

**REQUIREMENTS FOR VITAMIN C
AS AFFECTED BY EXERCISE**

by

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CHAPTER I

INTRODUCTION

Historical Background

Scurvy, a vitamin C deficiency disease, has been known in history as a plague, infesting armies, navies, and besieged towns. It was particularly common among the crusaders, on the voyages of exploration of the sixteenth century, among the colonists of the northern part of America, and throughout all history in the northern parts of Europe and Asia. The Greek, Roman, and Arabian writers do not seem to have been acquainted with the disease (53).

It has been known for over 300 years that fresh vegetables are a potent remedy for scurvy, but it was not known until the early part of the twentieth century that scurvy is an avitaminosis (86). In that same period, it was discovered by Bartenstein and, later, by Holst and Frolich, that the guinea pig could acquire scurvy. That discovery opened the way for some valuable experimentation relative to the nature of vitamin C deficiency.

Between 1918 and 1925, Zilva was able to obtain almost pure antiscorbutic vitamin from lemon juice. He was able also to determine the basic properties, such as molecular composition (empirical formula $C_6H_8O_6$), its

resemblance to the hexoses, and its instability to oxygen, especially in alkaline solution.

In 1928 Szent Gyorgyi isolated from adrenal glands, from oranges, and from cabbage, a strongly reducing substance which he called "hexuronic acid." In 1932, the identity of vitamin C with Szent Gyorgyi's "hexuronic acid" and with Zilva's "reducing factor" was discovered by various groups of workers, namely Waugh and King, Svirbely and Szent Gyorgyi, and Tillmans (86). The first papers by Waugh and King were based mainly on the isolation of their first antiscorbutic crystals in 1931. According to King (61),

These crystals, obtained from lemons, corresponded in type, melting point, solubility, titration value against oxidizing and reducing agents, rotation, electrical transference, and C -- H combustion, with the "hexuronic acid" which Szent Gyorgyi had isolated as a tissue respiratory factor from cabbages, oranges, and adrenal glands, at Cambridge University in 1928.

There seemed to be no reasonable basis for questioning the identity of the vitamin after it was demonstrated that Kendall's independent extract from adrenal glands and by an entirely different procedure had the same antiscorbutic value as the product of Waugh and King.

Reichstein and Haworth announced the first successful synthesis of ascorbic acid in 1933.

Chemical Properties of Ascorbic Acid

Vitamin C has an acid reaction, this property being due to an enolic hydrogen (59). The most significant reactions are those which involve oxidation and reduction. The oxidation of ascorbic acid is greatly affected by variations in pH and by the presence of catalysts, such as cupric, ferric, mercuric, silver, and manganese ions. The vitamin is reversibly oxidized by a great many reagents such as methylene blue, quinones, the indophenol dyes, hydrogen peroxide, and iodine. In aqueous solutions, the rate of aerobic oxidation is increased by exposure to light and by the presence of the hemochromogens. The oxidized form of the vitamin, dehydroascorbic acid, is fairly stable in solution below pH₇, but in more alkaline solution, another strong reducing substance forms, which on further oxidation gives rise to oxalic acid and l-threonic acid. Reduction of dehydroascorbic acid can be brought about by hydrogen sulphide, cysteine, and the "fixed - SH" groups of proteins. It has been found that glutathione in the tissues may act as a protective mechanism against oxidation of ascorbic acid by copper. According to Becker, ascorbic acid is considered the coenzyme for glutathione oxidation (6). Two of the enzymes which oxidize and reduce ascorbic acid respectively, can be separated from cabbage juice; the reductase has been found

to be much more labile than the oxidase (22).

Physiology of Ascorbic Acid

When ascorbic acid is withheld from the organism, the main changes that occur are in the intercellular substances surrounding the cells which start from the mesenchymal or middle layer of the embryo (24). Normally the fibroblast, or the basic cell of fibrous tissue, forms small fibrils in an amorphous ground substance, the fibrils being gathered together into wavy bands of collagen or connective tissue. In normal growth, the fibrils appear to be cemented together by a translucent ground substance; it is this formation of intercellular materials which is probably controlled by vitamin C. In scorbutic guinea pigs, the fibroblasts and the ground substance are present, but the fibrils and bands of collagen do not appear; when the animals are treated with sufficient vitamin C, the collagenous material appears in eighteen hours. The formation of the intercellular substance of teeth (dentin) and of bone (osteoid tissue) can be controlled in the same way, by supplying or withholding the vitamin.

The bony lesions in vitamin C deficiency are most common at the costochondral junctions, the distal and proximal ends of the long bone of the thigh, and the proximal end of the tibia and the wrist (26). It appears that, when vitamin C is not present, the osteoblasts return to their primitive form (fibroblasts) and try to form a

fibrous, rather than a bony, connection, between the epiphysis and diaphysis. This abnormal zone is called the "gerustmark" or "framework marrow," which is the characteristic lesion of infantile scurvy.

A deficiency of vitamin C results in changes in the teeth and gums (15,85). There are changes in the pulp and dentin of the teeth, and an inflammation of the gums results. Other parts of the body which may be affected by ascorbic acid deficiency are the eyes (13), the bone marrow (23), and glandular (23) and muscle tissue (10). Most lesions of scurvy can be traced to the inability of the mesenchymal cells to form supporting substance. The typical lesion of scurvy is the perifollicular petechial hemorrhage. The petechiae are most common on the legs or where there is pressure on the capillaries.

Crandon, Lund, and Bill (21) recently contributed a valuable study in vitamin C deficiency. Their subject, Crandon, was on a diet free from vegetables, fruits, and milk for about six months. At the end of 41 days, his plasma level of ascorbic acid was zero. The ascorbic acid content of the white-cell platelet blood fraction fell to zero after 82 days. On the 134th day, small perifollicular papules appeared over the buttocks and backs of the calves. These papules they considered to be the first signs of scurvy. Perifollicular hemorrhages appeared after 161 days. When the subject was in the deficient

state, an incision was made across the lumbar region of the back. This wound failed to heal until the ascorbic acid supplement was given. Winfield and Irvin (47) advise that the plasma ascorbic acid and the serum protein levels of patients be brought to normal before surgery.

When vitamin C rich foods are excluded from the diet, a vague condition of general poor health may result (69), the state often being accompanied by anemia (4,66,69). A deficiency may also be accompanied by a lowered resistance. King and Menten (62) found a definite lowering of the resistance of vitamin C deficient guinea pigs to injections of diphtheria toxin. Flori (34) demonstrated that ascorbic acid alone or with extract of adrenal cortex had some detoxifying action in vitro against diphtheria.

It has been suggested that a lack of adequate vitamin C may be the cause of hypersensitiveness to certain foods, particularly as it occurs in infancy (91,112).

There has been some evidence presented (82) that a vitamin C deficiency may lead to respiratory and other infections.

The effect of ascorbic acid deficiency on the resistance mechanisms of the body is not known. It is known that there is some relationship between ascorbic acid and blood complement (68). It has been found that there is a drop in the complement titer of the scorbutic guinea pig and that the value rises when vitamin C is administered.

In paired feeding experiments with guinea pigs, McHenry and Sheppard (70,92) found that with vitamin C deficiency there is a loss in weight, which is accompanied by an increased metabolic rate and a decrease in water retention. In the same experiments, they found that the body fat of the deficient animals was greater than that of the controls in spite of the increased metabolic rate.

Bessy, Montan, and King (10) made extensive histological studies on scorbutic guinea pigs, mainly with the aid of silver nitrate and fat staining materials applied directly to the tissues. They found that the silver nitrate was reduced much less rapidly when the animals were on a vitamin C free diet. In the scorbutic guinea pigs, they noted the disappearance of fat staining material and of doubly refractive cholesterol from the adrenal cortex. They also found fatty degeneration in parts of the myocardium and in skeletal muscle as well as fatty infiltration and fatty degeneration of the liver and sometimes congestion of the liver and hemorrhages.

Murakami (75) found that in guinea pigs fed on a diet deficient in vitamin C, the pigment excreting function of the liver and the capacity of the liver for detoxifying sodium santonicum and indole were decreased.

The oxidative reactions of ascorbic acid suggest that the vitamin may play an important part in tissue respiration. Kellie and Zilva reported that Harrison found an

uptake of oxygen by liver slices of scorbutic animals when vitamin C was added (56). Kellie and Zilva were able to repeat the experiment and found that the addition of ascorbic acid to liver tissue increased the surviving respiration even when the animals were not scorbutic. They also found that the increase in respiration was less in animals on a normal diet than in animals on a diet restricted in calories or in ascorbic acid. In further experiments, they found that the surviving respiration of the liver tissue of 95 per cent of their animals on a diet restricted in calories and 31 per cent of those on a normal diet was increased by the addition of ascorbic acid. The great reduction of oxygen uptake which was brought about by phloridzin, inhibitor of phosphorylation of carbohydrate, suggests a connection of ascorbic acid with any one of the stages of carbohydrate catabolism following the step of phosphorylation.

Stewart and Learmonth (98) found that ascorbic acid injected into cats which had been bled to 50 per cent of their original blood volume would, in some cases, prolong their lives. They suggested that the action may have been due to the increased supply of oxygen to the tissues by ascorbic acid and even suggested a possible practical application of this phenomenon to human subjects during war time.

Other biochemical functions of ascorbic acid have been suggested. Robertson, Ropes, and Bauer (83,84) found that ascorbic acid would reduce the viscosity of mucin in the presence of hydrogen peroxide. With further experimentation, they found that it acted on polysaccharides such as starch, pectin, flaxseed, mucilage, and the polysaccharides of synovial mucin and cartilage and that it destroyed the capsules of various types of pneumococci. Later they brought about the in vitro dephosphorylation of β -glycerophosphate by ascorbic acid and hydrogen peroxide. They suggested that a possible function of ascorbic acid may be the degradation of mucins and polysaccharides in physiological and pathological processes.

Noggle and Wynd (77) suggested that ascorbic acid may have a role in protein synthesis when they found that the ratio of insoluble to soluble nitrogen in the leaves of cereal grass increased with increasing amounts of ascorbic acid.

It has been found that ascorbic acid has some function in protein metabolism. In 1939, Levine, Marples, and Gordon (64) found that certain tyrosyl compounds (l-p-hydroxyphenylactic acid and a keto acid which they believed to be p-hydroxyphenylpyruvic acid) were excreted by premature infants. They found that the feeding of the aromatic amino acids to these infants would increase the

excretion and that the administration of ascorbic acid would prevent the excretion.

At about the same time, Sealock and Silberstein (89,90) were able to produce an experimental alcaptonuria by feeding vitamin C deficient guinea pigs tyrosine or phenylalanine. The condition was controlled with the feeding of the vitamin.

In later experiments, Sealock, Parkinson, and Basinski (88) found that homogentisic acid, alpha keto acids, and tyrosyl compounds, mainly p-hydroxyphenylpyruvic acid, were excreted from ascorbic acid deficient guinea pigs when either l-phenylalanine or phenylpyruvic acid was given. When one of the metabolites, p-hydroxyphenylpyruvic acid, was given, the vitamin was found to have little or no effect on the succeeding metabolites. From this they concluded that the point of attack of the vitamin on amine acid metabolism is confined to the early stages.

Beyer (12) was able to produce the inactivation of certain amines by ascorbic acid both in vivo and in vitro. He obtained up to a 55 per cent yield of ammonia when air was bubbled through a buffered solution of ascorbic acid and some amines. He also found a decreased excretion of amphetamine by dogs when they were given 22 to 400 mg of ascorbic acid.

Bourne (14) has suggested still another function of ascorbic acid. He points out that ascorbic acid is located in high concentration in the region of the Golgi apparatus of biologically active cells and that the Golgi apparatus is recognized as the synthetic center of the cell. He concludes that ascorbic acid probably has some function in the synthesis of chemical substances; he gives as the most likely explanation that the presence of ascorbic acid forms in the synthetic center of the cell a highly reducing area which prevents the synthetic products from being metabolized as soon as formed.

The relationship of ascorbic acid to specific hormones and enzymes has been studied in some laboratories. There is some evidence that the vitamin has an anti-thyretropic action, but the relationship has not been clearly demonstrated (95).

Sigal and King (93) found that the glucose tolerance of guinea pigs was definitely lowered by progressive ascorbic acid depletion.

Harrer and King (42) recently made a study of the relationship between ascorbic acid and four enzymes, two hydrolytic and two respiratory. For the study of the hydrolytic enzymes, the tissues used were incubated with the appropriate enzymic substrates and the products of the hydrolysis analyzed quantitatively. The activities of the respiratory enzymes were studied by measuring the

oxygen or hydrogen uptake of certain tissues in a unit time.

In this study, they found that the liver esterase activity of their scorbutic guinea pigs was one-third the activity of the normal animals. They found that phosphatase activity of intestinal mucosa and kidney cortex showed a moderate decrease in scurvy. The activities of the two respiratory enzymes as measured by this method were definitely lower in the scorbutic animals. The activity of succinic dehydrogenase in scorbutic heart tissue appeared to be 20 per cent less than that of normal tissue and 65 per cent less in scorbutic skeletal muscle than of normal skeletal muscle. The activity of cytochrome oxidase in scorbutic heart and skeletal muscle was about 45 per cent lower than that of the normal tissue.

Harrer and King (42) suggested that ascorbic acid may be important in cellular physiology as a regulating and protective agent. They write as follows: "By protecting active groups such as $R - SH$ (R being a protein), relatively small amounts of ascorbic acid might aid in the regulation of major hydrolytic and respiratory systems in the tissues without entering directly into the reactions concerned." They point out the apparent paradox that although scurvy is accompanied

by total increased body respiration and increased respiration of tissue slices, there is a distinct decrease in activity of two of the major respiratory enzymes.

The role of ascorbic acid in the metabolic processes of plants and animals is not clearly understood. There is practically no ascorbic acid to be found in dry seeds, but the vitamin appears where the sprouting processes are apparently initiated, in a few hours after the seed is moistened. It is found in high concentrations in the actively growing parts of the higher plants, and in all fresh green leaves. In the animal organism ascorbic acid is found in the greatest concentrations in the tissues of greatest activity; the pituitary body generally has the greatest concentration and, in decreasing order, the corpus luteum, adrenal cortex, young thymus, liver, and brain. The presence of the vitamin in tissues of highest metabolic activity indicates that it may have an essential metabolic role. King (61) states: "The high concentration of ascorbic acid in glandular tissues indicates a degree of correlation with the general rate of metabolism analogous to the relationship in plants." Heinemann (52) found that in blood kept in an atmosphere of nitrogen, the plasma ascorbic acid entered the cells at room or body temperature but not at low temperatures (7 degrees centigrade). From this he concluded that the

transfer into the cells is probably associated with some metabolic process.

Methods of Assay

The earlier assays for ascorbic acid were made with the use of the guinea pig, both by the preventive and curative methods. Another method that has proved to be very useful is the "guinea pig tooth method" suggested by Højer, in which the degree of scurvy present is determined by changes in the structure of the dentin of the teeth.

Tillmans, Hirsch, and Hirsch first developed a method for determining the presence of reduced ascorbic acid in blood. Their method was based on the titration of a trichloroacetic acid filtrate of blood plasma with the dye 2,6 dichlorophenol indophenol.

Emmerie and van Eekelen suggested reducing the ascorbic acid present in blood plasma with hydrogen sulfide and precipitating interfering reducing substances such as cysteine, with mercuric acetate (28). This method has proved to be rather uncertain, as it is necessary to remove the hydrogen sulfide completely with nitrogen in order to prevent excess reduction of the dye.

Farmer and Abt have modified the original dye procedure and have adapted it to the use of very small amounts of blood. They suggested using sodium tungstate and, later, metaphosphoric acid, as protein precipitants (30).

Pi Joan and Klemperer have suggested the use of potassium cyanide and potassium oxalate to prevent the oxidation of ascorbic acid in drawn blood samples (79). Other workers have found that there is no greater loss of ascorbic acid when potassium cyanide is not added and that some lots of the salt tend to decolorize the dye. (23,29).

Various other reagents have been suggested for ascorbic acid analysis, among which are ferrocyanide, phosphotungstic acid, methylene blue, and phosphomolybdic acid. For one reason or another, these reagents have proved to be not entirely satisfactory. A spectroscopic method and a procedure based on the use of oxidation-reduction curves have been suggested. Roe suggested the use of the reaction between aniline acetate and furfural, which is formed from ascorbic acid on treatment with concentrated hydrochloric acid.

Various potentiometric methods have been tried, but until now, these have been very limited in precision because of drifting potentials and indistinct end-points. Harris, Mapson, and Wang (44) have recently introduced a method for an electrometric analysis with the use of a platinum electrode coated with mercury. The method may prove to be a useful one for routine analysis, from the standpoint of accuracy and simplicity.

Mindlin and Butler (72) have adapted the micro dye method to the photoelectric colorimeter. Of the many micro methods tried by various workers, the indophenol titration method as modified by Farmer and Abt and the colorimetric method of Mindlin and Butler have been preferred by the greatest number of workers.

Methods of Determining Requirements

The first attempts at measuring human requirements were made by G  thlin in Sweden in 1931. His method was to find the smallest amount of orange juice which would correct a lowered capillary resistance, a condition which was revealed by petechiae appearing when pressure was applied to the arm (37,38,39). This method has been partly replaced by a method introduced by Dalldorf, whereby negative pressure is applied to an area of the arm and the number of petechiae counted (94).

Many workers since G  thlin's experiments were performed have found no correlation between capillary resistance and nutritional state (113,78,49,25). According to G  thlin, the reason that these workers have found no such correlation is that the method is not specific when plasma levels are much above 0.1 mg per 100 ml and that in the countries where these experiments have been carried out (except in Sweden), the average diet is comparatively rich in vitamin C, and plasma values as low

as that are seldom found. Dalldorf has made extended studies on the method, using his negative pressure technique, and is of the opinion that the method will give dependable information on the presence or absence of vitamin C deficiency but does not show the degree of deficiency. Dalldorf has found that the method will give falsely negative results when severe anemia is present. Sloan points out that the thickness of the skin and the position of the arm also affect the results (94).

There is some evidence now that lowered capillary resistance as shown by these tests may be due to the lack of vitamin P (65). Subjects of Levcovich and Batchelder had larger petechiae when on a low vitamin C diet supplemented with 40 to 120 mg of pure ascorbic acid than they did when on a freely chosen diet.

In 1937, Rotter introduced a direct test for determining nutritional state (87). He injected a small amount of the dye 2,6 dichlorophenolindophenol into the forearm of his subjects, measured its speed of decolorization, and concluded that that speed was proportional to the amount of ascorbic acid in the blood. Others since then have failed to find such a correlation (80) and advanced the theory that there may be other reducing substances in the skin, which would interfere with the test.

In 1932 Tillmans announced a relationship between the ascorbic acid content of food and its ability to reduce the dye 2,6 dichlorophenolindophenol. Later, this principle was applied to the measurement of ascorbic acid in urine. Since then, some progress has been made on the determination of minimum and optimum requirements for various age groups.

It has been shown by Harris and others that the urinary excretion of ascorbic acid is roughly proportional to the intake at certain levels (45,46,55,74). Johnson and Zilva (53) found that the excretion depends both upon the magnitude of the intake and upon the degree of saturation of the subject, a much smaller percentage of a single large dose being excreted if the reserves of the subject have been previously depleted.

For a time an attempt was made to assess nutritional status by determining the urinary excretion of ascorbic acid (43,113). The level of excretion corresponding to the "lower limit of normal" has been set at approximately 13 to 20 mg by various workers (43,112,32). Harris, Ray, and Ward, and Ahmad have estimated that the average daily excretion for normal subjects is about 30 mg (46,3). Harris and Abbasy (43) found that an intake of 25 mg of ascorbic acid would result in an excretion of 13 mg. One subject of Johnson and Zilva (55) excreted

80 to 150 mg of ascorbic acid when he regularly included in his daily diet two oranges besides green vegetables.

It has been shown that there is a "renal threshold" value or a definite concentration in the blood at which ascorbic acid begins to appear in the urine in large amounts (81,2,31,11). This renal threshold varies for different individuals, but the average appears to be about 1.0 to 1.4 mg per 100 ml of blood plasma. When the tissues become "saturated" with ascorbic acid and the intake is suddenly stopped, the excretion drops very rapidly, usually by the second day, to the low resting level commonly found in the subject on a freely chosen diet (55).

Johnson and Zilva (55) gave vitamin C deficient subjects gradually increasing doses of ascorbic acid and found no significant rise in excretion until the seventh day. The amount excreted gradually rose until the seventeenth day, when a constant level was reached; this point they considered to be the level at which the tissues are saturated with the vitamin.

Reinemann, van Wersch, and van Bekelen have attempted to measure the daily expenditure of ascorbic acid. (50,51). In describing their method, they say,

If a previously saturated subject is resaturated after having omitted all vitamin

C for a certain length of time, the dose of ascorbic acid required to produce saturation corresponds to the quantity used from the body stores during the vitamin C free period. Consequently this amount when divided by the number of days the experiment lasted, can be considered as the daily expenditure.

"Saturation" in their experiments is considered to be the condition when considerable portions of a test dose appear in the urine.

The response to a "test dose" of ascorbic acid has been widely used as a method of measuring nutritional state and requirements. A large dose (200 to 1000 mg) is ingested and the urine collected for the next 24 hours and analyzed for ascorbic acid. This method, in its many variations, is founded upon the principle that a large percentage of the test dose is excreted if the tissues are "saturated," while almost no rise occurs if the tissues are severely depleted. Sometimes, several days elapse before the excretion responds to repeated test doses (78).

Harris and Ray (45) found that low excretion and lack of response to test dose go parallel with a history of low ascorbic acid intake and with a state of ascorbic acid subnutrition as shown by capillary tests, but he recommends the test dose method as being a better method of measuring nutrition than the capillary resistance method because it is more truly specific and more accur-

ately quantitative and because repeated measurements can be made at any time.

Harris and Abbasy (43) found that the consumption of 25 mg of ascorbic acid daily will yield a response to the test dose generally on the first day and certainly on the second.

Ordinarily, the determination is made on the urine collected for the 24 hours following the test dose, but some have suggested short collections (3 to 5 hours) with the argument that the peak of excretion comes at about the fourth hour and drops rapidly for the rest of the twenty-four. Wright, Irving, Lillenfield, and MacLenathen (109) have found that 80 per cent of the excretion occurs in the first five hours.

The test doses used in different studies have varied greatly, and there is a great difference of opinion as to the percentage of excretion which indicates adequate and saturation levels of the vitamin in the tissues. Youmans (113) estimated that an excretion of 30 mg after a 600 mg test dose shows that the subject is in "normal" vitamin C nutrition. Abbasy, Harris, and Ray (1) set a 40 to 50 mg excretion of a 700 mg test dose as normal. Faulkner and Taylor (31) found that their "normal subjects" (blood levels averaging 1.7 mg per 100 ml) excreted 503 mg of a 1000 mg test dose in 24 hours. Van

Ekelen (104) defined "saturation" as the point at which a dose of 250 to 400 mg of ascorbic acid will result in a definite rise above 30 mg excretion. Wright, Irving, Lilienfield, and MacLenathan (109) report that a normal subject will excrete 500 mg of a 1000 mg test dose in 24 hours or 400 mg in five hours. For many requirement studies, "saturation" is considered the point at which 50 per cent of a test dose will be excreted (97).

Storvick and Hauck compared this method of determining requirements with a method in which the subject is given 200 mg of ascorbic acid daily for a preliminary "saturation period." The lowest response to a test dose after this period is taken as the measure of "saturation" for that subject in subsequent periods when the subject is kept on various levels of intake to determine the minimum requirement to produce "saturation." For three subjects, requirements as determined by the two methods were the same, while in two, they were slightly higher as determined by the second method.

In 1935, Farmer and Abt (29) reported that the amount of reduced ascorbic acid in the blood plasma closely follows the vitamin C intake. Since then, others have confirmed this correlation (113,17,40). Since the introduction of the plasma micro titration technique of

Farmer and Abt, the plasma level of ascorbic acid has been used to a great extent both in determining nutritional state and in calculating requirements. Some have tried to determine nutritional state by analyzing the total ascorbic acid content of the blood plasma after reduction with hydrogen sulfide or carbon monoxide and find no correlation between total ascorbic acid of the plasma and the dietary regime. Farmer and Abt stated that the amount of reduced ascorbic acid in the plasma is more significant than the level of total ascorbic acid as a measure of nutritional state.

A rather close correlation has been found between plasma levels and the amount of ascorbic acid excreted in the urine.

Goldsmith and Ellinger (35) made extensive comparisons of the vitamin C content of blood and urine on 22 normal subjects and some who had vitamin deficiencies. Of the group which showed a normal response to a test dose (40 to 50 mg of a 700 mg test dose), only one had a blood level of less than 0.65 mg per 100 ml, a level which they chose as indicating normal nutrition. They believed that the one low level may have been due to an unusually low intake of vitamin C for two or three days preceding the test. They stated that occasionally a blood level may not be indicative of the nutritional

status but believed that for the most part, the method was reliable.

Wortis, Liebeman, and Wortis (108) made extensive comparative studies on plasma, urine, and spinal fluid and concluded that a normal (0.7 mg per 100 ml) and a very low ascorbic acid content of blood plasma (below 0.4 mg per 100 ml) are almost invariably associated with corresponding normal and subnormal values for both spinal fluid and ascorbic acid excretion in five hours after a "test dose" and that, therefore, the blood plasma in these ranges furnishes an adequate and accurate index of vitamin C nutrition. In the range of 0.4 to 0.7 mg per 100 ml they recommend that all means be used for the clinical evaluation of scurvy.

Storvick and Hauck (99) found that there was a definite correlation between urinary and plasma ascorbic acid on intakes of 75 mg and less but not a significant correlation on levels above that.

There has been some question about which fraction of the blood is the best medium for determining nutritional state. It has been shown by Butler and Heinemann (18 and 52) that ascorbic acid, when it is first absorbed, appears in the plasma and then slowly penetrates the red blood cells. A few workers (104,76) have argued that an analysis of whole blood will give a better picture of vitamin C nutrition than either white cell-platelet layer

or plasma because it does not fluctuate as the others do. Butler and Gushman (18) recommend white cell-platelet layer analysis since they in cooperation with Crandon, Lund, and Dill (21) have found that the ascorbic acid level of the white cell-platelet layer is the last to become depleted in deficiency and the first to rise when ascorbic acid intake is resumed. It is evident that analysis of this layer would show the presence of a long continued severe deficiency better than would the plasma level. The latter, however, is a better measure of what the diet has been in the days preceding the test.

Goldsmith and Ellinger (35) found that when ascorbic acid was ingested, the plasma level rose in about an hour, then persisted or reached a peak at the end of three hours and fell slightly at the end of six hours. They recommended the determination of the nutritional state by using the height and general behavior of the blood curve after an oral dose of ascorbic acid.

Evaluation of Methods for Determining Nutritional State and Requirements

Much has been said for and against the various methods of determining requirements and nutritional state. In general, the blood tests have proved to be the most satisfactory and convenient.

Sloan (94) states that the determination of the vitamin C content of blood plasma is the simplest

dependable procedure; he also states that the most precise and dependable method of determining degree of saturation is the rate of disappearance of an injected dose from the blood stream and that the method based on rate of excretion of a test dose is less precise but quite dependable. Van Ekelen (104) believes that the determination of ascorbic acid in plasma is better than urinary studies for determining nutritional state because it is less complicated and only one or two tests are required, while the urinary test must be determined for a twenty-four hour output; he states that that output is very variable for different people. Farmer and Abt (29) believe the blood tests to be more informative than the urinary. Goldsmith and Ellinger (35) found in their experiments that there is a close correlation between urinary and blood findings as indicative of normal or deficient vitamin C nutrition, but they believe that the urine tests may be less reliable because of the high amount of interfering, non-ascorbic acid reducing substances in the urine in such conditions as diabetes or when the subject is on a high protein diet. Some workers have found that the great variation in response to a test dose in the same and different individuals makes the test unreliable (47,109,95). Some of these have found (95,109) that instead of progressive increases

in excretion with continued high doses of ascorbic acid, there is no further rise for several days, or until the doses are discontinued for several more days. This "plateau of excretion" Heinemann believed was due to a form of specific dynamic action, in which the excess of vitamin disappeared after the body stores were filled.

Johnson and Zilva (55) stated that the percentage excreted is higher on lower test doses than on high ones and that the amount retained is higher in the second case and is not indicative of the true requirement. These questions show the uncertainty of depending upon the test dose as a method of determining requirements.

Plasma Levels

There is some controversy over what constitutes "normal" values for the ascorbic acid content of blood. Many conclusions have been drawn by various workers after studies of plasma levels in relation to dietary intake.

Greenberg, Rinehart, and Phatek (40) studied the fasting plasma levels of 55 medical students. Their values ranged from 0.25 to 1.48 mg ascorbic acid per 100 ml of blood; the average was 0.72 mg per 100 ml. Forty-five per cent of the students had plasma levels below 0.72 mg. The experimenters concluded that values below 0.72 mg were probably suboptimal, that values between 0.7 and 0.9 appeared adequate, and that optimal

values were probably above this range. They stated that values below 0.5 mg per 100 ml must be considered low.

Youmans (113) found blood values of 1.66 to 4.31 mg per 100 ml in subjects on their usual or on a forced intake. Twelve patients, who were suspected of having a low intake, had values of 0.25 mg to 1.09 mg per 100 ml, the average being 0.539 mg.

Van Eekelen (95) concluded from his studies that values of from zero to 0.4 mg ascorbic acid per 100 ml are poor, from 0.4 to 0.8 mg moderate, 0.8 to 1.2 mg very good, and above 1.2 mg excellent.

Wright, Lilienfield, and MacLennan (109) found that the plasma values of their subjects who reported good to excellent dietary histories were 0.84 to 1.54 mg per 100 cc, that those who reported poor to fair dietary histories had values of from 0.27 to 0.68 mg. Some of their subjects who reported good dietary histories had levels of 0.31 and 0.37 mg, and two with diets considered fair to good had levels of 0.41 mg.

Sloan (94) considered 0.5 mg ascorbic acid per 100 ml to be the lower limit of normal and 0.8 to 1.00 mg the average normal fasting value. The average value for his subjects with the mildest cases of scurvy was 0.36 mg and for the severe cases 0.2 mg.

Taylor, Chase, and Faulkner (100) found the average plasma level of 10 scorbutic patients to be 0.245 mg;

the range was .11 to .55 mg per 100 ml.

There is some difference of opinion about what value shows tissue saturation. Ralli, Friedman, and Sherry (81) state that 1.0 mg per 100 ml or over indicates saturation, while Faulkner and Taylor (31) state that values over 1.4, and Goldsmith (35), values over 1.3 mg indicate saturation.

Wortis, Lieberman, and Wortis (108) used a value of 0.7 mg as the lowest limit of normal.

Drigalski (27) writes that values in blood over 1.0 mg ascorbic acid per 100 ml seemed to indicate good vitamin C nutrition, and less than 0.5, a mild state of deficiency.

Neuweiller considers values below 0.55 mg per 100 ml plasma as pathological and values below 0.4 mg as definitely scorbutic. He regards values of about 0.8 mg per 100 ml as good normal values without denoting saturation.

Goldsmith and Ellinger (35) concluded that an original level of 0.7 mg, reaching 2 mg or more after a test dose, shows normal vitamin C nutrition.

Regardless of the wide divergence of opinion over what constitutes "saturation" levels or "the lower limit of normal," it seems to be generally accepted that plasma levels of 0.7 or 0.8 mg ascorbic acid per 100 ml indicate a satisfactory nutritional state.

Requirements

In recent years, a great deal of work has been done on the determination of requirements for the protection of the organism from scurvy, for maintaining tissue saturation and for maintaining a "satisfactory" blood level.

In the days when scurvy was still a great problem to mariners, it was found by medical men in the British Navy that one ounce of lemon juice per day was sufficient to protect a man from scurvy. Kellie and Zilva (57) concluded from their experiments that 15 mg of ascorbic acid would protect from scurvy and maintain good health. Fifteen mg is approximately the amount of ascorbic acid found in one ounce of lemon juice. G8thlin found that 25 mg of pure ascorbic acid or the equivalent of that in orange juice was just sufficient to prevent lowered capillary resistance as shown by his positive pressure technique.

There appears to be a great difference between minimum requirements and values which are considered optimal. Fincke and Landquist (33) found that their four subjects required 1.1 mg, .7 to .8 mg, 1.1 mg, and 1.0 mg per kilo respectively to maintain a blood level of 0.8 mg per 100 ml of plasma.

Leveowitch and Batchelder (65), doing excretion studies on three subjects, concluded that the minimum ascorbic acid requirement of fairly active college women may lie at about 50 mg; with a 50 per cent margin of safety, the recommended intake would be 75 mg daily. Heinemann (50), using the method described previously, found that one of his subjects expended 62.6 mg per day for 37 days, while another used 63.3 mg for 53 days. He set 60 mg as the actual requirement for the adult male.

By the same method, van Bekelen (104) found his "daily expenditure" to be 34 mg after he had been on his ordinary diet and 63 mg when he had a fairly large store of the vitamin. Wersch (51) by the same method, calculated his daily expenditure as being 56 mg and that of an eighteen-year-old girl with scurvy as 44 mg daily.

Ralli, Friedman, and Sherry (81) found that in their subjects, the best retention was usually maintained on an intake of 100 mg daily.

Most of the work on requirements has been done with the purpose of determining the necessary amount of ascorbic acid to produce tissue "saturation." A summary of the results of some of these studies will be found in Table I.

It is not yet known whether or not it is advisable or of any value to keep the organism in a state of tissue

saturation, but there is a great deal of evidence that more than the minimum requirement to protect from scurvy is necessary to keep the organism in the best of health. When recommending requirements it is necessary to state whether those requirements are minimum or optimum as defined in the light of present knowledge. It is interesting to note that the recommended daily allowance for vitamin C was set at 75 mg for the adult man and 70 mg for the adult woman by the Committee on Foods and Nutrition of the National Research Council in May, 1941. It will be noted that these figures are located between the average reported optimum requirements and the average reported minimum requirements.

Table I

**Daily Intake of Vitamin C Required to Maintain Tissue Saturation,
as Reported by Various Experimenters**

Experimenters	No. of Subjects	Daily Requirement		Criterion of Saturation
		Total	Per Kilo	
Bryan, Hughes, Turner, Heine- mann	56		1.7-1.9 mg	Blood level of 1.0 mg per 100 cc
Todhunter and Robbins	3	90 mg	1.7	*Predetermined percent of excretion of test dose
		80	1.6	
		110+	1.6	
	1	120+		Blood level of 1.4 mg per 100 cc
Belzer, Hauck, and Storvick	2	70-85	about 1.0 to 1.6 mg per kg	*Predetermined percent of excretion of test dose
	3	85-100		
	2	100+		
Storvick and Hauck	5	100	1.4	*Predetermined percent of excretion of test dose
		75	1.5	
		100	1.3	
		150	2.8	
		125+	2.1	

Table I (Continued)

Experimenters	No. of Subjects	Daily Requirement Total	Per Kilo	Criterion of Saturation
	Same 5	90	1.3	50 percent excretion of test dose
		65	1.3	
		100	1.3	
		150	2.8	
		90	1.5	
Chen, Yu, Liu, Chiu	4 scorbutic		1.6 mg	Blood level of 1 mg per 100 cc
Faulkner and Taylor	1	300		Blood level of 1.4 mg per 100 cc
Fincke and Landquist	3	131	2.0	
		111	1.8	
		111	1.7	

*Method described, for Belzer, Hauck, and Storvick (7), in section entitled "Methods for Determining Requirements."

Factors Affecting Requirement

Study of the physiological functions of ascorbic acid suggests that certain factors may affect the requirements for the vitamin by man in various phases of health and activity.

The requirement for ascorbic acid is greatly increased during pregnancy and lactation as would be expected, since the vitamin is stored in the fetus and placenta. There is evidence that requirement for ascorbic acid bears no relation to age, height, or weight in the adult (81).

It has been found that the ascorbic acid requirement is raised considerably in certain febrile conditions such as tuberculosis, rheumatoid spondylitis, and pneumonia (82,41).

It has been suggested that the requirement is higher in other cases of heightened metabolism. Goldsmith, Ogaard, and Gove (36) were able to maintain a plasma level of 1.0 mg per 100 ml in 10 of 12 subjects with bronchial asthma by the administration of 50 mg ascorbic acid daily after a preliminary saturation. Of the two subjects who were not able to maintain a high plasma level, one was obese, and one was taking thyroid.

Koldasov and Gelman (63) found that the ascorbic acid content of a guinea pig muscle was decreased after

the application of an induction current whether the animals had received excessive, normal, or inadequate supplies of ascorbic acid. The reduction was somewhat less in the muscles of animals which received large doses of the vitamin than in animals which suffered a deficiency.

Tedhunter and Robbins (103) noticed some evidence from their experimental work that fatigue after long hours of study and laboratory work caused a lowering of blood and urinary vitamin C content.

Excretion studies in exercise experiments have, so far, brought only conflicting reports. Von Jezler and Haffter (105) studied the nightly urinary excretion of five participants before and after a ski race in which a height of 2450 meters was surmounted in a stretch of 50 km. The participants were given 300 mg Redoxon (synthetic ascorbic acid) for seven days before the race and for the six days following it. For each subject the excretion fell on the first or second day after the contest, the average nightly excretion for the five being 166 and 156 mg per 100 ml, for the day preceding and the day of the contest, and 115 and 116 mg per 100 ml for the two days following it.

This may be significant, but the drop may have been due to what Heinemann refers to as "the specific dynamic

action of ascorbic acid," in which the peak of excretion is not maintained after repeated high doses.

Hamel observed a definite drop in the excretion of his subjects on the first two days following their participation in a football game.

Brandt and Schussele (17) made excretion studies on seven young men subjects who were in good physical training. In five of the subjects, the reducing power of the urine rose and in two, it fell, after several forms of violent exercise; the change was in the same direction for each subject in each case. The experimenters could find no other explanation for this than that there is a "constitutional type, at least in what concerns the humerohormonal reactions in effort."

There is some evidence that a considerable amount of ascorbic acid may be lost in sweat. Cornbleet, Klein, and Pace (20) reported that from 0.55 to 0.64 mg of the vitamin is excreted with 100 ml of sweat. Bernstein (8) reported that from 0.5 to 1.1 mg. per 100 ml of sweat was lost by Bantu mine laborers. It was calculated on the basis of weight loss from evaporation that these laborers might have lost as much as 16 mg of ascorbic acid in an eight-hour day of work. On the other hand, Wright and MacLenathan (110) calculated the ascorbic acid loss from patients in artificial fever treatment to be about 0.041 mg per 100 ml of sweat. Calculated on the

basis of a loss through evaporation of 1750 to 5500 ml in a five-hour period, the most that could be lost would be two to three mg of ascorbic acid in that period.

From this they concluded that vitamin C is not lost in any appreciable amount through excessive perspiration. In the same fever periods, the urinary excretion of the vitamin fell to a greater degree than could be accounted for by the loss in sweat. This drop they attributed to a probable loss through increased metabolism.

There is also a conflict about the effect of increased dosage of ascorbic acid on physical performance. Wiebel (106) noted better performance in scarcely any of 44 female athletic students in a ski tournament when large doses of ascorbic acid had been supplied.

Basu and Ray (5), recording fatigue curves of the finger muscle on the hand ergograph for four subjects before and after the administration of large doses of ascorbic acid, reported that the onset of fatigue was definitely delayed for some time after the administration of the vitamin.

Crandon, Lund, and Dill reported that their subject could run for only sixteen seconds before fatigue set in when he was in a state of complete deficiency, while after ten days' ascorbic acid therapy, the time was increased to 66 seconds. This may be compared to the

performance of men of the same age, which averaged 270 seconds.

The disappearance of blood lactate was followed in the recovery period from the same fatigue tests, and it was found that the lactate disappeared more slowly during the recovery period when the subject was in the scorbutic state. The minute-oxygen consumption per kilogram of body weight, however, was no higher in the fatigue test after therapy than it was when the subject was in the scorbutic state.

Crandon, Lund, and Dill (21) did not find an increased metabolism in ascorbic acid deficiency, as some others have reported. There was a slight drop in metabolism in the scorbutic state. This drop, they believed was probably due to weight loss or inanition or both. They reported that, in the first four days of vitamin therapy, when the subject was becoming saturated with ascorbic acid, the basal metabolic rate was consistently lower one and a half to two hours after injection than it was before the injection; following the period of saturation, the basal metabolic rate was consistently higher two hours after the administration of ascorbic acid than it was before its injection.

With all of the conflicting reports in the literature, it is obvious that the mechanism of the action of

ascorbic acid in muscular activity and tissue respiration is not clearly understood, but there has been enough evidence presented to conclude that the vitamin does have some role in these metabolic functions.

CHAPTER II

PURPOSE OF THIS INVESTIGATION

The question has been opened as to whether or not human requirements for ascorbic acid are affected by muscular activity.

The issue has been suggested by the following factors:

1. Ascorbic acid appears to have an essential role in the general metabolic processes of the body.
2. Ascorbic acid appears to have a protective or regulatory action on at least two respiratory enzymes in skeletal muscle. Further evidence for its importance in tissue respiration is shown by the retarded disappearance of blood lactate in the scorbutic state.
3. Evidence has been presented by experimental work that the requirement for ascorbic acid is raised in conditions accompanied by an elevated metabolism, as in fever, obesity, thyroid therapy, and exercise.

It is the purpose of the following study to determine whether or not the human requirement of adults for ascorbic acid is affected by an increase in physical activity.

CHAPTER III

EXPERIMENTAL

General Procedure of Experiment

Three subjects were carried through two experimental periods of nine days each. Throughout the periods, the subjects were kept on a diet of known low ascorbic acid content, while a supplement of the crystalline acid was given. The basic diet consisted of cheese, beef, dried cooked prunes, and canned beets, carrots, pears, potatoes, and evaporated milk. Foods taken ad libitum were eggs, shredded wheat, Ry-Krisp, rice, flour, butter, nuts, sugar, coffee, tea, seasonings, and chocolate. The ascorbic acid contents of these foods will be found in Table II. The diet contributed 15 mg of ascorbic acid per day.

In the first period of the experiment, the subjects followed their regular routine of a fairly sedentary student's life. In the second period, the subjects exercised vigorously for about one hour per day.

For the first two days of the first experimental period, the subjects were given one pint of orange juice, one glass at breakfast and one glass at lunch, in addition to the constant diet. This was to make certain that the plasma ascorbic acid levels were brought well above

Table II
 Ascorbic Acid Content of Foods
 Used in Experimental Diet

Food	Weight	Ascorbic Acid Content
Canned beets	100 g	3.6 mg
Beet juice	10	.7
Canned Carrots	100	1.1
Carrot juice	10	.2
Canned pears	100	1.2
Pear juice	50	.2
Prunes	80	.2
Prune juice	10	.1
Evaporated milk	60	.2
Cheese	60	.0
Beef	100	.0
Potatoes	100	7.6
Total:		15.1

Foods taken ad libitum were eggs, shredded wheat, Ry-Krisp, rice, flour, butter, nuts, sugar, coffee, tea, seasonings, and chocolate.

0.8 mg per 100 ml, the figure which is usually taken as the level of "good nutrition." For the rest of the period, an ascorbic acid supplement of 50 mg daily was given to AR, the woman subject, and a supplement of 55 mg was given to KD and LR, the two men subjects. This made a total intake of 65 mg for the woman and 70 mg for the men. Plasma ascorbic acid levels were determined daily on each of the subjects. Between the two periods, there was an interval of four days, in which the subjects had a fair amount of vigorous exercise and in which they kept their ascorbic acid levels reasonably high by taking about two oranges per day. In the nine-day period of exercise, the ascorbic acid intake was the same as in the first period. Plasma content of the vitamin was again determined daily.

In deciding on the amount of ascorbic acid to be given to the subjects, it was judged best to use the recommended daily allowance as released by the Committee on Food and Nutrition of the National Research Council in May 1941. This figure was stated as 75 mg daily for men and 70 mg for women. A rough calculation of the basic diet preceding the experiment showed that it contained approximately 20 mg (about half of which was to come from canned potatoes). To make up the recommended allowance, then, supplements of 50 mg were given to the

woman and 55 mg to the men. Later a more careful analysis of the constituents of the diet, in which the potatoes were analyzed as prepared for serving, showed that the basic diet contributed 15.14 mg of ascorbic acid, 7.63 mg from the potatoes and 7.51 from the rest of the diet. Actually, then, the total daily ascorbic acid intake was 65 mg for the woman and 70 mg for the men. These intakes are above one milligram per kilogram; the intake which Fineke and Landquist (33) found would maintain the plasma levels of their subjects at 0.8 mg per kg.

Analysis of Foods Used in Diet

The foods used in the experimental diet were analyzed for ascorbic acid as follows: About 25 grams of the food were weighed, ground in a mortar with sand with a few cubic centimeters of three per cent metaphosphoric acid and centrifuged. The process was repeated twice and the extract diluted to 50 cc with the metaphosphoric acid. One-half of the extract was brought to a pH of 3.5 to 3.6 with a citrate buffer. To four ml of a solution of 2,6 dichlorophenolindophenol (8 mg in 500 ml) in a colorimeter tube were added 4 ml of the buffered extract. The tube was shaken and inserted in the Evelyn photoelectric colorimeter and the galvanometer reading recorded at 15 seconds and at 30 seconds (readings G_{s_1} and G_{s_2}). The excess dye was then completely reduced by an added crystal of ascorbic acid and the reading again recorded

(Gr). A colorimeter tube containing 4 ml dye solution with 4 ml of 3 per cent metaphosphoric acid buffered to the same pH as the extract and a crystal of ascorbic acid was placed in the colorimeter and the galvanometer adjusted to the reading Gr. With the instrument set in this position, a blank tube (4 ml of dye solution with 4 ml of buffered 3 per cent metaphosphoric acid) was placed in the colorimeter and the reading recorded (Gb). The concentration of ascorbic acid was calculated according to the following equation:

$$C = K (\log G_s - \log G_b)$$

The constant for this Evelyn instrument is 0.086.

Procedure for Determining Plasma Ascorbic Acid Level

The finger was pricked with a sterile lancet and several drops of blood (about .4 cc) were collected in a small phial containing crystals of lithium oxalate. The blood was stirred with one turn of the broad end of a toothpick and the phial stoppered and centrifuged for three minutes. One-tenth of a milliliter of the clear plasma was pipetted off and blown into a 15 ml conical centrifuge tube, 0.1 ml of recently boiled and cooled redistilled water added with the same pipette, and the protein precipitated with 0.2 ml of 5 per cent metaphosphoric acid. The tubes were shaken to insure mixing and were then stored in the refrigerator until the titration could be completed.

The centrifuge tubes containing the deproteinized plasma were removed from the refrigerator and centrifuged for five minutes. Two-tenths of a milliliter of the supernatant fluid were placed in a depression of a porcelain spot-plate and 0.2 ml of 2.5 per cent metaphosphoric acid solution in a neighboring depression. The dye was run into the plasma solution from a micro-burette until there appeared a faint pink color which lasted for fifteen seconds. The metaphosphoric acid blank in the neighboring depression was then titrated to a pink of similar intensity. The concentration of ascorbic acid in the plasma was calculated according to the following equation: $\text{ml dye} \times S \times 2000$, where S is the strength of dye expressed in mg of ascorbic acid equivalent to one ml of dye.

Standardization of Dye

The method of standardizing the dye directly against sodium thiosulfate as suggested by Menaker⁷⁰ and Geurra⁷¹ was adopted in preference to that in which the dye was standardized against ascorbic acid, and the ascorbic acid against iodine, the iodine being standardized every four weeks with a sodium thiosulfate solution, which was standardized with the primary standard potassium dichromate. The new method cut out several steps in the standardization procedure and had the added advantage

of using the sodium thiosulfate standard, which is stable when it has once reached equilibrium, rather than the less stable iodine solution. The three following methods of standardization were compared and good checks received: the methods just described and the method of standardizing dye against an ascorbic acid solution which had been titrated with a standard potassium iodate solution.

The procedure used for standardizing the dye is as follows: 25 cc of the dye solution were pipetted into an Erlenmeyer flask; 0.5 grams potassium iodide and 0.5 cc of dilute sulfuric acid (1:4) were added and the mixture shaken until all of the iodine was liberated; .001 N. sodium thiosulfate was run in from a 10 ml burette of small bore until the yellow iodine color had practically disappeared; then 2 cc of 2 per cent starch solution were added and the titration continued until the solution became colorless. One cc of 0.001 N iodine is equivalent to 1 ml of 0.001 N sodium thiosulfate, and it has been shown that 1 ml of 0.001 N iodine will react with .088 mg ascorbic acid, so 1 ml of 0.001 N sodium thiosulfate is equivalent to 0.088 mg of ascorbic acid. Therefore, the strength of the dye solution can be calculated according to the following equation:

$$\frac{\text{ml Na}_2\text{S}_2\text{O}_3 \times \text{N} \times 88}{25} = \text{mg ascorbic acid equivalent to 1 cc dye}$$

N is the normality of the thiosulfate solution.

Standardization of Sodium Thiosulfate

The sodium thiosulfate solution was standardized against 0.1 N potassium dichromate about every five or six days until the fresh solution had become stabilized. It was found advisable to make the thiosulfate solution several days before its first standardization.

The sodium thiosulfate standardization was carried out according to the procedure outlined by Fales and Kenney in "Quantitative Inorganic Chemistry." A 1.8 M potassium iodide solution (iodate free) was prepared; 10 ml of this was added to 200 ml of water, followed by 5 ml of 12 N hydrochloric acid. To this were added slowly with constant stirring 25 ml of standard potassium dichromate solution. This was allowed to stand out of direct sunlight for three minutes and then diluted to 400 ml and titrated with the sodium thiosulfate solution.

The normality of the thiosulfate solution was calculated according to the following equation:

$$\text{Normality of Na}_2\text{S}_2\text{O}_3 = \frac{N \text{ of K}_2\text{Cr}_2\text{O}_7 \times \text{ml K}_2\text{Cr}_2\text{O}_7}{\text{ml Na}_2\text{S}_2\text{O}_3}$$

Solutions

Dye. Twenty-eight mg of the dye 2,6 dichlorophenolindophenol were dissolved in 980 ml of hot water which had been distilled from glass. The solution was cooled

and 20 ml of phosphate buffer, pH 6.8 added. The solution was then filtered and stored in a dark bottle in the refrigerator. The dye was made fresh weekly.

Metaphosphoric Acid Solution. The sticks of solid acid were rinsed several times with redistilled water and dried to remove the coating of orthophosphoric acid which forms on the outside during storage. Five grams of the acid were dissolved in a small amount of boiled and cooled redistilled water and made up to 100 ml in volume. This was filtered and stored in the refrigerator. The solution was made fresh about every seven days.

Sodium Thiosulfate Solution. Approximately 24.82 g of crystalline sodium thiosulfate were dissolved in water and made up to one liter. This approximately 0.1 N solution was kept in stock and a 0.001 N solution made from it immediately before standardization of the dye.

Potassium Dichromate Solution (0.1 N). Approximately 4.9 g of pure dry potassium dichromate were accurately weighed, dissolved in distilled water and carefully made up to one liter.

Description of Subjects

Subject KD, a graduate assistant in chemistry (28 years of age) was five feet, ten inches in height and weighed 140 pounds. He appeared to be rather nervous

and complained of "indigestion" before the experiment began, but was in apparent good health otherwise. After the experiment had progressed, he announced that the indigestion had disappeared and that he had a great deal more vitality than he had had for some time. This state continued throughout the entire experiment. When questioned about his habits of eating, it was found that they were irregular and poor, his usual breakfast being a cup of coffee. This subject is the only one of the three who complained of being tired during the period of exercise.

Subject LR (26 years of age), the other man, also a graduate assistant in chemistry, was 5 feet, 10 inches in height and weighed 144 pounds. He, too, appeared to be nervous, and it was disclosed that there was something worrying him at the time of the experiment. It was often very difficult to obtain blood samples from him; this may have been because of his unusual nervous tension or because his fingers were scarred from burns. This subject complained of being tired on the last day of the experiment, but the exercise did not seem to tire him as it did subject KD.

Subject AR (19 years of age), the wife of LR, was 5 feet, 3 inches in height and weighed 104 pounds at the beginning of the experiment but gained two and one-half

pounds during the course of the experiment. She also appeared to be nervous and was very active, being a home-maker and student and working in a laboratory part of the time. The exercise never appeared to tire her.

CHAPTER IV

RESULTS AND DISCUSSION

The daily plasma ascorbic acid levels for the three subjects in the two experimental periods are shown in Tables III, IV, and V. The average of the last four days of each period was used as the level at which the plasma ascorbic acid content was maintained at that particular intake.

For subject AR, the average for the last four days of the control period was 0.72 mg ascorbic acid per 100 ml of plasma, and the corresponding average for the exercise period was 0.66 mg per 100 ml, the daily intake of ascorbic acid being the same in both periods. It will be noted that the level dropped to 0.57 mg on the last day of the exercise period. The average of the last four days of the control period for subject LR was 0.60 and for the exercise period 0.58 mg per 100 ml. The corresponding figures for subject KD were 0.78 mg per 100 ml for the first period and 0.69 mg per 100 ml for the exercise period.

For subject LR, there was no significant drop in the plasma level maintained in the second period. For subject AR, the average for the exercise period was 0.06 mg lower than in the first period, and for subject KD the average for the exercise period was 0.09 mg lower

than the average for the control period.

It is generally accepted that the titration method of measuring ascorbic acid in plasma is precise within about 0.1 mg per 100 ml. In view of this, it cannot be considered that the lower averages for subjects AR and KD for the exercise period are significant. It is interesting to note, though, that subject KD, whose average level was 0.09 mg lower in the period of exercise, is the only one of the three who complained of being fatigued by the exercise. It is worthy of note, also, that the trend of the plasma level in the exercise period was downward in the case of each subject.

Considering the minimum "level of good nutrition" to be 0.7 to 0.8 mg ascorbic acid per 100 ml of plasma, it is apparent that a daily intake of 65 mg for the woman and 70 mg for the men (figures which are 5 mg below the recommendation of the National Research Council) were sufficient to maintain this blood level for subject KD in both periods and for subject AR in the control period and insufficient for subject LR and AR in the exercise period. Subject LR required more than 70 mg in both periods.

As mentioned before, subject LR was worried at the time of the experiment. It has been noted in this laboratory and others that worry and nervous tension often

appear to be accompanied by a low plasma ascorbic acid level. That may have been a factor in the case of the apparent high requirement of LR.

It was revealed that KD included very little exercise in his regular routine, while LR and AR were accustomed to a fair amount of vigorous exercise.

It can be concluded from this investigation that the ascorbic acid requirements for the three subjects used were not raised significantly by about one hour of vigorous exercise per day.

In reviewing the literature on the subject of exercise in connection with vitamin C requirements, it is apparent that the experiments performed have brought conflicting results. Hamel (41) and von Jezler and Haffter (105) noted a drop in ascorbic acid excretion after exercise, while Brandt and Schussele (16) noted a drop in the excretion of two and a rise in that of five subjects.

The possibility of a loss of the vitamin through sweat in exercise should be considered. Calculating the hourly loss with the highest figures found in the literature, 0.5 to 1.1 mg, quoted by Bernstein (8), it would appear that the loss for the added hour of exercise for these subjects would not exceed 2 mg, an amount which could hardly be considered as raising the requirement significantly.

As mentioned previously, Koldaev and Gelman observed that there was a decreased ascorbic acid content of a guinea pig muscle after the application of an induction current. It is likely that the stimulus used resulted in the contraction of a far greater percentage of muscle fibers than would be used in any voluntary physical exercise, so their experiment is not comparable with this study. It is quite possible, however, that if this experiment were repeated with subjects who were able and willing to devote several hours a day to vigorous exercise, a definite drop in plasma ascorbic acid would be noted. It is possible, also, that a greater drop may have been noted had the experiment been allowed to run for a longer period of time.

Since the trend for the plasma levels of ascorbic acid is downward for each subject in exercise, it seems advisable to suggest that the intake of ascorbic acid be raised during hard physical labor or exercise.

CHAPTER V

CONCLUSIONS

The ascorbic acid requirements for the three subjects used were not raised significantly by about one hour of vigorous exercise per day, but the trend of the plasma level of ascorbic acid was downward in the exercise period for each subject.

A daily intake of 65 mg ascorbic acid was enough to maintain a level of 0.7 mg ascorbic acid per 100 ml for the woman subject in the control period and not quite enough to maintain this level in exercise.

A daily intake of 70 mg of the vitamin was sufficient to maintain this level for one of the men subjects in the control period and hardly sufficient for the exercise period. An intake of 70 mg was insufficient to maintain this level in the other man subject in either period.

Table III
Daily Plasma Ascorbic Acid Values
Subject AR

Date	Ascorbic Acid Intake	Plasma Level	Exercise
1/15	Orange juice	.56 mg/100 ml	
1/16	Orange juice	1.22	
1/17	65 mg	1.10	
1/18	65 mg		
1/19	65 mg	.92	
1/20	65 mg (+ 100 g pota- toes)	.73	
1/21	65 mg	.77	
1/22	65 mg	.71	
1/23	65 mg	.67	
1/24	65 mg	.72	
Average of last four days:		.72	
1/28	Orange juice	1.04	Badminton 50 min.
1/29	Orange juice	.61	Bicycling one hour
1/30	65 mg	.64	Bicycling one hour
1/31	65 mg	.75	Bicycling one hour
2/1	65 mg		Bicycling one hour
2/2	65 mg	.68	Bicycling one hour
2/3	65 mg	.71	Running 1 1/2 miles
2/4	65 mg	.68	Bicycling one hour
2/5	65 mg	.57	
Average of last four days:		.66	

Table IV
Daily Plasma Ascorbic Acid Values
Subject LR

Date	Ascorbic Acid Intake	Plasma Level	Exercise
1/15	Orange juice	.48 mg/100 cc	
1/16	Orange juice	.93	
1/17	90 mg	1.04	
1/18			
1/19	70 mg	.71	
1/20	70 mg (+ 100 g pota- toes)	.67	
1/21	70 mg	.68	
1/22	70 mg	.57	
1/23	70 mg	.53	
1/24	70 mg	.61	
Average of last four days:		.60	
1/28	Orange juice	.71	One hour handball
1/29	Orange juice	1.07	One hour handball
1/30	70 mg	.60	One hour handball
1/31	70 mg	.54	One hour handball
2/1			Running 40 min.
2/2	70 mg	.56	Running 40 min.
2/3	70 mg	.55	One hour handball
2/4	70 mg	.65	One hour handball
2/5	70 mg	.57	
Average of last four days:		.58	

Table V
Daily Plasma Ascorbic Acid Values
Subject KD

Date	Ascorbic Acid Intake	Plasma Level	Exercise
1/15	Orange juice	.57 mg/100 cc	
1/16	Orange juice	.86	
1/17	70 mg	1.13	
1/18	70 mg		
1/19	70 mg	.81	
1/20	70 mg (+ 100 g pota- toes)	.78	
1/21	70 mg	.78	
1/22	70 mg	.82	
1/23	70 mg	.73	
1/24	70 mg	.78	
Average of last four days:		.78	
1/28	Orange juice	1.16	One hour handball
1/29	Orange juice	1.23	One hour handball
1/30	70 mg	.72	One hour handball
1/31	70 mg	.58	One hour handball
2/1	70 mg		Running 40 min.
2/2	70 mg	.72	Running 40 min.
2/3	70 mg	.71	One hour handball
2/4	70 mg	.68	One hour handball
2/5	70 mg	.65	
Average of last four days:		.69	

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