Separation of Putrescine Oxidase and Spermidine Oxidase in Foetal Bovine Serum with the aid of a Specific Radioactive Assay of Spermidine Oxidase

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1. A sensitive and specific assay for spermidine oxidase is described. The method involves the separation of [^{14}C]spermidine (substrate) from [^{14}C]putrescine (product) and other ^{14}C -labelled products on a Dowex 50 cation-exchange column: 92% of the putrescine applied to the column was eluted by 2.3 M-HCl, but this treatment left 96% of the spermidine bound to the column. Unchanged spermidine could be removed from the column by elution with 6 M-HCl. 2. By means of this assay, foetal and adult bovine serum were each shown to contain spermidine oxidase activity, putrescine being a major product of the oxidation of spermidine by the serum enzymes. 3. In foetal bovine serum, spermidine oxidase activity is separable from putrescine oxidase activity by chromatography on a cadaverine-Sepharose column, by gel filtration and by ion-exchange column chromatography. Putrescine oxidase was purified 1900-fold and spermidine oxidase 130-fold by these procedures. The former oxidized putrescine but not spermidine, and spermidine oxidase exhibited no activity with putrescine as substrate.

Diamine oxidase (EC 1.4.3.6) is an enzyme or group of enzymes catalysing the oxidative deamination of histamine, benzylamine and aliphatic amines, including the polyamines, putrescine, spermidine and spermine (Zeller, 1963). The latter three compounds are considered to be intimately involved in growth and development (Cohen, 1971; Bachrach, 1973). Study of the degradation of these polyamines has not yet revealed whether a single enzyme catalyses the oxidation of all three compounds or whether separate enzymes each catabolize a single polyamine. The development of a sensitive diamine oxidase assay specific for each polyamine substrate would help resolve this controversy. Putrescine oxidase activity can be readily measured by a sensitive and specific liquid-scintillation assay by use of [^{14}C]putrescine, whose oxidized product, amino[^{14}C]butyraldehyde, cyclizes to 1-[^{14}C]pyrroline. This cyclic compound can be extracted with toluene and the ^{14}C determined (Okuyama & Kobayashi, 1961). However, such a separation of product from substrate cannot be used when spermidine and spermine are substrates, since their immediate products do not cyclize to become toluene-soluble.

The oxidation of spermidine is initially catalysed by diamine oxidase as follows (Tabor *et al.*, 1964):

In foetal bovine serum, the aminoaldehyde produced is then degraded to putrescine and acrolein ($\text{CH}_2=\text{CH}-\text{CHO}$), presumably through spontaneous β -elimination (Alarcon, 1970). The activity of diamine oxidase against spermine and spermidine has been measured by means of O_2 consumption or production of ammonia (Hirsch, 1953; Tabor *et al.*, 1954; Blaschko & Hawes, 1959). In rat liver, a polyamine oxidase has been assayed by electrophoretic separation of the radiolabelled putrescine and 3-aminopropionaldehyde products of spermidine and spermine degradation on paper (Holtta, 1977). Quash *et al.* (1979) assayed polyamine (i.e. spermidine) oxidase activity by using [^{14}C]spermidine and a coupled reaction in which exogenous and endogenous diamine (i.e. putrescine) oxidase converted the [^{14}C]putrescine produced directly to 1-[^{14}C]pyrroline. No attempt has been made to apply these assay procedures for spermidine oxidase,

independent of putrescine oxidase, to partially purified preparations containing diamine oxidase activity.

We have developed a procedure to separate and directly recover the ^{14}C -labelled products of spermidine oxidation and thereby assay spermidine oxidase by liquid-scintillation counting. We have applied this assay and that for putrescine oxidase simultaneously to identical fractions from several protein-separation procedures, using foetal bovine serum. These methods enabled us to ascertain that putrescine oxidase and spermidine oxidase activities are associated with separable protein species.

Experimental and Results

Materials

[1,4- ^{14}C]Putrescine dihydrochloride (sp. radioactivity, 96 Ci/mol) and [^{14}C]spermidine [*N*-(3-aminopropyl)[1,4- ^{14}C]tetramethylene-1,4-diamine trihydrochloride; sp. radioactivity, 10.6 Ci/mol} were obtained from New England Nuclear Corp., Boston, MA, U.S.A. Putrescine dihydrochloride and spermidine trihydrochloride were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., and aminoguanidine bicarbonate was a product of Aldrich Chemical Co., Milwaukee, WI, U.S.A. DEAE-cellulose DE-52 was purchased from Whatman, Clifton, NJ, U.S.A. CH-Sepharose 4B and Sephadex G-200 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Dowex 50W resin (8% cross-linked; 200–400 mesh) was purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A. Foetal bovine serum, purchased as foetal calf serum, was a product of Grand Island Biological Co., Grand Island, NY, U.S.A. Adult bovine serum, a generous gift from the Oscar Mayer Co. (Madison, WI, U.S.A.), was kindly prepared by Dr. Kazuto Kajiwara of this Institute. Adult bovine whole blood was filtered in cheesecloth, centrifuged in a cream separator, and the serum removed and frozen. It was then thawed and filtered in a no. 10 Sefas filter and frozen in small portions for future use. Human serum was obtained from subjects after fully informed consent. An Isocap scintillation counter (Nuclear-Chicago, Des Plaines, IL, U.S.A.) was used in all radioactivity measurements.

Protein determination

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

Putrescine oxidase assay

Putrescine oxidase activity was determined by liquid-scintillation counting as previously described (Gahl & Pitot, 1979). The standard reaction mixture contained enzyme, 1 μg of [^{14}C]putrescine substrate

(0.15 μCi) and buffer (0.07 M-potassium phosphate) to a final volume of 2.0 ml. The final concentration of putrescine was 3.1 μM . The pH was 8.0, which is optimal for the crude foetal bovine serum enzyme (Gahl & Pitot, 1979). Incubation was for 2 h at 37°C. For Figs. 3–5, 1 unit was defined as the amount of enzyme that converts 1 pmol of substrate to product in 1 h under the conditions described. In Tables 4 and 5 enzyme activity is expressed in international units.

Spermidine oxidase assay

The standard reaction mixture for assay of spermidine oxidase contained 8.94 μg of [^{14}C]spermidine (0.16 μCi), 0.07 M-potassium phosphate buffer, pH 8.0, and enzyme (serum) in a total volume of 2.0 ml. The final concentration of spermidine was 17.5 μM . This was incubated for 3 or 4 h at 37°C, the reaction was stopped with 10 ml of 2% (v/v) HClO_4 , and the mixture centrifuged at 12000 rev./min for 15 min in an RC-2 Sorvall centrifuge. The supernatant contained the ^{14}C -labelled substrate and product.

Separation of putrescine from spermidine by cation-exchange chromatography was done by a modification of the procedure of Inoue & Mizutani (1973). A 10 mm $\times$ 40 mm column of Dowex 50 resin was prepared, and a deproteinized sample placed on the column. The cationic polyamines bound tightly to the resin, which was washed with 40 ml of 0.1 M-sodium phosphate buffer, pH 8.0, containing 0.7 M-NaCl, to remove basic amino acids. This was followed by a second wash of 10 ml of 1 M-HCl; 15 ml of 6 M-HCl would elute all polyamines from the column, but this final wash was replaced by either a 1–4 M-HCl gradient or 40 ml of 2.3 M-HCl followed by 50 ml of 6 M-HCl, as described below. This removed all ^{14}C from the columns. Twenty columns were prepared and kept at room temperature in distilled water.

Fig. 1 shows the pattern of elution of [^{14}C]putrescine and [^{14}C]spermidine from the Dowex 50 cation-exchange column described above. A linear gradient of 1–4 M-HCl was used, 10 ml fractions were collected, and samples were counted for radioactivity. When [^{14}C]putrescine was applied separately to the column, label was recovered only in fractions 1–11 and not in the region of elution of spermidine. When [^{14}C]spermidine was applied, label was recovered only in fractions 12–21 and not in the region of elution of putrescine. The elution patterns were identical whether the polyamines were applied together or individually. Of the 2.55×10^6 d.p.m. of [^{14}C]putrescine placed on the columns, 2.37×10^6 d.p.m. was recovered in fractions 1–11 when putrescine alone was applied, and 2.40×10^6 d.p.m. was recovered in fractions 1–11 when both labelled polyamines were loaded.

Similarly, of 1.39×10^6 d.p.m. of [^{14}C]spermidine placed on the columns, 1.27×10^6 d.p.m. was recovered in fractions 12–21 when spermidine alone was applied and 1.23×10^6 d.p.m. was recovered in fractions 12–21 when both labelled polyamines were loaded. Fig. 1 indicates that the more positively charged spermidine is more tightly bound to the resin than putrescine; the overlap in elution fractions between putrescine and spermidine is insignificant.

In Fig. 2 is shown the conversion of [^{14}C]spermidine to [^{14}C]putrescine by 200 μl of foetal bovine serum. After incubation for 4 h at 37°C in the standard reaction mixture, the ^{14}C -labelled polyamines were applied to the Dowex 50 column and separated by the linear HCl gradient described above. A definite rise in radioactivity in the putrescine fractions accompanied the decline in radioactivity in the spermidine fractions in the presence of foetal bovine serum. When no foetal bovine serum was present, all recoverable radioactivity remained in the spermidine peak.

Since the separation of putrescine and spermidine by a linear HCl gradient is a laborious procedure, we determined the optimal molarity of HCl that would remove virtually all the putrescine from the cation-exchange column and leave all the spermidine bound for subsequent elution with a 6 M-HCl wash. To this end, either [^{14}C]putrescine or [^{14}C]spermidine was placed on separate but identical Dowex 50 columns, which were then washed with the sodium phosphate buffer and 1 M-HCl. (Consistently less than 5% of the recovered radioactivity for [^{14}C]putrescine and

[^{14}C]spermidine was eluted in each of these fractions.) Each column was then washed with 1.4, 1.7, 2.0, 2.3, or 2.6 M-HCl, and the radioactivity in each acid fraction determined. HCl at concentrations lower than 2.3 M eluted putrescine poorly; HCl concentrations higher than 2.3 M eluted significant amounts of spermidine; 2.3 M-HCl gave the best removal of putrescine (91.7%) without a decrement in spermidine recovery in the 6 M-HCl wash (96.1%). Results for the 2.3 M-HCl elution are shown in Table 1. The application of both labelled putrescine and spermidine did not alter the recovery of radioactivity in each peak. As 40 ml of 2.3 M-HCl eluted over 99.9% of the [^{14}C]putrescine eluted by 50 ml of the acid (results not shown), in all subsequent assays of spermidine oxidase activity 40 ml of 2.3 M-HCl was used to separate putrescine from spermidine.

Table 2 indicates the amount of [^{14}C]spermidine (8.94 μg , 0.16 μCi) converted to product by 50, 100 and 200 μl of foetal bovine serum in the standard reaction mixture. The total radioactivity in the product fractions of the blank (no foetal bovine serum) was 20.4×10^3 d.p.m. or less than 6% of the initial radioactivity in the substrate. The total radioactivity in the product increased linearly with the amount of foetal bovine serum present, and the radioactivity in the substrate (6 M-HCl) fraction decreased proportionately. The largest component of the product was in the 2.3 M-HCl fraction, which represented putrescine (Fig. 1, Table 1). ^{14}C label in the 1 M-HCl fraction probably represented putrescine that would have been eluted in the 2.3 M-HCl

Fig. 1. Elution of [^{14}C]putrescine and [^{14}C]spermidine from Dowex 50 with a 1000 ml linear gradient (1–4 M) of HCl and four columns

Points are the results of duplicate determinations. [^{14}C]Putrescine (1.7 μg , 1.0 μCi) and [^{14}C]spermidine (12.0 μg , 0.5 μCi) were dissolved in 10 ml of 2% HClO_4 and placed on a Dowex 50 column (40 mm $\times$ 19 mm). The column was washed with 40 ml of 0.1 M-sodium phosphate buffer, pH 8.0, containing 0.7 M-NaCl, and the gradient was applied. Aliquots (500 μl) from each 10 ml fraction were counted, and radioactivity per fraction was determined.

Fig. 2. Conversion of [^{14}C]spermidine to [^{14}C]putrescine by foetal bovine serum

[^{14}C]Spermidine (8.9 μg , 0.16 μCi) was incubated at 37°C for 4 h with (----) or without (—) 200 μl of foetal bovine serum in a reaction mixture brought to 2.0 ml with 0.07 M-potassium phosphate buffer, pH 8.0. The reaction was stopped with 10 ml of 2% HClO_4 , and the mixture was centrifuged. Each supernatant was placed on a Dowex 50 column, washed, and the 1–4 M-HCl gradient applied. Fractions were collected and radioactivity was counted as described in Fig. 1. Results are means of duplicate determinations.

Table 1. *Dowex 50 column separation of putrescine and spermidine by HCl washes*

Either [^{14}C]putrescine (0.54 μg , 0.32 μCi) or [^{14}C]spermidine (3.87 μg , 0.16 μCi) or both, in 10 ml of 2% HClO_4 , were placed in duplicate on Dowex 50 columns, which were then washed with 40 ml of sodium phosphate buffer and 10 ml of 1 M-HCl, as described in the Experimental and Results section. Less than 6% of the [^{14}C]putrescine radioactivity and less than 2% of the [^{14}C]spermidine radioactivity were recovered in these fractions. Each column was then washed with 50 ml of 2.3 M-HCl, followed by 50 ml of 6 M-HCl. Duplicate 500 μl portions of each eluate were counted and radioactivity in each wash was quantified. The percentage of the loaded radioactivity recovered in each wash is given.

	$10^{-3} \times$ Radioactivity loaded on column (d.p.m.)	2.3 M-HCl wash		6 M-HCl wash	
		($10^{-3} \times$ d.p.m.)	(% of loaded radioactivity)	($10^{-3} \times$ d.p.m.)	(% of loaded radioactivity)
[^{14}C]Putrescine	766	702	91.7	14.2	1.9
[^{14}C]Spermidine	2155	18.5	0.9	2070	96.1
Both	2835	685	92.1*	2010	96.1†

* Refers to percentage of loaded radioactivity attributable to [^{14}C]putrescine.

† Refers to percentage of loaded radioactivity attributable to [^{14}C]spermidine.

Table 2. *Recovery of ^{14}C in Dowex 50 eluates after incubation of [^{14}C]spermidine with different volumes of foetal bovine serum*

[^{14}C]Spermidine (8.94 μg ; 0.16 μCi) was incubated for 4 h at 37°C with 0, 50, 100 or 200 μl of foetal bovine serum in the standard reaction mixture. The reaction was stopped with 10 ml of 2% HClO_4 , the mixture centrifuged, and the supernatant placed on a Dowex 50 column. Each column was washed consecutively with 40 ml of sodium phosphate buffer (pH 8.0), 10 ml of 1 M-HCl, 40 ml of 2.3 M-HCl and 50 ml of 6 M-HCl. The substrate was eluted in the 6 M-HCl wash. Duplicate 500 μl portions of each eluate were counted, and total radioactivity recovered for each wash was calculated. Results are means of duplicate determinations. Individual values varied from the mean by less than 5%. The recovery (%) refers to the percentage of the initial [^{14}C]spermidine radioactivity recovered in the combined product (total) and substrate (6 M-HCl). Total refers to total radioactivity in sodium phosphate and 1 M- and 2.3 M-HCl washes.

Foetal bovine serum (μl)	$10^{-3} \times$ Radioactivity in each wash (d.p.m.)					Recovery (%)
	Sodium phosphate	1 M-HCl	2.3 M-HCl	Total	6 M-HCl	
0	14.2	1.5	4.7	20.4	388	100
50	29.7	4.7	34.9	69.3	317	95
100	34.5	8.9	60.4	104	243	85
200	56.8	15.7	101	174	146	78

wash, since it is a fixed percentage (15%) of the ^{14}C content. The radioactivity recovered in the sodium phosphate wash represents label in a breakdown product of putrescine. We have shown that foetal bovine serum contains considerable putrescine oxidase activity at pH 8.0 (Gahl & Pitot, 1979), and that the product of putrescine oxidation will be eluted from the Dowex 50 column in the sodium phosphate wash (W. A. Gahl, A. M. Vale & H. C. Pitot, unpublished work). All of the ^{14}C used in the initial reaction mixture was recovered in either product (sodium phosphate, 1 M-HCl and 2.3 M-HCl washes) or substrate (6 M-HCl wash) when no foetal bovine serum was added to the reaction mixture. The 100% recovery in Table 2 reflects this. However, as the amount of foetal bovine serum increased, the total recovery of ^{14}C decreased. This may have been

due to conversion of putrescine to a volatile product (e.g. $^{14}\text{CO}_2$) that is lost to recovery.

To prevent that loss, we considered adding aminoguanidine to the reaction mixture to inhibit putrescine oxidase but not spermidine oxidase activity. We have reported that foetal bovine serum putrescine oxidase activity is markedly inhibited by aminoguanidine, whose K_i for the enzyme is 0.01 μM . Table 3 shows that aminoguanidine at 0.1–100 μM prevents formation of a product that is washed from the Dowex resin with sodium phosphate, but allows formation of a product (putrescine) recoverable in the 2.3 M-HCl wash; this demonstrates that those concentrations of aminoguanidine employed inhibit putrescine oxidase but not spermidine oxidase, and do not alter the total amount of ^{14}C recovered. Aminoguanidine at 1 mM does inhibit spermidine

oxidase slightly. In the absence of aminoguanidine, some ^{14}C is probably lost as $^{14}\text{CO}_2$.

Since the conversion of spermidine to putrescine is stoichiometric (Holttä, 1977) and since the ^{14}C label in the 1- and 4-positions of the aliphatic backbone remains unaltered, the radioactivity recovered in the product can be converted to nmol by dividing by the sp. radioactivity (d.p.m./nmol) of the substrate [^{14}C]spermidine. One unit of spermidine oxidase activity can then be defined as the amount of enzyme that converts 1 pmol of spermidine to its product(s) in 1 h under the conditions of the assay.

The amount of product formed from spermidine by foetal bovine serum increased linearly as a function of incubation time (results not shown). With 35 nmol of spermidine substrate initially, the reaction remained linear for at least 6 h, with a mean rate of 3.2 nmol of spermidine converted to product/h at pH 8.0 under the described conditions. The performance of this assay procedure under sterile conditions did not alter the results (not shown); this

indicates that foetal bovine serum itself, and not a bacterial contaminant, is responsible for the degradation of spermidine.

Table 4 gives the measured spermidine oxidase activity of bovine sera in units/mg of protein. No aminoguanidine was used, since its effects on spermidine oxidase activity of sera other than foetal bovine serum are not known. A pH of 9.8 was chosen for the adult bovine serum, since that is the optimal pH for putrescine oxidase activity from this serum (Gahl & Pitot, 1979). The optimal pH for foetal bovine serum putrescine oxidase is 8.0 (Gahl & Pitot, 1979). Although foetal bovine serum exhibits considerable spermidine oxidase activity (approx. 8 international units), adult bovine serum has over 5 times that amount (Table 4).

For subsequent assays of spermidine oxidase activity in foetal bovine serum, the reaction mixture included 50 μM -aminoguanidine, since this concentration did not interfere with spermidine oxidase activity but did inhibit putrescine oxidation. In

Table 3. *Effect of aminoguanidine on formation by foetal bovine serum of [^{14}C]spermidine-breakdown products eluted from a Dowex 50 Column*

Foetal bovine serum (200 μl) was incubated at 37°C for 4 h in the presence of 4.3 nmol of [^{14}C]spermidine (0.19 μCi), 0.07 M-potassium phosphate buffer, pH 8.0, to a total volume of 2.0 ml, and the designated concentration of aminoguanidine. Details of the reaction mixture preparation and column elution are described in the text. In the absence of foetal bovine serum the sodium phosphate and 2.3 M-HCl washes together contained less than 16×10^3 d.p.m. and the 6 M-HCl wash contained 398.3×10^3 d.p.m. Recovery (%) is the total radioactivity compared with that recovered in the absence of foetal bovine serum.

[Aminoguanidine] (μM)	$10^{-3} \times {}^{14}\text{C}$ recovered (d.p.m.)				Recovery (%)
	Product minus control (no foetal bovine serum):			Substrate: 6 M-HCl wash	
	Sodium phosphate wash	2.3 M-HCl wash			
0	156	143	37.4	81	
0.1	18.8	321	42.1	92	
1	14.7	325	45.9	93	
10	26.0	335	35.8	96	
100	13.0	341	42.1	95	
1000	6.6	298	90.6	95	

Table 4. *Spermidine oxidase activity of bovine sera*

[^{14}C]Spermidine was incubated at 37°C for 2 h with various sera in the standard reaction mixture described in the Experimental and Results section. ^{14}C in the product is the sum of the counts in the sodium phosphate, 1 M-HCl and 2.3 M-HCl washes minus ^{14}C in comparable washes of blank (no foetal bovine serum). Product formed (nmol) was determined by dividing d.p.m. recovered in the product by the substrate sp. radioactivity, i.e. 12 779 d.p.m./nmol. One international unit of activity is defined as that amount of enzyme required to convert 1 μmol of spermidine to product/min per mg of protein. Results shown are means of duplicate determinations.

Serum	Volume (μl)	pH	$10^{-3} \times ^{14}\text{C}$ in product (d.p.m.)	Product formed (nmol/2 h)	Protein (mg)	$10^{-6} \times$ Activity (international units)
Blank	—	8.0/9.8	—	—	—	—
Foetal bovine serum	200	8.0	109	8.5	9.0	7.9
Adult bovine serum	50	9.8	366	28.6	5.5	43.3

addition, only the 2.3M-HCl wash was counted, since sufficient aminoguanidine was present to prevent conversion to a product eluted by the sodium phosphate wash (Table 3). Under these conditions, six consecutive repetitions of this assay using 50 μ l of foetal bovine serum on several different days gave activities all of which were within 18% of the mean. In view of the cumulative variance associated with different serum batches, Dowex columns, blanks, substrate batches, pipetting, elution and counting procedures, this reproducibility appears acceptable. The assay of eight 50 μ l samples of foetal bovine serum on a single day with an identical serum source, blank and substrate batch gave activities all of which were within 6% of the mean.

Separation of putrescine oxidase and spermidine oxidase in foetal bovine serum

Three different methods were used to distinguish foetal bovine serum putrescine oxidase from spermidine oxidase. The first was the affinity-chromatography procedure of Baylin & Margolis (1975), with the use of cadaverine bound to CH-Sepharose 4B. A flow rate of 200 ml/h was used for a 3 cm $\times$ 7 cm column, on which was placed 140 ml of foetal bovine serum diluted to 700 ml and dialysed against 0.01 M-potassium phosphate, pH 8.0. Since both bovine serum enzymes were eluted from the column by the high-salt washes before the heparin wash used by Baylin & Margolis (1975), a 1-litre linear (0–1 M-NaCl) gradient was employed to elute the enzymes. Fractions of volume 6 ml were collected and portions assayed for putrescine oxidase and

Fig. 3. Elution patterns of putrescine oxidase and spermidine oxidase activities from cadaverine-Sepharose 4B

Diluted dialysed foetal bovine serum (700 ml) was placed on the column, and a linear NaCl (0–1 M) gradient, total volume 1 litre in 0.01 M-potassium phosphate, pH 8.0, was applied; 500 μ l of each 6 ml fraction was assayed for putrescine oxidase (●) and spermidine oxidase (○). Results are shown in units/ml and represent single determinations. There was no activity of either enzyme in fractions 80–160.

spermidine oxidase. Fig. 3 shows that spermidine oxidase was eluted at low salt concentration (less than 0.15 M), although it did bind to the resin. The finding that putrescine oxidase activity was eluted by 0.15–0.40 M-NaCl perhaps reflected a greater affinity of that enzyme for cadaverine. Recoveries for each enzyme approximated to 70%.

The second method for separation of foetal bovine serum putrescine oxidase and spermidine oxidase employed gel-filtration chromatography. Fig. 4 shows the elution pattern of putrescine oxidase and spermidine oxidase from a Sephadex G-200 column on which 5 ml of foetal bovine serum was layered. The column (45 cm $\times$ 3 cm with a flow rate of 30 ml/h) was washed with assay buffer, 0.07 M-potassium phosphate, pH 8.0. Fraction 1 was recovered immediately after the void volume; each fraction was 3.8 ml. Putrescine oxidase activity was eluted immediately after the void volume, with a recovery of over 70%. Spermidine oxidase activity was eluted later, with over 60% recovery.

The third method, ion-exchange chromatography, employed DEAE-cellulose (microangular). A 3 cm $\times$ 12 cm column with flow rate of 200 ml/h was prepared. Fig. 5 shows the elution patterns for each enzyme. Pooled fractions from DEAE-cellulose of each enzyme (total volume 100 ml) from a prior elution were replaced on the column, which had been equilibrated with starting buffer, 0.001 M-potassium phosphate, pH 8.0. A linear gradient, consisting of 400 ml of starting buffer and 400 ml of starting buffer plus 0.3 M-NaCl, was made; 6.5 ml fractions were collected and portions assayed. Putrescine oxidase activity was consistently eluted with 0.10–0.17 M-NaCl and spermidine oxidase activity was

Fig. 4. Elution of putrescine oxidase and spermidine oxidase from Sephadex G-200

After 5 ml of foetal bovine serum was layered on the resin, 60 ml of 0.07 M-potassium phosphate, pH 8.0, was washed through at 30 ml/h. Fractions (3.8 ml) were collected, and 500 μ l portions assayed for putrescine oxidase (●) and spermidine oxidase (○). Results are in units/fraction and represent single determinations.

eluted with 0.15–0.23 M-NaCl. Recoveries were greater than 90% for each enzyme.

The cadaverine–Sephacel procedure yielded a 90-fold purification of putrescine oxidase (Table 5). Fractions from this column were subjected to the DEAE-cellulose separative procedure, giving 20 times greater purity, or a roughly 2000-fold purification. As Table 5 shows, spermidine oxidase was purified 3-fold by the cadaverine–Sephacel column, another 20-fold by DEAE-cellulose, and 2-fold by Sephadex G-200 to a final purification of 130-fold. Yields were low in these preliminary experiments because only the most active fractions were saved. Neither enzyme, as prepared, had activity against the other’s substrate.

Discussion

Spermidine oxidase activity can be measured by the separation of its ¹⁴C-labelled substrate, spermidine, from its ¹⁴C-labelled product, putrescine, by means of a Dowex 50 cation-exchange column and elution of putrescine with 40 ml of 2.3 M-HCl. A blank containing no enzyme must be included, since there is some ¹⁴C-labelled material that will be eluted by the sodium phosphate buffer and the 1 M-HCl washes (Table 2) in the absence of enzyme. When enzyme activity is measured, radioactivity recovered in the sodium phosphate wash must be considered product, since putrescine is further degraded to a product eluted by the sodium phosphate buffer. Although washes with sodium phosphate, 1 M-HCl and 2.3 M-HCl were performed to remove all ¹⁴C-labelled product, they need not be counted for radioactivity individually, but can be pooled and a sample counted. Moreover, when aminoguanidine is added to the reaction mixture to inhibit only putrescine oxidase and not spermidine oxidase, only the 2.3 M-HCl wash need be performed and counted for radioactivity. This circumstance obtained with foetal bovine serum, for which 50 μM-aminoguanidine was sufficient.

The radioassay for spermidine oxidase described above is simple, sensitive (easily detecting less than 1 nmol of product when up to 1 ml of enzyme sample was used) and specific (when [¹⁴C]spermidine was used as a substrate and the label detected in its products). Furthermore, there is 100% recovery of substrate ¹⁴C provided that the label is prevented from being converted from putrescine to its volatile product, CO₂, by an appropriate inhibitor such as aminoguanidine (W. A. Gahl, A. M. Vale & H. C. Pitot, unpublished observation). The procedure allows accurate differentiation of spermidine oxidase activity from putrescine oxidase activity, the assay of which is a well-established procedure (Okuyama & Kobayashi, 1961).

The assay for spermidine oxidase described here must be compared in different systems with that presented by Quash *et al.* (1979). That assay

Fig. 5. Elution of putrescine oxidase and spermidine oxidase from DEAE-cellulose. Partially purified fractions of each enzyme were pooled and placed on the same column. A linear 0–0.3 M-NaCl gradient in 0.001 M-potassium phosphate, pH 8.0, was utilized. Fractions (6.5 ml) were collected and 500 μl portions assayed for putrescine oxidase (●) and spermidine oxidase (○). No enzyme activity was eluted before fraction 30. Results are in units/ml of each fraction and represent single determinations.

Table 5. Purification of foetal bovine serum putrescine oxidase and spermidine oxidase. Spermidine oxidase activity (SO), in the presence of 50 μM-aminoguanidine, and putrescine oxidase activity (PO) were assayed as described in the Experimental and Results section. Only selected fractions from each separative procedure were used for the subsequent procedure.

	Activity (nmol/h)		Protein (mg)		10 ⁶ × Sp. activity (international units)		Purification (fold)		Yield (%)	
	PO	SO	PO	SO	PO	SO	PO	SO	PO	SO
Crude serum	753	2170	7455	7455	1.7	4.9	1	1	100	100
Sephacel-cadaverine	262	860	29	1030	151	13.9	89	3	35	40
DEAE-cellulose	14	670	0.07	40	3330	279	1960	57	2	31
Sephadex G-200	—	150	—	4	—	625	—	128	—	1

assumes that putrescine is stoichiometrically produced from spermidine, a fact verified by quantitative recovery of substrate ^{14}C as putrescine for foetal bovine serum (Fig. 2) but not for other systems. Moreover, as for all coupled assays, that assay demands a great excess of the coupling enzyme (putrescine oxidase) devoid of the test enzyme (spermidine oxidase) for quantitative measurement of the desired activity. In the future, in systems in which acrolein is a known product of spermidine or spermine degradation, measurement of acrolein fluorimetrically (Alarcon, 1968) may provide a sensitivity assay of polyamine-oxidizing activity.

Employing several purification processes and assaying putrescine oxidase and spermidine oxidase activities independently, we determined that spermidine oxidase and putrescine oxidase are not one and the same enzyme. In foetal bovine serum, the two enzyme activities are associated with two distinct proteins, separable by cadaverine-Sepharose (Fig. 3), Sephadex G-200 (Fig. 4) and DEAE-cellulose (Fig. 5) column chromatography.

We have verified that foetal bovine serum not only contains considerable spermidine oxidase activity (Table 4), but that the product of spermidine oxidation by the foetal bovine enzyme is putrescine (Fig. 2). This was previously suggested by Alarcon, who, although able to isolate acrolein as one product of spermidine catabolism by bovine serum (Alarcon, 1964), was unable to detect equivalent amounts of putrescine (Alarcon, 1970). This may have been due to the presence of substantial putrescine oxidase activity in the bovine serum (Gahl & Pitot, 1979). Nevertheless, putrescine appears to be both a precursor and a product of spermidine in foetal bovine serum. Whether the same is true of human pregnancy serum, the putrescine oxidase content of

which is high (Bardsley *et al.*, 1974), awaits application of these techniques to that source.

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