A STUDY OF THE COFACTORS REQUIRED BY THE TYROSINE OXIDASE SYSTEM OF LIVER*

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Recently considerable work has been reported on the enzyme system in mammalian liver which metabolizes tyrosine to fumarate and acetoacetate. This conversion has been shown to consist of several steps, involving first a transamination of tyrosine to *p*-hydroxyphenylpyruvic acid, and subsequent oxidation of this intermediate to 2,5-dihydroxyphenylpyruvic acid, then to homogentisic acid, and finally to fumaryl acetoacetate, which is hydrolyzed to fumarate and acetoacetate (1–5). Additional intermediate steps have not yet been elucidated, although they probably exist.

Ascorbic acid has been implicated as a possible cofactor for at least one step in tyrosine oxidation, although its exact point of action has not been demonstrated (6–8). With the exception of the expected cofactor for the transaminase step, pyridoxal phosphate, cofactors for the other possible enzymatic steps have not been indicated. Therefore, the authors felt it would be important to study the tyrosine oxidase system to attempt to locate the site of action of ascorbic acid as well as to uncover the existence of any additional cofactors that might be involved in the system. For these purposes our investigations have been initiated with a substance known to destroy reduced ascorbic acid, 2,6-dichlorophenol indophenol (DCPP), used as a tool. The studies carried out to explain some apparently anomalous results in these experiments have led to new and interesting facts concerning the site of ascorbic acid action and the implication of perhaps two new cofactors for the tyrosine oxidase system.

EXPERIMENTAL

Preparation of Enzyme System—To obtain a suitable and duplicable enzyme preparation, it was found that the total enzyme system metabolizing tyrosine to acetoacetate and fumarate was present completely in a soluble form in an isotonic sucrose-rat liver homogenate. 16.7 per cent rat liver homogenates (adult male Sprague-Dawley rats) were prepared in

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0.25 M sucrose (isotonic) and centrifuged at 0° for ½ hour at 25,000 × *g*. This procedure removes all particulate matter, including most of the microsomes, and leaves the completely soluble enzymes in the supernatant solution. This supernatant mixture of enzymes was then used as the enzyme preparation in the experiments reported in this paper. The liver particulate matter was found to be totally devoid of tyrosine oxidase.

Effect of DCPP on Tyrosine Oxidase—The standard system used for measuring tyrosine oxidase activity was prepared as follows. To duplicate Warburg vessels with double side arms, 1.0 ml. of Krebs-Ringer-phosphate buffer of pH 7.4 (9), 0.5 ml. of the enzyme preparation, and 0.7 ml. of water were added in the main compartment. According to the order of addition from the side arms (the contents of the first side arm were added first in all the experiments), 0.2 ml. of 0.15 M sodium α-ketoglutarate and 0.15 M L-tyrosine suspension or water were added to one side arm, and various volumes of test substances as indicated in Tables I and II were added to the other side arm. The total volume in the flasks was brought to 3.9 ml. with water. After the addition of the substrate, oxygen uptakes were followed manometrically for various lengths of time, depending upon the experiment. All the reagents were brought to pH 7.4. For all of the enzyme experiments reported in this paper, 37° was the temperature of the Warburg bath.

In the first preliminary experiments the effect of DCPP on the normal system was studied. Various levels of DCPP were incubated with the enzyme for 10 minutes before the addition of α-ketoglutarate and tyrosine. The results of these experiments are presented in Table I, Column 1. Here it can be seen that high levels of DCPP produce a marked inhibition of the system, which would be expected if the ascorbic acid in the system were being destroyed by the dye and if ascorbic acid were essential as a cofactor for a step in the system. At low levels of DCPP, however, a strikingly anomalous effect was produced in that a marked stimulation of activity occurred.

In order to observe whether these results were specific for DCPP or could be given by other substances, methylene blue was used in place of DCPP. These experiments indicated that methylene blue was inactive except to give a negligible stimulation of oxidation at all the stoichiometric levels employed.

Reversal of DCPP Inhibition—If the inhibition of tyrosine oxidase by DCPP is due to the destruction of ascorbic acid, the addition of an excess of the vitamin *in vitro* should reverse the inhibition of the dye.

Therefore, the effects of ascorbic acid added to the enzyme before and after the addition of DCPP were studied. When ascorbic acid was added after the DCPP, the dye was included in the main compartment of the

flask and ascorbic acid in the first side arm. When ascorbic acid was added before the DCPP, the ascorbic acid was included in the main compartment and the dye in the first side arm. A preliminary 10 minute incubation of either ascorbic acid or DCPP with the enzyme was carried out before the other substance and the substrate were added.

The results of these experiments are presented in Table I. Here it can be seen that, in the absence of the dye, a marked stimulation by ascorbic acid occurred which is about 3 times the control activity. At the low level of the dye, no further effect of ascorbic acid occurred, indicating that the low dye concentration stimulated the system to an apparently maximal extent. On the other hand, at the high level of DCPP marked inhibition of activity was observed, and this inhibition could not be overcome by

TABLE I

Relationship of DCPP Stimulation and Inhibition to Presence of Ascorbic Acid

Level of DCPP per flask	Ascorbic acid added			
	0 (1)	1000 γ after dye (2)	1000 γ before dye (3)	2000 γ before dye (4)
	Tyrosine oxidase activity,* μl. O_2 per 30 min. per flask			
γ	γ	γ	γ	γ
0	61	196	190	
50	230	200	219	
200	90			
550	12	3	135	139

* These figures are the averages of seven experiments.

ascorbic acid, once the dye had contacted the enzyme. However, if ascorbic acid was incubated with the enzyme before the DCPP was added, a partial reversal of inhibition could be demonstrated. Increasing the level of ascorbic acid to 2000 γ per flask did not increase this reversal to any greater extent. It is interesting to note that the stimulation by ascorbic acid in the absence of DCPP (190−61 = 129 μl. of O_2 per 30 minutes) was equal to the degree to which ascorbic acid reversed the inhibition by the high level of the dye (135−12 = 123 μl. of O_2 per 30 minutes). This is probably an important effect and will be discussed later in the light of succeeding experiments. Dehydroascorbic acid was tested and found to be completely inactive, both in the absence and in the presence of the dye.

Since ascorbic acid was unable to overcome the inhibition by DCPP completely but reversed the inhibition only to the extent to which ascorbic acid stimulated the system in the absence of the dye, it appeared that

ascorbic acid was not the only cofactor destroyed by DCPP. Therefore, in the next experiments a search was made for a substance which could also reverse the effect of the dye, and the activity of which in the tyrosine oxidase system would be different from that of ascorbic acid. Such a substance was found to be glutathione. In these experiments glutathione was incubated with the enzyme for 10 minutes before DCPP was added. The level of glutathione chosen was equivalent to 1000 γ of ascorbic acid. From Table II it can be seen that the effect of glutathione is quite different from that of ascorbic acid. In the absence of DCPP there was a slight stimulation of tyrosine oxidase, which, although small, was consistent in every experiment. With the low level of DCPP, glutathione showed little further effect. However, with the high level of DCPP, an almost complete

TABLE II

Relationship of DCPP Stimulation and Inhibition to Presence of Glutathione

Level of DCPP per flask	Glutathione added	
	0	3120 γ before dye
	Tyrosine oxidase activity,* μl. O_2 per 30 min. per flask	
γ	γ	γ
0	32	53
50	233	242
550	14	205

* These figures are the averages of three experiments.

reversal of the dye inhibition occurred. A possible explanation for this effect is that, if ascorbic acid were necessary as a cofactor for one step in tyrosine oxidase and glutathione, as a cofactor for a different step ascorbic acid could reverse the inhibition only of that step in which it was required, while glutathione could reverse the inhibition of both steps. This may be deduced from the fact that the oxidation-reduction potential of ascorbic acid is slightly higher than that of glutathione. Thus the excess glutathione could reduce any enzyme ascorbic acid oxidized by the dye, reduce the remaining dye, and also furnish reduced glutathione for that of the enzyme oxidized by the dye. Essentially the end-result would be the same as that of low DCPP conditions in which there are enough endogenous reducing agents in the enzyme preparation to reduce the low level of the DCPP added. In fact in all of the experiments in which the 50 γ level of DCPP was employed, the dye was decolorized immediately after mixing with the enzyme. The 550 γ level of the dye, however, was decolorized only when the excess ascorbic acid or glutathione was added. The fore-

going explanation will be implemented in later experiments in which it was found that the dye must be in a reduced form for stimulation to occur unless the enzyme system is protected by the prior addition of substrate. Therefore, if this explanation is essentially correct, the effect of glutathione on the oxygen uptake data in the presence of the high level of the dye would be to bring the oxidation up to the same level as that caused by adding the low level of the dye. That the effect of glutathione may be specific was shown by the fact that cysteine at an equivalent level in the presence of 550 γ of DCPP was unable to reverse the inhibition by the DCPP.

Studies on Stimulatory Effect of Low Concentrations of DCPP—From Table I it can be seen that two apparently different effects of DCPP occur, one stimulatory and the other inhibitory. From gross observation of the Warburg vessels used for obtaining the results in Table I, the low level of the dye (50 γ) was immediately decolorized upon being mixed with the enzyme, while the high level (550 γ) was unaffected by the enzyme and remained blue throughout. This indicated that the stimulation by the dye occurred if it was present in small amounts and kept mainly in the reduced form. This also suggested the possibility that the dye was able to substitute for a cofactor already present in the enzyme, but which was labile or present only in limiting amounts. To check the possibility that the stimulation by the dye occurred only if it was in the reduced form, experiments were carried out in which 0.5 ml. of a 20 per cent suspension of particulate matter of liver (containing the mitochondria) in sucrose was mixed with 0.1 ml. of 0.1 M sodium succinate and 550 γ of DCPP in one side arm of Warburg vessels with double side arms. The remaining components of the system were the same as those given earlier. In this way the high level of the dye which was ordinarily inhibitory to tyrosine oxidase would be reduced by the succinic dehydrogenase of the liver residue. The results of these experiments indicate that this level of the dye was no longer inhibitory but stimulated the tyrosine oxidase system, although the degree of stimulation was still not as great as with the low level of DCPP.

To show that the stimulatory effect of the dye on oxygen uptake did not occur simply by a chemical oxidation of a susceptible intermediate in tyrosine oxidation, the amount of acetoacetate produced in the absence and in the presence of the low level of DCPP was measured. Concomitantly with these experiments, the stimulatory effect of ascorbic acid was also studied to observe whether its stimulation produced an equivalent amount of acetoacetate from the tyrosine oxidized. In these experiments the effects of 50 γ of DCPP and 1000 γ of ascorbic acid separately on oxygen uptake and acetoacetate formation from tyrosine oxidation were studied. At the end of 1 hour acetoacetate was estimated manometrically by the method of Edson (10). From the results of these experiments in

Table III, it can be seen that the stimulation by DCPP as well as ascorbic acid gave acetoacetate production equivalent to the oxygen uptake obtained (2 moles of oxygen are required for the complete oxidation of tyrosine to 1 mole of acetoacetate).

Evidence for Relative Positions of Enzymes Activated by Ascorbic Acid and by Glutathione—Since ascorbic acid can only partially reverse the inhibition by the high level of DCPP, it appears that this lack of complete reversal

TABLE III

Effect of Ascorbic Acid and Low Level of DCPP on Acetoacetate Production from Tyrosine

System	Tyrosine oxidase activity, μl. O_2 per hr. per flask	Acetoacetate production, μl. CO_2 equivalent per hr. per flask	O_2-acetoacetate ratio, μl. O_2 uptake-μl. CO_2 equivalent of acetoacetate production
Control	90*	41	2.1
" + 50 γ DCPP	152	66	2.3
" + 1000 γ ascorbic acid	144	72	2.0

* These figures are the averages of three experiments.

TABLE IV

Experiments on Reversal of DCPP Inhibition of Tyrosine Oxidase by Ascorbic Acid

System	Tyrosine oxidase activity, μl. O_2 per hr. per flask	Acetoacetate production, μl. CO_2 equivalent per hr. per flask	O_2-acetoacetate ratio, μl. O_2 uptake-μl. CO_2 equivalent of acetoacetate production
Control	36	19	1.9
" + 550 γ DCPP	2	6	
" + 1000 γ ascorbic acid, followed by addition of 550 γ DCPP	98	29	3.4

is due to the failure of ascorbic acid to reverse the inhibition of the steps activated by glutathione. As pointed out earlier, this is probably due to the difference in oxidation-reduction potentials of ascorbic acid and glutathione. If this is true, the step activated by ascorbic acid would lie before the step activated by glutathione in the sequence of tyrosine oxidation reactions. Otherwise ascorbic acid would cause no reversal of the inhibition at all. One consequence of this hypothesis would be that the ascorbic acid reversal would allow the oxidation of tyrosine to proceed maximally only up to a certain point in the oxidation sequence, and the ratio of the oxygen uptake to acetoacetate formation would be significantly

greater than 2.0. An experiment designed to test this hypothesis was set up as follows. Ascorbic acid (1000 γ) was incubated with the enzyme for 10 minutes and 550 γ of DCPP were then added from one side arm and the mixture incubated for 10 minutes more. The substrate was added from the second side arm and the oxygen uptake recorded for 1 hour. Acetoacetate production was then estimated. The results of these experiments, which are presented in Table IV, indicate that the normal control oxygen to acetoacetate ratio was very close to the expected 2.0, while the system with the dye alone, as expected, gave only negligible tyrosine oxidase activity. In the system with ascorbic acid added, however, the oxygen to acetoacetate ratio is significantly greater than 2.0, substantiating the hypothesis presented in the foregoing.

DISCUSSION

From the experiments presented in this paper, the involvement of ascorbic acid indicated by other workers in activating liver tyrosine oxidase (6–8) has been substantiated from a different approach. Also it appears that glutathione may be a cofactor for the system in addition to ascorbic acid. The sites of activation by ascorbic acid and glutathione appear to be separate and distinct, the ascorbic acid-activated component lying earlier in the reaction sequence than glutathione. Experiments are now in progress to locate these points of activation more precisely.

The function of low concentrations of DCPP in stimulating the tyrosine oxidase system is somewhat obscure at present. Some possible explanations of this function are that (1) DCPP may substitute for a cofactor in the system which is labile or is present in limiting amounts, or that (2) it may act on a cofactor already present to maintain it in an active form. These possibilities are now being investigated, and preliminary results indicate that DCPP functions by substituting for a labile cofactor.

SUMMARY

1. The total tyrosine oxidase system of rat liver has been shown to be completely soluble in 0.25 M sucrose.

2. By using 2,6-dichlorophenol indophenol for the destruction of ascorbic acid and other substances of lower oxidation-reduction potential in enzyme preparations, the involvement of ascorbic acid in the system has been substantiated and glutathione has been implicated as a probable cofactor in addition to ascorbic acid. The relative positions of the sites activated by ascorbic acid and glutathione have also been indicated in these studies.

3. A unique phenomenon that low concentrations of DCPP markedly stimulate tyrosine oxidase, while high concentrations totally inhibit it, has

been investigated, and possible explanations for this effect have been discussed.

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