The Effects of Denervation on Protein Turnover of Rat Skeletal Muscle

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The effects of denervation on muscle weight, rates of protein synthesis and breakdown, and RNA concentrations were studied in the soleus and extensor digitorum longus muscles. Although the soleus underwent a true atrophy after section of the sciatic nerve, the extensor digitorum longus continued to grow, albeit at a lower rate than innervated controls. At 24 h after nerve section protein breakdown was increased in both muscle types when compared with internal controls, and remained so throughout the 10 days studied. The possibility that this increased catabolism might arise from conformational changes of proteins after denervation was not substantiated, as myofibrillar or soluble proteins of denervated and control tissues were equally susceptible to degradation *in vitro* by three proteinases. Tyrosine uptake into the denervated extensor digitorum longus was decreased throughout the 10 days studied, whereas two phases of increased transport of the amino acid were found in the soleus. Significant decreases in rates of protein synthesis were found 1 and 2 days after denervation, and results are presented that suggest that these changes may result from a decrease in ribosomal involvement in the translation process. These initial decreases were not maintained and the rate of protein synthesis was in fact increased when compared with controls, at 7 and 10 days. The increased synthetic rates of the 7-day denervated tissues were reflected as proportional increases in both myofibrillar and soluble proteins. It is suggested that the increase in synthesis at this time may result from an increase in both the availability and active involvement of ribosomes, and that these anabolic trends may be caused by spontaneous fibrillation and/or the amount of passive stretching of the denervated muscles.

It is well known that skeletal muscle can alter its size in response to different physiological demands. For example, increased work loads induce compensatory growth, whereas diminished muscular activity leads to atrophy. In spite of the obvious importance of this adaptive function, we know little of the cellular events and regulatory mechanisms that ultimately determine muscle size. Our limited knowledge of this aspect of metabolism hinders progress in understanding abnormal conditions such as the muscular dystrophies, steroid myopathies or heart failure.

Denervation is frequently used in the study of muscle atrophy (Gutmann, 1962). Since protein is such a large component of muscle, it is not surprising that atrophy is accompanied by a net loss of tissue protein. Exactly how this adjustment is made after denervation is not clear. Either decreased rates of protein synthesis or increased rates of protein breakdown, or both, could cause decreases in muscle size. Most previous investigators have concentrated on measuring changes of protein synthesis after denervation, but the results to date are confusing, with Schapiro *et al.* (1953), Padiou (1959), Dreyfus (1967) and Goldberg (1969) showing decreases and Slack (1954), Ferdman (1963) and Pater & Kohn (1967) increases in synthetic rates. Comparatively few studies (Goldberg,

1969; Pearlstein & Kohn, 1966) have been made of the alterations in degradative rates. In the present paper, the effects of denervation on rates of protein synthesis and degradation have been studied to try to clarify the existing confusion and elucidate the sequence of biochemical events and possible control mechanisms involved in this process of muscle wasting.

Materials and Methods

All experiments involved the use of young growing male rats (CD strain; approx. 50–80 g) obtained from Charles River U.K. Ltd., Manston, Kent, U.K. Under halothane (I.C.I., Alderly Park, Macclesfield, U.K.) anaesthesia the muscles of one hind limb were denervated by removal of approx. 1 cm of the sciatic nerve at a point close to the spine. The contralateral limb was simultaneously sham-operated and its muscles were used as internal controls for the denervated tissues.

Animals were killed by cervical dislocation and the appropriate muscles rapidly dissected out and placed in oxygenated Krebs–Ringer bicarbonate buffer (Deluca & Cohen, 1964). Excess of buffer was blotted off, and the wet weight of the tissue determined on a torsion balance.

Average rates of protein synthesis and protein breakdown were measured in these intact isolated muscles by the method of Fulks *et al.* (1975). Protein synthesis was determined by measuring the incorporation of tyrosine into muscle protein during a 2 h incubation in 3 ml of Krebs–Ringer bicarbonate buffer, containing glucose (10 mM), 0.05 μ Ci of L-[U- 14 C]tyrosine (483 mCi/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) and L-tyrosine hydrochloride (0.1 mM). Actual calculation of the tyrosine incorporated was made by dividing the incorporated [14 C]tyrosine counts by the specific radioactivity of the intracellular tyrosine pool. The latter value was obtained as follows:

$$\text{c.p.m./nmol} = \frac{(\text{acid-soluble c.p.m./mg of muscle}) - (10^{-2} \times E \times \text{c.p.m./}\mu\text{l of medium})}{(\text{acid-soluble nmol of tyrosine/mg of muscle}) - (10^{-2} \times E \times \text{nmol of tyrosine/}\mu\text{l of medium})}$$

where E is the extracellular space of the muscle (%). With the assistance of Dr. G. Goldspink (Department of Zoology, University of Hull) extracellular-space measurements were determined microscopically on unfixed sections of the appropriate muscles. These values were 10 and 6.5% of the extensor digitorum longus and soleus muscles respectively.

Average rates of protein breakdown were determined independently of protein synthesis by measuring the release of tyrosine (Waalkes & Udenfriend, 1957) into intracellular amino acid pools and into the surrounding medium (3 ml) during 2 h incubation in Krebs–Ringer bicarbonate buffer containing glucose (10 mM). Cycloheximide (0.5 mM) was added to the medium in this case to block protein synthesis, thus preventing reutilization of the tyrosine released by degradation of muscle protein.

To measure relative rates of synthesis of myofibrillar and soluble proteins, six denervated (7 days) and six control muscles were individually incubated in 3 ml of oxygenated ($\text{O}_2 + \text{CO}_2$; 95:5) Krebs–Ringer bicarbonate buffer, containing glucose (10 mM) and 2.0 μ Ci of L-[3,5- ^3H]tyrosine (49 Ci/mmol; The Radiochemical Centre). After a 2 h incubation, muscles were pooled into groups of denervated or control tissues, homogenized and fractionated into myofibrillar and soluble components (Goldspink *et al.*, 1971a). An equal volume of 20% (w/v) trichloroacetic acid was added to samples of the homogenate and the myofibrillar and soluble fractions. Acid-insoluble material was centrifuged down and retained, and supernatants containing free [^3H]tyrosine were discarded. Protein pellets were resuspended, washed in 3 $\times$ 5 ml of 10% trichloroacetic acid and resedimented. Washed pellets were dissolved in 0.5 M-NaOH. Scintillation medium (Patterson & Greene, 1965; 5 ml) was added to 0.5 ml samples of dissolved pellets and ^3H measured in a

Packard scintillation counter by using [1,2- ^3H]n-hexadecane (The Radiochemical Centre) as internal standard for quench correcting. Other samples of the dissolved pellets were used for protein determinations. Radioactivity incorporated per mg of protein of homogenate, myofibrillar or soluble fractions was calculated and expressed as a ratio of denervated/normal muscle for each fraction.

For degradation by proteinases *in vitro* myofibrillar and soluble proteins were prepared (Goldspink *et al.*, 1971a) from pooled denervated or pooled control muscles obtained from 12 rats. Portions (0.4 mg) of protein preparations were incubated with 20 μ g of trypsin, chymotrypsin or Pronase in Tris/HCl buffer

(100 mM), pH 8.0, for 60 min at 37°C. Reaction was stopped by the addition of an equal volume of ice-cold 10% (v/v) HClO_4 . Proteinase activity was measured as the increase in E_{224} of the acid-soluble material liberated during incubation (Goldspink *et al.*, 1971a).

The extraction and assay procedures for measuring muscle RNA, DNA or DNA synthesis have been described elsewhere (Goldspink & Goldberg, 1975). Muscle protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin (Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K.) as a standard.

Results

Two muscles were studied throughout these investigations, the soleus (a predominantly 'red' muscle) and the extensor digitorum longus (a predominantly 'white' muscle). Fig. 1(a) shows the percentage weight losses of both muscles relative to their internal controls (i.e. relative atrophy) as a function of time after denervation. In general, both muscles appeared to undergo a rapid initial atrophy, followed from about 5 to 7 days onwards by a more gradual loss of weight. However, it is important to recognize that such changes relative to internal controls can be misleading, especially in a highly dynamic state of growth as in this investigation. In accordance with the rapid growth of these young animals, the innervated extensor digitorum longus and soleus muscles increased in size in a linear manner with time, approximately doubling their weight over the 10 days of the study (Fig. 1b). The denervated extensor digitorum longus muscle did not show a loss of weight, but grew, albeit at a slower rate than the control tissue. Therefore the relative atrophy of the extensor digitorum longus muscle (Fig. 1a) actually represents the dis-

Fig. 1. Changes in wet weight and protein of muscles after denervation

(a) The percentage decrease in muscle weight after nerve section is calculated as the mean of individual differences in weight of denervated and internal-control muscles, i.e. relative atrophy. Each value is the mean $\pm$ S.E.M. of at least six denervated extensor digitorum longus ($\blacktriangle$) or soleus ($\bullet$) muscles compared with an equal number of innervated control muscles. (b) Wet-weight measurements were made on both innervated extensor digitorum longus ($\triangle$) or soleus ($\circ$) and denervated extensor digitorum longus ($\blacktriangle$) or soleus ($\bullet$) muscles, as a function of time. Each value is the mean $\pm$ S.E.M. of five muscles. (c) Total protein measurements were made on the muscles of (b) by the method of Lowry *et al.* (1951). Each value is the mean $\pm$ S.E.M. of five muscles.

proportionate growth between innervated and denervated muscles. The soleus muscle, however, did lose weight (i.e. underwent 24% absolute atrophy) during the first few days after nerve section, but slow growth at later times restored the tissue to its pre-denervation weight (Fig. 1b). The total amount of protein in the control and denervated muscles (Fig. 1c) correlated with changes in muscle size and always remained between 18 and 19% of the wet weight. These data indicate that changes in protein metabolism are a predominant feature of denervation.

Since the amount of tissue protein at any one time is regulated by rates of protein synthesis and protein breakdown, both of these parameters were measured to determine the contributory role of each in modifying muscle growth after denervation. Table 1 compares the average rates of protein synthesis and breakdown of normal extensor digitorum longus and soleus muscles. The actual nmol of tyrosine incorporated, or released, are average values from 12 muscles of each type. The percentage differences (Table 1) were obtained from the same experiment by using paired analysis of the parameters measured for extensor digitorum longus and soleus muscles from the same animal, i.e. internally controlled. These data indicate that higher rates of turnover are found in the 'red' soleus muscle. However, changes in these basal rates of protein synthesis and degradation of both muscles occurred in response to denervation.

Rates of protein breakdown of the extensor digitorum longus (Fig. 2), whether expressed per whole muscle or per mg of muscle, were elevated soon after nerve section. The earliest significant change was observed after 24 h and rates remained elevated thereafter for the 10 days investigated. These increased rates of protein breakdown measured *in vitro* are supported by studies *in vivo* in this laboratory (D. F. Goldspink, unpublished work) showing a greater loss of radioactivity (^3H leucine) from prelabelled muscle proteins from 4-day denervated extensor digitorum

Table 1. Basal rates of protein synthesis and protein breakdown of extensor digitorum longus and soleus muscles

Average rates of protein synthesis and breakdown were measured in intact isolated muscles. Synthesis was measured as the incorporation of tyrosine from the medium into muscle proteins after a 2 h incubation. The specific radioactivity of the intracellular tyrosine pool was measured and this value incorporated into the final calculation of rates of synthesis. Breakdown was measured as the release of tyrosine into intracellular amino acid pools and into the surrounding medium after 2 h incubation. Each value is the mean $\pm$ S.E.M. of determinations made on 12 extensor digitorum longus and 12 soleus muscles. Percentage difference values represent the mean $\pm$ S.E.M. of individual percentage differences of rates of synthesis, or breakdown, of the soleus relative to the extensor digitorum longus from the same animal. Statistical significance (* $P < 0.001$) of these differences was determined by the method of paired analysis.

	Protein synthesis (nmol of tyrosine incorporated/2h)		Protein breakdown (nmol of tyrosine released/2h)	
	per muscle	per mg of muscle	per muscle	per mg of muscle
Extensor digitorum longus	3.5 $\pm$ 0.2	0.09 $\pm$ 0.01	10.6 $\pm$ 0.6	0.39 $\pm$ 0.02
Soleus	4.1 $\pm$ 0.3	0.13 $\pm$ 0.01	14.8 $\pm$ 1.0	0.57 $\pm$ 0.04
Difference (%)	18 $\pm$ 4.9*	38 $\pm$ 4.1*	48 $\pm$ 9.1*	58 $\pm$ 12*

Fig. 2. Changes in rates of protein synthesis and breakdown of the denervated extensor digitorum longus muscle

Percentage changes of protein synthesis are expressed either per mg of muscle (Δ) or per whole muscle ($\bullet$), and percentage changes of breakdown per mg of muscle ($\triangle$) or per whole muscle ($\circ$). The changes in rates of synthesis and breakdown are expressed as the mean $\pm$ S.E.M. of the individual differences between denervated and control muscles from each rat, calculated as a percentage of the control value.

longus muscles. Such increases in protein catabolism correlate with the smaller gain of protein in the denervated tissue (Fig. 1c). Protein synthesis was significantly lower over the first 2 days of denervation (Fig. 2). These changes, like those of catabolism, correlate with the diminished growth of the denervated extensor digitorum longus muscle (Fig. 1b). However, the initial decreases in synthetic rates were not maintained. At about 4 days, and onwards, rates of protein synthesis were reversed, i.e. higher in the denervated extensor digitorum longus than in the control tissue.

As mentioned above, the calculations of rates of protein synthesis incorporate measurements of the specific radioactivity of intracellular tyrosine pools. Allowance is thus made for possible changes in pool sizes with respect to synthesis. However, it is interesting to consider these measurements of tyrosine pools independently. From 24h onwards significant percentage decreases in the specific radioactivity of the tyrosine pools were found in the denervated extensor digitorum longus muscle compared with the control tissues. Further, tyrosine uptake into the extensor digitorum longus is decreased after nerve section, since the amount of radioactive tyrosine/mg of muscle becomes progressively lower in the acid-soluble phase of the denervated tissue compared with controls (Fig. 3). This suggestion, that the uptake of tyrosine is decreased after nerve section, is further supported by statistically significant decreases (20–25%) in the uptake of cycloleucine into the extensor digitorum longus muscle 4 and 7 days after denervation. Cycloleucine is a non-metabolized

Fig. 3. Changes in specific radioactivity of intracellular tyrosine pools of the denervated extensor digitorum longus muscle

Percentage changes of specific radioactivity (∇), nmol of tyrosine ($\bullet$) and c.p.m. of [^{14}C]tyrosine ($\blacksquare$) of intracellular tyrosine pools/mg of extensor digitorum longus muscle are plotted as a function of time after denervation. The percentage changes are calculated as the mean of individual percentage differences of denervated muscles relative to their internal controls. Each value is the mean $\pm$ S.E.M. of a minimum of five denervated and five control extensor digitorum longus muscles.

amino acid believed to enter muscle cells by the same transport system as tyrosine. Measurements of tyrosine (i.e. radioactive and non-labelled)/mg of muscle did not differ significantly between denervated and control tissues, except at 7 and 10 days (Fig. 3). Presumably, for this to be so, the decreased uptake of tyrosine by the denervated extensor digitorum longus must be offset by increased entry of this amino acid into the intracellular pools as a result of the increased rates of protein catabolism (Fig. 2).

A broadly similar pattern of events was found for the soleus muscle after denervation (Fig. 4), with the following exceptions. After a transient initial increase (2 days), rates of protein degradation/muscle were generally slightly lower in the denervated tissue. Further, more rapid changes in protein synthesis occurred in the denervated soleus, i.e. as early as 12 h after nerve section (Fig. 4). Protein synthesis/whole soleus remained lower after denervation, but synthetic rates expressed per mg of muscle increased at the later time-points (Fig. 4) in a manner similar to that found for the extensor digitorum longus muscle (Fig. 2). In the soleus, where changes appear to be contradictory, depending on whether a parameter is expressed per whole muscle or per mg of muscle, the explanation appears to be that percentage losses in muscle weight exceed the individual percentage changes in synthetic or catabolic rates after denervation.

For the first few hours after section of the nerve supply to the soleus, the pool's specific radioactivity

Fig. 4. Changes in rates of protein synthesis and breakdown of the denervated soleus

Percentage changes of protein synthesis are expressed either per mg of muscle (Δ) or per whole muscle ($\bigcirc$), and percentage changes of breakdown per mg of muscle ($\square$) or per whole muscle ($\bullet$). All measurements and calculations were made in a manner identical with that of Fig. 2 on at least six denervated and six control soleus muscles.

Fig. 5. Changes in the specific radioactivity of intracellular tyrosine pools of the denervated soleus

Percentage changes in specific activity (Δ), nmol of tyrosine ($\bullet$) and c.p.m. of [^{14}C]tyrosine ($\blacksquare$) of intracellular tyrosine pools/mg of soleus are plotted as a function of time after denervation. These values were calculated in a manner identical with that of Fig. 3, on at least five denervated and five control soleus muscles.

increased significantly, but then returned to control values by 24h (Fig. 5). Further substantial increases reappeared 4–10 days after denervation. Although the tyrosine concentration of the pools was generally unchanged, the uptake of tyrosine by the tissue was enhanced after nerve section (Fig. 5). The latter finding was further supported by a $43 \pm 7.1\%$ increase

($P < 0.001$) in cycloleucine uptake/mg of soleus 7 days after denervation.

Investigating further the contrasting changes of protein synthesis after denervation (Fig. 2 or Fig. 4), we measured the RNA concentrations of both muscle types and attempted to relate these changes in RNA to the changes in protein synthesis. In keeping with the findings of others (Dreyfus, 1967; Goldberg, 1967), higher concentrations of RNA were found in the normal 'red' muscle ($8.4 \pm 0.6 \mu\text{g}$ of RNA-P/soleus or $0.25 \pm 0.01 \mu\text{g}$ of RNA-P/mg of soleus) compared with the normal 'white' muscle ($6.3 \pm 0.4 \mu\text{g}$ of RNA-P/extensor digitorum longus or $0.18 \pm 0.01 \mu\text{g}$ of RNA-P/mg of extensor digitorum longus). Since rRNA is more than 80% of total muscle RNA, changes in the concentrations of RNA probably reflect changes in the ribosomal content and therefore ribosomal capacity for involvement in protein synthesis (Millward *et al.*, 1973). An index of ribosomal efficiency, or active involvement, in the translation process can also be obtained from a measure of protein synthesis/unit of RNA. The effects of 1 and 7 days denervation on RNA concentrations, ribosomal efficiency and protein synthesis are shown in Table 2.

At 24h after nerve section, small decreases in RNA were found in the extensor digitorum longus (Table 2). Tyrosine incorporation/ μg of RNA-P of the denervated tissue was substantially lower (34%) at this time, suggesting a less active involvement of ribosomes in translation. This lower ribosomal efficiency was of a similar order of magnitude to the percentage decrease in protein synthesis/mg of muscle (Table 2). By 7 days of denervation the extensor digitorum longus showed a marked increase in RNA/mg of muscle (Table 2). This appears to be the result of a net accumulation of RNA/muscle (Table 2) against a diminishing muscle mass. Ribosomal involvement in protein synthesis, unlike that at 24h, was increased 7 days after denervation. Possibly the reversal of protein synthesis/mg of extensor digitorum longus, from being decreased (34%) at 24h to being increased (91%) 7 days after denervation (Table 2 and Fig. 2), may be explained by both the marked increase in ribosomal capacity (i.e. RNA/mg of muscle) and the complete reversal of ribosomal efficiency (Table 2).

Within 24h of denervation, small but significant decreases in RNA concentrations were found in the soleus muscle (Table 2). The lowered rates of protein synthesis at this time could therefore arise from a decreased availability of ribosomes and/or the decreased ribosomal efficiency as suggested by the lower values of protein synthesis/unit of RNA. By 7 days after denervation an even greater net loss of RNA/soleus had occurred, with the depletion of RNA proceeding at a slightly lower rate than the loss of muscle mass, hence the small percentage increase in RNA/mg of muscle (Table 2). As with the extensor digitorum

Table 2. Relationship between changes in RNA concentrations and rates of protein synthesis 1 and 7 days after denervation

Rates of protein synthesis were measured as described for Table 1. RNA concentrations and protein synthesis were determined simultaneously in the same muscles, thus enabling protein synthesis to be calculated per μg of RNA-P. Each value is the mean $\pm$ S.E.M. of individual percentage changes of measurements made on denervated tissues relative to their internal controls. A minimum of 15 denervated and 15 control extensor digitorum longus or soleus muscles were studied. Significance values (* $P < 0.001$; † $P < 0.025$) were determined by the method of paired analysis.

	Percentage changes in				
	RNA-P		Protein synthesis		
	per muscle	per mg of muscle	per muscle	per mg of muscle	per μg of RNA P
Extensor digitorum longus					
1-Day denervated	$-5.8 \pm 1.6^*$	$-5.8 \pm 1.8^*$	$-33 \pm 2.7^*$	$-33 \pm 2.6^*$	$-34 \pm 3.2^*$
7-Day denervated	$29 \pm 6.1^*$	$41 \pm 4.6^*$	$47 \pm 4.7^*$	$91 \pm 5.8^*$	$41 \pm 4.2^*$
Soleus					
1-Day denervated	$-12 \pm 1.4^*$	$-5.9 \pm 1.2^*$	$-35 \pm 2.8^*$	$-34 \pm 2.4^*$	$-35 \pm 3.8^*$
7-Day denervated	$-41 \pm 2.2^*$	$3.7 \pm 1.6^\dagger$	$-47 \pm 4.5^*$	$44 \pm 6.2^*$	6.4 ± 6.8

longus, changes in direction of RNA capacity and ribosomal efficiency may possibly explain the reversal of protein synthesis at the early (1 and 2 days) and later time-points (7 and 10 days) after denervation (Table 2 and Fig. 4).

Further investigating the elevated rates of protein synthesis of 7-day denervated extensor digitorum longus and soleus muscles, we attempted to obtain some information on the nature of the proteins being synthesized, e.g. myofibrillar or sarcoplasmic, or both. Muscles were incubated *in vitro* with [^3H]-tyrosine for 2 h. They were then pooled, homogenized and differentially centrifuged into myofibrillar and soluble components. The radioactivity incorporated/mg of protein of whole homogenate, myofibrillar or soluble fractions was expressed as a ratio of denervated/control muscles (Table 3). All ratios were greater than unity (Table 3), further indicating increased rates of protein synthesis in the denervated tissues compared with controls. Since within each muscle type there were no appreciable differences in the ratios for the homogenate, myofibrillar or soluble fractions, clearly no preferential synthesis of either myofibrillar or soluble proteins was taking place. Rather, the increased synthetic rates of protein in the intact denervated muscles are reflected as proportional increases in both myofibrillar and soluble protein components.

Appreciable evidence is now accumulating to suggest that the half-life of a protein is largely determined by the conformation of that particular protein (Dice *et al.*, 1973; Goldberg & Dice, 1974). Indeed, it has been known for several years that denaturation of a protein usually increases that protein's susceptibility to cleavage by proteolytic enzymes. Therefore the possibility that the increased rates of protein breakdown after denervation might arise from conformational changes in proteins of denervated muscle

Table 3. Comparison of the synthesis of myofibrillar and soluble proteins 7 days after denervation

Denervated and control muscles obtained from six rats (80 g) 7 days after operation were incubated individually for 2 h with $0.67 \mu\text{Ci}$ of [^3H]tyrosine per ml of Krebs-Ringer bicarbonate buffer. The six denervated, or control, soleus muscles were then pooled, homogenized and fractionated into myofibrillar and soluble protein components. Extensor digitorum longus muscles were treated similarly. Incorporated ^3H radioactivity was counted in solubilized samples of the appropriate fractions and the c.p.m./mg of protein expressed as a ratio of denervated/control muscles. Alterations in ratios should indicate changes in the relative rates of synthesis of myofibrillar and soluble proteins.

	Denervated/control (radioactivity per mg of protein)		
	Whole homogenate	Myofibrillar fraction	Soluble fraction
Extensor digitorum longus	1.84	1.89	1.73
Soleus	2.29	2.34	2.15

was examined. Crude fractions of myofibrillar and soluble proteins were prepared (Goldspink *et al.*, 1971a) from 12 denervated extensor digitorum longus muscles 4 days after nerve section. Similar preparations were obtained from an equal number of soleus muscles. Standard amounts (0.4 mg) of myofibrillar and soluble proteins were incubated with $20 \mu\text{g}$ of trypsin, chymotrypsin or Pronase for 60 min at 37°C . Proteolysis was measured as the increase in E_{224} of the acid-soluble material released during the incubation. The three proteolytic enzymes degraded myofibrillar or soluble proteins to the same extent regardless of whether they originated from denervated

or control muscles, suggesting that there was no preferential digestion of proteins from the denervated tissues. Interpretation of the data on the digestion of soluble proteins by proteinases is not without its complications. The soluble fraction of muscle is known to contain inhibitors of trypsin and chymotrypsin (Goldspink *et al.*, 1971c). This inhibitory activity of the soluble fraction is believed to be caused by the presence of serum proteinase inhibitors, which are also known to inhibit proteinases from microbial sources (Fossum, 1970). Changes in the vascular supply to muscles after denervation could conceivably lead to different concentrations of the serum proteinase inhibitors in the soluble fractions prepared from normal or denervated muscles.

Discussion

Caution has to be exercised when attempting to explain events *in vivo* by using data from systems *in vitro*. This point is illustrated in Table 1, where isolated muscles incubated in the absence of several plasma components are in negative nitrogen balance, i.e. breakdown exceeds synthesis, whereas these muscles are clearly growing *in vivo* (Figs. 1b and 1c). Such differences can be partially explained by previous findings, which indicate that protein synthesis of isolated muscles (Fulks *et al.*, 1975) or perfused hearts (Morgan *et al.*, 1971) is inhibited, and protein degradation enhanced, in the absence of insulin or plasma amino acids. These substances were absent from the incubation medium used in obtaining the data of Table 1. The major emphasis of this study lies in comparing changes in the denervated muscle relative to its control tissue under identical conditions *in vitro*, with the probable likelihood that such changes closely reflect events *in vivo*.

The initial smaller gain (extensor digitorum longus) or loss (soleus) of muscle mass (Fig. 1b) correlates with the rapid increases of protein breakdown and initial decreases of synthesis after nerve section (Figs. 2 and 4). Such changes occurring either separately, or simultaneously as here, would favour a smaller gain, or a net loss, of protein (Fig. 1c) in the economical remodelling of these disused tissues. The more rapid onset of relative atrophy of the soleus (Fig. 1a) may possibly arise from the earlier (12h) changes in protein synthesis in this tissue (Fig. 4) compared with the extensor digitorum longus (Fig. 2). Also the elevated rates of protein synthesis of 7- and 10-day denervated muscles compared with controls (Figs. 2 and 4, and Table 2) presumably decrease the difference in the growth rates of denervated and innervated muscles, thus explaining the slower phase of relative atrophy (Fig. 1a) from about 5 days onwards.

Clearly, denervation atrophy is a situation where modifications of degradative, as well as synthetic, rates are important in regulating amounts of tissue

protein (Fig. 1b), and therefore in influencing muscle size. Increased rates of protein catabolism have been found in the muscle-wasting conditions of the muscular dystrophies (Simon *et al.*, 1962), steroid myopathies (Goldberg, 1969), denervation (Pearlstein & Kohn, 1966; Dreyfus, 1967; Goldberg, 1969) and limb immobilization (D. F. Goldspink, unpublished work). Indeed, the true significance of breakdown pathways in regulating tissue size is gradually gaining in recognition since the early work of Schoenheimer (1942). However, our understanding of the regulation of protein degradation is at best rudimentary. Although several intracellular proteinases have been found in muscle, and their activities are known to change in various wasting conditions (Weinstock & Iodice, 1969; Goldspink *et al.*, 1971b; Pluskal & Pennington, 1973), the exact role of these enzymes in normal protein turnover remains uncertain. Another likely area of control relates to protein structure. In our experiments we were unable to show preferential degradation of proteins of denervated muscles by three proteinases of differing specificities. By themselves these negative findings do not exclude the possibility that changes in protein conformation *in vivo* may be a mechanism regulating the rates at which proteins are degraded by intracellular proteinases. One possible difficulty with our experimental approach is that only a relatively small proportion of the cellular proteins may need to undergo conformational changes to produce assayable changes in rates of catabolism, but such changes may be too small to be readily resolved by digestion *in vitro* by proteinases, particularly since the isolation procedures used may themselves induce physical changes in the proteins.

Most previous attempts to explain the muscle wasting caused by denervation have concentrated on studying changes in protein synthesis. These investigations, however, have produced conflicting findings of either decreased (Schapira *et al.*, 1953; Padieu, 1959; Dreyfus, 1967; Goldberg, 1969) or increased (Slack, 1954; Ferdman, 1963; Pater & Kohn, 1967) rates of synthesis after nerve section. Some of the conflict may be due to the use of different experimental procedures and in some instances the use of unsuitable amino acid tracers for studying muscle. However, our data may help to resolve some of the previous confusion, since many of these earlier measurements of synthesis were restricted to only one, or two, specific time-points after denervation. In the present study we have systematically investigated the changes occurring over the first 10 days of denervation, and as mentioned above have found this interesting reversal of trends for changes of protein synthesis (Figs. 2 and 4). Clearly, the random choice of time-points within our short study could lead to three different conclusions, i.e. decreased (1 or 2 days), unchanged (3 days) or increased (7 or 10 days) rates of synthesis after

denervation (Fig. 2 and 4). Indeed, many of the reports describing decreases of synthesis were investigations performed only a few hours, or 1 or 2 days, after denervation. Conversely, those describing increases of synthesis tend to be studies of longer duration, i.e. days to weeks after denervation. Viewed in this context, there is generally good agreement between the previous studies and our findings. However, caution has to be exercised in applying our data to other muscles of the body and to species other than the rat, since the time-course of atrophy appears to vary with animal age and size (Young, 1970).

Changes in tyrosine transport do not always move in the same direction as changes in protein synthesis after denervation suggesting different control mechanisms for these processes. Both tyrosine transport and protein synthesis in the extensor digitorum longus are decreased initially (Figs. 2 and 3), but unlike protein synthesis tyrosine uptake does not increase at the later time-points. The situation is reversed in the soleus, with postdenervation changes in tyrosine uptake and protein synthesis being in phase at later, but not earlier, time-points (Figs. 4 and 5). These different responses of the two muscle 'types' to tyrosine transport may be due to differences in blood supply, degree of passive stretching (see below) or degree of spontaneous activity (see below) after nerve section. These changes in tyrosine transport only partially agree with reported changes of α -aminoisobutyric acid transport after denervation (Goldberg *et al.*, 1974), i.e. agreement exists for the first few hours after denervating the extensor digitorum longus, or several days after denervating the soleus, but not otherwise. However, tyrosine is a more hydrophobic amino acid than α -aminoisobutyric acid and is transported by a different system. Further, these two amino acid-transport systems are known to respond differently in other physiological situations (Goldberg *et al.*, 1974).

The physiological significance of the increased synthesis of both myofibrillar and soluble proteins (Table 3) 7 days after nerve section is not entirely clear at the moment. Whatever the explanation, it is likely to have broad implications, since analogous increases in rates of protein synthesis, as well as degradation, have been described in dystrophic muscle also (Simon *et al.*, 1962; Coleman & Ashworth, 1959).

Although several control factors can be envisaged as regulating the observed changes of protein synthesis after denervation, our data relating to RNA concentrations (Table 2) enable us to speculate as to possible sites of control. For example, the initial fall in protein synthesis by both the extensor digitorum longus and soleus muscles (Figs. 2 and 4, Table 2) may be due to a decrease in the active involvement of ribosomes in the process of translation, as suggested by the decrease in tyrosine incorporated/ μ g of RNA (Table 2). In contrast, the increased synthetic rates of

the 7-day denervated muscles, compared with controls, may result from both a greater availability of ribosomes and an increase in ribosomal efficiency (Table 2). These estimates of ribosomal capacity and efficiency do, however, make the assumption that changes in total RNA concentrations reflect proportional changes in the various forms of RNA (Millward *et al.*, 1973).

Both the increase in rates of protein synthesis and the accumulation of RNA in the 7-day denervated extensor digitorum longus muscle are changes associated with growth rather than atrophy and are therefore in keeping with the continued growth of this tissue (Figs. 1b and 1c). In fact, these changes are not dissimilar to those found in the transient hypertrophy of the diaphragm after phrenicectomy (Turner & Garlick, 1974; Manchester & Harris, 1968). Unlike the diaphragm (Manchester & Harris, 1968), however, the incorporation of [3 H]thymidine into DNA is lower in the denervated extensor digitorum longus and soleus muscles compared with controls at 1 (Goldberg *et al.*, 1975) and 7 (D. F. Goldspink, unpublished work) days. The soleus, however, shows less marked tendency towards anabolism than the extensor digitorum longus 7 days after denervation (Table 2). Changes of protein synthesis, although increased per mg of muscle, are actually lower on a whole-muscle basis (Fig. 4 and Table 2). In addition, RNA concentrations tend to be maintained rather than accumulated, as in the extensor digitorum longus (Table 2). The increased concentrations of RNA in the extensor digitorum longus presumably result from increased rates of synthesis, since the activities of ribonuclease enzymes, which are presumed to degrade RNA, are known to be elevated in the extensor digitorum longus after denervation (Goldspink *et al.*, 1971b). The changes in RNA concentrations of the soleus are, however, very similar to changes reported for the gastrocnemius of the rat 7 days after nerve section (Lissack *et al.*, 1963).

The different responses of the soleus and extensor digitorum longus muscles described above may simply reflect metabolic difference of 'red' and 'white' muscles correlating with their various activities and different functions within the body. For example, 'darker' muscles show a greater uptake of amino acids (Dreyfus, 1967; Goldberg, 1967), higher basal rates of protein turnover (Table 1, and Goldberg *et al.*, 1974) and greater concentrations of RNA (Goldberg, 1967) than do 'paler' muscles. However, another significant factor explaining these differences may be the anatomical location of these muscles within the limb (see below).

Contractile activity is very important in determining muscle size (Goldspink, 1974; Goldberg *et al.*, 1975) and sarcomere number (Williams & Goldspink, 1971). Direct stimulation of muscle has also prevented some of the changes that normally occur after dener-

vation (Drachman & Witzke, 1972; Goldberg *et al.*, 1974). One feature associated with denervated muscle is its acquisition of spontaneous electrical activity as the cut motor nerve degenerates. If denervated muscle develops tension in response to such electrical activity this may well be a factor promoting anabolic responses in the otherwise disused tissue. Thesleff (1963) detected the appearance of spontaneous fibrillation in the rat diaphragm 3 days after phrenicectomy. If this time of onset of spontaneous fibrillation is applicable to leg muscles also, it would certainly correlate well with the reversal of changes in protein synthesis in the denervated muscles (Figs. 2 and 4).

Other possible explanations may relate to changes in tissue responsiveness to circulating hormones after denervation, or to the different anatomical positions of the two muscles in the hind leg and their exposure to unequal amounts of passive stretch. Stretch is believed to promote the transient hypertrophy of the denervated diaphragm (Sola & Martin, 1953) and the rapid growth of synergistic muscles in response to tenotomy of the gastrocnemius muscle (Goldberg *et al.*, 1975). We have noticed that the rat generally drags its denervated limb in an extended position with occasional mechanical flexing of the ankle. This means that the extensor digitorum longus probably experiences a much greater incidence of stretching (i.e. when leg is extended) than the soleus (when ankle is flexed) and this may be an important explanation of the more extensive atrophy of the soleus (Fig. 1).

Arguing against these hypotheses is the inability of Goldberg *et al.* (1975) to find any promotion of protein synthesis after short periods of direct electrical stimulation or stretching of muscle *in vitro*. However, studies in our laboratory (D. F. Goldspink, unpublished work) have shown that the 7-day denervated soleus atrophies more rapidly when the hind limb is immobilized in extension, i.e. minus possible stretching by ankle flexion. This appears to be caused by substantial decreases in protein synthesis both per mg of muscle (66%) and per whole muscle (88%) (compared with data of Table 2), and slightly higher rates of protein breakdown compared with the values presented in Fig. 4. In contrast, the normal innervated extensor digitorum longus hypertrophies when the limb is fixed in the extended position, a response similar to other muscles after stretch has been applied (Sola *et al.*, 1973). The 7-day denervated extensor digitorum longus under the same conditions of immobilization undergoes less atrophy than when the denervated limb is carried free. This greater resistance to atrophy appears to arise from additional increases in rates of protein synthesis (i.e. higher than those of Fig. 2 and Table 2) and lower rates of breakdown than those presented in Fig. 2. This presumably represents the muscles' response to being stretched.

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