CCCXIX. I. ON THE RELATION BETWEEN DIET AND URINARY OUTPUT OF THIOSULPHATE (AND ASCORBIC ACID). II. HUMAN REQUIREMENTS FOR VITAMIN C.

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PART I.

For several vitamins the quantity necessary to maintain health depends on the composition of the diet. Rats, for instance, need more vitamin D while on a diet with little phosphorus and with abundant calcium than with P and Ca offered in equal doses. It is known also that man requires more vitamin B₁ the greater the carbohydrate content of the food.

As to vitamin C, Ahmad [1936] and Chakraborty & Roy [1936] have recently shown that the reducing capacity of urine (against dichlorophenolindophenol) increases during a high protein diet. If there really exists a greater urinary excretion of ascorbic acid during a high protein diet, greater requirements of the organism, too, may be expected. Both authors titrated the urine without previous treatment; they did not eliminate interfering reducing substances other than ascorbic acid by precipitation with mercuric acetate [Emmerie, 1934]. Van Eekelen [1934; 1935, I] observed a normal amount of ascorbic acid in urine with a very high total reducing capacity (as determined by direct titration), the latter being chiefly due to an increased excretion of thiosulphate.

By evaluating during different diets the amounts of total reducing substances and also of ascorbic acid, conclusions have been reached which differ from those of Ahmad and Chakraborty & Roy.

EXPERIMENTAL.

Determination of ascorbic acid (and non-specific reducing substances).

The titrations were carried out in acid medium with 2:6-dichlorophenolindophenol [Tillmans *et al.*, 1932; Harris & Ray, 1933; Birch *et al.*, 1933; Johnson & Zilva, 1934], standardized against crystalline ascorbic acid.

As to the technique of eliminating interfering reducing substances by precipitation with mercuric acetate and reduction with H₂S we refer to Emmerie & van Eekelen [1934] and van Eekelen & Emmerie [1936].

The urine was both titrated directly and after treatment with mercuric acetate immediately after it had been voided. From the following table it may be observed that the reducing capacity may diminish even if the urine is kept with glacial acetic acid in the ice-box (as recommended by several investigators).

Direct titration:

1 ml. urine immediately	0.23;	after 16 hours	0.17	ml. indicator	
" "	0.25	" 11	" 0.20	"	
" "	0.17	" 8	" 0.17	"	
" "	0.26	" 6	" 0.22	"	

After precipitation with mercuric acetate:

Added immediately after voiding	10.0	ml. urine	1.32	ml. indicator	
" 6 hours	" 10.0	"	1.08	"	

Such a decline is the more important since the urine voided during the evening and during the night is generally kept for a considerable time and because particularly this urine is rather more concentrated (observed also by Harris *et al.* [1933]).

To determine the ascorbic acid in blood the method described by Emmerie & van Eekelen [1934] was used.

Diets. Two diets were used; the first contained 100 g. of rice with 50 g. of raisins, or 100 g. of spaghetti on alternating days, 600 g. of brown bread, 125 g. of butter, some honey, one egg and tea with sugar (per day). The second diet was composed of 750 g. beef, 6 eggs, 125 g. of butter, 400 g. of bread and tea per day. The caloric values of both diets were approximately equal (about 3200 kg.-cal.), the $\frac{\text{protein}}{\text{carbohydrate}}$ ratio was about $\frac{1}{8} \left(\frac{60}{500} \right)$ in the first, $\frac{1}{1} \left(\frac{217}{203} \right)$ in the second diet.

Before the experiments began a saturation test (*vide* Part II) was accomplished; the increased output in the urine following an intake of 500 mg. of "Redoxon" (pure ascorbic acid) (250 mg. on two successive days) indicates that the experimental subject was saturated.

Of the two diets the first is devoid of vitamin C, the second contains only those amounts present in beef (average 1.5 mg./100 g.). To avoid a too rapid decrease in the vitamin C content of the organism, a daily dose of 25 mg. of "Redoxon" was taken. In this way the vitamin C level in the blood did not become extremely low (7.2 mg./l.), nor did the urinary output of ascorbic acid decrease. Without a supply of vitamin C a more significant fall of the blood content had to be expected, demonstrating a depletion of the depots in the organism; the urinary output would have decreased likewise. In this way the experimental conditions might have been influenced so unfavourably, that an actual dietetic stimulation of the excretion of ascorbic acid could not become manifest.

Fig. 1. Influence of proteins on the urinary output of non-specific substances and ascorbic acid. —•—•— mg./l. blood. •—•—•— Daily urinary output of ascorbic acid. ○—○—○— Daily urinary output of total reducing substances (expressed as ascorbic acid). I=Low-protein diet. II=High protein diet. III=II without any carbohydrates.

The fact that the vitamin C level of the blood decreases in spite of the daily intake of 25 mg. of ascorbic acid is discussed later.

To be sure that the dietetic prescriptions were followed as strictly as possible and that any strain beyond the habitual work was avoided, the writer became the

experimental subject. A control of the body weight showed no change during the experimental period.

The experiment demonstrates that during a diet rich in proteins the total reducing capacity of the urine increases. This increase originates mostly from a greater excretion of thiosulphate, as could be proved by its elimination with barium acetate.

Direct titration	After barium acetate	After mercuric acetate
11.30	3.88	3.62*

* Calculated as ascorbic acid in 286 ml. of urine.

The values obtained do not always agree so closely, for, like other investigators, we have observed that the ascorbic acid can be excreted partly in the reversibly oxidized form; in this case the reducing capacity of urine becomes greater after treatment with H_2S .

1 ml. urine	After H_2S	After barium acetate	After barium acetate and H_2S
0.095	0.13 (+0.035)	0.045	0.09 (+0.045)
0.115	0.15 (+0.035)	0.055	0.085 (+0.03)
1.62*	1.75 (+0.13)	1.11	1.23 (+0.13)

* While saturated with ascorbic acid.

The very distinct influence of a high protein diet on the total reducing capacity does not exist for the output of ascorbic acid. No definite rise in the excretion of ascorbic acid (determined after precipitation with mercuric acetate) has been observed. A certain increase between 10 and 12 April will be recognized as preceding the beginning of the protein diet. Furthermore, the somewhat higher level of the ascorbic acid excretion (after 11 April) continues after the protein content of the diet has been lowered, while the total reducing capacity decreases immediately.

Fig. 2. Influence of protein and cystine on the urinary output of non-specific substances and ascorbic acid. —•—• mg./l. blood. —•—• Daily urinary output of ascorbic acid. ○—○ Daily urinary output of total reducing substances (expressed as ascorbic acid). I=Low protein diet. II=High protein diet.

After an intermediate period of 25 days of normal nutrition a second experiment under the same dietetic conditions was started. For reasons dealt with above a daily dose of vitamin C was taken, even larger than in the first

experiment: 37.5 mg. instead of 25 mg. To get an insight in the cause of the influences exercised by proteins the latter were replaced by an amino-acid containing sulphur and taken in larger doses; cystine (Hoffmann-La Roche) was tried first.

Again we observe, that the high-protein diet increases the total reducing capacity and that the amount of ascorbic acid in the urine is not influenced. Cystine, given *per os*, increases the total reducing capacity in quite the same way. During intake of cystine and while on the beef-egg diet a greater diuresis was observed (compared with the carbohydrate regimen). The quantity of urine, however, did not influence the amounts of thiosulphate or ascorbic acid either (observed also by Johnson & Zilva [1934]). Large quantities of tea or water have been taken without any change in the amount of ascorbic acid or other reducing substances.

Date		Ascorbic acid	Reducing capacity expressed as ascorbic acid
1936	ml. urine	mg.	mg.
9. iv.	880	15.6	24.8
10. iv.	1530	13.8	25.8
11. iv.	1090	16.3	27.0
7. v.	1140	21.9	25.4
8. v.	1566	29.8	35.9
9. v.	937	74.5	84.4

Hawley *et al.* [1936] emphasize the possibility of an influence of *pH* on the amount of ascorbic acid in urine. We have not observed such an influence; moreover, the diets used were both acidotic. In the experiments of Chakraborty & Roy [1936] a high fat diet produced the same increase in the "total daily urinary excretion of ascorbic acid" as did the high protein diet. It will be seen that a suddenly augmented intake of butter had no such effect (20 July; 210 g. butter additional to the daily quantity of 125 g. were taken).¹

PART II.

From measurements of the capillary resistance Göthlin [1931; 1932; 1934] concluded that about 25 mg. of ascorbic acid comprised the daily dose required for healthy adults weighing 60 kg. Referring to Stepp, Heupke [1936] gives an even smaller dose, viz. 10–20 mg. Stepp *et al.* [1936] now admit that this dose is "too low according to the most recent examinations". Diverging from this dose of 20–25 mg. van Eekelen [1935, 2; 1936, 1, 2] has found considerably greater requirements for man, his calculation being based on the results of a saturation test following a dietetic period devoid of vitamin C. The question being of practical importance a further contribution to it seemed desirable.

With regard to the results of Ahmad [1936] and Chakraborty & Roy [1936] and to the possible independence of requirements and urinary output (greater requirements with normal excretion?) two experiments were done on the same subject living on a diet devoid of vitamin C, but with different amounts of protein.

To ascertain the daily requirements the conditions of the experiments described in Part I were also suitable: a daily dose of ascorbic acid was taken equal to that postulated by Göthlin and formerly by Stepp; in the second experiment the dose was even larger.

¹ Chopra & Roy [1936] likewise missed any influence of large amounts of fat on the output of total reducing substances, whereas the increase following high protein diet was confirmed.

EXPERIMENTAL.

Determination of requirements. The daily requirements are calculated according to the method of van Eekelen [1935, 2; 1936, 1, 2]. In the experiments with M. H. daily doses of 25 or 37.5 mg. of ascorbic acid were given during the dietetic period.

Table I.

I. Requirements while on a diet poor in protein (M. E.; male, aged 25, height 173 cm., 68.5 kg.).

Date 1936	Ascorbic acid				Total reducing substances (expressed as ascorbic acid)	
	Intake mg.	Blood mg./l.	Urinary output	Extra excretion (normal average about 17)†	Urinary output	Extra excretion (normal average about 25)†
Normal nutrition poor in vitamin C:						
10. vi.	250	13.64	17.30	—	26.88	—
11. vi.	250	—	32.88	—	39.95	—
12. vi.	—	—	97.7	—	113.04	—
Diet poor in protein, devoid of vitamin C:						
13. vi.	—	—	30.37	13	31.76	7
18. vi.	—	12.20	—	—	—	—
27. vi.	250	8.42*	—	—	—	—
28. vi.	250	—	—	—	—	—
29. vi.	250	—	15.84	—	24.66	—
30. vi.	250	—	48.22	31	53.71	28
18 days	1000	Minus		44†	or	35†

* Before "Redoxon" was taken.

II. Requirements while on a protein diet (M. E.).

Protein diet devoid of vitamin C:	(Normal average about 20)†				(Normal average about 26)†	
1. vii.†	—	—	17.54	—	28.41	—
2. vii.	—	14.15	22.26	—	28.79	—
3. vii.	—	—	16.87	—	23.59	—
4. vii.	—	—	18.90	—	30.40	—
5. vii.	—	13.86	—	—	—	—
8. vii.	—	10.0	—	—	—	—
11. vii.	—	8.4	—	—	—	—
21. vii.	—	7.49	—	—	—	—
22. vii.	300	—	22.95	—	27.12	—
23. vii.	300	—	23.16	—	32.40	6
24. vii.	300	—	30.79	10	33.70	7
25. vii.	300	—	65.98	46	70.31	44
25 days	1200	Minus		56†	or	57†
	+ 150	(25 × 6 mg. in 300 g. beef)				
	1350					

Calculation based on the excretion of:

	Ascorbic acid	Total reducing substances
Exp. I	$\frac{956}{18} = 53.1$ mg.	$\frac{965}{18} = 53.6$ mg.
Exp. II	$\frac{1294}{25} = 51.8$ mg.	$\frac{1293}{25} = 51.7$ mg.

† No complicated calculation is needed, the final result being not essentially influenced by it. Furthermore, differences up to 5 mg. in the daily required dose can and should be neglected as unimportant.

‡ This experiment following on the final saturation of the first one, further saturation could be omitted.

Diets. The diets prescribed were similar to those described in Part I, on account of the longer duration of the high protein period (25 days) the daily quantity of meat was 300 g. (instead of 750 g.).

The data do not need further commentary, we learn, however, that no difference exists as regards the usefulness of saturation tests between direct titration and that after treatment with mercuric acetate—at least so far as these experiments are concerned. The daily dose required in Exp. II is almost equal to that of the first one with a comparatively low proportion of proteins. Thus, a high protein diet failed to increase markedly the requirements of ascorbic acid as it did not influence its urinary excretion.

Requirements of M. H. (aged 37, height 174 cm., 75 kg. while on a daily dose of 25 mg. of ascorbic acid (see Fig. 1)).

Calculation based on the excretion of ascorbic acid:

Experimental period = 37 days. Ascorbic acid taken during the experimental period = 2213 mg. ("Redoxon") + 180 mg. (12×15 mg. in 750 g. beef) = 2393 mg. minus 76.5 mg. (excreted above the average) = 2316.5 mg.

Daily requirements = $\frac{2316.5}{37} = 62.6$ mg.

Calculation based on the excretion of total reducing substances

$$\frac{2393 - 75}{37} = 62.6 \text{ mg.}$$

Requirements of M. H., while on a daily dose of 37.5 mg. of ascorbic acid (see Fig. 2).

Calculation based on the excretion of ascorbic acid:

Experimental period = 53 days. Ascorbic acid taken during the experimental period = 3312 mg. ("Redoxon") + 105 mg. (7×15 mg. in 750 g. beef) = 3417 mg. minus 63 mg. (excreted above the average) = 3354 mg.

Daily requirements = $\frac{3354}{53} = 63.3$ mg.

Calculation based on the excretion of total reducing substances:

$$\frac{3417 - 67}{53} = 63.2 \text{ mg.}$$

Daily doses of 25 or even 37.5 mg. of "Redoxon" failed to maintain the content of the blood at the level existing when the experiments began, nor did they prevent the development of a deficiency demonstrable in the course of saturation tests.

The subjects on whom the experiments were done were in good health; regarding the results of the saturation tests at the start of the experiments, their vitamin C conditions appeared to be normal.

DISCUSSION.

In the experiments described there has been no influence of high protein diets on the urinary output of ascorbic acid as suggested by Ahmad and Chakraborty & Roy. The different results are obviously caused by the fact that these authors did not remove interfering reducing substances. As to the total reducing capacity the observations agree, its increase by high protein diets having been confirmed. Furthermore, cystine exercises the same effect as does protein, and it appears very probable that not the proteins as a whole but rather their proportion of sulphur compounds causes the augmented reducing capacity. This point of view is further supported by the evidence that the excretion of

thiosulphate is principally augmented. On the days of cystine intake sodium nitroprusside tests were constantly negative (absence of cysteine, which also decolorizes dichlorophenolindophenol [Emmerie, 1934]). Whereas this influence of high protein diets has been partly confirmed, a sudden and considerable rise in the proportion of fat was not found to exercise any influence.

Chopra & Roy [1936], who are apparently unacquainted with the elimination of interfering substances by mercuric acetate, suggest that the increase of the "indophenol titre" (following high-protein diet, direct titration without previous treatment) is not due to ascorbic acid. In fact, there could be demonstrated "a significant relation between this reducing property and the uric acid excretion". Uric acid, however, does not decolorize dichlorophenolindophenol.

The requirements of two normal subjects were calculated by determining the dose required for a positive saturation test after dietetic periods without any vitamin C or with an insufficient supply (25 and 37.5 mg. daily). Examinations during dietetic periods with different proportions of protein resulted in equal requirements in spite of or together with normal urinary output of ascorbic acid. The values found agree closely with those calculated by van Eekelen [1935, 2; 1936, 1, 2] and van Wersch [1936]. The daily requirements for a subject weighing 70 kg. amount to about 60 mg. This value being twice that given by Göthlin [1934], we tend to regard it as the optimum dose—the *actual requirement*; while the smaller one of Göthlin may be only preventive against the manifestation of scurvy—the *indispensable minimum*. This interpretation seems the more justified if we note that this author supposes 19–25 mg. to be "the smallest daily dose of ascorbic acid which, given *per os*, protects a person weighing 60 kg. against the slightest objectively ascertainable prescorbutic symptoms—those in the capillaries."

SUMMARY.

The urinary output of ascorbic acid, determined after precipitation with mercuric acetate, is not influenced by the proportion of protein in the diet.

The total reducing capacity of urine rises and falls corresponding to the quantity of protein in the food and depends chiefly on the output of thiosulphate.

Cystine, taken in the course of low protein diet, has the same effect on the total reducing capacity of urine as have large quantities of protein. The sulphur-containing amino-acids can be considered as the source of thiosulphate in urine.

By means of saturation tests following dietetic periods the daily human requirements were calculated.

Equal requirements were observed during low- and high-protein diets on the same subject.

The daily requirement of man amounts to about 60 mg. for a body weight of 70 kg. The same amount was required when 25 or even 37.5 mg. of "Redoxon" per day had been taken.

Considering this daily dose as *actual requirement*, the suggestion that an essentially smaller quantity is sufficient to avoid scurvy—the *indispensable minimum*—has been discussed and affirmed.

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