XXXIII. EXPERIMENTS CONFIRMING THE ANTISCORBUTIC ACTIVITY OF DEHYDROASCORBIC ACID AND A STUDY OF ITS STORAGE AND THAT OF ASCORBIC ACID BY THE GUINEA-PIG AT DIFFERENT LEVELS OF INTAKE.

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HIRST AND ZILVA [1933] showed that the antiscorbutic value of dehydroascorbic acid prepared by oxidation of ascorbic acid with iodine was only slightly less than that of the ascorbic acid from which it had been prepared. Having devised a simple method for preparing dehydroascorbic acid free from contaminating substances [Fox and Levy, 1936] it was thought worth while to study this interesting observation in greater detail. At the same time advantage was taken of the chemical method now available for estimating ascorbic acid to try to learn something about the metabolism of these two substances at different levels of intake.

Exp. 1. 12 young guinea-pigs were placed on the scurvy-producing diet described by Bracewell *et al.* [1930]. The progress of the four groups into which these animals were divided is charted in Fig. 1.

The norite-orange juice (N.O.J.) was prepared by treating 250 ml. fresh juice with 10 g. of norite and allowing to stand for 15 min.; the liquid was then filtered through a thick layer of cotton-wool and stored in the ice-chest until required. No attempt was made to remove the last traces of norite. The N.O.J. was prepared every second day or rarely every third day during the course of the experiment, each batch being tested before use by means of the indophenol reagent and shown to be entirely free from reducing substance.

All the animals on the dehydroascorbic acid ration when examined *post mortem* were found to be entirely free from any signs of scurvy. It was, however, noticed that the adrenals of animals 9, 10, 12, 12 (b) and 12 (c) looked somewhat flabby and wrinkled. On microscopic examination it was found that each of these cases showed evidence in varying degrees of fatty change affecting the outer cortical zone, most marked just underneath the capsule. The majority of the cells resembled those found in xanthomatous degeneration, *i.e.* they were converted into foamy cells. In addition many of the cells were distended with definite large droplets of fat. Whilst the medulla invariably showed no abnormality, the cortex was in each case the seat of marked congestion.

Conclusion. This experiment confirms the observation that dehydroascorbic acid possesses antiscorbutic activity. Not only was it able to prevent the appearance of scurvy over a period of 14 weeks, but 2 animals gave birth to live young whilst on the diet and one of the young was successfully reared on the oxidised vitamin. It is not clear what significance can be attached to the changes observed in the adrenals.

In addition to the clinical evidence regarding freedom from scurvy obtained above it was decided to estimate the amount of antiscorbutic vitamin in the livers and adrenals of all the animals included in the experiment. For this purpose we used the method described by Birch *et al.* [1933].

Exp. 2. With the object of first finding out what the normal level of ascorbic acid in these organs might be we examined some animals that had been killed to provide complement for the Wassermann reaction. They had all been living on a liberal diet of cabbage and bran. As will

Fig. 1. Growth curves of guinea-pigs on a basal diet *plus* dehydroascorbic acid. A. Negative controls. Basal diet only; all animals died within 23-28 days, showing in each case well marked evidence of scurvy. B. Basal diet *plus* norite-orange juice (dehydroascorbic acid). Animals remained in excellent condition and showed no signs of scurvy when killed after 64 days. C. Similar diet to B but larger quantities of N.O.J. given after 64th day. No evidence of scurvy when killed after 100 days. D. Similar diet to B. 4 young born during experiment (64th day). Both animals died several days later from intercurrent infection, but showed no evidence of scurvy. D₁. Of the 4 young, 2 died within a day after birth; the other 2 were separated from the mother (guinea-pig 12) at the end of a week and were fed on the basal diet *plus* N.O.J. 1 remained alive for 2 weeks, whilst the other continued to grow and was in excellent condition when killed after having been reared for 40 days on the N.O.J. *Post mortem*, both these animals were found to be completely free from scurvy.

K = Killed.
N = Normal.
I = Intercurrent infection.
+ = Died.
No S = No Scurvy.

S = Scurvy.
B¹ = Birth of 1 young.
B¹¹¹ = Birth of 3 young.
↓ = Amounts of norite-orange juice given daily.

be seen from Table I (a) very fairly constant values were obtained for the 8 animals examined. These results are in good agreement with those recorded by Svirbely [1933], whose mean values for 4 animals fed on a basal diet *plus* excess of spinach were 0.23 and 1.24 mg. per g. for liver and adrenals respectively. Similarly Zilva [1935] obtained 0.25 mg. per g. of liver as an average for 4 animals on a mixed diet.

Substantially the same values were again obtained when the above diet was supplemented with 4-6 ml. of orange juice daily (Table I (b)).

These animals were all killed about 20 hours after their last feed of cabbage. Table I (c) shows that substantially higher concentrations were obtained when the animals had free access to cabbage until just before they were killed.

Table I. *Ascorbic acid content of liver and adrenals of normal guinea-pigs placed on various diets.*

Animal	Liver		Adrenals	
	Weight g.	Ascorbic acid per g. mg.	Weight g.	Ascorbic acid per g. mg.
(a) On a diet of cabbage and bran.				
1	19.0	0.26	0.160	1.70
2	17.5	0.17	0.346	1.30
3	17.9	0.21	0.500	1.24
4	33.5	0.31	0.852	1.48
5	19.3	0.32	0.390	1.23
6	20.0	0.23	0.722	1.10
7	18.8	0.28	0.293	1.62
8	24.8	0.23	0.440	1.62
9	15.4	0.24	0.165	1.68
10	23.6	0.20	0.181	2.06
Mean	21.0	0.25	0.405	1.50
(b) On the same diet plus 4-6 ml. orange juice per day.				
11	20.0	0.25	0.132	1.48
12	31.0	0.31	0.360	1.33
13	22.5	0.33	0.153	1.28
Mean	24.5	0.30	0.215	1.36
(c) On same diet, but with free access to cabbage until killed.				
14	20.9	0.52	0.120	2.14
15	25.6	0.45	0.185	2.02
(d) On same diet plus 100 mg. ascorbic acid per day for 8 days and with free access to cabbage until killed.				
16	18.9	0.42	0.147	2.06
17	25.8	0.38	0.153	1.96

In order to convince ourselves that this new level represented the maximum concentration obtainable, two more animals were given the same diet plus 100 mg. of ascorbic acid (redoxon) daily; this dose of more than 100 times the minimum protective dose was continued for 8 days and the animals were then killed after having access to cabbage overnight. Table I (d) shows that the concentration previously obtained was not exceeded; presumably this represents the saturation point for the liver of a guinea-pig.

Conclusion. From these experiments it is clear that the guinea-pig has a small and definite capacity for storing the vitamin in its liver; this maximum value can easily be reached on an ordinary herbivorous diet.

Exp. 3. In order to form some more definite idea as to the normal intake of ascorbic acid 12 guinea-pigs of about 350 g. weight were placed on a diet of bran plus cabbage *ad lib.* The weights of cabbage consumed on 4 successive days were found to be 240, 410, 370 and 330 g. giving an average consumption of about 30 g. per animal. Assuming a value of 0.4-0.6 mg. ascorbic acid per g. this would represent an intake of from 12 to 18 mg. *per diem*, or between 10 and 20 times the minimum protective dose. This definite but rather limited power of storage no doubt accounts for the well-known fact that the onset of scurvy in guinea-pigs cannot be delayed to any extent by the previous administration of large doses of the vitamin [see for example *Medical Research Council Report on Vitamins*, 1932].

Exp. 4. The amounts of ascorbic acid found in the animals used in Exp. 1 are recorded in Tables II and III.

It will be seen that animals dying from scurvy showed very low stores of the vitamin and indeed it is doubtful whether the very small titration obtained was due to ascorbic acid at all. This finding is again in agreement with those recorded by Svirbely [1933] for similar animals dying from scurvy [*cf.* Hamilton, 1933].

Table II. *Ascorbic acid contents of liver and adrenals of guinea-pigs dying from scurvy.*

Animal	Days on diet	Liver		Adrenals	
		Weight g.	Ascorbic acid per g. mg.	Weight g.	Ascorbic acid per g. mg.
1	28	10.0	0.014	0.44	≤ 0.024
2	25	13.2	0.016	0.60	≤ 0.017
3	25	9.7	0.021	0.44	≤ 0.023
4	26	15.0	0.018	0.34	≤ 0.030
5	23	11.3	0.025	0.38	≤ 0.026
	Mean	11.8	0.019	0.44	≤ 0.024
Animal treated with N.O.J. 2 days prior to death.					
6	25	12.4	0.064	0.50	0.21

Table III. *Ascorbic acid contents of liver and adrenals of guinea-pigs fed on norite-orange juice.*

Animal	Age in days	Days on N.O.J.	Dose per day ml.	Liver		Adrenals	
				Weight g.	Ascorbic acid per g. mg.	Weight g.	Ascorbic acid per g. mg.
7	—	64	8	32.7	0.021	0.41	0.10
8	—	66	8	29.7	0.065	0.15	0.56
9	—	100	32	34.2	0.100	0.49	0.80
10	—	99	32	23.5	0.120	0.35	0.70
11	—	62	8	29.0	0.038	0.41	≤ 0.03
12	—	77	8	30.3	0.066	0.52	0.13
			Mean	29.9	0.068	0.39	0.39
Young born during experiment.							
11 (a)	1	—	—	4.1	0.06	0.021	≤ 0.48
12 (a)	1	—	—	2.7	0.08	0.033	≤ 0.30
12 (b)	16	9	4	5.7	0.07	0.060	0.50
12 (c)	42	35	8	12.6	0.08	0.100	0.74

It was the quite unexpected finding that animals 7 and 8, although receiving plenty of N.O.J. and with no signs of scurvy, had such low reserves of vitamin that led us to feed the remaining animals for a longer time and on very generous quantities of dehydroascorbic acid. As will be seen, however, it made little difference, for the stores of animals 9 and 10 were very nearly as low as the preceding ones. Similar low results were also obtained with animals 11 and 12. Finally, the 4 young born during the experiment, including the animal which remained in excellent health until killed when 6 weeks old, also showed the same low reserve of vitamin C, yet were entirely free from clinical evidence of scurvy.

DISCUSSION.

In seeking an explanation for these surprisingly low reserves the following possibilities call for consideration.

(1) That the animals cannot have been living just above the scurvy border line owing to traces of ascorbic acid present in the basal diet is evident from the fate which so rapidly overtook the six animals that were on this diet alone.

(2) That similar traces of ascorbic acid were supplied by the norite-orange juice is also most unlikely since repeated controls showed that it had no action whatever on indophenol.

(3) That the liver was not storing the vitamin as dehydroascorbic acid was shown by the fact that preliminary treatment of the liver extract with hydrogen sulphide did not increase the titre.

(4) We therefore concluded that dehydroascorbic acid, though effective as an antiscorbutic, was not as easily stored as the parent substance.

With the hope of throwing more light on the ability of the guinea-pig to store ascorbic acid at different levels of intake three further experiments have been carried out.

Exp. 5. In order to determine the lowest level of storage that the guinea-pig can attain without clinical evidence of scurvy 5 animals were placed on the usual basal diet and killed after 10 days, *i.e.* at the time when the weight usually begins to fall [see also Siehrs and Miller, 1933; 1934]. Table IV shows that by this time the reserves are almost completely exhausted although no clinical evidence of scurvy was obtained.

Table IV. *Ascorbic acid contents of the liver and adrenals of guinea-pigs killed after 10 days on the scorbutic diet.*

Animal	Liver		Adrenals		Remarks <i>Post mortem</i> examination
	Weight g.	Ascorbic acid per g. mg.	Weight g.	Ascorbic acid per g. mg.	
1	13.4	0.037	0.34	0.24	No scurvy
2	13.7	0.017	0.18	0.11	Slight scurvy
3	19.8	0.028	0.26	0.19	No scurvy
4	22.0	0.017	0.39	0.26	No scurvy
5	21.0	0.030	0.23	0.22	No scurvy
Mean	18.0	0.026	0.28	0.20	

Exp. 6. In this experiment 4 guinea-pigs were placed on the basal diet *plus* a daily ration of 5 ml. of orange juice (equivalent to 2.5–3.0 mg. ascorbic acid as found by titration). On this amount, which was intermediate between the minimum protective dose and that present in a diet of unrestricted cabbage or similar food, it was found that the storage was again of a low order (Table V). As this diet had been continued for 2 months it is clear that had there been but a slight storage of the available vitamin day by day it would have soon led to quite a considerable reserve [*cf.* Hamilton, 1933].

Table V. *Ascorbic acid contents of the livers and adrenals of guinea-pigs fed for 2 months on basal diet plus 5 ml. orange juice ($\equiv$ 2.5–3.0 mg. ascorbic acid per day).*

Animal	Liver		Adrenals	
	Weight g.	Weight acid per g. mg.	Weight g.	Weight acid per g. mg.
1	21.8	0.063	0.28	0.41
2	25.2	0.104	0.24	0.58
3	22.0	0.054	0.21	0.51
4	25.6	0.073	0.33	0.48
Mean	23.6	0.073	0.27	0.50

Exp. 7. Further confirmation of the results of the foregoing experiment was obtained by supplying varying amounts of ascorbic acid to guinea-pigs, this time, however, in the form of lucerne leaves. The animals were on the same basal diet and were given weighed quantities of the leaves, the ascorbic acid content of which had been estimated previously by means of indophenol. The feeding was continued for nearly 100 days [see, for further particulars, Levy and Fox, 1935]. Here again it will be noticed (Table VI) that although the animals were fully protected from

Table VI. *Ascorbic acid contents of the livers and adrenals of guinea-pigs fed for 3 months on a basal diet plus lucerne leaves.*

(Average value of lucerne leaf, 1 g. = 2.67 mg. ascorbic acid.)

Animal	Liver		Adrenals		Remarks
	Weight g.	Ascorbic acid per g. mg.	Weight g.	Ascorbic acid per g. mg.	
(a) Controls (basal diet only).					
1	8.6	0.043	0.32	0.057	Died of scurvy
2	9.4	0.041	0.44	≤ 0.027	"
Mean	9.0	0.042	0.38	0.042	
(b) Basal diet <i>plus</i> 0.8 mg. ascorbic acid per day.					
3	25.1	0.044	0.32	0.22	Killed
4	19.4	0.042	0.50	0.09	Died of intercurrent infection
5	27.4	0.030	0.40	0.13	Killed
6	45.3	0.022	0.55	0.14	"
7	29.7	0.025	0.63	0.15	"
Mean	29.4	0.033	0.48	0.15	
(c) Basal diet <i>plus</i> 1.6 mg. ascorbic acid per day.					
8	25.8	0.10	0.45	0.13	Both animals died of intercurrent infection
9	17.1	0.12	0.28	0.17	
Mean	21.5	0.11	0.37	0.15	
(d) Basal diet <i>plus</i> 3.2 mg. ascorbic acid.					
10	20.3	0.062	0.27	0.45	Killed
11	27.1	0.054	0.28	0.37	"
12	30.9	0.053	0.42	0.28	"
13	40.7	0.052	0.33	0.21	"
14	29.1	0.043	0.25	0.29	"
Mean	29.6	0.053	0.31	0.32	

macroscopic evidence of scurvy, even when fed on very small quantities of the lucerne at these lower levels of intake of ascorbic acid, they were unable to build up any considerable reserve of the vitamin. It is also of interest to notice that the amounts found in liver and adrenals are closely similar to those obtained by feeding the same quantity of ascorbic acid contained in orange juice. This affords an interesting proof of the availability of the vitamin in green leaves as compared with a fruit juice.

SUMMARY.

1. The antiscorbutic value of dehydroascorbic acid has been fully confirmed in experiments with guinea-pigs lasting over a period of 14 weeks. One animal, born during the course of the experiment, was successfully reared on the oxidised vitamin.

2. Dehydroascorbic acid was reduced to ascorbic acid and stored to a small extent in the liver of these animals; the amount stored remained low, even when large doses were given over a considerable period.

3. On the other hand it is seen that with ascorbic acid the amount stored is closely dependent upon the actual level of intake. At levels little greater than the minimum protective dose hardly any storage takes place; at intermediate levels the storage is approximately proportional to the intake, whilst with larger amounts an upper limit soon appears to be reached.

How far these findings apply to the human being remains to be seen, but they are in general agreement with the conclusions arrived at by Johnson and Zilva [1934] and by Harris and Ray [1935] based on urinary excretion tests.

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