FURTHER STUDIES ON THE COFACTORS OF THE LIVER TYROSINE OXIDASE SYSTEM*

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In a previous communication (1) it was reported that, in addition to ascorbic acid, glutathione appeared to be required as a cofactor for a step in tyrosine oxidation. It was also shown that the site of action of ascorbic acid probably was earlier in the sequence of reactions than that of glutathione. These results were obtained with a substance known to be able to oxidize both reduced ascorbic acid and glutathione as a tool. An anomalous effect of this substance, 2,6-dichlorophenol indophenol (DCPP), was also observed, in that very low levels of the dye markedly stimulated the normal tyrosine oxidase system while much higher levels had to be employed to demonstrate the destruction of ascorbic acid and glutathione and thus to produce inhibition of the system. Some possibilities were conjectured to explain the stimulatory effect of the low DCPP concentration. The most likely among these appeared to be that the dye was able to substitute for a cofactor already present in the system, but which was either labile or present in limiting concentrations.

In the present paper these studies have been extended to explain more fully the stimulatory effect of low concentrations of DCPP on tyrosine oxidase as well as to clarify the functions of ascorbic acid and glutathione in the system.

EXPERIMENTAL

In all of these experiments, adult male rats of the Sprague-Dawley strain fed a complete stock ration were employed as experimental animals. The preparation of the tyrosine oxidase enzyme system from the livers of freshly killed rats has been described previously (1). Unless otherwise indicated, the enzyme was prepared fresh for each experiment. All the additions to the Warburg vessels except enzyme were brought to pH 7.4.

Study of Stimulatory Effect of Low DCPP Concentrations—The possibility that DCPP at low concentrations is able to substitute for a labile cofactor,

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or one present in limiting amounts, was first investigated. In these experiments the normal tyrosine system (1), containing 1.0 ml. of a Krebs-Ringer-phosphate buffer of pH 7.4 (2), 0.2 ml. of 0.15 M α-ketoglutarate, 0.2 ml. of 0.15 M L-tyrosine or water, and water to make 3.9 ml., was allowed to react for 1 hour. At the end of this period the oxidation of tyrosine had begun to fall off rapidly, indicating the possible destruction of a labile enzyme or cofactor. At that point 50 γ of DCPP were added to the system and the effect on further oxidation was measured. For the sake of comparison two other control systems were included in these experiments, one in which 50 γ of DCPP were added at the beginning of the experiment and the other in which the substrate was not added until after 1 hour to estimate the rate of inactivation of the enzyme system at 37° in the absence of substrate.

The results of these experiments are presented in Fig. 1. Here it can be seen that the normal control system rapidly decreased in activity after 1 hour of tyrosine oxidation (Curve I). When 50 γ of DCPP were added to a duplicate system after 1 hour of tyrosine oxidation, the decreased activity was immediately stimulated (Curve II). When DCPP was added at the beginning of the reaction (Curve III), the marked stimulation observed in previous experiments (1) was again demonstrated. From Curve IV, in which the tyrosine and α-ketoglutarate substrates were not added until after 1 hour, it can be seen that the enzyme system was very rapidly inactivated by incubation at 37°. From these results the following interpretations can be made: (1) The enzyme system is very labile when incubated at 37°; (2) the addition of DCPP returns some but not all of the original activity, indicating that the loss in cofactors is only partially replaceable by DCPP. The latter result is substantiated by the fact that the ultimate difference in oxygen uptake between Curves II and I is considerably less than that between Curves III and I. That portion of the lost activity not returned by DCPP has also been studied and will be reported in a later section of this paper. Since the stimulation by DCPP after the 1 hour incubation might have been due to a chemical (non-enzymatic) oxidation of an accumulated intermediate, the following experiment was carried out. After the 1 hour incubation of the enzyme with substrate, the flasks were removed and heated at 100° for 10 minutes to inactivate the enzyme. After returning the flasks to the bath, 50 γ of DCPP were added to the heated mixture from a side arm and oxidation was recorded. Since there was no further oxidation, it can be concluded that the effect of the DCPP occurs in conjunction with an enzyme.

After about 2 hours, Curves II and III also began to level off, indicating again that inactivation of the system was not completely reversible by DCPP; otherwise the oxygen uptake should not have leveled off until the

curve had reached 1340 μl. of O_2. The value of 1340 μl. of O_2 is the theoretical oxygen uptake, assuming that 4 atoms of oxygen are necessary for the complete oxidation of 1 mole of L-tyrosine to acetoacetate and fumarate. To show that the leveling off in Curves II and III is due to a loss in activity other than by the enzymatic destruction of the added DCPP, 50 γ more of DCPP were added to the flasks from which Curve III was ob-

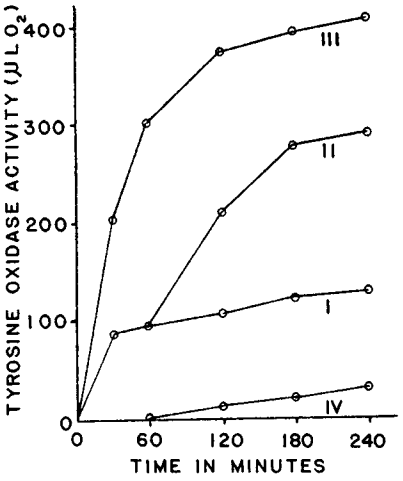


FIG. 1

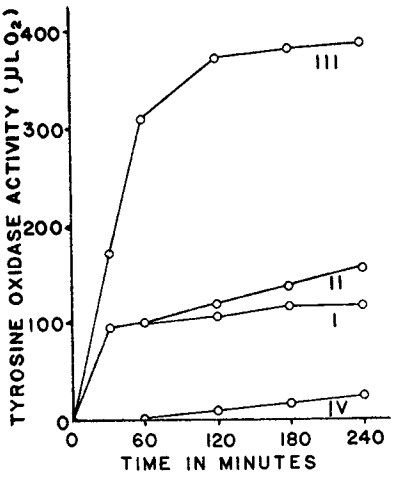


FIG. 2

FIG. 1. Effect of 2,6-dichlorophenol indophenol (DCPP) on liver tyrosine oxidase. Curve I, normal control; Curve II, effect of 50 γ of DCPP added after 1 hour; Curve III, effect of 50 γ of DCPP added at zero time; Curve IV, effect of incubating the enzyme without substrate for 1 hour. Substrate added after 1 hour. The results of five experiments are averaged together. There was no overlapping of values.

FIG. 2. Effect of ascorbic acid on liver tyrosine oxidase. Curve I, normal control; Curve II, effect of 1000 γ of ascorbic acid added after 1 hour; Curve III, effect of 1000 γ of ascorbic acid added at zero time; Curve IV, effect of incubating the enzyme without substrate for 1 hour. Substrate added after 1 hour. The results of three experiments are averaged together. There was no overlapping of values except in one case between Curves I and II.

tained. There was no further effect, either inhibitory or stimulatory, when this was done.

Since ascorbic acid is known to stimulate the tyrosine oxidase system (1, 3, 4), it was possible that the low level of DCPP might have been substituting for ascorbic acid which was inactivated during incubation. In the next studies, experiments analogous to those presented in Fig. 1 were carried out in which 1000 γ of ascorbic acid were substituted for DCPP. The results, which are presented in Fig. 2, indicate that ascorbic acid markedly stimulates the system, as observed before (1), when added at

zero time. However, when it was added after 60 minutes, when the normal tyrosine oxidation began to level off, ascorbic acid was able to stimulate the system to some extent, but not nearly as much as 50 γ of DCPP. This indicated that some endogenous ascorbic acid was lost during the incubation, but that the functions of ascorbic acid and DCPP in the system are separate and distinct and that the DCPP probably substitutes for a cofactor other than ascorbic acid.

In similar experiments with glutathione, it was shown that the functions of DCPP and glutathione are also distinct since glutathione, like ascorbic acid, was able to return very little activity to the system after the 1 hour incubation of enzyme with substrate.

Effect of Delayed Additions of High Concentrations of DCPP—In these experiments a test was made to determine whether, once the oxidation of tyrosine had proceeded, a high level of DCPP would inhibit further oxidation. In earlier experiments high concentrations of DCPP have been shown to inhibit the system totally when added at the beginning. Therefore, the normal system was allowed to oxidize tyrosine for 1 hour, and then 550 γ of DCPP were added and further oxidation was measured. The results of these experiments were almost identical with the corresponding curves in Fig. 1 (Curves I and II). In other words the high dye level no longer inhibited the system but stimulated it to the same extent as the delayed addition of the 50 γ level of DCPP. It appears then that, once the enzymes of the system are saturated with the intermediates of the reaction, substrate protection occurs and even the high level of the dye stimulates the oxidation.

Effect of Ascorbic Acid on Conversion of Homogentisic Acid to Acetoacetate and Fumarate—Because of the increased excretion of homogentisic acid and other hydroxyphenyl compounds (5–7) by scorbutic animals, it appears that one site of ascorbic function in tyrosine oxidation might be in the conversion of homogentisic acid to acetoacetate and fumarate. Evidence presented in this and a preceding paper (1) indicates that ascorbic acid also functions early in the scheme of tyrosine oxidation. Therefore, it is possible that ascorbic acid functions in more than one step in the sequence of reactions. There is also no reason to believe that ascorbic acid should function at only one step in tyrosine oxidation. To study the effect of the vitamin on the conversion of homogentisate to acetoacetate and fumarate, the following experiments were carried out. The enzyme system and other flask components were the same as in the preceding sections, except that 0.2 ml. of 0.15 M neutralized homogentisic acid was used as substrate and 0.2 ml. of water was substituted in place of α-ketoglutarate. In every case a control flask containing water in place of homogentisate was employed. The effects of 1000 γ of ascorbic acid, incubated with the

enzyme for 10 minutes before the addition of substrate, on the conversion of the substrate to acetoacetate were studied. Since autoxidation of homogentisate was so great as to give unreliable results in oxygen uptake, the formation of acetoacetate was used as the criterion for the oxidation of the substrate. The acetoacetate was estimated by the manometric method of Edson (8) after 1 hour of reaction. To rule out the possibility that homogentisic acid oxidase is the site of stimulation by DCPP, the effect of 50 γ of DCPP on acetoacetate formation from homogentisate was also studied. The results of these experiments are reported in Table I. Here it can be seen that ascorbic acid markedly stimulates the production of acetoacetate from homogentisic acid. On the other hand, DCPP inhibits the reaction, indicating that the point of DCPP stimulation lies earlier in the sequence of tyrosine oxidation than on the oxidation of homogentisate. Since other hydroxyphenyl compounds as well as homogentisic acid are

TABLE I

Effects of Ascorbic Acid and DCPP on Conversion of Homogentisic Acid to Acetoacetate

System	Acetoacetate formation
	μl. CO_2 equivalent per hr.
Control	32
" + 1000 γ ascorbic acid	70
" + 50 γ DCPP	13

excreted by scorbutic animals (5–7), this is further evidence that ascorbic acid may function at more than one step in tyrosine oxidation. The results of these experiments and previously reported work (1) indicate that this is probably the case.

Effects of Various Possible Cofactors on Tyrosine Oxidase of Aged Enzyme System—From Fig. 1, Curve IV, it can be seen that incubation of the enzyme at 37° for 1 hour in the absence of substrate causes a marked loss in tyrosine oxidase activity. As pointed out earlier, aging by incubation probably causes a loss in labile cofactors and possibly loss in activity from simple protein denaturation. In order to observe whether the possible cofactors, ascorbic acid, glutathione, DCPP, and an extract of boiled fresh enzyme, could return this activity lost during incubation, the following experiments were carried out. 0.5 ml. of the fresh enzyme was incubated in the Warburg vessels with 1.0 ml. of the Krebs-Ringer-phosphate buffer and 0.7 ml. of water for 1 hour. At the end of that time the effects of 1000 γ of ascorbic acid, 3120 γ of glutathione, 50 γ of DCPP, and 0.5 ml. of an extract of boiled fresh enzyme on the loss in activity were tested separately and in the various combinations reported in Table II. The

volumes of these substances added were adjusted so that, when 0.2 ml. of 0.15 M L-tyrosine and 0.2 ml. of 0.15 M sodium α-ketoglutarate were added, the final volume in the flask was 3.9 ml. The substrates were also added after the 1 hour incubation. The extract of boiled fresh enzyme was prepared by heating a fresh enzyme preparation for 7 minutes at 100° and centrifuging at 25,000 × *g* for 30 minutes. From the results in Table II it can be seen that aging in the absence of substrate markedly reduces the tyrosine oxidase activity of the fresh enzyme. The addition individually of ascorbic acid, glutathione, 50 γ of DCPP, or extract of boiled fresh enzyme significantly stimulates activity of the aged enzyme. When the various combinations of cofactors were added to the system, as indicated in

TABLE II

Effects of Various Cofactors on Tyrosine Oxidase of Liver Enzyme Aged for 1 Hour at 37°

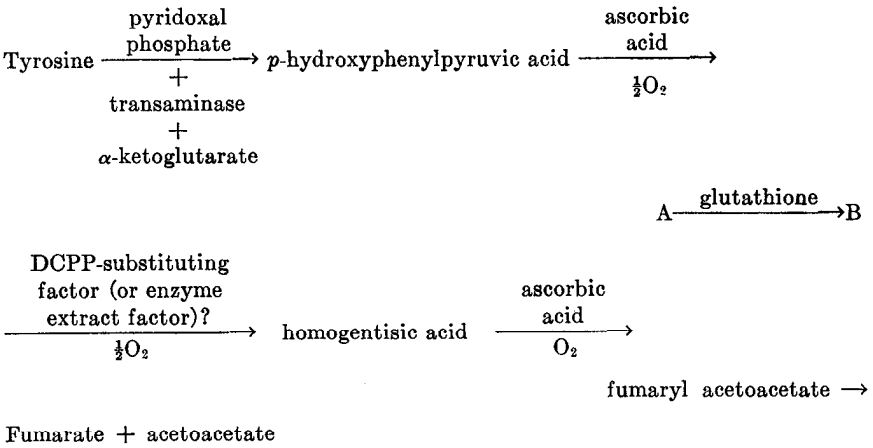
System	Tyrosine oxidase activity	Difference in aged control and experimental flasks	Sum of individual values
	μl. O_2 per hr.	*μl. O_2 per hr.*	
Normal control	153		
Control aged 1 hr.	72		
" " 1 " + 1000 γ ascorbic acid (AA)	164	92	
" " 1 " + 3120 " glutathione (GSH)	151	79	
" " 1 " + 50 γ DCPP	134	62	
" " 1 " + enzyme extract (EE)	98	26	
" " 1 " + AA + GSH	218	156	171
" " 1 " + " + " + EE	258	186	197
" " 1 " + " + " + DCPP + EE	329	257	259

Table II, in every case the effects were additive and closely approached the effects of the sum of the individual substances (Table II). Since very large excesses of both ascorbic acid and glutathione were added to the system, the stimulatory effects of the DCPP and the boiled enzyme extract were probably at a different site of the reaction sequence of the system. Otherwise addition of DCPP or enzyme extract would have caused no further stimulation over that contributed by ascorbic acid and glutathione. This reasoning also holds true for the additive effects of ascorbic acid and glutathione, which is additional evidence (1) that these two factors stimulate different steps in tyrosine oxidation.

DISCUSSION

From the results of a previous communication (1) and the present paper, it appears that the tyrosine oxidase system requires at least two more co-

factors than ascorbic acid. One, glutathione, appears to function later than the first site of activation by ascorbic acid. Also ascorbic acid, besides stimulating early in the series of reactions, appears to stimulate again later in the conversion of homogentisic acid to acetoacetate and fumarate. The stimulatory effect of DCPP must lie earlier than homogentisic acid oxidase since it inhibits that step, probably by destroying endogenous ascorbic acid under the conditions under which that step was studied. There is also evidence that a factor or factors in an extract of boiled fresh enzyme activate some step or steps other than those activated by ascorbic acid, glutathione, and DCPP, since, when it is added in addition to those substances, further stimulation of a partially inactivated enzyme occurs. The possibility must not be overlooked, however, that the labile factor for which DCPP is substituting may be the same as the factor in the enzyme extract. Further studies are now in progress to observe whether the two effects can be differentiated. According to the evidence presented in this and a preceding report (1), the accompanying scheme might be employed to locate the sites of action of the various cofactors.



Experiments are now in progress to characterize the factor in the extract of boiled enzyme and to extend the studies presented in this paper.

SUMMARY

1. Evidence has been presented to indicate that ascorbic acid functions in two places in the tyrosine oxidase system, one early in the scheme and the other in the conversion of homogentisic acid to acetoacetate and fumarate.

2. In addition to ascorbic acid, glutathione and a labile factor for which a low concentration of 2,6-dichlorophenol indophenol can substitute appear to be additional cofactors required by the system.

3. The probable sites of action of these cofactors in the oxidation of tyrosine have been indicated according to the evidence presented.

BIBLIOGRAPHY

1. Williams, J. N., Jr., and Sreenivasan, A., *J. Biol. Chem.*, **203,** 109 (1953).
2. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and tissue metabolism, Minneapolis (1949).
3. Painter, H. A., and Zilva, S. S., *Biochem. J.*, **46,** 542 (1950).
4. Sealock, R. R., Goodland, R. L., Sumerwell, W. N., and Brierly, J. M., *J. Biol. Chem.*, **196,** 761 (1952).
5. Sealock, R. R., and Silberstein, H. E., *J. Biol. Chem.*, **135,** 251 (1940).
6. Woodruff, C. W., Cherrington, M. E., Stockell, A. F., and Darby, W. J., *J. Biol. Chem.*, **178,** 861 (1949).
7. Salmon, R. J., and May, C. D., *J. Lab. and Clin. Med.*, **36,** 591 (1950).
8. Edson, N. L., *Biochem. J.*, **29,** 2082 (1935).