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Oxidation of Spermidine and Spermine in Rat Liver: Purification and Properties of Polyamine Oxidase†

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ABSTRACT: A novel enzyme responsible for the oxidation of spermidine and spermine has been found in rat liver. Spermidine is shown to be degraded to putrescine and 3-aminopropionaldehyde, and spermine to be cleaved to spermidine and 3-aminopropionaldehyde. A single enzyme catalyzing both reactions and designated as polyamine oxidase has been purified 4000-fold to electrophoretic homogeneity. Polyamine oxidase appears to be a flavoprotein, containing flavin adenine dinucleotide (FAD) as a prosthetic group. Hydrogen peroxide is evolved in the reaction and no other electron acceptors except molecular oxygen have been found. The molecular weight of the enzyme was approximately 60 000 and the sedimentation coefficient 4.5 S. The enzyme appears to be a single polypeptide chain since no evidence for structural subunits was obtained. Polyamine oxidase was sensitive to sulfhydryl and carbonyl group reagents. The optimum pH value for the oxidation of polyamines was close to 10. The reaction velocities were enhanced by various aldehydes, especially certain aromatic aldehydes. Polyamine oxidase appears to be localized in peroxisomes of liver cells, although the existence of an isoenzyme in the cytosolic fraction was not definitively ruled out. No marked changes were observed in the activity of polyamine oxidase in rat liver after partial hepatectomy, carbon tetrachloride poisoning, and after treatment with growth hormone or thioacetamide, conditions which are known to alter profoundly the metabolism and accumulation of polyamines.

The enzymic degradation of the polyamines spermidine and spermine in mammalian tissues is rather poorly understood. An exception is the amine oxidase in blood plasma of many ruminants, which acts on the primary amino groups of polyamines, and is fairly extensively studied and characterized. Brain of some mammalian species, connective tissue of bovine and chick aorta as well as bone are the other few tissues which reportedly contain polyamine oxidizing enzymes, probably similar to that found in ruminant plasma (Kapeller-Adler, 1970; Cohen, 1971; Tabor and Tabor, 1972; Bachrach, 1973).

In general, not much information is available of any other kind on the catabolism of spermidine and spermine in mammalian cells. Rosenthal and Tabor (1956) reported that 4–8% of parenterally administered spermine was excreted as spermidine into urine in the rat. Siimes (1967) observed some conversion of spermidine to putrescine, and spermine to spermidine in rat liver after injections of [^{14}C]polyamines to the animals. These findings in vivo were later confirmed and extended by Hölttä et al. (1973b). The hepatic conversion of [^{14}C]spermidine to labeled putrescine was markedly enhanced

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after partial hepatectomy and following treatments with growth hormone, thioacetamide, and carbon tetrachloride. The apparent conversion of spermidine to putrescine in vivo has served as a rational basis for a search of an enzyme catalyzing this reaction.

Unlike the oxidation of polyamines in ruminant blood plasma, different types of oxidation of spermidine and spermine have been reported in bacteria (Tabor and Tabor, 1972; Bachrach, 1973). Tabor and Kellogg (1970) purified a spermidine dehydrogenase from *Serratia marcescens* to homogeneity. This enzyme splits spermidine to 1,3-diaminopropane and Δ^1-pyrroline. Extracts of *Pseudomonas* strain, however, appear to be capable of cleaving the spermidine molecule at the secondary amino group, yielding putrescine and 3-aminopropionaldehyde, as briefly reported by Padmanabhan and Kim (1965). The enzymic formation of 3-aminopropionaldehyde has also been reported to occur in hog kidney (Quash and Taylor, 1970). It is not known whether the reaction pathway is similar to that occurring in *Pseudomonas*.

In the present work the enzymic oxidations of spermidine to putrescine and 3-aminopropionaldehyde and that of spermine to spermidine and 3-aminopropionaldehyde in rat liver have been investigated. The enzyme responsible for the oxidation of both polyamines has been purified to electrophoretic homogeneity and some of its properties have been described.

Experimental Procedures

Materials. Radioactive polyamines, [1,4-^{14}C]putrescine (17.5 mCi/mmol), [*tetramethylene*-1,4-^{14}C]spermidine (12.4 mCi/mmol), [*tetramethylene*-1,4-^{14}C]spermine (12.5 mCi/mmol), [3-*aminopropyl*-3-^{3}H(N)]spermidine (526 mCi/mmol), and [3-*aminopropyl*-3-^{3}H(N)]spermine (1.063 Ci/mmol) were purchased from the New England Nuclear Corp. Before use they were purified on a Dowex 50-H^+ column, and the purity was checked by paper electrophoresis according to Raina (1963). [*carbonyl*-^{14}C]Benzaldehyde and [^{14}C]dopamine were obtained from the Radiochemical Centre, Amersham. Unlabeled polyamines were purchased from Calbiochem. N^1-Acetylspermidine, $CH_3CONH(CH_2)_3NH(CH_2)_4NH_2$, was synthetized according to Tabor et al. (1971). Beef liver catalase, horseradish peroxidase, and fungal glucose oxidase were purchased from Sigma. Pargyline was a generous gift from Abbot A.B. (Stockholm) and NSD-1055 (4-bromo-3-hydroxybenzyloxyamine dihydrogen phosphate) from Smith and Nephew Research Ltd. All other chemicals were obtained commercially.

Enzyme Assays. The standard assay mixture for spermidine/spermine oxidase (designated as polyamine oxidase) contained 0.1 M glycine-NaOH buffer (pH 9.5 at 20 °C), 0.4 mM [^{14}C]spermidine/[^{14}C]spermine, 5 mM dithiothreitol, 5 mM benzaldehyde whenever used, and enzyme in a total volume of 0.25 ml. The incubation time was either 60 or 120 min at 37 °C. The reaction was stopped by the addition of 0.05 ml of 50% trichloroacetic acid. After sedimenting protein at low-speed centrifugation, an aliquot of the supernatant (20 μl) was subjected to paper electrophoresis according to Raina (1963). Unlabeled polyamines were used as markers in electrophoresis. When the formation of putrescine from spermidine was assayed, the electrophoretic separation was accomplished using 0.1 M citric acid buffer at pH 4.3. For the assay of the formation of spermidine from spermine, the electrophoresis was performed at pH 3.6. In most cases no contamination of the reaction products (migrating faster) with the substrates was observed. However, if found necessary the polyamines were separated on Dowex 50-H^+ columns prior to electrophoresis. After staining the paper strips with 0.1% ninhydrin spray, the polyamine bands were cut out and placed in vials containing toluene-based scintillator fluid, and the radioactivity was counted. One unit of polyamine oxidase is defined as the amount of enzyme catalyzing the formation of 1 nmol of putrescine or spermidine per min from spermidine and spermine, respectively.

Another assay of the enzyme activity was based on the measurement of 3-aminopropionaldehyde formation. 3-Aminopropionaldehyde was first converted to a thiosemicarbazone derivative (Padmanabhan and Kim, 195; Unemoto, 1963) and measured spectrophotometrically using a molar extinction coefficient of 1.96×10^4 M^{-1}. Thiosemicarbazide could not be added until at the end of the incubation due to the inhibition of the enzyme activity by carbonyl reagents. A radioisotopic technique for the measurement of 3-aminopropionaldehyde can be applied to polyamines labeled in the propyl moiety as substrates. Radioactive 3-aminopropionaldehyde formed in the reactions was reduced to the corresponding stable alcohol (3-aminopropanol) by an excess of borohydride. An aliquot of the labeled products was subjected to either paper electrophoresis or chromatography with authentic 3-aminopropanol as a marker. Owing to the instability of 3-aminopropionaldehyde, this method does not appear to be as reliable as the above assay based on the polyamine determination.

A third method for the enzyme assay was based on the spectrophotometric measurement of hydrogen peroxide evolved by peroxidase-coupled *o*-dianisidine reaction (Aarsen and Kemp, 1964; Gunther and Glick, 1967). This method was applicable to the enzyme preparations which had been partially purified. The amount of H_2O_2 formed during the incubation was calculated from a standard curve made in the presence of various amounts of exogenous H_2O_2 (titrated with $KMnO_4$ before use) in the standard incubation mixture for polyamine oxidase. The concentration of dithiothreitol, however, was reduced to 0.02 mM in these assays because higher thiol concentrations were found to be inhibitory for the peroxidase-coupled dye reaction. Similar technique was also applied to visualize polyamine oxidase on polyacrylamide gels (Hampton et al., 1972). After the electrophoresis the gel was immersed into a mixture of 0.1 M glycine-NaOH buffer (pH 9.5), 0.025% *o*-dianisidine, 0.002% horseradish peroxidase, 5 mM spermidine or spermine, and 5 mM benzaldehyde in a final volume of 5 ml. The incubation was allowed to continue in dark for 24 h.

Succinate dehydrogenase activity was measured according to Lee and Lardy (1965), acid phosphatase as described by Fishman and Lerner (1953), and catalase by following the disappearance of H_2O_2 at 240 nm (Aebi, 1970). Monoamine oxidase activity was assayed as reported by McCaman et al. (1965) and that of diamine oxidase according to the method of Okuyama and Kobayashi (1961), as modified by Tryding and Willert (1968). Spermidine and spermine synthase activities were analyzed as described earlier (Raina and Hannonen, 1971; Jänne et al., 1971; Hannonen et al., 1972a).

Analytical Methods. Ascending paper chromatographic analyses were carried out on Whatman No. 1 paper. The following solvent systems were used: (I) 1-butanol–glacial acetic acid–water (50:25:25) (Fink et al., 1963); (II) 1-butanol–acetic acid–pyridine–water (40:10:10:20); (III) 1-propanol–concentrated hydrochloric acid–water (30:10:10) (Dubin and Rosenthal, 1960); and (IV) ethylene glycol monomethyl ether–propionic acid–water (70:15:15) saturated with sodium chloride (Herbst et al., 1958). When labeled compounds were

chromatographed, unlabeled carrier compounds were added as markers to each spot. After development of the chromatograms, the papers were dried, stained with ninhydrin, and then cut into pieces and counted for the radioactivity.

Gel Electrophoresis. Analytical polyacrylamide gel electrophoresis was performed at two different pH values, pH 8.9 and 7.5, essentially as described by Davis (1964) and Maurer (1968) omitting the spacer gel. Electrophoresis on 7.5% separating acrylamide gels (0.5 × 5.5 cm) was run at 2 mA per gel column. Bromphenol blue was used as a marker. The gels were stained for 2 h with 0.1% Coomassie blue solution in 7.5% acetic acid, and destained in acetic acid–methanol–water solution (12:8:100).

Sodium dodecyl sulfate electrophoresis was carried out according to Weber and Osborn (1969). Enzyme (5 μg of purified preparation) was treated with 1% sodium dodecyl sulfate and 2-mercaptoethanol. The proteins used for calibration, viz., catalase, bovine serum albumin, aldolase, chymotrypsinogen, and cytochrome *c*, were treated similarly. The calculation of the molecular weight of polyamine oxidase was based on the migration of the calibration proteins.

Isoelectric Focusing. The isoelectric point of the enzyme was determined with an LKB (Bromma, Sweden) isoelectric focusing apparatus (Model 8101; volume, 110 ml) using enzyme preparation purified 480-fold. LKB Ampholines of both pH 3–10 and 4–6 ranges were used at 1% concentration with glycerol gradient containing 0.1 mM dithiothreitol as stabilizer. The focusing was continued for 3 days at 200 V, with continuous water cooling. Fractions of 3 ml were collected, and the pH was measured at 4 °C. Before the assay of the enzyme activity, the fractions were extensively dialyzed against 10 mM Tris[1]–0.1 mM EDTA–0.1 mM dithiothreitol–50 mM NaCl buffer (pH 7.8) for 24 h.

Sucrose Density Gradient Centrifugation. The centrifugations were carried out in a Spinco L-3 preparative ultracentrifuge using an SW 50 rotor at 40 000 rpm for 15 h. Linear sucrose gradients (5–20%) were prepared in a Beckman gradient former. Catalase (11.3 S), yeast alcohol dehydrogenase (7.4 S), and horse liver alcohol dehydrogenase (4.9 S) were used as marker proteins.

Molecular Weight Determination. The molecular Stokes radius of polyamine oxidase was determined as described by Ackers and Steere (1967). Molecular weight of the enzyme was calculated from the Svedberg equation based on the combined data from the sedimentation-velocity and gel filtration experiments. The molecular weight was also determined using molecular sieving according to the method of Andrews (1965). Blue Dextran 2000, potassium dichromate, catalase, yeast alcohol dehydrogenase, bovine serum albumin, chymotrypsinogen, and cytochrome *c* were used to calibrate the Sephadex G-100 column (Pharmacia, Uppsala, Sweden).

Protein Measurement. Protin was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Absorption Spectra. Absorption spectrum of the purified enzyme was measured in an Aminco DW-2 UV-VIS spectrophotometer; the other measurements were made with Gilford and Beckman B spectrophotometers. Fluorometric assay of flavin adenine dinucleotide was performed by the method of Bessey et al. (1949) using an Aminco Bowman spectrofluorometer. The fluorometric analysis was carried out in a 0.25-ml quartz cuvette with 45 μg of purified enzyme. Riboflavin was used as a standard.

Preparation of Subcellular Fractions. The livers from five rats were homogenized (Potter-Elvehjem homogenizer) in 9 volumes of 0.25 M sucrose–10 mM Tris (pH 7.4). The homogenate was first centrifuged at 600*g* for 10 min to sediment the cell debris and nuclei and then for 10 min at 3500*g* to pellet the heavy mitochondria. The resultant supernatant fraction was centrifuged at 15 000*g* for 10 min to sediment the light mitochondria, peroxisomes, and lysosomes, collectively designated as light mitochondrial fraction. The final supernatant fraction was centrifuged at 100 000*g* for 1 h to obtain the microsomal and cytosolic fractions. The pellet from each centrifugation was suspended in the homogenization buffer and resedimented. A portion of the homogenate was used to prepare pure nuclei according the Blobel and Potter (1966). The nuclei were sedimented through a 2.2 M sucrose cushion in a Spinco ultracentrifuge using an SW 25 rotor at 25 000 rpm for 75 min.

In order to achieve a separation of peroxisomes from lysosomes and mitochondria the method of Tolbert (1973), as modified by Kähönen (1976), was employed. The purity of the subcellular fractions was tested by using the following enzymes as markers for different fractions: succinate dehydrogenase as a marker for mitochondria, acid phosphatase for lysosomes, catalase for peroxisomes, and glucose-6-phosphatase for microsomes.

Animals. Female rats of the Wistar strain weighing 120–170 g were used. Partial hepatectomy was performed by the method of Higgins and Anderson (1931).

Results

Subcellular Localization of the Polyamine Oxidase Activity. The subcellular fractions obtained by differential centrifugation as described above were suspended in 10 mM Tris–0.1 mM EDTA–0.1 mM dithiothreitol buffer, pH 7.8 (buffer A) and made 0.5% with respect to Triton X-100 to solubilize the enzyme. Thereafter the fractions were centrifuged at 100 000*g* for 60 min, and the supernatant fractions were extensively dialyzed against buffer A before the assay of the enzyme activity. Although the cytosolic fraction shared the highest proportion of the total activity, a substantial part of polyamine oxidase activity resided in the light mitochondrial fraction containing the bulk of the peroxisomes and lysosomes (Table I). The highest specific activity of the polyamine oxidase was found in the light mitochondrial fraction (Table II). The crude nuclear fraction centrifuged at 600*g* for 10 min) showed some polyamine oxidase activity most probably due to the presence of unbroken cells since the purified nuclei (Blobel and Potter, 1966) did not exhibit any significant polyamine oxidase activity.

In order to study whether the polyamine oxidase activity was associated with peroxisomes, mitochondria, or lysosomes, these fractions were isolated according to Kähönen (1976). Figure 1 illustrates that the polyamine oxidase activity appears to be associated with particles exhibiting catalase activity. Hence polyamine oxidase is most likely to be a peroxisomal enzyme. A considerable proportion of the activities of both polyamine oxidase and catalase were found in the cytosolic fraction (at the top of the gradient). Half of the total catalase activity and 65% of the polyamine oxidase activity were in the cytosolic fraction in this particular experiment (cf. Table I).

Purification of Polyamine Oxidase. The enzyme was purified from the peroxisomal fraction. For the solubilization of

[1] Abbreviations used: FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; CoA, coenzyme A.

TABLE I: Subcellular Distribution of Polyamine Oxidase.[a]

Enzyme	% Enzyme Act. in the Subcellular Fractions					
	Crude Nuclear (Cell Debris)	Heavy Mitochondrial	Light Mitochondrial	Microsomal	Cytosol	Recovery[b]
Polyamine oxidase	14.8	17.2	25.5	3.4	38.7	99.6
Succinate dehydrogenase	25.0	52.1	11.7	5.3	3.5	97.6
Catalase	17.6	25.1	38.2	3.6	14.1	98.6
Acid phosphatase	12.3	16.5	23.6	14.5	27.5	94.4
Glucose-6-phosphatase	17.5	16.5	10.6	46.0	3.8	94.4

[a] The subcellular fractions were obtained by differential centrifugation of the homogenate and the enzyme activities were measured as described in Experimental Procedures. Polyamine oxidase was assayed from Triton X-100 treated, dialyzed (buffer B), subcellular fractions. Succinate dehydrogenase activity was measured from the dialyzed fractions and catalase, acid phosphatase (in the presence of 0.1% Triton) and glucose-6-phosphatase from the undialyzed fractions. [b] Recovery represents the percentage of the activity of the homogenate found in the subcellular fractions.

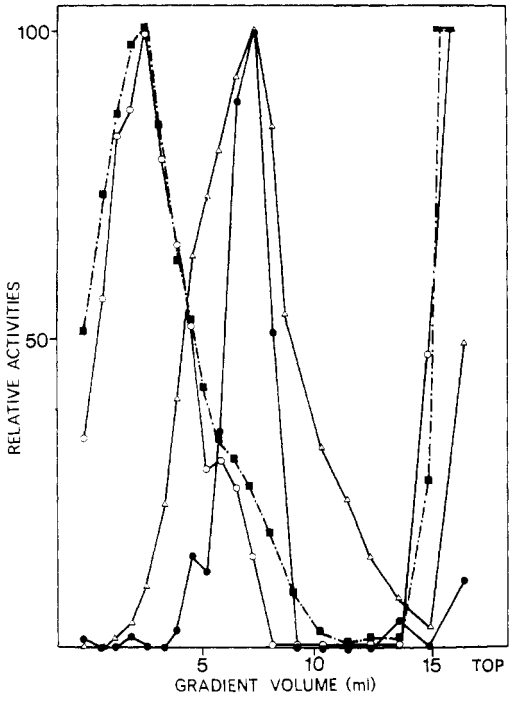

FIGURE 1: Subcellular distribution of polyamine oxidase. Isolation of the subcellular particles was performed according to the procedure of Tolbert (1973) as modified by Kähönen (1976). A hemoglobin-free liver piece was homogenized in a loose-fitting Potter-Elvehjem homogenizer with one stroke. The supernatant fraction, resulting from a centrifugation of the homogenate at 300*g* for 10 min, was subjected to sucrose density gradient centrifugation in SW-25 rotor at 78 000*g* for 2 h. Enzyme activities in the fractions of the gradient were measured as described in Experimental Procedures. (■ - · - ■) Polyamine oxidase; (○ — ○) catalase; (● — ●) succinate dehydrogenase; (△ — △) acid phosphatase.

TABLE II: Specific Activity of Polyamine Oxidase in Subcellular Fractions.[a]

Fraction	Spermidine as Substrate	Spermine as Substrate	
	+ Benzaldehyde	− Benzaldehyde	+ Benzaldehyde
Homogenate	0.071	0.064	0.440
Crude nuclear (cell debris)	0.060	0.067	0.430
Heavy mitochondrial	0.068	0.084	0.530
Light mitochondrial	0.150	0.140	0.950
Microsomal	0.005	0.010	0.064
Cytosol	0.028	0.023	0.150

[a] Specific activity is expressed as units/mg of protein. (See Experimental Procedures.)

the enzyme, a number of techniques were tested. Although sonication was found to be effective for this purpose, a treatment with Triton X-100 proved to be a better alternative. All the purification steps were carried out at 0–4 °C. The livers from 20 rats were homogenized in 5 volumes of 0.25 M sucrose–10 mM Tris buffer (pH 7.4). The homogenate was first centrifuged in a Sorvall RC-2B refrigerated centrifuge at 600*g* for 10 min, and the pellet was discarded. The supernatant was centrifuged at 15 000*g* for 10 min. The resulting pellet was used as the starting material for further purification. The pellet was suspended in 50 ml of buffer A and made 0.1% with respect to Triton X-100. The suspension was centrifuged at 25 000*g* for 30 min to obtain a soluble fraction, which was dialyzed overnight against buffer A. More than 90% of the total activity could be recovered in the supernatant fraction (fraction 1, 120 ml) that was applied to a DEAE column (Whatman DE-52, 3 × 40 cm) previously equilibrated with buffer A. After application of the sample, the column was washed with 150 ml of buffer A and then connected to a linear gradient of 0.1–0.4 M NaCl made in the same buffer (gradient volume was 1000 ml). The enzyme was eluted at about 0.25 M NaCl. The most active fractions were pooled (fraction 2, 83 ml) and poured on a hydroxylapatite column (Clarkson, Hypatite C, 3 × 12 cm) equilibrated with 10 mM potassium phosphate–0.1 mM dithiothreitol buffer (pH 7.8). The enzyme was eluted with a linear gradient of 0.01–0.3 M potassium phosphate (pH 7.8), containing 0.1 mM dithiothreitol; total gradient volume was 500 ml. Polyamine oxidase activity emerged after 200 ml of the fluid had passed through the column. The fractions showing the highest activity (fraction 3, 67 ml) were concentrated in an Amicon ultrafiltration chamber to a volume of 5 ml. Concentrated fraction 3 was subjected to molecular sieving on a Sephadex G-100 column (Pharmacia, 2.6 × 62 cm) equilibrated with 10 mM Tris–0.1 mM EDTA–0.1 mM dithiothreitol–50 mM NaCl buffer, pH 7.8 (buffer B). The polyamine oxidase activity appeared as a single symmetrical peak at 1.4 times the void volume. The fractions of highest activity were pooled (fraction 4, 24 ml). Ten milliliters of fraction 4 was concentrated in an Amicon ultrafiltration cell to a volume of 1.4 ml and further purified on polyacrylamide gels (1 × 10 cm) prepared by the method of Davis (1964). The enzyme was purified on two successive electrophoretic runs, 2 mA per gel column. After the run the gel was sliced into

TABLE III: Purification of Polyamine Oxidase from Rat Liver.[a]

Fraction	Total Act. (units)	Total Protein (mg)	Spec Act. (Spermine as Substrate) (units/mg)		Purification	Yield (%)	Spec Act. (Spermidine as Substrate) + Benzaldehyde (units/mg)
			− Benzaldehyde	+ Benzaldehyde			
Extract	220.0	6380	0.0345	0.34	1	100	0.054
DEAE	138.5	275	0.504	4.33	15	63	0.952
Hydroxylapatite	112.5	42.3	2.66	21.8	77	51	5.30
G-100	102.4	6.15	16.7	136	484	47	34.6
Gel electrophoresis	56.81	0.403	141	1380	4087	26	271

[a] Total activity (at pH 9.2), purification factor, and yield are calculated from experiments with spermine as the substrate, in the absence of benzaldehyde. For definition of enzyme unit, see the Experimental Procedures.

0.25-cm pieces, which were eluted with 0.5 ml of buffer B for 24 h. The two most active fractions from the gel slices were pooled and designated as fraction 5. The purification of polyamine oxidase is summarized in Table III.

Criteria of Purity. The purified enzyme (fraction 5) exhibited only one protein band (stained with Coomassie blue) in analytical acrylamide gel electrophoresis at two different pH values, 8.9 and 7.5 (Figure 2a,b). The location of polyamine oxidase on the gels run parallel was visualized with *o*-dianisidine dye as described above. As illustrated in Figure 2c, the formation of H_2O_2 in the presence of spermidine and spermine corresponds with the migration of the only protein band on the gels. In addition, the enzyme activity was measured by the standard assay (see Experimental Procedures) after slicing the gel into 0.25-cm pieces and eluting the enzyme as described above in preparative gel electrophoresis. Again the enzyme activity appeared to be localized in the position of the single protein band. The location of the enzyme activity on the gels was the same regardless of whether spermidine, spermine, or both together were used as substrates.

Sodium Dodecyl Sulfate Electrophoresis. On sodium dodecyl sulfate electrophoresis, only one major protein band corresponding to the molecular weight of 60 000 was found. In two experiments less than 5% of the total protein was also found in minor bands.

Isoelectric Point. A single peak of the enzyme activity at a pH value 4.9 was observed after the isoelectric focusing.

Stability of Enzyme. The purified enzyme at a concentration of 0.3 mg/ml was stable at least for 2 months at 4 °C without any loss of activity. The enzyme activity was unaffected by the freezing at −20 and −70 °C at least once. Exposure of the enzyme to 60 °C for 1 min resulted in a decrease of 50% in the activity, and after 5 min at the same temperature 75% of the original activity was lost. The stability of polyamine oxidase at different proton concentrations was tested by dialyzing the enzyme for 2 h (at 20 °C) in buffers of varying pH. The enzyme purified 480-fold was quite stable between pH 7 and 9, but a decrease in the enzyme activity was noticed outside this range of pH. On the alkaline side a closer analysis revealed that at pH 9.5 (glycine-NaOH buffer at 20 °C) only a slight (10%) reduction in the enzyme activity occurred. At pH 10 the stability of the enzyme was markedly reduced and the reaction ceased to be linear with respect to time.

Sedimentation Coefficient. A sedimentation constant of 4.5 S was obtained from the sucrose density gradient analysis. A similar sedimentation pattern for the enzyme was obtained whether the gradients were made with or without the inclusion of thiols. The addition of benzaldehyde to the gradient had no influence on the sedimentation of the enzyme.

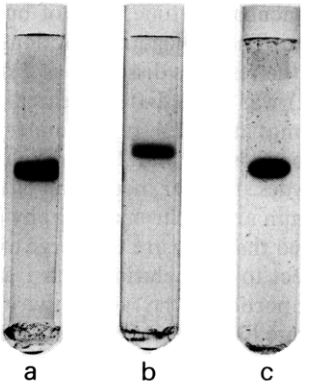

FIGURE 2: Gel electrophoresis of purified polyamine oxidase. Polyacrylamide gel electrophoresis was carried out as described in Experimental Procedures (a) at pH 8.9, 10 μg of protein, (b) at pH 7.5, 5 μg of protein, stained with Coomassie blue, (c) at pH 8.9, polyamine oxidase visualized by peroxidase-coupled dianisidine dye (see the text). Cathode is at the top, run from cathode to anode.

Molecular Weight of the Enzyme. A molecular weight of approximately 55 000 was obtained from the molecular sieving experiments on a Sephadex G-100 column according to Andrews (1965). A value of 61 000 was achieved from the calculations based on the combined data of molecular sieving and density gradient centrifugation employing the Svedberg equation and assuming a partial specific volume of 0.74 ml/g. As described earlier, a molecular weight of 60 000 was obtained from sodium dodecyl sulfate electrophoresis. The diffusion coefficient for polyamine oxidase was calculated to be 6.8 Ficks and the Stokes radius of the enzyme 3.12 nm, using yeast alcohol dehydrogenase (4.53 nm), bovine serum albumin (3.50 nm), and chymotrypsinogen (2.09 nm) as standards.

Identification of the Reaction Products. The reaction products from the oxidation of spermidine were identified by using [^{14}C]spermidine and [^{3}H]spermidine as the substrates in the parallel incubations. Borate buffer was used in these experiments, and the incubation time was reduced to 30 min, but otherwise the reaction mixture was similar to that described above. The reaction mixtures containing the enzyme purified 480-fold were applied, following deproteinization, to Dowex 50-H^+ columns (1 × 3 cm). The columns were successively eluted with H_2O (15 ml), 1 N HCl (25 ml), 2 N HCl (50 ml), and 6 N HCl (15 ml), monitoring the radioactivity in the eluates. The fractions containing radioactivity were evaporated to dryness under reduced pressure at 37 °C and the residues were dissolved in 250 μl of distilled water. In both incubations, 28% of the substrates (6 N HCl eluate) were converted to the reaction products. In the case of [^{14}C]sper-

midine, $NH_2CH_2CH_2CH_2NH^{14}CH_2CH_2CH_2{}^{14}CH_2NH_2$, as the substrate, 99% of the radioactive products were eluted with 2 N HCl. Practically all of the radioactivity in this eluate comigrated on paper chromatograms in solvents I–IV (R_f values 0.28, 0.25, 0.41, and 0.39, respectively) and electrophoresis (see Experimental Procedures) with authentic putrescine. Furthermore, the identity of the reaction product as putrescine is substantiated by the fact that the compound in the 2 N HCl eluate could be utilized as a substrate in the biosynthesis of spermidine by spermidine synthase from rat liver cytosol. Using [^{3}H]spermidine, $NH_2C^3H_2CH_2CH_2$-$NHCH_2CH_2CH_2CH_2NH_2$, as the substrate, 90% of the radioactive products were eluted with 1 N HCl. In addition, 4.5 and 5.5% of the labeled products were eluted with H_2O and 2 N HCl, respectively. However, the relative amounts of radioactivity eluted with 1 and 2 N HCl seemed to vary with respect to the incubation time, type of buffer, and enzyme preparation used. The radioactive fractions were evaporated to dryness and the residues dissolved as described above. An aliquot (150 μl) was immediately treated with an excess of borohydride (5 mg) at pH 7.4. In preliminary experiments, the untreated compounds in 1 and 2 N HCl eluates proved to be unstable aldehydes. Further incubation of the compounds at pH 9.5 for 30 min and rechromatography on Dowex 50-H^+ columns revealed that they are to a large extent interconvertible with respect to their elution with 1 and 2 N HCl. Although a major portion of the radioactivity in these fractions seemed to migrate like 3-aminopropionaldehyde (synthetized from 3-aminopropanol by horse liver alcohol dehydrogenase) in few chromatograms, the identification was complicated by the instability of the products. For further identification, the compounds reduced to stable alcohols by borohydride were used. To the borohydride-treated aliquot was added 1 ml of H_2O, 0.2 g of Na_2SO_4, and 0.05 ml of 10 N NaOH, and the solution was extracted twice with 3 ml 1-butanol. The combined butanol fractions were evaporated to dryness (after addition of few drops of concentrated HCl), and the residue was dissolved in distilled water. Recovery of the extracted radioactivity was 85%. Aliquots of the butanol extract were subjected to paper chromatography in solvents I–IV and electrophoresis, with authentic 3-aminopropanol as a standard. In each case 70–80% of the radioactivity comigrated with the standard (R_f values 0.61, 0.42, 0.68, and 0.72, respectively). The rest of the radioactivity migrated more slowly, presumably representing the condensation products of 3-aminopropionaldehyde formed during the incubation. In addition, the radioactive compound eluted with 1 N HCl could be oxidized with dichromate in dilute sulfuric acid to [^{3}H]-β-alanine, which was identified by the above techniques with an authentic standard. The product was further characterized as a thiosemicarbazone derivative according to published reports (Padmanabhan and Kim, 1965; Unemoto, 1963). A similar absorption spectrum (absorption maximum at 262 nm) was obtained from the reaction product and 3-aminopropionaldehyde synthetized from 3-aminopropanol by horse liver alcohol dehydrogenase.

The identification of the reaction products from the oxidation of spermine was carried out with [^{14}C]spermine and [^{3}H]spermine as the substrates (for the incubation conditions, see the oxidation of spermidine above). The reaction products were separated on Dowex 50-H^+ columns (0.2 × 5 cm) with successive elutions of H_2O (10 ml), 1 N HCl (50 ml), 2 N HCl (100 ml), and 4 N HCl (25 ml). In these experiments approximately 55% of the substrates (4 N HCl eluate) were converted to the products. The radioactive fractions were processed and analyzed with the same techniques as described above. In the case of [^{14}C]spermine, $NH_2(CH_2)_3$-$NH^{14}CH_2CH_2CH_2{}^{14}CH_2NH(CH_2)_3NH_2$, as the substrate, more than 98% of the radioactive products were eluted with 2 N HCl. Practically all of the radioactivity comigrated with authentic spermidine on paper chromatograms in solvents I–IV (R_f values 0.17, 0.18, 0.31, and 0.23) and electrophoresis. When [^{3}H]spermine, $NH_2C^3H_2CH_2CH_2NH(CH_2)_4$-$NHCH_2CH_2C^3H_2NH_2$, was used as the substrate, 4.8, 46.0, and 49.2% of the labeled reaction products were eluted with H_2O, 1 N, and 2 N HCl, respectively. The compound in 1 N HCl eluate was proved to be 3-aminopropionaldehyde, based on its identification after conversion to a thiosemicarbazone derivative and to the corresponding alcohol using the techniques described above. The product in 2 NHCl eluate comigrated (more than 95% of the radioactivity) with authentic spermidine on paper chromatograms and electrophoretograms. The identity of the compounds in 2 N HCl eluates as spermidine was also verified by their ability to serve as a substrate in the biosynthesis of spermine by spermine synthase from rat brain cytosol.

The formation of hydrogen peroxide in the oxidation of spermidine or spermine was established by the method described in Experimental Procedures. The analysis of the stoichiometry of the spermidine–spermine oxidation yielded a molar ratio of putrescine–spermidine to 3-aminopropionaldehyde to hydrogen peroxide of approximately 1.0:0.7:0.8.

The dependence of the enzyme activity upon molecular oxygen was tested by adding glucose oxidase (known to have a high affinity for oxygen) and glucose to the reaction mixture. Under these conditions, a total inhibition of the polyamine oxidase reaction was observed. Neither glucose nor glucose oxidase alone markedly inhibited the reaction. The inhibition was not attributed to the hydrogen peroxide formed (results not shown).

The oxidation of spermidine and spermine in rat liver thus occurs according to the following equations:

$$\underset{\text{spermidine}}{NH_2(CH_2)_3NH(CH_2)_4NH_2} + O_2 + H_2O \rightarrow \underset{\text{putrescine}}{NH_2(CH_2)_4NH_2} + \underset{\text{3-aminopropionaldehyde}}{NH_2CH_2CH_2CHO} + H_2O_2 \quad (1)$$

$$\underset{\text{spermine}}{NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2} + O_2 + H_2O \rightarrow \underset{\text{spermidine}}{NH_2(CH_2)_3NH(CH_2)_4NH_2} + \underset{\text{3-aminopropionaldehyde}}{NH_2CH_2CH_2CHO} + H_2O_2 \quad (2)$$

Identification of Flavin Adenine Dinucleotide as a Cofactor of Polyamine Oxidase. Fraction 4 enzyme (after gel-filtration step) was treated with acidic ammonium sulfate in the presence of KBr essentially as described by Srittmatter (1961) with the modifications described in the legend to Table IV. As shown, th apoenzyme thus obtained was partially reactivated by the addition of 0.01 mM FAD, which had no significant activating effect on the native enzyme. Attempts to dissociate FAD with ammonium sulfate alone at pH 4.0 were not successful, which might indicate a tight binding of the cofactor to the apoenzyme. FMN and riboflavin were without effect in the reconstitution experiments. The incubations containing exogenously added flavins were carried out in the dark because of the observed nonenzymic catalysis of the reaction by photons. The flavin content of the purified enzyme was also determined spectro-

TABLE IV: Inactivation of Polyamine Oxidase by Acidic Ammonium Sulfate Treatment and Reactivation by FAD.[a]

Enzyme	Flavin Added	Activity (units)
Native	None	7.27
Native	FAD	7.74
Resolved	None	1.03
Resolved	Riboflavin	1.02
Resolved	FMN	1.02
Resolved	FAD	4.33

[a] The enzyme purified 480-fold, 55 μg of protein in 250 μl of buffer B, was mixed with an equal volume of 3 M KBr. To this was added 1 ml of saturated ammonium sulfate mixed with 0.1 ml of 1 N H_2SO_4 during 1-min interval, with constant stirring. The supernatant was carefully removed. The precipitate was dissolved in 250 μl of buffer B and was allowed to stand at 0 °C for 30 min. Four aliquots (60 μl) were thereafter taken and 25 μl of 0.1 mM flavin solutions was added, and preincubation was continued for 2 h in an ice bath. The enzyme activity was assayed in the presence of benzaldehyde with [^{14}C] spermine as the substrate.

TABLE V: Effect of Various Amines on Polyamine Oxidase.[a]

Addition	Rel Act. of Polyamine Oxidase: [^{14}C]Spermine as Substrate, − Benzaldehyde	[^{14}C]Spermine as Substrate, + Benzaldehyde	[^{14}C]Spermidine as Substrate + Benzaldehyde
None	100	100	100
Propylamine	90	100	103
Diaminopropane	68	113	73
Putrescine	73	122	80
Cadaverine	43	117	61
Spermidine	27	89	
N^1-acetylspermidine[b]	20	77	31
Spermine			3
Benzylamine	141	101	90
Noradrenaline	94	100	108
Adrenaline	87	85	115
Dopamine	84	106	97
Tyramine	96	114	101
Histamine	88	109	89

[a] The enzyme activity was measured as described in Experimental Procedures, except that the concentration of [^{14}C]polyamines as substrates was 0.24 mM. The concentrations of the added amines were 1 mM in each case. Fraction 4 enzyme preparation was used in the experiments. [b] Determined in a separate experiment.

fluorometrically according to the method of Bessey et al. (1949). The amount of acid-extractable FAD was quite low in preliminary experiments, only about 2.6 nmol of FAD/mg of protein. The activity of the purified enzyme was, however, not stimulated by an addition of exogenous FAD. Neither did the enzyme assays of the previous purification steps provide any evidence for readily dissociable FAD in polyamine oxidase. The absorption spectrum of the purified enzyme did not show the typical three-banded spectrum for flavoproteins. Only a very small peak at 456 nm in addition to the major absorption peak at 275 nm was observed for the native enzyme. An explanation for this type of spectrum might be its perturbation by some additional cofactor (e.g., carbonyl compound) or the flavin being in reduced form or lost from the enzyme.

Kinetics of Polyamine Oxidase Reaction and Stimulation by Aldehydes. The enzyme reactions were linear with respect to the time and enzyme concentrations under the conditions used. The activity of the enzyme (60 min incubation) was maximal at about pH 10. The same pH optimum for the oxidation of spermidine and spermine in the presence or absence of benzaldehyde was obtained, but the ascending slope of the pH–activity curve for spermidine was steeper than that of spermine. The enzyme activities were, however, assayed at pH 9.5 because of the apparent lability of the enzyme at higher pH values (see above). Under these conditions 50–75% (spermidine–spermine) of the maximal activities were retained. Of a few buffers tested, glycine-NaOH, Tris-HCl, borate, and barbital buffers were equally good, and carbonate–bicarbonate buffer was slightly inferior to the other buffers at pH 9.5.

The apparent K_m values for spermidine and spermine were 0.05 and 0.02 mM, respectively. The reaction velocities were markedly stimulated by the addition of a number of aldehydes. When spermidine was used as the substrate, the activation-(-fold) of the reaction by different aldehydes were as follows: formaldehyde (2.3), acetaldehyde (1.7), propanal (5.3), butanal (9.0), hexanal (16.3), benzaldehyde (74.3), anisaldehyde (73.3), salicylaldehyde (3.7), *p*-hydroxybenzaldehyde (5.0), *o*-aminobenzaldehyde (3.0), phenylacetaldehyde (11.0), and pyridoxal (22.3). Benzaldehyde and anisaldehyde were thus the most potent activators of polyamine oxidase. The stimulatory effect of benzaldehyde was much greater with spermidine (about 40–100-fold, depending on the pH) than with spermine (only about 10-fold) as the substrate. The optimum concentration of benzaldehyde was found to be 5 mM in the presence of 5 mM dithiothreitol. The exact mechanism of the stimulation is not yet known. However, benzaldehyde appeared to slightly lower the K_m values for the substrates: those being 0.015 and 0.005 mM for spermidine and spermine in the presence of the activator. Nevertheless, this cannot account for the great differences in the extent of stimulation of the two reactions. Thiol groups seem to be involved in the benzaldehyde activation. In the presence of benzaldehyde the stimulation of the enzyme activity by dithiothreitol is greater with spermidine (16-fold) than with spermine (2-fold) as the substrate. Further, benzaldehyde may change the equilibrium of the reactions through Schiff base formation with the substrates or reaction products. As shown in Table V, the reaction products inhibited the enzyme activity in the absence of benzaldehyde, but the inhibition was almost abolished when benzaldehyde was present. Hydrogen peroxide evolved in the standard incubation conditions had no significant inhibitory action as judged by the inability of added catalase to increase the enzyme activity. By using [^{14}C]benzaldehyde, it was found that benzaldehyde was not oxidized to benzoic acid during the reaction. That benzaldehyde was not effectively bound to the enzyme, was revealed by the failure of [^{14}C]benzaldehyde to get eluted with the enzyme when the mixture was passed through a Sephadex G-25 column. Moreover, benzaldehyde could not be covalently reduced to the enzyme at pH 7–9.5.

Effect of Various Amines. Several aromatic amines, e.g., benzylamine, tyramine, and dopamine, stimulated the enzyme activity in crude enzyme preparations. It is likely that these effects are due to their oxidation to the corresponding aldehydes by monoamine oxidase since very little activation was observed with the purified enzyme. In the absence of benzaldehyde, diaminopropane, putrescine, cadaverine, spermidine, and N^1-acetylspermidine inhibited the enzyme activity measured with [^{14}C]spermine as the substrate, but addition of benzaldehyde almost abolished this inhibition (Table V).

TABLE VI: Effect of Carbonyl Reagents on Polyamine Oxidase.[a]

Carbonyl Reagent	Concn, Pre-incubation (mM)	Rel Act. −Benz-aldehyde	Rel Act. +Benz-aldehyde
None		100	100
Phenylhydrazine	10	2	0
Hydroxylamine	10	4	8
Semicarbazide	10	1	0
Isonicotinic acid hydrazine	10	93	111
Canaline	2	75	103
KCN	50	0	0
NSD-1055	10	0	0
$NaBH_4$	50	4	5

[a] The enzyme purified 480-fold was preincubated for 30 min at 37 °C in the presence of carbonyl reagents. The incubation was carried out as described in Experimental Procedures, except that the concentration of [^{14}C]spermine as the substrate was reduced to 0.2 mM. In the assay, the carbonyl reagents were diluted 1:50 (concentrations at which the carbonyl reagents did not affect the enzyme activity, except semicarbazide causing a 25% inhibition).

Spermine proved to be the most potent inhibitor of the reaction when assayed in the presence of benzaldehyde with [^{14}C]-spermidine as the substrate. The ability of the different amines to act as substrates for polyamine oxidase was tested by the measurement of H_2O_2 production in the presence of the amines. Only spermine (most active), N^1-acetylspermidine and spermidine were found to be utilized as substrates for the enzyme.

Electron Acceptors. A variety of electron acceptors, phenazine metosulfate, dichloroindophenol, ferricyanide, tetrazolium salts, cytochrome *c*, duroquinone, menadione, 2-hydroxynaphthoquinone, at 0.1 mM concentrations, did not markedly influence the enzyme activity assayed in the absence of benzaldehyde. Antimycin A (1 μg/ml) and rotenone (10 μM), inhibitors of electron transport chain, were without any significant influence on the enzyme activity. Thus no requirement for exogenous electron acceptors in the presence of molecular oxygen was found.

Essential Groups in the Enzyme. Polyamine oxidase contains sulfhydryl groups essential for its activity. Addition of dithiothreitol up to 5–10 mM increased the enzyme activity measured in the presence of 5 mM benzaldehyde. Dialysis of the enzyme against a buffer lacking thiols decreased the activity ([^{14}C]spermidine as the substrate) to hardly detectable level, but the activity could be restored (to variable extent) by the addition of 5 mM dithiothreitol (100%), mercaptoethanol (89%), and dihydrolipoic acid (51%), while glutathione was ineffective. Sulfhydryl reagents, Hg^{2+}, *p*-hydroxymercuribenzoate, *N*-ethylmaleimide, and iodoacetamide inhibited the enzyme activity.

Polyamine oxidase apparently contains carbonyl group(s) involved in the enzymic activity as judged from the profound inhibition of the polyamine oxidase activity by a number of carbonyl reagents (Table VI). The known inhibitors of pyridoxal phosphate enzymes isonicotinic acid hydrazine and canaline (Rahiala et al., 1971) did not markedly inhibit the enzyme activity. Free pyridoxal increased the activity of polyamine oxidase (see above), but addition of pyridoxal phosphate, up to 5 mM concentration, had no effect on the enzyme activity. The spectrum of the purified enzyme did not reveal the presence of pyridoxal phosphate.

Effect of Metal Chelators. Addition of 1 mM thiourea and diethyl dithiocarbamate were without any effect on the enzyme activity, substantiating the conclusion that copper, if present, does not participate in the reaction. Iron chelators α,α′-dipyridyl, 8-hydroxyquinoline, and *o*-phenanthroline at a concentration of 1 mM decreased the enzyme activity 12, 40, and 52%, respectively. EDTA up to 5 mM concentration, however, did not significantly reduce the enzyme activity. NaF (50 mM) and NaN_3 (10 mM) were also without any effect. Preliminary attempts were made to find out possible metal cofactor(s) of polyamine oxidase. Fraction 4 enzyme was treated with *o*-phenanthroline (4 mM) and cysteine (50 mM) for 12 h (at 4 °C), whereafter the enzyme mixture was dialyzed against 10 mM Tris–50 mM NaCl (pH 7.8). This treatment resulted in a decrease of 72% in the enzyme activity. Ferrous ions (Fe^{2+}) were found to partially restore (70%) the polyamine oxidase activity measured in the absence of thiols, whereas Mg^{2+}, Cu^{2+}, Zn^{2+}, and molybdenum (all at 0.1 mM concentration) were either inhibitory or without effect.

Effect of Various Compounds. All of the following agents structurally related to benzaldehyde, namely, benzoic acid and its *o*- and *p*-amino derivatives, benzyl alcohol, acetophenone, and benzophenone, were without effect on the purified enzyme preparation showing that aldehyde group is required for the stimulatory activity.

Since putrescine is reported to be degraded by monoamine oxidase after being acetylated (Seiler and Al-Therib, 1974), addition of acetyl coenzyme A to liver homogenate was tested to see a possible effect on the degradation of spermidine and spermine by polyamine oxidase. Acetyl-CoA (0.5 mM) did not markedly increase the enzyme activity whether added to the homogenate or to the purified enzyme.

The enzyme activity was unaffected by the addition of NAD, NADH, or NADPH, but NADP was somewhat (20%) inhibitory. Ascorbate (1 mM) similarly inhibited the enzyme activity by 20%. Mg^{2+}, Ca^{2+}, and Fe^{2+} (1 mM) were without influence, whereas Cu^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, and Ni^{2+} were clearly inhibitory when the enzyme activity was assayed in the absence of thiols.

Polyamine oxidase activity was considerably inhibited (80%) by 0.1 mM quinacrine, a known inhibitor for several flavoproteins (Haas, 1944). Pargyline, a potent inhibitor of monoamine oxidase (Taylor et al., 1960), was without effect at 0.1 mM concentration, and only a reduction of 20% was observed in the polyamine oxidase activity at 1 mM concentration of the compound. Methylglyoxal bis(guanylhydrazone), an inhibitor of diamine oxidase (Hölttä et al., 1973a), had no effect at a concentration of 0.1 mM, but caused a 40% inhibition of polyamine oxidase activity at 1 mM concentration.

Enzyme Activity under Various Growth Situations. The changes of the enzyme activity during liver regeneration after partial hepatectomy and carbon tetrachloride injury, as well as in liver following treatments with growth hormone or thioacetamide, were also investigated since an increased incorporation of radioactivity from spermidine to putrescine in vivo was detected earlier (Hölttä et al., 1973b). However, after these treatments no remarkable changes were observed in the polyamine oxidase activity assayed under optimum conditions employing dialyzed liver homogenates as the source of the enzyme. Furthermore, in undialyzed homogenates there were no changes in the enzyme activities if measured in the absence of benzaldehyde. In liver slices from carbon tetrachloride treated rats, an increased incorporation of the label from spermidine to putrescine was observed (results not shown), although the increase was not as marked as in vivo.

Discussion

The existence of a novel enzyme (polyamine oxidase) capable of forming putrescine from spermidine, and spermidine from spermine, is found in rat liver. This enzyme is possibly responsible for the observed interconversions of polyamines in vivo (Siimes, 1967; Hölttä et al., 1973b).

Polyamine oxidase is most probably localized in peroxisomes, where the highest specific activity of the enzyme was found. Thus, polyamine oxidase is a new addition to peroxisomal enzymes. The enzyme activity in the cytosolic fraction may be due to a leakage of the enzyme protein out of the peroxisomes during the isolation procedure. However, until a definite characterization of the cytosolic enzyme, the possibility of the existence of an isoenzyme cannot be ruled out.

The purification of the enzyme from rat liver peroxisomes resulted in an electrophoretically homogenous protein, which has not been characterized in mammalian tissues previously. A single enzyme is shown to catalyze the oxidation of both spermidine and spermine. These molecules are cleaved at the secondary amino groups yielding 3-aminopropionaldehyde as one of the reaction products. A similar cleavage of spermidine and spermine appears to occur in *Pseudomonas* (Padmanabhan and Kim, 1965) and *Mycobacteria* (Bachrach et al., 1960), whereas mammalian amine oxidases have been only reported to oxidize polyamines at the terminal primary amino groups (Tabor et al., 1964; Tabor and Tabor, 1972; Bachrach, 1973).

It appears that polyamine oxidase is a flavoprotein with FAD as a prosthetic group since the native enzyme could be resolved to an apoenzyme that was partially reactivated by FAD. Further, quinacrine (an inhibitor of many flavoenzymes) profoundly inhibited the polyamine oxidase activity. The discrepant ratio of acid-extractable FAD to protein for the purified enzyme (0.16 mol of FAD/mol of enzyme) requires further experimentation. Besides an eventual loss of the cofactor from the enzyme, the possibility of the presence of impurities with physical properties similar to those of polyamine oxidase should be kept in mind.

Polyamine oxidase seems to contain sulfhydryl group(s) essential for the enzyme activity since sulfhydryl reagents profoundly inhibited the activity and exogenous thiols were needed for full activity. Further, carbonyl group(s) are involved in the enzyme activity as judged from the susceptibility of the enzyme to carbonyl group reagents. NSD-1055, which has been reported to inhibit some pyridoxal phosphate requiring enzymes (Levine et al., 1965), abolished the polyamine oxidase activity. However, pyridoxal phosphate is not likely to be the carbonyl compound involved since typical inhibitors of pyridoxal phosphate enzymes (isonicotinic acid hydrazine and canaline) did not inhibit polyamine oxidase. Nor did exogenous pyridoxal phosphate influence the enzyme activity. The stimulation of polyamine oxidase by free pyridoxal is likely to be attributable to the aldehyde group in a hydrophobic environment like those in hexanal and benzaldehyde, which also increase the enzyme activity.

Polyamine oxidase activity was stimulated by various aldehydes, especially by certain aromatic ones, when the enzyme activity was measured with spermidine or spermine as the substrates (but not with N^1-acetylspermidine according to preliminary results). Further studies are needed to elucidate the mechanism of aldehyde action in the regulation of the polyamine oxidase activity.

Treatment with carbon tetrachloride increased the formation of putrescine from spermidine in rat liver slices. However, the activity of polyamine oxidase of liver homogenates did not show any changes after various treatments known to greatly enhance the conversion of spermidine to putrescine in vivo (Hölttä et al., 1973b). Whether intact cell structures are required for the enhanced conversion of spermidine to putrescine remains to be seen. Accumulation of radioactive putrescine after [^{14}C]spermidine administration under the above conditions might also be due to a decreased degradation of putrescine by monoamine or diamine oxidases. The activity of diamine oxidase is very low in rat liver, but the high activity of monoamine oxidase was reduced after carbon tetrachloride poisoning. The present results indicate that, in addition to spermine and spermidine, N^1-acetylspermidine could be utilized as a substrate for polyamine oxidase. The elucidation of the possible role of acetylation in the oxidation of spermidine and spermine by polyamine oxidase waits for further studies.

The significance of the liver polyamine oxidase in the metabolism of polyamines remains to be established. The activity of polyamine oxidase seems to be rather high when compared with the activities of the enzymes, involved in the biosynthesis of polyamines (Hannonen et al., 1972b). Thus polyamine oxidase may be of potential importance in regulating polyamine concentrations in mammalian tissues. The association of this enzyme with growth processes and malignant transformation is a possibility, which merits further exploration, since significant changes in the amount of 3-aminopropionaldehyde have been reported in the sera of cancer patients (Quash and Taylor, 1970).

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Properties of L-Methionine γ-Lyase from *Pseudomonas ovalis*†

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ABSTRACT: The distribution of bacterial L-methionine γ-lyase (L-methionine methanethiollyase (deaminating) (EC 4.4.1.11)) was investigated, and *Pseudomonas ovalis* (IFO 3738) was found to have the highest activity of enzyme, which was inducibly formed by addition of L-methionine to the medium. L-Methionine γ-lyase, purified to homogeneity from *Ps. ovalis,* has a molecular weight of about 173 000 and consists of nonidentical subunits (mol wt: 40 000 and 48 000). The enzyme exhibits absorption maxima at 278 and 420 nm, and a shoulder around 330 nm, which are independent of the pH (6.0 to 10.0), and contains 4 mol of pyridoxal 5′-phosphate per mol of the enzyme. The formyl group of pyridoxal 5′-phosphate is bound in an aldimine linkage to the ε-amino group of lysine residues of the protein. The holoenzyme is resolved to the apoenzyme by incubation with hydroxylamine, and reconstituted by addition of pyridoxal 5′-phosphate. The enzyme activity is significantly affected by both carbonyl and sulfhydryl reagents. L-Methionine γ-lyase catalyzes α,γ- and α,β-elimination reactions of, in addition to L-methionine, several derivatives of L-methionine and L-cysteine, e.g., L-ethionine, DL-methionine sulfone, L-homocysteine, and *S*-methyl-L-cysteine. The enzyme catalyzes also γ-replacement reactions of the thiomethyl group of methionine with various alkanethiols (C_2-C_7), arylthio alcohols (benzenethiol and β-naphthalenethiol) and the derivatives of ethanethiol (2-mercaptoethanol and cysteamine) to yield the corresponding *S*-substituted homocysteine. The thiomethyl group of *S*-methyl-L-cysteine also is replaced by ethanethiol to form *S*-ethyl-L-cysteine.

Two different metabolic pathways have been proposed for the production of methanethiol from methionine. One of them is a pathway where methionine is first deaminated and then dethiomethylated with the release of methanethiol as reported for various aerobic bacteria and soil fungi (Segal and Starkey, 1969; Ruiz-Herrera and Starkey, 1969a). Alternatively, methionine is deaminated-dethiomethylated simultaneously to form methanethiol. The first evidence for the occurrence of this pathway was obtained by Onitake (1938) using the dried cells

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