Spermine Synthesis in the Uterus of the Ovariectomized Rat in Response to Oestradiol-17 β

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We reported that spermidine and spermine pools in the uterus both doubled within 24h after oestradiol administration to castrated rats (Russell & Taylor, 1971). Now we have studied the enzymic synthesis of spermine (by spermidine-dependent *S*-adenosyl-L-methionine decarboxylase) and find that the activity of the enzyme(s) involved is elevated soon after hormone administration. Enzyme activity is increased within 4h and is five times that of controls within 24h. Cycloheximide or actinomycin D administered at the time of oestradiol injection completely blocked the increase in enzyme activity. The enzyme involved in spermine synthesis, *S*-adenosyl-L-methionine decarboxylase, with *S*-adenosyl-L-methionine and spermidine as required substrates, was partially purified on Sephadex and DEAE-cellulose columns. The decarboxylation of *S*-adenosyl-L-methionine could not be separated from the transfer of a propylamine moiety from the decarboxylated *S*-adenosyl-L-methionine to spermidine to form spermine. We were unable also to separate this system from the enzyme that formed spermidine when *S*-adenosyl-L-methionine and putrescine are used as substrates. Spermidine-stimulated *S*-adenosyl-L-methionine decarboxylase has an apparent half-life of 60min, identical with the half-life reported for putrescine-stimulated *S*-adenosyl-L-methionine decarboxylase. These results strongly suggest that the same enzyme(s) operate in the synthesis of both spermidine and spermine.

Of great importance are those early biochemical events which occur in hormone-responsive tissues to allow these tissues to proliferate. Enhancement of polyamine biosynthesis is one of the earliest events that occurs after stimulation of growth (Russell & Snyder, 1968; Jänne & Raina, 1968; Russell, 1970). This makes both the study of the control of the enzymes involved in the biosynthetic process and the study of the actual nature of these enzymes of great importance.

The biosynthetic pathway for spermidine and spermine synthesis in mammalian systems has been studied (Pegg & Williams-Ashman, 1969; Jänne & Williams-Ashman, 1971; Feldman *et al.*, 1971, 1972). For putrescine to be converted into spermidine there is first a decarboxylation of *S*-adenosyl-L-methionine and then the transfer of a propylamine moiety from the decarboxylated *S*-adenosyl-L-methionine to putrescine to form spermidine. The same two processes are involved in spermine formation; that is, for spermine formation, *S*-adenosyl-L-methionine first undergoes a decarboxylation and then a propylamine moiety is transferred from the decarboxylated *S*-adenosyl-L-methionine to spermidine to form spermine. The question at this time is whether these processes are catalysed by one enzyme or two enzymes. There is conflicting information on this question, as certain investigators have indicated that

they can separate these two activities although the system still remains putrescine-dependent (Jänne *et al.*, 1971*a,b*). Other investigators have been unable to separate the decarboxylation of *S*-adenosyl-L-methionine from the propylamine transferase even after purification by nearly 400-fold of the rat liver system, and we believe that this is one enzyme or one system of enzymes that cannot be separated (Feldman *et al.*, 1971, 1972). We were unable to separate the rat prostate system into components, also (Feldman *et al.*, 1972).

Since the same enzyme processes are involved in spermine formation, we wondered whether the same enzyme or enzyme complex operates in spermine synthesis as that found in that of spermidine. We have reported (Feldman *et al.*, 1972) the purification of putrescine-dependent *S*-adenosyl-L-methionine decarboxylase, and have found that if spermidine is substituted for putrescine, spermine formation ensues. The enzyme prefers putrescine as a substrate and the ratio of putrescine/spermidine preference remains constant through purification. However, other workers have studied this enzyme system in the brain and indicate that in late stages of purification two propylamine transferases are involved, one specific for putrescine and the other for spermidine (Raina & Hannonen, 1971).

In the present paper, we report that the enzyme or

enzymes involved in spermine synthesis are subject to activation or enhancement by the hormone oestradiol-17 β , and further that the enzyme or enzymes respond to inhibitors of protein and RNA synthesis exactly as does the spermidine-synthesizing system. Further, the half-life of the enzyme measured after cycloheximide administration is identical with that for the spermidine-synthesizing system. Therefore, there is evidence in both rat liver and rat uterus to support the concept of one complex which functions as both a decarboxylase and a propylamine transferase for the synthesis of both spermidine and spermine.

Experimental

Materials

DL-[1- 14 C]Ornithine (3.31 mCi/mmol), [1,4- 14 C]-putrescine dihydrochloride (11.3 mCi/mmol), [14 C]-spermidine trihydrochloride (10.7 mCi/mmol) and [14 C]spermine tetrahydrochloride (9.9 mCi/mmol) were obtained from New England Nuclear Corp., Boston, Mass., U.S.A. *S*-Adenosyl-L-[carboxyl- 14 C]methionine (47 mCi/mmol) was obtained from Amersham-Searle, Des Plaines, Ill., U.S.A. Oestradiol-17 β , cycloheximide, the hydrochloride salts of putrescine, spermidine and spermine, and *S*-adenosyl-L-methionine iodide were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Actinomycin D was supplied by Merck, Sharp and Dohme Research Laboratories, Rahway, N.J., U.S.A. Pyridoxal phosphate was purchased from Calbiochem, Los Angeles, Calif., U.S.A. NSD-1055 (4-bromo-3-hydroxybenzoyloxyamine dihydrogen phosphate) was a gift from Smith and Nephew Research Ltd., Welwyn Garden City, Herts., U.K.

Methods

Ovariectomized Sprague-Dawley rats (150–200 g) and controls were obtained from Zivic-Miller, Allison Park, Pa., U.S.A. All rats were maintained in a room with a 07:00–19:00 h photoperiod. Rats were

used 2 weeks after the ovariectomy for the following reasons: (1) to ensure recovery from the operation, and (2) to ensure that the hormones normally produced by the ovaries were cleared from the body. To avoid the possibility of cyclic enzymic changes, all animals were killed between 09:00 and 11:00 h.

Oestradiol-17 β was dissolved in a 1% ethanol–0.9% NaCl solution, at a concentration of 10 μ g/0.5 ml of solution. All injections were given intraperitoneally and controls received only the ethanol–NaCl solution without the oestradiol.

Tissues were excised, chilled on ice, weighed and within 10 min homogenized in 5 vol. of cold 0.05 M-sodium-potassium phosphate buffer, pH 7.2, containing 1 mM-dithiothreitol. The crude homogenate was centrifuged at 10000 g for 10 min and the supernatant solution was re-centrifuged at 100000 g for 90 min. The supernatant solution after 100000 g centrifugation was used in the enzymic assays, except in those cases specified, in which partially purified enzyme fractions were used.

Assay for spermidine-activated S-adenosyl-L-methionine decarboxylase activity. Enzyme activity was determined by measuring the liberation of 14 CO $_2$ from *S*-adenosyl-L-[carboxyl- 14 C]methionine as described by Pegg & Williams-Ashman (1969), with minor changes. Incubation mixtures consisted of 0.02 mM-*S*-adenosyl-L-[carboxyl- 14 C]methionine, 25–50 mM-sodium-potassium phosphate buffer, pH 7.2, 50 μ M-pyridoxal phosphate, 5 mM-spermidine, and 0.10–0.15 ml of enzyme supernatant solution (3–6 mg of protein) prepared as described above, to make a total volume of 0.2 ml. When putrescine-dependent *S*-adenosyl-L-methionine decarboxylase was assayed, 2.5 mM-putrescine was added instead of 5 mM-spermidine. Otherwise, the incubation mixtures were the same. The reaction rate was linear in this range of protein concentration and was linear for 30 min. The substrate concentration (0.2 mM as *S*-adenosyl-L-methionine) used as a routine was non-saturating. However, in some experiments, excess of *S*-adenosyl-L-[carboxyl- 14 C]methionine (2 mM) was used as substrate, and the same general changes were observed.

Table 1. Purification of spermidine-stimulated *S*-adenosyl-L-methionine decarboxylase isolated from rat uterus

Details are given in the Experimental section. Proteins were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard; 1 enzyme unit = 1 pmol of 14 CO $_2$ liberated in 30 min in the standard assay. At all stages of purification, this value is identical with that obtained when [14 C]spermidine and unlabelled *S*-adenosyl-L-methionine were used in the assay and the amount of [14 C]spermine formed was determined (see the Experimental section).

Fraction	Volume (ml)	Protein (mg)	Specific activity (units/mg of protein)	Purification	Recovery (%)
100000 g supernatant	7	56.0	103	—	—
Sephadex G-100 filtrate	35	21.4	419	4.1	150
DEAE-cellulose eluate	33	2.8	2000	20.0	95

All values obtained were corrected by comparison against a heated enzyme control. NSD-1055, an inhibitor of pyridoxal phosphate-dependent enzymes, was also used to corroborate the blank values.

Determination of [^{14}C]spermine formation when [^{14}C]spermidine was incubated with tissue extracts. Tissues were excised, immediately chilled, and homogenized in 4 vol. of 0.1M-HCl; 4 vol. of cold 10% (v/v) trichloroacetic acid was added, and then the mixture was centrifuged. The polyamines in the supernatant solution were extracted into alkaline butan-1-ol, separated by high-voltage electrophoresis, and located by drawing a glass rod dipped into a ninhydrin-acetone-acetic acid-cadmium acetate mixture (Heilmann *et al.*, 1957) along the chromatograms and developing the colour in a drying oven for 15 min. The spermine spot was then cut out and eluted into a vial with 5 ml of methanol. After 30 min, 10 ml of toluene phosphor was added and radioactivity was measured. This method is presented in detail elsewhere (Russell *et al.*, 1970).

Purification of the enzyme. The operations described below were done at 0–5°C. The purification at each step of a typical preparation is summarized in Table 1.

Crude extract. Uteri (2g) from Sprague-Dawley rats weighing 100–125g were homogenized with 4 vol. of 0.05M-phosphate buffer, pH 7.2, containing 1.0mM-EDTA, 0.1mM-dithiothreitol, 3 μM -pyridoxal phosphate and 0.5M-sucrose.

Cellular debris was removed from the crude homogenate by centrifugation at 40000g for 20 min. The resulting supernatant solution was re-centrifuged at 100000g (in a Beckman L3-50 ultracentrifuge) for 1 h and then passed through cheesecloth to remove the lipid layer.

Filtration on Sephadex G-100. A 9 ml portion of the 100000g supernatant solution was applied to a Sephadex G-100 column (45 cm $\times$ 4.9 cm²) which had been equilibrated previously with the homogenizing buffer. The enzyme was eluted with the equilibrating buffer under a hydrostatic pressure of 20 cm at a flow rate of 25 ml/h and the eluate was collected in 4.0 ml fractions. The nine most active fractions (20–28) were pooled.

DEAE-cellulose chromatography. The pooled enzyme fraction was dialysed for 1 h against two changes of 50 vol. of 0.01 M-phosphate buffer, pH 7.2, containing 1 mM-EDTA, 0.1 mM-dithiothreitol, 3 μM -pyridoxal phosphate and 0.5M-sucrose. Then 30 ml of the dialysed preparation were applied to a DEAE-cellulose column (25 cm $\times$ 4.9 cm²) which had been equilibrated previously with the same buffer as used in the dialysis. After the column had been washed with 90 ml of the equilibrating buffer, the active enzyme was eluted with 300 ml of a linear gradient of 0–1M-KCl in the equilibrating buffer. Fractions (4 ml) were collected, and seven of the most active

fractions (88–94) were pooled. At this stage enzyme activity was stable on storage at –20°C for more than 6 months.

Results

Time-course of spermidine-stimulated S-adenosyl-L-methionine decarboxylase activity after oestradiol-17 β administration

Oestradiol-17 β was administered intraperitoneally to 150–200 g rats and they were killed at various times thereafter, and a supernatant solution of their uteri was assayed for spermidine-stimulated S-adenosyl-L-methionine decarboxylase activity. Enzyme activity was 140% of that of controls within 4 h, was 400% of controls within 18 h, and within 24 h was over 500% of controls. Thereafter, enzyme activity declined and was well within the control range within 48 h (Fig. 1).

The endogenous concentrations of spermine increased significantly by 4 h after oestradiol injection (see legend of Fig. 1). The size of the spermine pool was doubled within 24 h. These increases are parallel with the detectable increases in enzymic synthesis shown in Fig. 1.

In the crude homogenate (100000g supernatant), there was a stoichiometric relationship between the evolution of $^{14}\text{CO}_2$ from carboxyl-labelled S-adenosyl-L-methionine and the formation of [^{14}C]spermine when [^{14}C]spermidine was used as the

Fig. 1. Time-course of spermidine-stimulated S-adenosyl-L-methionine decarboxylase activity in the uterus of the ovariectomized rat after an intraperitoneal injection of oestradiol-17 β (50 $\mu\text{g/kg}$)

Each point represents the mean $\pm$ S.E.M. for at least five rats. The corresponding spermine concentrations (nmol/g of tissue), as published by Russell & Taylor (1971), were: 0h, 222–235; 4h, 297–351; 24h, 377–440; 48h, 450–490; 72h, 500–560; 98h, 250–290.

substrate. Therefore in this fraction, *S*-adenosyl-L-methionine decarboxylase activity was determined by the evolution of $^{14}\text{CO}_2$ from carboxyl-labelled *S*-adenosyl-L-methionine.

Effects of cycloheximide on spermidine-stimulated and putrescine-stimulated S-adenosyl-L-methionine decarboxylase activity

When cycloheximide was administered to the rats immediately after oestradiol-17 β injection, and the enzyme activities were measured 18h later, the enhancement of both activities was essentially abolished (Table 2). When cycloheximide was

administered 2h after oestradiol administration, not only were the increases in enzyme activities abolished, but the activities were below control values.

The ratio of the preference of the enzyme or enzyme system for putrescine over spermidine remained relatively constant, i.e. 2.1–3.0 for the four systems reported in Table 2.

Effect of actinomycin D on enzyme activities

When actinomycin D was administered simultaneously with oestradiol-17 β and the enzyme activities were measured 18h later, activities were below those in the control system (Table 3). Also acti-

Table 2. *Effects of cycloheximide on spermidine-stimulated and putrescine-stimulated S-adenosyl-L-methionine decarboxylase activities of the uterus of the castrated rat after oestradiol-17 β treatment*

Rats received an intraperitoneal injection of the solvent system only (controls), oestradiol-17 β (50mg/kg), and some received also cycloheximide (50mg/kg) at those times specified in the Table. They were all killed 18h after the administration of solvent only or oestradiol-17 β . Results are means $\pm$ S.E.M. for at least five rats.

Treatment	<i>S</i> -Adenosyl-L-methionine decarboxylase activity (nmol/30min per g wet wt.)	
	Spermidine-stimulated	Putrescine-stimulated
Controls	2.3 $\pm$ 0.24	5.6 $\pm$ 0.83
Oestradiol only	11.9 $\pm$ 2.20	36.3 $\pm$ 5.10
Oestradiol and cycloheximide simultaneously	2.6 $\pm$ 0.48	6.4 $\pm$ 0.78
Oestradiol, then cycloheximide 2h later	1.3 $\pm$ 0.20	2.7 $\pm$ 0.41

Table 3. *Effects of actinomycin D on enzyme activities*

Rats received intraperitoneal injections of the solvent system only (controls), oestradiol-17 β (50mg/kg), and some received also actinomycin D (2.0mg/kg) at various times as indicated in the Table. The rats were all killed 18h after the initial injection of solvent or oestradiol. Results are means $\pm$ S.E.M. for at least five rats.

Treatment	<i>S</i> -Adenosyl-L-methionine decarboxylase activity (nmol/30min per g wet wt.)	
	Spermidine-stimulated	Putrescine-stimulated
Controls	2.5 $\pm$ 0.31	6.3 $\pm$ 0.90
Oestradiol only	11.4 $\pm$ 1.90	35.0 $\pm$ 4.70
Oestradiol, actinomycin D simultaneously	0.8 $\pm$ 0.13	2.6 $\pm$ 0.44
Oestradiol, actinomycin D 1h later	11.8 $\pm$ 2.30	37.1 $\pm$ 5.20
Oestradiol, actinomycin D 2h later	2.8 $\pm$ 0.45	5.6 $\pm$ 0.59
Oestradiol, actinomycin D 4h later	1.2 $\pm$ 0.17	2.7 $\pm$ 0.38
Oestradiol, actinomycin D 16h later	11.6 $\pm$ 1.30	35.8 $\pm$ 4.90

nomycin D, administered 2 and 4h after oestradiol, abolished enzyme responses. However, when actinomycin D was given 1h after oestradiol, activities were identical with those found 18h after injection of oestradiol only. This is rather puzzling, but appears valid, as these experiments were repeated four times with similar results in each case. In one experiment actinomycin D was administered 16h after the initial oestradiol administration, and in this case enzyme activities were the same 18h after oestradiol administration, as were those activities when oestradiol only was administered.

Estimation of the half-life of spermidine-stimulated S-adenosyl-L-methionine decarboxylase in the oestradiol-stimulated uterus

To evaluate the turnover of spermidine-stimulated S-adenosyl-L-methionine decarboxylase in the oestradiol-stimulated rat uterus, cycloheximide was administered at various times before the animals were killed and S-adenosyl-L-methionine decarboxylase was assayed in these tissues. The rat uterus was always assayed at 18h after oestradiol injection. Cycloheximide was administered to groups of five rats each at 15, 30, 45, 60, 75, and 90min before death and assay of uterine spermidine-stimulated S-adenosyl-L-methionine decarboxylase activity. The spermidine-stimulated S-adenosyl-L-methionine decarboxylase activity decreased, with a calculated half-life of about 60min (Fig. 2). This half-life is identical with that calculated for putrescine-stimulated S-adenosyl-L-methionine decarboxylase activity (Russell & Taylor, 1971). These results appear to indicate that the enzyme or enzyme systems involved in spermidine and spermine synthesis are the same.

Purification of spermidine-stimulated S-adenosyl-L-methionine decarboxylase isolated from rat uterus

The results of the procedure employed for purification of spermidine-stimulated S-adenosyl-L-

methionine decarboxylase are shown in Table 1. The final fraction represents a 20-fold purification with 95% recovery of enzyme activity. Sephadex-gel filtration was chosen as the initial step after ultracentrifugation because of the deleterious effects of ammonium sulphate on enzyme activity. Dithiothreitol, EDTA, pyridoxal phosphate, sucrose, and phosphate buffer, pH 7.2, were found to maintain the stability of the enzyme during purification. The DEAE-cellulose eluate was stable at -20°C for 6 months.

The following physical characteristics of the enzyme were determined. The pH optimum for decarboxylation was 7.2 in 0.1M-phosphate buffer,

Fig. 2. Decline of spermidine-stimulated S-adenosyl-L-methionine decarboxylase activity after the administration of cycloheximide

Cycloheximide was injected intraperitoneally to groups of five rats at various intervals before they were killed by cervical fracture. All groups received oestradiol-17β (50 μg/kg) 18h before death. The half-life of spermidine-stimulated S-adenosyl-L-methionine decarboxylase was calculated as 60min.

Table 4. Effect of polyamines on S-adenosyl-L-methionine decarboxylase from rat uterus

The fractions were prepared as described in the Experimental section.

Concentration of polyamines	S-Adenosyl-L-methionine decarboxylase activity (pmol of ¹⁴ CO ₂ /mg of protein)	
	Ultracentrifuged supernatant	DEAE-cellulose fraction
None	22	35
Putrescine (2.5mM)	110	895
Spermidine (5.0mM)	35	449
Spermidine (5.0mM) + putrescine (0.02mM)	58	563

and 7.35 in 0.1 M-tris-HCl buffer. The experimentally determined K_m for *S*-adenosyl-L-methionine was 3.5×10^{-5} M and the K_m for spermidine when the *S*-adenosyl-L-methionine concentration was kept at $0.28 \mu\text{M}$ was 2.1×10^{-3} M. The enzyme preparation after DEAE-cellulose chromatography required pyridoxal phosphate for maximal activity.

The spermidine-dependence of the decarboxylation reaction is demonstrated in Table 4; considerable variation in enzyme activity was observed as a direct result of the variation of spermidine (or putrescine) concentration. The addition of putrescine to reaction mixtures containing saturating concentrations of spermidine did not increase enzyme activity, as previously reported by Raina & Hannonen (1970). At each step of the purification, there remained a constant ratio of the preference of substrate, that is, enzyme activity was twice as great with putrescine as with spermidine as the substrate.

Discussion

It has now been shown that the entire polyamine-biosynthetic pathway is subject to stimulation by growth-promoting hormones. The most dramatic stimulation is obtained with putrescine-stimulated *S*-adenosyl-L-methionine decarboxylase, but the ratio of the preference of this enzyme for either putrescine or spermidine remains constant throughout stimulation. These results strongly suggest that the same enzyme or enzyme complex is operating in the biosynthesis of both spermidine and spermine. Further, we were unable to obtain any separation of the decarboxylation function from the propylamine-transfer function. Previously we reported extensive purification studies on the putrescine-stimulated *S*-adenosyl-L-methionine decarboxylase (purified 372-fold from rat liver) and that there was not a separate decarboxylase and propylamine transferase (Feldman *et al.*, 1971, 1972). With spermidine-stimulated *S*-adenosyl-L-methionine decarboxylase, as reported here, we were unable also to separate these two functions. Even after DEAE-cellulose chromatography, the decarboxylation is completely putrescine- or spermidine-dependent and is always inseparable from propylamine transfer.

Cycloheximide or actinomycin D, inhibitors of protein synthesis and DNA-dependent RNA synthesis respectively, administered simultaneously with oestradiol-17 β , completely inhibited the response of the polyamine-biosynthetic pathway to oestradiol. However, in all cases, they inhibited putrescine-stimulated *S*-adenosyl-L-methionine decarboxylase to the same extent as spermidine-stimulated *S*-adenosyl-L-methionine decarboxylase. Again, this indicates that the same enzyme may be active in the synthesis of both spermidine and spermine.

After cycloheximide treatment, the half-life of decline of spermidine-stimulated *S*-adenosyl-L-methionine decarboxylase activity was about 1 h. This is identical with the half-life previously reported for putrescine-stimulated *S*-adenosyl-L-methionine decarboxylase (Russell & Taylor, 1971). Studies of enzyme turnover rates based solely on the measurement of enzyme activity must be interpreted with caution, since drugs such as cycloheximide may block enzyme degradation as well as enzyme synthesis, and may produce effects unrelated to protein synthesis (Schimke *et al.*, 1968). However, for at least two hepatic enzymes, tyrosine transaminase (Kenney, 1962, 1967; Goldstein *et al.*, 1962) and tryptophan pyrrolase (Schimke, 1964; Schimke *et al.*, 1965, 1968), turnover rates calculated from the decrease in enzyme activity after treatment with inhibitors of protein synthesis appear to be valid measures since they are the same as when determined by changes in amount of isotopically labelled enzymes.

If only one enzyme operates in the synthesis of both spermidine and spermine there must be some rather sophisticated control mechanisms to maintain the ratio of spermidine to spermine. We know that in rat liver after partial hepatectomy spermine concentration actually is decreased (Jänne, 1967; Raina *et al.*, 1966; Russell *et al.*, 1970). The decrease appears to be due to spermine being converted into spermidine. Therefore, the enzyme must change its preference for putrescine and spermidine as possible substrates for the enzyme. At the present time, we have no clue about the mechanisms which operate in the cell to allow the enzyme to discriminate between these two substrates. Since this enzyme pathway is so important in terms of control of growth, new studies need to be initiated to give a better understanding of how a given tissue maintains the optimum concentration of spermidine and spermine for its particular growth requirements. These studies show that activation of spermine synthesis in the rat uterus after oestradiol injection is a much later event than either putrescine or spermidine synthesis. This is due in part to the affinity of the enzyme for putrescine and the high concentration of spermidine necessary to obtain maximal spermine synthesis.

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