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# DISTRIBUTION AND IMMUNOCHEMICAL PROPERTIES OF RAT KIDNEY L-AMINO-ACID OXIDASE, WITH A NOTE ON PEROXISOMES

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SUMMARY

An amino-acid oxidase in rat kidney cells has been located mainly in peroxisomes and the soluble fraction. The peroxisomes were isolated as a bright yellow pellet from the fraction comprising mitochondria and light mitochondria by sucrose density gradient centrifugation. The particles contained L-amino acid oxidase in the ratio to total peroxisomal protein of 1:4. The enzyme in the subcellular particles could be easily solubilized by treatment with Triton X-100 without causing inactivation or immunochemical changes. The enzyme was found to be essentially the same in all subcellular fractions, as judged by the immunochemical studies and electrophoretic data. No enzyme which catalyzes the dehydrogenation of L-lactate or L-α-hydroxyisocaproate without added $NAD^+$ or flavin cofactors, other than L-amino-acid oxidase, was present in the rat kidney cells. The soluble fraction in rat kidney cells contained an unknown dialyzable activator of L-amino-acid oxidase.

## INTRODUCTION

A highly purified enzyme which oxidizes a series of α-amino and α-hydroxy acids of the L configuration was first isolated from rat kidney by BLANCHARD *et al.*[1–3]. It was initially named L-amino-acid oxidase or L-hydroxy-acid oxidase, but the term mammalian L-amino-acid oxidase (L-amino acid:$O_2$ oxidoreductase, EC 1.4.3.2) would seem to be appropriate to distinguish it from L-amino-acid oxidase of snake venom and other L-hydroxy-acid oxidases.

Mammalian L-amino-acid oxidase has been crystallized in our laboratories from the mitochondrial as well as the soluble fraction of rat kindey[4,5]. Recent evidence from other laboratories[6,7] indicates that L-amino-acid oxidase is predominantly associated with peroxisomes, particularly with those which contain catalase. Since mammalian L-amino-acid oxidase also catalyzes the dehydrogenation of many L-α-hydroxy acids, it was uncertain whether a separate enzyme, such as hepatic aliphatic L-α-hydroxy-acid oxidase[8], having a specificity for only the L-α-hydroxy acids also existed in rat kidney.

In the present work the distribution of L-amino-acid oxidase in rat kidney in terms of enzyme activity and immunochemical properties of the enzyme in subcellular fractions in this tissue has been studied by differential centrifugation, on sucrose density gradients, and with enzyme–antibody reactions. The biochemical and morphological properties of the peroxisomes isolated from rat kidney are also the subject of this report.

## MATERIALS AND METHODS

### *Determination of enzyme activity*

(1) L-Amino acid oxidase activity was determined by the 3-hydrazinoquinoline method with L-α-hydroxyisocaproate as substrate[4]. One unit of activity is defined as that amount of enzyme which produces 1 nmole of α-ketoisocaproate in 20 min. Since the highly purified enzyme has a specific activity of 11 000 units/mg protein[5], the amount of enzyme (μg) in a crude preparation may be given as one eleventh of the total enzyme units, as defined above.

(2) Cytochrome oxidase activity was measured by the method of COOPERSTEIN AND LAZAROW[9]. 3 ml of solution contained $1.7 \cdot 10^{-5}$ M of reduced cytochrome *c* in 0.03 M sodium phosphate buffer at pH 7.4. In this system units of activity are based on: $\log (A_{t_1} - A_{ox}) - \log (A_{t_2} - A_{ox})/(t_2 - t_1)$, where $A_{t_1}$, $A_{t_2}$ and $A_{ox}$ represent absorbance at times $t_1$, $t_2$ and after addition of ferricyanide, respectively. In our studies $t_2 - t_1$ equals 1 min; the total volume is 3.1 ml.

(3) Acid phosphatase activity was determined by the method of KILSCHEIMER AND AXELROD[10]. One unit of enzyme is that quantity which liberates 1.0 μmole of nitrophenol per 15 min per 0.5 ml of reaction mixture.

(4) Catalase activity was measured in 0.1 ml of the enzyme preparation by the method of BEERS AND SIZER[11]. The units are based on: $\log A_0 - \log A_{10}$, where $A_0$ and $A_{10}$ represent absorbance (240 mμ) at 0 time and 10 sec, respectively.

### *Protein concentration*

Protein in soluble materials and in precipitated antigen–antibody complexes was measured by the phenol method[12]. Fractions which contained subcellular particles were first treated with deoxycholate and NaOH, and the protein concentration was assayed by successive additions of the $CuSO_4$ solution and phenol reagent[13].

### *Preparation of crystalline L-amino-acid oxidase*

All procedures were conducted at 0–5°. Rat kidneys (50–60 g) were ground with 4 vol. of 0.25 M sucrose in a glass homogenizer. The crude homogenate was then centrifuged at 600 × *g* for 10 min. The supernatant from this separation was then centrifuged at 20 000 × *g* for 2 h and the clear supernatant fluid ($S_1$) was saved. The precipitate was suspended in 100 ml of 0.05 M sodium phosphate buffer containing 1 mM EDTA (pH 7.9) and then treated with Triton X-100 at 0.2% at 0°. The resultant mixture was centrifuged at 20 000 × *g* for 2 h, and the supernatant was combined with $S_1$. Solid $(NH_4)_2SO_4$ was then added, and the mixture was stirred for 30 min. The protein fraction between 33 and 50% $(NH_4)_2SO_4$ saturation was dissolved in a small volume of 0.05 M sodium phosphate buffer which contained 1 mM EDTA

(pH 7.9). This solution was dialyzed against 3 l of 5 mM sodium phosphate buffer which contained 1 mM EDTA at pH 7.9 (Buffer A) for 18 h. The resultant clear solution, totaling 20–25 ml, was divided into equal volumes, and each aliquot was transferred to a DEAE-cellulose column (1.8 cm × 15 cm) which had been equilibrated with Buffer A. After washing the column with 100 ml of Buffer A, the enzyme was eluted with 200 ml of 0.1 M sodium phosphate buffer which contained 1 mM EDTA (pH 7.9). The enzyme was then concentrated and purified by successive $(NH_4)_2SO_4$ fractionation, DEAE-cellulose column chromatography (linear gradient elution), gel filtration on Sephadex G-200, and crystallization, according to the methods described previously[5]. These procedures yielded 4–5 mg of crystalline L-amino-acid oxidase with a specific activity of 11 000 units/mg protein under standard assay conditions. When the enzyme protein was examined by disc electrophoresis[14], only one band was discerned after staining with Amido Black (Fig. 1).

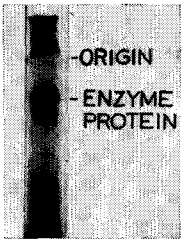



Fig. 1. Acrylamide gel electrophoresis of pure L-amino-acid oxidase. The gel was stained with Amido Black.

*Immunochemical assay*

The titration assay comprised incubation of antibody with the enzyme preparation at pH 7.4 for 18 h at 4°, and subsequent centrifugation of the mixture[2]. The clear supernatant and the antigen–antibody complex (washed twice with 0.02 M sodium phosphate buffer containing 0.15 M NaCl at pH 7.4 (buffered saline)) were used for the determination of enzyme activity and for the measurement of protein, respectively. The results of the titration assay were expressed in terms of the antibody unit, defined as that amount of antibody preparation which removed 1 unit of enzyme from the purified solution of enzyme. The Ouchterlony technique was used in the agar diffusion tests[15].

*Preparation of antibody*

Antisera were obtained from a rabbit as described previously[5], except that crystalline L-amino-acid oxidase obtained from whole kidney cells was used as the antigen in place of the enzyme from the soluble fraction of rat kidney cells. Saturated $(NH_4)_2SO_4$ adjusted to pH 7.4 with concentrated $NH_4OH$ was added to antiserum which had been diluted twice with 0.02 M sodium phosphate buffer containing 0.15 M NaCl (pH 7.4) until 50% saturation with this salt had been reached, and the mixture was stirred for 30 min at 0°. The precipitate which contained most of the globulins in serum was collected by centrifugation, dissolved in a small volume of 5 mM sodium phosphate buffer (pH 8.0) and dialyzed against 6 l of the same buffer at 4°.

This globulin fraction was then transferred onto DEAE-cellulose columns (1.2 cm × 20 cm) which had been equilibrated with 5 mM sodium phosphate buffer (pH 8.0). The protein was eluted successively with the same buffer, 0.02 M sodium phosphate buffer (pH 6.35) and 0.05 M $NaH_2PO_4$ (pH 4.3). The antibody was largely concentrated in the eluate obtained with 5 mM sodium phosphate buffer. Cellulose acetate electrophoresis[16] indicated that this was in the $\gamma$-globulins. Solid $(NH_4)_2SO_4$ was added to the eluate, which contained the antibody, to obtain its 50% saturation. The protein precipitate was collected and dialyzed against the buffered saline. This fraction contained 500–800 antibody units/mg protein.

*Cell fractionation*

Rat kidneys weighing 12–18 g were perfused with saline, homogenized in 0.25 M sucrose, and fractionated by differential centrifugation by the procedure of DE DUVE *et al.*[17] to separate the particles and the soluble fraction in liver homogenates. Centrifugal forces (at average radius) of 600 × *g* for 10 min, 3300 × *g* for 10 min, 10 000 × *g* for 20 min, and 105 000 × *g* for 30 min caused the sedimentation of nuclear, mitochondrial, light mitochondrial and microsomal fractions, successively. The nuclei were discarded, and the other particles were washed twice with 0.25 M sucrose. The fraction in which most of the components of the homogenate, excluding the nuclei, were present was designated the cytoplasmic extract. In some instances the rough-surfaced and the smooth-surfaced microsomes were isolated according to the method of CHAUVEAU *et al.*[18], designed for the fractionation of rat liver microsomes.

Subfractionation of the suspension which contained the mitochondria, lysosomes and peroxisomes was effected by density gradient centrifugation according to the method of BEAUFAY *et al.*[19] with some modifications. The gradients were prepared with a twin-syringe machine (Beckman density gradient former, model grad form) delivering two solutions of sucrose. The gradient obtained was between the limits set by the two solutions. After the suspension of particles in 0.25 M sucrose had been layered above the gradients, the tubes were centrifuged at 23 000 rev./min for 20 h at 4° in the swinging bucket rotor (RPS-25) of the Hitachi model 55-PA centrifuge. Samples of 1 ml were removed from the bottom of the centrifuge tube with a needle.

TABLE I

EFFECTS OF SOME DETERGENTS ON THE ACTIVITY OF L-AMINO-ACID OXIDASE

Pure L-amino-acid oxidase (320 units) was treated with detergents in the concentrations listed for 30 min at 0° and pH 7.4. Half of the original solution (1 ml) was then used for the assay of enzyme activity.

| *Reagents* | *Final concn. (%)* | *Activity recovered (%)* |
|---|---|---|
| None | — | 100 |
| Deoxycholate | 0.5 | 45 |
| Triton X-100 | 0.2 | 103 |
| | 0.5 | 100 |
| | 1.0 | 102 |
| Tween 20 | 0.5 | 74 |
| | 1.0 | 69 |

*Extraction of the enzyme from particles*

The effect of certain detergents on the pure enzyme was first examined. The results are summarized in Table I. Only Triton X-100 did not produce inhibition.

Each fraction containing subcellular particles obtained from 12–18 g kidneys was suspended in 0.05 M sodium phosphate buffer which contained 0.08 M NaCl (pH 7.4) in a total volume of 10 ml. It was then treated with Triton X-100 (final concentration 1.0%) at 0° for 30 min. The suspension was centrifuged at 105 000 × *g* for 60 min. Under these conditions more than 90% of the activity in the suspension was recovered in the clear supernatant.

*Reagents*

Reagents used in the present work were obtained from the following sources: L-α-hydroxyisocaproic acid, cytochrome *c*, phenazine methosulfate, *p*-nitroblue tetrazolium and bovine serum albumin were purchased from Calbiochem, Los Angeles; catalase (lyophilized) was from Worthington Biochemical Corp., Freehold; sodium 2,6-dichlorophenolindophenol was from Fisher Scientific Co.; polyvinylpyrrolidon, Kyorin Seiyaku; Triton X-100, Rohm and Haas. All other reagents used were of analytical grade.

TABLE II

DISTRIBUTION OF L-AMINO-ACID OXIDASE IN CYTOPLASMIC EXTRACTS OF RAT KIDNEY

The fractions were obtained by differential centrifugation of cytoplasmic extracts of rat kidney as described in the text. The concentration of the oxidase was calculated by dividing the enzyme activity by 11.0.

| *Fraction* | *Enzyme (μg/g tissue)* | *Protein (mg/g tissue)* | *Enzyme (μg/mg protein)* |
|---|---|---|---|
| Mitochondrial | 207 | 13.0 | 16.0 |
| Light mitochondrial | 205 | 8.8 | 23.3 |
| Microsomal | 83 | 18.8 | 4.4 |
| Soluble | 820 | 46.0 | 18.0 |

RESULTS

*Distribution of the enzyme in rat liver cells*

Results of representative differential centrifugation studies are shown in Table II. In 7 experiments 58–65% of the L-amino-acid oxidase activity of cytoplasmic extracts was found in the soluble fraction of cells; most of the activity remaining in the granular fractions was about equally divided between the mitochondrial and light mitochondrial moieties. Since the nuclear fractions were heavily contaminated with mitochondria, erythrocytes and intact cells, these fractions were discarded without attempting to measure their enzyme activity.

The following experiment was carried out to identify any artifacts which may have been introduced by the cell particles during homogenization and by the subsequent washing. Cytoplasmic extract obtained from the homogenate of rat kidney, diluted 1:6 in 0.25 M sucrose or in 8.5% sucrose–10% polyvinylpyrrolidon, was

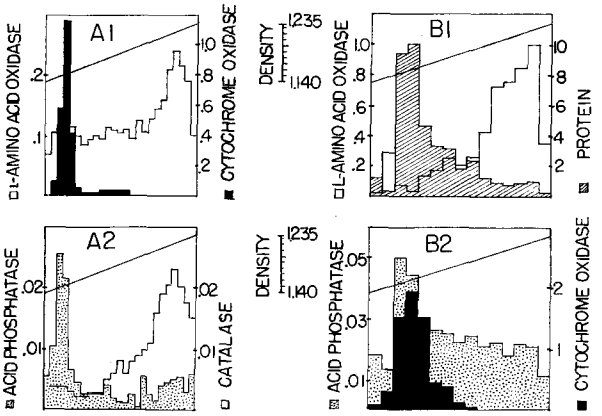



Fig. 2. Sucrose density gradient centrifugation of M + L fraction. 4 ml of the M + L fraction (protein concn. 0.165 mg/ml) were layered on top of a 30-ml gradient in the small-scale experiment (A1 and A2). 17 ml of M + L fraction (protein concn. 0.165 mg/ml) were layered onto 17 ml of the gradient (B1 and B2). The tubes were centrifuged and 1-ml samples were collected as described in the text. The sloped lines indicate sucrose density. L-amino-acid oxidase, units $\times$ $10^{-3}$/ml; cytochrome oxidase, units/0.1 ml; acid phosphatase, units/ml; catalase, units/0.1 ml; protein, mg/ml.

centrifuged at 105 000 $\times$ *g* for 30 min. The enzyme activity of the residual and the supernatant fractions was measured. In the experiments with 0.25 M sucrose approximately 53% of the enzyme activity in the total cytoplasmic extract was recovered in the supernatant fluid. The use of polyvinylpyrrolidone reduced the enzyme activity in the supernatant fluid to 85% of that recorded with the sucrose procedure. Density gradient centrifugation of the mixture of mitochondria and light mitochondria established that the density distribution of L-amino-acid oxidase was similar to that of catalase but differed from that of cytochrome oxidase and acid phosphatase (Fig. 2). From these data the median density of particles containing L-amino-acid oxidase or catalase was estimated to be 1.225, while those of particles containing cytochrome oxidase and acid phosphatase were calculated to be 1.16 and 1.15, respectively. In liver cells cytochrome oxidase, catalase and acid phosphatase are known to be the host enzymes of mitochondria, peroxisomes and lysosomes, respectively. It therefore appears that particles which contain L-amino-acid oxidase resemble peroxisomes rather than mitochondria or lysosomes in their density distribution.

As shown in Table III, most of the enzyme activity in microsomes is located in the rough-surfaced microsomes. Much less oxidase activity is demonstrable in the smooth-surfaced or agranular forms. In the latter the enzyme concentration per mg of protein is only one fifth of that found in the rough-surfaced microsomes.

*Some properties of renal peroxisomes*

The gradient fractions showing high activity of L-amino-acid oxidase, corresponding to the layers between densities of 1.20 and 1.230, were centrifuged at 105 000 $\times$ *g* for 1 h after having been diluted with $H_2O$ to a final sucrose concentration of 0.8 M. This procedure yielded particles which sedimented and formed a bright yellow pellet. Electron microscopy revealed that the pellet consisted mainly

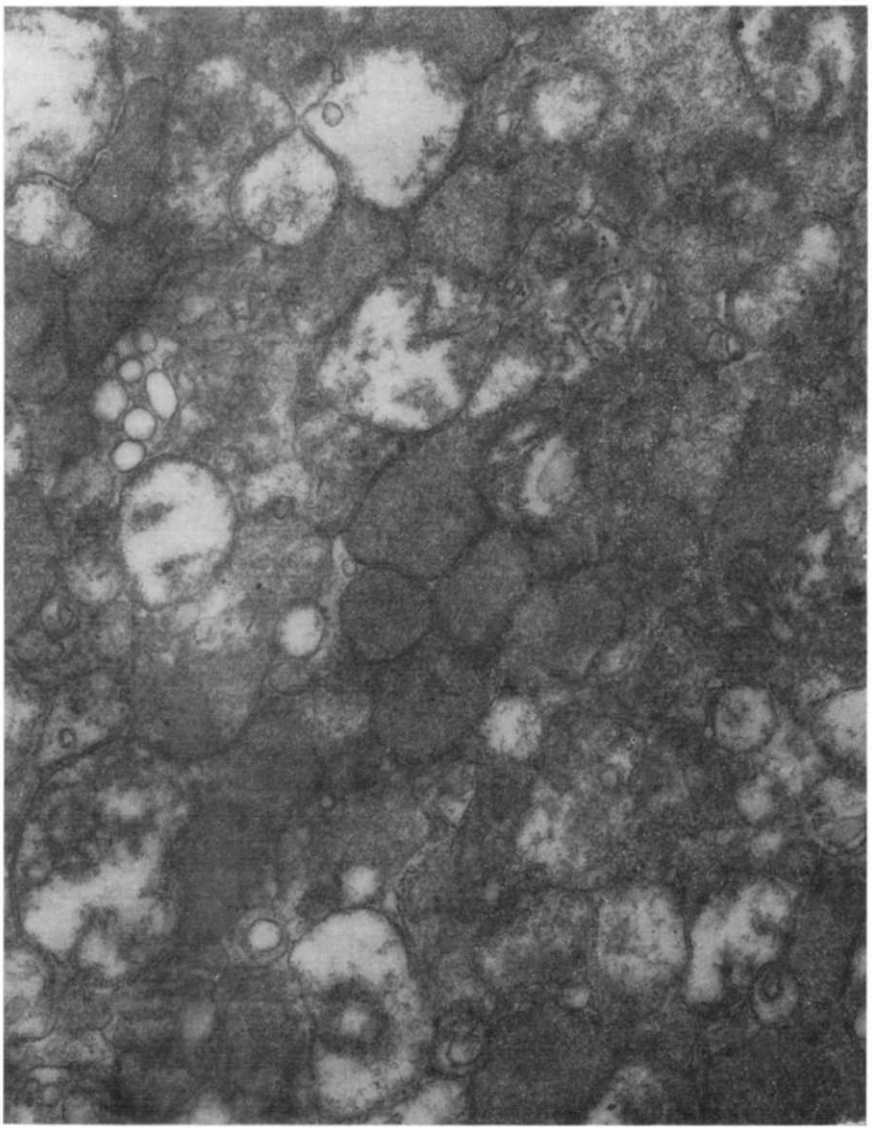


Fig. 3. Electron micrograph of peroxisome-rich pellet. 60 000 ×.

of membrane-limited cytoplasmic particles with a moderately dense granular stroma and another more dense area (Fig. 3). These ultrastructural characteristics closely resemble those described in the peroxisomes[7,20]. In some sections of the pellets observed by us mitochondria, microsomal membranes, and unidentified organelles were occasionally present as contaminants. The biochemical properties of peroxisomes which we isolated are listed in Table IV. Judging from the L-amino-acid oxidase and catalase contents, purified peroxisomes accounted for approx. 30% of the fraction comprising heavy mitochondria, lysosomes and peroxisomes (M + L fraction). In

TABLE III

L-AMINO-ACID OXIDASE IN ROUGH- AND IN SMOOTH- SURFACED MICROSOMES AND IN FREE RIBOSOMES

The submicrosomal fractions were prepared by the method of CHAUVEAU *et al.*[18]. See Table II for details of calculations of enzyme concentration.

| *Fraction* | *Enzyme (μg/g tissue)* | *Protein (mg/g tissue)* | *Enzyme (μg/mg protein)* |
|---|---|---|---|
| Rough-surfaced microsomes | 71.50 | 12.90 | 5.6 |
| Smooth-surfaced microsomes | 2.00 | 2.00 | 1.0 |
| Ribosomes (free) | 0.80 | 1.60 | 0.5 |
| Unsedimented fraction | 1.75 | 1.75 | 1.0 |

terms of the specific activities of these reference enzymes, the peroxisomes were purified almost 12-fold over the M + L fraction. Since the recovery of acid phosphatase in purified peroxisomal fractions equaled 4% of those in the M + L fraction, it can be concluded that the peroxisomes were still contaminated with lysosomes. The concentration of L-amino-acid oxidase in the purified peroxisomes represented one fourth of the total peroxisomal protein.

No glycolate oxidation activity was detected in the particles with or without added FMN by the following methods; 2,6-dichlorophenolindophenol assay[4], 2,4-dinitrophenylhydrazone assay[8] and 3-hydrazinoquinoline assay[4]. Since L-amino-acid oxidase is inactive to glycolate[4], a potent substrate for aliphatic L-α-hydroxy-acid oxidase in the light mitochondrial fraction from rat liver[18], the dehydrogenation of L-α-hydroxy-acids in the peroxisomes from rat kidney would be catalyzed by L-amino-acid oxidase rather than by L-hydroxy-acid oxidase.

*Immunochemical nature of the enzyme in cell fractions*

The reaction between antibody and crude extracts from particles or soluble fractions used as antigens was characterized by double diffusion in agar gel. When

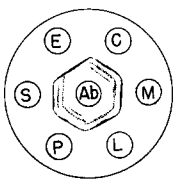
TABLE IV

BIOCHEMICAL PROPERTIES OF ISOLATED PEROXISOMES

Peroxisomes were isolated from M + L fractions (total protein, 62–64 mg) by the small-scale experiment of Fig. 2 ($A_1$ and $A_2$) using 4 sucrose–water gradients. The values given represent the averages of 2 experiments.

| *Materials* | *M + L fraction* | *Peroxisomes* |
|---|---|---|
| Recovery of protein, % | 100 | 2.4 |
| Recovery of L-amino-acid oxidase, % | 100 | 31.3 |
| Specific activity, units/mg protein | 205 | 2575 |
| Enzyme concn., μg/mg protein | 20.5 | 234 |
| Recovery of catalase, % | 100 | 31.7 |
| Specific activity, units/mg protein | 0.58 | 7.9 |
| Recovery of acid phosphatase, % | 100 | 4.0 |
| Specific activity, units/mg protein | 0.19 | 0.18 |

an excess of antibody against the enzyme in a crude extract or a soluble fraction was used, the product yielded at least 3 bands of precipitation in all but the control experiment, with pure enzyme as the antigen (Fig. 4). The main band nearest the antigen well, representing the enzyme–antibody reaction, was much more intense when crude preparations with high enzyme activity were used. Judging from precipitin reactions in agar gel, the minor bands are formed from components other than L-amino-acid oxidase which react with the antibody.



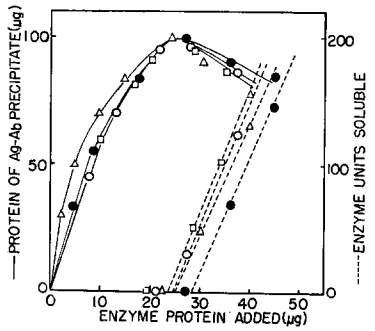
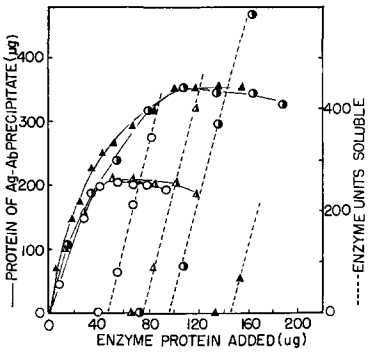




Fig. 4. Agar gel diffusion analysis of enzyme–antibody interaction. The well marked E contained 2200 units/ml of the pure enzyme; that marked M contained 2200 units/ml of extract derived from the heavy mitochondrial fraction; that marked L contained 2300 units/ml of extract from the light mitochondrial fraction; that marked P contained 165 units/ml of an extract of the microsomal fraction; that marked S contained 134 units/ml of the soluble fraction. The center well, Ab, contained 4400 units/ml of antibody.

Fig. 5. Antibody titration with purified enzyme and extracts of subcellular particles. Each tube contained 275 antibody units which neutralized 25 $\mu$g of pure enzyme. The enzyme concentration ($\mu$g) of pure L-amino-acid oxidase (○) and of extracts of subcellular particles such as the mitochondria (●), light mitochondria (△) and microsomes (□) was calculated by dividing the enzyme activity (units) by 11.0.

The results of titration assays based on extracts of subcellular particles and antibody are presented in Fig. 5. The results indicate that within the limits of experimental error, the equivalence point of the pure enzyme–antibody reaction is identical with that of the crude extract–antibody reaction. Also, the quantity of protein precipitated per $\mu$g of added enzyme is essentially the same at the equivalence point irrespective of whether the enzyme is added in a purified form or as a crude preparation. However, with crude preparations more protein precipitate was formed in the region of antibody excess. This indicates that the subcellular particles or the soluble fractions of kidney cells contain components unrelated to L-amino-acid oxidase which react with antibody. Such components are probably identical with those which complex with antibody in the agar plate and appear as precipitin bands (Fig. 4). Under conditions identical with those used with extracts from particles, the equivalence point of the enzyme in soluble fractions was markedly different from that obtained with pure L-amino-acid oxidase as the antigen (Fig. 6). The amount of protein precipitated at the equivalence point determined by pure enzyme–antibody reaction, however, was the same in both cases.

In 4 experiments, the equivalence point of the antibody reaction with the soluble fraction was found to be 1.5 ($\pm$ 0.2) times greater than that of the pure enzyme–antibody reaction in which the equivalence points were measured enzymati-



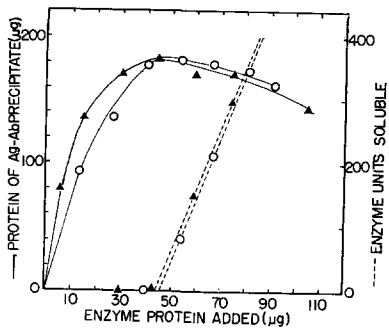




Fig. 6. Antibody titration with pure enzyme and undialyzed soluble extract. In small-scale experiments each tube contained 550 antibody units and the indicated concentration of pure (○) or crude enzyme (△). In large-scale experiments each tube contained 1045 antibody units and the indicated concentration of pure (◑) or crude enzyme (▲). See legend to Fig. 5 for calculations of enzyme concentration.

Fig. 7. Antibody titration with the pure enzyme and the dialyzed soluble fraction. Each tube contained 500 antibody units and the indicated concentration of pure (○) or crude enzyme (▲). See legend to Fig. 5 for calculations.

cally. When the soluble fraction was dialyzed against buffered saline and used as antigen, the equivalence point of the dialyzed preparation–antibody reaction proved to be identical to that of pure enzyme–antibody reaction (Fig. 7).

*Homogenity of the enzyme in subcellular fractions*

Polyacrylamide gel electrophoresis of clear supernatant prepared from particles or soluble fraction revealed a single migrating zone in all cases (Fig. 8) when stained with nitroblue tetrazolium reaction[6].

The migration of the zone was identical with that of the pure enzyme. This may indicate that only one species of L-amino-acid oxidase is present in subcellular fractions.

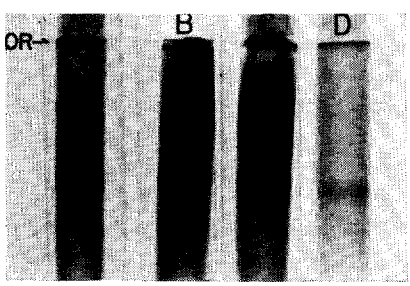



Fig. 8. Acrylamide gel electrophoresis of pure L-amino-acid oxidase and extracts of subcellular fractions. The gels were stained by incubation in a mixture of DL-lactate, nitroblue tetrazolium and phenazine methosulfate at 37° for 25 min. (A) 35 units of pure L-amino-acid oxidase. (B) 45 units of extract of light mitochondrial fraction. (C) 45 units of extract of mitochondrial fraction. (D) 45 units of soluble extract. OR, origin.

## DISCUSSION

ALLEN AND BEARD[6] investigated the intracellular localization of L-hydroxy-acid oxidase (L-amino-acid oxidase) in rat kidney by differential centrifugation and density equilibration in a sucrose gradient and concluded that the enzyme is largely associated with microbodies (peroxisomes).

Our data indicate, however, that the enzyme is located in the soluble fraction as well as in peroxisomes. The technique of cell fractionation used by the above authors is comparable to ours. However, the enzyme assay based on the 2,6-dichlorophenolindophenol or the 2,4-dinitrophenylhydrazone method employed by ALLEN AND BEARD[6] may have been inadequate. ROBINSON *et al.*[21] have reported that these methods yield erratic results in measuring L-hydroxy-acid oxidation activity of crude preparations. DE DUVE AND BAUDHUIN[7] have found that injured peroxisomes readily release their catalase and D-amino-acid oxidase in a soluble form. In our experiments, the repeated washing and resuspension of subcellular particles during the fractionation studies could have increased the L-amino-acid oxidase activity in the soluble fraction. Whether the enzyme found in isolated supernatant fluid existed in a soluble form in the cell or was accidentally released from peroxisomes during the cell fractionation is therefore unknown. However, the fact that the activity in a soluble fraction obtained by careful homogenization of kidney cells in sucrose or polyvinylpyrrolidon corresponded to about 50% of the activity in cytoplasmic extract suggests that L-amino-acid oxidase has a bimodal distribution in both soluble fraction and peroxisomes. Judging from the immunochemical studies and electrophoretic data, the enzyme in the soluble fraction appears to be essentially the same as that associated with subcellular particles or with isolated L-amino-acid oxidase.

Furthermore, these data suggest that no enzyme which catalyzes the dehydrogenation of L-lactate or L-$\alpha$-hydroxyisocaproate without added $NAD^+$ or flavin cofactors, other than L-amino-acid oxidase, is present in rat kidney cells.

It is of interest that L-amino-acid oxidase is activated about 1.5-fold by a dialyzable material present in soluble fraction. The nature of this material (an activator) or the activation mechanism is uncertain.

In previous work, we noted that nearly 100% of the L-amino-acid oxidase activity in a mitochondrial extract (a mixture of mitochondria, lysosomes and peroxisomes) or 60% of the activity in the soluble fraction is recovered in the precipitate when the preparation had been incubated with an excess of antibody[5].

The low recovery of enzyme activity in the enzyme–antibody complex precipitated from the soluble fraction may be due to the lack of an activator in this complex. The activator might have been eliminated from the soluble fraction during precipitation of the enzyme–antibody complex and the subsequent washing, and hence the recovery of enzyme activity in this complex would be lowered. On the other hand, the fact that the activity in the extract of crude mitochondrial fraction is recovered quantitatively in the enzyme–antibody complex would account for the absence of an activator in this extract. For this reason, the amount of enzyme in the soluble fraction must be corrected by dividing by an activation factor of 1.5. Thus, the true enzyme concentration of the soluble fraction is about 40% of that of the cytoplasmic extract.

The microsomal fraction also contained L-amino-acid oxidase, and most of this

activity was associated with the rough-surfaced microsomes. Even though the amount of enzyme in the rough-surfaced fraction is small compared with that in peroxisomes and the soluble fraction, this fraction may be important as a locus of L-amino-acid oxidase synthesis in kidney cells. Since DNA-synthesizing activity has not been found in liver peroxisomes[20], the peroxisomes of kidney may not be the site of L-amino-acid oxidase synthesis.

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