losing weight rapidly. Movements active on admission: general condition poor. Home circumstances: one other child. Father working regularly: been getting a little green vegetable daily, with one or two oranges every day and occasionally other fruit.

GROUP II B.

No.	Age in yrs,	Diagnosis	Length of time been in hospital	Previous diet		Home circum-stances	Ascorbic acid excreted in 24 hours before test dose	% of 1st T.D. excreted in 24 hrs.	% of 2nd T.D. excreted in 24 hrs.	No. of petechiae in circle.
				Home	Hospital					
3B	8	Recent acute tonsillitis (Cured chorea)	1 day	Poor	-	Poor	7.02	<10%	21%+	-
11	7	Constipation, Debility	2 wks.	Poor	Full	Fair	6.62	19%		3
14	8	Henoch's purpura	2 days	Very poor	-	Poor	8.03	16%		0
15	5	Aplastic anaemia (unknown aetiology)	5 days	Poor	Full+	Good	7.01	20%	35%	2
16	8	Habit spasm, debility	1 day	Poor	-	Poor	6.78	<10%	<10%	0
17	7/12	Constipation, loss of weight	1 day	?	-	Good	1.67	<10%		0
19	10	Post-diphtheritic paralysis	3 days	Good	Full	Good	6.32	20%		1
21A	12	Active chorea Rheumatism	3 days	Very poor	Full	Very poor	12.69	20%		1
21B	12	" plus recent acute tonsillitis	23 days	Very poor	Full	Very poor	9.1	<10%		-
24	10	Hysteria, Debility	5 days	Very poor	Full	Poor	9.12	18%		0
25	9	Active chorea	3 wks.	Very poor	Poor	Very poor	9.76	<10%	30%+	4
26A	9	Subacute rheumatism	1 day	Very poor	-	Very poor	6.9	<10%	<10%	0
26B	9	" plus recent acute tonsillitis	5 wks.	Very poor	Full	Very poor	-	<10%	<23%	-
27	7	Chorea, Bronchitis	9 wks.	Fair	Fair	Fair	7.64	<10%	32%	6
30	10	Rheumatism, Chorea	3 days	Fair	Full	Fair	11.43	17%	44%	1
34	11	Bronchitis, Debility	1 day	Very poor	-	Poor	7.9	<10%	<10%	2

CLINICAL NOTES of the CASES in GROUP II B.

Case 3 B. Chapman: Admitted 9:12:36: tested 9:12:36.

See previous notes on 3 A in Group I. Now four months since discharge from hospital. Has not been getting an adequate diet at home: looks pale and unkempt. Recently had an acute febrile attack of tonsillitis - tonsils still inflamed and injected.

Case 11. Fisher, age 7. Admited 13:8:36: tested 27:8:36. Constipation, Debility.

Been "poorly" for eight months - losing weight, off his food, constipated. Rather thin and pale, pulse rate fast. Persistently constipated in hospital despite treatment.

Home circumstances: Only child but father unemployed; gets little fruit or vegetables at home.

Case 14. Hayes, age 8. Admitted 5:9:36: tested 7:9:36. Henoch's purpura.

Known to have had purpura for at least three years but only recent symptom has been slight epistaxis. On admission had a very few (not recent) purpuric spots and a few r.b.c.s in the urine; the blood showed no abnormalities of cells or clotting and coagulation

times. Sore throat four days prior to admission. Home circumstances: not good, father unemployed, eight other children: patient did not get much fruit or vegetables at home.

Case 15. Stamps, age 5. Admitted 7:9:36: tested 12:9:36. Aplastic anaemia of unknown aetiology.

Became ill six weeks before admission with fever, vague pains in limbs, anorexia, tiredness: became gradually more pale. Temperature only raised at intervals after first week, but pulse rate has remained rapid. No focus of sepsis found; nothing found to account for the aplastic anaemia.
Home circumstances: satisfactory - father working, no other children. But patient did not like fruit juices nor fruit nor vegetables and had very little of these during the course of her illness.

Case 16. McMahon, age 8. Admitted 14:9:36: tested 15:9:36. Habit spasm, Debility.

Said to have had "twitchings" since a few months old and was backward at speaking and walking and is still backward at school. Never really well: no temperature on admission but pulse rate increased and has lot of mucopus in nasal and post-nasal spaces.

Home circumstances: poor - father unemployed, four other children: patient dislikes vegetables and won't eat them; gets occasional fruit, usually apples.

Case 17. Baby Godbehere, age 7/12. Admitted 9:9:36: tested 9:9:36. Constipation.

In last month lost 1 lb. weight: cries a lot; very restless: bowels constipated for some weeks; nothing else abnormal found.
Home circumstances: good, only child, father working. Been fed on Ostermilk - made up according to directions: no added orange juice ever been given.

Case 19. Board, age 10. Admitted 26:10:36: tested 29:10:36. Post-diphtheritic paralysis.

Discharged from fever hospital after diphtheria eight weeks ago: since then has developed weakness of legs, so bad she can scarcely walk; at same time she has had some pains in the limb joints and little lumps like erythema nodosum on the legs. Some cough; no temperature, no increased pulse rate on admission.
Home circumstances: fairly good - father working, one other child; been getting fairly good diet at home, with fair amount of fruit but no vegetables.

Case 21 A. Beaver, age 12. Admitted 2:11:36: tested 5:11:36. Active chorea and rheumatism.

In-patient two years ago, with same complaints. Symptoms on this occasion only been present for about one week; also has septic tonsils; no carditis. Home circumstances: very poor - father out of work seven years, seven other children. Patient said to get green vegetable daily but little fruit.

Case 21 B. As above. Tested 25:11:36.

Since last test has had acute febrile tonsillitis but has had full hospital diet.

Case 24. Eadon, age 10. Admitted 6:11:36: tested 11:11:36. Debility, Hysteria.

Has not been sleeping well for months, has bad dreams and wakes up herself and others in the night. Thin and under-nourished. No other symptoms or physical findings.
Home circumstances: very poor - father out of work, three other children; patient has had little fruit or vegetable at home.

Case 25. Abbis, age 9. Admitted 20:10:36: tested 13:11:36. Active Chorea.

Chorea for two years but been worse lately: no other rheumatic manifestations; nothing else abnormal

found.

Home circumstances: poor - only child but father not working regularly: not fond of fruit, did not get much vegetable at home. In hospital would take very little fruit or vegetable.

Case 26 A. Grice, age 9. Admitted 16:11:36: tested 17:11:36. Subacute rheumatism.

Been receiving treatment for "rheumatism" for three years: for last two months pains been worse, particularly in right knee and right forearm. History of frequent sore throats. Pale and thin and pulse rather rapid but no endocarditis.

Home circumstances: very poor - five of family, only have 32/- per week, consequently very little fruit or vegetable available.

Case 26 B. As above. Tested 20:12:36.

Since last test has been on full hospital diet but has had febrile sore throat lasting for three days three weeks ago.

Case 27. Luttrell, age 7. Admitted 17:9:36: tested 18:11:36. Chorea and bronchitis.

Choreic movements for four months prior to

admission but has also been attending a psychological clinic on account of screaming and bad temper. For a week has had cough and had temperature of 101° on admission. Temperature settled after two days in ward but choreic movements persisted for a long time: cough also persisted but there was no sputum; difficult child to deal with.

Home circumstances: only child, father working, said to have been getting a fair amount of fruit and vegetables at home; in ward was difficult and would not take all her diet.

Case 30. Mellors, age 10. Admitted 25:11:36: tested 28:11:36. Rheumatism and chorea.

Chorea been present for two years: rheumatic pains in thighs and upper arms for six months. No other rheumatic manifestations. Choreic movements still active on admission and some pains still present.

Home circumstances: fairly good, father working regularly, only child; said to get a little fruit and some vegetable almost every day.

Case 34. Grayson, age 11. Admitted 11:12:36: tested 12:12:36. Debility and bronchitis.

Patient collapsed at school on day of admission -

cause of collapse uncertain, probably due to insufficient diet plus poor state of health. Has had a cough for some time, but no sputum. On admission, temperature slightly raised but normal in a short while: pale and looks under-nourished, a few rhonchi in chest.

Home circumstances: poor (details lacking); patient told ward sister that he lived mostly on bread and dripping, with cocoa or tea to drink; patient's mother later contradicted this.

2. Discussion of the results of the urinary excretion test.

Observations on the method.

It has already been stated that the dye solution used in these experiments was obtained by dissolving a tablet of the indicator in a measured quantity (50 cc.) of distilled water: this method was employed because of its convenience and because of the lack of facilities for standardisation of dye solutions: the solution of the dye so prepared was renewed at least every third day. The amount of this dye solution recommended to be used at each titration is .5 cc., which is equivalent to .01 mgm. of ascorbic acid; Harris and Ray, on the other hand, used a much stronger dye solution, the amount taken at each titration being .05 cc., which contained the equivalent of .025 mgm. ascorbic acid. In order to comply with Harris and Ray's suggestion that the amount of urine used at each titration should be in the region of 1.5 - 2 cc., it was sometimes found necessary to use double the amount of dye solution, i.e., 1 cc. instead of .5 cc. This larger amount of solution, however, resulted in a still further dilution of the titration mixture and the end point of the titration became more difficult to estimate accurately;

it was therefore decided to use a double strength dye solution, i.e. two tablets of dye were dissolved in the 50 cc. of distilled water and .5 cc. of this solution used, this amount being now equivalent to .02 mgm. of ascorbic acid.

In a few cases where the urine was rather highly coloured, it was found more difficult to estimate the end point of the titration but in the average case little difficulty was experienced. (Before the tests reported here were begun, the writer performed a large number of titrations in order to become familiar with the method.) The cases which gave most difficulty were those in which the concentration of ascorbic acid in the urine was low.

It was found therefore that the two (or sometimes three) titration readings made in each case compared very favourably; in a large number of the titrations the two readings were identical and in others the difference was only one or two hundredths of a cc. In a few cases, however, the difference was such that on the total 24 hours output of vitamin C it might cause an error of 2% or 3%, the largest error noticed being 5% (in one case only). The percentage of possible error did not differ in the more concentrated specimens

(e.g. those after a test dose) as in these cases the urine was diluted before titration.

No difficulty was ever experienced in getting the titration finished within the specified time of two minutes.

Tabulation of the results.

These have been tabulated in three columns - (1) the output of ascorbic acid in the urine in milligrammes in the 24 hours before a test dose had been given; (2) the percentage excretion of the first test dose in the 24 hours following its administration; (3) the percentage excretion of the second test dose (if given) in the succeeding 24 hours.

It will be noted that in a few cases the figure for the total output, or for the percentage output of a test dose, is followed by the sign "$\pm$" - this indicates that the figure it accompanies is an approximate estimate due either to the fact that one specimen of urine had not been collected with the rest, or that a specimen had not been acidified immediately and had had to be discarded: in practically all cases the approximation can be accepted as being a very close one.

The figures for the amount of excretion of ascorbic

acid in the 24 hours before a test dose.

Starting with Group I it will be seen that these figures vary widely from a minimum of 5.67 mgm. to a maximum (in the case of the child patients) of 28.25 mgm. There is no correlation with the ages of the cases in the group but there is a noticeable agreement in many cases between the amount of the excretion and the previous history with regard to vitamin C intake. To take the four highest figures - Cases 6A, 9A, 28 and 33, it will be seen that in each of these cases the diet had contained unusually large amounts of vitamin C (being either "very good" at home or "full+" in hospital). Only one figure is noticeably lower than the others, namely case 13 (5.67 mgm.); in this case the result is contrary to the previous diet which was estimated to be "very good".

In Group II A it will be seen that again there is a fairly wide variation but that there are no such high totals as in Group I; the range is actually from 4.7 mgm. to 12.38 mgm. In this group it is noteworthy that in no case where the amount is higher than most or lower than most is it possible to correlate this difference with the previous diet; it is also to be noted that in the four cases where the diet is marked

as "full+" or "full++" there is no correspondingly large amount of ascorbic acid excretion.

In Group II B it is found that there is not such a wide range; in this case it is from 6.32 mgm. to 12.69 mgm. (the reading for case 17 (1.67 mgm.) must of course be kept out of this comparison as the subject is an infant of a few months only). In this group it is not possible to find any definite correlation between the previous diet and the figure of total excretion in 24 hours; several cases which were known to have had a diet "very poor" in vitamin C were found to excrete a larger amount of vitamin C than some whose previous diet had been adequate.

The percentage excretion of test doses.

It is in the comparison of these columns in the three groups that the most striking differences are seen in the results. It will be found that the percentage excretion of the first test dose gives.-

in Group I a percentage varying from 25% to 86%;
in Group II A a percentage of 10% or less;
in Group II B a percentage of 20% or less.

These figures require further explanation.

For the sake of convenience it was decided to express all these figures as percentages of the test

dose given (as the test dose varied in amount from case to case it is obvious that it would have been much more difficult to compare the results had they merely been expressed as milligrammes of ascorbic acid). It was subsequently found quite simple to express the percentage in Group I as in all these cases a fairly large amount of the test dose was excreted in the following 24 hours.

In Group II A, however, it was found that in many cases the amount of ascorbic acid excreted in the 24 hours following the test dose was almost the same as, or only a little greater than, the amount excreted in the 24 hours before the test dose. In some of these cases it was obvious there had been no response to the test dose, in others there had apparently been a response of 1%, 2% or 5%, etc., but the exact percentage was difficult to calculate on account of the uncertainty as to how much of the amount was really in excess over the normal excretion. When it was found therefore that the largest excretion in the group was 10% (in one case only), it was decided to overcome the dubiety about the other results by classing them all as less than 10%. Actually in ten cases out of the total number in Group II A there was apparently no response to the administration of a test dose.

In Group II B the highest percentage of excretion of a test dose is 20% (in three cases); several others show percentages of 10% to 20%, while rather more than half the cases (to the number of 10) show excretions of less than 10% of the test dose. Of the cases showing less than 10%, half the number had no appreciable increase in the figure for ascorbic excretion after a test dose and here the reason for failing to specify any percentage lower than 10 is the same as described in the cases in Group II A.

The percentage excretion of a second dose was only estimated in a few cases, consequently the results are only applicable to individual cases and no series of such readings is available for comparison between groups. It should be noted, however, that no second test doses were given in Group I as in every case the first test dose yielded a definite and adequate response.

In Group II A the second test dose in all cases where it was performed immediately after the first test dose gave very low responses, in some cases apparently no response and in others a definite but small response. The results in Group II B were only slightly better.

The interpretation and significance of the results.

It seems necessary at this point to lay stress on the fact that all the figures detailed in the three groups, relating to amounts of excretion of vitamin C and percentage excretions of test doses are not to be accepted as being absolutely accurate. In the earlier part of this work many criticisms of the method now being employed were brought forward and it was concluded that this method of estimating vitamin C in the urine could not hope to do more than give an approximate estimate of the amount of vitamin C actually present. At the same time it was suggested that with an unvarying technique the amount of error in the method might be kept somewhat the same from case to case; no suggestion as to the probable amount of the error could, however, be made.

As a result of his own work with the method, the writer has concluded that a further error of up to 5% was possible in the titration technique. So that although many of the values in the results have been expressed to two places of decimals, it must be remembered that these results are more qualitative than quantitative. To proceed with the analysis of the results:-

a. The values for excretion of vitamin C in 24 hours.

It is of some importance to determine whether there is any difference between these values in the three groups which might be significant of some biological or physiological variation. The averages of these values for the three groups were found to be 13.22 mgm. in Group I, 8.17 mgm. in Group II A, and 8.3 mgm. in Group II B; the average in Group I was thus found to be much higher than in either of the other groups. The "standard deviation" for the values in each column was then worked out, the results being seen in the following table:-

Group number	I	II A	II B
Average value for excretion of vitamin C in 24 hours	13.22 mgm.	8.17 mgm.	8.3 mgm.
Standard deviation of the series of values	7.16 mgm.	2.22 mgm.	1.92 mgm.

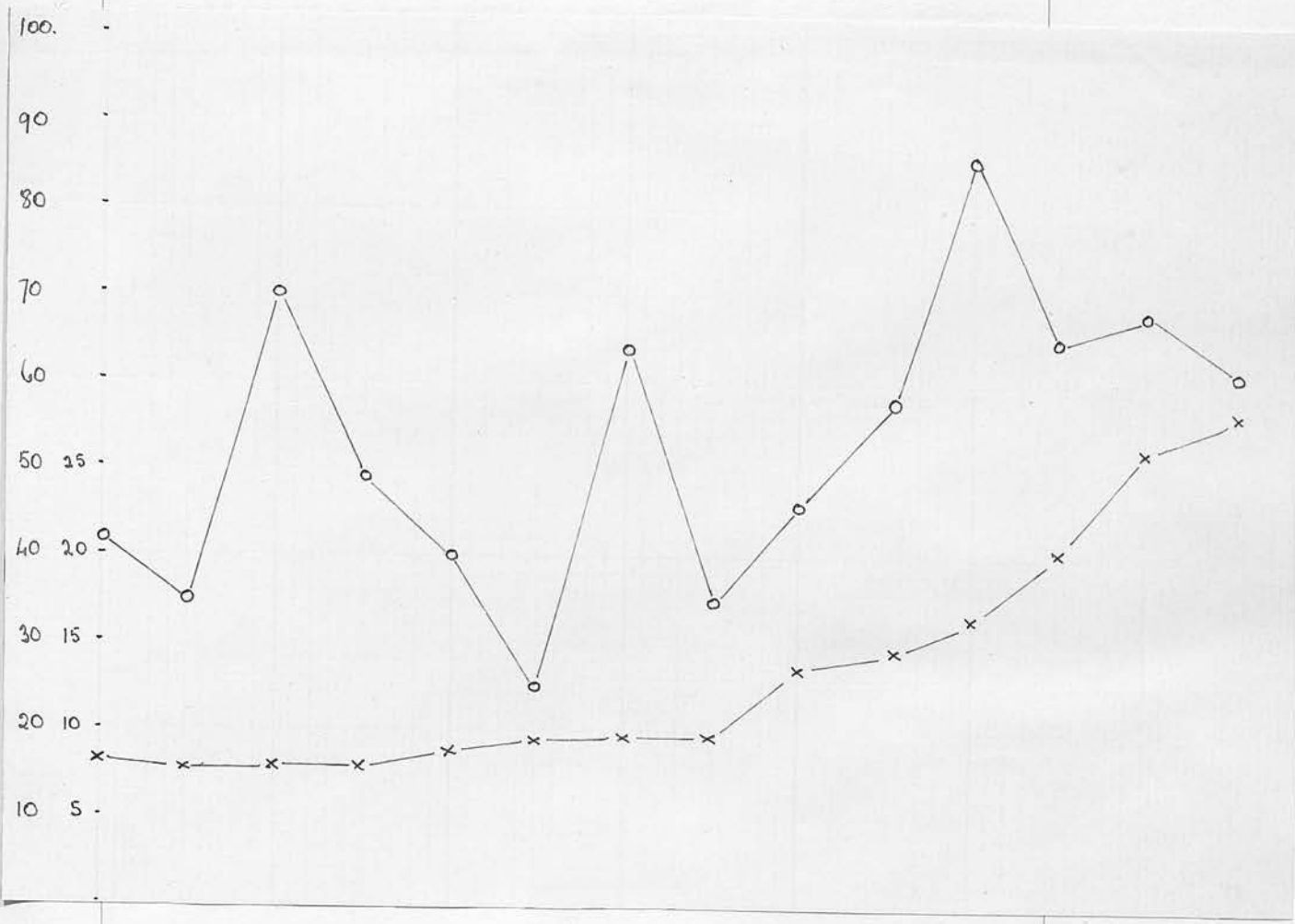
Little further calculation was needed to demonstrate that, on account of the wide deviations from the average in each group, these results expressed no significant difference between the groups, a finding which was perhaps only to have been expected after what

has just been said with regard to the possible errors in the technique.

b. The relation of the percentage excretion of the first test dose to the previous 24 hours excretion of vitamin C.

The next point to be investigated was whether the values for the 24 hours excretion of vitamin C bore any relationship to the corresponding values for the percentage excretion of the first test dose. It was at once obvious that no possible correlation existed between the two sets of values in Groups II A and II B so attention was focussed on Group I; the two sets of values have been plotted out in graphic form in order to make the comparison easier (see page 138). The graph has been constructed by first plotting out the 24 hours excretion values in ascending order and then filling in the corresponding value of percentage excretion. A study of the graph shows that while in some parts the two curves appear to bear no relationship to each other, in other parts the two curves show a decided tendency to follow one another.

It is somewhat difficult to draw any very definite conclusions from this graph: it would appear that in many cases, and especially where the amount of excretion of vitamin C is fairly high, there is a correlation



o — o = percentage output of first test dose.

x — x = excretion in 24 hours before test dose.

between the amount of the excretion and the percentage excretion of a test dose - the amount of the latter being graded in proportion to the amount of the former. There is no discernible explanation of why this correlation does not hold good in all the cases. (Such a correlation appears also to be present in the readings for the three adults included in this group - which figures do not appear on the graph.)

c. The percentage excretion of the first test dose in the three groups.

Comparison of the values for the percentage excretion of the first test dose in Groups I and II A yields some very interesting information. In Group I the percentages range from 25% upwards; the case (No. 20) showing the 25 percentage was one which while not showing any active disease had only been on full diet for ten days and as previously the diet had only been "fair" it is quite possible that a slight element of dietary deficiency was still present. The two next lowest excretions were 35% (twice) and 42%. It therefore appears quite justifiable to regard 30% as an approximate minimum figure for this group, a group showing no active disease and a vitamin C intake calculated to have been adequate.

In Group II A the same column shows a very different series of readings, which have already been commented on. This group, of cases which had had an intake of vitamin C calculated to be adequate and in many instances similar to cases in Group I, but cases which all showed some evidence of ill health, a chronic illness or a frequent recurrence of short febrile illnesses, or a chronic infective state of some part of the body, showed a percentage output of the test dose of 10% or less.

The only other point of difference between the two groups lies in the fact that the average length of stay in hospital prior to the tests was 41 days in Group I and 72 days in Group II A.

One is therefore justified in concluding that the poor or absent response to a test dose in the cases in Group II A is due to the increased need for, or increased utilisation of, vitamin C manifested by the disease processes which affected these cases. It is possible that a deficient absorption of the test dose might influence the figures in a few cases, but it could scarcely do so to the extent shown. Further support is lent to the above conclusion by a closer inspection of some of the cases and their results. For

example, in Group II A, Case 1 had been in hospital for three and a half months and during all that time had been getting extra supplies of fresh fruit, and especially orange drinks - yet the test dose yielded a less than 10% excretion. Case 2 at the first test had certainly not had a previously sufficient intake of vitamin C and this reading actually ought not to be in this group, but at the second and third tests after 12 and 19 weeks respectively, on full hospital diet, there was a very deficient response to the test dose, and at the third test even a second test dose did not yield a satisfactory response. Case 12, similarly, after 6 weeks and 11 weeks in hospital still showed a deficient response to the test dose. The clinical notes of these cases reveal that no apparent change took place in the clinical condition of the patients between the re-tests. And so it is with other cases in the series, e.g. Cases 32 and 35 had been in hospital for 7 months and 8 months respectively and Case 32 had all along received extra supplies of fruit drinks and fresh fruit, yet both showed a deficient excretion after the first test dose and a very poor response after the second test dose.

In some of these cases the physical signs of

disease were definite and obvious, e.g. physical signs in the chest or signs of acutely active chorea; in other cases the physical signs were not so obvious, yet, when detected, were sufficiently definite to leave no doubt that they existed and thus exclude the case from Group I. Case 35 is a case in point - as an example of this latter type: at first sight quite healthy apart from his spastic paralysis of the lower limbs (presumably of itself no longer an active disease process), closer examination revealed that he had very septic tonsils not yet quiescent from a recent tonsillitis and his history revealed that he had suffered from numerous febrile sore throats with patchy tonsils during the last few months.

The results in the same column in Group II B are also of great interest. In this series, with more than half the cases giving a less than 10% excretion of test dose and with no case giving a higher excretion than 20%, it is evident that, in comparison with Group I, the cases show a poor response. These cases have all one factor in common: it has been concluded in each case that the vitamin C intake has been deficient, in some cases to a very marked extent and in other cases to a lesser extent. The average length of stay

in hospital prior to the tests was only 10 days (as opposed to 41 and 72 days in the other groups) and it will be seen that a large number of the cases had had a "very poor" diet at home and had an estimate of "very poor" made of their home circumstances.

A number of these cases, however, in addition to the deficient diet factor had the complicating factor of impaired health in some form or other and therefore this series cannot be used to show the effects of deficient diet in contrast to those which had had an adequate diet.

A few cases in which the dietary deficiency was thought to be the principal factor, e.g. Cases 14, 21A and 24, yet managed to show a definite though not large response to the test dose; in other cases where the dietary deficiency was equally pronounced but in which there was in addition more active evidence of disease, the excretion was less than 10% of the test dose, e.g. in Cases 21A, 26A, 34. These figures suggest that deficient intake of vitamin C (of the severity met with in these cases) by itself does not cause such a poor response to test doses as does an equally deficient intake plus the factor of active disease, or even active disease itself in the presence of a sufficient intake

of vitamin C.

Case 17 is interesting in that it is the only infant tested: the child had not been having its feeds properly adjusted and was consequently becoming constipated and losing weight: fed on Ostermilk, it had never had any added orange juice. The test dose revealed no increase of excretion of vitamin C in the succeeding 24 hours; this poor response being apparently due solely to lack of vitamin C in the diet.

Case 3B appeared as 3A on Group I, when, as a convalescent chorea, it showed a response of 49% of the test dose. Now, having been at home for some weeks on poor diet and having just recovered from an acute tonsillitis, it shows a response of less than 10% to the same test dose. In this connection three other test dose results might be reported which do not appear on the group lists. All are re-tests done as out-patients on cases which had previously been in the ward. All three cases had originally appeared in Group I (as reference to their numbers will show) and they were re-tested at varying intervals after discharge from hospital. The comparison between their figures for percentage excretions is easily made by reference to the following table:-

No. of Case	%age excretion of test dose while in ward	%age excretion of test dose as out-patient	Length of time between discharge from hospital & re-testing	Date of re-test
4	35%	<10%	4 months	14:12:36
6	63%	10%	2 months	15:12:36
13	42%	<10%	1 month	7:12:36

In the interval between discharge from hospital and the re-testing as out-patients -

Case 4 had a "fair" diet and had had one "cold" recently;

Case 6 had kept well but had had a "poor" diet;

Case 13 had had a "good" diet but for two weeks prior to the re-test he had had a "cold".

It will be noticed that all these cases were re-tested in December, when colds were prevalent and when Vitamin C containing foods were most expensive.

Concluding remarks.

a. The writer would like to stress again the fact that the cases reported here have been grouped according to their clinical condition taken in association with the estimate of their previous vitamin C intake. On account of the suggestion

in recent publications that certain infections caused an increased "usage" of vitamin C, it was resolved to obtain one group of cases for comparison in which there was no evidence of acute or chronic infection of any sort - for this purpose the cases in Group I were chosen. The inclusion of two acknowledged cases of chorea in this Group has already been pointed out and some might suggest that these two cases were almost certainly suffering from a rheumatic infection (however mild). The two cases, however, were quite uncomplicated by any other evidence of rheumatism; in view of this fact, therefore (supported by the fact that the blood sedimentation rate in such cases is usually not raised (175)(176), see also (177)), it was decided to retain these two cases in Group I. Other cases of chorea tested were all either very acutely active with very marked movements and rapid pulse, or else were associated with some other evidence of ill health, and thus these cases could not be included in Group I.

b. The method used was found to be straightforward but a few points for criticism emerged:- The fact that at low concentration of ascorbic acid the end

point of the titration was more difficult to determine has already been mentioned. The amount of time taken up by the work is also a drawback, although this could be got over to some extent by delegating some of the preliminaries - such as acidification of urine specimens - to trained staff. In the writer's own case, where this work was being done in addition to the ordinary routine work in the hospital, it was found inconvenient to do tests on more than one case at a time, as the minimum time spent on each titration was 15 minutes and more often was considerably longer if, for example, the urine had to be diluted before titrating. The fact that loss of ascorbic acid occurred very rapidly from specimens of urine left exposed to air and light without being acidified, was the main reason for the writer undertaking so much of the acidification himself; during the summer months also, the ward staff was being frequently changed and it was felt that it would be best not to permit any source of error to arise at such an important point in the procedure. Nevertheless there is no reason why all specimens collected should not be measured and acidified by a reliable

ward sister or her deputy; this would simplify the work of the investigator enormously.

c. It has not been found possible to establish any "normal values" for 24 hours excretion of vitamin C, partly because of the error of the method and partly because of the variations which appear to exist between cases. It has been concluded, therefore, that under the conditions of the test as here described, it has not been found that the excretion of ascorbic acid in the urine for 24 hours on a controlled diet yields any indication of the state of vitamin C nutrition of the individual. Whether more accurate information would be available if each case were first of all to be put on a maintenance diet containing no vitamin C for four or five days, is another matter, and such a procedure is obviously outside the scope of a test which is being used as a clinical test.

d. The results of administration of test doses encourage the impression that a test dose by itself may yield the most helpful information as to the state of vitamin C nutrition. It has already

been noted that other workers have suggested that the results of the test dose are probably of more significance in helping to establish the position of a case, which from a single 24 hours estimate of the urine appears to be on the borderline between "satisfactory" and "unsatisfactory" vitamin C nutrition.

While Harris and Ray have simply stated that to be satisfactory a case must show a response to the test dose without specifying the amount of the response, Youmans and his colleagues (124), on the other hand, who used Harris's method and used the same size of test dose, suggested a minimum excretion of 30% of the test dose as being the lower limit of normal. It will be seen that in the cases of Group I in this study, all show an excretion of 30% or more of the first test dose. It is reasonable to assume therefore that these cases are all in a state of satisfactory vitamin C nutrition. According to this standard of comparison, all the cases in Group II A and Group II B are in a state of unsatisfactory vitamin C nutrition.

3. Discussion of the results obtained with the tourniquet test.

Observations on the method.

The technique of this test was found to be straightforward and simple to carry out. In each case the test took at least 20 minutes to perform and it was found to be essential that the operator should sit by the bed or couch throughout the duration of the experiment in order to keep a close watch on the manometer. Few of the patients were upset by the application of the tourniquet but it was noticed that (being children) they were easily upset by events taking place in the ward and in many cases the younger children had to be screened off during the time of the test.

Difficulty was occasionally encountered in deciding whether or not a doubtful spot should be classed as a capillary haemorrhage; in such doubtful cases the use of a magnifying glass was often most helpful. Provided any permanent marks on the skin had been noted beforehand, little difficulty was experienced in making a count.

The recording of the results.

In all the cases examined, the size of the circle used was only 40 mm. diameter, as opposed to the

"standard" size of 60 mm. Gothlin suggested that when the smaller circle had been used, then the number of petechiae counted should be multiplied by 2.25 in order to represent the equivalent of a reading in the larger circle; in the presentation of these results the writer has not multiplied each figure by 2.25 but has recorded the actual number of petechiae found in the smaller circle. This has been done because it was thought that, especially in many of the smaller children, multiplication of the actual number of petechiae by $2\frac{1}{4}$ would give an exaggerated reading quite out of proportion to the truth.

It was a little difficult to decide on a value dividing "satisfactory" from "unsatisfactory" results but it was eventually decided that any reading of five petechiae or over would be quite definitely outside the limits of normal. (Gothlin's original maximal normal figure was eight petechiae in the circle of 60 mm.; this would correspond to a figure of approximately four petechiae in the smaller circle; it is therefore evident that the choice of five petechiae as the outer limit of normal in this series leaves a fairly wide margin for normality.)

The interpretation of the results.

The tables show that many of the cases show no

petechiae at all; numerous others show one or two petechiae while the highest number reached was eight. Comparison of the three groups has not brought out any significant facts with regard to the number of petechiae, the average number of petechiae per case in each of the groups being almost identical. It will be seen that few or no petechiae were found equally amongst the cases of the different groups, and that several cases whose previous dietary with regard to vitamin C was adjudged "very poor" showed none or only single petechiae.

In three cases (Nos. 5. 6 & 12) an unusually high reading on one arm led to an immediate repetition of the test on the other arm for verification; in two cases the other arm yielded a considerably lower reading, while in the third case the second reading was a higher one. On the basis of the first readings alone, each of these cases would have fallen into the class of "subnormals"; the lower second reading in two cases, however, makes their ultimate classification very difficult. The remaining two definitely abnormal results occurred in cases in one of which the previous diet had been "fair" and in the other, full hospital diet had been taken for several weeks. In neither

case, however, was the child healthy.

It must be confessed that from these results very little helpful information has emerged. The equal distribution of none and single petechiae in all the groups under review, especially in those cases known to have had a definite lack of vitamin C in their diet for some time, is perhaps the most striking fact, and suggests at least that the results are not a quantitative expression of the state of vitamin C nutrition of the individuals. The variance between two consecutive readings in the different arms of the same patient has caused difficulty in classifying at least two of the results and casts doubt on the accuracy of all the other readings. Of the actual method itself it may be said that the technique is without serious difficulty but the method takes a fairly considerable time to perform.

SUMMARY and CONCLUSIONS.

I. A review has been presented of the present state of knowledge with regard to the origin, distribution, chemistry and physiological functions of vitamin C. It was demonstrated that several gaps still exist in our knowledge of the processes of absorption, utilisation and excretion of the vitamin, and of the factors controlling and influencing these processes.

II. A deficiency of vitamin C in the diet is known to be able to produce eventually symptoms of scurvy in adults or children. There is also considerable evidence to suggest that minor degrees of deficiency of the vitamin insufficient to cause symptoms are probably not uncommon.

III. For the detection of vitamin C deficiency, various tests have been elaborated; the history and present position of these tests has been studied and two tests selected which were thought to be sufficiently well established to justify their use (with reservations): these tests were (1) a method of estimating the resistance of the

skin capillaries, and (2) a method for the estimation of vitamin C in the urine.

IV. The two tests chosen have been applied to an unselected series of cases in a children's hospital and the results obtained have been tabulated and examined.

V. The tourniquet test was found to be unreliable and it yielded no helpful information as to the state of vitamin C nutrition of any of the cases to which it was applied.

VI. The test for excretion of vitamin C in the urine, while taking rather a long time for its performance, was found to yield useful information.

VII. Using this test it was found that a series of cases on a diet containing ample vitamin C and showing no active disease were all in a satisfactory state of vitamin C nutrition.

VIII. A further series of cases on similar diet but who were all suffering from some pathological condition, mostly a chronic infection, were found to be in a state of unsatisfactory vitamin

C nutrition.

IX. Further light has therefore been shed on the conditions which may cause an increased "utilisation" of vitamin C and it has been conclusively demonstrated that chronic, apparently afebrile, infective conditions may deplete the vitamin C reserves of the tissues.

X. There is some evidence to suggest that a deficient intake of vitamin C, of itself, does not cause so great a depletion of the vitamin C reserves as when some mild, often unsuspected, infection is present at the same time.

XI. The frequent combination of these two factors and the difficulty of assessing the relative importance of each has been shown in a group of in-patient cases and also in a small group of ex-in-patients re-tested some time after discharge from hospital.

XII. In only a very small number of cases found to be in a poor state of vitamin C nutrition was it thought that the main cause was a previous dietary deficiency of vitamin C.

XIII. The writer would therefore like to point out the need, in applying this test to any case or group of cases, for a very thorough clinical examination of the cases beforehand in order to eliminate any subacute or chronic or slowly healing infective condition.

XIV. This study suggests that the ordinary diet of the hospital concerned, while containing a sufficiency of vitamin C containing foods for many of the patients, requires to be supplemented with further vitamin C for many others.

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