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Polyamine degradation in foetal and adult bovine serum

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1. Using protein-separative chromatographic procedures and assays specific for putrescine oxidase and spermidine oxidase, adult bovine serum was found to contain a single polyamine-degrading enzyme with substrate preferences for spermidine and spermine. Apparent K_m values for these substrates were approx. 40 μM. The apparent K_m for putrescine was 2 mM. With spermidine as substrate, the K_i values for aminoguanidine (AM) and methylglyoxal bis(guanylhydrazone) (MGBG) were 70 μM and 20 μM respectively. 2. Bovine serum spermidine oxidase degraded spermine to spermidine to putrescine and N^8-acetylspermidine to *N*-acetylputrescine. Acrolein was produced in all these reactions and recovered in quantities equivalent to H_2O_2 recovery. 3. Spermidine oxidase activity was present in foetal bovine serum, but increased markedly after birth to levels in adult serum that were almost 100 times the activity in foetal bovine serum. 4. Putrescine oxidase, shown to be a separate enzyme from bovine serum spermidine oxidase, was present in foetal bovine serum but absent from bovine serum after birth. This enzyme displayed an apparent K_m for putrescine of 2.6 μM. The enzyme was inhibited by AM and MGBG with K_i values of 20 nM. Putrescine, cadaverine and 1,3-diaminopropane proved excellent substrates for the enzyme compared with spermidine and spermine, and *N*-acetylputrescine was a superior substrate to N^1- or N^8-acetylspermidine.

In ABS, amine:oxygen oxidoreductase (deaminating, copper-containing; EC 1.4.3.6) degrades polyamines and other amines to their aminoaldehydes, H_2O_2 and ammonia (Tabor *et al.*, 1964; Yasunobu *et al.*, 1976). The aminoaldehydes of spermidine and spermine subsequently undergo non-enzyme β-elimination to yield acrolein (Kimes & Morris, 1971) and the corresponding amine (Alarcon, 1970). The enzyme responsible for the initial reaction in ABS is an amine oxidase, but will be called spermidine oxidase here to designate its preferred substrate and to distinguish it from another amine oxidase, putrescine oxidase, found in addition to spermidine oxidase in FBS (Gahl *et al.*, 1980). The foetal-bovine-serum putrescine oxidase is an amine:oxygen oxidoreductase (deaminating), but it cannot be assigned to EC 1.4.3.6 yet because it has not been shown to contain copper. It has not been determined whether putrescine oxidase and spermidine oxidase activities exist as separable proteins in ABS, as in FBS.

In the present investigation polyamine-degrading enzyme activity was compared in foetal, newborn, calf and adult bovine serum. The kinetic properties, products, stoichiometry and substrate preferences of the adult enzyme were examined, and the FBS putrescine oxidase was characterized with respect to approximate molecular weight, substrate preference and kinetic parameters.

Abbreviations used: FBS, foetal bovine serum; ABS, adult bovine serum; AM, aminoguanidine hydrogen carbonate; MGBG, methylglyoxal bis(guanylhydrazone) dihydrochloride monohydrate [2,2′-(1-methyl-1,2-ethanediylidene)bis(hydrazinecarboximidamide)].

Experimental

Materials

[1,4-^{14}C]Putrescine dihydrochloride (sp. radioactivity 102 Ci/mol), [^{14}C]spermidine trihydrochloride (sp. radioactivity 98 Ci/mol) and [^{14}C]spermine tetrahydrochloride (sp. radioactivity 62 Ci/mol) were obtained from New England Nuclear Corp., Boston, MA, U.S.A. Unlabelled polyamines, *o*-dianisidine and horseradish peroxidase were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., AM and MGBG were products of Aldrich Chemical Co., Milwaukee, WI, U.S.A. CH-

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Sepharose 4B and Blue Sepharose CL-6B were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden, and DEAE-cellulose DE-52 was purchased from Whatman, Clifton, NJ, U.S.A. Dowex 50W resin (8% crosslinked; 200–400 mesh) and Bio-Gel Al.5 were obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. Foetal bovine serum was a product of Grand Island Biological Co., Grand Island, NY, U.S.A., newborn-calf serum was obtained from Irvine Scientific, Santa Ana, CA, U.S.A., and calf serum was purchased from KC Biological Inc., Lenexa, KS, U.S.A. Adult bovine serum, a generous gift from the Oscar Mayer Co. (Madison, WI, U.S.A.), was kindly prepared by the late Dr. Kazuto Kajiwara of this Institute as previously described. An Isocap scintillation counter (Nuclear-Chicago, Des Plaines, IL, U.S.A.) was used in all radioactivity measurements.

Methods

Polyamine separation. *N*-Acetylputrescine, N^1-acetylspermidine and N^8-acetylspermidine were synthesized as described by Tabor *et al.* (1971). The acetylated compounds were identified by melting points and by paper chromatography using authentic N^1- and N^8-acetylspermidine kindly provided by Dr. M. M. Abdel-Monem (University of Minnesota, Minneapolis, MN, U.S.A. N^1-Acetylspermidine was contaminated with 0.5% spermidine. Polyamines were separated by paper chromatography on Whatman no. 1 paper using both propan-2-ol/85% (v/v) formic acid/water (8:1:1, by vol.) (Menashe *et al.*, 1980) and n-propanol/HCl/water (3:1:1, by vol.) (Dubin & Rosenthal, 1960). N^1- and N^8-Acetylspermidine were separated on Schleicher and Schuell no. 507 paper using n-propanol/triethylamine/water (85:3:15, by vol.) (Dubin & Rosenthal, 1960). Dansyl (5-dimethylaminonaphthalene-1-sulphonyl) derivatives were prepared as described by Heby *et al.* (1973) and separated on silica-gel plates (Analtech Inc., Newark, DE, U.S.A.) using cyclohexane/ethyl acetate (3:2, v/v). When radioactive polyamines were used, the plates were apposed to films (Kodak X-ray Noscreen) for several days.

Protein determination. Protein was determined by the method of Lowry *et al.* (1951) with crystalline freeze-dried bovine serum albumin as standard (Sigma).

Acrolein measurement. For each reaction mixture to be assayed for acrolein, a portion (2ml) was placed in the outer well of a Conway diffusion vessel. Into the centre well was placed 1.5 ml of a mixture of *m*-aminophenol (10mg/ml) and hydroxylamine hydrochloride (12mg/ml) in 1M-HCl. Saturated KCl (2ml) was added to the outer well, and the vessel was sealed and shaken at 37°C for 2h. Duplicate 0.5ml portions were removed from the centre well and assayed fluorimetrically for α,β-unsaturated aldehydes, as described by Alarcon (1968). A standard curve with various amounts of authentic acrolein was run simultaneously with test samples.

Amine oxidase assays. When oxidase activity was measured by the production of H_2O_2, a modification of the *o*-dianisidine/peroxidase method of Gunther & Glick (1967) was employed. The standard substrate concentration was 1mM. The 2.0ml reaction mixture was incubated at 37°C for 30–300min. This assay was used when an enzyme exhibited a high K_m (mM range) for a particular substrate, and when non-polyamines were used as substrates. The assay was not appropriate for assays of crude serum because serum pigments resulted in substantial background absorption.

Spermidine oxidase was measured by the radioassay previously described (Gahl *et al.*, 1980). The final concentration of [^{14}C]spermidine was 17.5μM, incubation time was 1–4h and aminoguanidine was omitted from the reaction mixture. This specific assay was used when small amounts of spermidine oxidase activity were assayed in either crude serum or partially purified enzyme preparations.

Putrescine oxidase was measured by the liquid-scintillation counting method of Okuyama & Kobayashi (1961), as previously described (Gahl *et al.*, 1980). This sensitive assay, with a final [^{14}C]putrescine concentration of 3.1μM, was used when putrescine oxidase activity was low and when the enzyme to be assayed exhibited a low K_m (in the μM range) with respect to putrescine.

One unit of each enzyme activity was defined as the amount that converted 1pmol of substrate into product in 1h under the conditions of the assay.

Molecular-weight determination. Gel filtration was performed using a column (1.3cm × 103cm) of Bio-Gel Al.5 and a flow rate of 13ml/h. Elution buffer was 0.01M-potassium phosphate, pH8.0. Ferritin, catalase and aldolase were used as calibration proteins (Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A.). Log (molecular weight) was plotted against V_e/V_0, where V_e is the protein's elution volume and V_0 the void volume (Andrews, 1965).

Gel electrophoresis was performed with native slab gels at pH8.7 (Davis, 1964). The separating gel consisted of 7.5% or 5% acrylamide, whereas the stacking gel was 1.5% agarose to preserve enzyme activity. Each gel was run a length of 12cm over 6h at 14mA. Calibration proteins were stained with Coomassie Blue, and spermidine oxidase and putrescine oxidase activities were identified by incubating the gel in the standard *o*-dianisidine/peroxidase reacton mixture. The substrate was either 1mM-spermidine or -putrescine. A brown band indicated H_2O_2 production. The ratio of migration on 5%

acrylamide to migration on 7.5% acrylamide was a logarithmic function of molecular weight (Maurer, 1971).

Results

Adult bovine serum spermidine oxidase

Bovine serum was subjected to different protein separative procedures to determine whether two distinct proteins degrade putrescine and spermidine in adult serum, as in FBS (Gahl *et al.*, 1980). A column (2.6 cm x 7 cm) of cadaverine–Sepharose 4B was prepared as previously described (Baylin & Margolis, 1975), and 50 ml of adult bovine serum diluted to 250 ml with 0.01 M-potassium phosphate, pH 8.0, was placed on the column. The flow rate was 200 ml/h. An 800 ml linear (0–0.5 M-NaCl) gradient was run, and portions of 7.2 ml fractions were assayed for putrescine oxidase and spermidine oxidase activities by using the *o*-dianisidine/peroxidase reaction. Both enzyme activities were eluted in identical fashion at less than 0.18 M salt (Fig. 1), and measurement of each activity using the [^{14}C]putrescine and [^{14}C]spermidine assays verified the identical elution patterns. Recovery was 85%. The peak of putrescine oxidase activity eluting between 0.15 and 0.40 M-NaCl found in foetal serum (Gahl *et al.*, 1980) was absent from adult serum.

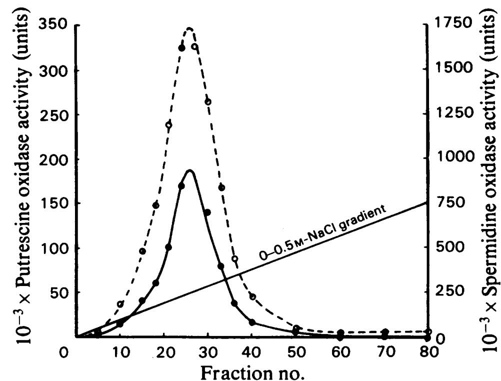

Fig. 1. *Elution patterns of ABS putrescine oxidase and spermidine oxidase from cadaverine–Sepharose 4B*
Diluted dialysed ABS (250 ml) was placed on the column and a linear NaCl (0–0.5 M) gradient, total volume 800 ml, in 0.001 M-potassium phosphate, pH 8.0, was applied; 1 ml portions of selected 7.2 ml fractions were assayed for putrescine oxidase (●), and 100 μl portions were assayed for spermidine oxidase (○). In each case, the substrate concentration was 0.5 mM, and the *o*-dianisidine/peroxidase assay was employed. Results are shown in units/ml and represent single determinations.

The adult serum enzyme, partially purified by the cadaverine–Sepharose methods, was further subjected to anion-exchange chromatography using DEAE-cellulose and gel filtration using Bio-Gel A1.5; putrescine oxidase and spermidine oxidase activities were eluted in a single peak compared with the two separate peaks found for FBS (Gahl *et al.*, 1980). Both newborn bovine serum and calf serum displayed enzyme elution patterns identical with those for ABS, lacking the putrescine oxidase peak characteristic of FBS.

Since the elution patterns of the adult bovine serum enzyme closely resembled those of FBS spermidine oxidase rather than FBS putrescine oxidase (Gahl *et al.*, 1980), spermidine oxidase activity in both foetal and adult serum was further investigated. To partially purify FBS spermidine oxidase, 100 ml of serum diluted to 500 ml with buffer (0.01 M-potassium phosphate, pH 8.0) was placed on a cadaverine–Sepharose 4B column (2.6 cm x 7 cm) and washed stepwise with 0.07 M-NaCl and 0.40 M-NaCl in buffer. The latter fraction was dialysed and washed through a column (2.7 cm x 9 cm) of Blue Sepharose CL-6B. Three preparations of this sort were pooled, placed on a column (3 cm x 8.5 cm) of DEAE-cellulose DE-52, and eluted with an 800 ml linear (0–0.3 M-NaCl) gradient. Active fractions were pooled, dialysed, placed on a hydroxyapatite column (2 cm x 17 cm), and eluted with a 200 ml linear (0–0.1 M-potassium phosphate) gradient. Freeze-dried fractions containing spermidine oxidase activity were layered on a column (1.7 cm x 43 cm) of Bio-Gel A1.5 and eluted. The final procedure was cadaverine–Sepharose 4B chromatography using a column of dimensions 1 cm x 9 cm and a 500 ml linear (0–0.5 M-NaCl) gradient. The composite yield was 10%, with a 200-fold purification to approx. 100000 units/mg of protein.

For ABS spermidine oxidase, 150 ml of serum was diluted to 750 ml, placed on a cadaverine–Sepharose 4B column (2.6 cm x 6.6 cm), and eluted with an 800 ml linear (0–0.5 M-NaCl) gradient. Fractions containing spermidine oxidase activity were pooled, dialysed and further purified by DE-52 DEAE-cellulose column chromatography as described above. The final yield was approx. 65%, with a 28-fold purification to 1100000 units/mg of protein. Both foetal and adult spermidine oxidase preparations were stable at 4 °C for at least 2 months.

Table 1 shows that the apparent K_m values with respect to spermidine and putrescine for the ABS spermidine oxidase were very close to those for the FBS spermidine oxidase. However, the $V_{max.}$ values indicate that ABS possessed roughly 100 times the activity found in FBS. Both AM and MGBG inhibited the adult and foetal spermidine oxidase activities competitively, with K_i values in the range of 20–70 μM. The ABS enzyme exhibited apparent

Table 1. *Kinetic parameters of spermidine oxidase from ABS and FBS*
ABS was purified 28-fold with respect to spermidine oxidase activity, and all kinetic parameters for this preparation were determined using the *o*-dianisidine/peroxidase assay for H_2O_2 production. The optimal pH for each substrate (spermidine, pH 7.6; spermine, pH 7.4; N^8-acetylspermidine, pH 8.0; putrescine, pH 8.6) was used. FBS was purified 200-fold with respect to spermidine oxidase activity, which was measured using the [^{14}C]spermidine radioassay. Activity against putrescine was determined by the liquid-scintillation counting method using as substrate [^{14}C]putrescine diluted with various amounts of unlabelled putrescine. Both inhibitors acted competitively, and kinetic parameters were obtained using Lineweaver–Burk plots.

	Spermidine		Spermine		N^8-Acetylspermidine		Putrescine	
	ABS	FBS	ABS	FBS	ABS	FBS	ABS	FBS
K_m (mM)	0.048	0.039	0.033	N.D.*	0.044	N.D.	2.3	1.8
$V_{max.}$ (μmol/h per ml of crude serum)	5.7	0.07	5.6	N.D.	2.7	N.D.	1.0	0.01
K_i for AM (mM)	0.070	0.066						
K_i for MGBG (mM)	0.029	0.014						

* Not done.

K_m values with respect to spermidine, spermine, N^8-acetylspermidine of approx. 40 μM. The apparent K_m with respect to putrescine was 500 times as great; both the ABS and FBS enzyme preparations degraded 40 times more putrescine when its concentration was 1 mM (colorimetric assay) than when the concentration was 3.1 μM (standard radioassay).

The substrate preferences for the ABS enzyme and the FBS spermidine oxidase, as well as the FBS putrescine oxidase, are shown in Table 2. The substrate pattern of FBS spermidine oxidase closely resembled that of the adult enzyme, which exhibited greatest activity against spermidine and substantial activity with spermine, N^8-acetylspermidine and benzylamine. Both the ABS and the FBS spermidine oxidase preparations displayed only slight activity against 1,3-diaminopropane and putrescine. Since the spermidine oxidase exhibited a relatively high apparent K_m for putrescine, that substrate was also tested at concentrations of 10 mM and 50 mM. At these concentrations of putrescine, the enzyme produced 20% and 38% of the H_2O_2 formed when 1 mM-spermidine served as substrate.

On the basis of similar kinetic parameters (Table 1), substrate preferences (Table 2) and elution patterns using affinity, gel-filtration and anion-exchange chromatography (Figs. 3–5 in Gahl *et al.*, 1980), the FBS spermidine oxidase and the ABS spermidine oxidase were considered to be identical. The products formed by the enzyme were further characterized.

[^{14}C]Putrescine was previously shown to be produced from [^{14}C]spermidine by crude FBS (Gahl *et al.*, 1980). The products of spermine degradation were investigated by incubating [^{14}C]spermine with FBS spermidine oxidase, placing the reaction mixture on a Dowex column and eluting with a linear HCl gradient as described above. Fig. 2 shows that after 30 min of incubation substantial portions of the [^{14}C]spermine were converted into ^{14}C-labelled products eluting with authentic [^{14}C]spermidine and [^{14}C]putrescine. By 3 h, most of the radioactivity was present in the putrescine peak, and very little

Table 2. *Relative rates of substrate oxidation by ABS spermidine oxidase and by FBS spermidine oxidase and putrescine oxidase*
The *o*-dianisidine/peroxidase assay was used with a substrate concentration of 1 mM. For the ABS spermidine oxidase (SO), 100 μl (110 μg of protein) of a 28-fold-pure preparation was reacted with each substrate for 30 min at 37°C. For FBS spermidine oxidase, 500 μl (170 μg of protein) of a 75-fold-pure preparation was reacted for 1 h. This enzyme preparation had negligible activity against putrescine when the radioassay was used with a [^{14}C]putrescine concentration of 3.1 μM. For the FBS putrescine oxidase (PO), 1.2 ml (135 μg of protein) of a 220-fold-pure preparation was incubated for 4 h at 37°C. This enzyme preparation had no activity against spermidine when the radioassay was used with a [^{14}C]spermidine concentration of 17.5 μM. Results shown are means of duplicate determinations.

	Activity relative to spermidine as substrate		
Substrate	ABS SO	FBS SO	FBS PO
Spermidine	1.00	1.00	1.00
Spermine	0.81	0.76	1.23
Putrescine	0.05	0.06	2.03
N^8-Acetylspermidine	0.66	0.65	0.70
N^1-Acetylspermidine	0.20	0.14	1.15
N-Acetylputrescine	0.05	0.02	1.60
Benzylamine	0.34	0.32	0.30
Histamine	0.01	0.03	0.63
1,3-Diaminopropane	0.07	0.09	2.21
1,6-Diaminohexane	0.03	0.02	0.36
1,7-Diaminoheptane	0.12	0.10	0.51
Methylamine	0.03	0.04	0
Ethylamine	0.08	0.09	0.57
Cadaverine	0	0	2.05
Arginine	0	0	0
Ethylenediamine	0	0	0

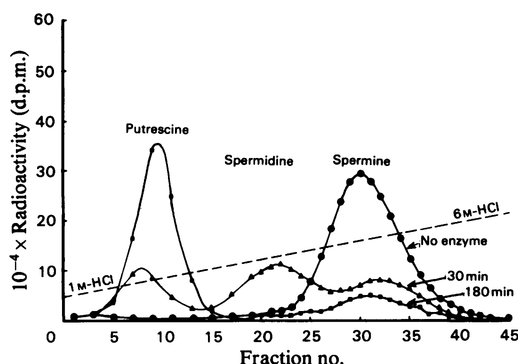

Fig. 2. *Elution pattern of radioactivity from Dowex 50W after incubation of* [14C]*spermine with FBS spermidine oxidase*

[14C]Spermine (1 μCi, 5.6 μg) was added to 9600 units (330 μg of protein, 75-fold pure) of FBS spermidine oxidase and 0.07 M-potassium phosphate, pH 8.0, in a 2.0 ml reaction mixture. After incubating at 37°C, 10 ml of 2% $HClO_4$ was added, and the mixture was placed on a Dowex 50W column (9 mm × 40 mm). A linear 400 ml HCl gradient (1–6 M) was run, 67 fractions were collected and 200 μl portions were measured for radioactivity. Incubation time was 30 min (▲) or 3 h (■). Areas labelled putrescine, spermidine and spermine indicate the elution patterns of [14C]putrescine (1 μCi, 1.6 μg), [14C]spermidine (1 μCi, 2.6 μg) and [14C]spermine (1 μCi, 5.6 μg), each incubated for 10 h in the absence of enzyme. Results are expressed in d.p.m. per 6.0 ml fraction. Negligible radioactivity was recovered from fractions 45–67.

remained as the substrate spermine. Dansylation, t.l.c. with ethyl acetate/cyclohexane (2:3, v/v) as solvent and autoradiography verified that radioactivity in fractions 9–11 represented putrescine and radioactivity in fractions 20–22 represented spermidine.

When 10 mg of N^8-acetylspermidine was incubated for 5 h with 3.5×10^6 units of ABS spermidine oxidase, a ninhydrin-positive substance different from N^8-acetylspermidine was produced. That substance (spot 2) migrated on Whatman no. 1 chromatography paper identically with *N*-acetylputrescine using two different solvent systems (Table 3).

Although putrescine and spermidine were recovered from the degradation of spermidine and spermine, one would also expect H_2O_2 to be formed in the oxidative deamination of polyamines, and acrolein to be formed non-enzymically from spermidine aminoaldehyde or spermine aminoaldehyde (Kimes & Morris, 1971). This was investigated by incubating 3 μmol of [14C]spermine,

Table 3. *Identification of N-acetylputrescine as a product of N^8-acetylspermidine degradation by ABS spermidine oxidase*

N^8-Acetylspermidine (10 mg) was incubated at 37°C for 5 h in the presence of 3.5×10^6 units (3.3 mg of protein; 28-fold pure) of ABS spermidine oxidase. The reaction mixture was brought to 5 ml final volume with 0.01 M-potassium phosphate, pH 8.0. Blank contained no enzyme. A 3 μl sample of each reaction mixture, and 5 μg of each standard, were spotted on Whatman no. 1 chromatography paper. Two spots were separable from the reaction mixture incubated in the presence of enzyme. Solvent A: n-propanol/HCl/water (3:1:1, by vol.), for 7 h. Solvent B: propan-2-ol/85% formic acid/water (8:1:1, by vol.), for 6 h.

	R_F value	
	Solvent A	Solvent B
Putrescine	0.45	0.11
Spermidine	0.31	0.03
N-Acetylputrescine	0.73	0.64
N^8-Acetylspermidine (no enzyme)	0.61	0.25
N^8-Acetylspermidine (plus enzyme)		
Spot 1	0.63	0.23
Spot 2	0.76	0.64

[14C]spermidine or N^8-acetylspermidine for 1 h at 37°C in the presence of 177×10^3 units of ABS spermidine oxidase. The final concentration of each substrate in the 6 ml reaction mixture was 0.5 mM. A 2 ml portion of each reaction mixture was assayed for H_2O_2, and a 2 ml portion was placed in the outer well of a Conway diffusion vessel for measurement of acrolein (see under 'Methods'). For [14C]spermidine, a 2 ml portion was removed, the [14C]putrescine product was separated and radioactivity was measured as previously described (Gahl *et al.*, 1980). For [14C]spermine, a 2 ml portion was treated as described in the legend to Fig. 2, and radioactivity in the peak corresponding to the [14C]-spermidine product was estimated. Table 4 shows that over 100 nmol of H_2O_2 and acrolein were formed from 1 μmol of spermidine in nearly stoichiometric fashion, and that 99 nmol of [14C]putrescine was produced. Similar stoichiometry was observed with spermine as substrate and H_2O_2, acrolein and spermidine as products. H_2O_2 and acrolein were produced in equivalent amounts from N^8-acetylspermidine. Similar results were obtained when partially purified FBS was the source of spermidine oxidase activity.

The molecular weight of the ABS spermidine oxidase was investigated by gel filtration and by gel electrophoresis. Gel filtration using Bio-Gel A1.5 gave an elution volume for ABS spermidine oxidase

Table 4. *Stoichiometry of products formed from spermidine, spermine and N^8-acetylspermidine by ABS spermidine oxidase*
Each substrate (3 μmol) was incubated with 177×10^3 units (160 μg of protein; 28-fold pure) of ABS spermidine oxidase at 37°C for 1 h. The 6 ml reaction mixture contained 0.07 M-potassium phosphate, pH 8.0. Spermidine and spermine were each labelled to a specific radioactivity of 1 Ci/mol; AM was present at 50 μM when spermidine was substrate. Peroxidase (60 μg) and o-dianisidine (750 μg) were added for the measurement of H_2O_2, which was performed on a 1 ml portion of the reaction mixture. Acrolein was measured by transferring a 2 ml portion of the reaction mixture to the outer well of a Conway diffusion vessel (see under 'Methods'). Standard curves for H_2O_2 and acrolein contained enzyme protein but no substrate and were run simultaneously with test samples. The product [^{14}C]putrescine was assayed on a 2 ml portion of the reaction mixture using the standard radioassay for spermidine oxidase. The product [^{14}C]spermidine was measured on 2 ml of the [^{14}C]spermine reaction mixture by using an HCl gradient as described in the legend to Fig. 2. It was estimated because the [^{14}C]spermidine shoulder overlapped with the [^{14}C]spermine peak. Controls contained no enzymes; no product was recovered.

	Products (nmol/h per 2 ml sample)			
Substrate	H_2O_2	Acrolein	Putrescine	Spermidine
Spermidine	111	127	99	—
Spermine	107	124	—	100*
N^8-Acetylspermidine	61	73	—	—

* Estimated.

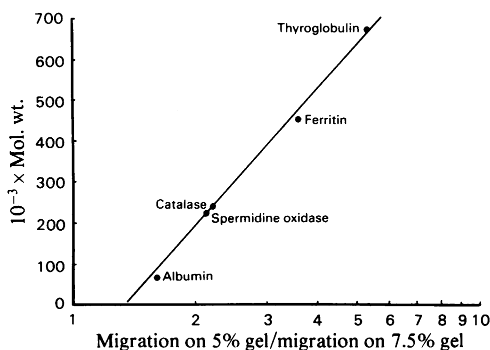

Fig. 3. *ABS spermidine oxidase molecular-weight determination by gel electrophoresis*
ABS spermidine oxidase (28-fold pure, 55 μg of protein) and four calibration proteins (10 μg) were run in separate wells on 7.5% and 5% acrylamide gels with a 1.5% agarose stacking gel. The portion of the gel containing marker proteins was stained with Coomassie Blue. The portion containing enzyme was placed overnight in 1 mM-potassium phosphate, pH 8.0, at 4°C. It was then incubated at 37°C in the presence of o-dianisidine, peroxidase and 1 mM-spermidine in 0.07 M-potassium phosphate, pH 7.5, as described under 'Methods'. Enzyme activity was identified as a brown band, which was not present when AM was added to the incubation mixture. Log (migration on 5% gel/migration on 7.5% gel) was plotted against molecular weight.

corresponding to a mol.wt. of 280000. Native slab-gel electrophoresis was performed at acrylamide concentrations of 7.5% and 5% to eliminate differences in migration due to different native conformations. The ratio of the migration distance of spermidine oxidase activity on the 5% gel to the migration distance on the 7.5% gel yielded a mol.wt. of 220000 (Fig. 3).

The relative amount of spermidine oxidase activity, measured by radioassay with substrate excess, in crude bovine serum of different ages is shown in Table 5. Spermidine oxidase activity was 30-fold greater in the neonatal period compared with late in gestation; activity tripled again as maturity was reached. However, the serum protein concentration also increased with maturation, so the enzyme's specific activity rose by only 39-fold from late in gestation to adulthood.

FBS putrescine oxidase

Filtered, dialysed FBS (100 ml) was diluted to 500 ml with standard buffer (0.01 M-potassium phosphate, pH 8.0) and placed on a cadaverine–Sepharose column (2.5 cm × 8.5 cm). A linear NaCl (0–0.5 M) gradient, total volume 800 ml in standard buffer, was used to elute the enzyme, and sequential 7.2 ml fractions were assayed for putrescine oxidase activity. Active fractions were dialysed against 1 mM-potassium phosphate and run through a column (2.7 cm × 9 cm) of Blue Sepharose CL-6B. The portion washing through (172 ml) was combined with a 1 mM-potassium phosphate wash and placed on a column (3 cm × 8.5 cm) of DEAE-cellulose DE-52. A 400 ml linear NaCl gradient (0–0.3 M) in standard buffer was run, and 6.2 ml fractions were assayed for putrescine oxidase. Active fractions (198 ml) were dialysed, and 95 ml was placed on a second column of cadaverine–Sepharose. A 400 ml linear NaCl gradient (0–1 M) was run; fractions containing putrescine oxidase activity were pooled. Purification results are shown in Table 6.

Table 5. *Spermidine oxidase activity in different bovine sera*

Crude bovine serum was incubated at 37°C for 1 h in the presence of [^{14}C]spermidine (0.5 μCi, 1 μmol) and 0.07 M-potassium phosphate, pH 8.0. The final concentration of spermidine was 0.5 mM, and the radioassay for spermidine oxidase activity was employed. One international unit of activity is defined as the amount required to convert 1 μmol of spermidine into product/min per mg of protein. Results shown are means of duplicate determinations.

Serum	Age	$10^{-3}\times$ Activity (units/ml)	Protein (mg/ml)	$10^{-6}\times$ Activity (international units)
Foetal	6–9 months gestation	42	36.0	19
Newborn	1–10 days	1362	55.5	409
Calf	16 weeks	2466	59.0	697
Adult	Mature	3926	88.0	744

Table 6. *Purification of FBS putrescine oxidase*

Putrescine oxidase was measured by the radioactive assay. Only one-half of the putrescine oxidase activity recovered from DEAE-cellulose was placed on the second Sepharose–cadaverine column.

	Activity (nmol/h)	Protein (mg)	Specific activity (nmol/h per mg)	Purification (fold)	Yield (%)
Crude serum	596	4475	0.13	1	100.0
Sepharose–cadaverine	401	22.4	17.9	135	67.2
Blue Sepharose	265	6.7	39.6	297	44.4
DEAE-cellulose	299	2.1	142.4	1071	50.2
Sepharose–cadaverine	39	0.01	570	4285	6.5

A portion of the DEAE-cellulose fraction (Table 6) was freeze-dried with loss of specific activity to 20 nmol/h per mg of protein. Approx. 500 μg of this putrescine oxidase preparation (150-fold pure) was layered on a Bio-Gel A1.5 column; enzyme activity eluted at 1.1 times the void volume. This was approximately where thyroglobulin (mol.wt. 670000) eluted, but was in the non-linear portion of the elution curve. Slab-gel electrophoresis of the native protein was performed using over 100 μg of protein (160-fold pure) obtained by batch elution with 0.5 M-NaCl from cadaverine–Sepharose 4B. The appearance of a brown band on the gel incubated with putrescine indicated H_2O_2 production. The 7.5% acrylamide gel revealed putrescine oxidase activity in heterogeneous species migrating between 2 and 15 mm, corresponding to a mol.wt. of over 450000. On 5% polyacrylamide, the colour pattern was too diffuse to see. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of 1000-fold purified putrescine oxidase revealed three bands, the brightest corresponding to a mol.wt. of 380000.

The substrate preferences of a 220-fold-pure preparation of FBS putrescine oxidase (the most active portions of the Sepharose–cadaverine column fraction; Table 6) were examined. This enzyme preparation exhibited no activity against spermidine using the radioassay with a spermidine concentration of 17.5 μM. However, when the substrate concentration was 1 mM, the putrescine oxidase showed substantial activity against spermidine, spermine and *N*-acetylputrescine (Table 2). The preferred substrates were the aliphatic diamines 1,3-diaminopropane, putrescine and cadaverine, with a rather broad range of acceptable substrates.

Table 7. *Kinetic parameters for FBS putrescine oxidase*

With putrescine as substrate, FBS putrescine oxidase (550 units, 44 μg of protein, 90-fold pure) was incubated for 2 h at 37°C with various concentrations of [^{14}C]putrescine (sp. radioactivity 2.46 Ci/mol) in the standard reaction mixture. K_i values were determined by Lineweaver–Burk plots using 40 nM-AM (competitive) or 50 nM-MGBG (non-competitive). With spermidine as substrate, FBS putrescine oxidase (3100 units, 61 μg of protein) was incubated for 5 h with various concentrations of spermidine, using the *o*-dianisidine/peroxidase assay for determination of H_2O_2 production.

Substrate ...	Putrescine	Spermidine
K_m (μM)	2.6	25.0
$V_{max.}$ (nmol/h per ml of crude serum)	9.5	3.6
K_i for aminoguanidine (μM)	0.022	N.D.*
K_i for MGBG (μM)	0.024	N.D.

* Not done.

Kinetic parameters for putrescine oxidase are shown in Table 7. The enzyme displayed an apparent K_m for putrescine (2.6 μM) that was one-tenth the apparent K_m for spermidine. The FBS putrescine oxidase was inhibited competitively by aminoguanidine and non-competitively by MGBG at concentrations in the range of 10 nM.

Discussion

Since ruminant sera were first shown to contain an enzyme that catalysed the oxidation of spermidine and spermine (Hirsch, 1953; Blaschko & Hawes, 1959), bovine serum has provided the source for the prototype of polyamine-degrading enzymes in animal serum. Tabor *et al.* (1954) termed the enzyme bovine plasma amine oxidase and purified it 200-fold; they later showed that H_2O_2, ammonia and an aminoaldehyde were produced from spermine and spermidine by the enzyme (Tabor *et al.*, 1964). Yamada & Yasunobu (1962) termed the enzyme monoamine oxidase and purified it approx. 500-fold. They characterized it as a protein of mol.wt. 255000 with three subunits of mol.wt. 85000 (Yasunobu *et al.*, 1976). This is the enzyme we termed spermidine oxidase and found it to have a mol.wt. of 220000–280000 and activity primarily against spermidine and spermine.

Spermidine oxidase was shown to be the only polyamine-degrading enzyme in adult bovine serum (Fig. 1), in contrast with the separate spermidine oxidase and putrescine oxidase proteins found in foetal serum (Gahl *et al.*, 1980). Spermidine oxidase was found in increasing quantities as maturation progressed from late in gestation through the newborn and weanling periods (Table 5). The enzyme displayed an apparent K_m for spermidine of $40\,\mu$M at 37°C as determined independently by H_2O_2 production and by [^{14}C]putrescine production from [^{14}C]spermidine. This K_m value differed markedly from the K_m for spermidine of $2.7\,\mu$M at 25°C reported by Allen *et al.* (1979), who employed a polarographic assay for O_2 consumption. The disparity may be a reflection of differences in assay procedure, temperature, buffer or enzyme purity.

The meagre degradation of putrescine by bovine serum spermidine oxidase in part reflects a high apparent K_m for putrescine. At 2mM, this differs from a previously reported apparent K_m for putrescine of $50\,\mu$M (Gahl & Pitot, 1979). However, that was determined in crude adult bovine serum at a different pH (9.8). Some substance present in crude serum, perhaps putrescine itself, may have altered the results. Nevertheless, the bovine serum spermidine oxidase is clearly specialized to degrade polyamines, not diamines. The degradation of N^8-acetylspermidine reflects the enzyme's preference for substrates with free propylamine moieties.

The role of acetylated polyamine derivatives has been examined more extensively in tissues than in serum. A rat liver cytosolic spermidine *N*-acetyltransferase produces N^1-acetylspermidine from spermidine (Matsui *et al.*, 1981), and N^1-acetylspermidine is a substrate for purified rat liver polyamine oxidase (Hölttä, 1977). Blankenship (1979) showed that a rat liver homogenate converted N^1-acetylspermidine into putrescine, and Bolkenius & Seiler (1981) identified both putrescine and 3-acetamidopropanal as products of N^1-acetylspermidine, which in turn acts as a substrate for a tissue polyamine oxidase producing putrescine. The spermidine. They suggested that spermidine degradation is facilitated by conversion into N^1-acetylhypothesis was based in part on previous demonstrations that acute liver damage in rats results in elevated N^1-acetylspermidine and putrescine levels (Seiler *et al.*, 1980; Matsui *et al.*, 1981; Abdel-Monem & Merdink, 1981).

The bovine serum spermidine oxidase differs from the rat liver polyamine oxidase in two major respects. First, it degrades N^8-acetylspermidine in preference to N^1-acetylspermidine. N^8-Acetylspermidine is not a substrate for the rat liver polyamine oxidase. Secondly, rat liver polyamine oxidase produces aminopropionaldehyde (Hölttä, 1977) or 3-acetamidopropanal (Bolkenius & Seiler, 1981), whereas the bovine serum spermidine oxidase produces acrolein (Kimes & Morris, 1971; Alarcon, 1970). It may be that cells avoid the production of acrolein, a compound with extensive toxicity (Izard & Libermann, 1978), whereas acrolein production in serum is permitted since proteins bind the compound and protect cells from contact with it.

The conversion of spermidine into putrescine in the presence of bovine serum has previously been demonstrated (Gahl *et al.*, 1980). We have shown here that [^{14}C]spermine is degraded rapidly to [^{14}C]putrescine by a partially purified preparation of bovine serum spermidine oxidase when the spermine concentration is $8\,\mu$M (Fig. 2). However, when [^{14}C]spermine is present in excess (0.5mM), the degradation stops at spermidine, whose catabolism by spermidine oxidase is hindered by competing spermine. Consequently, a single mole of acrolein is formed for each mole of spermine degraded and spermidine is formed rather than putrescine (Table 4).

The FBS putrescine oxidase, which disappears from bovine serum by birth, is clearly a different enzyme from the bovine serum spermidine oxidase. Its preference for three to five carbon aliphatic diamines (Table 2) may point to the importance of these compounds relative to spermidine and spermine for foetal growth. The apparent K_m for putrescine, $2.6\,\mu$M (Table 7), is low compared with the K_m for spermidine, and very low compared with the K_m for putrescine calculated for spermidine oxidase (2mM). As for spermidine oxidase, putrescine oxidase accepts acetylated polyamines as substrates. However, the putrescine oxidase is exquisitely sensitive to inhibition by the carbonyl reagents AM and MGBG, with K_i values for each of 20nM (Table 7). This is consistent with the enzyme's substrate preference for smaller molecules

when compared with spermidine oxidase, which exhibits K_i values for AM and MGBG three orders of magnitude higher (Table 1).

FBS putrescine oxidase appears to be a very large molecule, since it eluted just after the void volume from Bio-Gel A1.5. Moreover, activity against putrescine was noted over a wide range of molecular weights when a partially purified preparation of the enzyme was run on native polyacrylamide gels. This phenomenon suggests aggregation of smaller active protein units and requires further investigation.

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