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The Fixation and Retention of Ascorbic Acid by the Guinea-pig

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In this communication experiments are described which are an extension and an elaboration of an investigation on guinea-pigs and man which has been in progress for some time (Johnson & Zilva, 1934; Zilva, 1936; Kellie & Zilva, 1938, 1939; Zilva, 1941), the general object being to coordinate experimental data obtained on guinea-pigs with observations made on human beings. Since the scope of experimentation on man is for obvious reasons limited, many gaps in our knowledge can only be filled in by systematic and detailed experimental work on animals. Such information can not only throw light on the general metabolic problem but can also help in assigning proper value to observations made on man. The present experiments deal with the absorption and retention of ascorbic acid by the tissues of the guinea-pig and the bearing of this phenomenon on the development of scurvy.

TECHNIQUE

Experimental animals and diet. All animals employed in this investigation were young growing guinea-pigs weighing approximately 300 g. The basal scorbutic diet had the following composition (% by weight):

Bran	24
Barley meal	16
Weatings	21
Fishmeal	8
Oats (crushed)	26
Salt mixture	5

In addition each animal received reconstituted full cream milk which had been boiled for 1 hr. after the addition of

1 ml. 0.5% CuSO_4 /100 ml. of milk. This preliminary treatment of the milk was carried out since it was considered that the nature of the investigation required a basal diet as free as possible from vitamin C. On this diet, guinea-pigs increased in weight for about 14 days and then decreased rapidly. The first macroscopic lesions of scurvy appeared after c. 15 days and the animals succumbed to the disease in about 25 days.

Preparation and analysis of the tissues. The guinea-pigs were killed by stunning and bleeding and the blood was collected in oxalate. The tissues were then prepared for analysis as follows. Whole blood (5 ml.) was precipitated with 15 ml. 6% trichloroacetic acid. The liver, kidney and muscle samples and also the stomach and intestinal tissues, after removal of the contents and subsequent washing and drying the tissues with filter paper, were extracted twice with 2 parts by weight of 10% trichloroacetic acid and by grinding with sand and centrifuging. Measured portions of the resulting extracts (not more than 4 ml.) were diluted to 20 ml., the final trichloroacetic acid concentration being adjusted to 4%. The adrenal, spleen and marrow were extracted with 20 ml. 4% trichloroacetic acid. Since the amount of marrow obtained from one animal was small, the sample analyzed was taken from the femurs and tibias of three guinea-pigs killed at the same time. Leucocytes were obtained by a procedure similar to that employed by Kellie & Zilva (1938), the pooled white cells from six animals being used for each experiment. Eighteen hours before the guinea-pigs were killed, they were injected intraperitoneally with 30 ml. of meat infusion broth containing 1% peptone and 0.5% NaCl. The leucocytes thus obtained (yield, 0.5–0.8 g.) were extracted with 20 ml. 4% trichloroacetic acid. Ascorbic acid was then determined by the 2:4-dinitrophenylhydrazine method of Roe & Kuether (1943). Using this technique we have found that added ascorbic acid is recovered with an error of less than 5%. No interference

was detected when the 2:4-dinitrophenylhydrazine reaction was allowed to proceed for 15 min. at 25° instead of 3 hr. at 37°, i.e. the tissues did not contain any non-ascorbic acid substances described by us (Penney & Zilva, 1945) which are capable of simulating the reaction.

RESULTS

The gastric utilization of ascorbic acid by the guinea-pig

Guinea-pigs, unlike human beings, do not eliminate ascorbic acid in the urine when the vitamin is consumed orally unless the doses ingested are large (300–500 mg.). On the other hand the parenterally introduced vitamin is rapidly eliminated by guinea-pigs after very much lower doses (25–50 mg., cf. Zilva, 1935*a*, *b*; 1936). As the main object of this investigation was to facilitate the application of the present results to man, it was evident that some light had to be thrown on this different behaviour of these two species which are susceptible to scurvy and whose reaction to the disease is mostly very similar. It will be seen from the experiments to be described that the restricted capacity of the guinea-pig for absorption of ascorbic acid is partly responsible for this difference. A single dose of 25 mg., which is capable of saturating the tissues of vitamin C depleted animals when administered parenterally, failed to bring about saturation when given orally. The same total quantity of ascorbic acid administered by mouth in a number of small doses given at short intervals, led to a more efficient deposition in the tissues. The highest ascorbic acid concentration of the tissues was found to be attained when

average of three guinea-pigs in each group which were injected with single doses of 25 mg. and 14 mg., and the calculated values of the tissue concentration for theoretical saturation (to be discussed in a later section) are given as standards of comparison. All the animals were killed for analysis 15 hr. after the administration of the ascorbic acid. It will be seen that by administering 25 mg. of ascorbic acid orally in 0.5 mg. doses every 10 min., tissue concentrations were obtained which approximated those obtained by a single injection of 25 mg. or those obtained by calculation for theoretical saturation. On the other hand when 25 mg. were given orally in one dose the ascorbic acid tissue concentrations were less than half of the above standards and were even less than those obtained with a single injection of 14 mg. These results suggest that ascorbic acid above a certain low concentration is only partially absorbed during the time it remains in the guinea-pig stomach, where it is stable owing to the prevailing acidity. In the intestine conditions are unfavourable to the stability of the vitamin and, unless very large quantities are administered, in all probability little remains for absorption. In contradistinction to this, in man the gastric absorption of the vitamin is evidently much more rapid and efficient—for small or moderate doses it is probably almost as rapid as by injection. Consequently when in man the quantities of ascorbic acid which enter the blood stream by absorption from the stomach are well above the saturation dose some of it is quickly eliminated by the kidney as in the case of parenteral introduction.

Table 1. *Influence of oral and parenteral administration of ascorbic acid on the concentration in guinea-pig tissues*

Amount and method of administration		Blood	Liver	Kidney	Adrenal	Spleen	Stomach	Small intestine	Large intestine	Muscle	Marrow
		(mg./100 ml.)	(mg./100 g.)	(mg./100 g.)	(mg./100 g.)	(mg./100 g.)	(mg./100 g.)	(mg./100 g.)	(mg./100 g.)	(mg./100 g.)	(mg./100 g.)
25 mg. intramuscular injection	{Av.	0.36	9.1	5.2	72	33	8.3	14.9	9.5	2.7	9.1
	{S.D.	0.03	1.0	0.4	14	3	1.0	2.1	0.5	0.3	—
14 mg. intramuscular injection	{Av.	0.17	5.4	2.9	33	16	4.5	8.4	4.7	1.2	6.5
	{S.D.	0.01	0.4	0.3	11	4	0.9	1.8	0.7	0.1	—
25 mg. by mouth (0.5 mg. every 10 min.)	{Av.	0.30	8.8	6.0	50	30	8.0	14.0	8.6	2.7	9.5
	{S.D.	0.02	0.4	0.5	1	5	0.3	1.4	0.9	0.3	—
25 mg. by mouth (single dose)	{Av.	0.14	3.4	2.0	20	9.7	3.0	6.3	3.2	1.0	4.6
	{S.D.	0.02	0.5	0.1	5	1.2	0.7	0.7	0.4	0.1	—
Theoretical saturation value (calculated)		0.25	9.7	5.5	96	34	7.5	14.0	8.5	2.3	9.5

S.D. = standard deviation in this and subsequent tables.

doses of 0.5 mg. were given every 10 min. Table 1 shows the results of the final experiment. The guinea-pigs, three animals in each group, were depleted of the vitamin by maintenance on the scorbutic diet for 12 days before receiving the dose. The tissue concentrations of the animals, also the

The disappearance of ascorbic acid from the tissues of guinea-pigs subsisting on a scorbutic diet

In order to ascertain whether there was any definite relationship between the ascorbic acid content of the tissues and the development of

scurvy it was essential to obtain precise data regarding the decrease of the tissue ascorbic acid content of animals subsisting on scorbutic diet for various periods. The following experiments were therefore carried out: three groups of guinea-pigs, taken from stock and receiving a mixed diet, were subsequently maintained on the basal scorbutic diet. In addition the first group received cabbage *ad lib.* whilst the second and third groups were given oral doses of 25 and 50 mg. of ascorbic acid *per diem* respectively for six days preceding complete vitamin C depletion. During this preliminary period, group 2 therefore consumed the approximate oral minimum dose necessary to ensure saturation (Zilva, 1936) whilst groups 1 and 3 consumed much higher doses. The ascorbic acid content of the tissues was then determined after the animals had been maintained on the scorbutic diet alone for 1, 3, 8, 15 and 23 days respectively. As the results obtained in the three groups for the corresponding periods of vitamin C depletion were not significantly different, an average figure of all the results for each period of depletion is given in Table 2.

It will be seen that in all the tissues there is a rapid decrease in the ascorbic acid concentrations during the first eight days after which the loss becomes progressively smaller. In fact, when the $\log_{10}$ of the tissue concentration is plotted against the time of depletion the relationship is linear (Fig. 1). Furthermore the magnitude of the slopes of all the curves with the exception of those for blood and muscle are of the same order (0.07–0.08). It is also seen that at the stage of depletion when macroscopic scorbutic lesions usually appear on this basal diet and even in the premortal phase, ascorbic acid, as shown by the Roe & Kuether reaction, is still present. By the summation of the ascorbic acid contents of the above organs, it was found that when saturated a guinea-pig, weighing about 300 g., contained approximately 8.5 mg. of ascorbic acid, whilst after 8 days' depletion, when no macroscopic scorbutic lesions are to be observed, this value fell to 1.5–2.0 mg. After 15 days' depletion it was further reduced to 0.5 mg.; after 23 days traces of ascorbic acid could still be detected.

Attention must be drawn to the fact that in Fig. 1 the curves are extrapolated to zero time giving the

Table 2. *The disappearance of ascorbic acid from the tissues of initially saturated guinea-pigs subsisting on a scorbutic diet*

Days depletion ...	1	3	8	15	23
No. of animals ...	12	12	12	10	9
	(mg./ 100 g.)	(mg./ 100 g.)	(mg./ 100 g.)	(mg./ 100 g.)	(mg./ 100 g.)
Blood*	{ 0.26 S.D. 0.03	{ 0.20 0.05	{ 0.14 0.05	{ 0.10 0.02	{ 0.05 0.01
Liver	{ 9.9 S.D. 3.3	{ 5.4 1.1	{ 2.4 0.9	{ 0.63 0.10	{ 0.21 0.07
Kidney	{ 4.9 S.D. 1.0	{ 3.2 1.3	{ 1.3 0.3	{ 0.45 0.15	{ 0.14 0.04
Adrenal	{ 82 S.D. 24	{ 51 13	{ 20 10	{ 5.6 1.5	{ 1.3 0.3
Spleen	{ 29 S.D. 5.4	{ 19 10	{ 7.7 2.8	{ 1.6 0.5	{ 0.65 0.14
Stomach	{ 7.2 S.D. 1.2	{ 4.5 1.1	{ 2.0 0.9	{ 0.49 0.14	{ 0.18 0.06
Small intestine	{ 13.1 S.D. 3.2	{ 8.0 1.6	{ 3.0 0.7	{ 0.77 0.12	{ 0.28 0.07
Large intestine	{ 7.6 S.D. 2.0	{ 5.1 1.2	{ 2.1 0.7	{ 0.53 0.20	{ 0.19 0.06
Muscle	{ 2.2 S.D. 0.5	{ 1.5 0.6	{ 0.8 0.2	{ 0.33 0.13	{ 0.11 0.04
Marrow	{ 8.6 S.D. 0.7	{ 5.9 1.2	{ 2.4 0.5	{ 0.70 0.10	{ 0.21 0.07

* mg./100 ml.

true saturation values. In practice the initial values are very much higher as will be seen from Table 3 containing data obtained with guinea-pigs kept on a mixed diet which included cabbage *ad lib.* until just before analysis. These high values are due to the fact that quantities of ascorbic acid in the process of absorption from the food are still circulating in the blood stream and 'flush' the saturated tissues, raising for a short period their ascorbic acid concentration beyond the true saturation value. This was demonstrated by the following experiment. Ascorbic acid was determined in the blood of the following groups of animals: (1) guinea-pigs receiving a mixed diet containing cabbage *ad lib.* until killed for analysis; (2) after depleting such animals for 15 and 24 hr.; (3) after depleting for 15 and 24 hr. guinea-pigs previously maintained on a scorbutic diet and a daily dose of 25 or 50 mg.

Table 3. *A comparison of the tissue saturation values obtained experimentally for guinea-pigs on a mixed diet containing cabbage ad lib., with the calculated saturation values*

	Blood (mg./ 100 ml.)	Liver (mg./ 100 g.)	Kidney (mg./ 100 g.)	Adrenal (mg./ 100 g.)	Spleen (mg./ 100 g.)	Stomach (mg./ 100 g.)	Small intestine (mg./ 100 g.)	Large intestine (mg./ 100 g.)	Muscle (mg./ 100 g.)
Mixed diet { Av.	0.75	16.7	8.5	150	43	11.0	20.4	10.3	3.1
{ S.D.	0.07	2.7	1.2	12	3	2.9	3.3	1.2	0.6
Calculated saturation	0.25	9.7	5.5	96	34	7.5	14.0	8.5	2.3

Fig. 1. The disappearance of ascorbic acid from the tissues of initially saturated guinea-pigs subsisting on a scorbutic diet.

Table 4. *The influence on the blood ascorbic acid concentration of the intake and of the time after ingestion*

Ascorbic acid intake during the preliminary period	No. of guinea-pigs	Depletion (hr.)	Ascorbic acid concentration (mg./100 ml.)	Standard deviation (mg./100 ml.)
Cabbage <i>ad lib.</i>	8	0	0.61	0.12
Cabbage <i>ad lib.</i>	10	15	0.37	0.07
25 mg. ascorbic acid daily for 6 days	8	15	0.24	0.03
50 mg. ascorbic acid daily for 6 days	5	15	0.26	0.05
Cabbage <i>ad lib.</i>	10	24	0.25	0.05
25 mg. ascorbic acid daily for 6 days	9	24	0.26	0.06
50 mg. ascorbic acid daily for 6 days	9	24	0.27	0.05

ascorbic acid for six days. The results show (Table 4) that the average blood concentration of the animals receiving cabbage *ad lib.* and not previously depleted was 0.61 mg./100 ml. and that this decreased to 0.37 mg./100 ml. after 15 hr. depletion and 0.25 mg./100 ml. after 24 hr. depletion. The animals maintained on the daily doses of 25 and 50 mg. respectively for 6 days showed a value of this order even after 15 hr. depletion.* The results demonstrate the difficulty if not the impossibility of obtaining experimentally the true saturation value at zero time. In our opinion this can only be obtained with a fair degree of accuracy by extrapolation of the experimental depletion curve. Values obtained in this way were used by us as one of the standards for comparison in the experiments described in the preceding section.

Influence of intake on the ascorbic acid content of the guinea-pig tissues

The experiments described in this section are an extension and an elaboration of those already described by Zilva (1936). In the present experiments the doses investigated spread over a wide range and the use of Roe & Kuether's method instead of the direct indophenol titration procedure has enabled us to obtain results of greater precision. Daily doses of 0.5, 2.0, 6.0, 12.0 and 25 mg. of ascorbic acid were administered orally for 15 days to five groups of guinea-pigs, each consisting of three animals, existing on the scorbutic diet. These animals had previously received a mixed diet with cabbage *ad lib.* 24 hr. after the administration

* In a previous communication (Penney & Zilva, 1943b) it was reported that approximately 5 mg. of 2:3-diketogulonic acid per 100 ml. of plasma was obtained by the modified Penrose & Quastel reaction described by us (Penney & Zilva, 1943a) for animals depleted of vitamin C for 5 days. It was however pointed out at the time that the substance or substances which reacted were in all probability not diketogulonic acid. The very low blood ascorbic acid figures obtained in the present investigation showed definitely that it could not have been due to diketogulonic acid since the Roe & Kuether reaction is also given by this compound (Lloyd, Sinclair & Webster, 1945; Penney & Zilva, 1945).

of the last dose the guinea-pigs were killed and the tissues analyzed as previously described. In considering the results of these experiments (Table 5)

Table 5. *The influence of graded oral doses of ascorbic acid on the concentration in the tissues*

Dose (mg.)	...	0.5	2.0	6.0	12.0	25.0
		(mg./100 g.)	(mg./100 g.)	(mg./100 g.)	(mg./100 g.)	(mg./100 g.)
Blood*	{	0.10	0.13	0.14	0.20	0.22
	{S.D.	0.00	0.01	0.02	0.02	0.04
Liver	{	1.1	1.7	3.3	5.2	6.1
	{S.D.	0.1	0.5	0.8	1.7	1.3
Kidney	{	0.82	1.3	2.3	2.9	5.2
	{S.D.	0.04	0.1	0.4	0.3	2.1
Adrenal	{	10	21	32	34	65
	{S.D.	2	2	10	3	17
Spleen	{	3.8	9.3	11	16	25
	{S.D.	0.7	0.6	3	2	5
Stomach	{	0.84	1.7	3.0	3.9	5.7
	{S.D.	0.16	0.5	0.3	0.4	1.3
Small intestine	{	1.8	4.2	5.9	6.5	10.6
	{S.D.	0.2	0.7	1.3	1.3	2.5
Large intestine	{	1.1	2.2	3.8	4.1	6.1
	{S.D.	0.2	0.7	1.2	0.5	1.2
Muscle	{	0.44	0.44	1.0	1.0	1.3
	{S.D.	0.10	0.09	0.2	0.1	0.1
Marrow		2.1	3.2	6.6	6.9	8.9

* mg./100 ml.

it should be remembered that the ascorbic acid tissue contents are here a balanced effect of the depletion of the body and the deposition of the vitamin due to the absorption of the dose. It will be seen that the consumption of the daily dose of 0.5 mg. of ascorbic acid for 15 days raised the tissue concentration only very slightly as compared with the values after 15 days' depletion (cf. Table 2) and that a dose of 25 mg. brought up the concentration almost to the true theoretical saturation limit as obtained by calculation (cf. Table 3). These results are similar to those obtained by Zilva (1936) except that owing to the more sensitive method employed in these experiments it was found that a deposition of traces of ascorbic acid took place on the administration of doses lower than 5-8 mg. The higher

accuracy of these experiments has also enabled us to establish a mathematical relationship between the tissue concentration and the magnitude of the dose. It will be seen from Fig. 2 that a linear relationship exists between the $\log_{10}$ of the tissue concentration and the $\log_{10}$ of the corresponding dose for all the tissues and that the slopes of the curves, except those of the marrow, blood and leucocytes,

support to our observation that the capacity for absorbing higher oral doses by the guinea-pig is limited.

The more efficient absorption of ascorbic acid by the guinea-pig when introduced parenterally was also shown by the following experiment. Three groups of three saturated animals each, were placed on a scorbutic diet and 15 daily injections

Fig. 2. The influence of graded oral doses of ascorbic acid on the tissue concentration.

are of the same order, i.e. 0.43–0.48. The small difference in the slope of the marrow curve can be ascribed to imperfection of technique, but with blood the difference is significant and is probably due to the avidity of the other tissues under the prevailing experimental conditions. The ascorbic acid content of the leucocytes will be discussed in a later section. The slopes of the curves of all the tissues except the blood show that the tissue concentration is approximately proportional to the square root of the dose, and thus offers further

of 6, 9 and 12 mg. of ascorbic acid respectively, dissolved immediately before administration, were delivered intraperitoneally and intramuscularly into each of the four limbs consecutively. All the animals were killed 24 hr. after the injection of the last dose and analyzed as usual. It will be seen from Table 6 that a daily dose of only 6–9 mg. given parenterally will maintain saturation, whilst when the vitamin is given orally a daily dose of 25 mg. is necessary to attain this level of tissue saturation.

Table 6. *The influence of graded doses of ascorbic acid administered parenterally on the concentration in the tissues*

Ascorbic acid administered (mg.)	Blood (mg./100 ml.)	Liver (mg./100 g.)	Kidney (mg./100 g.)	Adrenal (mg./100 g.)	Spleen (mg./100 g.)	Stomach (mg./100 g.)	Small intestine (mg./100 g.)	Large intestine (mg./100 g.)	Muscle (mg./100 g.)	Marrow (mg./100 g.)
12 {	0.39	11.6	6.7	105	31.5	11.0	16.2	9.2	2.7	12.5
{ S.D.	0.04	0.5	0.4	15	2.2	0.3	2.4	0.4	0.3	—
9 {	0.33	12.3	6.3	102	31.0	9.7	16.6	8.7	2.8	12.0
{ S.D.	0.10	1.4	1.1	16	1.5	0.7	1.9	1.1	0.0	—
6 {	0.22	6.3	4.0	59	20.0	5.9	9.2	5.0	1.8	7.7
{ S.D.	0.02	0.8	0.4	11	0.7	0.7	0.6	0.3	0.2	—

Ascorbic acid tissue content and the development of scurvy in the guinea-pig

It has previously been shown by Zilva (1936) that saturation of the tissues does not prolong the period of survival of guinea-pigs on a scorbutic diet. It was thus found that the time taken by the animals to succumb to scurvy on a scorbutic diet was independent of their pre-experimental intake of vitamin C providing it exceeded 1 mg./day. Even when the experimental animals had received cabbage *ad lib.*, the preliminary diet did not delay the mortal termination of the disease. In view of the fact that the scorbutic diet employed in this investigation was more drastically depleted of vitamin C than the one used before, it was decided to repeat the above experiment. Daily ascorbic acid doses of 0.5, 1.0, 2.0, 3.0 and 4.0 mg. were given orally to five groups of guinea-pigs, each group containing four animals, maintained on a scorbutic diet for 10 days preceding complete vitamin C depletion. As previously found, no significant difference could be observed in the time of survival of any of the groups when compared with an initially saturated control group, except in the set receiving 0.5 mg. of ascorbic acid which succumbed somewhat earlier.

In view of these observations it was desirable to compare the tissue ascorbic acid concentrations of guinea-pigs which had existed on a scorbutic diet for various periods after receiving a daily dose of 2 mg. of ascorbic acid in the pre-experimental stage with those obtained by us for the tissues of animals depleted after saturation. These analytical data were obtained in an experiment in which the tissues of three groups of guinea-pigs, each consisting of three animals and receiving the above dose for 10 days before vitamin C depletion, were analyzed 1, 8 and 15 days after the last dose. These results are given in Table 7 and their significance is brought out clearly by comparing graphically (Fig. 3) the curves obtained here with those obtained in the vitamin C depletion experiments on saturated guinea-pigs. It is readily seen that the ascorbic acid concentrations of the tissues after 1, 8 and 15 days' complete vitamin C depletion in this experiment correspond approximately to the tissue concentrations obtained by

depleting saturated animals for 8, 15 and 23 days respectively. In spite of the similarity of the ascorbic acid tissue concentrations of the partially saturated guinea-pigs kept on a scorbutic diet for 8 days and those of the fully saturated animals treated similarly for 15 days, no macroscopic scorbutic lesions could be observed at the post-mortem examination of the former whilst 23 out of 26 of the latter showed marked signs of scurvy at autopsy. The ascorbic acid content of the tissues therefore seems to bear no definite relationship to the onset of well-declared scurvy any more than the high ascorbic acid content of the tissues of saturated animals has any influence on prolonging the time of survival of guinea-pigs placed on a scorbutic diet. Further experimental evidence supporting this view will be produced in a subsequent section.

Ascorbic acid content of the tissues of guinea-pigs existing for 3 months on a low intermittent intake

It was of further interest to establish the ascorbic acid content of the tissues of guinea-pigs subsisting on a scorbutic diet and receiving intermittent doses of vitamin C. Zilva (1941) showed that guinea-pigs kept on a scorbutic diet and receiving a weekly oral dose of 35 mg. grew well and when killed after 90 days showed no macroscopic signs of scurvy. This experiment was repeated utilizing the scorbutic basal diet described above which contained even smaller traces of vitamin C than the diet used in the original experiments. Two groups of animals each consisting of three guinea-pigs were killed after 84 days, the first group receiving its last dose 7 days and the second group 24 hr. before death. Table 8 shows that the ascorbic acid concentration of the tissues of the animals killed 7 days after the last dose corresponds to that observed by us in animals which had been depleted for 15–16 days (cf. Table 5). Administration of the last dose 24 hr. before death, yielded higher ascorbic acid tissue concentrations corresponding to those of animals depleted for only about 11 days (cf. Table 5). Of the 18 guinea-pigs used in this and another experiment to be discussed later only in one was any indication found which could be considered as a doubtful sign of scurvy, the remaining animals being free from macroscopic

Table 7. *The disappearance of ascorbic acid from the tissues of partially saturated guinea-pigs*

Days depletion	Blood (mg./ 100 ml.)	Liver (mg./ 100 g.)	Kidney (mg./ 100 g.)	Adrenal (mg./ 100 g.)	Spleen (mg./ 100 g.)	Stomach (mg./ 100 g.)	Small intestine (mg./ 100 g.)	Large intestine (mg./ 100 g.)	Muscle (mg./ 100 g.)	Marrow (mg./ 100 g.)
1	{Av. 0.13 {S.D. 0.01	2.5 0.5	1.5 0.2	19 8	8.8 2.5	2.6 0.5	4.8 0.7	2.3 0.3	0.68 0.07	3.7 —
8	{Av. 0.09 {S.D. 0.01	0.71 0.05	0.40 0.06	5.3 1.4	2.1 0.3	0.45 0.10	0.80 0.31	0.45 0.10	0.20 0.02	0.85 —
15	{Av. 0.05 {S.D. 0.01	0.31 0.03	0.14 0.00	0.87 0.11	0.50 0.00	0.14 0.00	0.30 0.02	0.15 0.04	0.07 0.01	0.25 —

Fig. 3. The comparison of the disappearance of ascorbic acid from the tissues of saturated (A) and partially saturated (B) guinea-pigs. 1, small intestine; 2, liver; 3, marrow; 4, large intestine; 5, stomach; 6, kidney; 7, muscle; 8, adrenal; 9, spleen.

Table 8. *The tissue concentrations of guinea-pigs receiving intermittent oral doses of ascorbic acid*

Time after last dose	Blood (mg./ 100 ml.)	Liver (mg./ 100 g.)	Kidney (mg./ 100 g.)	Adrenal (mg./ 100 g.)	Spleen (mg./ 100 g.)	Stomach (mg./ 100 g.)	Small intestine (mg./ 100 g.)	Large intestine (mg./ 100 g.)	Muscle (mg./ 100 g.)	Marrow (mg./ 100 g.)
7 days	{Av. 0.07 {S.D. 0.01	0.83 0.09	0.49 0.09	5.9 2.2	3.1 0.9	0.53 0.04	1.3 0.4	0.58 0.14	0.20 0.08	1.8 —
24 hr.	{Av. 0.09 {S.D. 0.01	1.9 0.3	1.5 0.1	12 0	6.5 0.2	1.9 0.1	2.7 0.4	1.5 0.3	0.43 0.06	2.2 —

signs of scurvy. On the other hand as has been already mentioned, of the 26 animals on the scorbutic diet not receiving any vitamin C for 15 days and showing a similar concentration in the tissues, in only three were no macroscopic lesions found at the post-mortem examination. This experiment therefore shows that the ascorbic acid concentration of the tissues bears no definite relationship to the onset of well-declared scurvy.

rapidly during the first 8 days and then reached a value which remained constant until the premortal stage. In this investigation more precise results were obtained by the Roe & Kuether method. After the initial loss during the first 10 days or so the ascorbic acid content of the cells remained constant until the twenty-second day when the experiment was terminated (Fig. 4). A comparison of the $\log_{10}$ ascorbic acid concentration/time of

Fig. 4. The ascorbic acid content of the guinea-pig leucocytes. $\odot$ — $\odot$ The depletion of ascorbic acid. $\odot$ — $\odot$ $\log_{10}$ of the depletion values. --- $\log_{10}$ of the small intestine ascorbic acid depletion values. Arrows on the depletion curve indicate the tissue concentration values observed under various conditions of ascorbic acid administration. Daily oral administration for 15 days. A, 0.5 mg.; B, 2 mg.; C, 6 mg.; D, 12 mg.; E, 25 mg. Single dose after 12 days' depletion. F, 50 mg. parenterally; G, 14 mg. parenterally; H, 50 mg. *per os*; I, 14 mg. *per os*.

The influence of ascorbic acid intake on the ascorbic acid content of guinea-pig leucocytes

Owing to the outstanding difference found between the behaviour of the leucocytes and of the other tissues with respect to ascorbic acid saturation and retention, the results are considered separately. Kellie & Zilva (1938) observed that the indophenol reducing capacity of leucocytes from guinea-pigs kept on a scorbutic diet decreased

ascorbic acid depletion curve plotted for leucocytes and for a typical tissue (small intestine) clearly shows the marked difference in the leucocytes during the depletion (Fig. 4). For the initial stages of the depletion the $\log_{10}$ curves are nearly parallel. After about 8 days however the slope of the leucocyte curve, unlike that of the other tissue, decreased and became zero as the leucocyte ascorbic acid concentration reached a constant value. In spite of this characteristic difference in vitamin C depletion

it would appear that the ascorbic acid content of the leucocytes, like that of the other tissues, cannot serve as an indication for the impending development of scurvy. This conclusion is further supported by the results of an experiment in which 35 mg. of ascorbic acid were administered weekly by mouth to 12 guinea-pigs which had been maintained on a scorbutic diet for 12 weeks. The ascorbic acid concentration of the leucocytes of these animals both 24 hr. and 7 days after the administration of the last dose, was only 0.8 mg./100 g., a value which falls on the horizontal section of the curve; yet post-mortem macroscopic signs of scurvy were absent in all the animals. Attention is also drawn to the experiment already described in which guinea-pigs received a daily oral dose of 2 mg. for 10 days before being placed on a scorbutic diet, when the initial ascorbic acid concentration of the tissues other than that of the leucocytes (not determined) corresponded in this case to the value obtained with saturated guinea-pigs depleted for about 8 days (Tables 5 and 7). As the $\log_{10}$ ascorbic acid/time of depletion curve of the leucocytes runs roughly parallel to that of the other tissues for the first 8 days, it may be deduced that in the case of the leucocytes the approximate initial value of the ascorbic acid concentration, after receiving a daily oral dose of 2 mg. for 10 days during the preliminary period, corresponds also to that obtained after 8 days' depletion or is probably even less owing to the smaller ascorbic acid capacity of the leucocytes. Such a value would almost correspond to a point on the horizontal section of the curve. In spite of this low initial ascorbic acid content of the leucocytes, the animals did not succumb to scurvy earlier than those which were saturated when the ascorbic acid was removed from the diet.

From other experiments on leucocytes several points of interest emerge of which the physiological significance cannot be fully explained at present. As already mentioned, when graded doses were administered orally, a linear relationship between $\log_{10}$ tissue ascorbic acid concentration and $\log_{10}$ dose was found, but the leucocyte curve had a smaller slope (Fig. 2). In other words, for the same blood ascorbic acid concentration the vitamin is taken up more readily by the other tissues. Similarly, when 50 mg. ascorbic acid was injected intramuscularly into the guinea-pigs maintained on a scorbutic diet for 12 days, the ascorbic acid content of the leucocytes was found to correspond roughly only to that obtained in the case of guinea-pigs maintained on a scorbutic diet for about 3 days (Fig. 4), whilst the other tissues under these circumstances were saturated. This confirms an earlier observation (Kellie & Zilva, 1938). On the other hand the leucocyte concentration after an intramuscular injection of 14 mg. corresponded to

that of the other tissues, being equivalent to that after 4 or 5 days' vitamin C depletion, and this was also the case when a single dose of 50 or 14 mg. of ascorbic acid was administered by mouth.

DISCUSSION

The experiments described offer an opportunity of obtaining a broad view of the bearing of saturation and of the tissue ascorbic acid content in general on the development of scurvy in the guinea-pig.

At the outset it is necessary to consider the specificity of the Roe & Kuether method. We have shown (Penney & Zilva, 1945) that certain substances can interfere in the determination and that this can be partially rectified if the reaction is carried out for 15 min. at 25° instead of for 3 hr. at 37°. No evidence of such interference was found to exist in any of the above experiments. The only known substances, the presence of which could have vitiated the results, were therefore compounds such as dehydroascorbic and diketogulonic acids and the analogues of ascorbic acid (cf. Penney & Zilva, 1945), but the current evidence is against their presence in tissues. There still remains the possibility of an unknown substance or substances capable of simulating the reaction. Our evidence however lends little support to this view, since the kinetics of the filling and depletion of most of the tissues suggested that a single substance, which could only be *l*-ascorbic acid, was involved in the process, dehydroascorbic and diketogulonic acids and the analogues being excluded on theoretical grounds. This argument does not apply however to the leucocytes since it was shown that although the loss during the first 8–10 days followed a similar course to that of the other tissues, after that time a residue remained which did not disappear with depletion even to the premortal phase. The residue is an indophenol reducing substance (Kellie & Zilva, 1938). This different behaviour therefore suggests that either a portion of the *l*-ascorbic acid of the leucocytes is held more retentively than in any other tissue so far investigated or that the residual indophenol reducing substance is not identical with *l*-ascorbic acid or any of the known substances capable of reacting like *l*-ascorbic acid.

With regard to the influence of the oral consumption of *l*-ascorbic acid on its deposition in the tissues of the guinea-pig, the results show beyond doubt that the restricted absorption of the vitamin in the stomach of this animal acts as a factor limiting its deposition in the tissues. Only when a dose was given orally in portions of 0.5–1.0 mg. at intervals of 10 min. was the deposition found to be as efficient as when the same total amount was administered as a single dose parenterally. The oral daily administration of doses to saturated

guinea-pigs led to a deposition in quantities which were proportional to the square root of the dose. This relationship, it must be noted, holds when the vitamin is given in solution. It is doubtful whether it would apply where the vitamin is consumed in solid foodstuffs such as vegetables. In these circumstances the food remains longer in the stomach and consequently the ascorbic acid would be released gradually. The chief point to bear in mind is that the gastric absorption of ascorbic acid in man is much more rapid and efficient than in the guinea-pig and under certain circumstances almost as rapid and efficient as when introduced parenterally. This fact must be considered when any attempt is made to apply the results obtained with guinea-pigs to man and it further suggests that under ordinary circumstances little can be gained in man by parenteral medication.

The experiments concerning the disappearance of ascorbic acid from the tissues of the guinea-pig maintained on a scorbutic diet have shed a new light on the process of the retention of the vitamin and consequently on the process of saturation. It was seen that the disappearance from all the tissues examined followed an exponential rule and, with the exception of the blood, the slopes of all the curves derived were similar. The initial vitamin values obtained were, however, always found to be higher than those obtained by extrapolating the curves ($\log_{10}$ content against time) to zero time (Fig. 1 and Table 3). This was demonstrated to be due to the 'flushing' of the saturated tissues by an excess of ascorbic acid circulating in the blood soon after the absorption of considerable quantities. Thus it was found that the blood ascorbic acid content fell to 0.25 mg./100 ml., the value obtained by extrapolation to zero time after 24 hr. depletion in the case of guinea-pigs previously receiving cabbage *ad lib.* and after 15 hr. in the case of animals receiving a daily dose of 25 or 50 mg. of ascorbic acid. At this blood concentration, equilibrium is reached between blood and tissue ascorbic acid. During the period of 'supersaturation', the mode of retention of the excess ascorbic acid by the tissues differs from that by which ascorbic acid is fixed in the process of saturation, since this excess disappears very rapidly and therefore masks the true loss from the tissues during the first 24 hr. or so. Once the excess is eliminated, depletion proceeds exponentially. Hence during the first few days of ascorbic acid depletion, the ascorbic acid concentration of the tissues falls considerably and reaches low values some time before scurvy manifests itself. As the disease develops when the curve is reaching asymptotic values of tissue ascorbic acid concentration for long periods of depletion (Fig. 3), it is evident that it would not be easy to utilize with any degree of reliance the above low values as an indica-

tion of a prescorbutic condition. The interpretation of such results would be further complicated by the fact, indicated by the present work, that there is no definite correlation between the tissue content and the onset of scorbutic lesions. In addition it must be noted that, as far as our present technique shows, the time of survival of initially saturated guinea-pigs when existing on a scorbutic diet is no longer than that of animals whose initial tissue concentration was about half of the saturation value. This fact suggests that at least the fraction of the tissue ascorbic acid lost in the initial stages of depletion does not act as a store of ascorbic acid for the protection against scurvy.

In view of our observation that the tissues of the guinea-pig can be 'supersaturated' by excess of ascorbic acid circulating in the blood after the absorption of considerable quantities of ascorbic acid from the stomach, it may be questioned whether the first signs of a continuous elimination of the vitamin by the kidney of man is the result of true saturation of the organism. We are inclined to believe that it is due to 'supersaturation', especially as the gastric absorption of the vitamin is much more efficient in man, and that the dose actually necessary to saturate a human being is much less than that usually ascertained by the urinary excretion technique. The elimination of ascorbic acid by the kidney is in all probability the extreme expression of overdosage.

SUMMARY

1. Oral doses of solutions of ascorbic acid above a certain limit (0.5–1.0 mg.) are only partially absorbed by the guinea-pig.
2. The daily oral administration of doses of ascorbic acid to initially saturated guinea-pigs on a scorbutic diet led after 15 days to a deposition in all the tissues except the leucocytes of quantities which were proportional to the square root of the dose.
3. The disappearance of ascorbic acid from the tissues of saturated guinea-pigs kept on a scorbutic diet follows an exponential rule and the slopes of most of the depletion curves are similar. When these curves are extrapolated to zero time, saturation values are obtained which are lower than the initial values usually observed by direct experiment. This is shown to be due to the fact that the saturated tissues are 'flushed' by an excess of ascorbic acid circulating in the blood while it is still in the process of absorption soon after the withdrawal of the vitamin from the diet. The mode of retention by the tissues of this excess of ascorbic acid is different to that of the fixation of the vitamin during the saturation process.
4. The loss of ascorbic acid from the leucocytes of guinea-pigs kept on a scorbutic diet follows a

course similar to that of the other tissues during the first 8–10 days, but after that time until the pre-mortal stage a residue persists which gives the Roe & Kuether reaction and which reduces indophenol.

5. Scurvy develops in guinea-pigs when the curve of the ascorbic acid tissue concentration reaches asymptotic values for long periods of depletion, and there is no definite correlation between the tissue content and the appearance of scorbutic lesions.

6. A high proportion of the ascorbic acid which is fixed by the tissues of saturated guinea-pigs and lost during the first few days of depletion on a scorbutic diet does not act as a store of vitamin C for protection against scurvy.

7. It is concluded that the first appearance of a

continuous elimination of ascorbic acid by the kidney of man is a result of 'supersaturation', the true saturation dose being lower than that ascertained by observation of urinary excretion, and consequently the elimination of ascorbic acid by the kidney is in all probability the extreme expression of overdosage. It is also suggested that, owing to the highly efficient gastric absorption of ascorbic acid by man, no advantage is gained under ordinary circumstances by parenteral rather than oral medication.

8. The specificity of the Roe & Kuether method as applied in this investigation is discussed.

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Spectrophotometric Study of the Excretion Products of Mepacrine compared with Synthetic Acridine and Diphenylamine Derivatives

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In the present investigation an attempt has been made to characterize some of the excretion products of mepacrine in human urine obtained from subjects on continuous mepacrine therapy. Scudi & Jelinek (1944) isolated four main fractions from dog urine. One of these they identified as unchanged mepacrine by chemical and spectrophotometric means, and they attributed acridine structures to the other three.

Extraction of large volumes of human urine with ethylene dichloride, and subsequent purification and chromatographic distribution, has yielded seven fractions which have been characterized spectrophotometrically. The general plan of fractionation is shown in Fig. 1. Some clue as to structure has been obtained by comparing the absorption curves of these purified fractions with those obtained from synthetically prepared acridine derivatives and related substances of known structure. The absorption spectra of the latter are also of interest from the point of view of acridine chemistry.

EXPERIMENTAL

Extraction of urine (Fig. 1). Large volumes of combined samples of urine from military personnel on a mepacrine regime were used. They were extracted in several lots of about 10 l. each, as follows. The urine was brought to pH 10 by addition of NaOH, and was shaken thoroughly with 2.5 l. of pure ethylene dichloride, allowed to separate, and the yellow ethylene dichloride layer removed. The residue was extracted with a mixture of 1 l. benzene, 500 ml. light petroleum and 500 ml. ether. This extract was only very slightly yellow and was not investigated further.

The ethylene dichloride extract was washed with water, centrifuging to break the gel, and the water discarded. It was then washed twice with 2.5*N*-NaOH, and twice with water. These aqueous alkaline washings were combined and designated the NaOH Fraction.

The remaining ethylene dichloride extract was shaken with three lots of 0.5*N*-HCl, which removed all its colour; the organic layer was then discarded. The acid solution was washed twice with ether to remove traces of ethylene dichloride, and the ether discarded. It was brought to pH 8.4–8.8 with NaOH and extracted with benzene. It was