STUDIES OF ASCORBIC ACID AND RHEUMATIC FEVER

I. QUANTITATIVE INDEX OF ASCORBIC ACID UTILIZATION IN HUMAN BEINGS AND ITS APPLICATION TO THE STUDY OF RHEUMATIC FEVER [1]

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In recent publications, Rinehart and Mettier (1933, 1934), and Rinehart, Connor and Mettier (1934) have offered evidence to the effect that chronic scurvy with superimposed infection in guinea pigs results in a histopathological picture "strikingly similar" to that found in rheumatic fever in human beings. Moreover, they have cited other experimental, epidemiological, and clinical data in favor of a theory that vitamin C deficiency may be a necessary accompaniment of the infection associated with this disease. Their experimental results with guinea pigs have, to some extent, been confirmed by Schultz (1936).

The experimental evidence in favor of this concept is inadequate because no one has yet succeeded in producing rheumatic fever experimentally in any species, and because two different species have been used in an attempt to detect a pathological similarity between two different diseases—scurvy, and rheumatic fever. Furthermore, the other factors tending to make apparent a relationship between latent scurvy and rheumatic fever possess the limitations imposed upon any evidence of a purely presumptive nature.

We have, therefore, endeavored to test this concept of Rinehart and his collaborators, by a direct study of the vitamin C [2] utilization in rheumatic and non-rheumatic patients. The present paper deals with the development of a method for the study of ascorbic acid nutrition in individuals, upon the basis of which, a quantitative criterion of adequacy or deficiency with respect to this substance has been established. With this index of nutrition as a base-line, a comparative study of rheumatic fever subjects and suitable controls has been made.

Eekelen, Emmerie, Josephy, and Wolff (1933) were the first to state that vitamin C could be detected in blood and urine. At about the same time, Harris, Ray and Ward (1933) independently demonstrated a correlation between the urinary excretion of ascorbic acid and the dietary intake, and suggested that their technique could be used for the diagnosis of hypovitaminosis-C in human beings. They administered a single large dose of orange juice to normal individuals and obtained an immediate marked rise in the daily urinary output of ascorbic acid, the level of which dropped off almost to normal on the following days when no dose was taken. Individuals previously on normal, adequate, diets excreted ascorbic acid at a steady rate even after the material was omitted from the diet for several days, thus indicating that a store of the substance was present in the body.

Hess and Benjamin (1934), studying presumably normal infants, found no appreciable amount of ascorbic acid in normally excreted urine, nor did they obtain the marked rise after feeding found by Harris, Ray, and Ward (1933), even after a daily dose of one pint of orange juice had been added to the diet. Although they themselves did not emphasize the difference between the reactions of infants and adults, it is apparent that their results are in agreement with those of the latter workers, in that the urinary output or response to a given dose, is a function of the body storage or degree of "saturation" which in turn is dependent upon the previous dietetic history and the demands of the organism. It is, therefore, probable that growing children require

[1] Presented in preliminary form, at the meeting of the American Society for Clinical Investigation, Atlantic City, N. J., May 6, 1935.

[2] The antiscorbutic substance, initially named vitamin C, has also been called hexuronic acid, ascorbic acid, cevitamic acid, "cebion," "cevita," and "redoxon." However, the term used by those who did the pioneer work in the identification of the substance, the elucidation of its structure, and finally, its synthesis, and the one now generally accepted by chemists, is ascorbic acid. We shall, therefore, as a rule, refer to it hereafter, by that name.

larger amounts in proportion to their size, than do adults.

Johnson and Zilva (1934) strengthened the idea that the daily urinary excretion of ascorbic acid is dependent not only upon the dose, but that the response to any given dose is a function of the amount stored in the body. Using orange or lemon juice, they found that for any adult the final level of urinary output attained (at "saturation"), is approximately constant, and proportional to the daily intake. However, the *percentage* excretion of ascorbic acid decreases with increase in dose.

More recently, Harris and Ray (1935) have applied the method of Harris, Ray and Ward (1933) to the detection of ascorbic acid deficiency in scorbutic infants and in other subjects ranging in age from eight months to eleven years. They found in scorbutic children that the low daily urinary output was increased to normal after cure. Furthermore, the feeding of large test doses of 100 mgm. of pure ascorbic acid to such patients induced responses entirely different, before and after cure. In the case of an adult (age not given), on an ascorbic acid-free diet, the addition of a daily intake of 220 cc. of orange juice, equivalent to 140 mgm. of ascorbic acid, resulted in only a slightly increased excretion over the amount previously excreted. When 280 mgm. of the pure substance were given daily (in four doses), however, the individual became "saturated" in 6 days, and finally excreted 225 mgm. daily, or 80 per cent of the intake. When the ascorbic acid was discontinued, the urinary output rapidly fell again. Harris and Ray also found that the ascorbic acid content of the previous diet had the same effect on the results of the Göthlin (1931) capillary premeability test, as on the urinary output results. In their conclusions, they suggested that test doses be graded according to the body weight of the subject.

It appears from the foregoing, that qualitatively, at least, a distinct correlation between the urinary output of ascorbic acid and the state of nutrition with respect to this substance had been well established. At the beginning of our work, we therefore undertook merely to apply the single daily dose technique of Harris, Ray and Ward (1933), as a means of studying and comparing the state of ascorbic acid nutrition of rheumatic and non-rheumatic subjects. In preliminary studies, however, we found the initial response to single, daily, test doses, of subjects on adequate diets, to be so irregular that it seemed imperative to find out what constituted the *normal* response to high dose tests, by which all subjects could be graded.

At about that time, the work of Johnson and Zilva (1934) appeared. Their results, which we found similar to ours, were also obtained by feeding *varying* daily doses of orange juice. From their work and ours, it was evident that this procedure would yield no standard of normal excretion. Some of the irregular results in such experiments could be ascribed to the variation in ascorbic acid content of different lots of orange juice some of which we titrated after feeding.

In order to detect latent scurvy, of subclinical severity, it seemed necessary to develop a standardized technique of greater sensitivity, and capable of yielding more accurate quantitative results, than any procedure then available. Results so obtained, under average, normal conditions, could then be used as a measure of the state of nutrition with respect to ascorbic acid. We finally found it best to follow, in all subjects, the output response to repeated, large, single, daily doses of the pure substance. Because orange juice varies considerably in its ascorbic acid content, we used synthetic ascorbic acid in daily doses of 250 mgm. (The synthetic product "Redoxon" of Hoffmann-La Roche, and of Abbott, was found by titration to be the same as a sample of the natural crystalline material kindly sent us by Merck and Co.) Furthermore, since experiments with orange juice indicated that most individuals reached the peak of urinary excretion within seven days, the above dose was given daily for such a period. In this way, the effect of individual variations in immediate reaction to the first dose, perhaps out of proportion to the available body store of ascorbic acid, was eliminated. Furthermore, the additional factors of the level of urinary excretion at saturation, and the rate of increase in urinary output in the approach to that level, were brought within the scope of analysis. The following is a description of the procedure and the results when applied to rheumatic and non-rheumatic subjects.

METHOD

Diet. Usually, immediately upon admission, the subject was given a diet comparatively low in ascorbic acid content, composed chiefly of cereals, eggs, cheese, and cooked lean meat. Milk, dried and canned fruit, and vegetables low in ascorbic acid, when fed, were thoroughly cooked. The amount of ascorbic acid in such a diet averaged *no more* than 12 mgm. daily, as calculated from the values for the various foodstuffs present which may still have retained some of the vitamin (Birch, Harris, and Ray (1933), Bessey and King (1933)). After 48 hours, the above diet was supplemented for the next seven days, by the addition of 250 mgm. of "Redoxon," taken after breakfast. The material was weighed out to ± 1 mgm. mixed with sugar in a spoon, and followed by a half glass of water. In order to obtain concentrated urine and a minimum number of samples, the fluid intake was restricted.

Collection of urine specimens. At the beginning of a test period, the urine voided at 8 a.m. was discarded. The last specimen for every 24 hour period was obtained at 8 a.m. It was not necessary to save all of the urine voided. *Immediately* after each voiding, a small sample was quickly transferred to a 30 cc. bottle which was stoppered tightly with a one-hole rubber stopper so that all air bubbles were driven out together with urine which overflowed through the aperture; this was then sealed by inserting a tightly fitting glass rod. The time of voiding and the volume were recorded, and the specimen was then put into the refrigerator. At the end of the 24 hour period, the collected samples were analyzed.

Titration of urine specimens. For analysis, one composite sample was prepared, representing a pooled, total 24 hour urinary output. Accordingly, from each sample bottle was pipetted a volume one-tenth or one-twentieth of the total amount of the patient's voiding represented by that sample. Thus, if five sample bottles were obtained from voidings of 180, 200, 300, 260, and 560 cc., samples of 9, 10, 15, 13, and 28 cc. were measured and mixed, giving a sample of 75 cc. of urine of the same composition as that which would have been obtained had all of the specimens been kept and mixed. In this way, the handling of large volumes of urine from several subjects, and the necessity of titrating each voided sample, were obviated. The proportionate samples were rapidly pipetted into a beaker containing mineral oil, quickly mixed by stirring, and immediately prepared for titration by the withdrawal of duplicate samples of 2 to 20 cc., depending upon the concentration of ascorbic acid present.

A volume of water to bring the total to about 48 cc., and 2.5 grams of trichloracetic acid were added, and the mixture was quickly titrated against a dilute solution of 2:6 dichlorophenolindophenol added from a 3 cc. microburette. The end-point reached was a definite pink persisting for *at least* 30 seconds. This procedure of titrating definite volumes of urine by the addition of the indicator, was the reverse of that used by most workers, who have titrated *definite* volumes of *indicator* with the unknown urine, to *discharge* the red color. The procedure employed by us, like that of Bessey and King (1933), seemed to yield a sharper end-point. Furthermore, when the titration was done in our way, the effect of trichloracetic acid on the indicator was minimized. *Under these conditions, we found no difference in end-point of urine samples, whether trichloracetic or glacial acetic acid was used for acidification.*

Standardization. The indicator solution was freshly prepared every 48 hours by repeated extraction of 0.15 gram of the solid material in warm water, and dilution to a volume of 200 cc. The solid, which dissolved almost completely, was the sodium salt of the indicator, prepared in this laboratory, according to the procedure of Gibbs, Cohen and Cannan (1925) as detailed by Bessey and King (1933). The solution was standardized, whenever used, against 5 cc. of a fresh solution of ascorbic acid, prepared by solution in water, and dilution to a total volume of 50 cc., of approximately 0.02 gram "Redoxon" accurately weighed. The indicator solution was then equivalent to from 0.35 to 0.4 mgm. ascorbic acid per cc., and urine samples required from 0.5 to 3.0 cc. of indicator per titration.[3]

Calculation. The standardization of the indicator was calculated from the equation

$$(1)\quad \frac{\text{Mgm. ascorbic acid weighed for standard} \times 0.1}{\text{Cc. indicator to titrate 5 cc. of standard sample}} = f = \text{mgm. ascorbic acid equivalent per cc. indicator.}$$

The total daily output of ascorbic acid was calculated according to the equation

$$(2)\quad \frac{\text{Cc. indicator to titrate urine}}{\text{Cc. urine sample}} \times f \times \text{cc. urine in 24 hours} = \text{mgm. ascorbic acid excreted in 24 hours.}$$

EXPERIMENTAL

Factors involved in the accuracy of the method

The specificity of the titration. It is well recognized that the titration of biological fluids with 2:6 dichlorophenolindophenol may not yield results absolutely specific for ascorbic acid. Error arising from this source would produce higher values for ascorbic acid. However, Harris and Ray (1935) state that it is very unlikely that there are constituents present in normal urine in sufficient amount to interfere with the titration. We have noted that substances which may reduce the indicator, as does the added trichloracetic

[3] The method of preparation as detailed, makes it impossible to avoid the presence of sodium chloride in the final material. Therefore, given weights of different batches of indicator may be expected to vary in their titration equivalent of ascorbic acid.

acid, do so at such a slow rate that their effect is rendered negligible by rapidity in titration. Tauber and Kleiner (1935) using a ferricyanide titration method, ascribe one-half of the total reducing power (=about 10 mgm. daily) of normal urine, to the presence of substances other than ascorbic acid. Ascorbic acid added to urine in their experiments was quantitatively recovered. The presence of substances other than ascorbic acid, capable of reducing the indicator in our titration, would make our results all high by a constant small amount, not affecting the comparative results for ingested ascorbic acid appearing in the urine.

The preservation of ascorbic acid in urine. Ascorbic acid in solution unprotected from air is very unstable, being rapidly oxidized. It seems that the stability of this substance in solution depends on the oxygen tension and the pH. Ascorbic acid in alkaline solutions is rapidly oxidized, but is relatively stable in the absence of oxygen. Acid solutions have been found to be but slightly affected by oxygen (Herbert, Hirst, Percival, Reynolds, and Smith (1933)).

In order to avoid or diminish loss by oxidation, other workers have found it necessary either to titrate all urine samples immediately after voiding, or to add acetic or sulfuric acid, as a preservative. In our studies, the former course proved to be impractical in the simultaneous study of several patients. Therefore, it was found necessary to determine the conditions under which small samples of every specimen passed, could be preserved for analysis on the following day. The results led us to adopt, as the most satisfactory from the standpoint of practicability coupled with accuracy, the method of collecting samples given in the preceding section.

The experiments in this connection indicated that in the preservation of ascorbic acid in urine, the three important factors were oxygen tension, temperature, and pH, the latter being of little consequence when the other two were properly controlled. Tables I, II, and III, representative of a larger number of similar results, serve to illustrate these points.

Table I indicates the decrease in ascorbic acid titration of urine samples left in contact with air for several hours. Acidification, either with acetic or sulfuric acid, did not prevent loss of titratable ascorbic acid.

It was found that at any pH from 3.0 to 7.6, loss of ascorbic acid on standing in contact with air could be considerably diminished by keeping the samples in a refrigerator. Table II contains the experimental evidence leading to this conclusion.

TABLE I

Change in ascorbic acid content of human urine exposed to air at room temperature, at different pH

Sample number	Time	pH	Indicator titration	pH	Indicator titration
	hours		*cc.*		*cc.*
1	0		2.25		
	4		1.88		
2	0		1.16		
	4		0.88		
3	0		0.12		
	4		0.07		
4	0	5.4	1.74	4.6	1.73
	6½		0.89	(+ acetic)	0.94
5	0	7.2	0.74	4.6	0.75
	6		0.24	(+ acetic)	0.24
6	0	6.2	0.72	3.0	0.70
	23		0.34	(+ sulfuric)	0.34

TABLE II

Change in ascorbic acid content of human urine exposed to air at 10° and 25° C.

Sample number	Time	pH	Indicator titration	
			At room temperature (25° C.)	At refrigerator temperature (10° C.)
	hours		*cc.*	*cc.*
1	0	6.7	0.86	
	6		0.55	0.79
	24		0.27	0.46
2	0	6.4	1.22	
	6		0.53	1.08
	24		0.36	0.99
3	0	4.2	0.67	
	23		0.43	0.58
4	0	7.6	0.67	
	23		0.52	0.56
5	0	6.1	0.72	
	24		0.34	0.52
6	0	3.0	0.70	
	24		0.34	0.42

TABLE III

Change in ascorbic acid content of human urine protected from air in mercury containers

Sample number	Time	pH	Indicator titration		
			Sample as voided kept at 25°	Sample saturated with H_2, kept at 25°	Sample as voided kept at 10°
	hours		*cc.*	*cc.*	*cc.*
1	0	6.4	1.20	1.20	
	6			1.14	
	24			1.11	
2	0	6.4			
	4			1.61	1.57
	6			1.61	1.57
	20			1.53	1.50
3	0	5.9	1.84		
	3		1.83	1.81	1.80
	5½		1.80	1.79	1.77
	22½		1.72	1.73	1.75
4	0	7.2	0.37		
	18		0.37		0.37
5	0	4.7	0.78		
	19		0.73		0.73
6	0	5.5	0.42		
	18		0.37		0.38
7	0	6.8	0.09		
	18		0.09		0.09
8	0	5.9	0.89		
	18		0.83		0.83

Finally, Table III indicates that the most important factor in preserving ascorbic acid in urine is undoubtedly the oxygen tension. In this series of experiments all samples were transferred immediately, after voiding under oil, to the sealed mercury containers of Austin *et al.* (1922) and kept under slight pressure of mercury. Air, therefore, had no access to the samples, and they were necessarily under their own oxygen tension (Table III, Columns 4 and 6). This tension, as Sendroy (1934) has observed, is quite low for most urines and sometimes diminishes on standing. Therefore, after exhaustion of the dissolved oxygen, either before or after voiding, barring a change to the reversible form, one would expect the ascorbic acid titer of urine to remain constant. As a matter of fact, we have found the titration value of several urines kept this way, even at room temperature, to be constant for two days, after a slight fall in the first 24 hours. Table III also shows the relative unimportance of temperature under these conditions.

In Column 5 of the same Table, there are other examples of the preservation of ascorbic acid in urine, at practically zero oxygen tension. Immediately after voiding, these samples were saturated with hydrogen to the complete displacement of air. This method, first used, is probably the best, but is impractical for clinical work.

The effect of amidopyrine and codeine dosage. In the treatment of patients with rheumatic fever, the use of amidopyrine, and of codeine to a lesser degree, was frequently found advisable. Experiments were performed to control the effect of these drugs on the titration of ascorbic acid in excreted urine. The subjects used for this purpose had all been kept for some time on a daily dose of 250 mgm. of ascorbic acid, the feeding of which was continued during and after the daily intake of up to four times the usual doses of amidopyrine and codeine. Table IV, in a comparison of control periods immediately before and after the dosage period of four days, indicates that amidopyrine had no effect on the results. The effect of codeine, if any, was to decrease the ascorbic acid output of the urine. In our studies, codeine was given in small doses, only when absolutely necessary. Error from this source would result in a slight tendency toward decreased ascorbic acid excretion for rheumatic fever cases in which the drug was used.

TABLE IV

The effect of amidopyrine and codeine dosage on the daily urinary output of ascorbic acid. Subjects in a state of saturation on a daily dose of 250 mgm. ascorbic acid.

Case number	Daily dose		Average daily urinary output of ascorbic acid		
	Amount	Substance	Previous control 3 days	Dosage period 4 days	Post control 4 days
			mgm.	*mgm.*	*mgm.*
16-A....	0.9 gram	Amidopyrine	216	210	215
8-B.....	0.9 gram	Amidopyrine	218	180	199
11-A....	1.8 gram	Amidopyrine	196	200	224
6-B.....	1.8 gram	Amidopyrine	178	187	
11-B....	128 mgm.	Codeine	208	179	204
6-C.....	128 mgm.	Codeine	203	164	168

The foregoing observations of factors affecting the accuracy of the results, constitute the basis of the technique adopted for these studies. Loss

of ascorbic acid in urine samples was kept at a minimum by storage in sealed containers at low temperature. The error under these conditions was of the order demonstrated in Table III. Such slight losses probably balanced the positive error which may have been caused by the presence of small amounts of reducing substances other than ascorbic acid. Taking into consideration all sources of error, we estimate that our titrations approximated, within 10 to 15 per cent, the total ascorbic acid content of the urine.

Results of urine studies

Classification of subjects. The complete data in connection with 33 studies made from January to April, 1935, of the state of ascorbic acid nutrition of 28 individuals, are given in Table V. The subjects are separated into three groups.

In the first group are patients, afflicted at the time of the test, with active rheumatic fever, and its several accompanying conditions, such as pyrexia, polyarthritis, carditis, etc. The diet of some of these subjects had previously been supplemented with ascorbic acid feeding.

In Group II are patients who were not suffering from rheumatic fever at the time of the test, but who had previously had the disease. In this group are a few convalescent patients who had also been tested (Group I) while the disease was at its height, and some subjects (inactive rheu-

TABLE V

Results of utilization tests of ascorbic acid on human subjects

1	2	3	4	5	6	7	8	9	10	11		12	13	14	15	16
Case number	Sex	Age	Body weight	Condition	Fever	Gastro-intestinal symptoms	Medication	Previous (ascorbic acid) diet	Daily urinary ascorbic acid excretion on low ascorbic acid diet (less than 12 mgm. daily)			Total 7 days excretion of ascorbic acid	7 days intake minus excretion of ascorbic acid	C = Ascorbic acid unexcreted per kgm. body weight	$\sqrt{\text{age}}$	I = Utilisation index = Column 14 × Column 15
									2 days preliminary	7 days following with addition of 250 mgm. ascorbic acid by mouth, daily						
		years	*kgm.*		°*F.*				*mgm.*	*mgm.*		*mgm.*	*mgm.*	*mgm.*		
GROUP I—ACTIVE RHEUMATIC FEVER DURING TEST																
1	M	4	19.5	Polyarthritis, pericarditis, carditis	100–104	Anorexia, repeated vomiting	Codeine	100 mgm. daily, 1 month	65	56	113	635	1199	61.5	2.00	123.0
2	M	7	21.5	Polyarthritis, pericarditis, pleurisy, pneumonia(?)	100–103.4	Anorexia, nausea, vomiting	Codeine	Good	16	16	24	224	1310	74.9	2.64	197.7
3	F	7	28.8	Polyarthritis, myocarditis	99–104.6	Anorexia	Codeine	Poor	24	17	14	754	1080	37.5	2.64	99.0
4	F	7	22.0	Polyarthritis, myocarditis	99–104.5	Anorexia, abdominal pain	Codeine	100 mgm. daily, 2½ months	35	38	60	973	861	39.1	2.64	103.2
5	F	9	41.4	Polyarthritis, pleurisy, myocarditis	99–104.5	None	None	Very good	43	24	32	1169	665	16.1	3.00	48.3
6	M	10	36.4	Slight polyarthritis, carditis	99–100.5	None	None	Good	25	26	55	1123	711	19.5	3.16	61.6
7	M	11	57.7	Polyarthritis, myocarditis	100–105	Anorexia	Codeine	Very good	43	41	57	643	1191	20.7	3.32	68.6
8	M	15	49.8	Polyarthritis, myocarditis, jaundice	100–104.5	Anorexia	Codeine	Poor	103	107	116	931	903	18.1	3.87	70.2
9	M	15	44.0	Myocarditis, congestive failure, pneumonia(?)	100.2–105	Anorexia, vomiting	Codeine, amidopyrine	100 mgm. daily, 3 months	122	83	61	884	950	21.6	3.87	83.6
10	M	16	45.0	Polyarthritis, myocarditis	99–103.5	Anorexia, vomiting	Codeine	Poor	33	22	53	859	975	21.7	4.00	86.8
11	M	17	54.2	Myocarditis, partial heart block	99–100	None	None	Good	25	26	33	947	887	15.4	4.12	63.4
12	F	19	59.0	Polyarthritis, carditis(?)	99–100.5	Anorexia	None	Very poor	20	22	21	238	1596	27.1	4.36	118.0
13	F	20	44.8	Myocarditis, low grade polyarthritis	99–100.5	Anorexia, vomiting	Codeine	250 mgm. daily, 4 months	40	18	84	795	1039	23.2	4.47	103.8

TABLE V—*Continued*

1	2	3	4	5	6	7	8	9	10	11						12	13	14	15	16
Case number†	Sex	Age	Body weight	Condition	Fever	Gastro-intestinal symptoms	Medication	Previous (ascorbic acid) diet	Daily urinary ascorbic acid excretion on low ascorbic acid diet (less than 12 mgm. daily)							Total 7 days excretion of ascorbic acid	7 days intake minus excretion of ascorbic acid	C = Ascorbic acid unexcreted per kgm. body weight	$\sqrt{\text{age}}$	I = Utilization index = Column 14 × Column 15
									2 days preliminary	7 days following with addition of 250 mgm. ascorbic acid by mouth, daily										
		years	*kgm.*		°*F.*				*mgm.*	*mgm.*						*mgm.*	*mgm.*	*mgm.*		
GROUP II—CONVALESCENT AND INACTIVE RHEUMATIC FEVER																				
3–A	F	7	28.8	Convalescent R.F.*	99.4–100	None	None	250 mgm. daily, 7 weeks	29	21	144	168	160	191	181	209	211	1264	570	19.8	2.64	52.3
14	M	8	25.5	No R.F. symptoms, varicella	99–100.5	None	None	250 mgm. daily, 7 weeks	29	20	137	146	156	173	188	176	157	1133	701	27.5	2.83	77.8
15	F	9½	29.0	Acute bronchitis	None	None	Digitalis 0.1 gram daily	100 mgm. daily, 3 months	17	22	152	170	207	174	198	181	190	1272	562	19.4	3.08	59.8
6–A	M	10	36.4	Convalescent R.F.	99.8–100	None	None	250 mgm. daily, 3 months	50	38	280	189	236	212	214	237	186	1555	279	7.66	3.16	24.2
16	F	10	30.1	Convalescent R.F.	99.5–100	None	None	250 mgm. daily, 5 weeks	29	23	169	171	186	196	181	212	191	1306	528	17.5	3.16	55.4
17	M	13	38.1	Convalescent pharyngitis	None	None	None	100 mgm. daily, 1 month	36	27	62	142	167	227	187	176	158	1119	715	18.8	3.60	67.6
8–A	M	15	53.4	Convalescent R.F. (No jaundice)	None	None	None	250 mgm. daily, 1 month	55	42	86	140	182	155	191	195	208	1156	678	12.7	3.87	49.1
18	M	17	56.0	Carditis?	99–101	None	None	100 mgm. daily, 6 weeks	38	38	47	83	127	147	198	177	195	974	860	15.4	4.12	63.2
19	M	17	60.4	Catarrhal jaundice	None	Anorexia	None	100 mgm. daily, 11 days	102	118	116	144	145	114	132	98	97	845	989	16.4	4.12	67.4
20	F	19	37.0	Pernicious anemia	None	Anorexia, nausea, vomiting	None	Poor	47	41	82	81	119	139	158	114	130	823	1011	27.3	4.36	119.0
20–A	F	19	38.5	Convalescent pernicious anemia	None	Vomited only once	None	Fair	53	56	94	125	156	154	173	165	167	1034	800	20.8	4.36	90.7
2–A	F	19	59.0	No R.F.	None	None	None	250 mgm. daily, 5 weeks	43	29	97	130	110	137	164	140	176	953	881	14.9	4.36	65.1
21	M	21	63.0	Recovered R.F. chronic valvular disease	None	None	None	100 mgm. daily, 1 month	27	30	53	88	124	184	192	188	200	1029	805	12.8	4.59	58.8

* R.F. = Rheumatic fever.

† Case 3–A = Case 3, after 50 days; Case 6–A = Case 6, after 3 months; Case 8–A = Case 8, after 1 month; Case 20–A = Case 20, after 1 month; Case 12–A = Case 12, after 5 weeks.

GROUP III. NORMALS AND NEGATIVE RHEUMATIC FEVER HISTORIES

22	F	3¾	15.6	Vaginitis	99–100	None	None	Very poor	98	88	12	14	26	139	189	113	120	614	1220	78.2	1.94	151.6
23	M	8½	25.7	Convalescent mumps	None	None	None	Good	16	15	175	140	170	217	209	169	186	1265	569	22.1	2.92	64.7
24	M	8¾	26.7	Convalescent varicella	None	None	None	Good	36	29	95	185	102	166	162	175	197	1083	751	28.1	2.96	83.2
25	M	17	46.1	Convalescent lobar pneumonia	None	None	None	Poor	31	24	29	35	40	75	95	146	182	602	1232	26.7	4.12	110.0
26	M	28	52.9	Convalescent lobar pneumonia	None	None	None	Good	41	21	80	172	190	167	170	194	176	1149	685	12.9	5.29	68.2
27	M	28	81.8	Normal		Indigestion 6th day	None	Good	37	49	75	52	85	125	151	120	131	740	1094	13.4	5.29	70.9
28	M	34	65.8	Normal			None	Good	37	37	66	139	168	175	177	188	193	1106	686	10.4	5.84	60.7

matic fever) who had been followed in our outpatient clinic, before being admitted to the wards for these studies. All of the members of this group, with one exception (Cases 20 and 20–A) had been taking ascorbic acid for varying lengths of time, prior to the test. This, and their freedom from their previous infection, made them desirable as control subjects.

Group III comprises two healthy individuals and several patients in a more or less advanced state of convalescence from diseases other than rheumatic fever. None of this second control group had ever suffered from rheumatic fever.

The selection of controls for the relative standard of normality of ascorbic acid nutrition. An *absolute* standard of the state of ascorbic acid nutrition in human beings, based on studies of urine excretion, would be the result obtained by a high dose study of individuals *previously* kept for some time on a controlled diet containing a definite amount of ascorbic diet very nearly the minimum adequate for the maintenance of good health. Results which varied markedly from the average of a large number of such studies could then be interpreted as an indication of a condition of ascorbic acid deficiency or excess. Since the establishment of such a standard offers obvious difficulties we decided to base our standard of ascorbic acid nutrition on the results obtained in a study of individuals previously on an average diet, the subjects being free from digestive or metabolic disturbances during the high dosage test. Since we were interested not in *absolute* so much as *relative* results, the use of control subjects taken from Groups II and III was justified, even though the intake of ascorbic acid of the former group, if not of the latter also, probably exceeded the actual minimum adequate requirement.

To arrive at an average normal standard, it seemed logical and necessary to exclude cases known to have been for a long period on a diet extraordinarily high or low in ascorbic acid. Cases Number 20, 22, and 25 were therefore omitted because of a previous dietary deficiency, and Cases 3–A, 6–A, 16, 8–A, and 12–A were also dropped here, because the subjects had taken over 250 mgm. ascorbic acid daily for several weeks. In our judgment, the other members of Group II had received wholly adequate, although not excessive, quantities of ascorbic acid, in that their habitual diets had been supplemented with 100 mgm. of ascorbic acid daily for periods of eleven to sixty days.

The calculation of a relative or reference standard of ascorbic acid nutrition. An inspection of Column 10, Table V, for all cases, shows that a study of the urinary excretion of ascorbic acid for two days on a low ascorbic acid diet is an unreliable and at best an insensitive indicator of the previous diet of the individual. Apparently, no reasonable quantitative standard of normal excretion can be derived from these results on any basis. In this, we are in agreement with previous workers.

If the procedure of Harris, Ray and Ward (1933), and of Harris and Ray (1935) be followed, and an attempt be made to determine the ascorbic acid saturation of the subjects by the response to *one* test dose (1st excretion value in Column 11, Table V) a qualitative index is obtained, unsuitable for quantitative comparisons. The latter authors have themselves suggested that it might be of more value to study the excretion further. This, we have done. For reasons previously discussed, it seemed that the total of seven days excretion on a daily high test dose of 250 mgm. would be more informative.

The total quantities excreted, however (Column 12, Table V), in themselves, reveal little that can be used in a systematic classification of these results. It seemed possible that the *difference between the amounts of intake and of excretion* during the test period might be of considerably greater importance and more distinctly characteristic of the body nutrition, than the excess (not used by or lost in the body) appearing in the urine (Column 13, Table V). Furthermore, since ascorbic acid, despite its relatively small total amount, is very widely, although irregularly, distributed throughout the body (Bessey and King (1933), Phillips and Stare (1934)), and seems necessary for the normal functioning of body processes (Bessey and King (1933), Szent-Györgyi (1934)), it appeared logical that this utilization, real or apparent, be calculated on a *body weight basis.* When this was done (Column 14, Table V), these *utilization coefficient* values for our eleven control cases apparently varied with the age of the subject.

Further examination indicated that there is an inverse relationship between the square root of the age of the subject and the *utilization coefficient*. Thus the results follow the equation

$$C \times \sqrt{\text{age}} = I, \quad (3)$$

where C is the *utilization coefficient,* or (mgm. ascorbic acid intake — mgm. ascorbic acid urinary output)/(kgm. body weight). Age is given in years, and I is the *utilization index*. Thus, the value of I for any subject, as compared with the average value of I for *control* subjects, is an indication of that individual's state of ascorbic acid nutrition. Values of I higher than the average indicate a *relative* ascorbic acid deficiency or subnutrition, and lower ones, a *relative* ascorbic acid excess.

A calculation of values of I (Column 16) for control subjects *previously* with an *approximately normal* ascorbic acid intake (with the omission of Case 20–A, which definitely seems outside of the normal limit of variation, and which will be discussed later), gives an average of 65.8 for the six such subjects of Group II, and of 69.5 for the five similar subjects of Group III. Thus it seems, from these results, that the state of ascorbic acid nutrition of the members of Group III on average, good, mixed diets, approximated the condition of members of Group II who had been taking over 100 mgm. of pure ascorbic acid daily, by mouth.

The control points, plotted in Figure 1, follow the curve of average ascorbic acid utilization in our method, represented by the equation

$$C \times \sqrt{\text{age}} = 67.5 \quad (4)$$

with an average deviation of ± 5.5. The two outer curves define the limits of "normal" results, at least, between the ages of 7 and 35 years. Areas D and E therefore correspond to conditions

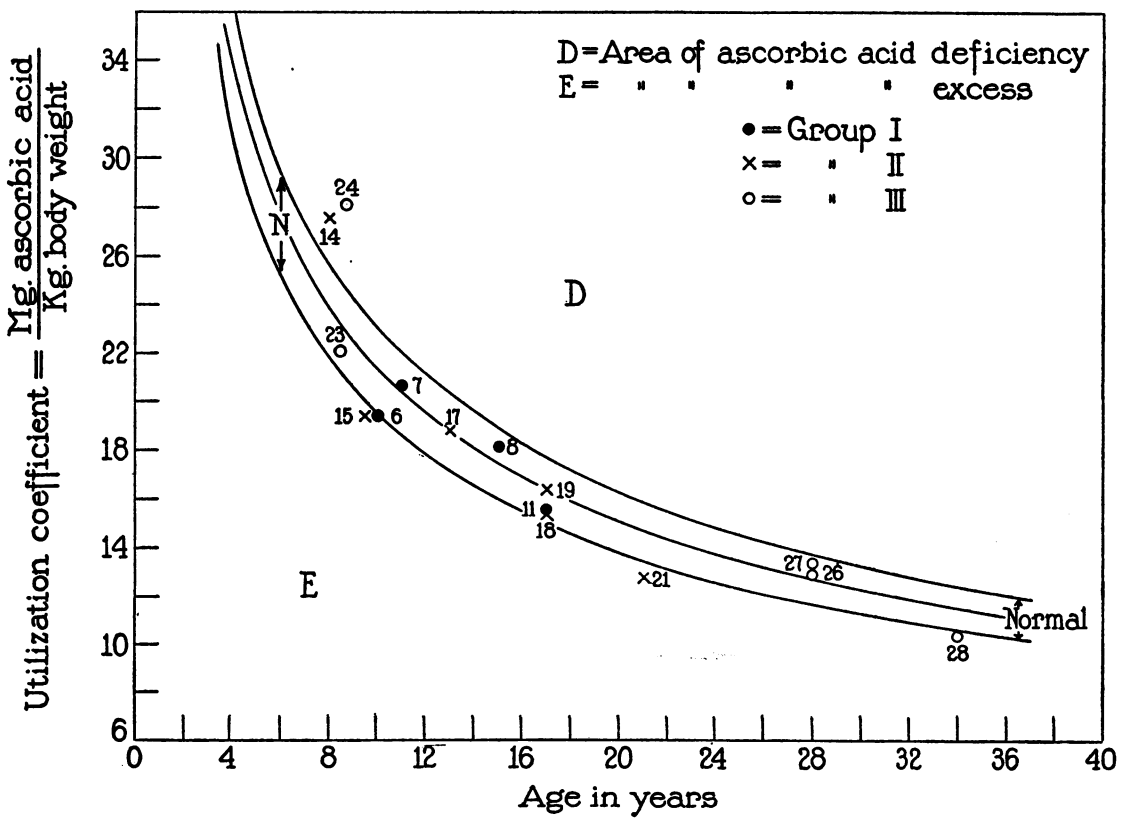

FIG. 1. DATA FROM TABLE V. THE VARIATION IN THE UTILIZATION OF ASCORBIC ACID FOR SUBJECTS OF GROUPS II AND III, ON AVERAGE GOOD DIETS WITHOUT DIGESTIVE DISTURBANCES.

Four cases of Group I showing normal results are also included. Case numbers are marked for each point.

of *relative* ascorbic acid deficiency and excess, respectively. Naturally, on the basis of these results, it cannot be claimed that the relationship of age to ascorbic acid utilization given by Equation 4, is the true mathematical expression for the physiological processes involved in the metabolism of ascorbic acid. The equation is a convenient, empirical expression which happens to fit the data well.

Since we had no opportunity of studying normal children below the age of seven, the course of the curve below that age is very doubtful. Indeed, the results of Hess and Benjamin (1934) indicate that there may be a marked difference in the ascorbic acid metabolism and requirement of infants, in comparison with adults.

Analysis of the results of ascorbic acid utilization of subjects with rheumatic fever. Of the 13 patients with rheumatic fever studied at the height of their infection, 8 were found relatively deficient (Cases 1, 2, 3, 4, 9, 10, 12, 13), 4 were normal (Cases 6, 7, 8, and 11), and one was above normal (Case 5), in ascorbic acid nutrition, as judged *solely* by the values of the utilization index (Table V, Column 16), or the position of the points plotted in Figure 2.

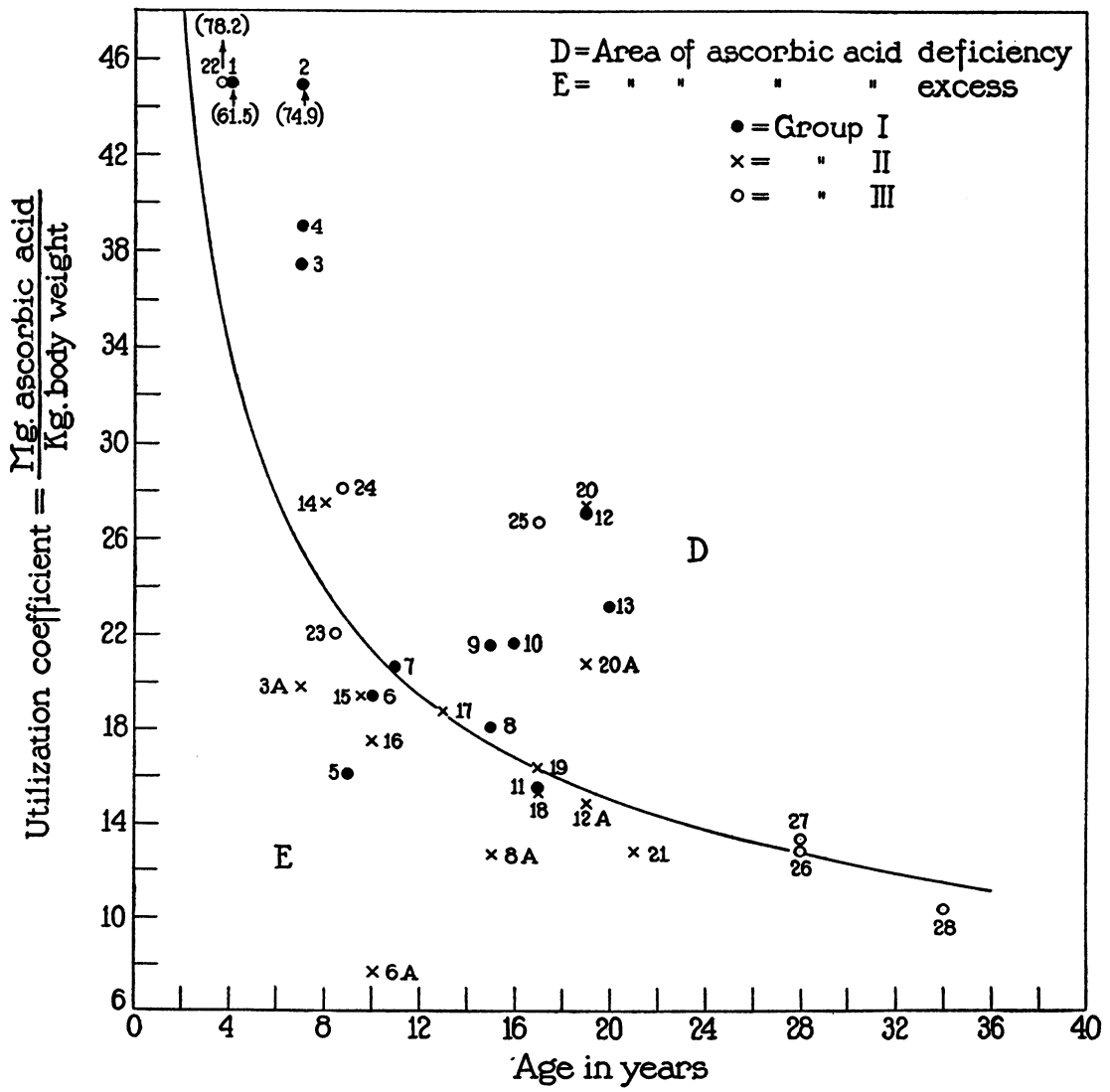

FIG. 2. DATA FROM TABLE V. THE RESULT OF ASCORBIC ACID UTILIZATION TESTS FOR ALL SUBJECTS STUDIED.

In our discussion of previous work, and the evaluation of a normal reference standard in the preceding section, it has been emphasized that the urinary excretion test of ascorbic acid is largely affected by two factors, the *first* of which is the *relative state of saturation* of the tissues of the individual at the beginning of the test. This, in turn, is mainly dependent on the ascorbic acid content of the previous diet, the proportion of the ingested material which was absorbed, and the proportion of the absorbed material which was stored. There is an additional possible factor, it seems, which would affect the results, but concerning which we have no definite indication, since the result would be the same as that indicating a low state of saturation caused by a poor supply or faulty absorption, namely, a condition in which ascorbic acid may be used up or metabolized in excess of normal amounts. It seems quite possible, from these considerations, that a condition of deficiency, as indicated by a relatively low state of saturation, may arise from causes other than a quantitative deficiency in the amount of ascorbic acid taken in the diet.

The *second* factor, it must be recalled, affecting the urinary excretion of ascorbic acid, is the *intake* in the diet of the subject *during the course of the test,* when the urine is being analyzed. In the preceding section it has been shown that if this second factor can be kept *constant,* by the ingestion *and absorption* of fixed high doses of ascorbic acid, then the results may be taken as a measure of the first factor, namely, the state of saturation. If this second factor is not kept constant, as in the feeding of orange juice of varying ascorbic acid content, or in the case of subjects incapable of assimilating the substance, the results will be invalid as an indication of the state of saturation.

In the search for a possible connection between the occurrence of rheumatic fever and any condition of ascorbic acid deficiency resulting in a low state of saturation relative to normals, the interpretation of the results requires that possible variations in the second factor be taken into consideration. Of the subnormal rheumatic cases, only *three* (Cases 3, 10 and 12) had been on a very poor ascorbic acid-containing diet, such as would be expected to lead to a condition of deficiency. Of the other subnormals, *previously on average to very good diets* with respect to ascorbic acid content, four exhibited conditions of vomiting, anorexia, and general gastric disturbance. There was no vomiting in Case 4, but anorexia.

We have no data as to how much, if any, ascorbic acid was lost during vomiting. But two conclusions are inevitable from those considerations, namely, that a gastro-intestinal disturbance, if it occurs during the feeding period of the test (second factor), invalidates the results; if it occurs (and reoccurs) for some period immediately preceding the test (first factor) it may well lead to an actual condition of relatively low ascorbic acid saturation, like that brought about by a dietary deficiency.

With five results invalidated on account of vomiting (but one of these had previously been on a poor ascorbic acid diet), we find (Table VI)

TABLE VI

The effect of previous diet and of digestive and nutritional disturbances on the result of the ascorbic acid feeding test

1	2	3	4	5	6		7	
				Result of nutrition test*				
Group number	Case number	Previous diet with respect to ascorbic acid	Nutritional disturbance during the test	Found	Expected on basis of diet		Expected on basis of diet and condition during test	
I	1	Good	Much vomiting, anorexia	D	N		D	X
	2	Good	Much vomiting, anorexia	D	N		D	X
	3	Poor	Anorexia	D	D	X	D	X
	4	Good	Malnutrition, anorexia	D	N		D	X
	5	Very good	None	E	E	X	E	X
	6	Good	None	N	N	X	N	X
	7	Very good	None	N	E		E	
	8	Poor	None	N	D		D	
	9	Good	Vomiting, anorexia	D	N		D	X
	10	Poor	Vomiting, anorexia	D	D	X	D	X
	11	Good	None	N	N	X	N	X
	12	Poor	Malnutrition, anorexia	D	D	X	D	X
	13	Very good	Vomiting, anorexia	D	E		D	X
II	3-A	Very good	None	E	E	X	E	X
	14	Good	None	N	N	X	N	X
	15	Good	None	N	N	X	N	X
	6-A	Very good	None	E	E	X	E	X
	16	Very good	None	E	E	X	E	X
	17	Good	None	N	N	X	N	X
	8-A	Very good	None	E	E	X	E	X
	18	Good	None	N	N	X	N	X
	19	Good	Anorexia	N	N	X	D	
	20	Very poor	Vomiting, anorexia	D	D	X	D	X
	20-A	Good	Vomiting, anorexia	D	N		D	X
	12-A	Very good	None	N	E		E	
	21	Good	None	N	N	X	N	X
III	22	Very poor	None	D	D	X	D	X
	23	Good	None	N	N	X	N	X
	24	Good	Anorexia	D	N		D	X
	25	Poor	Malnutrition, anorexia	D	D	X	D	X
	26	Good	None	N	N	X	N	X
	27	Good	None	N	N	X	N	X
	28	Good	None	N	N	X	N	X

* N = Normal.
D = Ascorbic acid deficiency.
E = Ascorbic acid excess.
X = Agreement with result found.

among the remaining eight cases of rheumatic fever, but *two* cases of deficiency caused by previous diet (Cases 3 and 12) and one (Case 4), with a condition of low saturation caused by some other factor, associated with a nutritional disturbance, before or during the test.

Table VI, Column 6, shows that in 23 out of 33 cases, the result of our test was in agreement with what would have been expected on the basis of our knowledge concerning the subjects' previous diet. When the probable depressant effect of digestive and nutritional disturbances on the ascorbic acid intake, and storage, is also taken into consideration (Column 7) the agreement is still closer, being 29 out of 33. The exceptions to this agreement are found in Cases 7, 8, 19, and 12–A, which will be mentioned again in the following sections.

The effect of icterus on the results of urinary excretion of ascorbic acid. It is interesting, and probably not without considerable significance, that Cases 8 and 19 were exact parallels in many respects. These two male patients, of about the same age and weight, were both jaundiced, showed bile in urine and serum, and had pyrexia. On the other hand, Case 8 had active rheumatic fever, and had previously been on a very poor diet, while Case 19 had no sign of the disease and had previously taken at least 100 mgm. ascorbic acid for 11 days. Yet, insofar as the ascorbic acid utilization test is concerned, both showed a parallel behavior quite different from those of any other class of patients.

The initial excretion for the first two days of substance reducing the titration indicator, without any intake of ascorbic acid, was high in both cases. Furthermore, the feeding of 250 grams for the next 7 days resulted in but a slight rise in the titration values in each case. A calculation of the utilization index showed values for both which were higher than those which would have been expected on the basis of diet alone. Furthermore, when Case 8 convalesced later (Case 8–A) and showed *no* icterus, the excretion picture was like that of the other patients of Group II, and the utilization index was entirely in line with the reresults of other subjects after a high feeding diet. In cases of icterus, the abnormal titration of the urine may be caused either by the increased excretion of reducing substances other than ascorbic acid, or by a real disturbance in the ascorbic acid excretion or utilization processes. This point bears further investigation.

The effect and significance of the results of intravenous injections of ascorbic acid following oral feeding. In a state of *saturation to any fixed dose* of ascorbic acid, an individual will approximate a condition in which there is only a relatively small, fairly constant difference between intake and urinary excretion, representing the amount stored, metabolized, or lost in the tissues. Such a condition was shown by but few of our subjects. Johnson and Zilva (1934) used as a criterion of saturation, the *constancy* of the level in urinary excretion finally attained on a constant daily intake. However, this did not seem satisfactory enough, since we were interested in obtaining some idea as to what happened to the material *unexcreted* in the urine when there was apparently a condition of saturation, requiring little further use or storage of the substance. Johnson and Zilva (1934) have already shown that this apparent loss could not be recovered or identified in the urine as dehydroascorbic acid.

In order to make certain of the intake, and to eliminate the factor of gastro-intestinal absorption, we tested some of our subjects by giving intravenous injections of 250 mgm. ascorbic acid for 2 days following the usual feeding period of 7 days. To prevent oxidation of the material during handling, the solutions used for injection were prepared and sterilized immediately before use, as follows: The ascorbic acid was accurately weighed out (500 mgm.), and placed in a 10 cc. calibrated Pyrex flask. A volume of 5 cc. of 1.8 per cent NaCl was added, *without stirring,* the neck was stoppered with cotton, and the flask was immersed to the volume mark in a Crisco oil bath at 120° C. After 10 minutes, during which time the powder dissolved completely, the flask was cooled and the solution was made up to volume with water, under sterile conditions. The solution was mixed and taken up in a syringe from which the subject received 5 cc. The entire procedure, from sterilization to injection, required no more than 20 minutes. Contrary to the assertion of Wright and Lilienfeld (1936) that " we are dealing with a substance which is destroyed by heat and hence cannot be completely sterilized," immediate titration of solutions prepared and used in this way

showed a maximum loss of only 2 per cent of the material weighed.

Table VII shows that every subject so tested, excreted more ascorbic acid in the urine while receiving the ascorbic acid intravenously, than during a previous feeding period. This is the case even for subjects who were presumably in a state of saturation at the time of the injections, i.e., they had reached a maximum in urinary output while being fed. These results indicate that there is *some* loss of the substance in the alimentary tract under all conditions of ascorbic acid nutrition.

TABLE VII

Results of intravenous injections of ascorbic acid following oral administration. Dose in all cases, 250 mgm., present in diet, 12 mgm.

Group number	Case number	Daily urinary excretion of ascorbic acid		Added increase in injection over previous period	Conclusion
		Period previous to injection	Injection period		
		mgm.	*mgm.*	*per cent*	
I	2	37, 45, 48	154	258	Loss in feeding
	4	60, 132, 185, 180, 144, 163, 110	255, 207	48	Poor absorption
	6	205, 164, 173	213, 274, 156, 243	23	Oral saturation
	7	104, 93, 96, 102	180, 224	104	Poor absorption
	9	212, 214	195, 298	16	Oral saturation
	10	220, 193	221, 251	14	Oral saturation
	11	228, 212, 188	234, 198, 195, 283	9	Oral saturation
	13	84, 138, 102, 144, 158, 71, 99	186, 211	68	Loss in feeding
II	6–A	236, 212, 214, 237, 186	219, 233	4	Oral saturation
	16	211, 191	220, 289	25	Oral saturation
	8–A	191, 195, 208	245, 219	17	Oral saturation
	18	198, 177, 195	233, 218	18	Oral saturation
	20–A	173, 165, 167	235, 229	38	Loss in feeding
	12–A	164, 140, 176	184, 221	25	Oral saturation?
	21	184, 192, 188, 200	210, 202	8	Oral saturation?
		Average of 10 cases saturated orally.....		16	

As judged by the excretion values for the two periods, of the 15 cases, 10 were near or at saturation by oral feeding, at the time of injection. For these cases the average increase in the daily urinary output of ascorbic acid after injections, was 16 per cent, in no case exceeding 25 per cent. On the other hand, the comparatively large increases in the others, seem to indicate either a previous greater loss of the substance in the alimentary tract, or a greater storage or loss in the tissues.

That it was a matter of absorption (associated with anorexia, etc.) is clearly shown by reference to Table V, Column 7, and to the sudden high level (in all cases but that of Case 2) reached on injection. Had the lowered excretion during feeding been the result of storage or destruction in the tissues, injection would not have caused such an abrupt cessation of the process. As a matter of fact, Cases 2, 13, and 20–A undoubtedly lost much of what was fed by vomiting. Case 2 apparently received so little during the feeding period, that the body stores were depleted and the injected material was retained to a much greater degree (Column 4), as compared with the excretion in the injection period of the other subjects of Table VII. In Cases 4 and 7 the inference is clear that for some reason there was a decreased absorption of ascorbic acid during the test. Considering the diet previously given, Case 12–A probably also had a subnormal absorption.

In this connection, it is interesting to note that Hou (1935) has found that the degree of protection given to guinea pigs on subminimal protective doses of ascorbic acid, was twice as much when the material was subcutaneously injected, as when given orally.

From these experiments, it seems that the feeding procedure for the determination of the state of ascorbic acid nutrition may sometimes indicate an apparent deficiency where there really is none. For this reason, it would be desirable to have for diagnostic purposes, a procedure which would eliminate the complicating factor of disturbances along the alimentary tract occurring *during the test.* The result of such a test would then be entirely a function of the state of saturation of the tissues. The factors of previous diet and of absorption of the ascorbic acid in that diet, contributing toward the result, could then be evaluated by further feeding tests, in order to determine whether oral or intravenous therapy was necessary to make up the tissue deficiency. Work on such a procedure will be undertaken in this laboratory.

DISCUSSION

Applied to the subjects with rheumatic fever, the excretion tests, relative to the controls, indicated to some degree, an apparent ascorbic acid deficiency in 8 out of 13 cases. Of these, the result in only 2 cases (Cases 3 and 12) could be ascribed solely to poor diet. In the other 6 cases, vomiting occurred, or else there was an incomplete absorption from or destruction of ascorbic acid

in the alimentary canal during the test (Case 4). Apparently, even in control cases, when there was a nutritional disturbance or anorexia during the test, the ascorbic acid, regardless of the previous diet, was not well assimilated, and was destroyed to a greater extent than usual.

Through digestive disturbances, patients with rheumatic fever evidently may develop a real hypovitaminosis on an ordinarily adequate diet. One would expect in such patients, that the tissues might be depleted not only of ascorbic acid but of other vitamins and essential food constituents of which there may not be large reserves in the body. However, even if we assume, contrary to the evidence of our experiments, that it is simply ascorbic acid deficiency that is associated with every case of rheumatic fever, it seems much more reasonable to regard the train of events, including digestive disturbances, leading to such depletion of the tissues, as caused by an infectious process, rather than to think of the ascorbic acid deficiency as initiating the infection. It seems certain that the factor of infection is present in all cases of rheumatic fever, whereas the signs of ascorbic acid subnutrition, if present, are probably incidental. Furthermore, it should be noted again that such signs of deficiency as have been found are only *relative* and not absolute. It has already been pointed out that our average results set up too high a standard of normality so that degrees of ascorbic acid deficiency, relative to the control cases previously on diets of about 100 mgm. ascorbic acid daily, would be exaggerated in the direction of ratings too low for ascorbic acid nutrition. When all of these factors are taken into consideration, it is difficult to accept subclinical scurvy as an etiological agent in rheumatic fever.[4]

The authors are indebted to Dr. Homer F. Swift for his advice and criticism during the course of the work, and in the preparation of this and the following paper.

[4] As this paper goes to press, we note the recent communication of Perry (1935), who used the single dose method of Harris, Day, and Ward (1933), studying the 12 hour urinary output after oral administration of 500 mgm. of ascorbic acid. He concludes from a study of 5 active, and 6 quiescent cases of rheumatic heart disease, that "vitamin C deficiency is not an important factor in the causation of acute rheumatism."

SUMMARY

The urinary excretion test for the adequacy of ascorbic acid nutrition has been improved and placed upon a quantitative basis by a chemical and clinical study of the various factors affecting the final results.

Applied to a comparison of patients with rheumatic fever and control subjects, the results of this method do not support the concept that a condition of ascorbic acid deficiency is a predisposing factor in the causation of this disease.

BIBLIOGRAPHY

Austin, J. H., Cullen, G. E., Hastings, A. B., McLean, F. C., Peters, J. P., and Van Slyke, D. D., Studies of gas and electrolyte equilibria in blood. I. Technique for collection and analysis of blood, and for its saturation with gas mixtures of known composition. J. Biol. Chem., 1922, **54**, 121.

Bessey, O. A., and King, C. G., The distribution of vitamin C in plant and animal tissues, and its determination. J. Biol. Chem., 1933, **103**, 687.

Birch, T. W., Harris, L. J., and Ray, S. N., A microchemical method for determining the hexuronic acid (vitamin C) content of foodstuffs, etc. Biochem. J., 1933, **27**, 590.

Eekelen, M. van, Emmerie, A., Josephy, B., and Wolff, L. K., Vitamin C in blood and urine. Nature, 1933, **132**, 315.

Gibbs, H. D. Cohen, B., and Cannan, R. K., Studies on oxidation-reduction. VII. A study of dichloro substitution products of phenol indophenol. Pub. Health Rep., 1925, **40**, 649.

Göthlin, G. F., A method of establishing the vitamin C standard and requirements of physically healthy individuals by testing the strength of their cutaneous capillaries. Skandinav. Arch. f. Physiol., 1931, **61**, 225.

Harris, L. J., and Ray, S. N., Diagnosis of vitamin C subnutrition by urine analysis. Lancet, 1935, **228**, 71.

Harris, L. J., Ray, S. N., and Ward, A., The excretion of vitamin C in human urine and its dependence on the dietary intake. Biochem. J., 1933, **27**, 2011.

Herbert, R. W., Hirst, E. L., Percival, E. G. V., Reynolds, R. J. W., and Smith, F., The constitution of ascorbic acid. J. Chem. Soc., 1933, Part 2, 1270.

Hess, Alfred F., and Benjamin, H. R., Urinary excretion of vitamin C. Proc. Soc. Exper. Biol. and Med., 1934, **31**, 855.

Hou, H. C., Comparison of oral and subcutaneous administration of protective doses of ascorbic acid (vitamin C). Proc. Soc. Exper. Biol. and Med., 1935, **32**, 1391.

Johnson, S. W., and Zilva, S. S., The urinary excretion of ascorbic and dehydroascorbic acids in man. Biochem. J., 1934, **28**, 1393.

Perry, C. B., Rheumatic heart disease and vitamin C. Lancet, 1935, **2**, 426.

Phillips, P. H., and Stare, F. J., The distribution of a

reducing substance (vitamin C) in the tissues of fluorine-fed cows. J. Biol. Chem., 1934, **104,** 351.

Rinehart, J. F., Connor, C. L., and Mettier, S. R., Further observations on pathologic similarities between experimental scurvy combined with infection, and rheumatic fever. J. Exper. Med., 1934, **59,** 97.

Rinehart, J. F., and Mettier, S. R., The joints in experimental scurvy and in scurvy with superimposed infection with a consideration of the possible relation of scurvy to rheumatic fever. Am. J. Path. (Proc.), 1933, **9,** 952.

Rinehart, J. F., and Mettier, S. R., The heart valves and muscle in experimental scurvy with experimposed infection. Am. J. Path., 1934, **10,** 61.

Schultz, M. P., Cardiovascular and arthritic lesions in guinea pigs with chronic scurvy and streptococcic infections. Arch. Path., 1936 (In press).

Sendroy, J., Jr., The oxygen tension and oxygen consumption of urine. J. Biol. Chem. (Proc.), 1934, **105,** lxxviii.

Szent-Györgyi, A., Vitamine und Ernährung Die medizinische Bedeutung des Vitamins C. Deutsche med. Wchnschr., 1934, **60,** 556.

Tauber, H., and Kleiner, I. S., A method for the quantitative determination of ascorbic acid (vitamin C). The vitamin C content of various plant and animal tissues. J. Biol. Chem., 1935, **108,** 563.

Wright, I. S., and Lilienfeld, A., Pharmacologic and therapeutic properties of crystalline vitamin C (cevitamic acid). Arch. Int. Med., 1936, **57,** 241.