THE USE OF A MICROMETHOD TO DETERMINE THE PLASMA ASCORBIC ACID LEVELS IN CHILDREN

by

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INTRODUCTION

Physiological Roles of Ascorbic Acid

Ascorbic acid has long been known to have a preventive and curative effect on scurvy. In man, an extreme deficiency in ascorbic acid resulted in scurvy. The symptoms of scurvy were anemia, pains in the joints, gingivitis, hemorrhages from the mucous membranes of the mouth and gastrointestinal canal, and hemorrhages in skin and muscle, and defects in skeletal calcification without much disturbance in mineral metabolism. Ascorbic acid also has been shown to be necessary for wound healing. These pathological changes have been considered to be the result of the reduction in the amount of intercellular substances because of the lack of ascorbic acid (1).

Besides aiding in formation of cement and supporting material, other important roles in the body's metabolism have been ascribed to ascorbic acid. It was found relatively high in some endocrine glands; e.g., adrenal cortex and corpus luteum (1.4 to 2.3 mg per gram) and fairly high in brain, liver, testes and ovary (0.1 to 0.4 mg per gram) (2). But the function of ascorbic acid in these tissues has not been clarified. Cio and Schteingart (3) observed that a simultaneous injection of gonadotropic hormone and ascorbic acid into a rat produced a greater increase in the size of the gonads than injection of the hormone alone. Ascorbic acid alone had no

effect on gonad size.

Ascorbic acid has been thought to be a part of one of the respiratory enzyme systems in tissues because of its oxidation-reduction potentials. It could be oxidized and reduced by glutathione which also might be a part of the system (1).

$$\text{Ascorbic acid} \xrightarrow[\text{enzyme}]{\text{GS-SG}} \text{Dehydroascorbic acid}$$

$$\xrightarrow{2\text{GSH}}$$

Ascorbic acid was described as a coenzyme for cathepsin and for liver esterase (1). It also was shown to have a direct relationship to the phosphatase content of serum and bone. Gould and Shwachman (4), and Shwachman and Gould (5) observed that a fall in activity of bone phosphatase and of serum phosphatase occurred in the scorbutic animal, and that with the administration of ascorbic acid there was a concomitant rise in the serum and bone phosphatase.

Ascorbic acid has been thought to influence the function of liver, because Beyer (6) observed that administration of ascorbic acid to guinea pigs protected them against experimental hepatic damage caused by hydrazine.

Ascorbic acid has been shown to aid the body in metabolizing tyrosine. Sealock and Silberstein (7) demonstrated that feeding of 0.5 gm or more of tyrosine per day to ascorbic acid deficient guinea pigs resulted in excretion of homogentisic acid, parahydroxyphenylpyruvic acid and parahydroxyphenyllactic

acid. Levine et al. (8) observed that administration of human milk or ascorbic acid corrected the defect of the premature infants who excreted parahydroxyphenyllactic acid and papa-hydroxyphenylpyruvic acid.

Although severe deficiency in ascorbic acid resulted in scurvy, in ordinary life such marked deficiency has seldom been observed (1) but it would seem reasonable to assume that deficiencies of various grades do occur and may threaten the optimum health even without obvious clinical symptoms, because ascorbic acid is related to so many aspects of normal function of the body.

Methods Used to Measure Ascorbic Acid Status

The nutritional status of a person in regard to ascorbic acid has been determined by his response to a large dose of ascorbic acid, by intradermal test, by examination of gums, by capillary strength test and by the determination of the plasma level of ascorbic acid.

Response to a Large Dose. After administration of a large dose of ascorbic acid, workers reported that there was a rise in the plasma level of ascorbic acid and large amounts were excreted in the urine. However the actual amount excreted, the time of the rise in plasma level, and the maximum levels obtained were found to depend on the individual's previous nutritional status in respect to ascorbic acid.

In urinary excretion tests, various standards for size of the test dose and the criteria for judging saturation have been suggested by various workers, Table 1. Fifty per cent of the test dose, 200-700 mg, excreted in 24 hours was used by most workers as the criterion of saturation.

In determinations of plasma ascorbic acid levels, Prunty and Vass (10) observed that after ingestion of synthetic ascorbic acid, a maximum rise occurred in the plasma within one to one and one-half hours, but if a natural source of ascorbic acid was employed, the peak of plasma ascorbic acid level occurred around two and one-half hours. Todhunter et al. (19) observed that ingestion of 50 mg ascorbic acid in pure form, or from orange juice or whole orange, led to an increase after half an hour, a maximum in one and one-half hours, and a return to the fasting level in three to three and one-half hours. However, the effects of ascorbic acid in strawberries and cauliflower were shown to be delayed.

The elevation of plasma ascorbic acid after administration of ascorbic acid was related to the status of tissue saturation. If the tissue ascorbic acid content was low, the ascorbic acid would flow from the plasma to tissue and the plasma level of ascorbic acid would not rise to a high level. Todhunter et al. (19) showed that a 50 mg intake caused no increase in plasma ascorbic acid level if the plasma fasting value was below 0.5 mg per 100 ml. If the tissue was saturated with ascorbic acid, the plasma level of ascorbic acid would rise markedly.

Table 1. Criteria of saturation based on urinary response to a large dose.

Worker	Test dose size (mg)	Criterion of saturation (amount of excretion)
Keith, cited in (9)	500	50 mg in 24 hours
Wedinbauer, cited in (9)	200-500	50% of half dose in 12 hours
Baumans, cited in (9)	25/kilo	29-50% of the dose in 24 hours indicated no deficiency
Prunty and Vass (10)	700	33.3% of dose in 24 hours
Engelfried (11)	200	20 mg in 4 hours or 30 mg in 6 hours - adequate. Less than 10 mg in 6 hours - inadequate
Goldsmith and Ellinger (12)	600	30% of the dose in 24 hours or 16.6% in 6 hours - saturation
Wortis et al. (13)	1000	400 mg in 5 hours - normal
Hathaway and Meyer (9) Lewis et al. (14) Roberts and Roberts (15) Haines et al. (16) Todhunter and Robbins (17) Storvick and Hauck (18)	500	50% of the dose in 24 hours

Farmer and Abt (20) observed that a large test dose of ascorbic acid led to a rise of plasma ascorbic acid level, usually reaching the peak in two hours; and the individual nutritional status could be determined by measuring the difference between the initial level and the height attained at the end of the second hour. The more saturated the individual, the greater was the span between the initial value and the peak attained.

Stotz et al. (21) derived four types of curves of plasma ascorbic acid response to a large dose of ascorbic acid, and used them to determine an individual's nutritional status. Type I curve, representing saturation, was characterized by a high initial plasma ascorbic level, a moderate rise, and a failure to decline. As the plasma level might exceed the kidney threshold, the urinary excretion might be high and the plasma level might fail to rise to higher levels. Type II curve, representing a high normal curve, was characterized by marked rises and various rates of falling. Type III curve, the low normal type, was characterized by a relatively low fasting plasma ascorbic acid level (0.35 to 0.70 mg per 100 ml) with only a slight or transitory rise. In type IV, the plasma level did not rise and the urinary excretion did not exceed 23 mg.

It was shown by Crandon et al. (22) that after injection of one gram of ascorbic acid to an individual being depleted of ascorbic acid, the plasma ascorbic acid content fell very

rapidly during the first three hours and reached zero at the end of the fifth hour. On each succeeding day the initial plasma level of ascorbic acid was higher and the rate of dropping down was slower. A very gradual drop of the plasma level of ascorbic acid might be taken as an indication of the body's saturation with ascorbic acid.

Estimation of number of days to saturate the body by a large test dose as a method to determine nutritional status was used by Harris (23). If the body were saturated with ascorbic acid, the amount of ascorbic acid excreted after the administration would attain a more or less constant level. The number of days required for this attainment of maximum urinary excretion was taken as an indication of the ascorbic acid saturation. If a person was in a satisfactory nutritional status in regard to ascorbic acid, saturation was reached in one day. If a person had a low intake of ascorbic acid, 3 to 5 days might be required for saturation. In cases of scurvy patients, 7 to 10 days might be required to attain saturation (24).

Intradermal Test. The degree of individual ascorbic acid saturation has been determined by the rate of decolorization of the blue wheal developed by the intradermal injection of dichlorophenol-indophenol solution.

Slobody (25) reviewed the work of Rotter in which he recommended the use of 0.01 ml N/400 dichlorophenol indophenol to raise a blue wheal (about 2 mm in diameter) which would disappear in less than 5 minutes if the tissue were saturated

with ascorbic acid but which would remain for 10 minutes if the tissue were deficient in ascorbic acid. But his method was found unreliable by some workers.

Slobody (25) revised the Rotter method by changing the amount and concentration of the dye solution and increasing the size of the wheal area because in the small area suggested by Rotter, the immediate area of needle puncture took a much longer time to be decolorized than the rest of the wheal. The results of his study on a group are shown in Table 2. Although long skin reduction time was associated with low plasma ascorbic acid level, Slobody (25) emphasized that the intradermal test indicated body saturation regarding ascorbic acid, and plasma ascorbic acid level reflected the recent dietary intake. The blood level of ascorbic acid was raised but the skin reduction time remained unchanged 30 minutes after administration of a 200 mg dose. On an ascorbic acid deficient diet the plasma ascorbic acid level fell and the skin test time was prolonged.

Examination of Gums. There were contradictory observations regarding the relationship between the individual's ascorbic acid nutritional status and the health of his gums. Kruse (26) recommended the gums as the tissue to be examined in mild deficiency of ascorbic acid because the gums would show early and progressive changes associated with the development of the deficient state. He reported that the first sign of ascorbic acid deficiency might be a slight dilation and en-

gorgement of the subsurface vascular papillae of the gum and that later the redness of the gum might be observed by edema and probably infiltration, distention and hypertrophy or atrophy of the papillae. But Crandon et al. (22) reported that appearance of petechiae on the skin and the change in gums occurred at the same time.

Table 2. Intradermal test as indication of ascorbic acid status (25).

No. of subjects	Skin reduction time (minutes)	Ascorbic acid status
102	3.5 - 9	less than 7 min. indicates complete saturation less than 9 min. indicates satisfactory ascorbic acid nutrition
104	9 - 13	mild degree of saturation
79	14 - 25.6	deficiency

Contradictory results were obtained from treatment of gingivitis. Campbell and Cook, whose work has been reviewed (27), and Kyhos et al. (28) found that ascorbic acid was effective in curing gingivitis in a short time. But Kruse (26) failed to cure gingivitis by increasing ascorbic acid intake. A review (29) of the work of Linghorn on the effect of ascorbic acid on gingivitis stated that he first removed existing infection around the teeth and then carried on the experimental

dietary work for eight months. The incidence of gingivitis was in inverse proportion to the amount of intake but the response was slow, and if gingivitis was established, an improvement of ascorbic acid nutrition for a few months did not eradicate the condition. These data indicate it was unreliable to determine the nutritional status of ascorbic acid only by examination of gums because ascorbic acid deficiency was not the only factor causing gingivitis, and gum disorder sometimes failed to appear at the early stage of ascorbic acid deficiency. However, it might serve as a further evidence to estimate the nutritional status when accompanied by another method.

The Capillary Strength Test. The strength of the skin capillaries was measured by applying pressure on the capillaries until they were unable to withstand the stress, ruptured and formed petechial hemorrhages. The relationship between ascorbic acid nutritional status and capillary strength was not obvious. A review (30) of the work of McMillan and Inglis stated that they found no relationship between nutritional status with regard to ascorbic acid and capillary strength in a group of 40 scurvy patients. Although 21 who showed abnormal capillary fragility before treatment showed normal capillary strength after ascorbic acid therapy, six were normal both before and after treatment while 13 were abnormal on both occasions. Todhunter et al. (19) observed that capillary resistance was not significantly affected by variation in known ascorbic acid intake. Munro et al. (31) found that two-thirds

of their cases of frank scurvy and one-third of their cases of undernourished subjects without scurvy showed capillary weakness. Contradictory results were obtained on administration of ascorbic acid to subjects with capillary fragility (13), but in some cases administration of fruit juice and vitamin P preparation improved the capillary strength. The fact that guinea pigs, rats or rabbits deficient in vitamin P had decreased capillary strength has been cited as a further indication of the positive relationship between vitamin P and capillary strength. It was very common to find a subject lacking both ascorbic acid and vitamin P because the sources of both of them are similar. They have been found abundantly in orange, lemon and other fruits and vegetables.

However, Cotereau et al. (32) observed that neither ascorbic acid alone nor vitamin P was effective in increasing capillary strength of guinea pigs, but a small dose of ascorbic acid accompanied by vitamin P helped to increase capillary resistance and led to a storage of a certain amount of ascorbic acid in organs. It was shown that substances capable of increasing the capillary strength in guinea pigs were able to protect ascorbic acid from oxidation in vitro (33). Vitamin P, a group of flavone glucosides, was considered to be a stabilizer of ascorbic acid (33) and to increase the utilization and effectiveness of ascorbic acid. Vitamin P was indispensable since without it the body would require ascorbic acid in much larger quantities than could be supplied by a normal diet.

Plasma Ascorbic Acid Level. The response to a large dose proved relatively troublesome to determine. The intradermal test was in its experiment stage, and both the gum examination and capillary strength test were not restricted to ascorbic acid nutritional status only. Therefore, blood ascorbic acid seemed to be an easy and accurate way to determine ascorbic acid nutritional status. The relationships between plasma ascorbic acid level and other methods have been summarized in Table 3.

The various criteria to determine nutritional status of ascorbic acid based on plasma ascorbic acid level established by previous workers have been listed in Table 4.

The plasma saturation level was indicated by the renal threshold. The renal threshold was the plasma ascorbic acid level corresponding to the first marked rise in urinary excretion (40). When the plasma ascorbic acid was below the renal threshold, the ascorbic acid level in urine was very low and tended to be constant. The subject was assumed to be saturated with ascorbic acid while his plasma ascorbic acid level was maintained at or above the renal threshold. It was showed by Crandon (22) that in cases of ascorbic acid deficiency the renal threshold might be lower than normal. The renal thresholds estimated by different workers have been summarized in Table 5.

Renal thresholds were shown to be different in different individuals. A person with a high renal threshold was able to

Table 3. Relationship of plasma ascorbic acid level to other methods of estimating ascorbic acid nutritional status.

Method	Subjects: No.	Subjects: Remarks	Plasma ascorbic acid level: mg per 100 ml	Reference: Workers	Reference: Date
Response to 1 gm dose	133	Normal excretion (400 mg in 5 hours) present in		Wortis et al. (13)	1938
		26 of 30 with	0.7 or more		
		11 of 35 with	0.4 - 0.69		
		7 of 68 with	less than 0.4		
Response to 600 mg dose	11	Normal - excrete 50 mg or more in 6 hrs.	0.43 - 1.98		
		- excrete 30 per cent or more in 24 hrs., 109 mg in 6 hrs.	1.3		
		- excrete 12 to 23 per cent in 24 hrs., 50 mg in 6 hrs.	0.65 - 1.3	Goldsmith, Ellinger (12)	1939
	14	Deficient - excrete less than 50 mg in 6 hrs.	0.05 - 0.52		
Gum study	49	With gingivitis			
		21 per cent	less than 0.2		
		43 per cent	0.2 - 0.6	Kruse (26)	1942
		10 per cent	0.6 - 1.0		
		26 per cent	more than 1.0		
Gum study	71	With gingivitis			
		77 per cent	less than 0.2	Kyhos et al. (28)	1944
		20 per cent	0.2 - 0.6		
		3 per cent	more than 0.6		
Intradermal test		Skin reduction time (minutes)			
	102	3.5 - 9	wide range of blood level		
	104	9 - 13	0.3		
	54	14 - 25.6	0.7	Slobody (25)	1944
	7	14 - 25.6	0.35 - 0.55		
	18	14 - 25.6			
Spinal fluid level	133	Normal levels of spinal fluid (1.8 mg per 100 ml) present in			
		29 of 30 with	0.7 or more		
		17 of 35 with	0.4 - 0.69	Wortis et al. (13)	1938
		6 of 68 with	less than 0.4		

Table 4. Criteria of nutritional status of ascorbic acid based on plasma ascorbic acid level

Workers	Nutritional status	Plasma ascorbic acid mg per 100 ml
Gyorgy, cited in (34)	normal	0.7
Youmans, cited in (34)	deficiency	less than 0.4
	borderline	0.4 - 0.7
	normal or better	0.7 - 1.2
Greenberg et al. (35)	low	less than 0.5
	suboptimal	less than 0.7
	adequate	0.7 - 0.9
Kaslin, cited in (26)	severe deficiency	less than 0.4
	mild deficiency	0.4 - 0.7
Bessey and Lowry (37)	poor	less than 0.4
	fair	0.4 - 0.6
	good	0.7 - 1.0
	excellent	more than 1.1
Abt and Farmer (38)	active scurvy	0.4 - 0.5
Ingalls, cited in (38)	active scurvy	0 - 0.15
	asymptomatic scurvy	0.15 - 0.30
Eckelen and Abt, cited in (39)	possibility of scurvy	0.0 - 0.5

Table 5. Renal thresholds for ascorbic acid.

Workers		Subjects no.	Plasma ascorbic acid mg per 100 ml
Faulkner	(14)	175	1.40
Goldsmith		22	1.40
Friedman		19	1.50
Lewis		12	1.10 - 1.30
Lewis		1	1.45
Lesis		1	1.80
Klosterman et al. (40)		12	1.0 - 1.3
Crandon et al. (22)		1 (deficiency)	0.85

maintain a higher plasma ascorbic acid level on a given intake than one with a low renal threshold could do.

Prolonged and severe ascorbic acid deficiency would result in scurvy. But the plasma ascorbic acid levels, corresponding to scurvy reported by various workers, were unreliable (Table 4). Prunty and Vass (39) emphasized the impossibility of diagnosing scurvy by determination of the plasma ascorbic acid level since a patient observed had a plasma ascorbic acid of 0.0 mg per 100 ml without symptoms of scurvy. Bessey and White (41) also reported that subjects with zero or near zero plasma ascorbic acid levels did not show clinical symptoms of scurvy. Crandon et al. (22) showed that their subject after 41 days on an ascorbic acid deficient diet had a plasma ascorbic acid level of zero, but developed the first symptom of small perifollicular hyperkeratotic papules only after 134 days,

or 10 days after the ascorbic acid content of the white cells was zero. In this case the diet was adequate in all aspects except ascorbic acid. Other factors, growth, infection or multiple avitaminosis, might hasten the appearance of symptoms of ascorbic acid deficiency.

Stephens and Hawley (42) observed that white blood cells and platelets were rich in ascorbic acid in normal individuals. These workers also reported that in patients with leukemia, the whole blood ascorbic level increased markedly and there was a striking difference between the ascorbic acid level of the whole blood and that of the plasma because of the predominance of leucocytes in such whole blood samples.

A review (43) of the work of Roe et al. stated that they obtained evidence to show that in normal persons the plasma ascorbic acid levels were below those of whole blood when the whole blood ascorbic level was less than 0.6 mg per 100 ml; both were approximately equal when whole blood ascorbic acid levels were between 0.6 - 0.9 mg per 100 ml; while when the whole blood levels exceeded 0.9 mg per 100 ml, the plasma ascorbic acid levels were higher than whole blood levels. But all the patients studied had more ascorbic acid in whole blood than in plasma without relation to the actual whole blood ascorbic acid levels. It was suggested that the disturbance in distribution of ascorbic acid in whole blood and plasma in pathologic conditions might be due to the result of impaired absorption, excessive excretion, alteration in the cell-plasma

equilibrium, or increased need. The phagocytic activity of leucocytes was shown to be related to their content of ascorbic acid (44, 45). Under pathologic conditions there might be a mechanism to increase phagocytic activity by increasing ascorbic acid content in the white blood cells.

The ascorbic acid level in white blood cells was considered to serve as a more precise index of nutritional status of ascorbic acid than plasma ascorbic acid level. Lowry et al. (46) obtained evidence that white blood cell ascorbic acid level was an index of total ascorbic acid content in the body, because the rate of increasing the content of ascorbic acid in white blood cells was the same as that of total body retention after administration of a large dose.

On the basis of these data on levels of ascorbic acid in leucocytes and plasma, it seems reasonable to formulate the following explanation. Administration of ascorbic acid to a person with a mild deficiency will enable ascorbic acid levels of plasma and white cells to rise rapidly to a saturation level for the white blood cell. In a severe deficiency state all the administered ascorbic acid will be absorbed first by the cells or tissues to maintain their proper function. This results in an ascorbic acid plasma level of zero. When ascorbic acid is administered to a person whose tissues are in a saturated state the cells will not take ascorbic acid from the plasma and the excess amount will be circulated in the plasma; if the plasma level exceeds the renal threshold, large amounts

will be excreted. In the intermediate stage the levels in the plasma and cells will be in equilibrium.

A comparison of the fasting level of plasma ascorbic acid with recent ordinary intake will indicate the individual's nutritional status of ascorbic acid to some extent. Although an ascorbic acid plasma level of zero or near zero may not indicate the presence of subclinical scurvy still it shows the individual to be in poor nutrition with respect to ascorbic acid. If the plasma ascorbic acid level is zero the white cell ascorbic acid level will serve as an index to show body depletion and to help to diagnose scurvy. If the plasma level is around the renal threshold it will indicate that the plasma is saturated with ascorbic acid. Tissue saturation may be indicated by the maximum ascorbic acid level of the white blood cells. A precise criterion of ascorbic acid status may be established from the plasma ascorbic acid level and white cell ascorbic acid level. But the white blood cell ascorbic acid level is relatively troublesome to determine. The whole body storage of ascorbic acid is reflected by dietary intake under normal conditions. Therefore plasma levels of ascorbic acid corresponding to different intakes may serve as a rough index to determine the individual's ascorbic acid status.

Ascorbic Acid Requirement of Children

Roberts and Roberts (15) estimated the ascorbic acid requirements of five children, aged 6 to 12 years, as based on various criteria for judging adequacy. The dietary intake required to maintain a blood level of 0.7 mg per cent was 63 mg per day, and an intake of 69 mg per day was required for saturation tested by urinary excretion after administration of a large dose. Both were lower than the 83 mg per day required to maintain a 40 mg resting level of urinary excretion, and the 109 mg per day required to maintain a maximum plasma value.

Bessey and White (41) estimated the ascorbic acid requirement of children by plotting the plasma ascorbic value against the dietary intakes. The minimum intake which would give the average maximum post-absorptive blood plasma level was the requirement. The results showed that the ascorbic value of blood plasma increased as the intake increased till the intake reached 40-50 mg. Beyond this point the rate of increase in plasma ascorbic acid value was slight.

The method used frequently to determine the requirement included three stages: 1) Previous saturation with a large test dose (200-400 mg); 2) Experimental period - the plasma ascorbic acid levels or the amount of urinary excretion corresponding to different levels of intake were measured to determine the nutritional status; 3) Resaturation period - a

large dose (usually 400 mg) was administered. The degree of saturation was estimated by the amount excreted in urine. Half of the administered dose excreted in 24 hours was usually chosen as an indication of saturation. The results obtained by using this method have been summarized in Table 6.

The reliability of some of the results might be questionable on the basis of the length of the experimental period. Doddsand Macleod (49) observed that the fasting levels of plasma ascorbic acid of all subjects showed daily fluctuations to reflect the adjustment to different levels of intake and was not affected immediately by an increase in intake. In their early work (49), they maintained the subjects at each level for periods long enough to indicate adjustment and took the weekly averages of daily plasma ascorbic acid levels. In 8 of 12 subjects the 82-85 mg intake by the end of two weeks produced average plasma ascorbic acid value approximately equal to or greater than those found later on the 107-110 mg intake, and one subject gave the first evidence of an amount great enough to supply body demands and to have an excess appear in the blood. Dodds and Macleod (50) in their recent work recommended that a 7 to 10 day adjustment period be allowed on each level and that the plasma values of two-day and six-day averages after the adjustment period be recorded. Haines et al. (16) used a six-week experimental period which they considered to be long enough to permit the demonstration

Table 6. The effect of different levels of intake on the plasma level response to a large dose, and on urinary excretion by the method including presaturation, experimental period and resaturation.

Reference	Subject	Diet intake mg per day	Plasma ascorbic acid mg per 100 ml	Saturation determined by urinary excretion (no. of subjects)	Length of experimental period
Todhunter & Robbins (17)	3 college women	1.6-1.7mg/kg		3	6 days
		60	0.7 (2)*		
		120	1.4 (3)*		
Storvick & Hauck (18)	2 men	200	1.07 - 1.58		5-7 days
	4 women	150		1 male	2-3 days for
		125	1.05 - 1.74	1 female	adjustment
		100	0.99 - 1.32	1 female, 1 male	
		90	0.84 - 1.40		
		75	0.95 - 1.17	1 female	
		50	1.01 - 0.85		
Kline, Eheart (47)	9 women	1.4 mg/kg		6	
		2.2 mg/kg		2	
		0.6 mg/kg		1	
Fincke, Lanquist (48)	4 college students	49)			
		38)	0.8		6 days
		59)			
		69)			
		111-131	1.0 - 1.2		
Dodds, Macleod (49)	12 adults	32-35	0.27 - 0.76		2 weeks for each level
		57-60	0.44 - 1.25		
		82-85	over 1.0 (2)*		
			over .75 (all)*		
		107-110	over 1.0 (6 of 8)*		
Dodds, Macleod (50)	41 women	32	0.34 - 0.62		7-10 days for adjustment The value was 2 days or 6 days average
		57	0.51 - 0.93		
		82	0.74 - 1.13		
		107	0.88 - 1.22		
Haines et al. (16)	6 men 6 women all under 46 years old	70	definite plateau	all had prompt response 4-5 days	6 weeks
		50, 53) 33)	no definite plateau		
Storvick et al. (51)	boys	61	0.6 (average)		1 week
		allowance	0.9		
	4 girls	41)	0.44 (1)*, 0.47 (1)*		
		)	0.23 (1)*, 0.20 (1)*		
		allowance)	0.9 (1)*, 1.2 (1)*		
		)	0.94 - 0.74 (1)*		
Johnson et al. (52)	24 manual workers	75	0.6 - 1.2		8 weeks (2-week preliminary control 60-100 mg)
		60 - 100	0.6 - 0.9		
		135 - 175	0.7 - 1.1		
		deficient	0.6 - 0.0		

* Number of subjects.

of differences in body stores of ascorbic acid on the various levels of intake used. It was shown that a 70 mg intake was not sufficient to maintain saturation but produced a stable state in which levels of plasma and urinary ascorbic acid had plateaued. At this level there was a prompt response of both plasma and urinary excretion values to resaturation. On a 33 mg intake, the plasma level and urine excretion had not reached a definite plateau in the six-week period and resaturation required a three-day period as judged by renal threshold, and a four- or five-day period as judged by urinary excretion. On 50 or 53 mg intake, the plasma level and urine excretion also did not reach plateaus but the levels were higher than those on the 33 mg intake. So the degree of depletion was smaller on 50 mg intake than 33 mg intake. These results seemed to indicate that a failure to maintain a constant plasma level of ascorbic acid on a fixed ascorbic acid intake meant that tissue stores of ascorbic acid were being depleted.

Johnson et al. (52) maintained the tested intake levels for an eight-week period to test the influences of various levels of intake on physical efficiency. Their results showed that total deprivation of ascorbic acid for a period of 8 weeks had no effect on physical efficiency and produced no untoward symptoms except minimal changes in the gums, but produced marked chemical unsaturation. A 75 mg daily intake maintained ample body stores of ascorbic acid for 8 weeks, and intakes of over 100 mg daily were of no demonstrable benefit other than to

increase the body stores.

According to the results of the studies made by these workers, an intake of about 75-85 mg per day can maintain the body in good nutritional status in respect to ascorbic acid, an intake of over 100 mg per day does not have obvious further benefits for the body besides increasing the degree of saturation.

Previous Surveys of the Plasma Ascorbic Acid Levels of Children

Results of previous surveys on ascorbic acid status of population groups of children are summarized in Table 7. Very few studies indicated the correlation between the plasma ascorbic acid levels and the diet intake. Some studies showed that the low plasma ascorbic acid level was associated with low citrus fruit and tomato intake. In the studies of seasonal variation, it was found that in the fall the number of subjects with low plasma ascorbic acid level was smaller than that in the spring for more vegetables and fruits were available in the fall than in the spring. It seemed that the economic standard had an effect on the nutritional status of ascorbic acid. The number of cases of low plasma ascorbic acid among the low income group was higher than that among the high or adequate income group.

Some studies included physical examinations. Crane and

Table 7. The incidence of different levels of ascorbic acid in children as found by eleven surveys.

Subjects No.	Age years	Plasma level mg per 100 ml	Per cent of subject	Diet comment	Reference workers, place, date
86	7-16		Fall / Spring	Subjects had citrus fruit or tomato on the days of test: 7 (fall), 16 (spring)	Crane and Woods (53) Maine, 1941
		below 0.4	46 / 63		
		0.4 - 0.6	23 / 22		
		0.6 - 1.0	23 / 4		
		above 1.0	8 / 9		
12	1 wk	0.53 - 0.77		Inadequate	Abt, Farmer, 1938 (20)
54	to 13 yrs.	0.75 - 2.42		Adequate	
28	6-15	0.26 - 0.4	25*	Cooked green vegetables and fruit were used in rare instances	Pijoan et al.(54), Papago Indians, 1943
		0.53	2*		
		0.66	1*		
	high school	less than .6	boy 6.7 / girl 4.2	High income	Wiehl, Kruse (55), 1941
			55.6 / 42.3	Low income	
34	high school	0.28	Average	Initial	Milam, Wilkins (56), North Carolina, 1941
		1.00	Average	After 6-weeks adjusted diet	
269	9-12	Exp. group	First soup	The soup provided daily allowance of vit. C of a 12-year old child	Harris et al. (57) Michigan, 1943
		less than 0.3	26.0 / 5.2		
		0.3 - 0.7	34.9 / 32.7		
		0.7 - 1.1	23.4 / 39.4		
		above 1.1	15.7 / 22.7		
207		Control group	first / last		
		below 0.3	33.6 / 23.3		
		0.3 - 0.7	30.2 / 35.5		
		0.7 - 1.1	21.4 / 28.5		
		above 1.1	15.6 / 11.7		
20	<2	.76±.14	mean	29 - 38 mg	Robinson et al. (58), Mexico City, 1944
	2-16	1.05±.03	mean		
206	1-12 (white)		white / negro	intake level mg / white / negro	Youmans et al. (59), Tennessee, 1945
115	1-12 (negro)	below 0.4	6.8 / 2.6		
		0.4 - 0.6	9.7 / 10.0	less than 30 / 32.4 / 50.0	
		0.6 - 1.1	34.0 / 37.4	30 - 49 / 29.4 / 31.6	
		above 1.1	40.8 / 46.1	50 - 69 / 17.2 / 8.2	
		unknown	8.7 / 4.3	70 - 99 / 13.2 / 11.2	
				100 or more / 7.8	
10	11	0.6	mean		Storvick et al. (51) 1947
19	12	0.53	mean		
21	13	0.62	mean		
18	14	0.56	mean		
6	15	0.43	mean		
4	16	0.62	mean		
3	17	0.72	mean		
386	school children		urban / rural	74% (rural) and 57% (urban) had citrus fruit	Pierce et al. (60) Vermont, 1945
		below 0.4	18 / 4		
		0.4 - 0.6	13 / 4		
		0.6 - 1.0	37 / 14		
		above 1.0	36 / 78		
1200	11-19	below 0.4	29.9		Bessey and Lowry (37), New York, 1947
		0.4 - 0.6	25.4		
		0.7 - 1.0	24.4		
		above 1.1	20.5		
200	(fall)		Fall / Spring		Moyer et al. (24) Michigan, 1948
143	(spring)	below 0.4	5 / 9.9		
		0.4 - 0.6	9 / 15.4		
		0.6 - 1.0	30.5 / 42.0		
		above 1.0	55.5 / 32.7		

* Number of subjects instead of percentage.

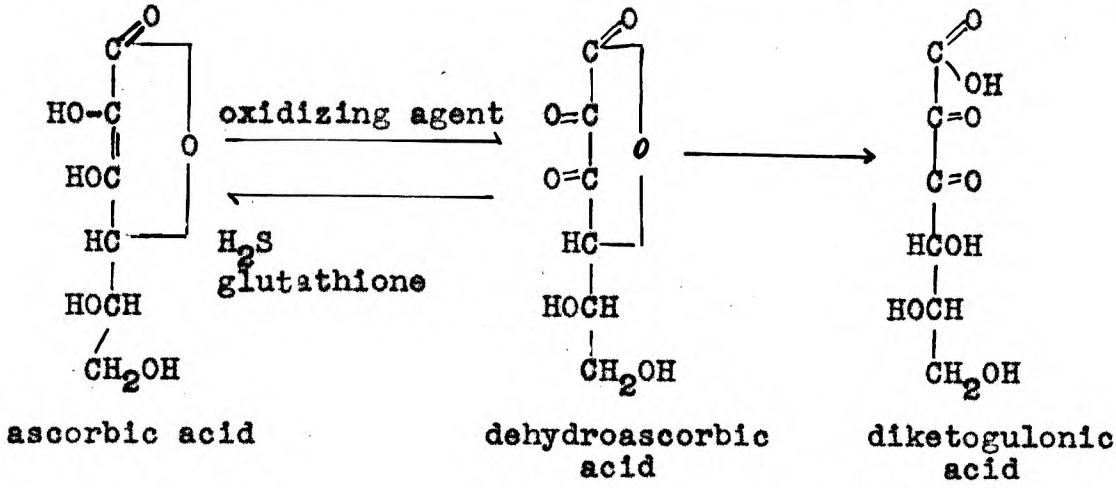
Woods (53) reported that 29 per cent of the 86 subjects in the fall and 51 per cent in the spring had gum inflammation which was found to be more frequent in children with consistently low plasma ascorbic acid levels. Forty per cent of the subjects in Vermont studied by Pierce et al. (60) had mild gingivitis. Youmans et al. (59), in their study, found a significant relationship between the presence of gingivitis and the plasma ascorbic acid values because 11 per cent of the 322 subjects with gingivitis had plasma ascorbic acid values below 0.3 mg per 100 ml while only 7.6 per cent of the subjects without gingivitis had so low a concentration. But the slight difference in serum ascorbic acid concentration between those with and without gingivitis suggested the existence of some other cause of gingivitis.

The results of these studies gave evidence that some subjects with a low plasma ascorbic acid level, which is generally believed to indicate deficiency, had no recognizable symptoms of a pathological state of ascorbic acid deficiency. In a North Carolina mill community studied by Milam (61), 27 per cent of the subjects had a zero level plasma ascorbic acid but only approximately 3 per cent were found to have mild cases of scurvy. Munsell et al. (62) did not find symptoms of scurvy or of severe ascorbic acid deficiency except pyorrhea and loss of teeth from a poor condition of the gums among 28 Puerto Rican subjects of whom 58 per cent had plasma ascorbic acid values below 0.5 mg per ml. Youmans et al. (59) found that

only 4 of the 10 subjects with plasma ascorbic acid below 0.1 mg per 100 ml had gingivitis, and a one-year-old child had X-ray evidence of mild scurvy.

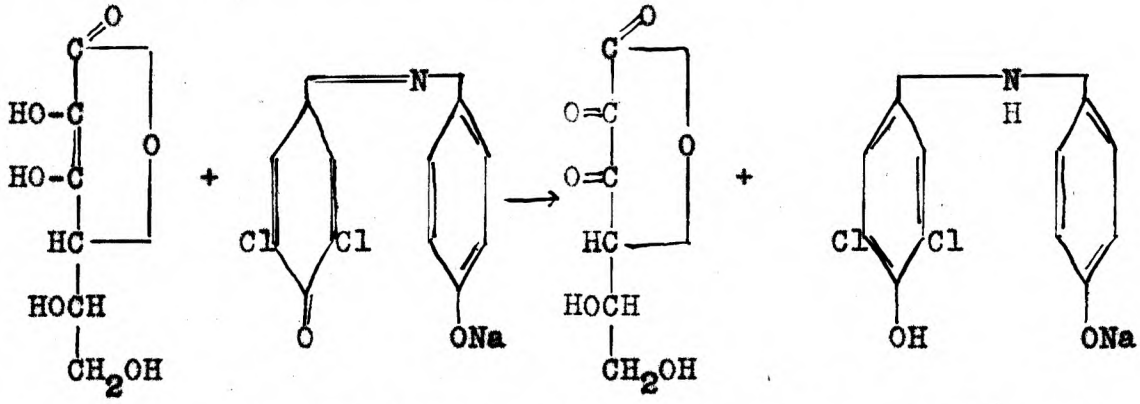
Methods Used to Determine Plasma Ascorbic Acid

The ascorbic acid in blood is free ascorbic acid (63). Ascorbic acid is an active reducing agent in the oxidation-reduction process. It is oxidized to dehydroascorbic acid. The conversion of ascorbic acid to dehydroascorbic acid is a reversible reaction and dehydroascorbic acid will change to ascorbic acid by treatment with a reducing agent such as hydrogen sulfide or glutathione. Therefore, dehydroascorbic acid also possesses antiscorbutic effect. But Penney and Zilva (64) observed that the dehydroascorbic acid was further changed into diketogulonic acid, which did not have antiscorbutic effect because the reaction is an irreversible one.



This reducing property has been used in the chemical quantitative determination of ascorbic acid. In two methods commonly used for blood analysis, the dichlorophenol-indophenol method and the methylene blue method, ascorbic acid is used to reduce the reagent and itself is oxidized to dehydroascorbic acid. In the other method, the dinitrophenylhydrazine method, the ascorbic acid is first oxidized by an oxidizing agent and then reacts with phenylhydrazine to produce an osazone.

Dichlorophenolindophenol Method. Dichlorophenolindophenol is a dye of blue color in alkali or neutral solution but of pink color in acid solution. It can be reduced mole for mole by ascorbic acid to a colorless compound.



ascorbic acid — dichlorophenol-indophenol — dehydroascorbic acid — colorless compound

Farmer and Abt (66) used dichlorophenolindophenol as a reagent for microanalysis of blood. The blood from the fingertip was oxalated and centrifuged. Then 0.1 ml of the plasma was diluted with 0.1 ml water, deproteinized by 0.2 ml 5 per cent

metaphosphoric acid and then centrifuged. Next 0.2 ml clean deproteinized filtrate was pipetted out and titrated with a standardized dye to faint pink color by using a special microburette. Then 0.2 ml of 2.5 per cent metaphosphoric acid was used as blank. Trichloracetic acid (66) and tungstic acid (66, 67) were tried as deproteinizing agents; but ascorbic acid was shown to be more stable in metaphosphoric acid which prevented the aerobic oxidation in the presence of ferric and cupric ions (68).

As this method was based on the reducing property of ascorbic acid, it was found that previous oxidation of ascorbic acid led to a negative error. Mindlin and Butler (69) recommended the addition of cyanide to the blood immediately after collection to prevent oxidation, but King (68) showed that the addition of cyanide was unnecessary as ascorbic acid was fairly stable in blood if hemolysis was prevented.

Mindlin and Butler (69) showed that an error might be introduced because of the fading of the dye and the rate of fading of color was dependent on the pH of the solution. They suggested a method in which sodium acetate was used to buffer the solution.

This method was suitable either for visual titration or photoelectric measurement. The addition of sodium acetate to the dye solution brought the pH of the final reacting solution to 4.1 which was not low enough to cause rapid significant fading of the dye and was not high enough to cause an immediate

reduction of the dye by reducing substances other than ascorbic acid. The dye at pH 4.1 was stable for at least five minutes. Mindlin and Butler (69) also showed that the conversion of the metaphosphoric acid to orthophosphoric acid decreased the pH and caused rapid fading of the dye. The metaphosphoric acid solution remained stable for two weeks when stored in the refrigerator.

Substances present in the sample and having a reduction potential lower than that of the dye, such as cysteine and glutathione which contain a sulfhydryl group, and some carbohydrate decomposition products, reduced the dye and acted as interfering substances. Mindlin and Butler (69) and Evelyn et al. (70) showed that those substances reacted with the dye at a considerably slower rate, that a reading at 30 seconds represented only the ascorbic acid, and that there was no need for correction for other interfering substances. King (68) suggested using an end point at five seconds or less for visual titration and taking two or more readings at 15 second intervals with the photoelectric colorimeter measurement.

Bessey (71) applied this method to determine dehydroascorbic acid. He converted the dehydroascorbic acid to ascorbic acid by treating it with hydrogen sulfide at pH 3.7 - 3.5 for two hours, then the hydrogen sulfide gas was removed by bubbling with nitrogen.

Stotz (72) recommended using xylene to extract the dye after its treatment with the sample for the short time necessary

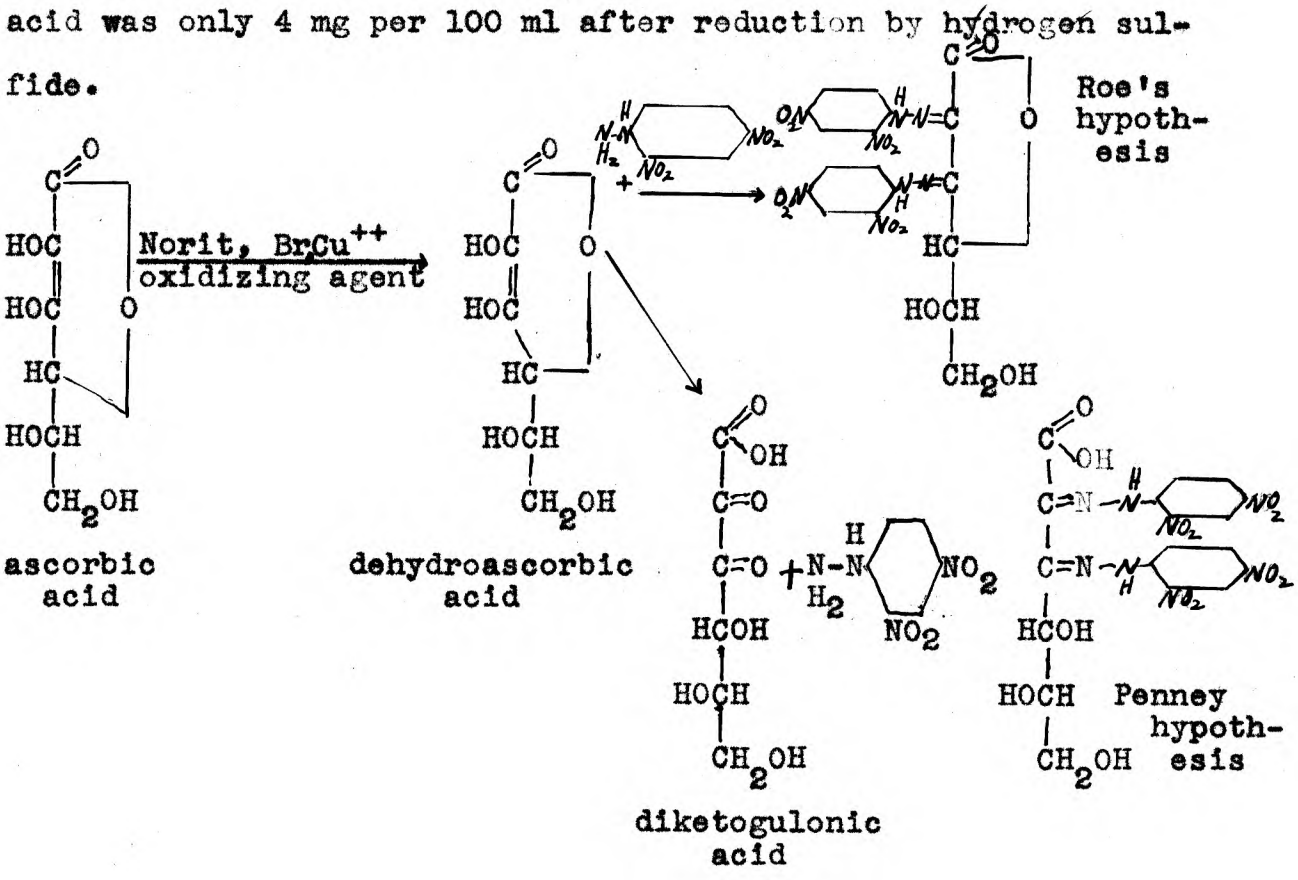
for the reaction. The ascorbic acid content was determined by measuring the color change in photocolorimeter. The color of the dye in xylene was stable for a few hours as it was no longer subjected to an acid medium or to slowly reducing substances.

Methylene Blue Method. Butler et al. (73) developed a micromethod using methylene blue in the presence of strong light (560 - 680 mu) as the agent to oxidize ascorbic acid in blood. As the methylene blue was decolorized by ascorbic acid, the amount of ascorbic acid was determined by means of color change measured in the photoelectric colorimeter. This reaction was quite specific for ascorbic acid since methylene blue was not reduced by uric acid, glutathione, cysteine, creatine, creatinine, urea, adenine, guanine, hypoxanthine, xanthine, glucose, and other carbohydrate metabolism products.

2-4 Dinitrophenylhydrazine Method. It was shown by Roe (74) that this method determined dehydroascorbic acid. But Penney and Zilva (64) and Pijoan and Gerjovich (75) gave evidences that diketogulonic acid rather than ascorbic acid itself was determined.

Roe (74) reported that a dehydroascorbic acid osazone derivative was formed when ascorbic acid was first oxidized with norit or bromine or copper and then treated with dinitrophenylhydrazine. But Penney and Zilva (64) observed that the dehydroascorbic acid was further changed into diketogulonic acid, and that ascorbic acid, dehydroascorbic acid, and diketogulonic

acid formed the same osazone (m.p. 279-281° C. decomposed), and they suggested that the osazone (m.p. 257-259° C.) obtained by Roe (74) might be a diketogulonic acid derivative with some impurity which caused the lower melting point. Pijoan and Gerjovich (75) also observed that dinitrophenylhydrazine method measured the amount of diketogulonic acid, a further breakdown product of dehydroascorbic acid since the aerated orange juice with estimation of 60-70 mg per 100 ml of dehydroascorbic acid by dinitrophenylhydrazine method did not protect guinea pigs from scurvy and the content of ascorbic acid was only 4 mg per 100 ml after reduction by hydrogen sulfide.



Roe and Kuether (76, 77) observed that a reddish color (maximum absorption bands at 500-550 mu and 350-380 mu) developed when the 2-4 dinitrophenylhydrazine derivative was treated with concentrated sulfuric acid. Roe and Kuether (77) developed a method based on this color reaction to determine the content of ascorbic acid in blood. A trichloracetic acid filtrate of blood, after shaking with norit, was treated with 2-4 dinitrophenylhydrazine with addition of thiourea for three hours at 37° C. to form the osazone. Then 85 per cent sulfuric acid was added to develop the red color which was read in the colorimeter after 30 minutes and was stable for at least 40 minutes.

Bolomey and Kemmerer (78, 79) recommended the substitution of glacial acetic acid for 85 per cent sulfuric acid to eliminate the charring effect of 85 per cent sulfuric acid which might lead to a positive error. They also omitted the use of thiourea. Their results showed that the reaction with acetic acid was only about half as sensitive as that with 85 per cent sulfuric acid at 540 mu but the reverse was true at 520 mu for determination of orange juice and pure ascorbic acid. Mills and Roe (80) observed that parallel curves were obtained for pure ascorbic acid and orange solution oxidized with norit or bromine in the presence of thiourea when sulfuric acid was used, but dissimilar curves for pure ascorbic acid or orange juice were obtained when acetic acid was used. They thought that close agreement of the curves obtained by Bolomey and

Kemmerer (79) at 520 mu was fortuitous. They indicated the importance of using sulfuric acid because it formed a reddish colored product only with dehydroascorbic acid derivatives, and the importance of addition of thiourea because it prevented the reaction with other possible interfering substances such as the ferric ion and hydrogen peroxide.

Bessey, Lopez, and Lowry (81) and Bessey, Lowry, and Brock (82) eliminated the charring effect of sulfuric acid by reducing the concentration from 85 per cent to 65 per cent and they achieved a further improvement by using copper sulfate as the oxidizing reagent in place of norit which tended to stay as a layer on top of the solution and give nonuniform results.

The substances such as glucose, pentose, fructose, and glucuronic acid, which also formed osazones, might be interfering substances. Roe and Kuether (77) showed that the amount of glucose in normal blood was too small to introduce an error because glucose in a concentration of 150 mg per cent in the reacting solution might interfere. If the blood sample was diluted one to four according to the procedure, the glucose would not interfere unless its original concentration was 600 mg per cent which was above the normal range. Roe and Kuether (77) also observed that the blood sugar of diabetic patients (600 - 1200 mg per cent) might give a positive error of only 0.008 - 0.030 mg per cent.

Roe and Kuether (76) and Mills and Roe (80) reported that the gluosazones, dissolved in sulfuric acid, produced brown to

yellow colors which gave practically complete transmission in the wave lengths at which the red color obtained from dehydroascorbic acid was compared. Furthermore, the glucosazone was split by sulfuric acid at the hydrazine linkage with formation of the original uncoupled products later (80). Mills and Roe (80) recommended the use of a 540 mu filter for analysis of tissues which contained a fair amount of interfering substances and the use of a 520 mu filter for blood analysis since a very small amount of interfering substance was present.

Roe and Kuether (77) gave evidence that oxidizing agents such as the ferric ion and hydrogen peroxide produced interfering color with 2-4 dinitrophenylhydrazine but reducing agents such as thiourea, and the ferrous or stannous ions did not interfere. They used thiourea or stannous chloride to reduce those oxidizing agents which might be present.

Roe and Kuether (77) showed that the 2-4 dinitrophenylhydrazine method was not only satisfactory for serum determination but also for whole blood determination; while the dichlorophenol-indophenol method and methylene blue method were applicable to whole blood analysis only when the oxyhemoglobin, an oxidant of ascorbic acid, was rendered inert by saturation with carbon dioxide or by reduction.

Although the dichlorophenol-indophenol method is easily carried out in clinical analysis work, its use is limited in nutrition survey work. The amount of plasma required by the

dichlorophenolindophenol method recommended by Farmer and Abt (67), 0.1 ml, is 10 times that of the dinitrophenylhydrazine method recommended by Bessey, Lopez, and Lowry (81). Farmer and Abt showed that the deproteinized plasma-metaphosphoric filtrate used in the dichlorophenolindophenol method was stable only for holding overnight in 4° C. The 2-4 dinitrophenylhydrazine method is suitable for nutrition survey work as well as for clinical use. Bessey, Lopez, and Lowry (81) showed that the trichloracetic acid filtrate with precipitate was stable for several days in 4° C. and several weeks at -20° C., and that the supernatent acid extract was stable for several weeks at 4° C. and indefinitely at -20° C.

Statement of Purpose

The purpose of this study was threefold. The first was to develop the technique to adapt the Bessey's micromethod for determining plasma ascorbic acid to the available apparatus. The second was to study the effect of different methods of blood storage on the stability of plasma ascorbic acid. The third was to estimate the ascorbic acid status of a group of school children from the information concerning plasma level and dietary intake of ascorbic acid. The effect of school lunch on the ascorbic acid status was also observed.

EXPERIMENTAL METHOD

Procedure

The ascorbic acid content of the plasma was determined by the micromethod recommended by Bessey, Lowry, and Brock (82) and modified in the Manual for Nutritional Appraisal (83).

Equipment Used. Constriction pipettes of 10, 30, 40, 50, 60, 100 cu mm capacity were made according to the directions given by Bessey et al. (84).

Beckman spectrophotometer fitted with special diaphragm and absorption cells to permit the use of 60 cu mm fluid volume.

6 x 50 mm serological tubes fitted with rubber stoppers.

Melting point capillary tubes (10 cm in length) boiled in 1:1 dilute nitric acid and then rinsed with water thoroughly for several times and then filled with 0.2 per cent sodium oxalate solution and dried in oven at 60 - 70° C.

Mechanical vibrator composed of a nail mounted on an electric motor.

Wooden block for storage of the sample.

Metal block for holding the tubes during chilling in ice water.

Water bath pan for ice water.

Incubator at temperature of 37 - 38° C.

Centrifuge, portable, for use in field as well as in

laboratory.

Home freezer unit at temperature of -15° C. (4° F.).

Food jar with dry ice to be used for transporting the sample from the field to the laboratory.

Reagents Used. Ditrophenylhydrazine-copper sulfate-thiourea solution (reagent 1) was prepared from 20 vol. of 2.2 per cent dinitrophenylhydrazine in 10N H_2SO_4, 1 vol. of 0.6 per cent $CuSO_4 \cdot 5H_2O$ water solution, and 1 vol. of 5 per cent thiourea water solution. It was centrifuged if there was precipitate developed. This solution was stable for at least two weeks.

65 per cent sulfuric acid was made by adding 70 cc conc. sulfuric acid to 30 cc water.

5 per cent trichloracetic acid solution.

4 per cent trichloracetic acid solution.

General Procedure. 1. One volume ascorbic acid solution was mixed with four volumes 5 per cent trichloracetic acid in the 6x50 mm tube, and centrifuged if precipitate was formed.

2. 30 cu mm of this clear ascorbic acid solution was transferred by means of a constriction pipette to another tube and mixed with 10 cu mm reagent 1 by using the mechanical vibrator.

3. The tubes were capped and incubated 4 hours at 38° C.

4. The tubes were chilled in ice water and mixed with 50 cu mm of cold 65 per cent sulfuric acid by using the mechanical vibrator. The tubes were allowed to stand at room temper-

ature for 30 minutes to 4 hours (82).

5. Approximately 90 cu mm of the solution was transferred to an absorption cell of the spectrophotometer and the optical density was read at wave length 520 mu against pure water.

6. The corrected optical density corresponding to an ascorbic acid solution was obtained by subtracting the blank reading against water from the observed reading of the corresponding solution against water.

Standardization of the Optical Density with Ascorbic Acid Solution of Known Concentration

Procedure. The optical densities of 107 samples of ascorbic acid solutions with concentrations ranging from 0.15 to 2.10 mg per 100 ml were observed by carrying on the general procedure. The relationship between the ascorbic acid concentration and optical density was described by an equation of regression line obtained by statistical analysis. The results are recorded in Table 8.

Results. Statistical study showed that the regression line passed through the origin with an equation $X = 10.93\ Y$, where X was the concentration and Y was the optical density. This equation later was used to predict the concentration of ascorbic acid of unknown solutions from the optical densities observed.

Table 8. The relation of optical density (Y) to concentration (X), mg per 100 ml, in standard solutions of ascorbic acid.

X	Y	X	Y	X	Y
.175	.011	.203	.020	2.180	.142
.175	.010	.203	.022	.527	.038
.175	.012	.605	.068	.297	.011
.525	.047	.605	.066	.297	.012
.525	.044	.605	.068	.297	.016
.525	.039	1.015	.119	.495	.038
.875	.082	1.015	.119	.495	.030
.875	.085	1.421	.163	.792	.060
1.230	.111	.527	.042	.792	.058
1.230	.114	1.421	.163	.792	.058
1.230	.113	2.030	.227	.990	.079
1.750	.158	2.030	.227	.990	.083
1.750	.155	2.030	.233	.990	.080
.175	.015	.210	.014	1.485	.125
.175	.012	.210	.018	1.485	.127
.525	.051	.630	.052	1.485	.131
.525	.050	.630	.056	.531	.036
.875	.095	1.050	.107	.531	.038
.875	.088	1.050	.108	.531	.036
.875	.087	1.050	.106	.885	.080
1.230	.121	1.470	.148	.885	.071
1.230	.115	1.470	.146	1.239	.112
1.230	.107	2.100	.185	1.239	.105
1.750	.163	2.100	.179	1.239	.113
1.750	.158	2.100	.184	1.770	.158
.182	.020	.218	.013	1.770	.149
.182	.025	.218	.015	1.770	.153
.546	.066	.218	.011	.177	.005
.546	.069	.654	.052	.177	.003
.910	.084	..654	.054	.177	.011
.910	.086	.654	.054	.318	.012
1.270	.129	1.090	.095	.318	.012
1.270	.129	1.526	.135	.318	.018
1.270	.130	1.526	.138	.424	.034
1.820	.168	2.180	.139	.424	.027
1.820	.169	.527	.035		

Mean $\bar{X}$ = .9284 mg per 100 ml $\bar{Y}$ = .0837 Total item 107

Sx^2 = 36.6026 Sy^2 = .3523 Sxy = 3.4677

Sd^2yx = .0239 $S^2y.x$ = .0002276

Regression equation Y = .097 X -.0042

Test of regression equation passing through the origin:

$$\frac{a}{\sqrt{s^2y.x\left[\frac{1}{N-2}+\frac{(\bar{X})^2}{Sx^2}\right]}} = 1.54$$

1.54 was less than the 5 per cent level (2.00) therefore the hypothesis that the regression line passed through the origin was accepted (85).

Regression line passing through origin

Y = .0915 X or X = 10.93 Y

Study of the Effects of Different Storage Conditions on the Stability of Ascorbic Acid in Plasma

Bessey, Lowry, and Brock (82) showed that plasma treated with trichloracetic acid was stable for several days in the refrigerator and for several weeks at -20° C. The supernatant acid extract was stable for several weeks in the refrigerator and indefinitely at -20° C. In the present experiment it was preferable to do the minimum amount of work possible in the field. Therefore, a study of effects of different storage conditions on the stability of plasma ascorbic acid was done to decide in which form the blood samples would be transported from the field to the laboratory.

Procedure. Blood samples were classified into four groups: whole blood, centrifuged blood, filtrate with protein precipitate, and protein-free filtrate. The plasma ascorbic acid values in these samples before storage were compared with their values after storage either in the freezer or refrigerator for 18 or 48 hours. The organization of the four parts of this experiment are shown in Table 9.

Results. The results of part 1 indicated that there was no loss of ascorbic acid value when the filtrate, either before or after removal from the precipitate, was stored at lowered temperatures.

There were variable changes in ascorbic acid value when whole blood or centrifuged blood was stored 18 hours in the refrigerator as indicated in part 2.

Table 9. Effects of different storage conditions on plasma ascorbic acid value.

Part	Storage condition: Form	Storage condition: Place	Storage condition: Time hrs.	Ascorbic acid value mg per 100 ml: Sample number 1	2	3	4	5	6	7	8
1	standard			.36	.64	.46	.56				
	filtrate, pro.-free	freezer	48	.35			.65				
	filtrate, pro. ppt.	refrigerator	18		.63	.47					
2	filtrate, pro.-ppt.	refrigerator	18	.26	.34	.63	.90	.13	.78	.18	
	whole blood	refrigerator	18	.54	.09	.53	.70				
	centrifuged blood	refrigerator	18	.20		.37	.89	.24	.48	.13	
3	filtrate	freezer	48 or 18	.75	.29	.63	.37	.72			
	centrifuged blood	freezer	18	.72	.32	.60	.41	.60			
4	centrifuged blood	freezer	18	1.28	.30	.55	.46	.73	.60	.28	.60
	centrifuged blood	freezer	48	1.26	.45	0	.48	.60	.55	.23	.48

When, in part 3, centrifuged blood was stored in the freezer for 18 hours, the ascorbic acid value was not significantly different from that of sample stored as filtrate, Table 10.

However, consistent results were not obtained in part 4 when centrifuged blood was stored as long as 48 hours in the freezer. This may have been due to the hemolysis present in varying degrees in these samples.

Observations made in this experiment indicated that there was no decrease in plasma ascorbic acid value of blood centrifuged immediately and stored in a frozen state for a period up to 18 hours. This procedure was used in the succeeding experiment.

Table 10. Analysis of variance of plasma ascorbic acid value of blood stored as filtrate and as centrifuged blood in deep freezer for 18 hours.

Source of variance	Degree of freedom	Mean square
Treatment	1	31.2
Persons	4	
Discrepance	4	26.87
Error	10	8.35

Test of significance of treatment:

$$F = \frac{31.2}{26.87} = 1.16 \text{ nonsignificant}$$

A Study on a Small Group of Children

Procedure: The sample of 29 children comprised the third, fourth, fifth and sixth grades in the Alma Elementary School where a school lunch program had been operated for about one semester prior to this study in May, 1949. The school lunch provided one meal a day, five days a week. There was a serving of orange or grapefruit juice in most of these meals. Twenty of the 29 children participated in the school lunch program, and nine did not.

The plasma ascorbic acid value for each child was determined on a blood sample taken at midmorning on Monday (16 children) and Wednesday (13 children) of the experimental week. Blood from a fingertip puncture was collected in a 10 cm capillary tube coated with sodium oxalate. After being sealed and centrifuged, the tube was stored in a jar refrigerated with dry ice, and transported 30 miles to the laboratory in the afternoon of the same day. The plasma was deproteinized and the filtrate was stored in a freezer for one to three days. The plasma ascorbic acid value was determined according to the general procedure previously described.

The daily ascorbic acid intake for each of 26 children was computed from a three-day diet record representing Tuesday, Wednesday and Thursday of the week in which the blood sample was taken. Dietary records were not obtained from three children.

Results and Discussion. The mean plasma ascorbic acid value for these 29 children was 0.65 ± 0.068 gm per 100 ml with a range of 0.14 to 1.60 mg per 100 ml (Table 11). Although in this experiment blood samples were taken after breakfast, data from the daily records seemed to justify the assumption that the plasma ascorbic acid values found were comparable to the fasting levels reported by other investigators. According to the three-day diet record, only 12.5 per cent of all the breakfasts had orange or other ascorbic acid-rich food. Citrus fruit appeared in breakfasts of only 2 of the 13 children whose records included the breakfast eaten just prior to the taking of the blood sample.

According to the standards for plasma ascorbic acid levels proposed by Bessey and Lowry (37), approximately one-third of these children rated poor, another one-third good, one-sixth fair, and another one-sixth excellent (Table 12). Based on the percentages rated poor and rated excellent, the Kansas children were inferior in ascorbic acid status to children studied in Vermont (60), and Michigan (34); superior to children studied in Maine (53); and similar to the 1200 children studied in New York (37).

The mean daily ascorbic acid intake for 26 children was 81.1 ± 6.02 mg. Intakes ranged from 3 to 254 mg per day, with intakes of individuals fluctuating greatly from day to day. However, a statistical analysis showed that the differences in ascorbic acid intakes between individuals were significant in

Table 11. Plasma ascorbic value and ascorbic acid intake of 29 children in the elementary school at Alma, Kansas.

Subject no.	Plasma ascorbic acid mg per 100 ml	Ascorbic acid intake mg per day[1] Day 1	Day 2	Day 3	Av.	Participation in school lunch	Citrus fruit in breakfast
1	.14	100	121	15	79	x[2]	no
2	.21	80	32	34	49		no
3	.22	44	120	24	63	x[2]	
4	.25	71	70	14	52	x[2]	
5	.25	3	91	72	55		no
6	.26	75	12	25	37		
7	.31	22	20	29	24	x[3]	no
8	.34	49	70	32	50		no
9	.38	128	254	114	165	x	
10	.42	29	127	31	62	x[2]	no
11	.52	95	73	124	97		no
12	.52	13	105	19	46	x[2]	no
13	.52					x	
14	.60	130	133	35	99	x	no
15	.61	138	68	69	92	x	
16	.62	132	106	17	85	x	
17	.66	120	159	134	138		yes
18	.70	124	118	53	98	x	
19	.71	69	182	73	108		
20	.74	42	29	21	31		
21	.92	119	67	18	68	x	no
22	.93	138	195	36	123	x	
23	.97	129				x	
24	.97	71	65	143	93	x	
25	1.00	7	109	24	47		no
26	1.09	153	159	124	145	x	yes
27	1.14	121	136	20	92	x	
28	1.16	208				x	
29	1.60	90	164	80	111	x	

Plasma ascorbic acid:
mean 0.65 mg per 100 ml
standard deviation 0.36 mg per 100 ml
standard error 0.068 mg per 100 ml

Ascorbic acid intake (based on three-day average) (mg per day)
mean 81.1
standard deviation 36.60
standard error 7.18

Ascorbic acid intake (mg per day)
mean 81.1
standard deviation 53.17
standard error 6.02

Correlation coefficient of plasma ascorbic acid value and ascorbic acid daily intake (three-day average)

$$r = \frac{Sx_1 x_2}{(Sx_1^2 \, Sx_2^2)^{\frac{1}{2}}} = .4956 \text{ with 24 degrees of freedom}$$

[1] The dietary ascorbic acid intake were calculated from the daily dietary record by using the figure given in references (86, 87, 88).
[2] The subjects rejected ascorbic acid-rich food in some of their meals.
[3] The subject did not participate in the school lunch on the three days tested.

comparison to the measurement of experimental errors, (Table 13).

The menu and computed ascorbic acid value of the three-day school lunch recorded in Table 14 showed that one of the three school lunches was low in ascorbic acid. But the average ascorbic acid intake of these three days, 49 mg, was more than one-third of the daily intake recommended for children of this age group.

The mean values for ascorbic acid intake and for plasma ascorbic acid of the group having school lunch were higher than those of the group of children having no school lunch, but differences in their means were not significant (Table 15). Of the 20 children who participated in the school lunch program, 9 did not eat all of the ascorbic acid-rich foods provided. This number included three with incomplete diet records and one who did not eat at school on days studied. Therefore, in respect to ascorbic acid intake, only 11 of the children actually participated in the school lunch. These 11 children had a significantly higher mean ascorbic acid intake and plasma ascorbic acid value than those children who did not participate in the lunch program at all (Table 16).

There was a positive trend between plasma ascorbic acid value and the ascorbic acid intake as shown in Table 17. By a statistical analysis the correlation coefficient was found to be 0.4956 with 24 degrees of freedom which was significant at the 5 per cent level but barely nonsignificant at the 1 per cent level. Though this positive correlation existed between

Table 12. Comparison of plasma ascorbic acid value of children in this study with that of other states.

Grade	Plasma ascorbic acid: mg per 100 ml	Per cent of subjects: New York: No.	New York:	Maine: No.	Maine: Fall	Maine: Spring	Vermont: No.	Vermont: Urban	Vermont: Rural	Michigan: No.	Michigan: Spring	Michigan: Fall	This study: No.	This study:
Poor	below 0.4	1200	29.9	86	46	63	386	18	4	200 (fall)	9.9	5	29	31.1
Fair	0.40-0.59 or 0.4 -0.6		25.4		23	22		13	4		15.4	9		17.2 24.1
Good	0.60-0.99 or 0.7 -1.0		24.4		23	4		37	14	143 (spring)	42.0	30.5		37.9 31.0
Excellent	1.00 and above or 1.1 and over		20.5		8	9		36	78		32.7	55.5		13.8 13.8

Table 13. Analysis of variance of ascorbic acid intake.

Source of variance	Degree of freedom	Mean square
Persons	25	4035.9*
Days	2	18964
Errors	50	1577.6

* Test of significance of persons: $F = \frac{4035.9}{1577.56} = 2.56$ significant

Table 14. The ascorbic acid value of the school lunch menus.

1st day	2nd day	3rd day
Orange juice	Orange juice	Potato salad
Macaroni & cheese	Scalloped potatoes with ham	Wiener with cheese on bun
Cabbage slaw	Lettuce and French dressing	Dill pickle
Peanut butter sandwich	Bread and butter sandwich	Sweet roll
Tapioca pudding with chocolate sauce	Prunes	Milk
Milk	Spice cookies	
	Milk	
	Ascorbic acid content	
71 mg	61 mg	14 mg

Table 15. Comparison of plasma and intake values of ascorbic acid for children who participated in the lunch program with values for those who did not.

		Participating in school lunch: Yes	No
		Number of subjects: 20	9
Plasma ascorbic acid mg/100 ml	mean	.70	.52
	standard error	0.085	0.092
Ascorbic acid intake mg/day	mean	88.1 (17 subjects)	68
	standard error	8.61	12.34

Test of significance of difference:

plasma ascorbic acid $t = \frac{.70 - .52}{\sqrt{.0084 + .0073}} = 1.44$ D/F 27 nonsignificant

ascorbic acid intake $t = \frac{88.06 - 68.0}{\sqrt{152 + 74.10}} = 1.33$ D/F 24 nonsignificant

Table 16. Comparison of plasma and intake values of ascorbic acid for children who ate ascorbic acid sources in school lunch with values for those who did not participate in the school lunch program.

		Having school lunch	
		Yes	No
		Number of subjects	
		11	9
Plasma ascorbic acid mg/100 ml	mean	.87	.52
	standard error	.100	.092
Ascorbic acid intake mg/day	mean	106.4	68
	standard error	8.46	12.34

Test of significance of the difference:

Plasma ascorbic acid value $t = \frac{.87 - .52}{\sqrt{.0104 + .0084}} = 2.55$ D/F 18 significant

Ascorbic acid intake $t = \frac{106 - 68}{\sqrt{71.57 + 152}} = 2.54$ D/F 18 significant

Table 17. Comparison of the ascorbic acid value in plasma and in diet.

Plasma ascorbic acid mg per 100 ml	Number of subjects: Ascorbic acid intake, mg/day (3-day av.)					
	below 25	25-50	50-75	75-100	above 100	Total
below 0.4	1	2	4	1	1	9
0.40-0.59		1	1	2		4
0.60-0.99		2	1	4	3	10
0.10 and above				1	2	3
Total	1	5	6	8	6	26

the plasma ascorbic acid value and ascorbic acid intake, it was quite weak and indicated that only 25 per cent of the variation in individual plasma ascorbic acid value might be due to the differences in ascorbic acid intake. Factors influencing the response of an individual's plasma ascorbic acid level to ascorbic acid intake may include individual differences in absorption, utilization, requirement of the tissue and its previous saturation with ascorbic acid.

Dodds and Macleod (49) showed that the plasma ascorbic acid value fluctuated daily to reflect the adjustment to different levels of ascorbic acid intake. In this study, the three-day diet record showed that the ascorbic acid intake varied greatly from day to day. Determinations of plasma ascorbic acid level made on several days might have given a more accurate picture of a child's ascorbic acid status than the single-day's determination. In a sample including less than 30 cases the effect of one extreme case might be very obvious, but would be unnoticed in a large size sample.

CONCLUSIONS

1. In Bessey's microdetermination of plasma ascorbic acid as carried out in this study, the linear relationship between the ascorbic acid concentration and the optical density of the solution being tested can be expressed by the equation: $X = 10.93\ Y$ where X is the concentration of the ascorbic acid and Y is its optical density.

2. The centrifuged blood sample may be stored over night in the freezer without any resulting decrease of its ascorbic acid value.

3. In spite of the fact that the mean plasma ascorbic acid value for the 29 elementary children in this rural community was rated good, the one-third of the subjects with plasma ascorbic acid values below the marginal value of 0.4 mg per 100 ml were rated in poor nutritional status with respect to ascorbic acid.

4. Although there is a positive relationship between plasma level and dietary intake of ascorbic acid, the correlation coefficient (0.4956) indicates that other factors besides the present ascorbic acid intake influence the plasma ascorbic acid level.

5. Since the children who consistently ate sources of ascorbic acid available in the school lunch had significantly higher plasma ascorbic levels than those children who did not eat the school lunch, it may be fair to conclude that a school lunch adequate in ascorbic acid will improve the ascorbic acid status of children similar to those studied in this experiment.

SUMMARY

The Bessey micromethod for plasma ascorbic acid was adapted to available equipment, and an equation was developed to express the linear relationship between the ascorbic acid concentration and optical density of the test solution. Determinations of the ascorbic acid retention of blood samples during various methods of storage showed that the centrifuged blood sample, when frozen, could be stored over night without loss of ascorbic acid. The plasma ascorbic acid levels of 26 elementary children were shown to be partially dependent on the ascorbic acid intake, and the school lunch was shown to contribute significantly to the children's ascorbic acid status.

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