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STUDIES ON BACTERIAL URATE:OXYGEN OXIDOREDUCTASE
I. PURIFICATION AND PROPERTIES OF THE ENZYME

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SUMMARY

1. The urate oxidase (urate:oxygen oxidoreductase, EC 1.7.3.3) of *Arthrobacter pascens* which was isolated from soil was purified.

2. The purified enzyme has shown to be homogeneous by ultracentrifugation and Tiselius electrophoresis, and some properties were investigated.

3. Ultraviolet spectrum and Michaelis constant (K_m) were shown to be different from those of the urate oxidase of pork liver.

4. The enzyme has a pH optimum at 9.2 and is stable at high pH.

5. The enzyme showed reversible inactivation in low buffer concentration, which was considered to be parallel with the conformational change of the enzyme protein from some evidence obtained.

6. Among the effects of metals and chelating agents on the activity of the enzyme, Cu^{2+} showed remarkable inhibition and Fe^{3+} showed slight stimulation. Strong inhibitory action of neo-cuproin (2,9-dimethyl-1,10-phenanthroline) and weak inhibitory action of *o*-phenanthroline and α,α'-dipyridyl were observed.

These properties of *A. pascens* enzyme were compared with the liver enzyme.

INTRODUCTION

In 1909, BATELLI AND STERN[1] first isolated urate oxidase (urate:oxygen oxidoreductase, EC 1.7.3.3) formerly known as uricase, which oxidized uric acid to unidentified products in animal tissue. Since then, numerous investigators[2–11] have reported purification procedures, the properties of the enzyme and the reactionproducts of this enzyme. In 1955, MAHLER *et al.*[9] first succeeded in isolating the enzyme in a purified form (mol.wt. 100000), from the mitochondrial fraction of pork liver cells, and showed that the enzyme contained 1 atom of copper per molecule of enzyme. They also proposed that Cu^{2+} which would be tightly bound to the enzyme was necessary for the enzyme activity and provided a means of the attachment of the sub-

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strate on the enzyme. Since then, urate oxidase has been presumed to be a cuproprotein.

The urate oxidase in microorganisms has been observed in many microbes, and was first purified in *Neurospora crassa* by GREEN AND MITCHELL[12]. They reported that 400 times purified enzyme had, in many respects, the same properties as the enzyme prepared from the pork liver cells by MAHLER.

In our present investigation we tried to prepare this enzyme from the bacterial cells in order to use it clinically as a reagent for the quantitative determination of uric acid in blood. The enzyme of the bacterial cells showed some interesting properties which were different from those of the mammalian liver enzyme. Moreover, urate oxidase seemed to be a good material with which to study the activation mechanism of molecular oxygen in oxidase or oxygenase reactions. So we studied the biochemical properties of this bacterial urate oxidase.

In the present paper, we report the purification of the urate oxidase of *Arthrobacter pascens* and some of its properties, which are compared with those of mammalian liver enzyme.

METHODS

1. *The growth of bacteria*

Arthrobacter pascens, which was isolated from the soil, was grown in the following nutrient medium (in g/l): powdered meat extract, 20; glucose, 5; uric acid, 0.05; soybean oil as an antifoaming agent, 2; and adjusted to pH 7.5. 9 l of preincubated culture were inoculated into 350 l of sterilized medium and cultured for 28 h at 30° with the aeration of 350 l of air per min. The cells were collected by centrifugation using a Sharples super centrifuge. The cell paste (13 kg wet wt.) was inoculated again into 400 l of sterilized 0.5% uric acid solution containing KH_2PO_4, 0.1%, Fries mineral solution[13], 0.1% (v/v), yeast extract, 0.05%, in distilled water at pH 7.5. After 13 h of incubation at 26° with aeration, the cells were harvested in the same way, yielding 12 kg of wet cells, and stored in the deep-freezer until use.

2. *Assay method*

Urate oxidase was assayed spectrophotometrically by the measurement of the disappearance of uric acid. A suitable volume (0.02–0.5 ml) of the enzyme solution was added to a quartz cuvette (1.0-cm light path) containing 0.2 M borate buffer (pH 8.6) to give 2.0 ml vol. The reaction was initiated by the addition of 1.0 ml of a freshly prepared solution of $5 \cdot 10^{-4}$ M uric acid (neutralized to pH 8.6 with LiOH) and followed by the measurement of the decrease in absorbance at 295 mμ of the reaction mixture at 30° against a blank. After the first 20 sec, $A_{295\ m\mu}$ was read at 20-sec intervals for 80 sec to ascertain if the reaction kinetics were of zero order. If the $\Delta A_{295\ m\mu}$/40 sec was in the range of 0.010–0.080, the reaction was proportional to the enzyme concentration. This assay method was a modification of MAHLER's[9] method which was not entirely suitable for the assay of our bacterial enzyme.

Enzyme activity was conveniently expressed as $\Delta A_{295\ m\mu}$/40 sec, or as units which were defined as μmoles of oxidized uric acid per min at 30°. In our assay method, 1 unit corresponded to $\Delta A_{295\ m\mu}$/40 sec $\times$ 3/8. Specific activity was defined as units per unit of absorbance at 280 mμ conveniently, or units/g protein, as calculated by the use of the Warburg–Christian equation[14].

RESULTS

1. Preparation of crude enzyme solution

The bacterial cell paste (5 l) was mixed with 2 l of 0.2 M borate buffer (pH 8.6), and was sonicated in 60–80-ml portions for 15 min in a Kubota (Japan) 200 W, 10 kcycles/sec sonicator. The sonicated cells were then centrifuged for 40 min at 40 000 $\times$ *g* in a Spinco model L centrifuge. The enzyme was contained in the supernatant fraction (yield 5 l).

2. Purification

The following operations were carried out between 0–4° unless otherwise described.

Step I. Ammonium sulfate fractionation.

The supernatant solution (5 l) described above was diluted with an equal volume of 0.2 M borate buffer (pH 8.6). 3040 g of ammonium sulfate were slowly added with stirring. After 3 h the precipitate formed was removed by centrifugation (40 000 $\times$ *g*, 20 min). An additional 1970 g of ammonium sulfate was added. In these salting out steps, the pH of the fluid was kept at pH 8.0–9.0 with 1 M NaOH solution. After another 3 h the resulting precipitate, which contained almost all the activity of the enzyme, was recovered by centrifugation (40 000 $\times$ *g*, 20 min) and dissolved in about 500 ml of 0.2 M borate buffer (pH 8.6) to obtain a volume of 570 ml.

Step II. Acetone fractionation

415 ml of cold acetone (Analar) which was previously cooled in the deep-freezer to −20°, was slowly dropped into the salting out preparation (570 ml) with stirring. After 5 h at 0°, the precipitate formed was removed by centrifugation (10 000 $\times$ *g*, 15 min). An additional 520 ml of cold acetone was added to the above supernatant (910 ml). After 3 h at 0°, the resulting precipitate, which contained the enzyme activity, was recovered by centrifugation and suspended in about 200 ml of 0.2 M borate buffer (pH 8.6). After another 16 h, the denatured protein was removed by centrifugation (10 000 $\times$ *g*, 15 min) to obtain clear supernatant fluid. This fraction was dialysed overnight against 1 l of 0.2 M borate buffer containing 0.1 M ammonium sulfate (pH 8.6) with gentle stirring. The dialysed solution (267 ml) was then subjected to a second acetone fractionation, recovering the supernatant of 50% (v/v) acetone and the precipitate of 60% (v/v) acetone. The enzyme solution obtained was then dialysed against 0.2 M borate buffer containing 0.1 M ammonium sulfate (pH. 8.0).

Step III. DEAE-cellulose column chromatography

Acetone-fractionated preparation (90 ml) was adsorbed to a DEAE-cellulose column (10 cm^2 $\times$ 50 cm) which had been equilibrated with 0.1 M borate buffer (pH 8.0) containing 0.1 M ammonium sulfate. After washing the column with 1100 ml of the starting buffer, the elution was carried out by the application of a linear gradient of ammonium sulfate. The mixing chamber contained 550 ml of 0.1 M borate buffer *plus* 0.1 M ammonium sulfate (pH 8.0), and the reservoir held the same volume of this buffer containing 0.4 M ammonium sulfate (pH 8.0). 10-ml fractions were collected automatically at an hourly flow of 5 ml/cm^2 of effluent.

The enzyme was eluted in the range of ammonium sulfate concentrations between 0.14 and 0.18 M. The peak fractions (40 ml) were combined and condensed by salting out of ammonium sulfate and dialysed overnight against 1 l of the same buffer.

TABLE I

YIELD AND ACTIVITY OF *A. pascens* URATE OXIDASE IN FRACTIONATION
In this table the specific activity was expressed as units/$A_{280\ m\mu}$ unit.

Preparation	*Total unit (units)*	*Recovery (%)*	*Total* $A_{280\ m\mu}$	*Specific activity (units/* $A_{280\ m\mu}$*)*
Cell fluid supernatant	6 400			5
Salting out product	10 740	100	50 600	212
Acetone fraction I	7 400	69	4 000	1 850
Acetone fraction II	6 100	57	1 350	4 500
DEAE-cellulose prepn.	1 400	13	177	7 900
DEAE-Sephadex prepn.	1 260	11.7	62.5	20 500

Step IV. DEAE-Sephadex column chromatography

The enzyme solution (21 ml) obtained above, was introduced into 3 $cm^2 \times 30$ cm column of DEAE-Sephadex A-50 which had been equilibrated with 0.1 M borate buffer containing 0.1 M ammonium sulfate (pH 8.0). The elution procedure was almost the same as that on DEAE-cellulose column chromatography. From the elution pattern the enzyme activity almost overlapped the protein curve. The active eluate was condensed and submitted to Tiselius electrophoresis and ultracentrifugation. The results of the above purification procedures were summarized in Table I. The purified enzyme preparation was stored until use at $-15°$ in 0.1 M borate buffer (pH 8.0) containing 0.1 M or more concentrated ammonium sulfate.

3. Properties of the purified enzyme

(a) Examination of purity

Sedimentation. Ultracentrifugation experiments were carried out in a Hitachi Model UCA-1 Ultracentrifuge. Schlieren patterns of the urate oxidase showed a single and almost symmetrical sedimentation boundary (Fig. 1). The sedimentation constant

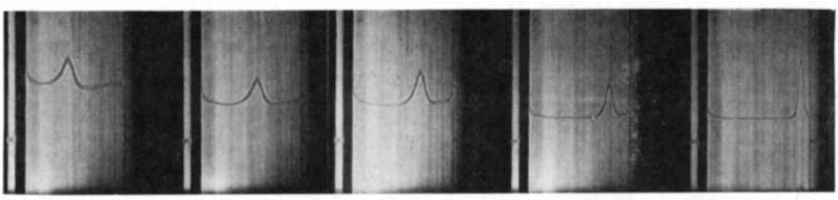

Fig. 1. Schlieren pattern of *A. pascens* urate oxidase in the centrifugation at 60 000 rev./min. Photographs at 0, 20, 40, 60 and 80 min after reaching final speed, from right to left. The concentration of the protein was approx. 7 mg/ml in 0.1 M borate buffer *plus* 0.1 M ammonium sulfate (pH 8.0); average temperature was 13.0°.

at this temperature, protein concentration and solution buffer system, was calculated as 5.47 ± 0.15 S, which is very similar to that of the liver enzyme ($s^{\circ}_{20,w}$ of 5.5).

Tiselius electrophoresis. Electrophoretic patterns of the enzyme at pH 8.0 and pH 9.0 of 0.1 M borate buffer *plus* 0.1 M ammonium sulfate showed single and symmetrical boundaries (Fig. 2 showed the electrophoresis at pH 8.0).

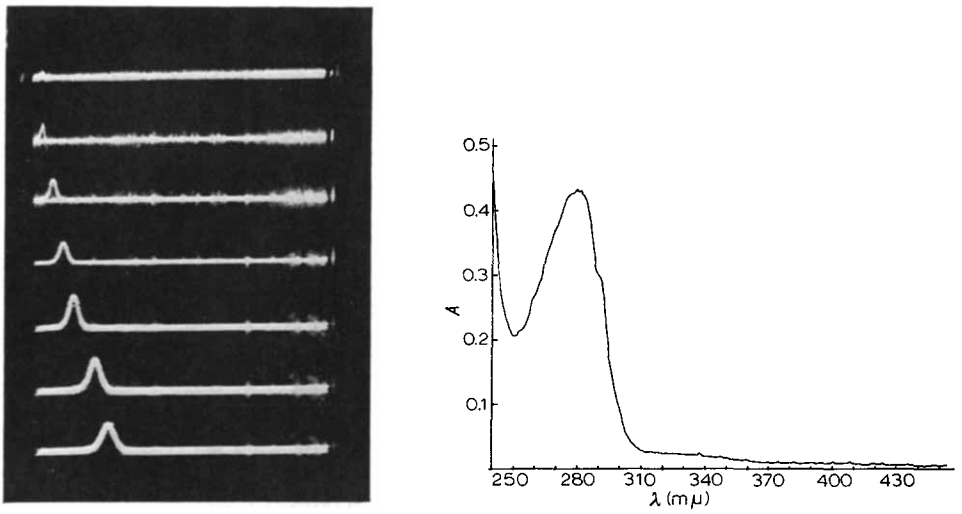

Fig. 2. Tiselius electrophoretic pattern of *A. pascens* urate oxidase at pH 8.0. Photographs at 0, 12, 24, 36, 61 and 76 min after starting with 4.4 mA, 20 V. The concentration of the protein was approx. 5 mg/ml in 0.1 M borate buffer *plus* 0.1 M ammonium sulfate (pH 8.0).

Fig. 3. Absorption spectrum of *A. pascens* urate oxidase. The enzyme was dissolved in 0.2 M borate buffer (pH 8.0).

(b) Absorption spectrum of the enzyme

Ultraviolet and visible absorption spectrum of the enzyme was recorded by the Cary Model 14 automatic spectrophotometer from 450 mμ to 240 mμ (Fig. 3). It was

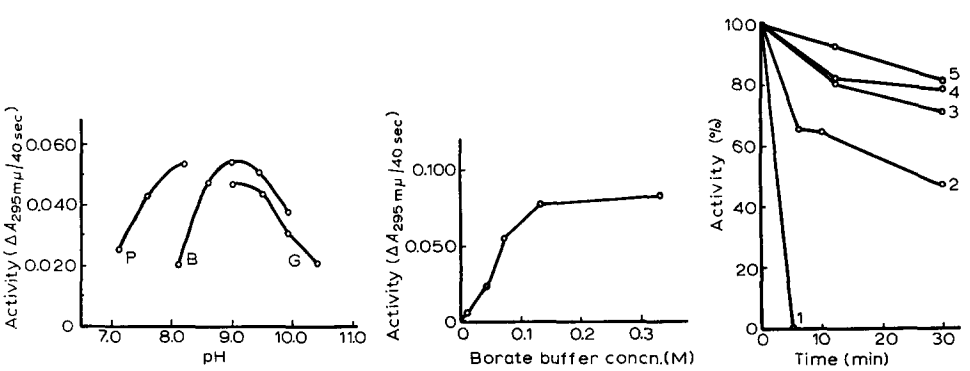

Fig. 4. Influence of pH on the activity of *A. pascens* urate oxidase. Reaction mixtures contain the enzyme solution, 0.05 ml, substrate solution ($5 \cdot 10^{-4}$ M), 1 ml and 0.2 M buffer, 1.95 ml: phosphate buffer (P), borate buffer (B), glycine–NaOH buffer (G). pH was determined by pH meter.

Fig. 5. Influence of buffer concentration on the activity of *A. pascens* urate oxidase. Activity was measured in the following reaction mixtures: enzyme solution, 0.1 ml, substrate solution ($5 \cdot 10^{-4}$ M), 1 ml, and each of borate buffers (pH 9.0) 1.9 ml. Final buffer concentrations were illustrated in the abscissa of the graph.

Fig. 6. Influence of buffer concentration on the stability of *A. pascens* urate oxidase. Enzyme solution (0.32 unit) was dissolved in each borate buffer (pH 9.0); its final concentration was as follows: 1, below 0.01 M; 2, 0.02 M; 3, 0.06 M; 4, 0.11 M; 5, 0.21 M. Incubation was at 40°. At intervals 0.2-ml samples were taken and activities were assayed.

observed that the enzyme had λ_{max} at 280 mμ and λ_{min} at 250 mμ and no absorbance at 450 mμ, which indicates that there is no flavin cofactor in this enzyme. Differences between the ultraviolet and visible spectra of our bacterial enzyme and the liver enzyme[9] were as follows: first, the liver enzyme had λ_{min} at 260 mμ instead of our 250 mμ; second, our enzyme had a ratio $A_{280\ m\mu}:A_{330\ m\mu}$ of approx. 20, the same value as that for ordinary proteins. The liver enzyme ,however, had a ratio of 5–10, which supports the hypothesis that the liver enzyme is a copper protein.

(c) Determination of K_m

The Michaelis constant (K_m) of this enzyme for our standard assay method was determined as usual. The reaction velocities at each of 4 uric acid concentrations (170, 83, 63 and 42 μM) in routine enzyme assay system (see METHODS) were measured. The K_m value was calculated to be about $2 \cdot 10^{-4}$ M, which was ten-fold larger than the K_m ($2 \cdot 10^{-5}$ M) of the liver enzyme. This difference in K_m between bacterial and liver enzyme will be discussed later.

(d) Effect of pH on the enzyme activity

The influence of pH on the enzyme activity was tested in 0.13 M phosphate, borate and glycine buffer. As shown in Fig. 4, the pH–activity curves were bell-shaped and the optimal pH of the enzyme appeared to be about 9.2. It was observed that the enzyme activity in phosphate buffer was higher than the others. The stability of the enzyme at different pH's was tested. It was found that the enzyme was more stable at alkaline pH region.

(e) Effect of the buffer concentration on the enzyme activity

The influence of the buffer concentration on the enzyme activity was tested in borate buffer (pH 9.0) as shown in Fig. 5. A strong effect of the buffer concentration on the enzyme activity was observed, that is, there was no activity at very low buffer concentration. The same effect was observed when the activity was measured at various concentrations of ammonium sulfate added in dilute borate buffer, instead of changing borate concentration. These observations seemed to show that the effect of buffer concentration on the activity resulted from that of ionic strength of the reaction mixture. The stability of the enzyme at different buffer concentrations was tested. From Fig. 6, it was observed that the stability was highly dependent on buffer concentration. In order to know the reasons of the inactivation of the enzyme at low buffer concentration, we examined the state of the enzyme protein at the process of the inactivation. Then the absorption at 280 mμ of the enzyme solution was constant during the inactivation of the enzyme. This seemed to show that the inactivation of the enzyme in the buffer of low ionic strength was not caused by the insolubilization of the enzyme protein, at least in early stage of the inactivation. Next, the change of ultraviolet spectrum of the enzyme after the inactivation by prolonged dialysis at low buffer concentration was examined. Ultraviolet spectrum of the supernatant of which activity reduced about one tenth of the enzyme preparation before dialysis, was shown in Fig. 7 with that of the native enzyme. It was observed that after the inactivation absorbances at 250 mμ (λ_{min}) and in the range from about 300 mμ to 380 mμ were raised in comparison with $A_{280\ m\mu}$ (λ_{max}). These changes in spectrum of the enzyme seemed to show some conformational change of the enzyme protein by the inactivation. This idea was supported by the fact that the inactivated enzyme by the low buffer concentration was reactivated gradually, by exposing the enzyme in

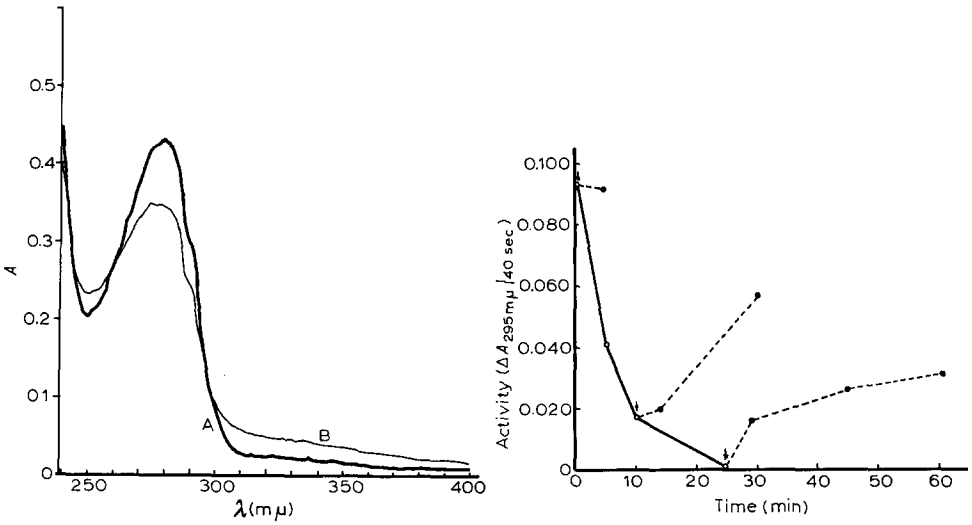

Fig. 7. Ultraviolet spectrum of the *A. pascens* urate oxidase before and after the inactivation by prolonged dialysis at low buffer concentration. The enzyme was dialysed against 0.01 M borate buffer (pH 8.0) at 4°, for 48 h and the little precipitate formed was removed by centrifugation (105 000 × *g*, 30 min) to obtain a clear fluid preparation for Cary Model 14 spectrophotometer. A, before dialysis; B, after dialysis. Note: Precipitated protein was reactivated to one fifth of the original specific activity by incubation at 4° in the final 0.2 M borate buffer containing 0.7 M ammonium sulfate for 3 days.

Fig. 8. Effect of ammonium sulfate on the reactivation of the urate oxidase of *A. pascens* inactivated at low buffer concentration. The enzyme was inactivated in dilute borate buffer (lower than 0.01 M ,pH 8.0) at 40° (○—○). At 0, 10 and 25 min after the incubation, an aliquot of the enzyme solution was taken, mixed with equal volume of 2 M ammonium sulfate in 0.02 M borate buffer (pH 9.0) (↓) and incubated again at 40°. At intervals its enzyme activity was measured and expressed as the original activity levels (●— —●).

TABLE II

EFFECT OF METAL IONS ON THE ACTIVITY OF *A. pascens* URATE OXIDASE

$Fe_2(SO_4)_3 \cdot (NH_4)_2SO_4$, $CuSO_4 \cdot 5\,H_2O$, $MnSO_4 \cdot 4\,H_2O$, $Co(NO_3)_2 \cdot 6\,H_2O$ and $MgSO_4 \cdot 7\,H_2O$ were dissolved in 0.03 M borate buffer (pH 9.0) and added to the reaction mixture in the final concentrations shown in the table. The enzyme activity was measured in 0.03 M borate buffer (pH 9.0), in which the effect of metals was amplified.

Metal ion	*Concentration (M)*	*Stimulation (%)*
Fe^{3+}	$5.4 \cdot 10^{-6}$	25
	$1.1 \cdot 10^{-5}$	38
	$2.2 \cdot 10^{-5}$	33
Cu^{2+}	$4.7 \cdot 10^{-7}$	−55
	$4.7 \cdot 10^{-8}$	−33
Mn^{2+}	$5.4 \cdot 10^{-6}$	−7
	$1.1 \cdot 10^{-5}$	−12
Co^{2+}	$5.5 \cdot 10^{-6}$	−7
Mg^{2+}	$1.3 \cdot 10^{-5}$	5
	$2.7 \cdot 10^{-5}$	6

TABLE III

EFFECT OF CHELATING AGENTS ON THE ACTIVITY OF *A. pascens* URATE OXIDASE

Each of chelating agents were dissolved in 0.13 M borate buffer *plus* 0.15 M ammonium sulfate (pH 8.6) and added to the reaction mixture in the final concentrations shown in the table. The enzyme activity was assayed in 0.13 M borate buffer *plus* 0.15 M ammonium sulfate (pH 8.6). The effect of preincubation with the enzyme and chelating agents was also examined. During the preincubation, the reaction mixture without substrate solution was set at 30° for 10 min.

Chelating agent	*Concentration (M)*	*Preincubation (min)*	*Inhibition (%)*
Neo-cuproin	$3.3 \cdot 10^{-4}$	0	59
	$3.3 \cdot 10^{-4}$	10	53
o-Phenanthroline	$3.3 \cdot 10^{-4}$	0	27
	$3.3 \cdot 10^{-4}$	10	15
α,α'-Dipyridyl	$1.7 \cdot 10^{-4}$	0	11
	$1.7 \cdot 10^{-4}$	10	10
8-Oxyquinoline	$3.3 \cdot 10^{-4}$	0	0
	$3.3 \cdot 10^{-4}$	10	0
Diethyldithiocarbamate	$3.3 \cdot 10^{-4}$	0	0
	$3.3 \cdot 10^{-4}$	10	0
Fluoride	$1.0 \cdot 10^{-3}$	0	0
	$1.0 \cdot 10^{-3}$	10	0

high ionic strength atmosphere again. Fig. 8 showed this partial reactivation of the inactivated enzyme. A need of time for reactivation seemed to support some reversible conformational change of the enzyme protein.

(f) Effect of metals and chelating agents on the enzyme activity

As shown in Table II, Cu^{2+} inhibited the enzyme activity at low concentration. Fe^{3+} stimulated the enzyme activity which had not been reported in the liver enzyme. Other metals had a slight inhibitory action on the enzyme, but that was not remarkable.

Some chelating agents for iron and copper, were examined. As shown in Table III, neo-cuproin inhibited the enzyme activity remarkably. But except for neo-cuproin, chelating agents which had more affinity for Fe^{3+} (or Fe^{2+}) than Cu^{2+} (or Cu^{+}) were apt to inhibit the enzyme activity.

DISCUSSION

Our bacterial enzyme was compared with the liver enzyme of MAHLER, HÜBSCHER AND BAUM[9–11] at some points. Specific activities of the purified enzyme preparations were compared. Correcting the factors of the assay conditions the specific activity of the liver enzyme was calculated to be 21 000 in units/mg protein, which was very similar to 20 500 for our bacterial enzyme. From the good resemblance of the sedimentation constants, both enzymes seemed to have the similar molecular size. But some different properties between these two enzyme preparations were observed. We were very interested in different behaviors in ultraviolet absorption spectrum of the enzyme protein and on the effects of Fe^{3+} and the chelating agents on the enzyme activity, that is, these results made us consider that our bacterial enzyme might differ

in its prothetic metal from the liver enzyme. As for the effect of ionic strength on the enzyme activity and stability, our bacterial enzyme showed peculiar behavior different than that of the liver enzyme.

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