Effect of Denervation on the Synthesis of Ribonucleic Acid and Deoxyribonucleic Acid in Rat Diaphragm Muscle

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1. The effect of unilateral denervation of rat diaphragm muscle on its content of nucleic acids and their incorporation of precursors was investigated. 2. After denervation the paralysed hemidiaphragm hypertrophies and within 3 days its content of RNA increases considerably. The concentration of DNA/unit mass remains fairly constant. 3. During this period there is some increase in the rate of incorporation of [^{14}C]adenine into RNA, whereas there is some diminution in the rate of incorporation of [^{14}C]orotic acid. 4. Incorporation of [^{14}C]adenine and [^3H]thymidine into DNA is much increased in the paralysed tissue, reaching its maximum by about the third day, but returning to normal by the tenth. 5. The significance of these results in relation to the hypertrophy after denervation is discussed.

Unilateral section of the phrenic nerve in several species causes a transitory hypertrophy of the denervated hemidiaphragm, which is later followed by atrophy (Sola & Martin, 1953; Stewart, 1955; Stewart & Martin, 1956). The hypertrophic response begins soon after the nerve section and by the third day the paralysed hemidiaphragm is considerably enlarged in comparison with its innervated control (Sola & Martin, 1953; Buse, McMaster & Buse, 1965). Analysis of the tissue indicates that the relative proportions of soluble, myofibrillar and connective-tissue proteins do not significantly change during the hypertrophy (Stewart, 1955), and the increase in weight is not explicable as imbibition of water. During the hypertrophy there is thus considerable synthesis of new muscle protein and, as is to be expected, an elevation of incorporation of amino acids into protein can be observed during the first few days after the operation (Buse *et al.* 1965; Harris & Manchester, 1966). The hypertrophy and enhancement of amino acid incorporation is not prevented by adrenalectomy or hypophysectomy before nerve section, though the response is possibly more sluggish in the latter case (K. L. Manchester & E. J. Harris, unpublished work). After 8–10 days the rate of incorporation of the paralysed tissue returns to that of the control hemidiaphragm, though the latter at this stage may be undergoing compensating work hypertrophy.

Since protein synthesis is controlled by nucleic acid metabolism we decided to investigate the

concentrations and rates of synthesis of RNA and DNA in denervated muscle.

MATERIALS AND METHODS

Diaphragm muscle was taken from non-starved albino rats (about 100 g. body wt.) and incubated as described by Manchester (1961) for 2 hr. at 37° in the presence of the labelled nucleotide. No glucose or oxidizable substrate was added to the medium (Krebs–Ringer bicarbonate) and all solutions were gassed with $\text{O}_2 + \text{CO}_2$ (95:5) before incubation, the incubation vessels being sealed during incubation. At the end of the incubation period the tissue, which had been weighed before incubation, was removed from the incubation vessel, blotted to remove adhering fluid and placed in 3 ml. of ice-cold 1 N- HClO_4 .

Left unilateral phrenicotomy was carried out the stated number of days before removal of the diaphragm. The operation was performed under ether anaesthesia, the phrenic nerve being exposed through a thoracotomy. As normally dissected the left lateral hemidiaphragm tends to be smaller than the right. By 2 days after phrenicotomy the weights were similar and by the third day the denervated hemidiaphragm was invariably heavier than its control (Table 1). From the second day the denervated tissue was visibly paler and had a turgid glistening appearance not normally associated with this tissue.

Radioactive nucleotide precursors came from The Radiochemical Centre, Amersham, Bucks. They were used undiluted in the quantities stated in the Tables, and had specific radioactivities: [$8\text{-}^{14}\text{C}$]adenine, 31.5 mc/m-mole; [$2\text{-}^{14}\text{C}$]uracil, 61.0 mc/m-mole; [$6\text{-}^{14}\text{C}$]orotic acid, 44.5 mc/m-mole; [$2\text{-}^{14}\text{C}$]thymine, 40.2 mc/m-mole; [$\text{Me-}^3\text{H}$]thymidine, 2000 mc/m-mole, the last-named being used within a few weeks of purchase (Wand, Zeuthen & Evans, 1967).

Table 1. *Change in weight of the left hemidiaphragm after unilateral denervation*

The left hemidiaphragm, both anatomically and as normally dissected, weighs less than the right. Thus the denervated left hemidiaphragm, despite hypertrophy, remains smaller than the right-hand portion until about the third day. Values are derived from the experiments of Table 2 and from unoperated animals used for other purposes.

Time (days) elapsed since phrenicotomy	$\left(\frac{\text{Wt. of left hemidiaphragm}}{\text{Wt. of right hemidiaphragm}} \right) \times 100$	Increase after denervation (%)
0	81	—
1	91	12
2	96	19
3	111	37
10	106	31

Determinations. The RNA content of the tissue was measured by a modification of the alkali-extraction procedure of Fleck & Munro (1962). The tissue (80–120 mg.) was broken up in a mortar in 3 ml. of 1 N-HClO₄ at 4° and then transferred to glass homogenizing tubes with a motor-driven glass pestle and finely ground. The insoluble material was collected by centrifugation and washed four times by resuspension in 3 ml. of 1 N-HClO₄. These operations were carried out as near 0° as possible and served to remove soluble and non-specifically adhering radioactivity, as the experiments with thymine (see below) showed.

To extract RNA from the residue, it was suspended in 3 ml. of 0.5 N-NaOH and left at 37° for 1 hr., after which dissolved protein was reprecipitated by the addition of 2 ml. of cold 1 N-HClO₄. The precipitate was collected by centrifugation and further extracted for DNA. The RNA in the supernatant was determined from its extinction at 260 m μ and 275 m μ by the equation:

$$\text{RNA P } (\mu\text{g./ml.}) = 11.87E_{260} - 10.40E_{275}$$

Additionally the nucleotide content of the supernatant was estimated colorimetrically with orcinol (Umbreit, Burris & Stauffer, 1957). Only the values from the two-wavelength method are quoted. On the basis of RNA containing 10% of phosphorus, the orcinol values agreed closely (to within a few per cent) with those derived from the $E_{260} - E_{275}$ values and they invariably showed the same qualitative changes.

DNA was extracted from the precipitate from the above procedure. The precipitate was first washed in 3 ml. of cold 1 N-HClO₄ and then extracted for 20 min. at 90° with 3 ml. of 1 N-HClO₄. The DNA content of the supernatant was estimated from its extinction at 268 m μ and 284 m μ by the method of Tsanev & Markov (1960), by the equation:

$$\text{DNA P } (\mu\text{g./ml.}) = 8(E_{268} - E_{284})$$

and these are the values quoted.

The DNA content of the supernatant was also estimated colorimetrically with diphenylamine by the method of Burton (1956). The values obtained with diphenylamine were on average only about 75% of those from the two-wavelength method, but the divergence varied in different experiments for unknown reasons. The two determinations gave the same results for the effects of denervation.

Radioactive determinations. Samples of the supernatant containing RNA and DNA were counted in a liquid-scintillation counting system of 1 ml. of extract plus 10 ml. of scintillator [0.4% 2,5-bis-(5-*tert.*-butylbenzoxazol-

2-yl)thiophen in toluene-Triton X-100 (2:1, v/v)]. This scintillation system has great resistance to quenching (Patterson & Greene, 1965) and counts ¹⁴C in HClO₄ with 60–70% efficiency and ³H with 15–20% efficiency with the Beckman and Nuclear-Chicago equipment used.

That washing with cold HClO₄ extracted unincorporated radioactivity was clear from the observation that there was no incorporation of label from [¹⁴C]thymine into the RNA or DNA fractions despite its penetration into the tissue. Some ¹⁴C from adenine and orotic acid was found in the DNA fraction (Table 4). A small portion of the [³H]thymidine (3–5% of that in the DNA fraction) was extracted by alkali.

The residue remaining after hot acid extraction contained negligible radioactivity.

RESULTS

Effect of denervation on RNA content. The concentrations of RNA found in normal diaphragm muscle were between 130 and 180 $\mu\text{g.}$ of RNA phosphorus/g. of tissue (Table 2). Similar values have been reported previously (Margreth & Novello, 1964; Manchester, 1967). The high results from the extinction values corresponded to high results from orcinol determinations, but whether the variation from one experiment to another was methodological or was indicative of variability in the tissue content is not known. The RNA content of diaphragm denervated for 1 day showed a slight increase by comparison with its control (Table 2). After 2 days the increase in RNA content was appreciable and by the third day it was substantial. Table 2 shows the RNA content/g. of tissue; not only had there been an increase in the RNA content of the hemidiaphragm because of the hypertrophy, but the actual concentration of RNA had also risen about 50%. Thus after 3 days, when the weight of the denervated tissue had increased by 30–40% (Table 1), the total RNA content had about doubled. After 10 days the RNA content was still elevated (Table 2).

By contrast, the concentration of DNA did not change markedly (Table 2), though again the fact

Table 2. *Nucleic acid content of rat diaphragm muscle in the period after denervation*

Each value is the mean $\pm$ S.E.M. with the number of observations in parentheses, and each represents analysis of a single hemidiaphragm. The columns headed 'Difference' show the change of the denervated hemidiaphragm related to its paired innervated control hemidiaphragm. Thus *P* values, quoted when <0.05 , are determinable both on the differences alone and on the differences between the two groups.

Time (days) elapsed since phrenicotomy	Nucleic acid content (μ g. of nucleic acid P/g. wet wt. of tissue)							
	RNA				DNA			
	Paralysed hemidiaphragm	Innervated control	Difference	Increase (%)	Paralysed hemidiaphragm	Innervated control	Difference	Increase or decrease (%)
1	179 $\pm$ 2.5 (24) <i>P</i> = 0.001	168 $\pm$ 1.6	11.4 $\pm$ 2.8 <i>P</i> < 0.001	7.1	170 $\pm$ 1.2 (6)	165 $\pm$ 2.3	4.8 $\pm$ 1.8 <i>P</i> < 0.05	3.0
2	196 $\pm$ 3.7 (18) <i>P</i> < 0.001	159 $\pm$ 2.8	37.4 $\pm$ 3.2 <i>P</i> < 0.001	23	145 $\pm$ 6.9 (6)	135 $\pm$ 3.8	9.7 $\pm$ 4.9	6.6
3	239 $\pm$ 2.8 (18) <i>P</i> < 0.001	155 $\pm$ 2.8	84 $\pm$ 3.6 <i>P</i> < 0.001	54	154 $\pm$ 2.5 (12)	150 $\pm$ 2.6	3.6 $\pm$ 4.5	2.7
10	200 $\pm$ 2.6 (12) <i>P</i> < 0.001	163 $\pm$ 2.6	37 $\pm$ 2.7 <i>P</i> < 0.001	23	128 $\pm$ 2.4 (12)	132 $\pm$ 2.6	3.5 $\pm$ 1.8	-2.6

that the concentration remained similar despite hypertrophy indicates that a net synthesis of DNA was taking place. There was a hint of an increase in concentration per unit of tissue in the early stages of denervation. The concentrations of DNA in the innervated tissue noted in Table 2 showed more variations than those for RNA. This reflects partly a smaller number of samples analysed for RNA than for DNA, but also the fact that the concentration of DNA in diaphragm muscle (per g. of wet tissue) declines markedly with increasing age (K. L. Manchester, unpublished work). Some variation of size of animal was unavoidable, particularly in the long-term experiments.

Incorporation of precursors into RNA. An increase in RNA synthesis would be expected to be reflected in an enhanced incorporation of labelled precursor. This expectation was only partly realized. Thus when incorporation of ^{14}C from adenine, uracil or orotic acid was measured 24 hr. after section of the phrenic nerve, i.e. before the nucleic acid content was measurably changed, the incorporation of label from adenine and uracil was enhanced in the denervated tissue by comparison with its control (Table 3). This was evident both from the specific radioactivity of the extracted RNA and when the results were expressed as incorporation/unit weight of tissue. On the other hand, incorporation of ^{14}C from orotic acid expressed in terms of specific radioactivity was depressed.

By the second day an enhanced incorporation of ^{14}C from adenine was still apparent (Table 3), and the value for orotic acid was equivocal, depending on whether specific radioactivity or incorporation/unit weight of tissue was considered. But by the third day the specific radioactivity of RNA labelled even with adenine was below that of the control, although the large increase in tissue RNA raised the value for tissue incorporation. The values for orotic acid both showed a decrease.

Incorporation of precursors into DNA. Incubation of diaphragm with [^{14}C]adenine and [^{14}C]orotic acid led to labelling of the DNA fraction (Table 4). Incorporation of label into DNA was much enhanced 2-3 days after phrenicotomy, but showed a decline after 10 days. The early stimulation was a noteworthy observation, since incorporation into RNA at this time showed a decline when expressed in terms of specific radioactivity. Although this dichotomy in itself provides evidence that adenine incorporation with the DNA fraction is indeed into DNA rather than into possible contaminating RNA, further investigations of the effect of denervation on DNA metabolism were carried out with thymidine to avoid possible confusion.

Incorporation of [^3H]thymidine into DNA was elevated within 24 hr. of phrenicotomy (Table 4), but the stimulation was much greater by the second and third day, and by the tenth day incorporation had returned to near the control value. Although the number of disintegrations incorporated appears high, it of course represents a very small actual quantity of thymidine. For example, 4000 disintegrations/min. incorporated/mg. of tissue (Table 4), assuming a specific radioactivity of 4×10^9 disintegrations/min./ μ mole, indicates an incorporation of 1 μ mole/g. of tissue/2 hr. If the denervated tissue expands 30% in 3 days and the DNA concentration remains constant there must be synthesis of about 0.5 mg. of DNA/g. of tissue. This represents an incorporation of about 0.5 μ mole of thymidine/g./72 hr. or about 7 μ moles/g./hr. Thus the isotope figure is actually a considerable underestimate of the rate of DNA synthesis, but this is presumably explicable by a lowering of the specific radioactivity of [^3H]thymidine on entering the pool of thymidine and thymidylic acid in the tissue.

No radioactivity from [^{14}C]thymine was found in the DNA fraction, confirming the observations of Plentl & Schoenheimer (1944) and Holmes, Prusoff

Table 3. *Incorporation from labelled adenine, uracil and orotic acid into RNA of isolated denervated rat diaphragm*Each value in the mean $\pm$ s.e.m. of six observations. Each precursor was added to the incubation medium undiluted to give a radioactivity of about $0.4 \mu\text{C}/\text{ml}$.

Time (days) elapsed since phrenicotomy	Isotope precursor	Radioactivity in extracted RNA (disintegrations/min. incorporated/mg. of tissue)				Sp. radioactivity of extracted RNA (disintegrations/min./ μg . of P)			
		Paralysed hemidiaphragm	Innervated control	Difference	Increase or decrease (%)	Paralysed hemidiaphragm	Innervated control	Difference	Increase or decrease (%)
1	[8- ^{14}C]Adenine	565 ± 40 $P < 0.05$	450 ± 16	115 ± 35 $P = 0.02$	27	3878 ± 27 $P < 0.05$	3152 ± 82	726 ± 211 $P < 0.02$	23
	[2- ^{14}C]Uracil	25.6 ± 1.7	21.1 ± 1.3	4.5 ± 1.7 $P = 0.05$	21	135 ± 10.9	111 ± 7.1	24 ± 10	22
	[6- ^{14}C]Orotic acid	59.8 ± 1.7	60.3 ± 4.2	0.4 ± 5.4	-0.8	309 ± 11	341 ± 20	32 ± 22	-9.4
2	[8- ^{14}C]Adenine	514 ± 24 $P < 0.001$	362 ± 18	152 ± 27 $P < 0.01$	42	2572 ± 184 $P < 0.01$	2238 ± 81	334 ± 218	15
	[6- ^{14}C]Orotic acid	83.4 ± 4.8	75.0 ± 2.7	8.4 ± 4.1	11	446 ± 13 $P < 0.02$	509 ± 13	63 ± 16 $P < 0.02$	-12
3	[8- ^{14}C]Adenine	409 ± 5.5	342 ± 11	67 ± 5.8 $P < 0.001$	20	1717 ± 50	2173 ± 38	456 ± 58 $P < 0.001$	-21
	[6- ^{14}C]Orotic acid	45.4 ± 1.6	55.4 ± 2.8	10.0 ± 2.7 $P < 0.02$	-18	179 ± 5.5 $P < 0.001$	318 ± 13	139 ± 12 $P < 0.001$	-44
10	[8- ^{14}C]Adenine	281 ± 15	278 ± 16	3.5 ± 23	1.3	1460 ± 73 $P < 0.05$	1777 ± 89	317 ± 140	-18

Table 4. *Incorporation from labelled adenine and thymidine into DNA of isolated denervated rat diaphragm*

Each value is the mean $\pm$ s.e.m. of six observations except where another number of observations is given in parentheses (3). [^{14}C]Adenine was present in the incubation medium at a concn. of $0.4\text{ }\mu\text{C}/\text{ml}$. and [^3H]thymidine at $8\text{ }\mu\text{C}/\text{ml}$.

Time (days) elapsed since phrenicotomy	Isotope precursor	Radioactivity in extracted RNA (disintegrations/min. incorporated/mg. of tissue)				Sp. radioactivity of extracted RNA (disintegrations/min./ μg . of P)			
		Paralysed hemidiaphragm	Innervated control	Difference	Increase or decrease (%)	Paralysed hemidiaphragm	Innervated control	Difference	Increase or decrease (%)
3	[$8\text{-}^{14}\text{C}$]Adenine	36.2 ± 1.8 $P < 0.001$	13.9 ± 1.2	22.3 ± 1.9 $P < 0.001$	161	262 ± 15 $P < 0.001$	97 ± 8.1	165 ± 16 $P < 0.001$	170
10		14.2 ± 1.3 $P < 0.05$	21.3 ± 2.6	7.1 ± 2.2 $P < 0.05$	-33	112 ± 8.6	174 ± 20	62 ± 16	-36
2	[$6\text{-}^{14}\text{C}$]Orotic acid	2.07 ± 0.25 $P < 0.01$	0.78 ± 0.09	1.29 ± 0.35 $P < 0.02$	165	—	—	—	—
1	[$Me\text{-}^3\text{H}$]Thymidine	6860 ± 1150	4350 ± 808	2510 ± 445 $P < 0.01$	58	40400 ± 6700	26500 ± 5100	13900 ± 2630 $P < 0.01$	52
2		12550 ± 830 $P < 0.001$	4440 ± 690	8110 ± 550 $P < 0.001$	182	81300 ± 7300 $P < 0.001$	32900 ± 5300	48400 ± 4400 $P < 0.001$	147
3		12430 ± 1610 (3) $P < 0.01$	2690 ± 106	9740 ± 1690 $P < 0.05$	360	71030 ± 5940 $P = 0.001$	19730 ± 880	53300 ± 6360 $P < 0.02$	300
10		1556 ± 226	2212 ± 542	656 ± 409	-30	11870 ± 1790	15980 ± 3670	4110 ± 2340	-26

& Welch (1954) of the very limited capacity of the rat to use thymine.

DISCUSSION

When a tissue hypertrophies it is to be expected that nucleic acid synthesis will rise, to maintain a constant tissue composition ratio. This appears to be the situation with DNA in denervated diaphragm and is consistent with proliferation of nuclei (Boeke, 1916; Chor, Dolkart & Davenport, 1937; Gutmann & Young, 1944). Similarly the increased concentration of RNA is indicative of a very large stimulus of RNA synthesis, which may be related to the enhanced rate of protein synthesis after denervation. Simple measurement of RNA does not determine its nature (whether ribosomal, transfer etc.), but, taking advantage of the high E_{280} value for cytosine, we measured the E_{280}/E_{240} ratio for a number of RNA extracts and found a slight increase in samples from denervated tissue. This could be indicative of an increase in ribosomal RNA, which is rich in guanine and cytosine. Higher concentrations of both DNA and RNA have been reported for muscles that have atrophied after denervation (Graff, Hudson & Strickland, 1965a; McCaman & McCaman, 1965; Gutmann, 1963), indicating probably for DNA the maintenance of a constant quantity per total muscle, but interpretation is complicated by the extent of fibrosis under these conditions.

The conflicting findings for incorporation of precursors into RNA are unexpected but reminiscent of the difference in influence of insulin on incorporation of ^{14}C from adenine, uracil and orotic acid noted previously (Manchester, 1967). Without knowing the size of the pool of nucleotides in the cell, it is not possible to know how much dilution the precursor undergoes on entry. An increased intracellular pool of precursors accompanying an increased synthesis of RNA is quite likely. Such an occurrence could explain the decrease in incorporation of label in some cases into RNA, but would equally mean that the increased synthesis of DNA was underestimated. Moreover, there is no evidence of a change after denervation in the size of the adenine nucleotide pool, and preliminary experiments showed no change in the extent of intracellular accumulation of labelled precursors 3 days after denervation (K. L. Manchester & E. J. Harris, unpublished work). Imrie & Hutchison (1965) showed that adrenocorticotrophic hormone could increase the concentration of adrenal RNA by inhibition of ribonuclease activity. It is possible in the present experiments that part of the increase in RNA concentration results from inhibition of RNA breakdown. It is also possible that lowered incorporation of label results from decreased activity of enzyme-catalysed conversion reactions in the paralysed tissue, for example from orotic acid into

UMP and CMP. Gallucci, Novello, Margreth & Aloisi (1966), in a study of regenerating rat muscle, found a diminished activity of many enzymes by comparison with the fully differentiated tissue, and a similar phenomenon may occur in denervation hypertrophy. On the other hand, McCaman & McCaman (1965) found no diminution in the activity of purine nucleoside phosphorylase after denervation. Change in tissue size results in synthesis and reorganization of membrane material, whose synthesis conversely declines in atrophy (Graff, Hudson & Strickland, 1965b). An immediate consequence of denervation may be a change in enzyme pattern towards that characteristic of the tissue when in its embryonic state, as described by Gallucci *et al.* (1966) for a muscle mince inserted *in vivo* in the anatomical site of the muscle. These changes are associated with increased amounts of nucleic acid.

We had considered the possibility that increased protein synthesis might be preceded by RNA synthesis and perhaps by increased turnover of DNA. However, Buse *et al.* (1965) showed that leucine incorporation increases within 24 hr. of denervation, that is as soon as we see evidence for stimulation of RNA synthesis. The incorporation of thymidine into DNA is still increasing 2–3 days after nerve section, and so evidently the enhancement of DNA synthesis is not a primary event. Feng & Lu (1965) have provided evidence that the almost unique hypertrophic response of diaphragm results from its anatomical position, in that unilateral denervation leads to regular stretching of the paralysed tissue by the contracting innervated portion. They suggest that the hypertrophy results primarily from stretching as opposed to nerve section. Diaphragm muscle has mixed fibre types (Nachmias & Padykula, 1958; George & Susheela, 1961) and it appears that it is primarily the red muscle (that with greater succinate dehydrogenase activity) that hypertrophies (Feng & Lu, 1965). If this is so, the quantitative expansion of the red muscle type must be greater than the overall results suggest, because it represents only a portion of the total mass, the other major constituents, the white fibres, undergoing atrophy. However, in either instance the primary biochemical stimuli remain to be elucidated.

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