The Influence of L-Ascorbic Acid on the Disappearance of the Phenolic Group of L-Tyrosine in the Presence of Guinea Pig-Liver Suspensions

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It is now well established that when high doses of L-tyrosine are consumed by guinea pigs or men, the phenolic group in the amino-acid is less readily metabolized if they subsist on a diet low in L-ascorbic acid than if a diet containing sufficient of this compound to saturate the tissues is offered (cf. Sealock & Silberstein, 1940; Levine, Marples & Gordon, 1941*a, b*). Evidence has, however, been produced (Painter & Zilva, 1947, 1948) which justifies the conclusion that this interaction between L-ascorbic acid and L-tyrosine is a physiological response to an unusual situation and has no important bearing on normal nutrition. The part played by L-ascorbic acid is, nevertheless, of biochemical interest, and the *in vitro* experiments described in this communication throw some light on the problem.

Pioneer work on the degradation of the molecule of L-tyrosine in the presence of animal tissue has been performed by Bernheim & Bernheim (1934), Krebs (1935) and Felix, Zorn & Dirr-Kaltenbach (1937). In these investigations the oxygen absorption and the evolution of ammonia techniques were employed. In the present work, as we were entirely concerned with the metabolism of the phenolic group of the molecule, we limited ourselves to the use of the Millon reaction only.

Darby, DeMeio, Bernheim & Bernheim (1945) were unable to establish any difference in the rate of disappearance of the phenolic group of L-tyrosine when incubated with liver tissue from normal and from scorbutic guinea pigs for 4 hr. As will be seen from the following experiments, under our experimental conditions we had no difficulty in demonstrating that such a difference does exist, and we were in consequence in a position to investigate the problem in some detail.

EXPERIMENTAL

Technique

Experimental animals and diet. All animals employed in this investigation were healthy young growing guinea pigs weighing approx. 300 g. The vitamin C-saturated animals were maintained on a diet of oats, bran and cabbage *ad lib.* and are referred to in text as normal or saturated animals.

All the deficient animals received a scorbutic diet (Penney & Zilva, 1946) for 21 days.

Preparation of liver suspensions. The guinea pigs were killed by stunning and bleeding. The liver was removed and ground thoroughly in a mortar with a small quantity of sand according to the procedure of Bernheim & Bernheim (1934). Usually the livers of two animals were pooled. After adding the requisite quantity of 0.05 M- $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$ buffer (pH 7.8), the mixture was further ground and then squeezed through fine muslin. The resulting suspension, which was readily transferable by means of a pipette, was used as soon as possible after preparation. Felix *et al.* (1937), Felix & Zorn (1941) and Sealock & Goodland (1949) suggest that in order to stabilize the system more completely the concentration of the buffer should be 0.2 M. Experiments in which parts of the same livers were ground with 0.05 and 0.2 M-buffers yielded in our hands similar results, and we therefore employed the former concentration.

Determination of hydroxyphenyl compounds. This was carried out by means of the Folin and Ciocalteu method (Painter & Zilva, 1947), except that a photoelectric instead of a visual colorimeter was employed in this investigation. L-Ascorbic acid did not appreciably influence the colour production unless the quantities added exceeded 10 mg./flask when the colour tended to be low.

Method of incubation. Liver suspension (2 ml.) was pipetted into 25 ml. conical flasks which also contained 1 ml. of buffer or 1 ml. of a buffered solution of L-ascorbic acid according to requirements. A buffered solution (1 ml.) containing 2 mg. of L-tyrosine or an amount of glycyl-L-tyrosine which gave a colour value equivalent to that of the tyrosine was then added to each flask, except the control flasks. Duplicate flasks were used in each experiment. The unstoppered flasks were shaken 80 times/min. with an amplitude of 12 cm. in a bath at 37.5°. At the requisite time the reaction was stopped by the addition of 4 ml. of 5% (w/v) HPO_3 , after which 1 ml. of the L-tyrosine or dipeptide solution was added to the control flasks. After centrifuging the contents of the flasks, the hydroxyphenyl compounds were determined using 0.5 ml. for the test.

Preliminary experiments have shown that the optimum activity lies between pH 7.3 and 7.5, an observation which is in agreement with that made by Bernheim & Bernheim (1934) and by Sealock & Goodland (1949). We therefore maintained our reaction media at pH 7.3-7.5.

As it was found that the variation of the individual activity values about the mean was quite large even when the 0.2 M-buffer was used (cf. Sealock & Goodland, 1949), we employed experimental groups which contained six or more animals.

L-Tyrosine and glycyl-*L*-tyrosine. See Painter & Zilva (1947).

L-Ascorbic acid. Kindly supplied by Roche Products Ltd. Solutions of the acid were prepared immediately before use.

D-Glucoascorbic acid. Sample kindly presented by Prof. E. L. Hirst. Brownish amorphous powder, purity approx. 85%.

RESULTS

The disappearance of the phenolic group of L-tyrosine and glycyl-L-tyrosine in the presence of suspensions of normal and of scorbutic guinea pig livers

We found that the diminution of the phenolic reaction during the first hour of incubation could not be accurately differentiated between the two groups by our technique, owing no doubt to the small quantity of *L*-tyrosine degraded during this period and we therefore omit these figures. Fig. 1 gives the

Fig. 1. The rate of disappearance of the phenolic group of *L*-tyrosine in the presence of suspensions of guinea pig livers; $\times$ — $\times$, livers from normal animals; $\circ$ — $\circ$, livers from scorbutic animals; each point represents the mean value of ten pairs.

results obtained after periods varying from 1 to 10 hr. The percentage of *L*-tyrosine which had disappeared after 3 hr., when normal livers were used, was 72 (S.D. 10.8), and in the case of scorbutic livers the percentage was 58 (S.D. 9.5). Combined results of experiments in which the liver suspensions from a larger number of animals, namely forty-six pairs of normal and forty-eight pairs of scorbutic guinea pigs, was used showed that after 3 hr. incubation figures of the same order as above were obtained (68, S.D. 10.5 and 54, S.D. 11.8, respectively). By

using Student's *t* test, the differences were shown to be statistically significant ($P < 0.01$ in the former set of experiments and < 0.001 in the latter). Similarly, the differences observed between the two groups after longer periods of incubation (Fig. 1) were also found to be significant. Thus it is perfectly plain that the phenolic group disappeared at an appreciably higher rate in the presence of liver suspensions originating from the normal saturated animals than from scorbutic animals, the tissues of which were almost completely devoid of *L*-ascorbic acid. In both cases the logarithmic shape of the curves was similar to that obtained by Bernheim & Bernheim (1934) and by Felix *et al.* (1937), who studied the absorption of oxygen by liver suspensions from normal animals only. It may be noted that even with such suspensions the reaction proceeds comparatively slowly, which is also in consonance with the results obtained by the above workers. Lan & Sealock (1944) have observed that while liver slices from normal guinea pigs absorbed extra oxygen in the presence of *L*-tyrosine the same tissue from scorbutic animals did not. This apparent deviation from our results is in our opinion due to the difference in technique employed in the respective investigations. This view is strengthened by the observation made later by Sealock, Goodland & White (1947) that the oxygen uptake by liver suspensions from scorbutic guinea pigs is influenced by the ratio of liver tissue to the substrate in the reacting medium more than when liver suspensions from normal animals are used. Further, it may also be pointed out that our tests did not measure the total oxidation of the *L*-tyrosine molecule, but indicated only the disappearance of the phenolic group.

We also found that when glycyl-*L*-tyrosine was used as the substrate the phenolic group, as in the case of *L*-tyrosine, disappeared after 3 hr. to a greater extent when suspensions of livers from normal animals were used. The metabolism of this substrate after longer periods was not investigated.

The disappearance of the phenolic group of L-tyrosine in the presence of suspensions of livers from scorbutic guinea pigs injected with L-ascorbic acid

This experiment was instituted in order to ascertain whether the higher rate of the disappearance of the phenolic group in the presence of suspensions of livers from normal animals was due to their higher content of *L*-ascorbic acid *per se*. For this purpose a number of guinea pigs were placed on a scorbutic diet for 21 days. They were injected intraperitoneally with 25 mg. of partially neutralized *L*-ascorbic acid 18 hr. before being killed. After this treatment such guinea pigs usually become saturated, whilst the quantity of *L*-ascorbic acid circulating in the system is not unduly high (Penney & Zilva, 1946). On the other hand, the macroscopic post-mortem picture in

such instances does not visibly differ from that observed in animals kept for 21 days on a scorbutic diet which were not previously treated with L-ascorbic acid. This post-mortem picture was observed in all our animals. Table 1 shows the L-ascorbic acid content of the liver and the percentage

normal animals were used, except that here no statistical significance was observed until 2.5 mg. of L-ascorbic acid were added, the percentage being raised from 65 (S.D. 9.7) to 78 (S.D. 11.7) ($P < 0.05$). Nevertheless, beyond a certain limit of concentration, namely when 2.5 mg. were added, the L-ascorbic

Table 1. *The disappearance of the phenolic group of L-tyrosine after incubation for 3 hr. in the presence of suspensions of livers from scorbutic guinea pigs injected with 25 mg. of L-ascorbic acid 18 hr. before death*

L-Ascorbic acid content of liver (mg./100 g.)	Individual values								Mean	S.D.
	12.9	12.8	9.1	18.8	11.3	12.7	16.7	15.1		
Percentage of L-tyrosine disappeared	70	93	66	50	76	69	73	60	68	12.6

of L-tyrosine metabolized in 3 hr. For the determination of L-ascorbic acid a portion of the liver suspension was treated with trichloroacetic acid, centrifuged and analysed by the modified Roe and Kuether method (cf. Penney & Zilva, 1945). It will be seen from the figures that the L-tyrosine was metabolized to the same extent as that observed in the case of normal guinea pigs (mean of injected animals was 68 (S.D. 12.6); the mean of the normal animals was 68 (S.D. 10.5). Furthermore, there is a significant difference between the mean value obtained in this experiment and that found previously when livers from scorbutic animals were used (i.e. 54, S.D. 11.8, $P < 0.01$). The result of this experiment therefore suggests that the presence of L-ascorbic acid in the liver is responsible directly for the acceleration in the rate of the metabolism of L-tyrosine. Subsequent experiments will offer further support to this view.

The influence of graded quantities of L-ascorbic acid on the disappearance of the phenolic group of L-tyrosine and glycyl-L-tyrosine in the presence of suspensions of livers from normal and scorbutic guinea pigs

As the presence of L-ascorbic acid in the guinea pig-liver tissue is capable of accelerating the rate of metabolism of L-tyrosine it was of interest to establish to what extent the acceleration of the reaction was influenced by the quantity of L-ascorbic acid present. The influence of the addition of quantities ranging from 0 to 10 mg. to the reaction mixtures was therefore investigated after 3 hr. incubation. Control flasks containing L-tyrosine and L-ascorbic acid showed that the substrate remains unaltered in the absence of liver suspensions. The results are given in Fig. 2. As was to be expected, the addition of L-ascorbic acid to the suspensions of livers from scorbutic animals accelerated the reaction. It was found, for example, that when 1 mg. was added the percentage of L-tyrosine that had disappeared was raised from 50 (S.D. 12.2) to 66 (S.D. 11.7) the difference between the two values being significant ($P < 0.05$). This was also the case when livers of

acid not only failed to exercise any further accelerating action, but seemed to inhibit somewhat the rate at which the phenolic group of the substrate disappeared. It is also to be noticed that as much as

Fig. 2. The influence of graded amounts of L-ascorbic acid on the disappearance of the phenolic group of L-tyrosine in the presence of suspensions of guinea pig livers after 3 hr. incubation; x—x, livers from normal animals; each point represents the mean value of seven pairs; o—o, livers from scorbutic animals; each point represents the mean value of seven pairs.

1 mg. of L-ascorbic acid had to be added to the liver suspensions from the scorbutic animals in order to attain the same degree of L-tyrosine degradation after 3 hr. incubation as that observed with liver

suspensions from normal animals without the addition of L-ascorbic acid. This latter reaction mixture contained only about 0.2 mg. of L-ascorbic acid all of which originated from the liver tissue.

The above experiments were repeated with glycyl-L-tyrosine as the substrate, and results very similar to those obtained with L-tyrosine as substrate were observed except that the initial values were lower.

Fig. 3. The effect of 2.5 mg. of L-ascorbic acid on the rate of disappearance of the phenolic group of L-tyrosine in the presence of suspensions of livers from scorbutic guinea pigs; x—x, no L-ascorbic acid added; each point represents the mean value of seven pairs; o—o, L-ascorbic acid (2.5 mg.) added; each point represents the mean value of seven pairs.

Fig. 3 shows the rate at which the phenolic group of L-tyrosine disappeared after the addition of the optimum amount of 2.5 mg. of L-ascorbic acid to liver suspensions from scorbutic guinea pigs. It will be seen that the curve obtained is almost identical in shape with that found when liver suspensions from normal animals were used (Fig. 1). In this connexion it is of interest to mention that Lan & Sealock (1944), using slices of livers from scorbutic guinea pigs and L-tyrosine as substrate, found an increase in the oxygen uptake by adding 0.3 mg. of L-ascorbic acid to the reaction mixture.

The influence of D-glucoscorbic acid on the disappearance of the phenolic group of L-tyrosine in the presence of suspensions of livers from normal and scorbutic guinea pigs

As it was probable that the influence of L-ascorbic acid in accelerating the metabolism of L-tyrosine in this reaction was due to its oxidation-reduction potential, an experiment was instituted in which L-ascorbic acid was replaced by D-glucoscorbic acid. Six normal and four scorbutic animals were used for this purpose. It will be seen from Table 2 that when

Table 2. *The effect of D-glucoscorbic acid in vitro on the disappearance of the phenolic group of L-tyrosine after 3 hr. incubation with guinea pig-liver suspensions*

(Results expressed as percentages of L-tyrosine originally present.)

Normal animals		Scorbutic animals	
No addition	D-Glucoscorbic acid equivalent to 2.5 mg. of L-ascorbic acid added	No addition	D-Glucoscorbic acid equivalent to 5.0 mg. of L-ascorbic acid added
62	80	42	59
79	87	52	61
50	74	67	71
79	90	59	94
73	80	—	—
62	75	—	—
Mean 67	81	—	—
S.D. 11.5	6.4	—	—

a quantity of D-glucoscorbic acid, equivalent to 2.5 mg. of L-ascorbic acid, was added to the reaction mixtures in which suspensions of livers from normal guinea pigs were used the percentage of L-tyrosine which was metabolized in 3 hr. was significantly raised from 67 (s.d. 11.5) to 81 (s.d. 6.4) ($P < 0.05$). Unfortunately, in the case of the scorbutic guinea pigs, we were unable to use a number of animals which would yield figures for a satisfactory statistical analysis. The results, however, indicate that in this case the disappearance of the substrate was also accelerated by the presence of D-glucoscorbic acid.

DISCUSSION

It emerges from this investigation that the phenolic group in L-tyrosine, when present in a concentration higher than that which is likely to occur in any of the tissues *in vivo* under normal conditions, is degraded slowly *in vitro* in the presence of guinea pig-liver suspensions. The rate of degradation is accelerated by the presence of L-ascorbic acid, whether the acid originates from the liver or whether it is added to the reaction mixture in the form of the synthetic compound. This is in consonance with the observations

made *in vivo* on guinea pigs which received high doses of L-tyrosine. It would also appear that the higher rate of degradation observed, when livers from saturated guinea pigs were used than when those from scorbutic guinea pigs were employed, is due to the higher quantities of L-ascorbic acid present in the former and that the vitamin acts directly *per se*. Lan & Sealock (1944) have shown that the livers from scorbutic guinea pigs which had been previously dosed with 20 mg. of L-ascorbic acid daily for 6 days reacted like livers from normal guinea pigs. The animals after such treatment must have been, as is usual, free from the reversible lesions which make up the syndrome of scurvy. In our experiments, on the other hand, owing to the short time that elapsed between the injection of the L-ascorbic acid and the death of the animals, the post-mortem picture which we observed was similar to that obtained with scorbutic animals not previously treated with L-ascorbic acid; yet the action of the liver suspension was the same as that from livers of normal animals. It is therefore reasonable to assume that this was due to the saturation of the tissues with the vitamin.

The results obtained in the experiment in which D-glucoscorbic acid replaced L-ascorbic acid show that the accelerating action of the compounds is in all probability due to their oxidation-reduction potential. D-Glucoscorbic acid does not possess any antiscorbutic activity, because unlike its active enantiomorph it is not retained by the tissues but is entirely excreted by the kidneys (Zilva, 1935). It is therefore interesting to find that when brought into contact with the substrate *in vitro* it functions like the antiscorbutically active L-ascorbic acid. This offers an explanation why D-araboscorbic acid, which is partially retained by the tissues of the guinea pig (cf. Zilva, 1935), when administered to these animals with high doses of L-tyrosine, was found by Sealock & Silberstein (1940) to be not as efficient as L-ascorbic acid in accelerating the disappearance of the phenolic group of L-tyrosine.

That L-ascorbic acid acts as a catalyst in this re-

action is evident from the fact that the quantities of L-ascorbic acid which disappeared during the experiment in the flasks in which the reaction took place was found by us to be the same as that which disappeared in the control flasks from which L-tyrosine was absent, in other words, none of the acid was used up in the actual reaction.

The evidence produced in these experiments does not offer sufficient information to formulate a complete hypothesis of the part played by L-ascorbic acid in the mechanism of the metabolism of L-tyrosine by the enzyme system of the liver, especially as the degradation of only one part of the molecule was investigated by us. The results invite further investigation.

SUMMARY

1. The rate of disappearance of the phenolic group of L-tyrosine in the presence of suspensions of livers from normal or from scorbutic guinea pigs was investigated over periods up to 10 hr. The reaction was found to be slow and proceeded at a higher rate when suspensions from livers of normal animals were used. Glycyl-L-tyrosine could replace the L-tyrosine as substrate.

2. Suspensions of livers from scorbutic guinea pigs injected with 25 mg. of L-ascorbic acid 18 hr. before death functioned like those from normal animals.

3. The accelerating effect of graded quantities (0.5–10 mg.) of L-ascorbic acid was investigated in the presence of liver suspensions from normal and scorbutic animals using L-tyrosine and glycyl-L-tyrosine as substrates. The addition of 2.5 mg. of the acid was found to produce the optimum acceleration. No L-ascorbic acid was used up in the reaction.

4. D-Glucoscorbic acid, which is antiscorbutically inactive, accelerated the above degradation *in vitro* to the same extent as equivalent amounts of L-ascorbic acid.

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