[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

The Effect of Ascorbic Acid on the Enzymatic Oxidation of Monohydric and *o*-Dihydric Phenols[1]

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Several studies in these laboratories in recent years have dealt with the enzymatic action of tyrosinase on various substrates in the presence of *l*-ascorbic acid.[3,4,5,6]

In these studies, *l*-ascorbic acid (AH_2) had been assigned the role of a reducing agent for any *o*-quinone produced in the action of the enzyme on the various substrates. The AH_2 was assumed to act according to the scheme

$$\text{(1) Catechol} \underset{\text{Tyrosinase, } O_2}{\rightleftharpoons} \text{o-quinone}$$

$$\uparrow 2H$$

$$\text{(2) Ascorbic acid} \longrightarrow \text{Dehydroascorbic acid}$$

In this investigation, the enzymatic oxidation of tyrosine, β-(3,4-dihydroxyphenyl)-alanine (dopa) and protocatechuic acid in the absence and presence of ascorbic acid has been measured by manometric means. In other experiments presented, the oxidation of tyrosine and 3,4-xylenol in the absence and presence of AH_2 has been followed by analyzing aliquots of the reaction mixture. The results indicate that AH_2, in addition to its function as a reducing agent for *o*-quinone (reaction 2), is acting in another capacity. AH_2 appears to function both as a pro-oxidant and anti-oxidant in these systems and the indications are that it becomes oxidized in part by an induced reaction.

Experimental

The Enzyme Preparations.—These were prepared by Mr. Stanley Lewis of these laboratories as described.[7] Preparation C259A2 contained 7000 catecholase units and 194 cresolase units/ml.[8] Preparation C259A3 contained 3500 catecholase units and 97 cresolase units/ml. Later in the course of this work, this preparation was found to have degraded and on the basis of tyrosine and dopa oxygen uptake measurements had an activity of 2740 catecholase units/ml.

***l*-Tyrosine.**—The *l*-tyrosine used was Eastman Kodak Co. pure grade recrystallized from water. For the respirometer experiments, the tyrosine was used as a 2.0 mg./ml. suspension.

***l*-Ascorbic Acid.**—The ascorbic acid was obtained from Merck & Co., Inc., and was shown to be more than 99% pure by titration with standard potassium iodate.[9] The acid was weighed out on an analytical balance immediately before using.

β-(3,4-Dihydroxyphenyl)-alanine (Dopa).—The dopa for these experiments was isolated from the Georgia Velvet bean according to the method of Miller.[10] *Anal.*[11] Calcd. for $C_9H_{11}O_4N$: C, 54.6; H, 5.6; N, 7.1. Found: C, 54.4; H, 5.8; N, 7.0.

3,4-Xylenol.—The xylenol was a pure grade from Fraenkel and Landau, Berlin; m. p. 64–66°; b. p. 225–226°.

Results

The Effect of Ascorbic Acid on the Oxidation of Monohydric Phenols.—Figure 1 shows the effect of ascorbic acid on the oxygen uptake in the enzymatic oxidation of tyrosine. The point C represents the point of coloration and thus the point at which the ascorbic acid has become completely oxidized. The oxygen uptake at point C was found to be 140 ± 5 cu. mm.[3] (4 determinations), 12 cu. mm.[3] more than that needed to oxidize the ascorbic acid present according to equation 2 (128 cu. mm.[3]). It is apparent that the dopa produced in the system does not bind the enzyme completely and stop the tyrosine from oxidizing. This view is supported by the direct analysis experiment (Fig. 3). These results are contrary to those of Robinson and Nelson[6] who showed that tyrosine was not oxidized and attributed this result to the complete binding of the tyrosinase by the small amount of dopa formed; the latter was oxidized by the enzyme and caused the oxidation of the ascorbic acid *via* a shuttling mechanism (equations 1 and 2).

Further evidence that dopa does not have a high affinity for the enzyme compared to the affinity of tyrosine for the enzyme has been obtained in this investigation by comparing the dissociation constants of the substrate-enzyme complexes. The Michaelis–Menten[12] constants determined by the method of Lineweaver and Burk[13] were found to be 0.0005 and 0.0006 mole/liter for the dopa–enzyme and tyrosine–enzyme complexes, respectively.

Furthermore, the rate of oxygen uptake when tyrosine is oxidized in the presence of ascorbic acid is dependent on the tyrosine concentration. When the tyrosine concentration was lowered (curve 1, Fig. 2) the rates of oxygen uptake became progressively lower, showing that the rate is

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(3) W. H. Miller and C. R. Dawson, THIS JOURNAL, **63**, 3375 (1941).

(4) L. Roth, Dissertation, 1944, Columbia University, New York, N. Y.

(5) W. H. Miller, M. F. Mallette, L. Roth and C. R. Dawson, THIS JOURNAL, **66**, 514 (1944).

(6) E. S. Robinson and J. M. Nelson, *Arch. Biochem.*, **4**, 111 (1944).

(7) M. F. Mallette, S. Lewis, S. R. Ames, J. M. Nelson and C. R. Dawson, *ibid.*, **16**, 283 (1948).

(8) M. Adams and J. M. Nelson, THIS JOURNAL, **60**, 2472 (1938).

(9) R. Ballentine, *Ind. Eng. Chem., Anal. Ed.*, **13**, 89 (1941).

(10) E. Miller, *J. Biol. Chem.*, **44**, 481 (1920).

(11) The C and H analyses were carried out by Miss Lois May and the N was determined by the micro-Kjeldahl method.

(12) L. Michaelis and M. Menten, *Biochem. Z.*, **49**, 339 (1913).

(13) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).

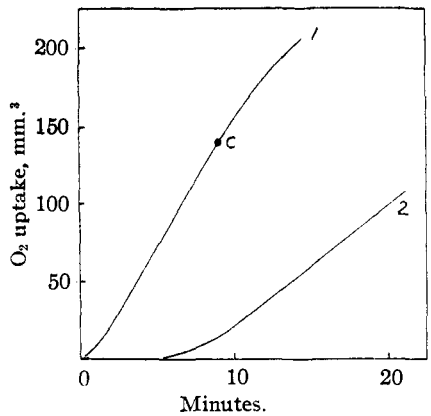

Fig. 1.—The effect of ascorbic acid on the enzymatic oxidation of *l*-tyrosine and the determination of the point of complete oxidation of ascorbic acid in the enzymatic oxidation of *l*-tyrosine in the presence of ascorbic acid. Reaction mixtures: curve 1, 4.0 ml. 0.2 *M* pyrophosphate–HCl buffer—final *p*H 6.0, 5.0 mg. *l*-tyrosine, 2.0 mg. ascorbic acid and 0.5 ml. 1 → 3 dilution of enzyme preparation C259A3 (2740 catecholase units/ml.), rate 16.5 cu. mm./min.; curve 2, 4.0 ml. 0.2 *M* pyrophosphate–HCl buffer—final *p*H 6.0, 4.0 mg. *l*-tyrosine (optimum concentration) and 0.5 ml. 1 → 5 dilution of the above enzyme preparation, rate 4.6 cu. mm./min. This rate corrected to correspond to the enzyme concentration used in curve 1 amounts to 7.7 cu. mm./min. and the latter rate is represented by curve 2. Warburg manometers were used to measure the oxygen uptake. The experiments were carried out at 25.00 ± 0.03° in a total reaction volume of 9.7 ml. Two-tenths ml. of 20% KOH along with a filter paper wick was used in the center well of the manometer flasks to absorb CO_2. For curve 1, manometer readings were made every two minutes except near the point of coloration, C, where readings were made every minute and the manometers were lifted out of the bath for a few seconds every 0.5 minute to note the point of coloration. For curve 2, readings were made every three minutes. The rates were determined from the straight portion of the O_2 *versus* time plot and duplicate rate determinations were made. The autoxidation of ascorbic acid in pyrophosphate buffer at *p*H 6.0 was found to be of the order of 0.1 cu. mm./min.

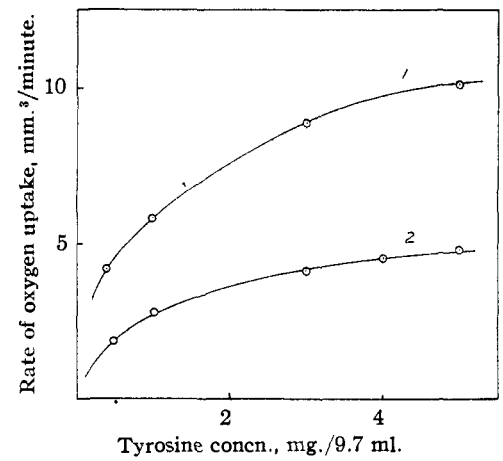

Fig. 2.—The effect of ascorbic acid on the oxidation of *l*-tyrosine at various tyrosine concentrations. Reaction mixtures: curve 1, 4.0 ml. 0.2 *M* pyrophosphate–HCl buffer—final *p*H 6.0, 2.0 mg. ascorbic acid, indicated amounts of *l*-tyrosine and 0.5 ml. 1 → 5 dilution of enzyme preparation C259A3 (2740 catecholase units/ml.); curve 2, 4.0 ml. 0.2 *M* pyrophosphate buffer—final *p*H 6.0, indicated amounts of *l*-tyrosine and 1.0 ml. 1 → 25 dilution of enzyme preparation C259A2. The final enzyme concentration here is essentially that used for curve 1. Oxygen uptake measured as described in Fig. 1.

dependent on the tyrosine concentration and not on any small amount of dopa formed in the reaction. If dopa were bound to the enzyme and controlled the oxidation, the rate of oxygen uptake would be independent of the tyrosine concentration.

An interesting feature of the tyrosine–enzyme–ascorbic acid system is revealed by the following considerations.

As was shown previously, the amount of tyrosine oxidized by the enzyme in the presence of ascorbic acid (curve 1, Fig. 1) corresponded to 12 cu. mm. Presumably this amount of tyrosine was oxidized to 0.2 mg. of dopa.[14]

It became of interest to see if this small amount of dopa in the presence of ascorbic acid would give the rate observed in curve 1, Fig. 1. The results shown in Table I indicate that the dopa produced in a system containing tyrosine and ascorbic acid cannot account for the rate obtained. In the presence of the much greater amount of tyrosine, the dopa probably would not interact with all the enzyme and the rate due to the dopa would be even smaller than the rate of 12.5 cu. mm./min. observed.

TABLE I

Flask contents	Rate of oxygen uptake, cu. mm./min.
Tyrosine	6.3
Tyrosine + 2.0 mg. ascorbic acid	16.7
0.2 mg. dopa + 2.0 mg. ascorbic acid	12.5

Experiments were conducted in duplicate under the same conditions as described in Fig. 1. The enzyme was a purified preparation diluted to give rates comparable to those in Fig. 1.

On the other hand, the rate of 16.7 cu. mm./min. cannot be entirely accounted for by the enzymatic oxidation of tyrosine. Allowing for complete use of the enzyme by tyrosine, the rate in the absence of ascorbic acid was 6.3 cu. mm./min. As will be shown below (Fig. 3), the rate of disappearance of tyrosine was increased 40% by the addition of ascorbic acid. Behm and Nelson[15] found that in the enzymatic oxidation of phenol, the rate of oxygen uptake of the reaction can be taken as a direct measure of the corresponding rate of disappearance of the phenol. That this is true for the tyrosine system can be shown by comparing the rate of oxidation for the manometric experiment of curve 2, Fig. 1, with that of the direct analysis experiment of curve 1, Fig. 3. On the basis of one atom of oxygen uptake for one mole of tyrosine, the rate of oxidation for the

(14) 1.0 mg. of dopa is equivalent to 57 cu. mm. of oxygen on the basis that one mole of tyrosine reacts with one atom of oxygen to yield one mole of dopa.

(15) R. Behm and J. M. Nelson, THIS JOURNAL, 66, 709 (1944).

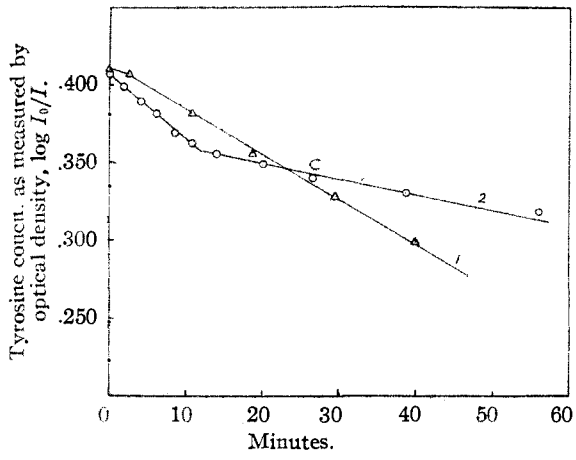

Fig. 3.—The effect of ascorbic acid on the rate of disappearance of *l*-tyrosine when oxidized by means of tyrosinase: curve 1, no ascorbic acid; curve 2, 60 mg. ascorbic acid. In the absence of ascorbic acid, the reaction mixture became colored about 0.25 min. after the addition of the enzyme. In the presence of ascorbic acid, color appeared at point C. The oxidation was carried out in a 500-ml. 3-neck flask containing the following: 110 ml. H_2O, 60 mg. *l*-tyrosine, 50 ml. 0.2 *M* KH_2PO_4, 20 ml. 1/15 *M* Na_2HPO_4 (final *p*H 6.05), 25 ml. gelatin soln.[16] (1.0 mg./ml.) and 0.5 ml. enzyme preparation C259A3 (3500 catecholase units/ml.) added at zero time. In the oxidation in the presence of ascorbic acid, the same reaction mixture was used and crystalline *l*-ascorbic acid was added and dissolved before addition of the enzyme. The flask was immersed in a constant temperature bath at 25.00 ± 0.05°, and the contents of the flask were mixed by a stream of filtered air. Before addition of the enzyme and at intervals during the course of the oxidation, 2.0-ml. samples were pipetted from the reaction mixture and run into 15-ml. centrifuge tubes containing 1.0 ml. 10% H_2SO_4 to stop the enzymatic action. The tyrosine in the tubes was determined by a modification of Lugg's method[18] as follows: 3.0 ml. H_2O and 1.0 ml. $HgSO_4$ reagent (30 g. of $HgSO_4$ dissolved in 200 ml. 5 *N* H_2SO_4 and filtered) was added, the tubes heated in a boiling water bath for five minutes, cooled for at least an hour and centrifuged. The supernatants were filtered through small cotton plugs into 25-ml. volumetric flasks containing 1.0 ml. 10% H_2SO_4 and 1.0 ml. $HgSO_4$ reagent. It was found that the use of the two latter reagents in the dilution prevented cloudiness in the final soln. Any precipitates in the centrifuge tubes were treated with 1.0 ml. H_2O and 1.0 ml. $HgSO_4$ reagent for 15 min. at boiling water temperatures. This procedure was found necessary for the precipitates tended to occlude some tyrosine and this process served to remove it. The tubes were then cooled, the contents filtered into the volumetric flasks and the tubes and funnels finally rinsed with H_2O. 1.0 ml. 0.4% $NaNO_2$ was added, and the flasks diluted to the mark. The color reached its maximum intensity in about 20 min., and the optical density was read on a Beckman spectrophotometer at 490 mμ in matched 1.00 cm. cuvettes. Beer's law held from very low concentrations of tyrosine to the highest value read. In separate experiments, the determination of tyrosine in the presence of various reagents was shown to be affected as follows: ascorbic acid, −0.5%; dehydroascorbic acid,[19] +1%; tyrosine oxidation products, +0.3 to −2%. Preliminary experiments also showed that with or without ascorbic acid in the absence of the enzyme, tyrosine was not oxidized. Two determinations of the rate of disappearance of tyrosine (curve 1) gave values of 0.426 and 0.431 mg./min.; in the presence of ascorbic acid, the rates of disappearance ranged between 0.58 and 0.61 mg./min.

(16) In some of the earlier experiments in this investigation, manometric experiment is 0.00027 mg./min./enzyme unit. The value in similar units for the direct analysis experiment is 0.00025.

Assuming, therefore, that the increase in the rate of disappearance of tyrosine appears as a proportional increase in the rate of oxygen uptake, the rate due to the enzymatic oxidation of tyrosine in the presence of ascorbic acid would amount to 9 cu. mm./min. (6.3 × 1.4). This rate does not account for the rate of 16.7 cu. mm./min. observed in the experiment of Table I.

The experiments described in Fig. 3 show that tyrosine disappears from the system in the presence of ascorbic acid (curve 2) and agree with the results of Fig. 1.

In addition, this experiment revealed other pertinent information. In the first place, the presence of ascorbic acid in the reaction mixture caused an increase in the rate of disappearance of tyrosine in the early stages of the reaction. At present no direct data are available to account for this increase in rate which appears to be enzyme dependent. By comparing experiments 1 and 2 with 4, 5 and 6 in Table II, it can be seen that doubling the enzyme used, doubled the initial rate of disappearance of the tyrosine. The initial rate (R_1) does not appear to be affected by wide variances in the ascorbic acid concentration (compare experiments 3 and 4, Table II).

TABLE II

THE EFFECT OF ASCORBIC ACID CONCENTRATION AND ENZYME CONCENTRATION ON THE RATE OF OXIDATION OF TYROSINE

Expt.	Ascorbic acid, mg.	Enzyme, ml.	R_1	R_2
1	180	1.0	1.32	0.23
2	120	1.0	1.10	.14
3	360	0.5	0.63	.12
4	60	.5	.58	.23
5	60	.5	.61	.14
6[a]	60	.5	.63	.20
7	0	.5	.43	..

[a] 100 mg. of tyrosine used. Reaction mixtures and methods for determining the rate of disappearance of tyrosine similar to those experiments represented by curves 1 and 2, Fig. 3. R_1 = initial rate of disappearance of tyrosine in mg./min.; R_2 = rate of disappearance of tyrosine after the sharp break in the course of the reaction.

Secondly, a marked retardation in the rate of disappearance of tyrosine was brought about by the addition of ascorbic acid. This secondary rate (R_2) was more difficult to reproduce and with the present data, no correlation seemed possible.

Thirdly, the presence of ascorbic acid caused the

gelatin was used because of the indications[17] that it would keep denaturation to a minimun. However, later, manometric measurements showed that gelatin did not have any effect on the enzymatic oxidation of tyrosine or dopa.

(17) W. H. Miller and C. R. Dawson, THIS JOURNAL, 63, 3368 (1941).

(18) J. Lugg, *Biochem. J.*, 31, 1430(1937).

(19) Made by bubbling air through a soln. of ascorbic acid until the KIO_4 titer dropped from 5.95 to 1.0 ml.

elimination of the induction period. This is evident in Fig. 3 and more so in Fig. 4 where 3,4-xylenol was used as the substrate.

The Effect of Ascorbic Acid on the Oxidation of *o*-Dihydric Phenols.—Ascorbic acid also affects the rate of oxygen uptake in the enzymatic oxidation of certain *o*-dihydric phenols. In Fig. 5 are plotted the results of a series of manometric experiments in which varying amounts of dopa were oxidized by means of tyrosinase in the presence and absence of ascorbic acid. The rates of oxygen uptake in the presence of ascorbic acid (curve 2) are higher than the rates in the absence of ascorbic acid (curve 1) except where the concentration of dopa approached very high values. The maximum rate of oxygen uptake in the presence of ascorbic acid, however, is about twice the maximum rate in the absence of ascorbic acid.

An increase in the rate of oxygen uptake due to ascorbic acid also occurred in the enzymatic oxidation of protocatechuic acid. After correcting for the autoxidation of ascorbic acid, the rate of oxygen uptake using the optimum concentration of the substrate in a phosphate buffer at *p*H 6.0 was increased by 40%.[20]

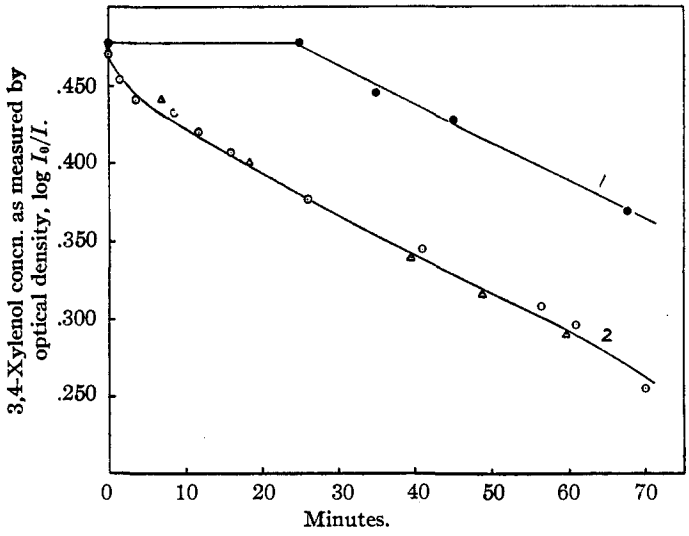

Fig. 4.—The effect of ascorbic acid on the rate of disappearance of 3,4-xylenol when oxidized by means of tyrosinase: Curve 1, no ascorbic acid. This curve represents a single experiment. To verify the presence of this long induction period, an experiment was run at a later time using an amount of enzyme which gave a rate of disappearance 25% lower. The induction period observed was 40 min. compared with 25 min. for curve 1. Curve 2, 120 mg. of ascorbic acid. This curve represents two separate experiments denoted by O and △. The oxidation was run as described in Fig. 3. The flask contents were as follows: 240 ml. H_2O, 40 ml. 0.5 *M* KH_2PO_4, 40 ml. 1/15 *M* Na_2HPO_4 (*p*H of final soln. 6.0), 25 ml. xylenol soln. (5.0 mg./ml.) 50 ml. gelatin soln. (1.0 mg./ml.) and 5.0 ml. of a 1 → 10 dilution of enzyme preparation C259A2. The procedure for determining the xylenol was essentially the same as that followed in Fig. 3, except that the tubes were heated initially for 10 min. The color was measured at 480 mμ immediately after mixing with $NaNO_2$. The optical density was found to be proportional to the concentration of xylenol in the range used. In separate experiments, ascorbic acid and dehydroascorbic acid[19] were shown to affect the colorimetric determination of xylenol by not more than ±1%. Tests made on the reaction mixture before enzyme was added showed that xylenol in the absence and presence of ascorbic acid did not disappear from the reaction mixture.

Discussion

The results of the manometric experiments represented by Figs. 1 and 2 indicate that in the enzymatic oxidation of tyrosine in the presence of ascorbic acid, the oxygen uptake is not controlled by the dopa produced in the reaction. Dopa does not have a high affinity for the enzyme and this is borne out by the magnitude of the dissociation constant for the dopa–enzyme complex. Thus the eventual retardation of tyrosine oxidation must occur by a mechanism differing from that postulated by Robinson and Nelson.[6]

The results of Table I indicate that the rates of oxygen uptake are higher than can be accounted for directly from tyrosine or dopa oxidation. The experiments with dopa (Fig. 5) and protocatechuic acid also show an increased rate of oxygen uptake that cannot be accounted for directly through the enzymatic oxidation of these substrates. According to equation (1), ascorbic acid does not take part in the oxidation of the *o*-dihydric phenol. If ascorbic acid caused an increase in the effective dopa concentration by a simple shuttling process (equations 1 and 2), it could not cause an increase in rate above the maximum rate obtained with high concentrations of dopa alone. It appears that ascorbic acid has another type of action in this system.

In the experiments represented by Fig. 3, ascorbic acid has a two-fold effect on the course of the oxidation of tyrosine. In the early stages, the rate of disappearance was 40% higher than the rate in the absence of ascorbic acid. The latter has some type of pro-oxidant effect on the system. Its reaction with quinone cannot account for this, except through its ability to keep the *o*-dihydroxy compound from disappearing from the system and thus priming the enzyme to a higher capacity. If this priming mechanism came into play, an induction period would be noted. Curve 2, Fig. 3, and curve 2, Fig. 4, show that this is not the case and in all the experiments in the presence of ascorbic acid noted in Table II, the disappearance started in at the increased rate immediately upon the addition of the enzyme. Manometric experiments[21] have also shown that the priming of the monophenolase reaction by the addition of catechol does not increase the rate of oxidation.

The removal of the induction period as in-

(20) Unpublished results of Professor J. M. Nelson, Dept. of Chem., Columbia University.

(21) D. Gregg and J. M. Nelson, THIS JOURNAL, 62, 2506 (1940).

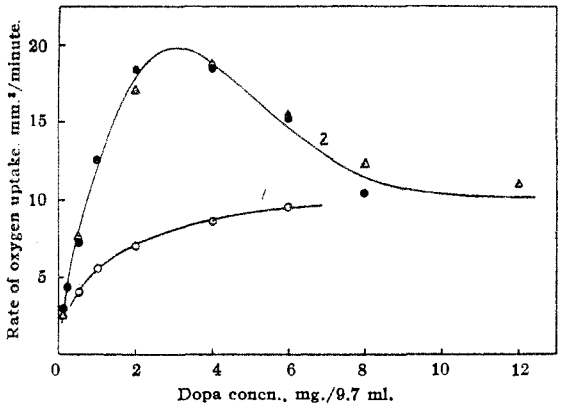

Fig. 5.—The effect of ascorbic acid on the rate of the enzymatic oxidation of β-(3,4-dihydroxyphenyl)-alanine (dopa): Curve 1, indicated amounts of dopa with 1.0 ml. 1 → 125 dilution of enzyme preparation C259A2. A small correction was applied to make this curve, obtained with 56 units of enzyme, comparable to curve 2 which was obtained by using 58 units of enzyme. Curve 2, indicated amounts of dopa with 2.0 mg. of ascorbic acid and enzyme as follows; Series No. 1 (●) was obtained by using 0.5 ml. 1 → 30 dilution of enzyme preparation C259A3 (3500 catecholase units/ml.) The maximum rate observed was 19.0 cu. mm./min.; Series No. 2 (Δ) was obtained later in the course of the investigation by using 0.5 ml. 1 → 30 dilution of preparation C259A3 which had become somewhat inactivated for the maximum rate was 14.5 cu. mm./min. These rates were corrected to bring the maximum rate to 19.0 cu. mm./min. for comparison with series No. 1. The activity of this partially inactivated enzyme was checked by a measurement of its activity toward tyrosine. Warburg manometers were used to measure the oxygen uptake. The experiments were carried out at 25.00 ± 0.03° in a total reaction volume of 9.7 ml. with 2.0 ml. 0.2 *M* phosphate buffer—final *p*H 6.1. Two-tenths ml. of 20% KOH along with a filter paper wick was used in the center well of the flasks to absorb CO_2. Rates were determined from the O_2 *versus* time plots which were linear in the initial stages of the reaction. In the presence of ascorbic acid, the rates were corrected for the autoxidation of ascorbic acid which under these conditions was of the order of 0.9 cu. mm./min.

dicated in Figs. 3 and 4 appears to be another facet of the pro-oxidant nature of ascorbic acid. This induction period (also shown by curve 2_1 Fig. 1) always occurs in the enzymatic oxidation of monohydric phenols. In the absence of ascorbic acid, the monophenolase activity becomes evident only after the enzyme has oxidized some of the *o*-dihydric compound.[22] However, in the present experiments, in the presence of ascorbic acid, the phenolase activity comes into effect immediately although at the start of the oxidation, no *o*-dihydric compound can be present. A more fundamental approach to this aspect of the problem may lead to a better understanding of the mechanism of the activation of the enzyme toward monophenols.

In the later stages of the reaction represented by curve 2, Fig. 3 retardation of the rate of disappearance of tyrosine was noted. This retardation set in after some tyrosine had become oxidized. In view of the essential equality of the dissociation constants of the tyrosine and dopa–enzyme complexes, it would seem improbable that the small amount of dopa produced would have the ability to displace the larger amount of tyrosine from the enzyme and stop its oxidation. To further substantiate this view, an experiment was run similar to the one represented by curve 2, Fig. 3, except that a small amount of dopa (10% of the tyrosine used) was added initially. Here, as in curve 2, Fig. 3, there was a sharp initial drop in the tyrosine concentration amounting to 9% of the tyrosine used, followed by retardation. Enzyme inactivation is not causing the inhibition, for the experiment in curve 1, Fig. 1, shows that the enzyme is fully active up to and beyond the point of the complete oxidation of the ascorbic acid. It appears that ascorbic acid or more likely some oxidation product of ascorbic acid is acting as an anti-oxidant in this system. The anti-oxidant property of ascorbic acid has been noted previously by Mattil, *et al.*,[23] and Calkins and Mattill.[24] In the present experiments, the fact that the retardation did not start until some ascorbic acid had become oxidized and that it continued after the ascorbic acid had become completely oxidized (point *C*, curve 2, Fig. 3) lends support to the view that an oxidation product of ascorbic acid is causing the retardation.

Weissberger and co-workers[25] have noted that ascorbic acid can act in some cases as an inhibitor and in other cases as an accelerator of autoxidation reactions. Inhibition of oxygen uptake was said to be due to the lowering of the concentration of the reactive semiquinone; acceleration was caused by the production of a high concentration of the reactive semiquinone. Whether inhibition or acceleration occurred depended on the rate of reduction of the quinone by the reducing agent added. It would be difficult to say whether or not enzymatic oxidations can be considered in relation to this hypothesis. Enzymatic oxidations take place at *p*H's much lower than those needed to effect autoxidations. The steps involved in the two types of oxidations are somewhat different especially with regard to the fate of oxygen. Hydrogen peroxide is formed in autoxidations but it is not a product in the enzymatic oxidations under consideration. Nevertheless, it would appear possible that the reactive semiquinone mechanism in conjunction with enzymatic action could account for the increased rate of oxygen uptake demonstrated in Table I and in the experiments with the *o*-dihydric compounds.

Another comparison between the systems under discussion and many common autoxidation proc-

(22) J. M. Nelson and C. R. Dawson, "Advances in Enzymology IV," Interscience Publishers Inc., New York, N. Y., p. 99, 1944.

(23) K. F. Mattil, L. J. Filer, Jr., and H. Longenecker, *Oil and Soap*, **21**, 160 (1940).

(24) V. Calkins and H. A. Mattill, THIS JOURNAL, **66**, 239 (1944).

(25) A. Weissberger, D. S. Thomas and J. E. LuValle, *ibid.*, **65**, 1489 (1943).

esses[26,27] is interesting. In autoxidation reactions, certain substances will cause inhibition of oxidation by processes which involve their own oxidation. This type of system has been described lately in detail by Kolthoff and Medalia.[28] In the present work, inhibition by ascorbic acid is indicated in the direct analysis experiments, while the concurrent induced oxidation of the ascorbic acid is indicated by the increased rate of oxygen uptake as demonstrated in Table I and in the experiments with the *o*-dihydroxy compounds.

Addendum.—While this manuscript was in preparation, Kendal[29] published some results which are closely related to the present work. It was shown that in the presence of ascorbic acid certain monophenols, including tyrosine, were oxidized by tyrosinase without an induction period. The view was presented that the ascorbic acid effect was not "dependent on its favoring *o*-dihydroxyphenol accumulation." No explanation can be offered, however, to account for Kendal not finding retardation of tyrosine oxidation during the later stages of the reaction.

(26) L. P. Hammett, "Physical Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1940, p. 370.

(27) W. A. Waters, "The Chemistry of Free Radicals," The Claredon Press, Oxford, 1946, p. 232.

(28) I. M. Kolthoff and A. I. Medalia, THIS JOURNAL, **71**, 3777 (1949).

(29) L. Kendal, *Biochem. J.*, 44, 442 (1949).

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Summary

The effect of ascorbic acid on the enzymatic oxidation of *l*-tyrosine, 3,4-xylenol, β-(3,4-dihydroxyphenyl)-alanine (dopa) and protocatechuic acid has been described. The rate of oxygen uptake in the oxidation of *l*-tyrosine, dopa and protocatechuic acid is increased by the addition of ascorbic acid. In the case of the tyrosine oxidation, it has been shown that the increase in the rate of oxygen uptake could not be completely attributed to the oxidation of the dopa formed in the reaction. Both an increase and a retardation in the rate of disappearance of tyrosine during the course of its oxidation in the presence of ascorbic acid has been found. The results indicate a parallel between the effect of ascorbic acid on the systems studied and the effect of inhibitors on autoxidation reactions.

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[CONTRIBUTION FROM THE CHEMICAL LABORATORIES OF THE UNIVERSITY OF CALIFORNIA]

The Physical Properties of Elinin, a Lipoprotein from Human Erythrocytes[1]

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Introduction

The structure and chemical composition of red blood cells have been the subject of repeated investigations. The results of this work have been adequately summarized in several reviews.[3a,b,4,5]

Some time ago, work was begun in these laboratories for the purpose of concentrating or isolating the Rh factors present in human blood. It was thought that a concentrated or pure Rh preparation might offer an avenue of approach for therapy in cases of *erythroblastosis fetalis*. A preliminary communication[6] reported the initial results of this project. The present work describes subsequent investigations of elinin, a lipoprotein preparation derived from the red cell membrane, which when prepared from Rh positive or type A or type B blood is capable of reacting specifically (in a serological sense) with antibodies to these factors. The method of preparation of elinin and its serological properties are described elsewhere.[7]

General Properties of Elinin Solutions

An elinin solution is a transparent, viscous, yellow-brown liquid having easily visible Tyndall scattering and double refraction of flow. A concentration of about 1% or greater is needed to demonstrate the latter property readily. At elinin concentrations of about 5% or more, gels result. These can easily be prepared from a

(1) A preliminary report of the present work was presented before the American Association for the Advancement of Science, *Chem. Eng. News*, **26**, 2118 (1948).

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