The influence of nerve section on the metabolism of polyamines in rat diaphragm muscle

Dominique HOPKINS* and Keith L. MANCHESTER

Department of Biochemistry, University of the Witwatersrand, Johannesburg 2001, South Africa

(Received 17 December 1980/Accepted 19 January 1981)

Concentrations of spermidine, spermine and putrescine have been measured in rat diaphragm muscle after unilateral nerve section. The concentration of putrescine increased approx. 10-fold 2 days after nerve section, that of spermidine about 3-fold by day 3, whereas an increase in the concentration of spermine was only observed after 7–10 days. It was not possible to show enhanced uptake of either exogenous putrescine or spermidine by the isolated tissue during the hypertrophy. Consistent with the accumulation of putrescine, activity of ornithine decarboxylase increased within 1 day of nerve section, was maximally elevated by the second day and then declined. Synthesis of spermidine from [^{14}C]putrescine and either methionine or *S*-adenosylmethionine by diaphragm cytosol rose within 1 day of nerve section, but by day 3 had returned to normal or below normal values. Activity of adenosylmethionine decarboxylase similarly increased within 1 day of nerve section, but by day 3 had declined to below normal values. Activity of methionine adenosyltransferase was elevated throughout the period studied. The concentration of *S*-adenosylmethionine was likewise enhanced during hypertrophy. Administration of methylglyoxal bis(guanyldrazone) produced a marked increase in adenosylmethionine decarboxylase activity and a large increase in putrescine concentration, but did not prevent the rise in spermidine concentration produced by denervation. Possible regulatory mechanisms of polyamine metabolism consistent with the observations are discussed.

A considerable amount of literature indicates that polyamines increase in concentration in rapidly growing cells (for references see Raina & Jänne, 1975; Tabor & Tabor, 1976). Though their specific functions are unknown, the polyamines undoubtedly interact with nucleic acids, and part of the increase in their concentration in growing tissue is likely to be in conjunction with increased synthesis of nucleic acids (for references see Cohen, 1971; Bachrach, 1973). It is uncertain whether changes in polyamine concentrations are causative of the increased synthesis of nucleic acids and protein, or of growth processes, or are merely coincident in time. Increasing evidence, however, suggests that various inhibitors of polyamine biosynthesis cause suppression of macromolecule synthesis and inhibition of cellular growth (Jänne *et al.*, 1978; Mamont *et al.*, 1978; Alhonen-Hongisto *et al.*, 1979; Hölttä *et al.*, 1979).

Diaphragm muscle possesses separate innervation

of its two hemidiaphragms. After nerve section of one hemidiaphragm the denervated tissue undergoes a transient hypertrophy in the following 3–5 days (Sola & Martin, 1953; Feng & Lu, 1965). This hypertrophy is accompanied by a substantial increase in RNA, though questionably of DNA (Manchester & Harris, 1968; Zak *et al.*, 1969). The precise sequence of molecular events that prompts hypertrophy after nerve section is not known. The purpose of the present experiments has been first to investigate whether polyamine concentrations rise during the hypertrophy of the denervated diaphragm and the temporal relationship of such changes to the phases of the hypertrophy, and secondly to consider what factors may influence polyamine concentrations.

Materials and methods

Materials

Radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

* Present address: Department of Physiology, Faculty of Medicine, University of Natal, Durban 4001, South Africa.

Cadaverine dihydrochloride, putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, L-arginine hydrochloride, S-adenosyl-L-methionine iodide and actinomycin D came from Sigma Chemical Co., St Louis, MO, U.S.A. Methylglyoxal bis(guanyldiazide) was from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Cation-exchange resin Dowex 50 (50W; X8; 50–100 mesh) came from Baker J. T. Chemical Co., Phillipsburg, NJ, U.S.A., and cation-exchange cellulose Cellex P (0.94 m-equiv./g; H⁺ form) was from Bio-Rad Laboratories, Richmond, CA, U.S.A.; it could be substituted with a cellulose phosphate cation exchanger (0.95 m-equiv./g; medium mesh) from Sigma Chemical Co. All other materials came from reputable suppliers.

Male and female Sprague–Dawley rats, weighing 80–100 g on slaughter, were used in all experiments. The animals were maintained under regularly alternating periods of light and dark and were allowed food and water *ad libitum*.

Denervation

Left unilateral phrenicectomy was performed under diethyl ether anaesthesia by means of thoracotomy incision. The phrenic nerve was severed after being exposed with a thin glass hook. After saturation the denervated animals were kept for the appropriate number of days until slaughter by decapitation. In sham-operated animals the nerve was exposed but not cut.

Measurement of polyamine concentrations

Preparation of diaphragm homogenate. Hemidiaphragms were dissected out, rinsed in ice-cold 0.9% NaCl, blotted and weighed. The muscle was cut in small pieces and homogenized in ice-cold saline with an Ultra-Turrax homogenizer. Polyamines were extracted by addition of 2 vol. of 0.4 M-HClO₄. The precipitate was removed by centrifugation and the supernatant was neutralized with 1 M-KOH. After removing further precipitate the extract was freeze-dried, then dissolved in a small volume of the buffer used for chromatography.

Chromatographic separation and quantitative analysis. Polyamines were separated by column chromatography. Two different procedures were used. The first method, performed manually and similar to that described by Shull *et al.* (1966) for separation of S-adenosylmethionine, was suitable for complete separation of spermidine and spermine but was not adequate to detect the low concentration of putrescine present in rat tissue. This was possible with an automatic amino-acid analyser, which had considerably greater sensitivity. In this case the procedure was carried out as described by Marton *et al.* (1973).

Measurement of uptake and metabolism of [¹⁴C]putrescine and [¹⁴C]spermidine

For study of the uptake of labelled amines, hemidiaphragms were incubated for various periods in a shaking water bath at 37°C in the presence of the amines. Each hemidiaphragm was incubated in 1 ml of Krebs–Ringer bicarbonate buffer containing ¹⁴C-labelled amine in a vessel gassed with O₂/CO₂ (19:1). After incubation the tissue was removed and blotted, then placed for 10 min in 2 ml of boiling water. Extract (1 ml) and appropriate portions of incubation medium were counted for radioactivity by scintillation counting.

For study of metabolism of the labelled amines, six to eight hemidiaphragms, either control or denervated tissue, were incubated together. The tissue after blotting was homogenized as described above. Chromatographic separation of polyamines was carried out manually and each fraction was counted for radioactivity.

Spermidine formation in muscle extracts

Hemidiaphragms, control and denervated, from 10 to 12 rats were homogenized with an Ultra-Turrax homogenizer in 1 vol. of ice-cold 25 mM-sodium phosphate buffer, pH 7.2, containing 5 mM-dithiothreitol and 0.1 mM-EDTA. The homogenate was centrifuged for 10 min at 5000 g and the supernatant for 1 h at 105 000 g. The resulting supernatant was passed through a column (1 cm × 4 cm) of Sephadex G-25 equilibrated with homogenizing buffer.

For formation of [¹⁴C]spermidine from [¹⁴C]-putrescine and methionine the reaction mixture, based on that of Pegg & Williams-Ashman (1969), in a final volume of 600 μl, consisted of 100 mM-sodium phosphate buffer, pH 7.2, also containing 5 mM-dithiothreitol, 3.3 mM-ATP, 15 mM-MgCl₂, 20 mM-methionine, 0.18 μCi (3 nmol) of [¹⁴C]-putrescine, with or without unlabelled 0.5 mM-putrescine, and 400 μl of muscle supernatant, which contained 3–5 mg of protein.

Incubation was carried out at 37°C for 4 h in conical centrifuge tubes and stopped by the addition of 1 ml of 10% (w/v) trichloroacetic acid containing 0.5 μmol of spermidine. Precipitated protein was removed by centrifugation and the supernatant was applied to a small column (4 cm × 0.2 cm) of Dowex 50 (H⁺ form). Separation and estimation of newly formed spermidine was carried out as described by Pegg & Williams-Ashman (1969).

For formation of [¹⁴C]spermidine from [¹⁴C]-putrescine and S-adenosylmethionine the reaction mixture, based on that of Pegg & Williams-Ashman (1969), in a final volume of 600 μl, consisted of 100 mM-sodium phosphate buffer, pH 7.2, also containing 0.2 mM-S-adenosylmethionine, 2.5 mM-meth-

ionine, 0.18 μ Ci (3 nmol) of [14 C]putrescine, with or without unlabelled 0.5 mM-putrescine, and 400 μ l of muscle supernatant containing 3–5 mg of protein. Separation and estimation of amines was as above.

The incubation time of 4 h was used to increase the measured counts. However, spermidine formation over this period was not strictly linear. Although initial rates are greater than the values quoted, we believe these values fairly represent the comparative rates for different samples.

Assay of ornithine decarboxylase (EC 4.1.1.17). Extracts of diaphragms were prepared as for measurement of spermidine formation. The reaction mixture, based on that of Jänne & Williams-Ashman (1971), contained in a total volume of 300 μ l of 100 mM sodium phosphate buffer, pH 7.2, also containing 0.2 mM-pyridoxal 5-phosphate, 5 mM-dithiothreitol, 0.2 mM-L-ornithine, 0.3 μ Ci (5 nmol) of DL-[1- 14 C]ornithine and 200 μ l of muscle extract containing 2–3 mg of protein. Incubation was for 2 h at 37°C with shaking in tubes equipped with a small glass centre well attached to the rubber cap. The reaction was stopped by injecting 0.5 ml of 50% (w/v) trichloroacetic acid through the cap. A portion (0.1 ml) of Hyamine hydroxide was injected into the centre well to absorb released $^{14}\text{CO}_2$. The tubes were shaken for another 30 min at 37°C to ensure complete absorption of evolved CO_2 into the alkaline solution. The Hyamine hydroxide was removed from the well, which was washed twice with 0.3 ml of methanol. The combined Hyamine hydroxide and methanol was transferred to a scintillation vial and counted for radioactivity. The results were corrected by subtracting the value obtained when no enzyme was present. The method gave a 70–80% recovery of $^{14}\text{CO}_2$ from $\text{NaH}^{14}\text{CO}_3$.

Assay of adenosylmethionine decarboxylase (EC 4.1.1.50). Extracts of diaphragms were prepared as for measurement of spermidine formation. The reaction mixture, based on that of Pegg & Williams-Ashman (1969) and Eloranta *et al.* (1976a), contained in a total volume of 300 μ l of 100 mM-sodium phosphate buffer, pH 7.2, also containing 2.5 mM-putrescine, 2.5 mM-methionine, 0.2 mM-S-adenosylmethionine {62 μ Ci (1 nmol) of S-adenosyl[carboxy- 14 C]methionine} and 200 μ l of muscle extract (2–3 mg of protein). Incubation and measurement of $^{14}\text{CO}_2$ was exactly as described for ornithine decarboxylase.

Assay of methionine adenosyltransferase (EC 2.5.1.6). Extracts of diaphragms were prepared as for measurement of spermidine formation. The assay was performed essentially by the methods of McKenzie & Gholson (1973) and Eloranta *et al.* (1976a). The incubation mixture in a final volume of 300 μ l consisted of 150 mM-Tris/HCl buffer, pH 7.6, containing 150 mM-MgCl₂, 250 mM-KCl, 5 mM-dithiothreitol, 5 mM-ATP, 2 mM-[35 S]methionine (sp.

radioactivity 4.5 Ci/mol) and 200 μ l of tissue extract (1.5–3 mg of protein). Incubation, carried out in conical centrifuge tubes, was for 2 h at 37°C with shaking. The reaction was stopped by heating the samples in boiling water for 2–3 min, which resulted in the precipitation of macromolecules while the pH remained near neutrality, as was required for the subsequent assay.

Separation of newly formed S-adenosylmethionine from methionine was performed by chromatography on P81 ion-exchange paper as described by McKenzie & Gholson (1973). Recovery of S-adenosyl[14 C]methionine was about 80% and that of [35 S]methionine 0.2%.

Assay of S-adenosylmethionine. Hemidiaphragms from eight to ten animals were weighed and homogenized in 2 vol. of ice-cold 0.9% NaCl. They were then mixed with an equal volume of 0.8 M-HClO₄. The precipitate was removed by centrifugation and the supernatant was neutralized by addition of 1 M-KOH. After discarding further precipitate, the supernatant was freeze-dried, dissolved in 1 ml of 10 mM-HCl and any residual precipitate removed by centrifugation. The supernatant was applied to a column (1 cm $\times$ 7 cm) of Cellex-P (H⁺ form) equilibrated with 10 mM-HCl and connected to a u.v. detector and recorder. Thereafter separation and estimation of S-adenosylmethionine was performed as described by Eloranta *et al.* (1976b). The procedure was tested with known amounts of S-adenosylmethionine and the recovery was found to be greater than 80%.

Determination of protein. Protein was measured by the method of Lowry *et al.* (1951) with reference to standard bovine serum albumin solutions ranging between 0 and 100 μ g/ml.

Results

Polyamine concentrations of diaphragm after denervation

The two portions of the normal rat diaphragm are unequal in size, the left hemidiaphragm being smaller and weighing about 20% less than the right half (Sola & Martin, 1953; Manchester & Harris, 1968; Turner & Manchester, 1972). Left unilateral phrenicectomy causes an enlargement of the denervated hemidiaphragm, which by the third to fifth post-operative day increases in mass by about 45%. At this time the denervated portion is appreciably heavier than its innervated contralateral control.

As described in the Materials and methods section polyamines of diaphragm muscle were measured by two different procedures, a manual and an automated method, the latter being more sensitive. The concentrations of putrescine, spermidine and spermine in the control innervated hemidiaphragm averaged (10 observations) 12 ± 5 , 148 ± 30 and

201 ± 20 nmol/g respectively when assayed by the automated procedure, and 116 ± 9 and 102 ± 5 nmol/g (14 observations) for spermidine and spermine when measured manually.

Table 1 shows the changes in putrescine, spermidine and spermine concentrations and contents, measured by the automated procedure, in the left hemidiaphragm at various times after unilateral phrenicectomy. The concentration of putrescine showed a very marked increase in the first 3 days after nerve section, but by 7 days had returned to near normal values. Spermidine concentration likewise rose in the early days after denervation, the greatest percentage increase relative to control, of the time intervals studied, being at 3 days. Spermine, by comparison, increased in concentration much less after denervation and only at about 10 days, thus indicating little correlation with the change in spermidine. Since the denervated tissue gained in weight both absolutely and relative to its paired control, the values for content showed larger proportional increases than for concentration. The values in Table 1 confirm similar observations obtained by the manual method (results not shown).

As a consequence of the delay in any increase in spermine concentration the ratio [spermidine]/[spermine] shows a 2–3-fold increase by the third day after nerve section when the hypertrophy of the denervated hemidiaphragm is most evident, as indicated by the weight ratio (Table 1). Both ratios had returned to their original value by day 10. Polyamine concentrations in the innervated right hemidiaphragm showed no clear trends or changes in the periods after denervation.

Uptake of putrescine and spermidine and synthesis of spermidine by isolated diaphragm

Isolated normal and denervated hemidiaphragms showed no significant difference in their rate of uptake of putrescine or spermidine when incubated for 1 h with radioactively labelled amine. This was taken to indicate that the increase in polyamine concentration of the muscle after nerve section is unlikely to be the result of enhanced uptake of either putrescine or spermidine from the circulation. It was observed, moreover, that a detectable proportion of the radioactivity taken up by the diaphragm after 4 h incubation with [¹⁴C]putrescine was recovered in spermidine and that the amount of newly formed [¹⁴C]spermidine was almost doubled in muscle of rats operated on 3 days previously (results not shown).

Activity of ornithine decarboxylase

A likely reason for the enhanced concentration of putrescine in the denervated hemidiaphragm is increased activity of ornithine decarboxylase. Activity appeared to be enhanced by 1 day after nerve section and remained increased to some extent until day 7 (Table 2). There is thus a good correlation between putrescine concentration and activity of the decarboxylase.

Synthesis of spermidine by diaphragm muscle extract

Synthesis of [¹⁴C]spermidine from [¹⁴C]-putrescine in liver cytosol has been reported by Jänne (1967). Similar experiments with diaphragm

Table 1. *Effect of unilateral phrenicectomy of the left hemidiaphragm on its weight and contents and concentrations of putrescine, spermidine and spermine*

Each value is the mean for two measurements carried out by the automated procedure described in the Materials and methods section. Increase in concentration is the concentration of polyamine in the left denervated hemidiaphragm divided by that of the right innervated muscle of the same animal. Increase in content is the amount of polyamine in the whole left hemidiaphragm divided by the content of the right hemidiaphragm. The value for [spermidine]/[spermine] for day 0 is the average of all the values for innervated tissue.

		Amines in denervated muscle									
		Putrescine			Spermidine			Spermine			
Days elapsed since nerve section	Weight ratio of left/right hemi- diaphragm (%)	Increase in concn. (nmol/g)		Increase in content (%)	Increase in concn. (nmol/g)		Increase in content (%)	Increase in concn. (nmol/g)		Increase in content (%)	[Spermidine]/ [spermine]
		Concn. (nmol/g)	(%)		Concn. (nmol/g)	(%)		Concn. (nmol/g)	(%)		
0	84										0.74
1	98	22	175	221	181	28	50	177	—9	6	1.02
2	100	79	887	1075	241	119	161	179	1	20	1.35
3	111	58	346	490	452	201	298	238	—3	28	1.90
7	108	20	5	35	277	52	96	228	15	48	1.21
10	96	13	8	24	199	28	47	342	81	107	0.58

extract also led to formation of [^{14}C]spermidine from [^{14}C]putrescine and either methionine or *S*-adenosylmethionine. The rate of spermidine synthesis was found to be dependent on the concentration of putrescine present in the assay mixture as might be expected since the diamine is both substrate for spermidine synthase and activator of adenosylmethionine decarboxylase (Williams-Ashman *et al.*, 1972).

Table 2. *Activity of ornithine decarboxylase in denervated diaphragm muscle*

Each value is the mean for three observations except * where $n = 2$. The value of P is indicated when <0.05 . The 'difference' values are means $\pm$ S.E.M.

Days elapsed since nerve section	$^{14}\text{CO}_2$ formation in denervated tissue (pmol/mg of protein per 2 h)	Difference from paired control (pmol/mg of protein per 2 h)	Increase over control (%)
0.125	65	8 ± 12	14
0.5	64	9 ± 4	16
1	343	282 ± 20 $P < 0.01$	462
2	426	369 ± 16 $P < 0.01$	647
3	203	145 ± 16 $P < 0.02$	250
7*	136	75 ± 8 $P < 0.02$	123

Table 3 shows the influence of denervation on the synthesis of spermidine by diaphragm extracts. Although the technical effort involved precludes clear statistical results at all time intervals studied, spermidine formation from putrescine and either methionine or *S*-adenosylmethionine at first increased after nerve section, was most elevated at around day 1 and subsequently declined. Similar changes were observed when spermidine synthesis was assayed with higher concentrations of putrescine (results not shown; higher concentrations led to enhanced spermidine formation, but were not always practical because of excessive dilution of the specific radioactivity of [^{14}C]putrescine). The peak elevation of spermidine synthesis was around 1–2 days after nerve section. Its subsequent decline contrasts with the tissue concentration of spermidine, which appeared to reach a peak about 3 days after denervation (Table 1). Table 3 also shows that spermidine formation returned to near normal values by the third day after nerve section when methionine was added to the assay, whereas it was below normal when *S*-adenosylmethionine replaced methionine. This difference may be explicable in terms of the changes in the activities of adenosylmethionine decarboxylase and of methionine adenosyltransferase.

Activity of adenosylmethionine decarboxylase after denervation

Table 4 shows the change of activity of adenosylmethionine decarboxylase in denervated muscle at

Table 3. *Synthesis of [^{14}C]spermidine from [^{14}C]putrescine and methionine or *S*-adenosylmethionine by extract of denervated diaphragm muscle*

The concentration of putrescine in the assay mixture was $5\mu\text{M}$ and that of other reagents was as stated in the Materials and methods section. Each value is the mean (and, where shown, $\pm$ S.E.M.) of observations shown in parentheses. The value of P is indicated when <0.05 . *Sham-operated control.

Formation of [^{14}C]spermidine

Days elapsed since denervation	With methionine				With <i>S</i> -adenosylmethionine			
	Denervated activity (pmol/mg of protein per 4 h)	Increase (%)	Paired differences (pmol/mg of protein per 4 h)		Denervated activity (pmol/mg of protein per 4 h)	Increase (%)	Paired differences (pmol/mg of protein per 4 h)	
0.125*	(3) 1.39	42	0.41 ± 0.40		(3) 12.4	1	0.1 ± 1.3	
0.125	(3) 1.45	134	0.83 ± 0.20		(3) 13.9	18	2.1 ± 3.7	
0.25	(1) 1.66	86	0.77		(1) 20.0	-3	-0.6	
0.5	(1) 2.26	78	0.99		(1) 29.3	82	13.2	
1	(4) 2.42	130	1.37 ± 0.28 $P < 0.02$		(3) 46.6	84	21.3 ± 1.6 $P < 0.01$	
2	(3) 2.93	97	1.44 ± 0.82		(3) 22.6	17	3.3 ± 1.7	
3	(5) 1.78	39	0.50 ± 0.19 $P < 0.05$		(4) 18.4	-33	-9.0 ± 2.1 $P < 0.05$	
5	(1) 1.75	13	0.020		(1) 12.0	-33	-5.8	
7	(3) 1.44	32	0.35 ± 0.50		(2) 7.9	-37	-4.7 ± 2.4	
10	(2) 1.45	7	0.09 ± 0.01		(2) 14.3	-55	-17.7 ± 6.2	

Table 4. *Activity of adenosylmethionine decarboxylase in denervated diaphragm muscle*

Each value is the mean ($\pm$ S.E.M., where indicated) for the number of observations shown in parentheses. The value of *P* is indicated when <0.05 . *Sham-operated control.

Days elapsed since nerve section		$^{14}\text{CO}_2$ formation		
		Denervated tissue (pmol/mg of protein per 2 h)	Increase (%)	Paired difference (pmol/mg of protein per 2 h)
0.125*	(2)	85	3	2 ± 12
0.125	(2)	76	33	19 ± 9
0.5	(2)	110	62	42 ± 5
1	(4)	396	132	225 ± 62 $P < 0.05$
2	(3)	138	55	49 ± 12 $P < 0.05$
3	(9)	96	-39	-62 ± 11 $P < 0.01$
5	(3)	59	-61	-95 ± 28
7	(4)	50	-64	-91 ± 21 $P < 0.05$
10	(5)	44	-67	-90 ± 10 $P < 0.001$

Table 5. *Activity of methionine adenosyltransferase in denervated diaphragm muscle*

Each value is the mean ($\pm$ S.E.M., where indicated) for the number of observations indicated in parentheses. The value of *P* is indicated when <0.05 .

Days elapsed since nerve section		^{35}S Adenosylmethionine formation		
		Denervated tissue (pmol/mg of protein per 2 h)	Increase (%)	Paired difference (pmol/mg of protein per 2 h)
1	(3)	230	96	113 ± 22 $P < 0.05$
2	(3)	250	54	98 ± 22 $P < 0.05$
3	(4)	230	90	109 ± 28 $P < 0.05$
5	(2)	298	38	82 ± 42
7	(3)	377	86	174 ± 8 $P < 0.01$
10	(3)	329	51	111 ± 8 $P < 0.05$

various periods after unilateral phrenicectomy. Activity was substantially enhanced 1 day after nerve section, possibly earlier. Thereafter it declined, reaching below normal values by day 3. The increased formation of spermidine by extracts of the denervated tissue in the first 2 days after nerve section (Table 3) thus showed a close correlation with the measured activity of adenosylmethionine

Table 6. *Concentration of S-adenosylmethionine in denervated diaphragm muscle*

Each value is the mean ($\pm$ S.E.M., where indicated) for the number of observations indicated in parentheses. The value of *P* is indicated when <0.05 .

Days elapsed since nerve section		S-Adenosylmethionine concentration		
		Denervated tissue (nmol/g wet wt.)	Increase (%)	Paired difference (nmol/g wet wt.)
0.5	(2)	25.2	5	1.3 ± 1.9
1	(3)	27.4	30	6.3 ± 0.8 $P < 0.02$
2	(3)	26.9	29	6.1 ± 2.1
3	(3)	24.3	23	4.6 ± 0.9 $P < 0.05$
5	(2)	24.6	32	6.0 ± 4.3
7	(2)	23.9	37	6.5 ± 2.6
10	(2)	20.9	21	3.6 ± 1.0

decarboxylase. Moreover, the decline to subnormal values in decarboxylase activity was reflected in subnormal rates of spermidine synthesis from putrescine and S-adenosylmethionine.

Activity of methionine adenosyltransferase and concentration of S-adenosylmethionine

Activity of methionine adenosyltransferase was increased at all time intervals studied after nerve section (Table 5). Thus synthesis of spermidine, when methionine as opposed to S-adenosylmethionine was added, would reflect combined changes in the activities of both methionine adenosyltransferase and the decarboxylase, the rise in the former counteracting the fall of the latter and thus explaining the difference in the results of Table 3 from day 3 after denervation.

In view of the increase in RNA synthesis after nerve section (Manchester & Harris, 1968), there is likely to be an increased requirement for S-adenosylmethionine for methylations. Notwithstanding this demand the tissue concentration of S-adenosylmethionine showed no decline after nerve section; rather it tended to be enhanced (Table 6).

Factors influencing adenosylmethionine decarboxylase activity

Like the enzyme studied in other tissues (Williams-Ashman *et al.*, 1972; Morris & Fillingame, 1974) adenosylmethionine decarboxylase from diaphragm extract was found to be sensitive to putrescine activation; 2.5 mM-putrescine resulted in an approx. 3-fold stimulation of decarboxylase activity.

Injection of actinomycin D at the time of nerve section abolished the increases in S-adenosylmethionine decarboxylation and spermidine synthesis

Table 7. Effect of administration of methylglyoxal bis-(guanyldihydrazone) (MGBG) on the concentration of putrescine in innervated and denervated hemidiaphragms. Each value is the mean for duplicate experiments.

Days elapsed since denervation	Putrescine concn. (nmol/g wet wt.)			
	Innervated		Denervated	
	Control	MGBG	Control	MGBG
1	8	71	22	188
2	8	37	79	415
3	13	16	58	39

that occur in response to denervation, thereby suggesting that enhanced decarboxylase activity might be the consequence of synthesis *de novo* of enzyme protein.

Adenosylmethionine decarboxylase from normal and denervated muscles was equally sensitive to inhibition by methylglyoxal bis(guanyldihydrazone) *in vitro*, 2 μ M resulting in an approx. 75% decrease in activity. Conversely, in animals treated with methylglyoxal bis(guanyldihydrazone) (8 mg/100 g body wt.) 2 days previously, there was a 10-fold increase in adenosylmethionine decarboxylase activity, consistent with the view that methylglyoxal bis(guanyldihydrazone) markedly decreases the rate of adenosylmethionine decarboxylase degradation as observed in other tissues (Pegg *et al.*, 1973; Hölttä *et al.*, 1973). Methylglyoxal bis(guanyldihydrazone) treatment caused no obvious changes in the concentrations of spermidine and spermine in either normal or denervated hemidiaphragms and it did not prevent the accumulation of spermidine associated with denervation (results not shown). Methylglyoxal bis(guanyldihydrazone) treatment, however, produced a marked rise in the concentration of putrescine after 24 h (Table 7) as already noted by Hölttä *et al.* (1973) in rat thymus. Although in proportional terms the increase was similar in both normal and denervated muscles, as a consequence the concentration of putrescine in the denervated tissue rose at 2 days to 40–50 times that found in normal diaphragm of untreated rats.

Discussion

The results presented show that the denervation-induced hypertrophy of the rat diaphragm is associated with an accumulation of polyamines and a stimulation of their synthesis. As in other rapidly growing systems (for references see Raina & Jänne, 1975), a temporal correlation is observed between increase in RNA (Manchester & Harris, 1968) and

spermidine concentration, and an enhanced [spermidine]/[spermine] ratio is measured during the growth period of the denervated muscle.

Ornithine decarboxylase, adenosylmethionine decarboxylase and methionine adenosyltransferase each catalyse effectively irreversible reactions that are unlikely to be at near equilibrium. The rates of reaction will thus be responsive to the amounts of enzyme protein present. Since ornithine, methionine and *S*-adenosylmethionine are all present in relatively low concentrations in muscle, it is likely that the rates of formation of putrescine and *S*-adenosylmethionine and its decarboxylated form in muscle will all also respond in varying degrees to changing concentrations of their respective substrates. In the denervated hemidiaphragm the observed rises in the concentration of methionine (Turner & Manchester, 1973), of putrescine (Table 1), which is a substrate for spermidine synthesis as well as an activator of adenosylmethionine decarboxylase, and of *S*-adenosylmethionine (Table 6), may each contribute towards the stimulation of polyamine production.

The concentration of spermidine in the denervated muscle by day 3 after denervation is enhanced by about 300 nmol/g of tissue. If no further metabolism of spermidine were taking place, there needs to be an increase in the rate of spermidine synthesis from the moment of nerve section of about 4 nmol/g of tissue per h to bring about the observed increase in concentration. Since the activity of adenosylmethionine decarboxylase declines markedly after the first post-operative day and reaches subnormal values when the accumulation of spermidine is maximal, the transient rise in enzyme activity appears insufficient to account for the observed increase in spermidine concentration. It is possible that our measured activities are much below tissue activities because, for example, of inadequate extraction. Our activities are not, however, grossly dissimilar to values reported by other workers (Raina *et al.*, 1976; Kremzner *et al.*, 1978). Examination of the published activities of enzymes involved in polyamine synthesis in cardiac hypertrophy (Feldman & Russell, 1972), regeneration after partial hepatectomy (Russell & Lombardini, 1971; Hölttä & Jänne, 1972) and oestrogen stimulation (Eloranta *et al.*, 1976a) suggests that these values appear equally inadequate to explain observed increases in spermidine content. From available data it is not possible to conclude whether there are other sources of polyamines. Kano & Oka (1976) have suggested that uptake of exogenous polyamines is important. We failed to observe increase in uptake of labelled putrescine or spermidine by the denervated diaphragm and it is unclear whether enhanced uptake of polyamines could explain the discrepancy between enzyme

activity measurements *in vitro* and enhanced polyamine concentrations in all the cases cited above.

The exact role of polyamines in cells is as yet unknown. It is probable that these strongly cationic molecules are largely associated with the negatively charged nucleic acids but in equilibrium with free species. Red muscle contains higher concentrations of RNA (Goldberg, 1967) and polyamines (Kremzner *et al.*, 1978) than white. A hypertrophy in the denervated diaphragm largely confined to the red fibres (Feng & Lu, 1965) would be consistent with the increase observed in both RNA and spermidine, the latter being possibly a corollary of the former. As a working hypothesis to explain our results in terms of the changes in spermidine concentration and enzyme activities we suggest that an increase in nucleic acid synthesis leads to an increased binding of intracellular polyamines, particularly spermidine. This binding leads to a decline in the amount of free polyamines. We have shown elsewhere that exogenous spermidine appears to decrease the activity of adenosylmethionine decarboxylase in muscle (Hopkins & Manchester, 1980). This binding of polyamines, by lowering the pool of free molecules, may lead to derepression of enzyme synthesis and enhance further polyamine formation until the concentrations of free polyamine are restored, when repression re-occurs.

We thank the South African Council for Scientific and Industrial Research, the Atomic Energy Board and the Muscular Dystrophy Associations of America for support for this work.

References

- Alhonen-Hongisto, L., Pösö, H. & Jänne, J. (1979) *Biochim. Biophys. Acta* **564**, 473–487
- Bachrach, U. (1973) *Function of Naturally Occurring Polyamines*, Academic Press, New York
- Cohen, S. S. (1971) *Introduction to the Polyamines*, Prentice-Hall, Englewood Cliffs, New Jersey
- Eloranta, T. O., Mäenpää, P. H. & Raina, A. M. (1976a) *Biochem. J.* **154**, 95–103
- Eloranta, T. O., Kajander, E. O. & Raina, A. M. (1976b) *Biochem. J.* **160**, 287–294
- Feldman, M. J. & Russell, D. H. (1972) *Am. J. Physiol.* **222**, 1199–1203
- Feng, T. P. & Lu, D.-X. (1965) *Sci. Sin.* **14**, 1772–1784
- Goldberg, A. L. (1967) *Nature (London)* **216**, 1219–1220
- Hölttä, E. & Jänne, J. (1972) *FEBS Lett.* **23**, 117–121
- Hölttä, E., Hannonen, P., Pispä, J. & Jänne, J. (1973) *Biochem. J.* **136**, 669–676
- Hölttä, E., Jänne, J. & Hovi, T. (1979) *Biochem. J.* **178**, 109–117
- Hopkins, D. & Manchester, K. L. (1980) *FEBS Lett.* **109**, 299–302
- Jänne, J. (1967) *Acta Physiol. Scand. Suppl.* **300**, 1–71
- Jänne, J. & Williams-Ashman, H. G. (1971) *J. Biol. Chem.* **246**, 1725–1732
- Jänne, J., Pösö, H. & Raina, A. (1978) *Biochim. Biophys. Acta* **473**, 241–293
- Kano, K. & Oka, T. (1976) *J. Biol. Chem.* **251**, 2795–2800
- Kremzner, L. T., Tennyson, V. M. & Miranda, A. F. (1978) *Adv. Polyamines Res.* **2**, 241–256
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mamont, S. P., Duchesne, M. C., Grove, J. & Bey, P. (1978) *Biochem. Biophys. Res. Commun.* **81**, 58–66
- Manchester, K. L. & Harris, E. J. (1968) *Biochem. J.* **108**, 177–183
- Marton, L. J., Vaughn, J. G., Hawk, I. A., Levy, C. C. & Russell, D. H. (1973) in *Polyamines in Normal and Neoplastic Growth* (Russell, D. H., ed.), pp. 367–380, Raven Press, New York
- McKenzie, R. M. & Gholson, R. K. (1973) *Anal. Biochem.* **53**, 384–391
- Morris, D. R. & Fillingame, R. H. (1974) *Annu. Rev. Biochem.* **43**, 310–325
- Pegg, A. E. & Williams-Ashman, H. G. (1969) *J. Biol. Chem.* **244**, 682–693
- Pegg, A. E., Corti, A. & Williams-Ashman, H. G. (1973) *Biochem. Biophys. Res. Commun.* **52**, 696–701
- Raina, A. & Jänne, J. (1975) *Med. Biol.* **53**, 121–147
- Raina, A., Pajula, R. L. & Eloranta, T. (1976) *FEBS Lett.* **67**, 252–255
- Russell, D. H. & Lombardini, J. B. (1971) *Biochim. Biophys. Acta* **240**, 273–286
- Shull, K. H., McConomy, J., Vogt, A., Castillo, A. & Farber, E. (1966) *J. Biol. Chem.* **241**, 5060–5070
- Sola, O. M. & Martin, A. W. (1953) *Am. J. Physiol.* **172**, 324–332
- Tabor, C. W. & Tabor, H. (1976) *Annu. Rev. Biochem.* **45**, 285–306
- Turner, L. V. & Manchester, K. L. (1972) *Biochem. J.* **128**, 789–801
- Turner, L. V. & Manchester, K. L. (1973) *Biochim. Biophys. Acta* **320**, 352–356
- Williams-Ashman, H. G., Jänne, J., Coppoc, G. L., Geroch, M. E. & Schenone, A. (1972) *Adv. Enzyme Regul.* **10**, 225–245
- Zak, R., Grove, D. & Rabinowitz, M. (1969) *Am. J. Physiol.* **216**, 647–654