THE STABILITY AND ACTIVATION OF THE LIVER TYROSINE OXIDASE SYSTEM*

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In recent communications from this laboratory (1, 2) it has been reported that ascorbic acid appears to activate the liver tyrosine oxidase system at two points, one early in the sequence of reactions and the other in the conversion of homogentisic acid to acetoacetate and fumarate. In addition, glutathione has been shown to activate at least one step in tyrosine oxidation, and the indications were that still another labile cofactor for which low concentrations of 2,6-dichlorophenol indophenol could substitute was involved in the system. An extract of boiled fresh enzyme was also shown to reactivate an aged enzyme preparation over and above the stimulation by excess ascorbic acid and glutathione.

In the present paper we wish to report further studies on the rôles of ascorbic acid, glutathione, the indophenol-substituting cofactor, and the enzyme extract factor in the reactivation of partially inactivated tyrosine oxidase preparations. In these studies the effects of aging and dialysis on the enzyme system and the restoration of activity by the various possible cofactors have been investigated.

Several workers have indicated that folic acid may be involved as a cofactor in the tyrosine oxidase system (3, 4). However, Gabuzda (5) has observed that the *Leuconostoc citrovorum* factor has no effect on the excretion of tyrosyl derivatives by scorbutic patients. We have reinvestigated this problem in our system and the results are included in this paper.

EXPERIMENTAL

Comparison of Effects of Aging and Dialysis on Liver Tyrosine Oxidase—Normal, adult male rats of the Sprague-Dawley strain were used as experimental animals. The tyrosine oxidase enzyme preparations used in all of the studies reported in this paper were obtained as previously outlined (1). In brief, this consisted of making 16.7 per cent rat liver homogenates in 0.25 M sucrose, centrifuging at 0° (25,000 × *g*) for 30 minutes, and using the supernatant fluid as the source of tyrosine oxidase. This system, in-

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cluding the transaminase required for the first step in the sequence of reactions, was found to be located entirely in the supernatant fluid.

The enzyme was divided into two portions. One portion was aged at 5° in a stoppered flask and the other portion was dialyzed against distilled water at 5° for various lengths of time, depending upon the experiment. The normal system to test the activity of enzyme was measured manometrically as follows. To the main compartment of Warburg vessels with double side arms were added 1.0 ml. of Krebs-Ringer-phosphate buffer of pH 7.4 (6), 0.7 ml. of water, and 0.5 ml. of the enzyme preparation. The substrate (0.2 ml. of 0.15 M L-tyrosine suspension and 0.2 ml. of 0.15 M sodium α-ketoglutarate) and 0.2 ml. of water were placed in one side arm. For the control flask, 0.2 ml. more of water was substituted in place of L-tyrosine. When the effect of other substances on the activity of the enzyme was studied, they were introduced into the second side arm. Water was added to the second side arm in all cases to bring the final volume in the flask, exclusive of 0.2 ml. of 10 per cent potassium hydroxide in the center well, to 3.9 ml. After a 10 minute temperature equilibration period, the contents of the side arms were added to the main compartment and the oxygen uptake was recorded for 1 hour. All the solutions were brought to pH 7.4 before being added to the flasks. The results of a typical experiment of this type are shown in Fig. 1. Here it can be seen that both aging and dialysis markedly decrease activity of the system. At the end of 72 hours, however, the activity of the dialyzed system is somewhat lower than that of the aged system. This difference is significant since, in every experiment continued over 24 hours, the aged enzyme activity was higher than that of the dialyzed enzyme. These results support previous experiments (2) in which it was found that incubation of the enzyme for 1 hour at 37° in the absence of substrate markedly decreased the activity.

If the lability of the enzyme system during aging is due to destruction of labile cofactors, addition of these cofactors to the aged enzyme should return some of the lost activity. Similarly, if the decrease in activity of the dialyzed enzyme is due to removal of dialyzable cofactors, the activity should be returned if the correct cofactors are added to the dialyzed system, unless the loss in activity is due entirely to protein denaturation. Therefore, in the next experiments the effects of adding ascorbic acid and 2,6-dichlorophenol indophenol (DCPP) to aged and dialyzed enzymes were studied. In these experiments 1000 γ of ascorbic acid or 50 γ of DCPP were added to the enzyme with the substrate and the resulting activity was measured. The enzyme preparation had been aged or dialyzed for 56 hours. The results of these experiments, presented in Table I, indicate that both ascorbic acid and DCPP stimulate the aged preparation but that only ascorbic acid stimulates the dialyzed preparation. The stimulation

by ascorbic acid is considerably greater in the aged preparation than in the dialyzed preparation. This might be expected since dialysis would remove

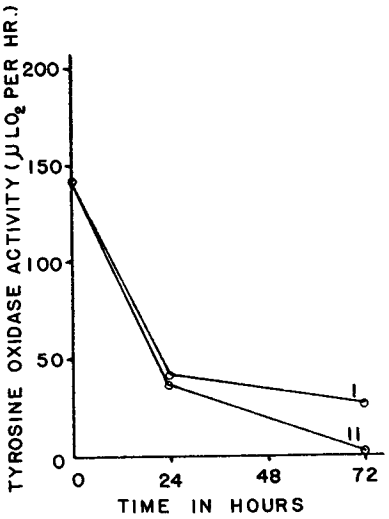


FIG. 1. A comparison of the effects of aging and dialysis at 5° on tyrosine oxidase activity of rat enzyme preparations.

TABLE I

Comparison of Aged and Dialyzed Tyrosine Oxidase Preparations with Respect to Stimulation by DCPP and Ascorbic Acid

System	Tyrosine oxidase activity* per flask of fresh enzyme and enzyme aged or dialyzed for 56 hrs.	
	Fresh	After 56 hrs.
	μl. O_2 per hr.	*μl. O_2 per hr.*
Dialyzed, control	100	16
" " + DCPP	212	17
" " + ascorbic acid	178	52
Aged, control	100	28
" " + DCPP	212	45
" " + ascorbic acid	178	86

* Average of four experiments.

other cofactors required for the complete conversion of tyrosine to acetoacetate and fumarate, while in the aged preparation at least small amounts of the cofactors besides ascorbic acid would probably still be present. Therefore, the addition of an excess of one of these cofactors, in this case

ascorbic acid, would allow the total reaction to proceed at a higher rate than in the dialyzed preparation. The lack of stimulation of the dialyzed preparation by DCPP is also to be expected since previous work has shown that stimulation by the dye occurred only if the dye was kept mainly in the reduced form (1), or if the substrate was in contact with the enzyme for some time before the dye was added (2). Since dialysis would remove reducing substances, normally present in the enzyme preparation, which could maintain the oxidized form of the dye at a low level, the DCPP would no longer stimulate the system. It should be pointed out that stimulation by DCPP might occur through its oxidized form and that the purpose of reducing it first is to maintain the oxidized form at a very low level. Undoubtedly some oxidation of the reduced dye by air occurs even in the presence of excess reducing agents.

Effects of Other Known Cofactors on Tyrosine Oxidase System—In the next experiments various substances known to be required by certain oxidative enzymes were studied for their effects on tyrosine oxidase. Diphosphopyridine nucleotide (DPN) has been reported (7) to be required for the enzymatic conversion of phenylalanine to tyrosine. Flavin-adenine dinucleotide (FAD) is known to be the prosthetic group of certain soluble terminal oxidases of liver, *e.g.* xanthine oxidase and D-amino acid oxidase. Folic acid has been reported to stimulate tyrosine oxidation *in vitro* (3, 4).

The effect of DPN was studied by adding 2 mg. of the coenzyme to tyrosine oxidase preparations partially inactivated by incubation for 1 hour at 37°. The results of these experiments indicated that the aged enzyme with added DPN gave the same activity as in its absence. Therefore, DPN is probably not a cofactor of the system.

It has been shown that quinine markedly inhibits the activity of FAD-activated enzymes (8, 9). Therefore, if FAD is one of the cofactors of the tyrosine oxidase system, the addition of quinine to a normal system should inhibit its activity. When 2240 γ of quinine sulfate were added to the system containing fresh enzyme, no effect was obtained.

Because of conflicting reports in the literature concerning a possible function of folic acid in activating the tyrosine oxidase system (3–5), we have investigated the *in vitro* effect of folic acid and the *L. citrovorum* factor (LCF) on the enzyme systems. Since LCF can be synthesized enzymatically from ascorbic acid and folic acid, the effect of a combination of ascorbic acid and folic acid was also studied. The levels of folic acid, ascorbic acid, and synthetic LCF (leucovorin) added to the normal system, with a fresh enzyme preparation, are presented with the results in Table II. Here it can be seen that folic acid appears to stimulate the oxidation of tyrosine. Whenever it was included, either with or without ascorbic acid, the stimulation due to folic acid was the same. LCF had no demon-

strable effect. In these experiments it was noted that the endogenous respiration of the control flask (without substrate) was almost completely inhibited by the added folic acid. This suggested the possibility that the apparent stimulation by folic acid could be explained by its inhibitory effect on endogenous respiration, which for some reason was inhibited to a greater extent in the flasks without tyrosine substrate than in the flasks containing the substrate. It is known that ordinary solutions of folic acid are decomposed by light to give a photolytic product of the vitamin, 6-formylpteridine (10–12). This product strongly inhibits liver xanthine oxidase, which is a soluble enzyme and, therefore, is present in the tyrosine oxidase preparations used in these experiments. If much of the endogenous respiration of the enzyme preparation is due to oxidation of endogenous

TABLE II

Effects of Folic Acid, LCF, and 6-Formylpteridine on Tyrosine Oxidase

System	Tyrosine oxidase activity per flask*
	μl. O_2 per 30 min.
Control	41
" + 200 γ folic acid	71
" + 1000 γ ascorbic acid	182
" + 200 γ folic acid + 1000 γ ascorbic acid	216
" + 200 γ LCF	38
" + 2 γ 6-formylpteridine	79

* Average of three to six experiments.

purine substrates via xanthine oxidase, folic acid would inhibit it because of the presence of 6-formylpteridine in the vitamin. Therefore, this point was checked by studying the effect of purified 6-formylpteridine on the tyrosine oxidase system. The results of these experiments, also presented in Table II, indicate that the same stimulation was given by 2 γ of 6-formylpteridine as by 200 γ of folic acid. Therefore, it appears that the "stimulation" of tyrosine oxidase by folic acid is an artifact and can be explained on the basis of the inhibition of endogenous respiration rather than by a positive stimulation of the system. Why there should be more inhibition of endogenous respiration in the flasks without substrate than in those with tyrosine added is still open to question, however.

Addition of Cofactors to Dialyzed Enzyme—Since dialysis may remove cofactors from the tyrosine oxidase system, a means was offered for studying the effects of various cofactors believed to be involved in the system. Thus the positive effects of ascorbic acid, glutathione, and unknown cofactors in an extract of boiled fresh enzyme could be studied. Since di-

alysis would remove pyridoxal phosphate, which is the coenzyme for the transamination of tyrosine, this coenzyme was included to make the list as complete as possible. To check the specificity of glutathione, the effect of cysteine was also studied. In these experiments a fresh enzyme preparation was dialyzed against distilled water for 72 hours. The following substances were then tested for their activity in the system: ascorbic acid 1000 γ, glutathione 1560 γ (equivalent to 500 γ of ascorbic acid), cysteine hydrochloride 890 γ (equivalent to 500 γ of ascorbic acid), pyridoxal phosphate 10 γ, and 0.5 ml. of an extract of fresh enzyme prepared by heating the fresh enzyme at 100° for 10 minutes and centrifuging at 25,000 $\times$ g for

TABLE III

Response of Dialyzed Tyrosine Oxidase to Various Possible Cofactors

System	Tyrosine oxidase activity per flask*
	µl. O_2 per hr.
Dialyzed enzyme control	5
Plus ascorbic acid (AA)	32
" glutathione (GSH)	5
" extract of fresh enzyme (EE)	7
" pyridoxal phosphate (B_6PO_4)	5
" AA + GSH	53
" " + cysteine	28
" " + GSH + B_6PO_4	75
" GSH + B_6PO_4	0
" AA + GSH + EE	103
" " + " + " + B_6PO_4	99

* Average of six experiments.

30 minutes to remove coagulated protein. These substances were tested in various combinations, as shown in Table III. The results of these experiments show that ascorbic acid alone stimulated the system to some extent, while glutathione, pyridoxal phosphate, and the enzyme extract had no effect when added individually. However, a combination of ascorbic acid and glutathione gave an increased stimulation over ascorbic acid alone which was further increased by pyridoxal phosphate. When cysteine was added with ascorbic acid, no effect except that of ascorbic acid was observed, indicating that the stimulation by glutathione is not due to a non-specific sulfhydryl effect. The combination of glutathione and pyridoxal phosphate showed no activity over the dialyzed control. When ascorbic acid, glutathione, and the fresh enzyme extract were added, the maximal stimulation occurred which was not further increased by adding pyridoxal phosphate. It should be emphasized that the levels of ascorbic

acid and glutathione employed are in large excess in the system, so that effects of other substances added with them should be indicative of distinctive effects of those substances. An interpretation of these results can be summarized as follows. If the first oxidative step in the series of reactions is activated by ascorbic acid, some stimulation of oxygen uptake by ascorbic acid alone would be expected, although the complete oxidative pathway to acetoacetate and fumarate would still not be opened. This supports previous conclusions (1, 2) that one of the sites of action of ascorbic acid is an early step in tyrosine oxidation. If the activation by glutathione occurred at a step later than that activated by ascorbic acid, no effect of glutathione alone would be expected. Even a combination of glutathione and pyridoxal phosphate would not increase oxidation if no ascorbic acid were present to activate the first oxidative step. The results of Table III support these conclusions. The observation that the enzyme extract alone had no effect on the activity can probably be explained by the destruction of ascorbic acid and glutathione during the heat extraction of the enzyme. Reduced glutathione concentration was found by analysis to be very low in the heated extract. Since the heated enzyme extract in combination with excess ascorbic acid and glutathione, however, gave the maximal stimulation in these experiments, it appears to contain a third cofactor necessary for tyrosine oxidase activity. Undoubtedly the heated extract contains pyridoxal phosphate, but since pyridoxal phosphate with ascorbic acid and glutathione gave less activity than ascorbic acid, glutathione, and the heated extract, the extract appears to contain both pyridoxal phosphate and a factor different from pyridoxal phosphate. This conclusion is supported also by the fact that the addition of pyridoxal phosphate to a combination of ascorbic acid, glutathione, and the heated extract gave no more stimulation than if pyridoxal phosphate were omitted.

Similarity of Effects of Enzyme Extract Factor and DCPP—In these experiments a combination of ascorbic acid (1000 γ), glutathione (3120 γ), pyridoxal phosphate (10 γ), and fresh enzyme extract (0.5 ml.) were added to a dialyzed enzyme (72 hours) and the reactivation compared with the effects of the same combination plus 50 γ of DCPP. If the enzyme extract factor acted in a manner similar to DCPP, no further stimulation by DCPP would be observed over the complete combination of other factors. The results of these experiments after 30 minutes of tyrosine oxidation were as follows: (1) all the factors except DCPP, 33 μl. of O_2; (2) all the factors including DCPP, 34 μl. of O_2. After 1 hour, all the factors except DCPP gave 61 μl. of O_2, and with DCPP 69 μl. of O_2. These results again support the conclusion that the enzyme extract factor is the same as the DCPP-substituting factor. The observation that, after 1 hour, an effect of DCPP began to appear indicates that the enzyme extract factor

is somewhat labile to incubation as reported previously (2) and that the dye can begin to take over its function as the natural factor becomes inactivated.

Ionic Nature of Heated Enzyme Extract Factor—In these experiments, the heated enzyme extract was passed through an ion exchange column to see whether it possessed enough ionic nature to be removed. An enzyme extract was made by heating a fresh enzyme preparation at 100° for 10 minutes as before. A portion of the extract was passed through a 100 mg. Dowex 1 column and used in the following experiments. The flask components were the same as those listed previously. A fresh tyrosine oxidase preparation was dialyzed against distilled water at 5° for 56 hours and used as the source of enzyme. The effects of 0.5 ml. of fresh enzyme extract, treated with Dowex and untreated, were studied in combination with 1000 γ of ascorbic acid, since ascorbic acid is necessary for the initial oxidative reaction of the system. The results of these experiments are shown in Table IV. Here it can be observed that the extract treated with Dowex alone has no stimulatory effect on the dialyzed enzyme preparation but is slightly inhibitory. In combination with ascorbic acid some stimulation is obtained, which is probably due mainly to the ascorbic acid. The untreated extract alone gives some stimulation in contrast to the results of Table III, although this can be explained by the shorter dialysis in the present experiment in which all of the enzyme ascorbic acid may not have been removed. This is substantiated by the higher activity of the dialyzed enzyme control in Table IV as compared to Table III. When a combination of untreated extract and ascorbic acid was added, a marked stimulation occurred which was much greater than when the treated with Dowex extract was employed. Therefore, these results indicate that the factor in

TABLE IV

Effect of Passing Extract of Fresh Enzyme (EE) through Dowex 1 upon Its Ability to Stimulate Dialyzed Enzyme

System	Tyrosine oxidase activity per flask*
	μl. O_2 per hr.
Dialyzed enzyme control	28
" " " + Dowex 1-treated EE	15
" " " + " " + ascorbic acid	48
" " " + untreated EE	41
" " " + " " + ascorbic acid	99

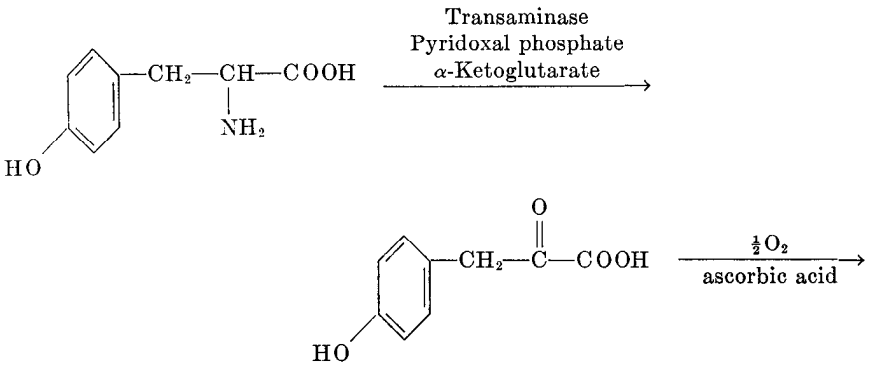
* Average of three experiments.

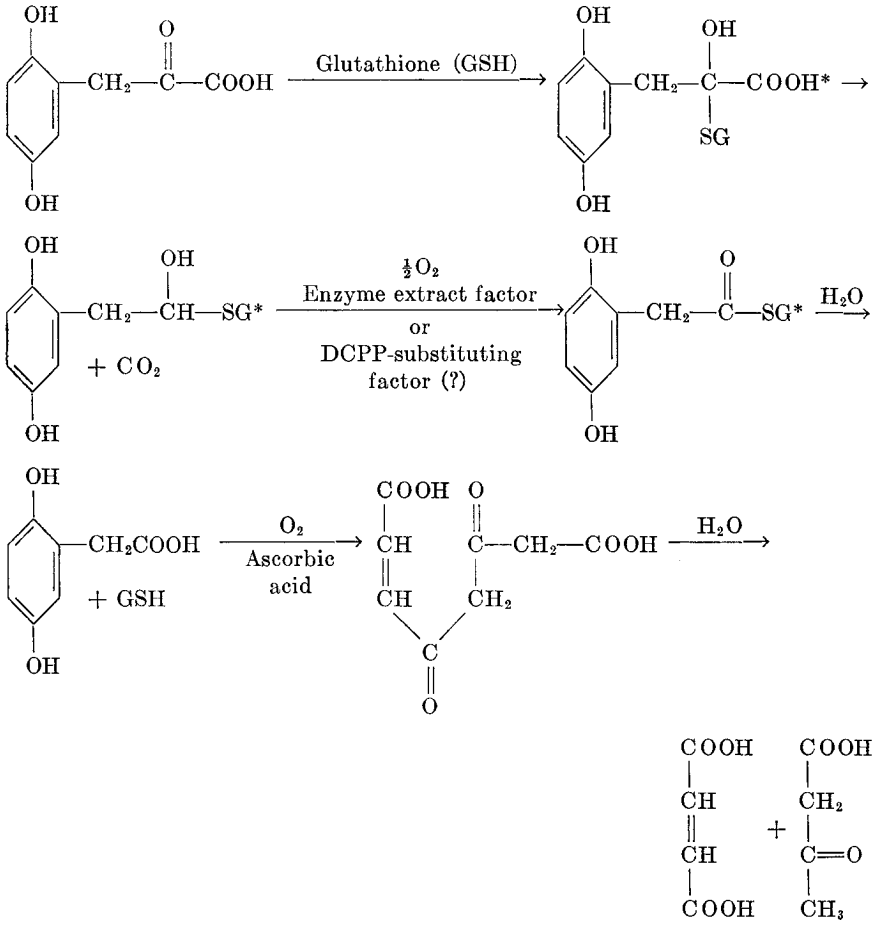
the enzyme extract responsible for activating a step in the tyrosine oxidase system is anionic in nature, since it can be completely removed by passing through Dowex 1.

DISCUSSION

The results of this paper confirm and extend the results of previous reports (1, 2) in that ascorbic acid appears to be entirely essential for tyrosine oxidation and the activation by glutathione occurs at a step later than the early activation by ascorbic acid. Ascorbic acid was also shown earlier (2) to activate homogentisic acid oxidation, so that the vitamin appears to act at two points in the reaction sequence, one at the first oxidative step and the other after the formation of homogentisic acid. The stimulation by low levels of 2,6-dichlorophenol indophenol has been explained on the basis that it substitutes for a labile cofactor which is different from either ascorbic acid or glutathione. Experiments have also been reported which indicate that the dye must be reduced before stimulation can occur. The factor in the heated enzyme extract which gives activation in addition to ascorbic acid, glutathione, and pyridoxal phosphate may be identical with the DCPP-substituting factor. The anionic nature of the heated enzyme extract factor may place it in the same category as other anionic cofactors, but the evidence indicates that it is probably not DPN or FAD.

A summary of the possible sites of action of the cofactors studied in this and previous papers (1, 2) may be outlined as follows. Some of the intermediates listed in the series are known, while others, indicated with an asterisk, are surmised by the authors and remain to be definitely proved. The general mechanism of action of glutathione as presented here is analogous to that suggested by Racker and Krimsky for the function of glutathione in glyceraldehyde-3-phosphate dehydrogenation (13).





SUMMARY

1. Rat liver tyrosine oxidase is rapidly inactivated by aging and dialysis.

2. By using the dialyzed enzyme, the activity can be returned by adding a combination of ascorbic acid, glutathione, and an extract of heated fresh enzyme. In addition to furnishing pyridoxal phosphate for tyrosine transamination, the enzyme extract contains a factor besides ascorbic acid and glutathione necessary for maximal tyrosine oxidation. If low concentrations of 2,6-dichlorophenol indophenol are reduced and kept mainly in the reduced form, stimulation of the system occurs. The DCPP-substituting factor and the enzyme extract factor are possibly identical. The unknown factor has been shown to be anionic in nature.

3. The interrelationships of the various cofactors for tyrosine oxidase

have been discussed, and their possible sites of action in the oxidation of tyrosine to acetoacetate and fumarate have been tentatively indicated.

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