The Localization of α-Hydroxy Acid Oxidase in Renal Microbodies

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ABSTRACT The enzymatic properties and cellular localization of α-hydroxy acid oxidase have been studied in renal tissue of the rat. The enzyme was most active in the presence of D, L- α-hydroxy valeric acid and D, L- α-hydroxy butyric acid and was inactive in the presence of glycolic acid and D-alanine. It appeared to have no co-factor requirement. When subjected to electrophoresis in acrylamide gels, followed by cytochemical development, the enzyme was visualized as a major form accompanied by a minor component. The properties of the major electrophoretic form as determined by cytochemical reaction and densitometric scanning were similar to those determined by quantitative biochemical assay. When subjected to differential centrifugation and density equilibrium centrifugation, particles containing α-hydroxy acid oxidase sedimented with particles containing D-amino acid oxidase; α-hydroxy acid oxidase, therefore, is associated with renal microbodies. The fact that α-hydroxy acid oxidase transfers electrons to Nitro blue tetrazolium or to Tetra nitro blue tetrazolium made it possible to localize the enzyme by a light microscopic cytochemical procedure. The enzyme was found by this method to be localized predominantly in cells of the proximal tubule. It was present in particles approximately 0.5 μ to 1.0 μ in diameter mainly situated in the basal portions of these cells. These cytochemical preparations probably accurately reflect the cellular distribution of α-hydroxy acid oxidase and, hence, of those microbodies which contain the enzyme.

Microbodies are subcellular particles characterized by their content of D-amino acid oxidase, urate oxidase, and catalase (Beaufay et al., '64). While the metabolic function of microbodies is unknown, it appears significant that two of these enzymes, D-amino acid oxidase and urate oxidase, produce hydrogen peroxide while the third, catalase, breaks down this product. Recently, a fourth enzyme, α-hydroxy acid oxidase, has been identified in microbodies derived from renal tissue of the rat and from Tetrahymena (Allen and Beard, '65; Baudhuin et al., '65), which like D-amino acid oxidase and urate oxidase, produces hydrogen peroxide. Thus the suspicion that microbodies are engaged in the metabolism of hydrogen peroxide is further strengthened. Our studies of the properties of α-hydroxy acid oxidase from renal tissue of the rat, the sedimentation behavior of particles containing this enzyme, and its localization by a microscopic cytochemical method form the subject of this paper.

MATERIALS AND METHODS

All work was carried out using the kidneys of 90–180-day-old male rats.

The following methods were used in biochemical characterization and identification studies:

α-Hydroxy acid oxidase by 2, 6-dichlorophenolindophenol (DIP) reduction, modified from Robinson et al. ('62); 0.05 M Sörenson's phosphate buffer, pH 7.5; 0.5 M sodium L-lactate or 0.05 M L-isomer of any other α-hydroxy acid; 33 μM DIP; 33 μM N-methylphenazonium methosulfate ("phenazine methosulfate," PMS). The change in optical density was measured at 600 mμ at 25°C.

α-Hydroxy acid oxidase by determination of α-keto acid formation, modified from Robinson et al. ('62); 0.05 M Sörenson's phosphate buffer, pH 7.5; 0.5 M L-lactic acid or 0.05 M L-isomer of any other α-hydroxy acid; 33 μM PMS. After incubation at 37°C the reaction was stopped by the addition of one-half volume of 15% trichloroacetic acid. α-Keto acid in the supernatant fluid obtained by low-speed centrifugation was determined by the formation of 2,4-dinitrophenylhydra-

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zones as prescribed by Kachmer and Boyer ('53). The optical density was measured at 510 mμ. Contrary to the finding of Kachmer and Boyer, sucrose (Mallinkrodt, Analytical Reagent) did not interfere with α-keto acid determination. Therefore, samples which contained sucrose were not dialyzed prior to measurement by this method.

D-*Amino acid oxidase* by determination of α-keto acid formation; 0.05 M Sörenson's phosphate buffer, pH 8.0; 0.1 M D-alanine; 20 μM flavinadenine dinucleotide (FAD). After incubation at 37°C the reaction was stopped by the addition of one-half volume of 15% trichloroacetic acid and α-keto acid was determined according to Kachmer and Boyer ('53).

Succinic dehydrogenase by DIP reduction coupled to PMS as suggested by Singer and Kearney ('57); 33 mM Sörenson's phosphate buffer, pH 7.5; 33 mM sodium succinate; 3 mM potassium cyanide; 33 μM DIP; 33 μM PMS. The change in optical density at 25°C was measured at 600 mμ.

Acid phosphatase by determination of α-naphthol liberated from sodium α-naphthyl acid phosphate at pH 5.0 and 25°C exactly as specified by Allen and Gockerman ('64).

Protein nitrogen by the biuret reaction standardized against purified bovine serum albumin (Armour) (Layne, '57).

The following methods were used for the localization of enzyme activity in sections or in gels after electrophoresis:

α-Hydroxy acid oxidase; 0.05 M Sörenson's phosphate buffer, pH 7.5; 0.5 M L-lactic acid or 0.05 M L-isomer of any other α-hydroxy acid; 50 μM PMS; 2 mM Nitro blue tetrazolium (NBT) or Tetra nitro blue tetrazolium (TNBT) (ca. 2 mgm per ml). Sections and gels were incubated in the dark at 37°C.

α-Hydroxy acid oxidase by coupled peroxidatic oxidation of 3-amino-9-ethylcarbazole, modified from Graham and Karnovsky ('65); 0.05 M Sörenson's phosphate buffer, pH 7.5; 0.1 M D, L- α-hydroxy valeric acid; 1 mM 3-amino-9-ethylcarbazole; 2 mgm horseradish peroxidase per 5 ml (Sigma, Grade II). Sections and gels were incubated at 37°C.

Succinic dehydrogenase; reaction mixture and conditions of incubation were identical to those specified for α-hydroxy acid oxidase except that a final concentration of 0.05 M sodium succinate was substituted for the α-hydroxy acid.

Acid phosphatase; exactly as specified by Barka and Anderson ('63), using Naphthol AS MX phosphate as the substrate. Sections were incubated at 25°C.

Electrophoresis in acrylamide gels was carried out at 25°C according to Davis ('64). A constant-current power supply delivered 2.5 mA per gel tube. Current was allowed to flow until the marker dye (bromphenol blue) had migrated 4 cm (approximately 55 minutes). Gels were immersed in an appropriate cytochemical reaction mixture immediately after electrophoresis was completed. These gels were incubated at 25°C. After cytochemical development these gels were stored in 7.5% acetic acid prior to photographing or densitometric scanning.

The device described by Johnson and Starkweather ('62) was employed for densitometric scanning. For scanning, gels were immersed in 7.5% acetic acid in an appropriate cuvette. The optical density of gels developed in reaction mixtures containing NBT or TNBT was measured at 540 mμ. Under the conditions of development and scanning employed by us, the rate of formazan deposition was linear over a reasonable time of incubation and range of enzyme concentration. For these quantitative studies TNBT was the preferred oxidation indicator since the build-up of optical density was more rapid than with NBT.

Tissue for centrifugation analysis was homogenized in smooth bore glass homogenizers with loose fitting (ca. 0.01 inch clearance) Teflon pestles. Initial homogenization was limited to three rapid up-down strokes during which the pestle was rotated approximately 600 rpm. Subsequent resuspensions were achieved by a single up-down stroke of a non-rotating pestle. α-Hydroxy acid oxidase was particularly prone to release by rough mechanical treatment during homogenization and resuspension. All homogenization and centrifugation procedures were carried out near 4°C. Homogenates were prepared in

0.25 M sucrose at a tissue concentration of 15 gm wet tissue weight dispersed in 100 ml of suspending medium.

Differential centrifugation was carried out as follows: Nuclear fraction, 270 g for ten minutes followed by two resuspensions and two washes at 270 g for ten minutes each; Mitochondrial fraction, 3020 g for ten minutes followed by resuspension and one wash at 2,600 g for ten minutes; Lysosomal fraction, 22,000 g for 20 minutes; Microsomal fraction, 10^5 g for 60 minutes. Treatment of the mitochondrial pellet after the initial centrifugation affected the distribution of α-hydroxy acid oxidase (fig. 2). This pellet was three-layered. The bottom layer was dark brown, the middle layer was tan, and the upper layer was beige. In certain cases the beige layer was removed by pipette and was incorporated with the supernatant fluid from which the lysosomal pellet was later derived. In other cases this beige layer was allowed to remain undisturbed. Sedimentation of the nuclear, mitochondrial, and lysosomal fractions was done with the Servall SS 34 rotor. The microsomal fraction was sedimented in the Spinco no. 40 rotor.

Density equilibrium centrifugation was done according to Beaufay et al. ('64) using the Spinco SW 39 rotor operated at 39,000 rpm for 2.25 hours exclusive of acceleration and deceleration times. Gradients were prepared and fractions were removed by the procedure of Martin and Ames ('61). The linearity of gradients was confirmed by spectrophotometric measurement of the distribution of DIP added during their preparation. Gradients were stored at 4°C for 6–18 hours prior to use. Duration of storage did not affect the distribution of particles within the gradients. Four hundred microliters of an appropriate sample were layered on top of these gradients to yield a total volume of 5 ml in the centrifuge tube. This volume was collected in 14 equally-sized fractions on the basis of drop counting. Fraction 14 was considered to contain solubilized-enzyme with minimal amounts of particle associated activity. The small per cent of the total succinic dehydrogenase activity recovered in this fraction supports this contention (table 1). Samples used for density equilibrium centrifugation consisted of the lysosomal fraction derived from the above scheme of differential fractionation. The samples layered on the gradients contained particles derived from 0.12 gm of fresh kidney tissue. Recovery of enzyme activity with the exception of D-amino acid oxidase, approached 100% (table 1). Low recoveries of D- amino acid oxidase have also been reported by others (Beaufay et al., '64). It is unlikely that this error affects the conclusions presented below.

Tissue for cytochemical study was fixed as specified by Walker and Seligman ('63). After fixation these tissues were quenched in isopentane immersed in a bath of liquid nitrogen. They were then sectioned at 2 μ

TABLE 1

Recovery data from centrifugation analysis

	Number of determinations	α-Hydroxy acid oxidase	D-Amino acid oxidase	Succinic dehydrogenase	Acid phosphatase
		%	%	%	%
Differential centrifugation [1,3]	15	97.4	93.0	100.5	99.9
Density gradient centrifugation [1,3]	10	103.1	80.9	105.1	93.2
Fraction 14 from density gradient centrifugation [2,3]	10	12.0	6.6	4.8	17.5

[1] Column entries refer to per cent of activity recovered from differential or density gradient centrifugation fractions as compared to the activity present in an appropriate sample of the whole homogenate (differential centrifugation) or of the "lysosomal" fraction (density gradient centrifugation).

[2] Column entries refer to the per cent of activity recovered in this fraction as compared to the activity recovered in the entire gradient.

[3] Recovery values are based on pooled data from all schemes of differential centrifugation or density gradient centrifugation.

or 4 μ in a microtome cryostat maintained at − 20°C and mounted on cover glasses. Some sections were dried approximately one minute in a current of air prior to immersion in cytochemical reaction mixtures. Other sections were used immediately after thawing. Either procedure produced equivalent results. In some cases tissues were sectioned without prior fixation. These were quenched, sectioned, and mounted exactly as specified for fixed tissues. Unless otherwise stated all observations reported below refer to fixed tissues.

RESULTS

Biochemical properties of α-hydroxy acid oxidase

α-Hydroxy acid oxidase was characterized biochemically using the supernatant fluid obtained by brief blending of homogenates prepared in 0.1 M Sörenson's phosphate buffer at pH 7.5 followed by centrifugation at 35,000 g for 30 minutes at 4°C in the Servall SS 34 rotor. These preparations, which contained over 90% of the α-hydroxy acid oxidase in the soluble phase, were also used in electrophoretic studies.

The properties of α-hydroxy acid oxidase as determined by DIP assay are given in tables 2 and 3. The enzyme (table 2) showed approximately equal activities in the presence of L-lactic acid, D, L-α-hydroxy butyric acid, and D, L-α-hydroxy valeric acid. Activity was slightly less with D, L-α-hydroxy caproic acid and tended to be decreased in the presence of branched chain substrates. Substitution of the phenolic radical inhibited activity. The enzyme showed no measurable activity in the presence of glycolic acid or D-alanine. With this assay system (table 3), the presence of flavin mononucleotide (FMN), FAD, or PMS did not influence the activity of the enzyme. The effect of nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) could not be assessed by this system because of the presence of other enzymes (such as lactate dehydrogenase) which require these cofactors. Enzyme activity was inhibited about 25% by quinacrin (atabrin), about 50% by equimolar concentrations of α-keto acid (α-keto valeric acid in the presence of α-hydroxy valeric acid), and nearly totally by parachloromercuribenzoic acid (PCMB).

α-Hydroxy acid oxidase could be readily identified after electrophoresis in acrylamide gels by virtue of its ability to transfer electrons from appropriate substrates to either NBT or TNBT. The enzyme appeared as a single major electrophoretic form (fig. 1c) accompanied by a minor form. In these electrophoretic preparations, α-hydroxy acid oxidase could be easily con-

TABLE 2
Substrate specificity of α-hydroxy acid oxidase

Substrate [1]	Crude supernatant [2]	Density gradient fraction [3]	Acrylamide gel [4]	Section [5]
0.05 M and 0.1 M Glycolic acid	0.00		0.00	
0.5 M L-Lactic-acid	0.99	1.05	0.46	+
0.5 M D-Lactic acid	0.00	0.00	0.00	−
0.10 M D, L-β-phenyllactic acid	0.22	0.44	0.22	±
0.10 M D, L-α-hydroxybutyric acid	1.30	1.05	0.98	++
0.10 M D, L-α-hydroxyisobutyric acid	0.01	0.00	0.00	−
0.10 M D, L-α hydroxyvaleric acid	1.00	1.00	1.00	++
0.10 M D,L-α-hydroxyisovaleric acid	0.29	0.38	0.39	
0.10 M D, L-α-hydroxycaproic acid	0.70	0.71	0.82	++
0.10 M D, L-α-hydroxyisocaproic acid	0.97	0.62	0.80	
0.10 M D-alanine	0.00		0.00	

[1] Reaction mixture contained, in addition to the indicated final concentration of substrate, 0.05 M Sörenson's phosphate buffer, pH 7.5; 78 μM PMS; 33 μM DIP or 2 mM Nitro blue tetrazolium.
[2] Assay by DIP reduction. Column entries refer to ratio of activity in presence of 0.1 M D, L-α-hydroxy valeric acid.
[3] Sample for assay derived from fraction 8 of a gradient extending from a density of 1.17–1.26. Assay by DIP reduction. Column entries refer to ratio of activity in presence of 0.1 M D, L-α-hydroxy valeric acid.
[4] Electrophoresis of crude supernatant followed by cytochemical development in reaction mixture containing NBT. Column entries refer to activity in presence 0.1 M D, L-α-hydroxy valeric acid.
[5] Formol-Hank fixed tissue, sectioned at 4 μ and immersed in appropriate reaction mixture containing valeric acid for 15 minutes at 37°C. ++, activity in presence 0.1 M D, L-α-hydroxy valeric acid; +, moderate activity; ±, just detectable activity; −, no discernible activity.

TABLE 3

Effect of adjuvants on α-hydroxy acid oxidase

Assay system	Crude supernatant [2]	Density gradient fraction [3]	Acrylamide gel [4]	Section [5]
Standard [1]	1.00	1.00	1.00	++
S PMS	1.07	1.05	0.61	+
C 50 μM FMN	1.01	1.10	1.04	++
C 50 μM FAD	1.04	0.80	0.88	++
C 1 mM NAD			1.00	
C 1 mM NADP			1.01	
C 1 mM KCN	1.00	1.30	0.85	+
C 0.1 M D, L-α-ketovaleric acid	0.40	0.45	0.33	±
C 1 mM parachloromercuri-benzoic acid	0.00	0.00	0.25	−
C 1 mM quinacrin	0.74	0.80	0.86	+

[1] Standard reaction mixture contained 0.05 M Sörenson's phosphate buffer, pH 7.5; 0.1 M D, L-α-hydroxy valeric acid; 78 μM PMS; 33 μM DIP or 2 mM Nitro blue tetrazolium. Column entries refer to additions (C) or to deletions (S) from this reaction mixture.
[2] Assay by DIP reduction. Activity expressed as ratio to that of "Standard."
[3] Sample for assay derived from fraction 8 of a gradient extending from a density of 1.17–1.26. Assay by DIP reduction. Activity expressed as ratio to that of "Standard."
[4] Electrophoresis of crude supernatant followed by cytochemical development in reaction mixture containing NBT. Activity expressed as ratio to that of "Standard."
[5] Formol-Hank fixed tissues, sectioned at 4 μ, and immersed in appropriate reaction mixture for 15 minutes at 37°C. ++, maximal activity based on activity in "Standard" reaction mixture; +, moderate activity; ±, just detectable reaction; −, no discernible reaction.

fused with either lactic dehydrogenase Isozyme 3 or 4) (fig. 1g) or with lactate dehydrogenase "X" (Zinkham et al., '63) of testis (fig. 1h). Lack of a requirement for NAD (see below), however, made positive identification of α-hydroxy acid oxidase a simple matter.

The properties of the major form of α-hydroxy acid oxidase as determined by photometric scanning of gels after electrophoresis and cytochemical development are presented in tables 2 and 3 and figure 1. Flavin mononucleotide, FAD, NAD, and NADP did not increase the activity of α-hydroxy acid oxidase but its activity was increased by PMS (table 3 and fig. 1). The substrate specificity and response to adjuvants of the major electrophoretic form were similar to those determined by DIP assay of crude supernatant material (tables 2 and 3). Activity in the presence of L-lactic acid, however, was an exception. With this material, activity as measured by DIP assay was about twice as great as that measured by tetrazole reduction. The activity of the minor electrophoretic form was too low to permit photometric measurement. However, visual observation suggested that its properties were similar to those of the major form.

Production of hydrogen peroxide by both major and minor forms of α-hydroxy acid oxidase was demonstrated by the method of Graham and Karnovsky ('65). The position of the reaction product, produced by peroxidatic oxidation of 3-amino-9-ethylcarbazole, corresponded exactly to that produced by α-hydroxy acid oxidase mediated tetrazole reduction (fig. 1c, f).

Distribution of particles containing α-hydroxy acid oxidase after differential centrifugation

Results of differential centrifugation analyses are given in figure 2. In these experiments, α-hydroxy acid oxidase, D-amino acid oxidase, and acid phosphatase behaved similarly and showed highest relative specific activities in the "lysosomal" fraction. Succinic dehydrogenase activity was concentrated in the "mitochondrial" fraction. When the beige colored upper layer of the "mitochondrial" pellet was carried over into the "lysosomal" fraction (fig. 2, II, A, B, and D), an enrichment of the activities of α-hydroxy acid oxidase, D-amino acid oxidase, and acid phosphatase in the "lysosomal" fraction was achieved.

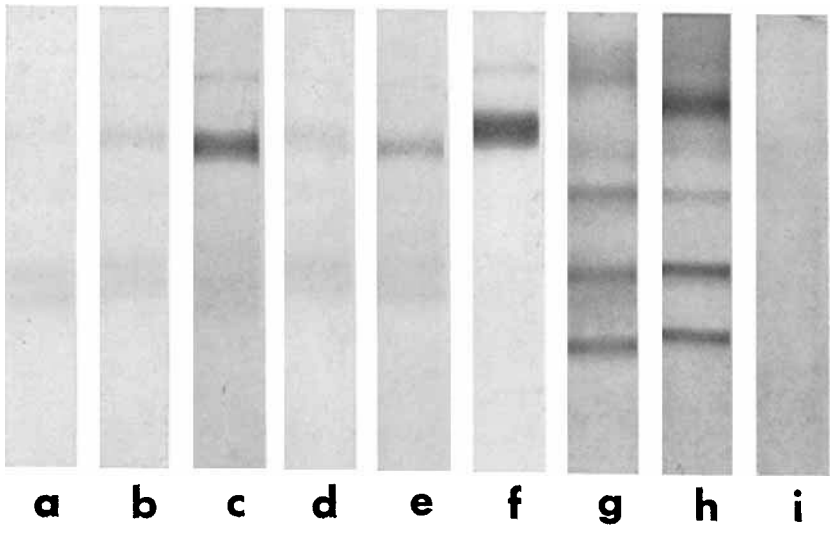

Fig. 1 Electrophoretic separation of α-hydroxy acid oxidase, lactate dehydrogenase, and lactate dehydrogenase "X" in acrylamide matrices. (*a*) gel incubated in a reaction mixture for the demonstration of α-hydroxy acid oxidase which contained 0.5 M L-lactic acid but which lacked PMS; (*b*) gel incubated in reaction identical to that used to produce "a" but which contained PMS; (*c*) gel incubated in reaction mixture identical to that used to produce "b" but which contained 0.1 M D, L-α-hydroxy valeric acid; (*d*) identical to "c" but contained 0.1 M D, L-β-phenylactic acid (*e*) identical to "c" but also contained 0.1 M D, L-α-keto-valeric acid; (*f*) gel incubated in the reaction mixture of Graham and Karnovsky ('65) containing 0.1 M D, L-α-hydroxy valeric acid; (*g*) identical to "b" but also contained 1 mM NAD; (*h*) identical to "c" but also contained 1 mM NAD; (*i*) gel incubated in reaction mixture lacking substrate. Material for electrophoresis consisted of crude supernatant preparations (see Materials and Methods). All preparations were derived from kidney except "h" which was from testis. All gels were incubated five minutes at 25°C except "f" which was incubated 30 minutes at 37°C. In all cases the cathode is at the top of the figure and migration of enzyme was toward the anode (bottom). Photographs enlarged 2 fold. The close correspondence between the properties derived for α-hydroxy acid oxidase by this method of analysis (compare b, c, d, and e) and those derived by DIP assay indicate that the same enzyme was measured in both cases.

Distribution of particles containing α-hydroxy acid oxidase after density equilibrium centrifugation

The behavior of particles containing α-hydroxy acid oxidase, D-amino acid oxidase, succinic dehydrogenase, and acid phosphatase after density equilibrium centrifugation in three different gradients is presented in figure 3. The average median densities of particles containing these enzymes are presented in table 4. In these gradients, α-hydroxy acid oxidase and D-amino acid oxidase displayed similar average median densities ($P > 0.05$). In gradients extending from densities of 1.12–1.26 and 1.15–1.26 these enzymes had similar density distribution profiles. However, in gradients extending from density 1.17–1.26, the density distribution profiles of α-hydroxy acid oxidase and D-amino acid oxidase were not strikingly similar. The density distribution profiles and the average median densities of particles containing α-hydroxy acid oxidase and D-amino acid oxidase were in all instances distinct from those of particles containing succinic dehydrogenase or acid phosphatase ($P < 0.01$ for average median densities).

α-Hydroxy acid oxidase derived from density gradient fractions having high relative activities showed substrate specificities and other properties nearly identical to those determined by assay of crude supernatant material (tables 2 and 3). After electrophoresis of such fractions, the sites of enzymatic activity seen following cytochemical development for the localization

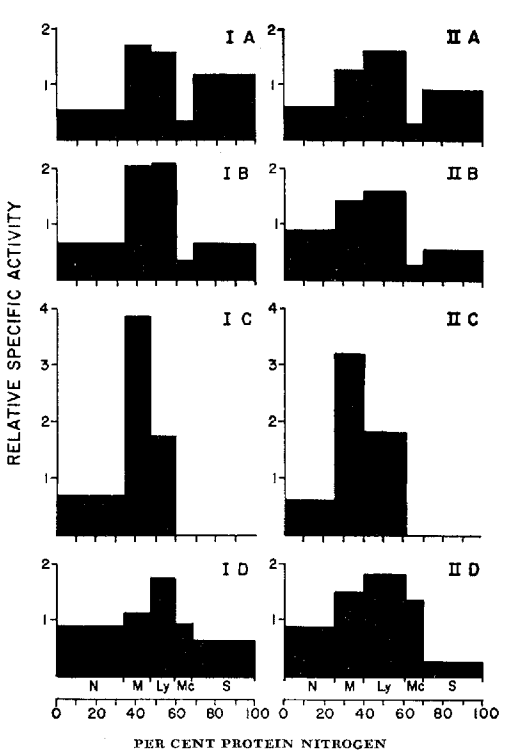

Fig. 2 Distribution of α-hydroxy acid oxidase (A), D-amino acid oxidase (B), succinic dehydrogenase (C), and acid phosphatase (D) after differential centrifugation. N, nuclear fraction; M, mitochondrial fraction; Ly, lysosomal fraction; Mc, microsomal fraction; S, soluble. I, centrifugation scheme in which the upper biege layer was left with the mitochondrial fraction; II, centrifugation scheme in which the upper biege layer of the initial mitochondrial fraction was carried over into the lysosomal fraction. Relative specific activity equals the per cent of total enzyme activity divided by per cent of total protein nitrogen (Biuret). Particles (microbodies) containing α-hydroxy acid oxidase and D-amino acid oxidase and those containing acid phosphatase (lysosomes) behave similarly and show highest relative specific activities in the "lysosomal" fraction. Succinic dehydrogenase, to the contrary, is concentrated in the "mitochondrial" fraction. This analysis indicates that particles containing α-hydroxy acid oxidase resemble lysosomes and microbodies rather than mitochondria in their sedimentation behavior.

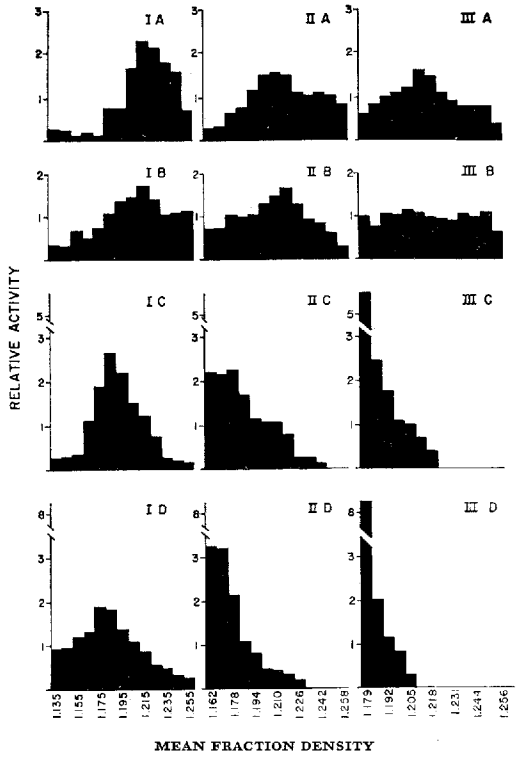

Fig. 3 Distribution of α-hydroxy acid oxidase (A), D-amino acid oxidase (B), succinic dehydrogenase (C), and acid phosphatase (D) after density equilibrium centrifugation in sucrose-water gradients of density 1.12–1.26 (I), 1.15–1.26 (II), and 1.17–1.26 (III). Relative activity equals enzyme activity in fraction divided by mean recovered enzyme activity per fraction. Particles containing α-hydroxy acid oxidase and D-amino acid oxidase (microbodies) show similar density equilibrium behavior. This behavior is different from that shown by particles containing succinic dehydrogenase (mitochondria) or acid phosphatase (lysosomes). Particles containing α-hydroxy acid oxidase, thus, resemble microbodies rather than mitochondria or lysosomes.

of α-hydroxy acid oxidase were identical to those derived from crude supernatant material. Electrophoresis and subsequent development for α-hydroxy acid oxidase followed by photometric scanning showed that fractions having the highest relative activities based on DIP assay also showed the highest rates of tetrazole reduction.

Microscopic cytochemical localization of α-hydroxy acid oxidase

The ability of α-hydroxy acid oxidase to transfer electrons to appropriate tetrazolium compounds made it possible to design a specific microscopic cytochemical test for the enzyme. Sections reacted for the localization of α-hydroxy acid oxidase showed a distribution of reduced tetrazole restricted primarily to the epithelial cells

TABLE 4

Average median particle densities

Density range of gradient	Number of gradients analyzed	α-Hydroxy acid oxidase	D-Amino acid oxidase	Succinic dehydrogenase	Acid phosphatase
1.12–1.26	3	1.218	1.213	1.191	1.185
1.15–1.26	3	1.218	1.211	1.187	1.181
1.17–1.26	4	1.217	1.218	1.184	1.192

of the proximal tubule[2] (fig. 12). The cytochemical reaction was most intense in the distal portions of this segment of the nephron. In all cases the dye deposits were spherical and were most frequently found in the basal regions of the cell although isolated granules were occasionally noted in the supranuclear region of the cytoplasm (figs. 4 and 7). These deposits ranged from 0.5 μ to 1.0 μ in diameter. The granules located in cells of the distal portions of the proximal tubule were larger than elsewhere. Equivalent preparations were obtained with either NBT or TNBT. As far as could be determined from cytochemical preparations, the properties of the enzyme responsible for the reduction of tetrazole under these conditions were similar to those observed by DIP assay of crude supernatant material or by analysis of electrophoretic material (tables 2 and 3).

Although the most distinct localizations of α-hydroxy acid oxidase were obtained with fixed tissues, it was possible to obtain similar results with unfixed material (figs. 8, 9). In such preparations, diffuse deposits of formazan were found in the cytoplasm adjacent to discrete, spherical deposits of the dye. These diffuse deposits suggest that the enzyme had leached out of its containing particle into the adjacent cytoplasm where it then mediated tetrazole reduction. When unfixed sections were washed for 12 hours in 0.25 M sucrose at 4°C and then reacted for the localization of α-hydroxy acid oxidase, the nature of the cytological image was altered. The enzyme was no longer present in solid-appearing spherical structures but was localized in the walls of seemingly empty vesicles (fig. 9).

The relative intracellular distributions of α-hydroxy acid oxidase and acid phosphatase or succinic dehydrogenase were demonstrated by dual microscopic cytochemical localizations. The intracellular localizations of acid phosphatase and α-hydroxy acid oxidase are shown in figure 5. The acid phosphatase containing particles (lysosomes) were restricted to the apical portions of the cells of the proximal tubule while the α-hydroxy acid oxidase containing particles were restricted to their basal portions. Careful scrutiny of many such preparations failed to uncover a single instance in which a particle could be said to contain both red (acid phosphatase) and blue (α-hydroxy acid oxidase) dye deposits.

Double localizations of α-hydroxy acid oxidase and succinic dehydrogenase were less satisfactory than those of the oxidase in combination with acid phosphatase. Initial incubation in reaction mixtures for the localization of succinic dehydrogenase inhibited α-hydroxy acid oxidase activity. The reverse procedure resulted in poor preservation of mitochondrial morphology. Cells of the proximal portions of the proxmal tubule were identified by the presence of numerous, elongate mitochondria which stained in reaction mixtures containing succinic acid. These cells when subsequently immersed in reaction mixtures containing α-hydroxy valeric acid, usually showed no deposition of reaction product. However, in some cases, deposition of reaction product in the form of round granules occurred. These granules were similar in their size and location to those described above and were in contrast to the form and location of the mitochondria. Cells of distal portions of the proximal tubule, after staining in reac-

[2] Identification of the segments of the tubule with which α-hydroxy acid oxidase was associated was made on the basis of morphological criteria and on the relative intensities and the distributions of cytochemically determined succinic dehydrogenase, acid phosphatase, and alkaline phosphatase (Wachstein, '55). The previously reported association with distal convoluted tubules was in error (Allen and Beard, '65).

tion mixtures containing succinic acid, showed the presence of numerous, spherical mitochondria distributed throughout the basal two-thirds of the ctyoplasm (fig. 6). These cells, after exposure to reaction mixtures containing α-hydroxy valeric acid, showed additional reaction product in the form of spherical particles restricted to the basal one-third of the ctyoplasm (fig. 6). These particles were approximately the same size as the mitochondria but were less numerous.

Several procedures were employed to confirm the validity of the cytochemical localization of α-hydroxy acid oxidase. Non-enzymatic reduction of tetrazole was tested by placing sections in reaction mixtures lacking substrate, and by reacting heat-inactivated sections (dry heat at 90°C for one hour or distilled water at 90°C for 15 minutes) in complete reaction mixtures. These sections showed no deposition of formazan.

Non-random adsorption of tetrazole to cellular structures followed by enzymatic or non-enzymatic reduction was investigated. Sections were immersed for 30 minutes at 37°C in reaction mixtures lacking substrate. After washing for one minute in distilled water they were immersed in 0.1 M cysteine at pH 7.5 or were exposed to ammonium sulfide vapor for one minute (fig. 10). These sections showed a generalized deposition of formazan but did not show a localization of this material in particles resembling those seen after incubation in reaction mixtures containing α-hydroxy valeric acid. Other sections were heat-inactivated as above and were then immersed in complete reaction mixtures at 37°C for 30 minutes. These sections were washed one minute in cold distilled water and were then placed in reaction mixtures containing all components with the exception of the tetrazolium salt. Two-hundred microliters of crude supernatant material (see Material and Methods) was added to 10 ml of reaction mixture and the system was incubated at 37°C for 30 minutes. These sections showed slight generalized deposition of formazan but did not show a localization of the dye restricted to certain cellular areas nor was this dye deposited in spherical particles.

Non-random adsorption of solubilized α-hydroxy acid oxidase to cellular structures was checked in the following fashion. Sections which had been heat treated as described above, were immersed in crude supernatant material (see Materials and Methods) at 4°C for 15 minutes. These sections were quickly rinsed in ice cold 0.05 M Sörenson's phosphate buffer and then were placed in complete reaction mixtures containing α-hydroxy valeric acid at 37°C for 15 minutes. These sections showed no deposition of formazan.

The possibility of lipid adsorption of reduced and non-reduced tetrazole was examined. Sections were stained with Oil Red O in isopropanol (Lillie, '54) and with Sudan Black B in propylene glycol (Lillie, '54). These sections showed no deposits of lipid in renal tubule cells with size and form, or distribution similar to the particles containing α-hydroxy acid oxidase. Other sections, both fresh and fixed, were extracted with acetone at 4°C and were then stained in reaction mixtures for the localization of α-hydroxy acid oxidase. These sections showed a distribution of formazan identical to that seen in unextracted sections.

Attempts were made to apply the method of Graham and Karnovsky ('65) in microscopic cytochemical studies (fig. 11). In our hands, this reaction system did not produce satisfactory cytochemical results. The end product was in the form of small spheres which were distributed homogeneously in all areas of the section and on the glass adjacent to the section. Careful microscopic examination of the distribution of these particles suggested that much of the reaction product was deposited on the surface of the section in no apparent relation to any cellular structure.

DISCUSSION

Microbodies were first proposed as a distinct class of intracellular entities on the basis of morphological observations by Gansler and Rouiller ('56) and Rouiller and Bernhard ('56). More recent electron microscopic observations (Afzelius, '65; Baudhuin et al., '65; Bruni and Porter, '65; de Duve, '65; Hruban and Swift, '64) define them as varying in size from about

0.5 μ to 1.0 μ in diameter, of roughly spherical shape, surrounded by a single membrane, and containing a moderately dense matrix. In some cases, microbodies contain a dense core of crystalloid nature (Afzelius, '65; Baudhuin et al., '65; Bruni and Porter, '65; de Duve, '65; Hruban and Swift, '64). This core has been correlated with the presence of urate oxidase (Hruban and Swift, '64). Thus far, microbodies have been identified morphologically only in hepatic cells of birds and mammals and in renal cells of rodents (Afzelius, '65; Ericsson, '64). On the basis of morphological observations, a variety of functional roles have been proposed for these structures. They have been equated with lysosomes (Essner and Novikoff, '61), suggested as precursors of mitochondria (Rouiller and Bernhard, '56), implicated in the intracellular transport of protein, and associated with lysosomes as members of an intracellular catabolic system (Bruni and Porter, '65).

On the basis of biochemical study, microbodies have emerged as structures with enzymatic and physical properties distinct from those of mitochondria and lysosomes (de Duve, '65). Microbodies derived from hepatic tissue of the rat are characterized enzymatically by the presence of D-amino acid oxidase, α-hydroxy acid oxidase, urate oxidase, and catalase (Baudhuin et al., '65). Physically, microbodies from this source appear to lack an osmotic space, possess a large sucrose space, and a membrane permeable to small molecules (de Duve, '65). Because of these properties, microbodies, in contrast to lysosomes and mitochondria, do not show the property of latency and display a characteristic density dependent behavior upon centrifugation (de Duve, '65). The biochemical characterization of microbodies has recently been extended to those derived from renal tissue of the rat (Allen and Beard, '65; Baudhuin et al., '65) and Tetrahymena (Baudhuin, et al., '65). In these cases, their enzymatic content was similar to that found for the rat's liver except for the absence of urate oxidase.

The data presented above confirms and extends the evidence for the presence of α-hydroxy acid oxidase in microbodies derived from renal tissue of the rat. Differential centrifugation and density equilibrium centrifugation indicated that particles containing this enzyme sedimented independently of mitochondria. Differential centrifugation did not effect a separation of particles containing α-hydroxy acid oxidase from lysosomes but clear separations were achieved upon density equilibrium centrifugation. In all instances, particles containing α-hydroxy acid oxidase were similar in their sedimentation behavior to particles containing D-amino acid oxidase. They showed similar average median densities, similar density distribution profiles, and exhibited similar density dependent behavior. Since D-amino acid oxidase is well documented as a microbody marker (Beaufay et al., '64) we conclude that α-hydroxy acid oxidase is also a constituent of these particles.

The cytochemical localization of α-hydroxy acid oxidase achieved by us represents one of the first attempts to determine the intracellular distribution of microbodies. As such, it should be examined critically in terms of its reliability. It is generally agreed that tetrazolium methods have the capability for high resolution light microscopic localization of structures containing appropriate electron transferring systems. There are, however, reasons for skepticism toward claims of localization by these methods. Of the possible artifacts to which tetrazolium methods are subject, the more important are: (a) non-random adsorption of the tetrazole to cellular structure and its subsequent reduction by enzymatic or non-enzymatic means; (b) non-enzymatic reduction of the tetrazole; (c) solubilization and subsequent crystallization of formazan by cellular lipid deposits adjacent to sites of enzymatic reduction; (d) solubilization of enzyme followed by diffusion and readsorption to adjacent cellular structure. The process of fixation and the control procedures employed by us appear to refute the existence of these sources of artifact.

The greatest uncertainty associated with our localization of microbodies is the lack of definitive morphological correlation between the cytochemically produced image and that achieved by other means. Light

microscopic identification of microbodies after routine staining has not been achieved. Detailed electron microscopic study of microbodies has, thus far, been limited to hepatic cells. However, Ericsson ('64) reported their distribution in cells of the proximal tubule of the rat kidney. In these cells, they were preferentially situated in the supranuclear region and adjacent to the basement membrane. This intracellular distribution is compatible with that observed by us following cytochemical localization of α-hydroxy acid oxidase. Centrifugation analysis (de Duve, '65) indicates that microbodies from hepatic cells are approximately 0.5 μ in diameter. Direct measurements from electron micrographs (Afzelius, '65; Bruni and Porter, '65; de Duve, '65; Hruban and Swift, '64) indicate diameters ranging from 0.1 μ to 1.5 μ. These diameters are consonant with those found in the present study (0.5 μ–1.0 μ) by direct measurement of structures appearing after cytochemical development for α-hydroxy acid oxidase.

The reducing system responsible for the production of the cytochemical image is enzymatic in nature. The close correspondence between its properties and those determined on the basis of routine quantitative and electrophoretic analysis argues that the same enzyme was involved in all cases. On the basis of this correspondence, the lack of artifactual reactions, and the compatibility of dimensions, and intracellular distribution we conclude that the cytochemical method employed by us accurately reflects the localization of α-hydroxy acid oxidase and, hence, of those microbodies which contain this enzyme.

The distribution of cytochemical reaction product produced by our method for the localization of α-hydroxy acid oxidase differed from that which we obtained with the coupled peroxidase method of Graham and Karnovsky ('65). The latter method, in our hands, produced a random distribution of reaction product. Often, this reaction product was associated with the surface of the section or the glass adjacent to it. Production of this reaction product did not occur when substrate was omitted from reaction mixtures. Thus, we do not contest the enzymatic nature of this reaction but, rather, the accuracy of the localization it effects. Hydrogen peroxide, the important intermediate in this cytochemical reaction, is a highly diffusible molecule and its oxidation by catalase (coupled to 3-amino-9-ethylcarbazole) is not an instantaneous process. It is our opinion that the diffusion of hydrogen peroxide prior to peroxidatic oxidation is responsible for the random distribution of reaction product observed by us. It is possible, in instances where hydrogen peroxide formation is rapid, that this localizing system will have utility. For the localization of hydrogen peroxide producing enzymes after electrophoresis in gels it is ideal. Problems of diffusion of reaction intermediates under these circumstances are much less severe than in microscopic applications.

Because of similar properties there is little doubt that the enzyme studied by us is the same as that described by Blanchard et al. ('44, '45) and more recently by Baudhuin et al. ('65). In our studies, the rate of lactate oxidation, when measured by DIP reduction, was equivalent to that found for α-hydroxy valeric acid. However, when analyzed by photometric scanning after electrophoresis and cytochemical development, the rate of lactate oxidation was about one-half that of α-hydroxy valeric acid. We feel that discrepancy to be due to the presence of lactate dehydrogenase in crude supernatant preparations. Unpublished observations suggest that sufficient NAD is present in such preparations to yield measurable reaction rates upon the addition of L-lactic acid. Thus, the rates of lactate oxidation measured by DIP assay or by the formation of pyruvate (Baudhuin et al., '65) are probably deceptively high.

Robinson et al. ('62) have identified two categories of α-hydroxy acid oxidases. One of these, the "short chain" oxidase, is maximally active with glycolate and is minimally active with longer chain α-hydroxy acids. The other, the "long chain" oxidase, is minimally active with glycolate and is maximally active with a variety of longer chain α-hydroxy acids. The α-hydroxy acid oxidase of renal microbodies described here and by Baudhuin et al. ('65) appears to be of the "long chain" variety as does the enzyme present in the micro-

bodies of Tetrahymena (Baudhuin et al., '65). The enzyme present in microbodies of liver (Baudhuin, '65) is of the "short chain" variety. Preliminary study in cooperation with S. L. Allen, indicates that at least two electrophoretically distinct α-hydroxy acid oxidases are present in Tetrahymena. Electrophoretic analysis of α-hydroxy acid oxidase from a variety of mammalian kidneys has shown that the enzyme, in several cases, exists in multiple molecular form and that species differences exist in terms of electrophoretic mobilities. The α-hydroxy acid oxidase of rat renal tissue showed a different electrophoretic mobility than the enzyme from rat hepatic tissue. Such data suggest that a variety of α-hydroxy acid oxidases exist. The extent to which these are present only in microbodies remains to be determined.

The function of microbodies is obscure. The catalytic activities of the enzymes associated with them are diverse and it is doubtful that they function in any unified metabolic role. The presence of three hydrogen peroxide-producing enzymes in association with catalase is suggestive. Perhaps microbodies serve as devices for sequestering hydrogen peroxide-producing systems and for the detoxification of this end product. Such a function would relegate the microbody to a role in cellular catabolism not unlike that of lysosomes. Baudhuin et al. ('65) have suggested that microbodies may serve in the disposal of electrons produced by soluble dehydrogenases. In this case, they would serve as shuttle systems for the transfer of electrons from one cellular compartment to another. A role as a shuttling device would not preclude a function in cellular catabolism as well. The extent to which microbodies cooperate with lysosomes in cellular catabolism as suggested by Bruni and Porter ('65) remains to be determined. The spatial separation between microbodies and lysosomes as indicated by our cytochemical preparations suggests that such cooperation is tenuous.

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PLATE 1

EXPLANATION OF FIGURES

4 α-Hydroxy acid oxidase in cells of the distal portion of the proximal tubule is localized within vesicular bodies 0.5 μ to 1.0 μ in diameter. Formol-Hank fixation, 4 μ cryostat section, incubated 15 minutes at 37°C in a reaction mixture containing 0.1 M D, L-α-hydroxybutyric acid and 2 mM NBT. × 1,500.

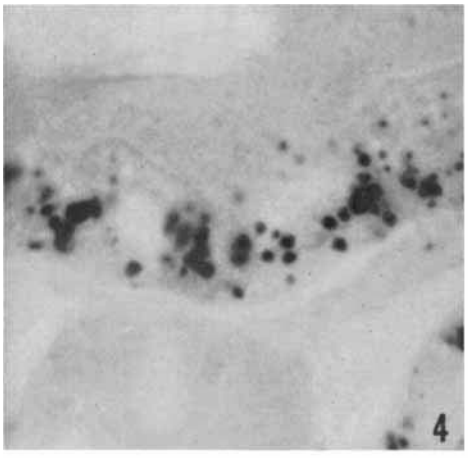
5 Double localization of α-hydroxy acid oxidase (a) and acid phosphatase (b) in cells of the distal portion of the proximal tubule. Acid phosphatase is localized in lysosomes situated in the apical portions of these cells while α-hydroxy acid oxidase is present in microbodies distributed in the basal areas of the cytoplasm. Structures containing both types of reaction product (i.e. red and blue) were never observed. This suggests that the biochemical properties of lysosomes and microbodies are distinct and that fusions between these particles, if they occur, are rare events. Preparation as for figure 4. First incubated for the localization of α-hydroxy acid oxidase and then incubated 30 minutes at 25°C in a Barka and Anderson ('63) reaction mixture for acid phosphatase localization which contained Naphthol AS MX phosphate. × 1,500.

6 Double localization showing the distribution of α-hydroxy acid oxidase (arrow) and succinic dehydrogenase. Elongate mitochondria of the proximal portion of the proximal tubule (bottom of figure) stain intensely for succinic dehydrogenase. Round mitochondria of the distal portion of the proximal tubule (top of figure) stain less intensely for this enzyme. Particles containing α-hydroxy acid oxidase (arrow) are located more basally than are the majority of mitochondria. Preparation as for figure 4. First incubated 15 minutes at 37°C for the demonstration of succinic dehydrogenase in a reaction mixture containing 0.05 M sodium succinate and 2 mM TNBT and then incubated in a reaction mixture for the demonstration of α-hydroxy acid oxidase as specified in figure 4. × 1,500.

7 α-Hydroxy acid oxidase in cells of distal portion of the proximal tubule. Preparation identical to that for figure 4 except the reaction mixture contained 0.1 M D, L-α-hydroxy valeric acid. × 1,000.

8 α-Hydroxy acid oxidase localization in unfixed cryostat section. Particulate distribution of the enzyme compares with that shown in figure 7 but diffuse deposition of reaction product is present around the particulate deposits. Such preparations suggest that the enzyme has escaped from the particles and has become secondarily adsorbed to adjacent cytoplasmic material. Incubation as specified for figure 7. × 1,000.

9 Preparation identical to that seen in figure 8 except that the section was washed 12 hours in 0.25 M sucrose at 4°C prior to cytochemical staining. The reaction product is deposited in the walls of empty vesicles. Such preparations suggest that the washing process has removed α-hydroxy acid oxidase from the particles. Staining in the walls of the vesicles is probably due to adsorbed α-hydroxy acid oxidase. This preparation and that seen in figure 8 illustrate a type of behavior analogous to that shown by acid phosphatase under similar conditions (Holt, '59; Misch, '62). The extent to which this behavior should be considered a demonstration of enzyme latency remains to be determined. × 1,000.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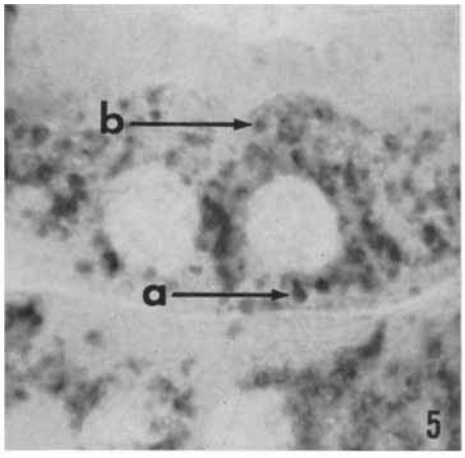
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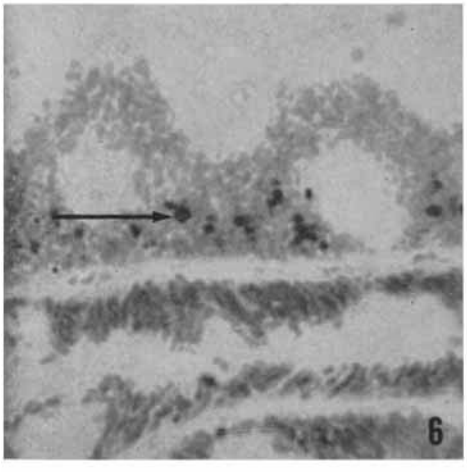
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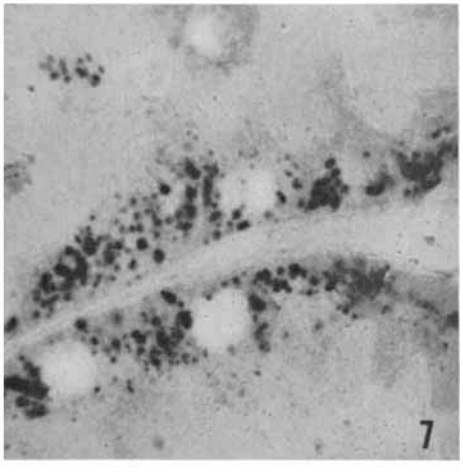
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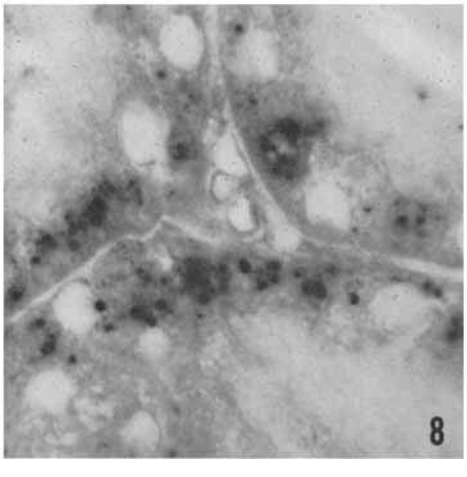
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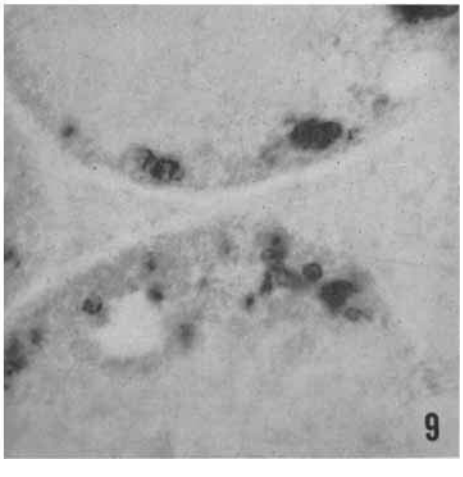
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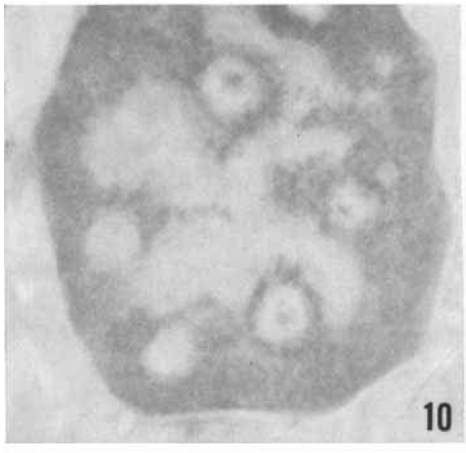

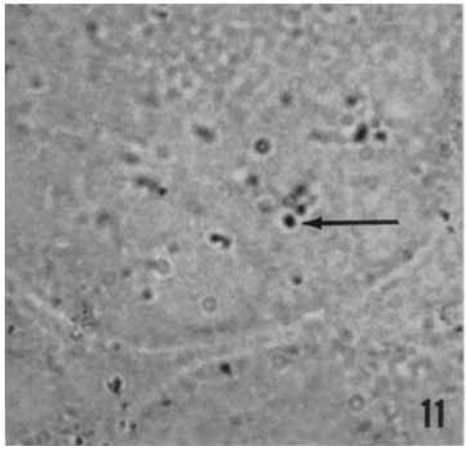

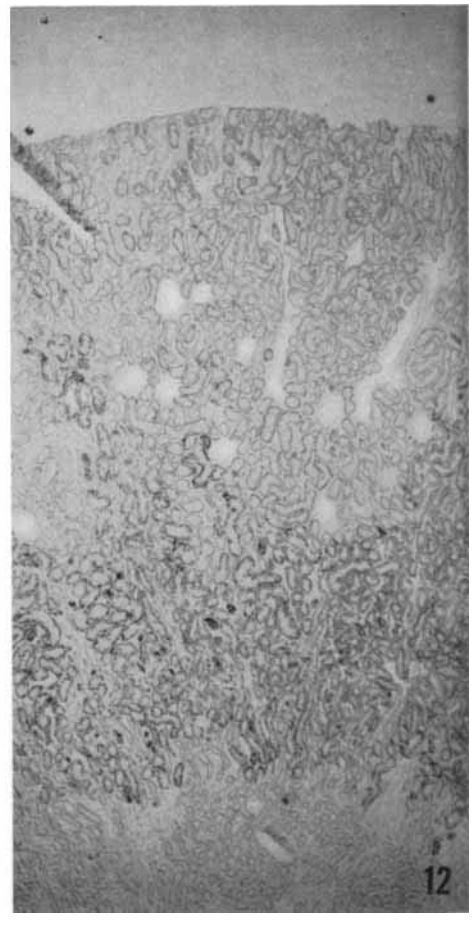

EXPLANATION OF FIGURES

10 Control preparation illustrating a section which had been immersed in a cytochemical reaction mixture lacking only substrate. After incubation in this solution for 15 minutes at 37°C the section was washed in two changes of distilled water for one minute each. Following washing it was exposed to ammonium sulfide vapor for one minute. Modest deposition of reduced tetrazole is seen particularly in areas of the cells which contain large numbers of mitochondria (compare to figure 6). No deposition of reaction product in a form comparable to that seen in figures 4 or 7 is evident. × 1,000.

11 Localization of α-hydroxy acid oxidase by the method of Graham and Karnovsky ('65). Granular reaction product (arrow) is distributed randomly throughout the section. Formol-Hank fixation, 8 μ cryostat section, 15 minute incubation at 37° C in reaction mixture containing 0.1 M D, L-α-hydroxy valeric acid. × 1,000.

12 Distribution of α-hydroxy acid oxidase in kidney cortex. Reaction product is most concentrated in the distal portions of the proximal tubule (inner cortex). Preparation as for figure 7. × 30.