Spermidine oxidase in human pregnancy serum

Probable identity with diamine oxidase

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Diamine oxidase was previously measured in human pregnancy serum with putrescine or histamine as substrate. We have now documented the presence of spermidine oxidase activity in pregnancy serum by means of a specific radioactive assay with [^{14}C]spermidine as substrate and Dowex 50 cation-exchange chromatography to separate products from substrate. The apparent K_m of a partially purified preparation of this enzyme for spermidine was $10.9\ \mu\text{M}$ and the K_i for aminoguanidine was $0.8\ \mu\text{M}$. The pH optimum (pH 9.0) and temperature optimum (55°C) were identical with those for diamine oxidase. Spermidine oxidase activity and diamine oxidase activity eluted in a concerted fashion when pregnancy serum was subjected to cadaverine–Sepharose chromatography, gel filtration and ion-exchange chromatography. Spermidine oxidase became detectable in serum during pregnancy in the human approx. 8 weeks after the last menstrual period and increased with gestational age in concert with the increase in diamine oxidase activity, reaching a plateau at 20 weeks of gestation. Foetal-cord serum displayed virtually no activity of either enzyme. A 400-fold-purified preparation of diamine oxidase retained the same diamine oxidase/spermidine oxidase ratio as exhibited by crude pregnancy serum. These data suggest that in pregnancy serum, unlike foetal bovine serum, spermidine oxidase and diamine oxidase activity may be a single enzyme protein.

Marked increases in maternal serum diamine oxidase activity during human pregnancy have been amply documented (Ahlmark, 1944; Carrington *et al.*, 1972). These investigations have employed histamine or putrescine as substrates, but Bardsley *et al.* (1974), using a high substrate concentration (3 mM), found that pregnancy-plasma diamine oxidase also catalysed the oxidation of spermidine. Recently, spermine oxidase activity was demonstrated in maternal serum as early as 10 weeks of gestation (Illei & Morgan, 1979). We present evidence for a serum spermidine oxidase activity appearing in the first 8 weeks of human pregnancy using a modification of the sensitive radioactive assay for spermidine oxidase previously described (Gahl *et al.*, 1980).

The oxidative deamination of the polyamines spermine and spermidine and of the diamine putrescine by diamine oxidase (EC 1.4.3.6) produces H_2O_2 , ammonia and the aminoaldehyde of the substrate (Tabor *et al.*, 1964). Human pregnancy serum diamine oxidase, which is identical with the amniotic-fluid enzyme (Tufvesson, 1978) and is

produced by placental decidual cells of maternal origin (Swanberg, 1950; Weisburger *et al.*, 1978), also possesses histaminase activity in a single protein (Tryding & Willert, 1968). The enzyme from human placenta (Bardsley *et al.*, 1974; Crabbe *et al.*, 1976), pregnancy plasma (Baylin & Margolis, 1975) and amniotic fluid (Tufvesson, 1978) has been purified and characterized. Although the enzyme's substrate preferences have been examined (Bardsley *et al.*, 1974), the question of whether a single enzyme degrades all three polyamines or separate enzymes each catalyse the oxidation of a single polyamine has not been pursued. In foetal bovine serum, separate enzyme proteins possessing putrescine oxidase activity and spermidine oxidase activity have been shown to exist (Gahl *et al.*, 1980). To determine whether two different enzyme proteins in human serum degrade putrescine and spermidine, we applied protein-separative procedures to pooled pregnancy sera and assayed elution fractions simultaneously for diamine oxidase and spermidine oxidase activity. Kinetic parameters for each enzyme activity were determined on partially purified

preparations of pooled pregnancy serum, and a 400-fold purified preparation of diamine oxidase was tested for the ability to degrade spermidine. We also compared the rises in maternal serum diamine oxidase and spermidine oxidase activities with increasing gestational age.

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]butane-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 hydrogen carbonate was a product of Aldrich Chemical Co., Milwaukee, WI, U.S.A. CH-Sepharose 4B was 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. Pregnancy sera from women of different gestational ages were submitted by physicians for routine testing by the Wisconsin State Laboratory of Hygiene. Pooled or individual specimens were frozen at -20°C , numbered and kindly supplied by Dr. R. Golubjatnikov. Assay of the anonymous specimens was approved by the University of Wisconsin Committee for the Protection of Human Subjects. 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).

Diamine oxidase assay

Diamine oxidase activity was determined by liquid-scintillation counting as previously described (Gahl *et al.*, 1980). The standard reaction mixture contained enzyme, [^{14}C]putrescine ($3.1\text{ }\mu\text{M}$, 24.2 Ci/mol) and buffer (0.07 M-potassium phosphate, pH 9.0) to a final volume of 2.0 ml. Incubation was for 2 h at 37°C . Results are reported in enzyme units where 1 unit is defined as the amount of enzyme that converts 1 pmol of substrate into product in 1 h under the conditions described. In Table 3 enzyme activity is expressed in international units.

Spermidine oxidase assay

Spermidine oxidase activity was measured as described for foetal bovine serum (Gahl *et al.*, 1980), with some modifications. [^{14}C]Spermidine ($17.5\text{ }\mu\text{M}$,

sp. radioactivity 14.3 Ci/mol) was incubated with 0.07 M-potassium phosphate in a total volume of 2 ml for 4 h at 37°C . HClO_4 (2%, v/v; 10 ml) was added and the mixture was centrifuged for 15 min in an RC-2 Sorvall centrifuge. The supernatant was placed on a Dowex 50 cation exchange column (10 mm $\times$ 35 mm) (Inoue & Mizutani, 1973) and washed with 40 ml of 0.1 M-sodium phosphate, pH 8.0, with 0.7 M-NaCl, followed by 40 ml of 2.0 M-HCl and 50 ml of 4.0 M-HCl. Over 95% of the radioactivity in 0.16 μCi of [^{14}C]putrescine was recovered in the phosphate or 2.0 M-HCl, whereas over 98% of the radioactivity in 0.5 μCi of [^{14}C]spermidine was retained by the columns until elution with 4.0 M-HCl. The sum of radioactivity in the phosphate and 2.0 M-HCl washes was considered product, and was converted into pmol by dividing by the specific radioactivity (d.p.m./pmol) of the substrate [^{14}C]spermidine. One unit of spermidine oxidase activity was defined as the amount of enzyme that converted 1 pmol of spermidine into its products in 1 h under the conditions of the assay.

Table 1 shows the radioactivity recovered in each fraction washed from Dowex 50 columns after incubation of the standard reaction mixture with [^{14}C]spermidine in the presence and absence of pooled pregnancy serum. Of the total product formed by the pregnancy-serum enzyme, approx. 11% was recovered in the 2.0 M-HCl wash and represents putrescine; the bulk of the product's radioactivity was eluted by sodium phosphate and represents a breakdown product of putrescine. This probably occurred because spermidine oxidase and diamine oxidase were inseparable and because the enzyme degraded putrescine rapidly, as it was formed from spermidine (see below).

Fig. 1 indicates that the pH optimum of spermidine oxidase, pH 9.0, was identical with that of diamine oxidase in pooled human pregnancy serum. However, at pH 7, diamine oxidase activity was 24 times that of spermidine oxidase, whereas at pH 11, spermidine oxidase activity was 4 times that of diamine oxidase. This discrepancy may have been due to different enzyme conformations at non-optimal pH values favouring one or the other substrate. Incubation at 55°C gave the greatest amount of both spermidine oxidase and diamine oxidase activity in crude pregnancy serum (Fig. 2). Again, the temperature curves for the two activities were not identical, perhaps reflecting temperature-dependent differences in stability and conformation. Both enzymes were routinely assayed at 37°C and pH 9.0. Under these conditions, with 35 nmol of [^{14}C]spermidine substrate (sp. radioactivity 14.3 Ci/mol) in a 2 ml reaction mixture, the spermidine oxidase assay was linear with time and amount of pooled pregnancy serum up to at least 12 nmol of product formed. Linearity was obtained in the same

Table 1. Recovery of ^{14}C in Dowex 50 washes after incubation of [^{14}C]spermidine with pooled human pregnancy serum [^{14}C]Spermidine (8.9 μg , 0.5 μCi) was incubated for 4 h at 37°C with or without pooled pregnancy 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 0.1 M-sodium phosphate, pH 8.0, containing 0.7 M-NaCl, 40 ml of 2 M-HCl and 50 ml of 4 M-HCl. Duplicate 500 μl portions of each eluate were counted for radioactivity, and total radioactivity recovered for each wash was calculated. Controls (no serum) were performed in duplicate. Pregnancy-serum results are means $\pm$ s.d. for eight separate determinations using eight different Dowex 50 columns. Total radioactivity recovered for the serum samples was over 98% of the radioactivity recovered in the absence of serum, which was 100% of the radioactivity initially in the reaction mixture.

Pooled pregnancy serum (μl)	$10^{-3} \times \text{Radioactivity (d.p.m.)}$			
	Product from:		Substrate from	Total
	Sodium phosphate wash	2 M-HCl wash	4 M-HCl washed	
—	40.8	23.6	1281.8	1346.2
400	194.2 $\pm$ 16.6	42.5 $\pm$ 8.3	1087.5 $\pm$ 35.3	1324.2 $\pm$ 30

Fig. 1. pH curve of the activity of human pregnancy-serum diamine oxidase and spermidine oxidase Pooled human pregnancy serum was assayed for diamine oxidase activity (50 μl) and spermidine oxidase activity (250 μl) at different pH values. Results are means of triplicate determinations for diamine oxidase (●) and duplicate determinations for spermidine oxidase (○).

Fig. 2. Activities of human pregnancy-serum diamine oxidase and spermidine oxidase as a function of temperature Diamine oxidase activity in 100 μl of pooled human pregnancy serum was measured by incubating the standard reaction mixture for 1 h at pH 9.0 at different temperatures. Spermidine oxidase activity in 500 μl of pooled human pregnancy serum was also assayed at pH 9.0 for 1 h. Results are means of triplicate determinations for diamine oxidase (●) and duplicate determinations for spermidine oxidase (○).

range of product formed when the assay was performed at 55°C. Subsequent assays were performed within this linear range of spermidine oxidase activity and displayed a day-to-day coefficient of variability of 12.6% ($n = 7$). The coefficient of variability for eight identical pregnancy serum

samples assayed on the same day was 10.8%. This was in part due to small differences in flow rate and height of the cationic-exchange columns. Both spermidine oxidase and diamine oxidase activities

were found to be stable in crude pregnancy serum at -20°C for at least 6 months. A 10-fold purified preparation of each enzyme was stable at 4°C for 3 months.

Results and discussion

Attempts to separate diamine oxidase and spermidine oxidase activities in human pregnancy serum

In an attempt to distinguish diamine oxidase from spermidine oxidase in human pregnancy serum, cadaverine was bound to CH-Sepharose 4B (Baylin & Margolis, 1975) and a column ($3\text{ cm} \times 7\text{ cm}$) was poured. Pooled pregnancy serum (280 ml) was dialysed against 0.05 M-sodium phosphate, pH 7.2, and diluted to 1400 ml with the same buffer. This was placed on the cadaverine-Sepharose column and eluted at a rate of 100 ml/h, and a 500 ml linear (0–0.5 M-NaCl) gradient in 0.05 M-sodium phosphate, pH 7.2, was applied at a rate of 200 ml/h. Portions of sequential 7.2 ml fractions were assayed simultaneously for diamine oxidase and spermidine oxidase activity. Both enzyme activities eluted together (Fig. 3). The flattening of the diamine oxidase peak at fraction 35 represents an artefact due to substrate limitation in the presence of large amounts of enzyme in each 200 μl portion. The low spermidine oxidase activity relative to diamine oxidase activity in peripheral fractions may reflect

Fig. 3. *Elution pattern of diamine oxidase and spermidine oxidase activities from cadaverine-Sepharose 4B* Pooled and dialysed human pregnancy serum (280 ml) was diluted to 1400 ml with 0.05 M-sodium phosphate, pH 7.2, and placed on a column ($3\text{ cm} \times 7\text{ cm}$) over 14 h. A linear NaCl (0–0.5 M) gradient, total volume 500 ml, was applied; 200 μl of each 7.2 ml fraction was assayed for diamine oxidase (●) and 750 μl was assayed for spermidine oxidase (○). Results are means of single determinations on fractions from two different elutions performed on the same column.

the relative insensitivity of the spermidine oxidase assay at very low activity, or salt-related differences in the substrate preferences of the enzyme. The cadaverine-Sepharose column may have acted as an ion-exchange column as well as an affinity column.

Partially purified pregnancy-serum enzyme preparations were layered on a Sephadex G-200 column ($3\text{ cm} \times 40\text{ cm}$) and a Bio-Gel A 1.5 column ($1.6\text{ cm} \times 43\text{ cm}$) and eluted with 0.07 M-potassium phosphate, pH 8.8. Portions were assayed for both diamine oxidase and spermidine oxidase. Neither gel-filtration procedure separated the two enzyme activities. Ion-exchange chromatography with DEAE-cellulose and column chromatography using hydroxyapatite also failed to separate the two enzyme activities. Each separative procedure was performed at least twice.

Pregnancy-serum diamine oxidase was purified 400-fold by sequential cadaverine-Sepharose and hydroxyapatite column chromatography, without regard to spermidine oxidase activity. The partially purified diamine oxidase clearly retained spermidine oxidase activity (Table 2), converting [^{14}C]spermidine into a product that was eluted from Dowex 50 by the sodium phosphate wash. Because of the long incubation time, approx. 5% of the ^{14}C label was not recovered, and may have been converted into $^{14}\text{CO}_2$. The ratio of diamine oxidase activity to spermidine oxidase activity in the partially-purified preparation was 6.7, similar to the diamine oxidase/spermidine oxidase ratio in pooled pregnancy serum (7.0; see Table 3). Further purification with respect to diamine oxidase activity yielded a 500-fold-purified preparation with a diamine oxidase/spermidine oxidase activity ratio of 7.3 (Table 3).

Kinetic parameters of pregnancy serum diamine oxidase and spermidine oxidase

Preparations of pooled pregnancy serum purified 20-fold with respect to diamine oxidase and spermidine oxidase were examined for their kinetic parameters and for inhibition by aminoguanidine, a specific inhibitor of diamine oxidase in contrast with monoamine oxidase (Shore & Cohn, 1960). The apparent K_m for diamine oxidase with respect to diamine was $2.5\text{ }\mu\text{M}$, which compares well with the K_m of $3.4\text{ }\mu\text{M}$ reported by Tufvesson (1978). For spermidine oxidase the apparent K_m with respect to spermidine was $10.9\text{ }\mu\text{M}$. Both enzyme activities were competitively inhibited by aminoguanidine, with K_i $3.0\text{ }\mu\text{M}$ for putrescine as substrate and $0.8\text{ }\mu\text{M}$ for spermidine as substrate. Since any concentration of aminoguanidine that completely inhibited diamine oxidase also completely inhibited spermidine oxidase, aminoguanidine could not be included in the reaction mixture for assay of human pregnancy-serum spermidine oxidase.

Table 2. Recovery of ^{14}C in Dowex 50 washes after incubation of [^{14}C]spermidine with partially purified diamine oxidase from pregnancy serum

Human-pregnancy-serum diamine oxidase was purified 400-fold and $26\mu\text{g}$ was incubated in the standard 2ml reaction mixture containing [^{14}C]spermidine ($35.7\mu\text{g}$, $2.0\mu\text{Ci}$) at 37°C for 24h. Sodium azide (1 mM) was added to prevent bacterial contamination. Controls contained no enzyme. The reaction mixtures were treated as described for Table 1. In the absence of enzyme, 100% of the initial radioactivity was recovered. Results are means of duplicate determinations. The value in parentheses indicated percentage recovery.

Diamine oxidase enzyme protein (μg)	$10^{-3} \times \text{Radioactivity (d.p.m.)}$			
	Product from:		Substrate from 4 M-HCl wash	Total
	Sodium phosphate wash	2 M-HCl wash		
—	209.2	99.0	4679.6	4987.8
26	573.8	118.7	4054.5	4747.0 (95.2)*

* With respect to total radioactivity recovered in the absence of enzyme.

Putrescine oxidase and spermidine oxidase in pregnancy serum at different gestational ages

Maternal serum specimens were divided into groups of eight according to time between the last menstrual period and sampling. Each of six groups spanned 2 weeks of gestation from 7 to 18 weeks; one group included specimens from 19 to 26 weeks, and the final group consisted of specimens obtained 27–36 weeks after the last menstrual period. Mean gestational age was determined within each group of eight serum specimens, each of which was assayed for diamine oxidase and spermidine oxidase activity. Spermidine oxidase was assayed at 55°C , so smaller portions of the limited serum could be used. Fig. 4 shows that both activities appeared approx. 8 weeks after the last menstrual period and increased linearly with gestational age to approx. 20 weeks of gestation, at which time each activity reached a plateau.

Post-partum spermidine oxidase activity in the serum of a single subject decayed with a $t_{1/2}$ of 18.8 h, compared with a $t_{1/2}$ of 18.3 h for diamine oxidase activity in the same woman.

Four cord-blood specimens displayed diamine oxidase activities of 0, 9, 13 and 27 units/ml, compared with a mean term maternal serum activity of over 20 000 units/ml. The same cord-blood specimens revealed no spermidine oxidase activity. Seven specimens of adult non-pregnancy serum (four male, three female, ages 26–70) and four specimens from children aged 5 months to 15 years also displayed no spermidine oxidase activity.

In summary, several lines of evidence suggest that spermidine oxidase activity and diamine oxidase activity are present within a single enzyme protein. First, both activities eluted together from chromatography columns, which clearly separated spermidine oxidase from putrescine oxidase in foetal bovine serum (Gahl *et al.*, 1980). Secondly, spermidine oxidase activity in pregnancy serum rose in

Table 3. Specific activities of diamine oxidase and spermidine oxidase in crude and partially purified human pregnancy serum

Pooled human pregnancy serum (crude serum, 160 ml) was diluted to 800 ml with 0.05 M-sodium phosphate, pH 7.2, placed on a cadaverine-Sepharose column and eluted with a linear 0.05 M-NaCl gradient. Fractions containing diamine oxidase activity were pooled, dialysed, placed on a hydroxyapatite column ($3.5\text{cm} \times 7\text{cm}$) and eluted with a linear gradient of 0.001 M–0.3 M-potassium phosphate, pH 9.0. The hydroxyapatite fraction was the 6 ml fraction representing the peak of diamine oxidase activity. Remaining active fractions were pooled, dialysed, freeze-dried, resuspended in 0.01 M-potassium phosphate, pH 9.0, and placed on a Bio-Gel A1.5 column ($1.3\text{cm} \times 103\text{cm}$) with a flow rate of 2.5 ml/h. Active fractions were pooled and constitute the gel-filtration fraction. Enzyme preparations were assayed in duplicate as described in the text.

Fraction	$10^6 \times \text{Specific activity (international units)}$		
	Diamine oxidase (a)	Spermidine oxidase (b)	(a)/(b)
Crude serum	5	0.7	7.0
Hydroxyapatite	1940	290	6.7
Gel filtration	2550	350	7.3

concert with diamine oxidase activity as gestation progressed and plateaued at 20 weeks of gestation (Fig. 4), as previously reported (Ahlmarm, 1944; Carrington *et al.*, 1972). Thirdly, spermidine oxidase appeared to co-purify with diamine oxidase activity (Table 3). In addition, no preparation of spermidine oxidase allowed the product putrescine to accumulate. Rather, the products of [^{14}C]spermidine degradation appeared largely in the Dowex 50 washes representing the products of putrescine

Fig. 4. *Diamine oxidase and spermidine oxidase activity in human pregnancy serum of different gestational ages* Mean gestational age in weeks since last menstrual period was determined for each group of eight pregnancy-serum specimens. Individual serum samples were assayed for both diamine oxidase (●) and spermidine oxidase activity (○). Diamine oxidase was assayed on 50 μ l of serum at 37°C in triplicate. Spermidine oxidase was assayed on 75 μ l or 100 μ l of serum at 55°C in duplicate. Points represent means and bars represent S.E.M. for eight separate serum specimens. Lines are best least-squares fits for the groups of specimens obtained from 7 to 18 weeks after the last menstrual period.

degradation (Tables 1 and 2). Fourthly, the enzymes exhibited identical pH and temperature optima.

Quash *et al.* (1979) used [14 C]spermidine to measure cellular polyamine oxidase in the presence of excess diamine oxidase. The [14 C]putrescine product of the polyamine oxidase was oxidatively deaminated and cyclized to form 1-[14 C]pyrroline; this was extracted by toluene and counted for radioactivity, as described by Okuyama & Kobayashi (1961). This method may not be appropriate for assay of polyamine oxidase activity in foetal bovine serum, where spermidine oxidase and putrescine oxidase are distinct and separable proteins, since complete degradation of the product putrescine to 1-pyrroline cannot be relied on. However, it may be the preferable method for measuring spermidine oxidase in human pregnancy serum, since all preparations of the enzyme further degrade 85–95% of the putrescine produced even without the addition of exogenous diamine oxidase. The situation in tissues awaits determination of whether separate enzymes degrade each polyamine there, but spermidine oxidase activity in human pregnancy serum may be monitored by the simpler diamine oxidase assay.

It has been suggested that the interaction of polyamines and polyamine oxidase prevents the rejection of the foeto-placental unit, an allograft (Morgan & Illei, 1980). This hypothesis is based in part on studies *in vitro* that showed decreased mouse lymphocyte responsiveness to phytohaemagglu-

tinin, pokeweed mitogen and concanavalin A when spermidine and spermine were present (Byrd *et al.*, 1977). The culture medium contained foetal bovine serum, which is rich in spermidine oxidase. Gaugas & Curzen (1978) demonstrated a similar effect using spermine or putrescine in the presence of human pregnancy serum. Spermidine was not tested, and polyamines had no effect in the presence of foetal-cord serum. The present data indicate not only that spermidine might inhibit lymphocyte responsiveness in the presence of pregnancy serum, but also that appropriate concentrations of amino-guanidine may prevent the inhibition.

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