CCCXVIII. ON THE AMOUNT OF ASCORBIC ACID IN BLOOD AND URINE. THE DAILY HUMAN REQUIREMENTS FOR ASCORBIC ACID.

By MARIE VAN EEKELEN.

From the Laboratory of Hygiene, University of Utrecht.

(Received 17 September 1936.)

THE investigations described in this contribution were undertaken [van Eekelen, 1936] to answer the following questions: (1) Do the ascorbic acid content of the blood and the daily urinary output of it depend on the amount of vitamin C stored in the human organism? Is it possible to get an insight into the state of saturation of a given subject, for the special purpose of detecting hypovitaminosis, by determining the ascorbic acid in blood or in urine? (2) What are the normal daily requirements for ascorbic acid of human adults?

Van Eekelen *et al.* [1933, 1] and Johnson & Zilva [1934] have shown that, following intake of large doses of ascorbic acid, the organism can be saturated to such an extent that a further dose of ascorbic acid is partly or entirely excreted in the urine. In this way the storage of ascorbic acid in a certain subject can be investigated by determining the amount of ascorbic acid required to reach the point of saturation. We shall consider this point to be reached when a daily dose of 250–400 mg. ascorbic acid results in a definite rise of urinary output (about 30 mg. above the normal average). This does not indicate complete saturation, in which case nearly the total amount taken in is excreted.

The questions arise: What is the amount of ascorbic acid in the blood when this point of saturation has been reached? Do the amount in the blood and the daily urinary output of ascorbic acid decrease when a saturated experimental person lives for some time on a diet devoid of vitamin C? When a decrease results an increase can be expected by saturating the person again.

By this experiment it is possible also to calculate the daily requirements for the vitamin supposing that the quantity of ascorbic acid necessary to saturate the subject at the end of the dietetic period equals the amount required during this period. No reasons against this supposition could be detected. To be sure that the dietetic prescriptions were followed as strictly as possible and that the urinary examinations could be done in time the writer himself was the experimental subject.

METHODS.

Determination of ascorbic acid in urine. The ascorbic acid was determined by titration with 2:6-dichlorophenolindophenol standardized against crystalline ascorbic acid (about 10 ml. indicator corresponds to 1 mg. ascorbic acid). Urine contains besides ascorbic acid other substances reducing the indicator, e.g. ergothioneine [Sullivan & Hess, 1933] and thiosulphates [van Eekelen, 1934]. For this reason it is necessary to remove these interfering substances by precipitation with mercuric acetate [Emmerie, 1934; Emmerie & van Eekelen, 1934; van Eekelen & Emmerie, 1936]. The urine is examined immediately after it has been voided.

Determination of ascorbic acid in blood. In blood also reducing substances other than ascorbic acid are present (e.g. ergothioneine, glutathione) which can be removed likewise by precipitation with mercuric acetate. Thus, the blood being precipitated with trichloroacetic acid, mercuric acetate is added, the mixture neutralized with CaCO_3 until slightly acid (Congo red paper remaining unaltered, blue litmus paper turning to red) and treated with H_2S [Emmerie & van Eekelen, 1934].

Hardly any reducing substances are present when the blood filtrate is titrated after precipitation with trichloroacetic acid (before the treatment with mercuric acetate and H_2S) even when pure ascorbic acid has been added previously. From this experiment we have earlier concluded [van Eekelen *et al.* 1933, 2] that the ascorbic acid in the blood is present in the reversibly oxidized state and that an added quantity of ascorbic acid will be reversibly oxidized by the blood. The following determinations however demonstrate in agreement with Kellie & Silva [1935; 1936] that this supposition is not true [van Eekelen, 1935].

Table I. *mg. ascorbic acid per 10 ml. ox blood, plasma and erythrocytes.*

	After precipitation with CCl_3COOH	After reduction with H_2S	After precipitation with CCl_3COOH and $\text{Hg}(\text{CH}_3\text{COO})_2$ and reduction with H_2S
Blood	0	0.28	0.042
Plasma	0.035	—	0.034
Erythrocytes	0	—	0.050
Blood + 0.9 mg. ascorbic acid	0.15	—	0.93
Blood, centrifuged; in 5 ml. plasma	0.75	—	0.75
Blood + 4.92 mg. ascorbic acid	3.31	—	4.91*
Plasma + 4.92 mg. ascorbic acid	4.89	—	4.91†

* Calculated 4.95.

† Calculated 4.94.

Table I shows: (1) Ascorbic acid is present both in the plasma and in the erythrocytes. (2) Precipitation with mercuric acetate is necessary because after reduction with H_2S without precipitation with mercuric acetate a larger amount of reducing substances is found. This demonstrates the presence of reducing substances other than ascorbic acid. (3) In plasma ascorbic acid is present in the reduced state. (4) During the precipitation with trichloroacetic acid, the ascorbic acid becomes reversibly oxidized if erythrocytes are present. This oxidation occurs especially when the blood is oxygenated. After treatment with CO_2 or CO only a small part of the ascorbic acid is oxidized (Table II).

Table II.

	After precipitation with CCl_3COOH	After precipitation with CCl_3COOH and $\text{Hg}(\text{CH}_3\text{COO})_2$ and reduction with H_2S
10 ml. aerated blood + 4.92 mg. ascorbic acid	3.31	4.91
10 ml. blood saturated with CO_2 + 4.92 mg. ascorbic acid	4.72	4.89
10 ml. blood saturated with CO + 4.92 mg. ascorbic acid	4.72	4.89

Thus we have offered sufficient evidence that the determination of ascorbic acid in blood is unreliable without treatment of the trichloroacetic extracts with mercuric acetate and H_2S . From the data above results the possibility of determining the vitamin in serum directly, without treatment with mercuric

acetate and H_2S [Gabbe, 1934; Farmer & Abt, 1935; Abt *et al.*, 1936; Taylor *et al.*, 1936]. However, it is advisable to apply the mercuric acetate and H_2S procedure because in serum also other interfering reducing substances may occur or the ascorbic acid may be partly reversibly oxidized (e.g. after haemolysis).

Experimental details. The experimental person was a man, aged 30 years, height 178 cm., weight 90 kg. The investigations were done from August to December 1935. The diet devoid of vitamin C contained bread, butter, cheese, eggs and water. For the saturation tests crystalline ascorbic acid (Hoffmann-La Roche) was taken.

The course of the experiment is given in Table III and Fig. 1.

Table III.

Date (1935)	mg. ascorbic acid taken in	ml. urine excreted	Daily urinary output mg. ascorbic acid	mg. ascorbic acid per l. blood	g. faeces excreted per day	mg. ascorbic acid in faeces	Number of petechiae. Left arm (L), right arm (R)
29. vii	—	—	—	—	—	—	—
30	—	742	13.1	—	—	—	—
31	—	875	12.4	—	—	—	—
1. viii	—	833	13.4	11.2	175	4.6	—
2	250	929	16.6	11.7	—	—	—
3	250	780	23	16.8	120	3.6	—
4	—	793	17.2	15	140	3.8	—
5	250	769	45.6	17	143	4.3	—
6	—	675	15.8	15	140	4.1	—
7	—	754	13.7	—	76	3.2	—
8	—	—	—	—	128	3.3	—
13	—	—	—	9.6	—	—	—
17	—	1046	10.5	—	—	—	—
18	—	903	12	—	—	—	—
19	—	770	10.2	—	—	—	—
21	—	—	—	6	—	—	—
22	—	847	11.9	—	—	—	—
23	—	819	11.2	—	—	—	—
28	—	902	10.2	—	—	—	—
29	—	830	10.1	—	—	—	—
30	—	—	—	4.3	—	—	—
4. ix	—	—	—	—	—	—	L 0
7	—	910	9.2	—	—	—	—
8	—	845	8.4	—	143	4.2	—
9	—	—	—	3.7	—	—	—
13	—	855	9.1	—	—	—	—
14	—	777	8	—	—	—	L 0
15	—	790	7.6	—	—	—	—
16	—	—	—	3.1	—	—	—
23	—	713	9	2.5	—	—	—
24	—	807	8.1	—	—	—	—
28	—	889	8	—	—	—	L 2, R 1
29	—	—	—	—	142	4.7	—
1. x	—	—	—	3	—	—	—
4	—	727	6.7	—	—	—	—
9	—	769	7.8	—	—	—	—
11	—	—	—	2.3	—	—	—
12	—	—	—	—	—	—	L 3, R 2
21	—	660	7.2	2.2	—	—	—
26	—	—	—	—	—	—	R 1
28	—	789	7	1.9	145	7.4	—
29	2 × 250	678	8.8	—	—	—	—
30	2 × 250	764	9.1	—	—	—	—
31	2 × 250	699	10.9	5	—	—	—

Table III (cont.).

Date (1935)	mg. ascorbic acid taken in	ml. urine excreted	Daily urinary output mg. ascorbic acid	mg. ascorbic acid per l. blood	g. faeces excreted per day	mg. ascorbic acid in faeces	Number of petechiae. Left arm (L), right arm (R)
1. xi	2 × 250	688	15.1	—	186	8.7	—
2	250	682	15.9	8.9	—	—	—
3	250	688	12.9	—	—	—	—
4	—	678	12.2	9.4	—	—	—
5	250	656	14.5	—	—	—	—
6	250	727	19	—	—	—	—
7	250	646	35.5	13.3	—	—	—
8	—	594	15	14.4	—	—	—
9	—	761	14.4	—	—	—	—
11	—	556	12.9	—	—	—	—
12	—	—	—	10.6	—	—	—
16	—	614	10.5	8.3	—	—	—
21	—	—	—	6.6	—	—	—
26	—	625	10.1	—	—	—	—
27	—	607	9.2	5.1	—	—	—
28	250	661	9.5	—	—	—	—
29	250	728	10.3	—	—	—	—
30	250	651	10.7	—	—	—	—
1. xii	250	683	13.3	—	—	—	—
2	250	737	15.8	—	—	—	—
3	250	604	21.6	—	—	—	—
4	250	556	39.5	13.2	—	—	—

Fig. 1. •—• mg. ascorbic acid, daily urinary output.
•- - • mg. ascorbic acid per l. blood.

From this table it will be seen that the diet deprived of vitamin C began on 29 July. On the next 3 days the amounts of ascorbic acid excreted in the urine were determined (about 13 mg. per day). After an intake of 250 mg. daily during 3 days a definite rise in the urinary output was observed (a surplus excretion of about 30 mg. on 5 August).

By determining the ascorbic acid content of the faeces it was shown that an increased excretion with the faeces did not occur when large doses of ascorbic acid were given (see Table III).

Following this saturation the diet devoid of vitamin C was continued until 29 October. At intervals the ascorbic acid content of the blood and the daily urinary output were determined. The amount in the blood being 11.2 mg./l. before the experiment began was 11.7 mg./l. after 250 mg. had been taken and increased to 16.8 mg. after the second dose of 250 mg. (determination 3 hours after intake of ascorbic acid). On the day of this increased amount in the blood a somewhat larger urinary excretion was observed (23 mg. instead of 13 mg.). The next day (4 August) no ascorbic acid was taken in; the amount in the blood decreased to 15 mg./l. On 5 August 250 mg. were taken in again and a greatly increased excretion (45.6 mg.) resulted. Compared with the amount on 3 August the content of the blood only rose to 17 mg./l. (3.5 hours after intake). This increase was transitory, the next day 15 mg. were found. So it appeared that a surplus excretion of ascorbic acid in the urine occurs when a certain level in the blood (about 15 mg./l.) is surpassed. In the course of the dietetic period the daily urinary output slowly decreases; after 83 days from about 13 mg. to about 7 mg. with, however, large fluctuations. Compared with this observation the decrease of the amount in the blood is more important however only during the first period of the experiment (after 25 days from 15 mg. to 4.3 mg./l.). During the next period the decrease was less marked (after 59 days from 4.3 to 1.9 mg./l.).

After 84 days of the regimen ascorbic acid was again taken. To reach the point of saturation more quickly two doses of 250 mg. were taken daily during the first days, then daily doses of 250 mg. were taken (as at the beginning of the experiment) to avoid the point of saturation being suddenly largely exceeded. 3250 mg. were necessary to restore saturation (7 November, 35.5 mg. were excreted in urine). In the course of the saturation the amount in the blood and in the urine increased. At the point of saturation the amount in the blood was 14.4 mg./l. The regimen was continued for another 20 days; the amount in the blood decreased to 5.1, the urinary output to 9–10 mg. per day. By taking 1750 mg. of ascorbic acid saturation was again reached. The amount in the blood rose to 13.2 mg.

From these experiments it follows that together with the development of a vitamin C deficiency the amounts of ascorbic acid in the blood and in the urine decrease. This agrees with the findings of Abt *et al.* [1936] and Taylor *et al.* [1936] on the ascorbic acid content of blood serum. It appears to be possible to get an insight in the state of saturation of a given subject by determining the ascorbic acid content of the blood or of the urine. For this purpose the estimation in the blood is preferable, for it is less complicated because it needs only one examination whilst the excretion in the urine has to be determined during 24 hours and immediately after voiding. Furthermore there are rather large fluctuations in the daily output of the same subject. Moreover, the daily output is very unequal in different subjects with about the same state of saturation, in the nearly saturated state varying from 9 to 27 mg. The amount in the blood decreases more and more quickly than that of the urine. The relation between

the state of saturation and the content in the blood however is not linear.¹ For this reason three classifications only appear reasonable: about or above 13 mg./l. signifies saturation, below 4 mg. has to be considered as insufficient, between 13 and 4 mg. more or less sufficient.

In illustration of the facts described above some determinations are given of the ascorbic acid content of the blood before and after saturation with ascorbic acid of three patients probably suffering from scurvy.

In two patients the initial ascorbic acid contents were 3.8 and 2.2 mg./l. After saturation with daily oral doses of 300 and 400 mg. to an amount of 3300 and 4000 mg. ascorbic acid the concentrations in the blood increased to 12.8 and 13 mg./l. In the third case the patient was saturated with 400 mg. ascorbic acid daily given *per os* or intravenously. The amount in the blood was estimated just before the daily dose of ascorbic acid was given, except on 25 February (5 hours after administration). The ascorbic acid content of the urine was investigated during 5 hours after administration of the ascorbic acid. The results are given in Fig. 2.

Fig. 2. i = Intravenous. o = per os.

The figure shows that after intravenous administration of ascorbic acid an increased urinary excretion already results before the point of saturation is reached. This can be explained by the supposition that after the injection the amount of ascorbic acid in the blood rises for a short period above the excretion level.

¹ Note added 4 December 1936. Determining the concentration of ascorbic acid in the blood and the quantity necessary to saturate different subjects I have found that the relation between these values is linear. A concentration in the blood of 4 mg. per l. corresponds with a saturation dose of about 2 g.; 8 mg. per l. corresponds with a dose of 1 g. ascorbic acid (for subjects with a body weight of 70 kg. The decrease of ascorbic acid in the blood during a dietetic period (see Fig. 1) is not linear because the use of ascorbic acid decreases during this period, as has been demonstrated in this paper.

The capillary resistance test.

According to Göthlin [1931] and Dalldorf [1933] the capillary resistance depends on the state of saturation; a lowered capillary resistance should indicate a deficiency.

During the first experiment described above some changes in the capillary resistance might have been expected at the end of the dietetic period. A considerable deficiency could be demonstrated by the saturation test (viz. 3250 mg. ascorbic acid had to be taken to reach the point of saturation). Yet the capillary resistance remained normal (see Table III). The technique of the measurements was that given by Göthlin (50 mm. Hg pressure during 15 min.). It appears noteworthy that at the end of the experiment (after 84 days) no other symptoms of vitamin C deficiency than fatigue and irritability could be observed; the body-weight remained constant.

Daily human requirements.

During the first experiment lasting from 6 August to 7 November, 3250 mg. ascorbic acid were taken (Table III). To calculate the amount stored or used in the body from the quantity taken must be subtracted the amount of ascorbic acid excreted above the normal average during the last few days of intake (about 30 mg.). The experiment lasting 94 days the daily requirements amount to

$$\frac{3220}{94} = 34 \text{ mg.}$$

This result differed somewhat from that of a similar informative experiment done in 1934. In this experiment the ascorbic acid content of the urine was estimated directly, without treatment with mercuric acetate and H_2S . This omission did not influence the main result of the experiment because the aim was the registration of a definite peak in the urinary output of ascorbic acid. That experiment lasted 40 days, 1850 mg. were required, the daily requirements amounted to 45 mg.

The difference between these two experiments can possibly be accounted for by their different durations. The decreased urinary output of ascorbic acid at the end of the dietetic period eventually indicates restricted requirements. The requirements should be dependent on the amount of the vitamin stored in the organism. To test this supposition another experiment was done lasting only 27 days, from 7 November to 4 December (Table III, Fig. 1). During this period the organism has not been depleted appreciably (the amount in the blood became 5.1 mg./l.). 1750 mg. minus 38 mg. (surplus excreted) were required to saturate the organism again. From this determination a daily requirement of 63 mg. can be calculated ($1712/27$). Thus we may suppose an adaptability of the daily requirement to the amount of ascorbic acid stored in the organism. The requirement decreases when the organism becomes depleted. During a period without strongly depleted depots the amount used daily is 63 mg.

According to this method [van Eekelen, 1936] van Wersch [1936, 1, 2] has determined the daily requirements for himself (man 38 years, height 168 cm., weight 72 kg., duration of the experiment 31 days) and for a female patient suffering from degeneration of the skeleton probably caused by chronic scurvy (age 18 years, height 136 cm., weight 53 kg., duration of the experiment 17 days). Requirements amounting to 56 and 44 mg. respectively resulted (the concentrations in the blood after saturation were respectively 13 and 13.7 mg./l.). Evaluating the daily requirements of these three experimental persons per kg. body weight, a close agreement can be observed when the weights are corrected to the body lengths (calculated from Shall's [1933] height-weight-age table). (See Table IV).

Table IV.

	Height cm.	Weight kg.	Corrected weight	Daily require- ments mg. ascorbic acid	Daily require- ments per kg.	Daily requirements per kg. corrected weight
Van Eekelen	178	90	74.6	63	0.70	0.84
Van Wersch	168	72	67	56	0.78	0.84
Patient J	136	53	?	44	0.83	0.83

From this the conclusion may be drawn that corpulent people require per kg. body weight less ascorbic acid than slender ones. Possibly the adipose tissues require little ascorbic acid.

From the data presented it follows that the daily requirements for healthy adults weighing 70 kg. amount to about 60 mg.

For a further discussion of the daily human requirements I will refer to the communication of Heinemann in this number of the *Journal* (p. 2299).

SUMMARY.

The content of ascorbic acid in blood and its urinary output depend on the amount of vitamin C taken in and on the quantity stored in the organism. Saturation of the organism coincides with a certain level in the blood (about 13 mg./l.). This level being surpassed surplus excretion in the urine results. To estimate the rate of saturation the determination of ascorbic acid in the blood has proved to be the most reliable method (besides the saturation test). A method to determine daily human requirements for ascorbic acid is described.

The daily requirements are dependent upon the amount stored in the organism, they are largest when the subject does not become markedly unsaturated.

The daily dose required for adults weighing 70 kg. amounts to about 60 mg. under normal conditions.

REFERENCES.

- Abt, Farmer & Epstein (1936). *J. Pediat.* **8**, 1.
 Dalldorf (1933). *Amer. J. Dis. Child.* **46**, 794.
 van Eekelen (1934). *Acta brev. neerl. Physiol.* **4**, 137.
 — (1935). *Nature, Lond.*, **136**, 144.
 — (1936). Thesis, Utrecht.
 — & Emmerie (1936). *Biochem. J.* **30**, 25.
 — — — — — Josephy & Wolff (1933, 1). *Acta brev. neerl. Physiol.* **3**, 168.
 — — — — — (1933, 2). *Nature, Lond.*, **132**, 315.
 Emmerie (1934). *Biochem. J.* **28**, 268.
 — & van Eekelen (1934). *Biochem. J.* **28**, 1153.
 Farmer & Abt (1935). *Proc. Soc. exp. Biol., N.Y.*, **32**, 1625.
 Gabbe (1934). *Klin. Wschr.* **13**, 1389.
 Göthlin (1931). *Skand. Arch. Physiol.* **61**, 225.
 Johnson & Zilva (1934). *Biochem. J.* **28**, 1393.
 Kellie & Zilva (1935). *Biochem. J.* **29**, 1028.
 — — — — — (1936). *Biochem. J.* **30**, 361.
 Shall (1933). *Nahrungsmitteltabelle* 63. (Leipzig: Kabitzsch.)
 Sullivan & Hess (1933). *J. biol. Chem.* **102**, 67.
 Taylor, Chase & Faulkner (1936). *Biochem. J.* **30**, 1119.
 van Wersch (1936, 1). *Ned. Tijdschr. Geneesk.* **80**, III, 3653.
 — (1936, 2). *Acta brev. neerl. Physiol.* **6**, 86.