Changes in the Protein Composition of Muscles of the Rat in Hypertrophy and Atrophy

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Following denervation of skeletal muscle a number of changes occur. The most obvious of these is the progressive decrease in size or atrophy of the muscle. This involves a decrease in dry as well as wet weight of the tissue. That increase in size of a muscle may occur following section of the motor nerve was demonstrated in this laboratory by Martin & Sola (1948) and by Sola & Martin (1953). Approximately 7 days after section of the left phrenic nerve, increases in wet weight of from 25 to 55% were observed in the hemidiaphragm of the rat. These increases in wet weight were accompanied by increases in total dry weight and the term hypertrophy was therefore applied to the phenomenon. The hypertrophy proved to be transient; the muscle had returned to normal weight by about 30 days and this was followed by typical atrophy of denervation. An attempt to duplicate the results in another laboratory was reported in a brief paper by Thomson, Morgan & Brodish (1951). These workers found no increase in dry weight, although an increase in wet weight was observed following denervation of the left hemidiaphragm. They suggested the increase in weight was the result of oedema.

In view of the lack of confirmation of the entire result, a more complete analysis appeared desirable, and in any case so unusual a response to denervation warranted further investigation. To be considered a true hypertrophy an increase in protein content, involving the fractions normally present, should be demonstrated. An increase of protein in muscle induced to hypertrophy by the administration of hormones has been described. Bigland & Jehring (1952) obtained hypertrophy of the quadriceps muscle of the rat by injection of growth hormone, but found no increase in total tension development. This led to the suggestion that the new material laid down was not contractile material. In part because of this observation, Hume (1952) repeated the work and confirmed the increase in total protein but found no change in the relative amounts of sarcoplasmic, contractile and connective tissue proteins. Similar results were obtained with

muscles in which hypertrophy was induced by growth hormone plus testosterone. Because a relative decrease in myofibrillar ATPase activity was observed which was not accompanied by a relative decrease in contractile protein, Hume suggested that the new protein at this stage might be a precursor of myosin. It was the purpose of the present research to determine whether the protein components of the denervated diaphragm increase during hypertrophy and whether in hypertrophy or atrophy there are changes in relative amounts of connective tissue, contractile and sarcoplasmic proteins. For comparison, analyses were made of the protein fractions in the normal levator ani muscle and in this muscle after hypertrophy induced by the administration of testosterone (Eisenberg, Gordon & Elliott, 1949).

EXPERIMENTAL

Materials and dissection techniques. Albino rats of the Sprague-Dawley strain were used for the analyses. Weights of the animals used were between 260 and 400 g. Denervation of the left hemidiaphragm was accomplished by resection of a portion of the left phrenic nerve, using the thoracic approach of Sola & Martin (1953). Hemidiaphragms from five normal animals and from five or six animals at each of the intervals of 7, 15, 30 and 45 days after denervation were analysed in these experiments. The animals were decapitated and the diaphragms removed in a cold room at 2°. The procedure of Sola & Martin (1953) was used for dissection of the hemidiaphragms. After removal of the dorsal slips each hemidiaphragm was weighed on a torsion balance. The calculation of the percentage hypertrophy or atrophy of the muscle was based on these weights as described below. The central tendon and the posterior one-sixth of each hemidiaphragm were removed and discarded. These parts contain relatively much more connective tissue than the rest of the hemidiaphragm. The more muscular portion of the organ was then weighed, minced with scissors in a small evaporating dish and a sample part taken to be used for the determination of total protein nitrogen. The remainder, which weighed from a minimum of 89 mg. in an atrophied hemidiaphragm to a maximum of 281 mg. in one of the normal hemidiaphragms, was used for the protein fractionation. Calculation of the percentage composition of the muscle was based on the wet weight of the more muscular portion of each hemidiaphragm.

The other muscle used in this work, the levator ani, was induced to hypertrophy by subcutaneous implantation of

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testosterone pellets 30 days before killing. The method of Eisenberg *et al.* (1949) was used for dissection of this muscle. After mincing, a sample of the tissue was removed for the total protein nitrogen determination and the remainder used for the protein fractionation. The levator ani muscles from five normal animals were analysed to determine the protein composition of the normal muscle.

Protein fractionation procedure. The method of fractionation was that of Robinson (1952) with the slight modification that the direct determination of the contractile protein was omitted because of the small amount of muscle available. A sample was taken for total protein nitrogen and the remainder was analysed for sarcoplasmic and connective tissue nitrogen and the contractile proteins were determined by difference.

The minced tissue was washed from the evaporating dishes with about 10 ml. of the cooled extractant solution into 15 ml. Ten Brock Pyrex glass tissue grinders. The extractant solution had the composition: 0.1M-KCl, 0.0277M- K_2HPO_4 , 0.0163M- NaH_2PO_4 , a pH of 7.1, ionic strength 0.2. After homogenizing by hand (the outer part of the homogenizer was held near the top while the ground glass plunger was rotated and moved slowly and uniformly up and down) for 5 min. at 2° the material was centrifuged at 2000 rev./min. (693 g) for 1 hr. at 0°. The supernatant solution containing the sarcoplasmic proteins was decanted into a 25 ml. volumetric flask. Second and third extractions, each for 2.5 min., with approximately 7 ml. of extractant solution were carried out in similar fashion. The three extracts were combined and made up to a volume of 25 ml. Samples of this were diluted with an equal volume of 30% (v/v) ethanol added from a fine capillary at 0°. The small precipitate of actomyosin was removed by centrifuging at 2500 rev./min. (1080 g) for 40 min. at 0°. Samples of the supernatant solution were taken for N determination. The residue remaining after extraction of the sarcoplasmic proteins was homogenized with 0.1N-NaOH for 2.5 min. and then centrifuged at 2500 rev./min. (1082 g) for 40 min. at 0°. The extraction was repeated twice, and after the third extraction the tissue was allowed to stand overnight in 0.1N-NaOH. The remaining material consisting mostly of connective tissue was next washed with 0.1N-NaOH solution into 50 ml. Pyrex glass centrifuge tubes and centrifuged. The supernatant solution was again discarded and the tissue allowed to stand one more night in 0.1N-NaOH at 2°. The residue was then washed with 5% trichloroacetic acid and digested in 2 ml. of a mixture of 120 ml. concentrated sulphuric acid and 2 ml. selenium oxychloride. The 50 ml. centrifuge tubes were used in the digestion to avoid transferring the protein to digestion flasks. Nitrogen determinations were made in duplicate by the method of Ballentine & Gregg (1947).

Evaluation of results. For an estimate of the degree of hypertrophy or atrophy it was necessary to know the original weight of the left hemidiaphragm with a minimum of error. Sola & Martin (1953) determined the theoretical weight before denervation from the maximum body weight exhibited by the experimental animal and from the relationship between body weight and hemidiaphragm weight in a series of normal animals. In the work reported here twenty-two normal male rats were killed and the weights of the right and left hemidiaphragms determined after removal of the dorsal slips. Since the coefficient of variation for calculation of hemidiaphragm weights from body weight in this normal series was 7.4%, another method was used

which permitted greater accuracy. The ratio, left hemidiaphragm weight/right hemidiaphragm weight, was determined for the series of normal rats and was found to increase with increasing body weight. The coefficient of correlation for this relationship is 0.56. The ratio of the weight of the left to that of the right hemidiaphragm was plotted against body weight and a regression line fitted to the data by the method of least squares. The equation of the line is $y = 0.00022x + 0.832$, where y represents the ratio left/right and x the body weight at the time of death. Then from the ratio obtained from this equation for any particular animal weight, and from the weight of the normally innervated right hemidiaphragm, the original weight of the denervated left half was computed. The coefficient of variation for calculation of hemidiaphragm weight by this method was 4.1%.

As described above, the calculation of the percentage change in wet weight was based on the weight of the hemidiaphragm including tendon, whereas only the more muscular portion was analysed. In the following calculations it was assumed that the entire hemidiaphragm had the composition of the muscular portion analysed. Since the calculation of both the theoretical initial amount and the amount present after denervation was based on this assumption, any error introduced becomes negligible.

In order to determine the extent of any change in total protein content during the periods of hypertrophy and atrophy it was necessary to know the amount present initially. From the theoretical initial weight of the hemidiaphragm and the percentage total protein N in the normal left hemidiaphragm, the initial total protein N was calculated. It was further desirable to know which protein fractions contributed to any change in total protein content. Therefore the distribution of the protein fractions in the normal and in the denervated hemidiaphragms was determined. From this information it was possible to calculate the amount present in each fraction initially and at each interval after denervation. These amounts were compared and the extent of change in each fraction was calculated.

For the determination of the extent of hypertrophy induced in the levator ani muscles by the administration of testosterone it was necessary to compute the original weight of the muscle. The weight of the levator ani muscle in fifteen normal male rats was determined and plotted against body weight. A line was fitted to the points by the method of least squares. The equation of the line is: $y = 0.000655x + 0.029$, where y represents the normal weight of the levator ani muscle and x the body weight at the time of death, the standard error of estimate, $S_y = 36.95$. The theoretical initial weight of each muscle was determined from this relationship and the percentage hypertrophy calculated. The coefficient of variation was 13.2%.

RESULTS

The mean values for the protein N of the three fractions expressed as a percentage of the total protein N are presented in Table 1 for the normal right hemidiaphragm and the normal levator ani muscle of the rat. The normal levator ani muscle contains relatively more protein N and contractile protein nitrogen and less connective tissue and sarcoplasmic protein nitrogen than the diaphragm. That these differences are significant is shown by the

fact that the probability of their occurrence by chance is below the 2 % probability level (column *d*, Table 1).

The percentage composition of the hypertrophied levator ani muscle is also shown in Table 1. The average extent of hypertrophy of the five muscles analysed was 39.25 %, s.e.m. ± 5.65 , s.d. ± 12.64 . Comparison of the relative compositions of the normal and hypertrophied levator ani muscles indicates no significant difference in the two cases (column *e*, Table 1). Therefore, in this type of hypertrophy, the three protein fractions appear to increase during hypertrophy in proportion to the amount present initially in each fraction. Owing to the initially greater bulk of the contractile proteins, this fraction contributed the most to the increase in total protein.

While a 30-day period was allowed for the hypertrophy of the levator ani muscle, the hemidiaphragm had hypertrophied to almost as great an extent by

7 days after nerve section and by 30 days was already well atrophied. The results of protein analyses of the normal hemidiaphragms and of the experimental hemidiaphragms at the different intervals of time after denervation are given in Table 2. The percentage of the wet weight of the muscle represented by the total protein N did not change significantly through the period studied and, since a highly significant increase in wet weight occurred at 7 days, it is clear that synthesis of new protein occurred.

The results of the analyses of the connective tissue, the smallest fraction, were quite variable. Unless there were synthesis of new connective tissue protein, a decrease in relative amount of this fraction might be anticipated. A decrease is shown in the table but, because of the variability of the data, the decrease did not prove to be significant. By 30 days, at which time an atrophy of 19 % had occurred, the connective tissue as a percentage of

Table 1. *Protein composition of the normal right hemidiaphragm, normal levator ani and hypertrophied levator ani muscles*

Total protein nitrogen is expressed as mean percentage of the wet weight of muscle tissue $\pm$ s.e.m. Connective tissue, sarcoplasmic and contractile protein nitrogen values are expressed as mean percentages of the total protein nitrogen $\pm$ s.e.m. s.d. in parentheses.

	Normal right hemidiaphragm (a)	Levator ani		Probability of difference being due to chance alone	
		Normal (b)	Hypertrophied (c)	(d) (a and b)	(e) (b and c)
Total protein N	2.71 $\pm$ 0.03 (0.08)	3.02 $\pm$ 0.04 (0.09)	3.04 $\pm$ 0.03 (0.06)	<0.01	0.7
Connective tissue	9.27 $\pm$ 0.64 (1.42)	6.24 $\pm$ 0.51 (1.14)	5.75 $\pm$ 0.76 (1.71)	<0.01	0.6
Sarcoplasmic protein	29.20 $\pm$ 0.62 (1.39)	26.84 $\pm$ 0.43 (0.96)	26.06 $\pm$ 0.42 (0.94)	0.015	0.2
Contractile protein	61.53 $\pm$ 0.89 (2.00)	66.92 $\pm$ 0.42 (0.94)	68.17 $\pm$ 0.72 (1.62)	<0.01	0.2

Table 2. *Protein composition of the left hemidiaphragm before denervation and at 7, 15, 30 and 45 days after denervation*

Total protein nitrogen is expressed as mean percentage of the wet weight of muscle tissue $\pm$ s.e.m. Connective tissue, sarcoplasmic and contractile protein nitrogen values are expressed as mean percentages of the total protein nitrogen $\pm$ s.e.m. The probabilities of the differences from normal being due to chance alone are included. s.d. in parentheses.

	Normal	7 days	15 days	30 days	45 days
Percentage change in weight		+33.86 $\pm$ 2.03 (4.55)	+15.64 $\pm$ 2.32 (5.67)	-19.61 $\pm$ 2.94 (6.59)	-42.38 $\pm$ 3.06 (7.51)
Total protein N	2.69 $\pm$ 0.05 (0.12)	2.56 $\pm$ 0.04 (0.09) <i>P</i> = 0.1	2.62 $\pm$ 0.03 (0.08) <i>P</i> = 0.3	2.61 $\pm$ 0.02 (0.05) <i>P</i> = 0.2	2.56 $\pm$ 0.06 (0.15) <i>P</i> = 0.2
Connective tissue	10.49 $\pm$ 1.13 (2.53)	8.96 $\pm$ 0.63 (1.40) <i>P</i> = 0.3	9.89 $\pm$ 1.03 (2.51) <i>P</i> = 0.7	13.80 $\pm$ 0.52 (1.17) <i>P</i> = 0.03	20.45 $\pm$ 1.07 (2.62) <i>P</i> < 0.001
Sarcoplasmic protein	28.92 $\pm$ 0.52 (1.16)	30.76 $\pm$ 1.07 (2.40) <i>P</i> = 0.2	29.53 $\pm$ 0.86 (2.10) <i>P</i> = 0.6	29.88 $\pm$ 0.86 (1.93) <i>P</i> = 0.4	28.64 $\pm$ 0.73 (1.78) <i>P</i> = 0.8
Contractile protein	60.59 $\pm$ 1.13 (2.53)	60.28 $\pm$ 0.57 (1.27) <i>P</i> = 0.8	60.57 $\pm$ 1.39 (3.39) <i>P</i> = 1.0	56.32 $\pm$ 0.97 (2.17) <i>P</i> = 0.02	50.90 $\pm$ 1.16 (2.83) <i>P</i> = 0.002

the total protein had begun to increase and by 45 days the connective tissue represented about one-fifth of the total protein. These changes were significant. The sarcoplasmic proteins showed no really significant change in relative amount through either the stage of hypertrophy or atrophy. Therefore the sarcoplasmic proteins increased in proportion to the total protein increase during the period of hypertrophy and decreased in proportion to the decrease in total protein during the period of atrophy. The contractile proteins as a percentage of the total protein showed no significant variation from the normal at 7 days after denervation, this fraction also increasing in absolute amount in proportion to the increase in wet weight of muscle. From 7 to 15 days there was a decrease in the wet weight of the muscle. The fact that the percentage of contractile protein nevertheless remained constant may be taken as evidence that there was a decrease in the amount of contractile protein which was in proportion to the weight loss. By 30 days, however, at which time weights below normal were observed, this fraction as a percentage of the total protein had begun to decrease. Thus during atrophy the contractile proteins decreased as a percentage of the total protein as well as in absolute amount.

Thus far only the percentage composition of the muscle at the various intervals has been presented.

To show clearly the effects of weight changes on the absolute amount of protein in each fraction, the percentage changes from the theoretical initial amounts have been calculated and the results presented in Fig. 1. In Fig. 1A are shown the percentage changes from the initial amount of total protein nitrogen and from the initial wet weight at each period. Since the change in total protein nitrogen very closely paralleled the change in wet weight the hypertrophy may be accounted for by the increase in protein.

The amount of connective tissue did not change significantly during either hypertrophy or atrophy (Fig. 1B), the probabilities of the occurrence by chance of the differences between the mean value at 0 days and the means at each of the intervals after denervation all being above 40%. Therefore the apparent increase in connective tissue at 30 and 45 days previously described is only a relative increase. In Fig. 1C and D are shown the respective changes in the sarcoplasmic and contractile protein fractions. Similar percentage increases were observed in these fractions at 7 days. However, owing to the greater bulk of the contractile proteins, they contributed more to the total increase in protein at this time. Approximately 60% of the increase in protein was contributed by the contractile protein fraction and 40% by the sarco-

Fig. 1. A, change in total protein nitrogen, $\circ$ — $\circ$, and change in weight, $\bullet$ — $\bullet$, from the theoretical initial values following denervation of the left hemidiaphragm; B, change in total connective tissue nitrogen from the theoretical initial amount; C, change in sarcoplasmic protein nitrogen from the theoretical initial amount; D, change in contractile protein nitrogen from the theoretical initial amount.

plasmic protein fraction. These fractions contributed 70 and 30 % respectively to the loss in weight which had occurred at 45 days.

DISCUSSION

Results of fractionation of muscle proteins have been reported by a number of investigators. Unfortunately most of them cannot be compared to the results presented here, since the different fractionation procedures have not resulted in the same degree of separation. Directly comparable are the values for chick muscle reported by Robinson (1952), whose method of analysis was used in this work on the rat diaphragm. Values which he obtained in chicken breast muscle 1 month after hatching were: contractile protein, 62 %; sarcoplasmic protein, 33 %; connective tissue protein, 5 % of the total protein. The values obtained here for two normal muscles of the rat, the diaphragm and the levator ani, are very similar to his values.

Results of fractionation of the proteins of the quadriceps muscle from rats treated with testosterone plus growth hormone have been reported (Hume, 1952). This treatment resulted in an increase in weight but did not alter the relative amounts of the three fractions: connective tissue, sarcoplasmic protein and contractile protein. Similarly, no change in the relative amounts was observed in the levator ani muscle in this work but no more direct comparison can be made since she did not report numerical values.

Hines & Knowlton (1933) undertook a study of changes in protein composition during atrophy of the gastrocnemius muscle of the rat through 7, 14 and 20 days. They used phosphate solutions of different ionic strengths as extractant solutions, but their work suffered from the fact that insufficient knowledge of the solubility of proteins was available at that time. Where Robinson used a solution of an ionic strength of 1.45 to extract both sarcoplasmic and contractile proteins, the ionic strengths of the solutions used by them varied from 2.62 to 4.72. A separation into sarcoplasmic and contractile protein groups probably was not accomplished. The relative amounts of the protein fractions in their study, which did not include connective tissue, did not vary during the period of atrophy. In the present work on the diaphragm, during the period of hypertrophy the sarcoplasmic and contractile proteins increased in absolute amount in proportion to the extent of the hypertrophy. After the onset of protein loss, both of these fractions decreased in absolute amount, again in proportion to the loss of weight. The normal distribution of the protein was retained during the period of hypertrophy and the period of return to normal weight. However, with further loss of

weight the contractile protein not only continued the process of decrease in absolute amount but began to constitute a smaller percentage of the tissue, a percentage loss compensated by a simultaneous increase in percentage of connective tissue. The atrophy, which amounted to 42 % loss in wet weight at 45 days, was still not sufficient to have resulted in a decreased percentage of sarcoplasmic protein.

Fischer & Ramsey (1946) and Fischer (1948) studied the changes in the properties of myosin after denervation. The actomyosin and myosin of denervated muscle were less readily extracted and only a small fraction of that extracted was precipitated by procedures ordinarily adequate for precipitation. Because the contractile proteins were not analysed directly this change was not observed in the work on the diaphragm. Although the contractile proteins were found to increase in amount with hypertrophy, it is possible that the properties of the new protein were different.

A number of histological examinations of denervated muscle have been made. Chor, Dolkart & Davenport (1937) reported that by the end of 2 weeks, longitudinal striations are apparent as a result of cleavage of small bundles of myofibrillae in the denervated gastrocnemius muscle of the monkey. They found that portions of the myofibrillae break down, the regions being replaced by a coarsely granular substance. From their histological investigation they concluded that the sarcoplasm is the first protein to be involved, but that the myofibrillae also are affected in atrophy due to denervation. Tower (1935), after studying the cat interosseus muscle, suggested that the nuclei and sarcoplasmic structures are affected first by denervation and reported no change in the myofibrillae for weeks after denervation. In a later report (Tower, 1939) it was suggested that the depletion may involve all parts equally since at the time when a 50–80 % reduction in mass has occurred the fibrils must be depleted although the cross and longitudinal striations are still in fairly good order.

The results reported here do not support these views. The decrease in the sarcoplasmic proteins occurs at the same rate as the decrease in weight. The contractile proteins are lost more rapidly, so that with progressing atrophy this group of proteins comes to represent a smaller and smaller fraction of the total protein present. Apparently a large part of the contractile protein can be lost and yet leave the striations clearly visible.

SUMMARY

1. The gain in weight of the hemidiaphragm of the rat caused to hypertrophy by denervation was shown to be associated with an increase in protein.

2. The increase in protein in the hypertrophy caused by hormones was confirmed for the levator ani muscle of the rat.

3. The percentage composition of the muscle was unchanged during the periods of hypertrophy and return to normal weight. With further loss of weight, the percentage of contractile proteins decreased and a corresponding increase in percentage of connective tissue occurred.

4. At 7 days after denervation a 36% increase in absolute amount of sarcoplasmic protein accompanied by a 27% increase in absolute amount of contractile protein was observed. At 45 days decreases in absolute amount of these two fractions of 45 and 54% respectively had occurred.

5. There was no significant change in absolute amount of connective tissue during the period studied.

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A Constituent of the Unsaponifiable Portion of Animal Tissue Lipids ($\lambda_{\max}$ 272 m μ .)

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The distribution of 7-dehydrosteroid (i.e. material showing ultraviolet absorption similar to that of provitamin D) in animal tissues has been studied by Glover, Glover & Morton (1952) and by Festenstein & Morton (1955). The small intestine is a relatively rich source in some species (e.g. guinea pig) but not in others.

In the course of their work Festenstein & Morton noted the presence in the intestines of horse and pig (but not sheep or ox) of a constituent of the unsaponifiable fraction which showed an absorption maximum at 272 m μ .

The selectively absorbing substance was concentrated by chromatography and characterized in terms of qualitative and quantitative ultraviolet absorption spectra in various solvents and by its adsorptive properties. Although horse intestine appeared to contain possibly 20 mg./kg. the yield of the richest concentrates was not very promising. A similar substance was found by Cain & Morton (1955) in horse liver but the investigation has been

hampered by the presence of other very persistent congeners.

Some time later the '272 m μ . substance' was again encountered. Unsaponifiable fractions from various tissues of both normal and vitamin A-deficient rats were chromatographed on alumina and a 'rat 272 m μ . material' was obtained and identified with the 'horse and pig 272 m μ . substance'. More extensive study of various tissues from xerophthalmic rats and normal rats showed the substance to occur in kidney, liver, intestine and submaxillary gland, but to be absent from similarly separated fractions of bladder, testis and vagina. It is hoped in due course to comment upon the relative amounts of '272 m μ . material' in the tissues of control rats and vitamin A-deficient rats. It is clear, however, that the livers and kidneys of vitamin A-deficient rats are a fairly good source, and the same substance has been observed in hen liver and kidney. It also occurs (Kantiengar, Lowe, Morton & Pitt, 1955) in cockerel liver and kidney and in guinea pig liver (Leat, private communication). The present work records properties aiming at

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