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# ALIPHATIC L-α-HYDROXYACID OXIDASE FROM RAT LIVERS PURIFICATION AND PROPERTIES

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## SUMMARY

L-α-Hydroxyacid oxidase has been purified 350-fold from a cytoplasmic extract of rat livers. Purification involved homogenization of rat livers, isolation of a light mitochondrial fraction, sonic treatment, ammonium sulfate fractionation, dialysis, chromatography on DEAE-cellulose, and gel-filtration through Sephadex G-200. The enzyme is specific for many L-aliphatic-α-hydroxy acids and glycolic acid and does not affect α-hydroxyisobutyric acid or aromatic hydroxy acids.

Inhibition studies indicate that a sulfhydryl group is essential for enzyme activity. Diethyldithiocarbamate and KCN have been found to be powerful inhibitors of the enzyme which follow the kinetics of competitive and uncompetitive types, respectively. From the mode of actions of two inhibitors, typical metal binding and metal chelating agents on the enzyme, it seems likely that the enzyme does not require a metal cofactor. The spectrophotometric studies of the highly purified enzyme and attempts to split off the prosthetic group have proved no evidence of functional flavin group or of $NAD^+$.

The reaction catalyzed by this enzyme is:

$$R\text{–}CHOH\text{–}COOH + O_2 \rightarrow R\ CO\text{–}COOH + H_2O_2$$

The enzyme was found to be located principally in the light mitochondrial fraction of rat liver homogenates.

## INTRODUCTION

We have reported that the L-amino-acid oxidase crystallized from rat kidney mitochondria and soluble fraction catalyzes the deamination of L-amino acids and the dehydrogenation of L-α-hydroxy acids[1,2]. Recently, L-amino-acid oxidase was demonstrated to be located in peroxisomes rather than mitochondria of rat kidney[3]. Further study of the intracellular distribution of this oxidase in rat liver cells with L-α-hydroxyisocaproate or L-α-hydroxyvalerate as substrate revealed that the activity was located

mainly in the light mitochondrial fraction (a mixture of light mitochondria, lysosomes and peroxisomes), whereas L-leucine oxidase activity was extremely low in this moiety. This finding suggests that rat liver cells contain an oxidase with a high specificity toward L-α-hydroxy acids. This report describes the partial purification of this enzyme, a L-α-hydroxyacid oxidase, from rat livers. This L-α-hydroxyacid oxidase differs from mammalian L-amino-acid oxidase[1,2] as well as long and short chain L-α-hydroxyacid oxidases of hog kidney cortex with regard to substrate specificity and other properties[4].

## EXPERIMENTAL PROCEDURE

### *Reagents*

Most of the α-hydroxy acids (L and DL configurations), DL-β-hydroxybutyric acid, α-ketovalerate, α-ketobutyrate, α-ketoisovalerate, lactic dehydrogenase ($NAD^+$ dependent), sulfhydryl reagents (*o*-iodosobenzoate and *p*-chloromercuribenzoate) were obtained from Sigma. Other α-hydroxy acids (D-lactate, L-lactate, DL-β-indol lactic acid, DL-mandelic acid, α-hydroxyisobutyric acid), FAD, FMN, $NAD^+$, α-ketocaproic acid, glyoxylic acid, horseradish peroxidase, and catalase were purchased from Cal. Biochem. Phenylpyruvic acid and *p*-hydroxyphenylpyruvic acid were obtained from Mann and from Tokyo Daiichi Seiyaku, respectively. Metal chelating and metal binding agents were purchased from Kanto kagaku. Sodium α-ketoisocaproate and α-ketocaproate were prepared by enzymatic deamination of their corresponding L-amino acids with crude L-amino acid oxidase (*Agkistrandom piscivorus*)[5].

Sodium salts were prepared from commercial free keto acids and crystallized from ethanol–water. Calcium or barium salts of hydroxy acids were also converted to sodium salts by passage through a column of Dowex-50 ($H^+$) and subsequent titration with standard NaOH.

### *Assay of L-hydroxyacid oxidase*

*3-Hydrazinoquinoline assay.* The assay was based on the formation of a keto acid–hydrazinoquinoline complex[1,6]. The enzyme preparation was incubated during 20 min at 37° in a mixture containing 0.0313 M sodium phosphate buffer (pH 7.9), 0.313 mM EDTA, 6.25 mM L-hydroxy acid (12.5 mM DL-hydroxy acid) and 40 μg of crystalline catalase (total volume of 1.6 ml). The reaction was stopped by the addition of 0.7 ml of 30% trichloroacetic acid. 1 ml of the deproteinized solution was added to 0.5 ml of 1 M NaOH and adjusted to pH 2.0 by the addition of 1 M NaOH or 1 M HCl. After adjusting the volume to 1.6 ml with water, 1 ml of 0.0116% 3-hydrazinoquinoline dihydrochloride in water was added to the acidified solution, followed by incubation for 30 min at 37°. 10 min after the addition of 1.0 ml of 0.1 M HCl the absorbancy of the resulting solution was determined at 305 mμ. Under the conditions cited the following molar extinction coefficients of the main keto acid–hydrazinoquinoline complexes were obtained: pyruvic acid and α-ketovaleric acid, 21 400; α-ketoisocaproic acid, 18 500; α-ketocaproic acid, 18 500; α-keto-*n*-butyric acid, 14 700.

*2,4-Dinitrophenylhydrazone assay.* The assay of KACHMAR AND BOYER[7] as modified by ROBINSON *et al.*[4] is based on the formation of the 2,4-dinitrophenylhydrazones of keto acids. The components of the incubation media were the same as

those of assay 1. After incubation for 20 min at 37°, the reaction was terminated by the addition of 0.7 ml of 30% trichloroacetic acid. 2 ml of the deproteinized solution were added to 0.7 ml of 0.025% 2,4-dinitrophenylhydrazine in 2 M HCl and incubated at 37° for 10 min. Exactly 20 min after addition of 1.4 ml of 4 M NaOH, the absorbancy of the resulting solution was measured at 435 mμ. Under these condition, the following molar coefficients of the various hydrazones at 435 mμ were obtained: glyoxylic acid, 17 100: pyruvic acid, 14 160; α-ketovaleric acid, 12 750; α-ketocaproic acid, 12 000; α-ketoisocaproic acid, 12 000 and α-keto-*n*-butyric acid, 6950.

*Borate-enol (aromatic) keto acid assay.* The dehydrogenation activity in the presence of aromatic α-hydroxy acid was measured by the borate-enol aromatic keto acid method as described in our previous report[8].

One unit of activity is defined as the amount of enzyme which produces 1 mμmole of α-keto acid per 20 min under the assay conditions. The specific activity is stated in units per mg of protein.

Unless otherwise noted, enzyme activity (specific or total) was expressed in mμmoles of α-ketoisocaproate formed from α-hydroxyisocaproate in 20 min at 37°.

*Assay of the other enzymes*

Acid phosphatase[9], uricase[10], cytochrome oxidase[11], catalase[12] and L-amino-acid oxidase[1] were determined by established methods.

*The protein determination*

Protein concentrations were measured by the method of LOWRY *et al.*[13].

*Tissue fractionation*

The fractionations were done in 0.25 M sucrose according to the procedure of DE DUVE *et al.*[14]. Wistar male rats approximating 300 g in body weight were stunned and bled. The liver was removed immediately and immersed in a tared beaker containing ice-cold sucrose. After washing the tissue was cut into pieces and dispersed in sucrose by means of a motor-driven Potter–Elvehjem homogenizer with a teflon pestle. The nuclear fractions, which may be contaminated with intact cells and cell debris, was discarded and the cytoplasmic extract was used as the starting material for the enzyme preparation. The cytoplasmic extract was diluted with isotonic sucrose to a final volume equal to 10 times the weight of the original tissue.

The washed granules obtained by the differential centrifugation of cytoplasmic extract were taken up in small volumes of isotonic sucrose to a final dilution approximately equal to 2 ml per gram of wet tissue in the studies of the mitochondrial fraction, and the microsomal fraction.

## RESULTS

*Distribution of L-α-hydroxyacid oxidase in liver cells*

The intracellular distribution of L-α-hydroxyacid oxidase is shown in Fig. 1. Nearly 70% of the total L-α-hydroxyacid oxidase activity in the cytoplasmic extract was found in the mitochondrial and light mitochondrial fractions, with the remainder largely in the soluble fraction. Hence, the distribution pattern of the enzyme resembles that of catalase or uricase, but differs from that of cytochrome oxidase. These findings

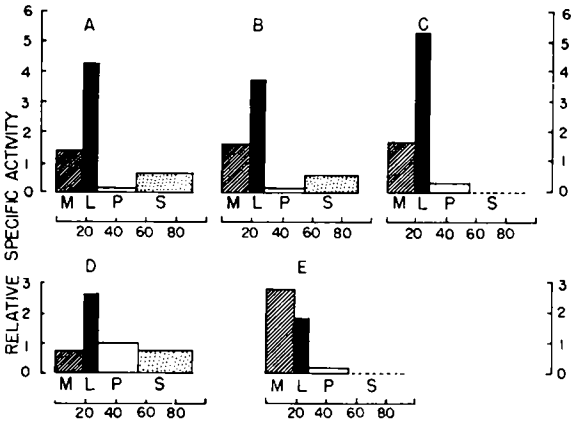


Fig. 1. Distribution patterns of enzymes. (A) L-Hydroxyacid oxidase[5]; (B) catalase[4]; (C) uricase[5]; (D) acid phosphatase[5]; (E) cytochrome oxidase[3]. Fractions are represented in the order of their isolation, *i.e.* (from left to right) mitochondrial (M), light mitochondrial (L), microsomal (P), and the soluble fraction (S). Each fraction is represented separately on the ordinate scale by its own relative specific activity (percentage of total activity in cytoplasmic extract/percentage of total activity protein in cytoplasmic extract). Along the abscissa scale each fraction is represented by its protein content, expressed as a percentage of the total protein. The numbers in parentheses refer to numbers of experiments.

suggest that a large part of the enzyme is associated with non-mitochondrial particles, *i.e.* in lysosomes or uricase containing particle (peroxisomes).

*Extraction of* L-*α-hydroxyacid oxidase from the light mitochondrial fraction*

The light mitochondrial fraction obtained from rat livers was suspended in 0.25 M sucrose ($^1/_5$ of the volume of cytoplasmic extract) and disintegrated with a sonic oscillator at 10 kcycles for 8 min at 4 6°. The sonically treated suspension was then centrifuged at 100 000 × *g* for 30 min at 4° and the clear supernatant fluid ($S_1$) was removed and stored. The sediment was then suspended in the same volume of 0.05 M sodium phosphate buffer (pH 7.9) containing $10^{-3}$ M EDTA, homogenized, and centrifuged at the same speed for another 30 min. The clear yellow supernatant ($S_2$) was collected. As shown in Tables I and II, approx. 70% of the L-α-hydroxyacid oxidase

TABLE I

RECOVERY OF L-HYDROXYACID OXIDASE FROM LIGHT MITOCHONDRIAL EXTRACTS

Livers (12 g, wet wt.) from two male rats were used in this experiment.

| *Preparation* | *Isocaproic* | *n-Valeric* | *n-Caproic* | *n-Butyric* | *Lactic* |
|---|---|---|---|---|---|
| Cytoplasmic extract | 110 500 | 69 500 | 46 900 | 56 900 | 42 200 |
| Light mitochondrial fraction | 61 600 | 37 000 | 20 600 | 22 500 | 11 600 |
| Sonic treated light mitochondrial suspension | 48 800 | 32 100 | 18 350 | 21 300 | 10 320 |
| Light mitochondrial extract ($S_1$ + $S_2$) | 46 950 | 23 100 | 15 300 | 14 300 | 6 970 |
| Recovery* | 75.5 | 62.5 | 74.0 | 64.0 | 60.2 |

* $\frac{\text{Activity in light mitochondrial extract}}{\text{Activity in light mitochondrial fraction}} \times 100$

TABLE II

PURIFICATION OF L-HYDROXYACID OXIDASE

Livers (approx. 145 g, wet wt.) from 15 rats were used in this experiment. The enzyme activity at each step of the purification was measured by the formation of α-keto isocaproate from L-hydroxyisocaproate under the assay condition.

| *Preparation* | *Vol. (ml)* | *Protein* | | *Enzyme activity* | | *Recovery (%)* |
|---|---|---|---|---|---|---|
| | | *(mg/ml)* | *(mg)* | *Specific* | *Total* | |
| Cytoplasmic extract | | | | 72–112 | | |
| Light mitochondrial extract | 458.0 | 3.4 | 1584.0 | 690 | 1093 000 | 100 |
| $(NH_4)_2SO_4$ ppt. | 19.5 | 17.2 | 337.0 | 2 450 | 825 000 | 76 |
| 1st DEAE-cellulose column eluate | 14.0 | 2.0 | 28.2 | 14 150 | 399 000 | 37 |
| 2nd DEAE-cellulose column eluate | 4.5 | 1.0 | 4.5 | 25 430 | 114 500 | 10.5 |

activity in the light mitochondrial fractions, measured with various substrates, was recovered in the $S_1$ and $S_2$ fractions with an increase in specific activity.

PURIFICATION OF THE ENZYME

Unless otherwise noted, all procedures were carried out at 0–4°; enzyme preparations treated with ammonium sulfate were centrifuged at 10 000 × *g* for 20 min. Dialysis was performed in 0.005 M sodium phosphate buffer containing $10^{-3}$ M EDTA at pH of 7.9 (Buffer A). The results of a typical procedure are listed in Table II.

*Ammonium sulfate fractionation*

The soluble extracts ($S_1$ and $S_2$) representing the light mitochondrial fraction from 140 to 150 g of rat livers were fractionated by the successive addition of solid ammonium sulfate and adjustment of the pH to 7.0 with 2 M $NH_4OH$. In each fractionation the salt was added during a 10-min interval with continuous mechanical stirring; the stirring was then continued for 20 min before centrifugation. The precipitates obtained by increasing the concentration of ammonium sulfate from 0 to 35% and from 35 to 50% saturation had little oxidase activity and were discarded. The yellow supernatant fluid contained most of the oxidase activity present in the soluble extract. The enzyme was precipitated from the supernatant fluid by increasing the ammonium sulfate to 70% saturation. The precipitate formed was centrifuged, dissolved in 10 ml of 0.05 M sodium phosphate buffer containing $10^{-3}$ M EDTA (pH 7.9), and dialyzed for 16 h against 3 l of Buffer A.

*First DEAE-cellulose fractionation*

The above enzyme preparation, containing 120–137 mg of protein, was transferred onto a DEAE-cellulose column (2 cm × 11 cm) which had been equilibrated with Buffer A. The column was washed successively with 80 ml of the same buffer and 80 ml of 0.02 M sodium phosphate (pH 7.9) buffer containing $10^{-3}$ M EDTA. The two washings were discarded. The enzyme was then eluted from the column with 80 ml of 0.05 m sodium phosphate buffer (pH 7.9) containing $10^{-3}$ M EDTA and precipitated by the addition of solid ammonium sulfate to 75% saturation. The precipitate was collected, dissolved in 5 ml of 0.05 M sodium phosphate buffer containing $10^{-3}$ M EDTA (pH 7.9), and dialyzed against 3 l of Buffer A for 16 h.

*Second DEAE-cellulose fractionation*

The above preparation containing 30–97 mg of protein was transferred onto a DEAE-cellulose column (2 cm × 11 cm) which had been equilibrated with Buffer A. Elution was effected by a linear gradient of 200 ml of Buffer A in the mixing chamber and 200 ml of 0.075 M sodium phosphate buffer (pH 7.9) containing $10^{-3}$ M EDTA in the reservoir at an average flow rate of 1 ml/min. Effluent fractions containing approx. 3.7 ml were collected. The results are presented in Fig. 2. The first 50 eluates were yellow in color and contained no oxidase activity. The oxidase was usually found in the subsequent eluates. The active fractions were combined, and the enzyme was precipitated by the addition of solid ammonium sulfate to 75% saturation. The precipitate was collected by centrifugation and dialyzed against 3 l of Buffer A for 16 h. The specific activity at this stage ranged between 19 000 and 25 800.

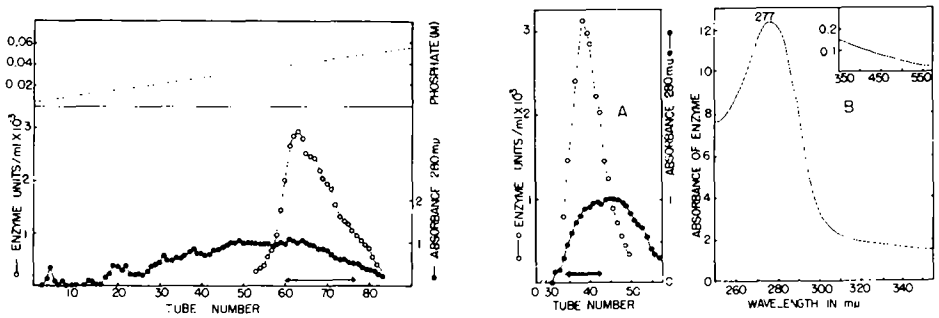


Fig. 2. DEAE-cellulose chromatography. A total of 28 mg of protein (specific activity of 14 150) were added to the column (2 cm × 11 cm) and purified by linear gradient elution as described in the text with a total volume of 400 ml of sodium phosphate buffer. The arrows identify the fractions which were pooled.

Fig. 3. A. Elution profile of enzyme on Sephadex G-200. 0.5 mg of enzyme protein (specific activity 19 200) was loaded onto the column of Sephadex G-200 and eluted with Buffer A as described in the text. The arrows identify the fractions which were pooled. B. The light absorption spectrum of purified enzyme (specific activity, 32 800). Purified enzyme (Fig. 3A) was dissolved at a protein concentration of 0.87 mg/ml in 0.005 M sodium phosphate buffer (pH 7.9) containing $10^{-3}$ M EDTA.

*Gel filtration*

Further purification was achieved by gel filtration on a column of Sephadex G-200 (2.0 cm × 50 cm) which had been equilibrated with Buffer A. Elution was carried out with the same buffer, and 3-ml fractions were collected. The peak of the enzyme activity was recovered in 120 ± 2 ml of effluent and enzyme activity was distributed almost symmetrically in a single peak (Fig. 3A). Fractions with specific activity of more than 25 000 in terms of units of absorbance at 280 mμ were combined.

The enzyme was slightly yellow at this stage of purification, but showed no specific absorption peak in the region between 320 and 490 mμ (Fig. 3B). The absorption ratios $A_{280\ m\mu}/A_{260\ m\mu}$ and $A_{280\ m\mu}/A_{330\ m\mu}$ for two preparations of the purified enzyme were approx. 1.38 and 6.4, respectively. The value of $A^{1\%}_{330\ m\mu}$ was 2.3. These characteristic features resemble those of uricase[15].

PRODUCTS OF THE REACTION

The products of the oxidation catalyzed by the enzyme were found to be keto acids corresponding to the hydroxy acid substrates. The reaction mixture contained 1 ml of 0.1 M sodium phosphate buffer (pH 7.9) containing $10^{-3}$ M EDTA, 24 000 units of enzyme (specific activity, 14 400), 1 mg of catalase and 50 μmoles of L-α-hydroxy acid (100 μmoles of DL-hydroxy acid) in a total volume of 1.6 ml. The reaction was carried out in air for 3 h at 37° with continuous shaking and terminated by the addition of 0.5 ml of 60% perchloric acid. After centrifugation the supernatant fluid was collected and 1 ml of 0.1% 2,4-dinitrophenylhydrazine in 2 M HCl was added to the supernatant fluid. The mixture was incubated at 37° for 30 min and extracted successively with 6 and 3 ml of ethylacetate. The 2,4-dinitrophenylhydrazone in the pooled ethylacetate extract was transferred to 7 ml of 10% $Na_2CO_3$. After acidification with 5 M HCl, the dinitrophenylhydrazone was again extracted into 10 ml of ethylacetate, which was then dried *in vacuo*. The residue was dissolved in 0.5 ml of ethanol and the aliquots were chromatographed on Whatmann No. 1 paper in *n*-butanol–ethanol–water (4:1:5, v/v/v) at 25° for 15 h. Yellow zones were located by spraying the chromatogram with 2% NaOH in ethanol. When L-α-hydroxy-*n*-butyrate, L-α-hydroxyisocaproate or L-lactate was used as the substrate under the conditions cited, chromatography revealed a single color zone with an $R_F$ value which was the same as that of the authentic hydrazone of the corresponding α-keto acid.

STOICHIOMETRY OF THE OXIDASE REACTION

This was determined by the following procedures. A reaction mixture consisting of 2880 units of enzyme (specific activity, 20 670), and 0.5 ml of 0.1 M sodium phosphate buffer (pH 7.9) containing $10^{-3}$ M EDTA, 80 μg of catalase and 2.94 μmoles of L-lactate in a final volume of 0.9 ml, was incubated for various intervals of time at 37.5° in air. The reaction was stopped by adding 0.1 ml of 70% perchloric acid. Pyruvic acid formation was measured by the 2,4-dinitrophenylhydrazone method. The acidified replicate reaction mixtures used for the measurement of L-lactate were neutralized with 4 M NaOH. The lactate was then determined with lactic dehydrogenase in the

TABLE III

STOICHIOMETRY OF L-LACTATE OXIDATION

The experiments were carried out as described in text.

| *Incubation time (min)* | *Lactate remained (μmoles)* | *Pyruvate formed (μmoles)* | *Lactate* consumed (μmoles)* |
|---|---|---|---|
| 0 | 2.94 | 0 | 0 |
| 30 | 2.52 | 0.38 | 0.42 |
| 60 | 2.34 | 0.55 | 0.60 |
| 120 | 2.10 | 0.81 | 0.84 |
| 180 | 1.88 | 0.99 | 1.06 |
| 240 | 1.72 | 1.19 | 1.22 |

* The value was obtained by substraction of the amount of lactate remaining from the initial amount of lactate.

presence of $NAD^+$ (ref. 16). As shown in Table III the amount of pyruvate formed during 4 h of incubation almost coincided with the amount of lactate consumed (the difference was less than 10%); the amount of the lactate consumed usually exceeded the amount of pyruvate formed. The oxygen consumed during the oxidation of substrate was measured by manometry. The experimental flasks contained highly purified enzyme of specific activity 20 000 (3100–4500 units), 1.3 ml of 0.1 M sodium phosphate buffer (pH 7.9) containing $10^{-3}$ M EDTA, 4 or 6 $\mu$moles of L-$\alpha$-hydroxyisocaproate and water in a total volume of 2.0 ml; center wells had 0.3 ml of 20% KOH. Vessels were also prepared which contained excess of catalase, KCN (inhibitor of catalase), ethanol or a combination of ethanol and excess of catalase in addition. After 3 h incubation at 37°, trichloroacetic acid was added. $\alpha$-Ketoisocaproate in an aliquot of the supernatant solution was measured by the 2,4-dinitrophenylhydrazone assay. The systems containing both catalase and ethanol have been used to identify indirectly $H_2O_2$ generation in the reaction between L-amino acid and crude L-aminoacid oxidase[17]. If $H_2O_2$ is actually formed in the reaction involving L-$\alpha$-hydroxy acids, the effect of adding ethanol to the incubation media composed of the L-hydroxyacid oxidase, L-hydroxyisocaproate and excess of catalase will be to change the ratio of moles of oxygen consumed to moles of $\alpha$-ketoisocaproate formed from 1:2 to 1:1. The results of the experiments are shown in Table IV, suggesting that the enzyme requires 1 mole of oxygen

TABLE IV

STOICHIOMETRY OF L-HYDROXYISOCAPROATE OXIDATION

Reactions were carried out in the Warburg manometer using Enzyme A (3100 units) or Enzyme B (4500 units) and 100% $O_2$ as gas space, as described in the text. A crystalline catalase (200 $\mu$g), KCN ($8 \cdot 10^{-4}$ M) or ethanol (2%) was added to the vessels indicated. The reaction was almost complete after 3 h incubation under these conditions, except in the experiment with KCN.

| *Incubation media* | *$\alpha$-Hydroxyisocaproate used ($\mu$moles)* | *Oxygen consumed ($\mu$moles)* | *$\alpha$-Ketoisocaproate formed ($\mu$moles)* | *Mole ratio of $O_2$ consumed to keto acid formed* |
|---|---|---|---|---|
| Enzyme A | 4.0 | 2.0 | 3.7 | 0.54 |
| Enzyme A, KCN | 4.0 | 1.85 | 2.0 | 0.92 |
| Enzyme A, catalase | 4.0 | 2.2 | 3.9 | 0.56 |
| Enzyme B | 6.0 | 2.9 | 5.1 | 0.57 |
| Enzyme B, ethanol | 6.0 | 4.5 | 5.1* | 0.90 |
| Enzyme B, ethanol, catalase | 6.0 | 5.4 | 5.1* | 1.06 |

* Since acetaldehyde, which should be formed from ethanol during the interaction between catalase and $H_2O_2$, may interfere with the assay of the keto acid formed from the substrate by method 2, the value of 5.1 $\mu$moles obtained with Enzyme B alone was conveniently used as the amount of keto acid formed in the experiment with ethanol or combination of ethanol and catalase.

to convert 1 mole of substrate to 1 mole of the corresponding keto acid with formation of $H_2O_2$. This suggestion could also be supported from the effect of KCN on the ratio of moles of oxygen consumed to moles of $\alpha$-ketoisocaproate formed during the enzyme reaction. In addition the enzyme preparation used for the experiments is considered to be contaminated with sufficient catalase to decompose any $H_2O_2$ formed to $H_2O$ and $O_2$.

TABLE V

SUBSTRATE SPECIFICITY OF L-HYDROXYACID OXIDASE

The incubation and assay 1 were the same as those described in the text. Enzymes with specific activities of 595 (Enzyme A) and 24 000 (Enzyme B) were used in these experiments. L-α-Hydroxyisocaproate was the reference substrate.

| *α-Hydroxy acids* | *Enzyme A (350 units)* | *Enzyme B (335 units)* |
|---|---|---|
| L-Hydroxyisocaproic | 100 | 100 |
| DL-Hydroxyvaleric | 49 | 45 |
| DL-Hydroxy-*n*-caproic | 32 | 25 |
| DL-Hydroxy-*n*-butyric | 33 | 22 |
| L-Lactic | 15 | 15 |
| DL-Hydroxyisovaleric | 5 | 3 |
| D-Lactic | 2 | 0 |
| Glycolic | — | 88 |

PROPERTIES OF THE ENZYME

*Substrate specificity*

The relative rates of oxidation of various hydroxy acids by the enzyme are listed in Table V. α-Hydroxyisocaproate and glycolate were effective substrates for the enzyme.

The highly purified oxidase was inactive toward D-lactate, DL-phenyllactate, DL-indol-β-lactate, DL-mandelate, DL-hydroxyphenyllactate, DL-β-hydroxybutyrate, α-hydroxyisobutyrate, L-leucine and glycine. Under the conditions employed, the formation of keto acid analogues from their corresponding L-α-hydroxy acids proved to be proportional to the concentration of enzyme which produced 50–500 mμmoles of keto acid.

*Identity of L-α-hydroxyacid oxidase and glycolate oxidase*

L-α-Hydroxyisocaproate and glycolate were chosen as the substrates for L-α-hydroxyacid oxidase and glycolate oxidase for testing the possible identity of the two

TABLE VI

RATIO SPECIFIC ACTIVITIES OF GLYCOLATE OXIDASE/HYDROXYISOCAPROATE OXIDASE

Hydroxyacid oxidases were incubated at 37° and pH 7.9 under standard conditions. Enzyme activities were assayed by method 2.

| *Fraction* | *Ratio specific activity glycolate oxidase / hydroxyisocaproate oxidase* |
|---|---|
| 1. Light mitochondrial extract | 1.01 |
| 2. Ammonium sulfate precipitate (50–70% saturation) | 1.00 |
| 3. First DEAE-cellulose column eluate | 0.84 |
| 4. Second DEAE-cellulose column eluate | |
| Tube No. 69 | 0.84 |
| Tube No. 79 | 0.81 |
| 5. Combined active fractions of 4 | 0.88 |
| 6. Sephadex G-200 filtrate | 0.85 |

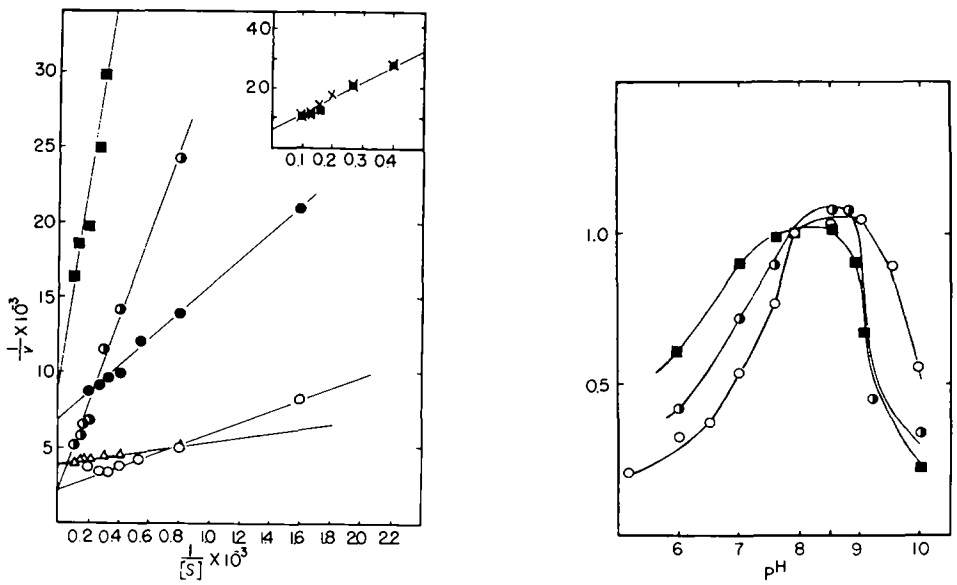


Fig. 4. Concentration of L-hydroxy acid in relation to the formation of the corresponding keto acid. In each experiment the standard reaction mixture was employed, except that the concentration of substrate was varied as indicated. The formation of the keto acid was assayed by method 2. An enzyme preparation with a specific activity of 14 400 (protein 28 $\mu$g) was used. The initial velocity, $v$, is expressed as the molar concentration of $\alpha$-keto acid formed per 20 min; [S] represents the molar concentration of L-$\alpha$-hydroxy acid. The effect of D-lactate ($6.25 \cdot 10^{-1}$ M) on L-lactate oxidation by the enzyme (specific activity, 20 450; activity, 540 units) is presented in the inset. ○—○, L-$\alpha$-hydroxyisocaproate; ●—●, DL-$\alpha$-hydroxycaproate; ◑—◑, DL-$\alpha$-hydroxybutyrate; ■—■, L-lactate; × × , L-lactate *plus* D-lactate; △ △, glycolate.

Fig. 5. Effect of pH on the rate of oxidation of L-hydroxy acid. The enzyme (320 units) of specific activity 14 500 was used for all these experiments. The conditions of incubation and assay 1 were as described in the text, except that the pH of the reaction mixture was varied as indicated. Sodium acetate buffer (pH 6.5 or less), sodium phosphate buffer (pH 7–8.0) and glycine NaOH buffer (pH 8.5–10) were used in these experiments. The results were shown relative to specific activity at pH 7.9, taken as unity. ○ ○, L-$\alpha$-Hydroxyisocaproate; ◑—◑, DL-$\alpha$-hydroxybutyrate; ■—■, L-lactate.

enzymes. The activity of both enzymes was determined at each stage of purification. The results are presented in Table VI. The ratio of L-$\alpha$-hydroxyacid oxidase to glycolate oxidase appeared to be constant at each step of purification, keeping in mind the limited accuracy of the assay.

*Effect of substrate concentration*

Fig. 4 indicates that L-$\alpha$-hydroxyisocaproate inhibited the activity of the enzyme at high concentrations, whereas the other compounds tested did not. $K_m$ and $V_{max}$ values for $\alpha$-hydroxy acids, calculated from intercepts and slopes of the lines in Fig. 4, are summarized in Table VII. The effect of the D-hydroxy acid on its L configuration was tested with both stereoisomers of lactate (Fig. 4, inset). The D-isomer did not inhibit the oxidation of L-lactate under the conditions employed.

*Effect of pH*

The activity curves of the enzyme with three different substrates exhibited an optimum pH at approx. 8.5 (Fig. 5).

TABLE VII

MICHAELIS CONSTANTS AND MAXIMAL VELOCITIES OBTAINED WITH L-HYDROXYACID OXIDASE

| *Substrate* | $K_m$ *(mM)* | $v_{max.}$ */20 min (mM)* |
|---|---|---|
| L-α-Hydroxyisocaproate | 1.65 | 0.455 |
| DL-α-Hydroxycaproate | 1.34 | 0.147 |
| DL-α-Hydroxybutyrate | 12.70 | 0.455 |
| L-Lactate | 8.50 | 0.125 |
| Glycolate | 0.50 | 0.260 |

*Cofactors and inhibitors*

Neither of the flavin analogues (FMN and FAD) nor $NAD^+$ accelerated the enzyme reaction. After treatment with acidified ammonium sulfate solution by a modification of the method of WARBURG AND CHRISTIAN[18] the enzyme retained approx. 40% of its initial activity. It was not reactivated by the addition of FMN or FAD. When the enzyme was treated with washed Norit and passed through a Millipore filter[19], the specific activity in the filtrate was found to be the same as that of untreated enzyme. Stimulation of hydroxy acid oxidation in the filtrate by $NAD^+$ was not observed.

The enzymatic formation of keto acid analogues from glycolate and L-α-hydroxyisocaproate was not affected in the presence of cytochrome *c* ($1 \cdot 10^{-5}$ M) or phenazine methosulfate ($1 \cdot 10^{-5}$ M). In the presence of purified enzyme, there was no appreciable reduction of 2,6-dichloroindophenol by the substrates, glycolate and L-α-hydroxyisocaproate. No enzyme activity could be detected with or without added cytochrome *c* or phenazine methosulfate under an atmosphere of nitrogen.

The results obtained on the effect of possible inhibitors on the oxidation of glycolate and L-α-hydroxyisocaproate are summarized in Table VIII. Most of the metal binding and chelating agents, such as NaF, thiocyanate, α,α'-dipyridyl, *o*-phenanthroline and EDTA, were found to inhibit slightly or to be without effect. Quinacrine, an inhibitor of some enzymes with flavin prosthetic groups, was not inhibitory. The enzyme was, however, markedly inhibited by reagents such as *p*-chloromercuribenzoate, *o*-iodosobenzoate and $CuSO_4$ which react with sulfhydryl groups. Ascorbate enhanced the enzyme activitjes slightly. Cysteine was without effect on L-α-hydroxyisocaproate oxidation and rather inhibitory on glycolate oxidation under the assay conditions. The latter may be attributable to a partial formation of a glycolate cysteine complex which is resistant to hydrolysis at ordinary temperatures and does not react with 2,4-dinitrophenylhydrazine[20]. The different degrees of inhibition of the oxidation of glycolate and L-α-hydroxyisocaproate were also found with KCN.

A few of the inhibitors of the enzyme were investigated further by studying the effects of the inhibitors at different substrate concentrations. Competitive type inhibition was observed with diethyldithiocarbamate ($K_i$ - - 0.77 mM) (Fig. 6A). The nature of the inhibition on glycolate oxidation by KCN was found to be of an uncompetitive type ($K_i$ = 0.88 mM) (Fig. 6B). The enzyme which had been preincubated with $2 \cdot 10^{-3}$ M KCN for 2 h at room temperarure in the absence of added glycolate was almost completely recovered by gel-filtration on a Sephadex G-25 column, with a layer

TABLE VIII

EFFECT OF POSSIBLE INHIBITORS ON L-HYDROXYACID OXIDASE

Group 1, metal chelating agents; 2, metal binding agents; 3, reducing agents; 4, sulfhydryl agents; 5, flavin inhibitor. In all the experiments, the compound was added to the 0.1 ml of enzyme (400 units of specific activity 20 200) and 0.0313 M sodium phosphate buffer (pH 7.9) and preincubated for 20 min at 28°. The reaction was started by the addition of glycolate (10 $\mu$moles) or L-$\alpha$-hydroxyisocaproate (10 $\mu$moles). Keto acids in the reaction mixtures were determined by the 2,4-dinitrophenylhydrazone method.

| *Group* | *Compound* | *Final concn. (M)* | *Inhibition (%) of* | |
|---|---|---|---|---|
| | | | *Glycolate oxidation* | *Hydroxyisocaproate oxidation* |
| 1 | Diethyldithiocarbamate | $1 \cdot 10^{-3}$ | 55 | 51 |
| | Allylthiourea | $1 \cdot 10^{-3}$ | 0 | 0 |
| | 8-Hydroxyquinoline | $1 \cdot 10^{-3}$ | 17 | 8 |
| | *o*-Phenanthroline | $1 \cdot 10^{-3}$ | 5 | 14 |
| | $\alpha,\alpha'$-Dipyridyl | $1 \cdot 10^{-3}$ | 0 | 0 |
| | EDTA | $1 \cdot 10^{-3}$ | 0 | 0 |
| 2 | KCN | $1 \cdot 10^{-4}$ | 36 | 0–20 |
| | KCN | $1 \cdot 10^{-3}$ | 91 | 6–30 |
| | Sodium thiocyanate | $1 \cdot 10^{-3}$ | 0 | 0 |
| | NaF | $1 \cdot 10^{-3}$ | 0 | 0 |
| 3 | Cysteine | $1 \cdot 10^{-3}$ | 28 | 0 |
| | Ascorbate | $1 \cdot 10^{-3}$ | −26 | −17.5 |
| 4 | *p*-Chloromercuribenzoate | $1 \cdot 10^{-5}$ | 75 | 99.5 |
| | *o*-Iodosobenzoate | $1 \cdot 10^{-5}$ | 15 | 9.5 |
| | *o*-Iodosobenzoate | $1 \cdot 10^{-4}$ | 46.5 | 45.5 |
| | $CuSO_4$ | $2.5 \cdot 10^{-5}$ | 99 | 99 |
| 5 | Quinacrine | $1 \cdot 10^{-3}$ | 6 | 6 |

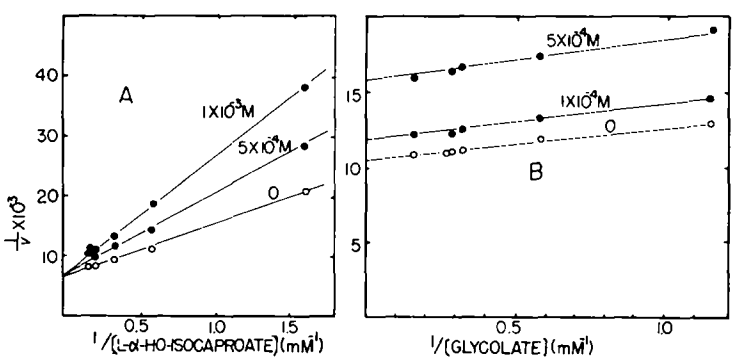


Fig. 6. Inhibition of (A) L-$\alpha$-hydroxyisocaproate oxidation by diethyldithiocarbamate and (B) glycolate oxidation by KCN. The conditions of the incubation and assay were the same as those described in the legend to Fig. 4, except that inhibitor was added to the reaction mixture. The enzyme preparation of specific activity 20 000 (10 $\mu$g of protein) was used .Numbers over straight line represent the concentration of the inhibitor. Velocity, V, is expressed as the molar concentration of keto acid from the corresponding hydroxy acid formed for 20 min with or without added inhibitor.

of chelex 100 at the bottom[21], without decrease of the specific activity and uncontaminated with free cyanide. From these results, it seems likely that cyanide couples with the enzyme substrate complex (especially with the oxidase glycolate complex) but not with the oxidase alone.

*Stability*

The purified enzyme (in sodium phosphate buffer (pH 7.9) with or without EDTA, 1 mg of protein per ml) can be stored frozen for at least two months without significant loss of activity. On the other hand, dilute enzyme solutions lose their activity on freezing and thawing and by vigorous shaking, but can be protected by the addition of crystalline human albumin to a concentration of about 200 $\mu$g of protein per ml.

*Molecular weight*

Gel filtration of the purified enzyme (specific activity, 32 500) was studied with a view to estimating its molecular weight. The enzyme, in Buffer A, was loaded on a column of Sephadex G-200, calibrated according to the method of ANDREWS[22], with crystalline mammalian L-amino-acid oxidase*, crystalline beef liver catalase and human $\gamma$-globulin. Elution was performed with Buffer A and fractions were collected and assayed for enzyme activity. The elution volume of the enzyme indicated a molecular weight of about 300 000.

## DISCUSSION

It has been reported by BLANCHARD *et al.*[24] that mammalian L-amino acid oxidase present in the livers and kidneys of rats catalyzes the deamination of L-amino acids as well as the dehydrogenation of L-α-hydroxy acids. Studies with the crystalline L-amino-acid oxidase obtained from rat kidneys confirmed their finding[1,2]. A long chain L-α-hydroxyacid oxidase from hog kidney cortex has been purified and identified as a flavoprotein which catalyzes the dehydrogenation of aliphatic and aromatic hydroxy acids but does not deaminate amino acids[4]. Apparently, no enzyme with the specificity of the aliphatic L-α-hydroxyacid oxidase herein described has been reported previously. This enzyme is similar however to the short chain hydroxyacid oxidase (a flavoprotein) obtained from hog kidney in that glycolate and L-α-hydroxyisocaproate are potent substrates of both enzymes[4].

Recently, DE DUVE AND BAUDHUIN have isolated "Peroxisomes" from rat liver homogenate by the sucrose gradient method and postulated that the particles contain the peroxide-producing enzymes, D-amino acid oxidase, uricase and L-α-hydroxyacid oxidase[3]. Peroxisomes, however, are not clearly separated from lysosomes and mitochondria by the differential centrifugation of rat liver homogenate in 0.25 M sucrose. Judging from our finding that the L-α-hydroxyacid oxidase purified from the light mitochondrial fraction generates $H_2O_2$, this enzyme could be located in the peroxisomes

* The molecular weight of mammalian L-amino-acid oxidase has already been reported to be 88 900 which is extremely low compared with its sedimentation coefficient[1]. A corrected value was recently reported to be 315 000, by two methods: the meniscus depression method[23], and by gel filtration[22].

rather than in the lysosomes of rat liver cells. It seems likely, therefore, that the enzyme presented here is identical with the peroxisomal L-α-hydroxyacid oxidase which has been suggested to be a flavoprotein[3]. Most oxidases which produce $H_2O_2$ have FMN or FAD as prosthetic groups. To date, however, spectrophotometric studies of the highly purified enzyme and attempts to split off a prosthetic group have provided no evidence of a functional flavin group or of $NAD^+$.

KCN and diethyldithiocarbamate were found to be the potent inhibitors of this enzyme. The kinetic study of cyanide inhibition suggests that it may combine with the enzyme–substrate (especially glycolate) complex but not with enzyme alone. Competitive type inhibition was observed with diethyldithiocarbamate which has been known to inhibit enzymes dependent for activity on amino groups, on sulfhydryl groups or on copper[25]. Even though the absorption spectrum of the enzyme is reminiscent of uricase, a copper protein[15], the mode of action of KCN and diethyldithiocarbamate on the enzyme differs from those to uricase and to other typical metalloproteins. Thus no positive evidence could be found for the existence of a metal cofactor.

Thus, it will be of interest to determine whether the enzyme possesses any cofactor or prosthetic group.

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