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## Turnover of Structural Protein Fractions in Denervated Muscle.* (32124)

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Very little is known about mechanisms of protein degradation in degenerative processes, or in normal turnover. Studies utilizing denervated muscle as a model have indicated that the rapid loss of some of the structural protein is due to accelerated degradation rather than retarded synthesis(1,2). The interpretation of these results, as well as the planning of future investigations of this system, require the resolution of certain questions. First, the possibility must be considered that structural proteins such as myosin occur in different pools which have different rates of turnover, and that myosin extracted by the usual methods may not be representative of the other structural proteins nor of the less soluble myosin. If this were the case, a given protein in fractions of varying solubility could be related on a precursor-product basis, as in the case of collagen where the soluble protein is transformed into the insoluble(3). Furthermore, only some of the fractions, for example, ones representing final products, might be degraded in atrophy. In this case only certain fractions would be suitable substrates for studying the mechanism of degradation. Finally, there is the difficulty of distinguishing between changes in protein turnover in atrophying muscle which are due to the cellular content of factors required for protein synthesis and degradation, and those which are related to functional status of muscle in the animal.

To attempt to answer some of these questions and to provide preliminary data which might suggest possibilities for further investigation of this system the following experiments were performed: 1) The turnover of a radioactive amino acid was compared between control and atrophying muscles in 4 differently soluble fractions of skeletal muscle structural protein; 2) Myosin was extracted from similar fractions, and turnover was compared among the 4 fractions themselves in control and atrophying muscles, as well as between the control and atrophic representatives of each fraction; 3) Incorporation into structural protein was followed

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in incubated muscle slices to test the comparative synthetic capabilities of the control and atrophic muscles in the absence of functional effects.

*Materials and methods.* Lower leg muscles of male rats were denervated as previously described(4). Opposite legs with intact innervation served as controls. The experimental procedures were begun 7 days after denervation. At this time each of 14 rats was injected intraperitoneally with 20 $\mu$c U-$^{14}$C-glycine in 2 ml Ringer's solution. At 1, 4, 8, 12, 24, 36 and 48 hours after injection a pair of rats was sacrificed by blows on the head. The tibialis anterior and gastrocnemius muscles were removed, dissected free of connective tissue, and weighed, the muscles from each rat being kept separate throughout the procedure. The gastrocnemii were minced in 50% glycerol and stored at $-20$°C in this solution. The tibialis anterior muscles were immediately washed and then fractionated by procedures based on those developed by Perry for the solubilization of structural protein(5). Each muscle was homogenized in 10 ml of 0.25 M sucrose with 0.005 M EDTA (pH = 7) for 2 minutes at a setting of 70 on the VirTis "45" homogenizer. The homogenate was centrifuged 6 minutes at 600 $\times$ *g* and the resulting precipitates washed 2 times with 10 and 30 ml of the sucrose solution respectively with centrifugations at the same time and speed. Precipitates were then washed 2 times with 20 ml each time of 12% sucrose with 0.005 M EDTA (pH = 7). Centrifugations were at 1000 $\times$ *g* for 10 minutes. This last procedure was found by interference microscopy to remove large amounts of granules. The resulting precipitates, containing structural proteins, were then extracted serially with KCl-$PO_4$ buffer (0.3 M KCl, 0.1 M $KH_2PO_4$, 0.05 M $K_2HPO_4$—pH 6.5), 0.9 M LiCl, and Weber's solution (0.6M KI, 0.01 M $Na_2CO_3$, 0.04 M $NaHCO_3$). Two 10-minute extractions with 5 ml of extractant, followed each time by 10 minutes centrifugation at 10,000 $\times$ *g* were carried out with each of these solutions. Each pair of supernatants was combined. The final residue was washed in 5% TCA (trichloracetic acid) and centrifuged at 1000 $\times$ *g* for 15 minutes. The supernatants were dialyzed 48 hours against 14 volumes of distilled water and then centrifuged at 10,000 $\times$ *g* for 10 minutes. The precipitates which formed were also washed in 5% TCA. Four structural protein fractions were thus obtained from each muscle.

The gastrocnemius muscles were treated in the same manner except that the precipitates after dialysis were extracted for myosin according to standard procedures(6), slightly modified by the addition of ATP at one stage to increase the yield of myosin. The precipitates were dissolved in equal volumes 1 M KCl (pH = 6.6), and then brought to a KCl concentration of 0.3 M with water. After centrifugation at 1000 $\times$ *g* for 15 minutes, the resulting precipitates were discarded and the supernatants diluted to 0.04 M KCl and allowed to stand 48 hours. The material insoluble at this dilution was brought down by centrifugation and then treated in the same manner as the original precipitates except that the water used in the dilution to 0.3 M KCl contained 0.004 M ATP (pH = 6.6). The precipitates forming at 0.04 M KCl were saved as myosin and were washed with 5% TCA. All precipitates from both the tibialis and gastrocnemius muscles were then extracted for 15 minutes with 5% TCA at 90°C and for 20 minutes with ethanol at 60°. The final precipitates were dissolved in 1 N NaOH and aliquots taken for protein determination by the method of Lowry, Rosebrough, Farr, and Randall(7). The remaining solutions were neutralized with 10 N HCl, suspended in a thixotropic gel and counted in a liquid scintillation spectrometer, utilizing internal standards for determination of quenching. Specific activities (CPM/mg protein) were calculated for each fraction.

For determination of protein synthesis *in vitro,* slices of control and atrophying tibialis anterior muscles from rats unilaterally denervated seven days previously were utilized. Slices were prepared with a Stadie-Riggs microtome, washed quickly in Ringer's solution at 37°C, and incubated at 37° in Krebs Ringer-bicarbonate solution, gassed with 95% $O_2$ and 5% $CO_2$, and containing 1 mg glucose and 2 $\mu$c U-$^{14}$C-glycine per ml. At various

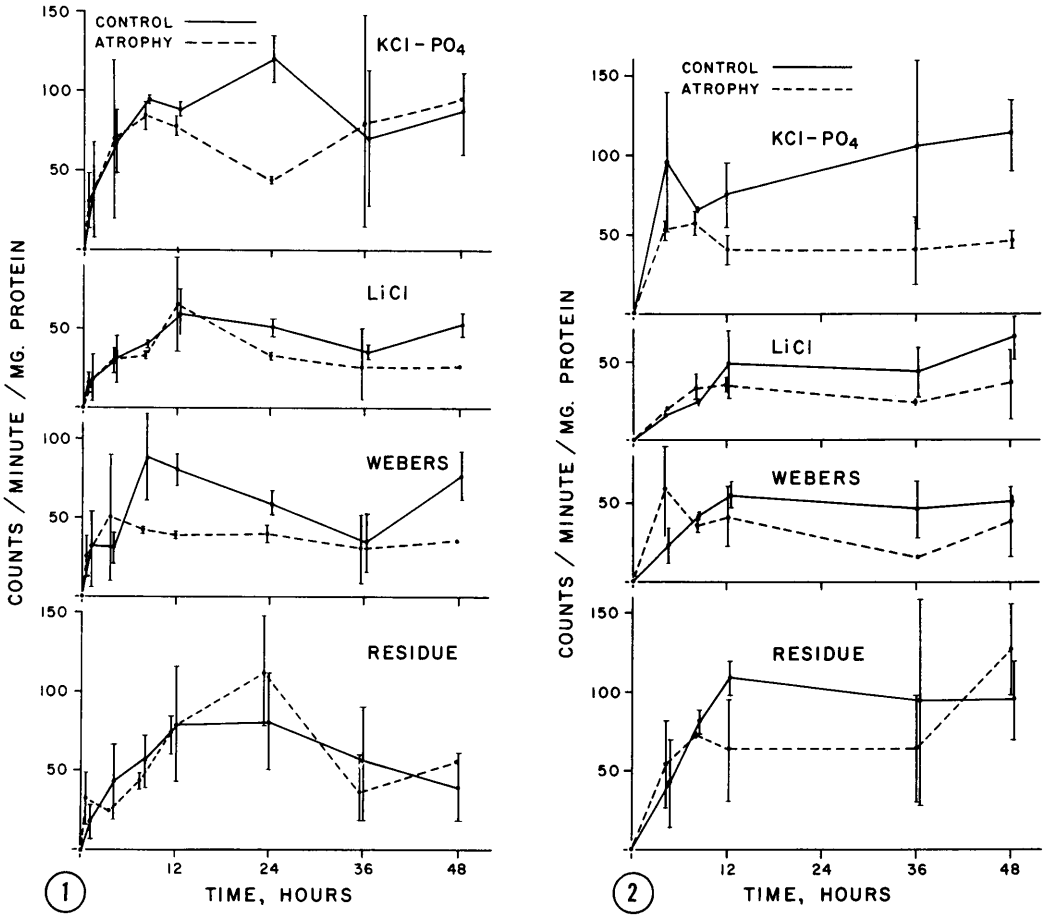


FIG. 1. Specific activity in fractions of control and atrophying tibialis anterior muscles at various times after the administrations of $^{14}$C-glycine. KCl-$PO_4$, LiCl, and Weber's refer to fractions extracted with these solvents and RESIDUE to the fraction remaining after extraction.

FIG. 2. Specific activity in preparations of myosin from 3 soluble fractions from control and atrophying gastrocnemius muscles after administration of $^{14}$C-glycine, as well as that in the fraction remaining after extraction. KCl-$PO_4$, LiCl, and Weber's refer to fractions extracted with these solvents.

times, slices were removed, washed in Ringer's solution, homogenized and extracted for structural protein fractions as described above. Structural protein fractions were counted, as described, and activity was expressed as counts per minute per mg protein.

*Results.* Specific activity as a function of time for each of the structural protein fractions from atrophying muscle was compared to that of similar fractions from control muscles. In some cases there was a great deal of variation between corresponding fractions from the duplicate muscles. Where excessive variation was observed, especially 1, 4 and 36 hours, most of the values were lower for one rat than for the other, both in the atrophic and the control muscle. This is presumably due to differences in efficiency of injection or absorption of the label.

The general pattern was one of a rapid uptake of label into all fractions, followed by either a slower increase, a levelling off or slow decrease in activity depending on the fractions and muscle of origin (Fig. 1, 2). Decreases were most apparent in the tibialis anterior fractions (Fig. 1). In all cases, except for the KCl-$PO_4$-extracted samples of myosin, the fractions from atrophying muscle showed equal or greater initial incorporation than the corresponding control fractions.

After 8-12 hours, however, the control muscle fractions maintained or increased their activity levels while the specific activity of the atrophic fractions tended to decrease. After 12 hours, specific activity of each control fraction was generally higher than that of corresponding fractions from atrophying muscle. An exception to this generalization is the tibialis anterior residue fraction where the atrophic muscle showed higher activity at 24 hours.

The 3 solvents extracted proteins with different patterns of activity. In all 3 structural protein preparations, and in both control and atrophying muscles, the KCl-$PO_4$-extracted fractions had the most rapid uptake of label and continued to have the highest level of activity over the time period covered by the experiment (Fig. 1, 2). Incorporation differences between the Weber's and LiCl extracts were not marked. In both the atrophying and the control tibialis anterior samples, the Weber's fractions showed an earlier and higher incorporation and an earlier loss of label than the corresponding LiCl fractions (Fig. 1). In the other series no consistent differences in labelling of proteins extracted in LiCl and in Weber's solution were apparent.

The residue fraction was included for comparison in the tibialis anterior and myosin preparations on the basis that it might be metabolically related to the other fractions extracted from the same muscles, although its protein composition is unknown. In the atrophying tibialis anterior muscles the residue fraction showed a slow uptake of label but eventually reached the highest level of activity (Fig. 1). In the control fractions from these muscles specific activity of the residue fractions was intermediate between the KCl-$PO_4$-extracted fraction and the LiCl and Weber's fractions both in rate of uptake and eventual concentration of label (Fig. 1, 2).

Comparison of the 4 fractions obtained in the myosin preparations, when plotted together for the atrophying and control muscles, respectively (from Fig. 2 data), indicated that activity in soluble fractions from control and atrophying muscle did not demonstrate any

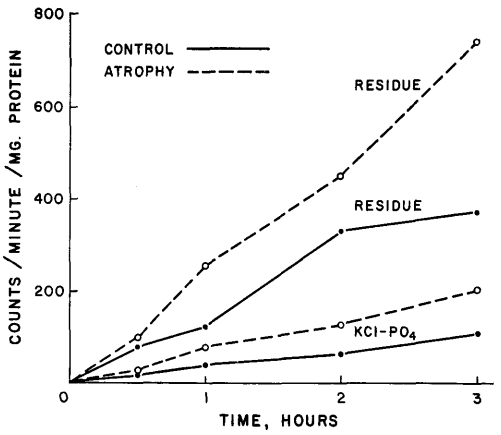


FIG. 3. Specific activity as a function of time of structural proteins from slices of control and atrophying tibialis anterior muscle incubated with $^{14}$C-glycine. KCl-$PO_4$ refers to proteins extracted with this solvent; RESIDUE refers to the insoluble protein remaining after additional extractions with LiCl and Weber's solutions.

precursor-product relationships. In the case of control muscle, however, there appeared to be an inverse relationship between the KCl-$PO_4$-extracted and residue fractions after 4 hours, such that the curves crossed at 2 points.

Active protein synthesis occurred in muscle slices. High levels of specific activity were observed in the KCl-$PO_4$-extracted fractions and the residue fractions. Yields and activities of proteins extracted by LiCl and Weber's solutions were too low to provide useful information. In the case of the KCl-$PO_4$-extracted and residue fractions, protein synthesis in atrophying muscle was significantly greater than that in control muscle. Results of a representative experiment are shown in Fig. 3.

*Discussion.* In the *in vivo* study the curves of specific activity indicate that the 3 extractable fractions obtained in each of the series of structural protein preparations represent different populations insofar as rates of synthesis and degradation of proteins are concerned. In the cases of the tibialis anterior preparations, this might be explained on the basis that the 3 solvents extracted varying proportions of the different proteins undoubtedly present in these preparations. The myosin preparations, however, should have been pure enough for these results to re-

present non-homogeneity within the protein itself.

On similar grounds, while the probability of varying protein populations being present within the different fractions of the tibialis preparations precludes the evaluation of possible precursor-product relationship between them, such a consideration is probably justified in the case of the myosin fractions. Since it was prepared from the same muscle and might be related metabolically to the extractable fractions, the residue fraction is also treated in this context, although its myosin content is unknown. The results obtained in the myosin series indicate that the 4 fractions turn over independently in the atrophying muscles, that is, there is no apparent progression of label from one fraction to another. In the control muscles, although the extracted myosin fractions again appear to be independent, there may be a precursor-product relationship between the KCl-$PO_4$-extracted and residue fractions. This possibility needs further investigation. The lack of evidence for precursor-product relationships among the extracted fractions may be a function of the limited time span of the experiment. If, for example, the myofibril were synthesized in layers, it would require some time before a recently synthesized portion would become incorporated into the interior. The solubility of a fraction might reflect its position in the myofibril, in which case the time required for movement of label from one fraction to another could be longer than the duration of this experiment. In any case the evidence obtained over the time course of this experiment does not exclude independent breakdown of any of the extractable fractions studied, and thus, any of these could serve as a suitable substrate for further investigation of mechanisms of degradation.

In regard to the differences shown in this experiment between control and atrophic muscles, with reference to mechanisms of degradation in atrophy, the reservations made above concerning the tibialis anterior preparations probably need not be made. In this case, comparisons are being made between fractions extracted by the same solvent, and, thus, presumably of the same protein population. There is evidence, both from the work of other investigators(8,9) and from the fact that some of the fractions in this experiment showed an increase in activity in the 36-48 hour period, that, under the conditions present here, labelled amino acids of relatively high specific activity were available for incorporation into muscle throughout the time period studied. Thus, the curves of specific activity obtained from these studies represent the sum of more than one process and cannot be regarded as simply reflecting incorporation and loss of label in one cycle of a system.

In every case but one, the early incorporation of label into fractions from atrophying muscle was equal to, or somewhat greater than, that in similar control preparations. This indicates that the ability to synthesize protein in the various fractions of atrophying muscle is not impaired, a result confirming those of earlier experiments (1,2). At later periods after isotope administration, however, the atrophying muscle preparations showed, with one exception, lower levels of activity than the control muscles. In every case where this occurred, except for the LiCl fraction extracted from the tibialis anterior muscle, there was a time period where the control muscle was gaining activity while the atrophying was losing label or maintaining a constant level. This is difficult to explain since the gain in activity in the control muscles implies that the specific activity of the available amino acid pool was higher than that of the already synthesized muscle protein. Under the condition of continuing synthesis in the atrophying muscle—as the evidence presented above indicates to be the case—it is, thus, impossible to explain the loss or even the levelling off of specific activity unless it is assumed that the atrophying muscle is synthesizing from a pool of lower activity than the control muscle or that protein in the atrophying muscle is selectively degraded. That is, recently synthesized protein with high specific activity is preferentially removed or degraded. Evidence from a previous study(2) and the unlikelihood of muscle amino acid pools with different specific activities in the same animal

favor selective degradation as a more probable explanation.

It can be concluded from the *in vivo* studies that loss of structural protein in denervated rat skeletal muscle is a result, not of decreased synthesis, but of accelerated degradation, as has previously been indicated (1,2). The mechanism of this degradation remains to be elucidated.

The observation that structural protein synthesis in slices of atrophying muscle was greatly increased over synthesis in control slices, while, *in vivo,* similar fractions from atrophying muscle showed only a slight, or no increase in early label incorporation over that in control muscle may be of use in understanding the relationship between function and protein synthesis. It would appear from the slice data that factors, possibly including a larger amino acid pool (10), required for protein synthesis are at a higher concentration or are operating more efficiently in atrophying muscle when both control and atrophying muscle are not functioning. This is presumably a compensation for accelerated protein degradation. In the animal, however, function in control muscle might result in an acceleration of the protein synthesizing apparatus so that the level of synthesis approaches that in the non functioning atrophying muscle. An alternative explanation is that *in vivo,* selective degradation of recently synthesized protein occurs in atrophying muscle, as proposed above, lowering the specific activity to the approximate level of control muscle; and that such a difference in degradation does not occur in slices when both control and atrophying muscle are non-functional.

*Summary.* Studies were made of the incorporation of a radioactive amino acid into various fractions of rat skeletal muscle structural proteins *in vivo* and *in vitro,* in order to compare protein turnover in different fractions within a muscle and to characterize the differences in protein turnover between similar fractions from denervated atrophying, and control muscles. The *in vivo* studies indicated that fractions of structural protein extracted by different solvents had different patterns of incorporation and loss of label. No precursor-product relationships were found among the 3 extracted fractions of myosin. The *in vivo* study also revealed, in general, an uptake of label into fractions from atrophying muscle equal to, or greater than, that of the comparable control muscle fractions, followed by decreasing levels of activity in the fractions from atrophying muscle while activity remained higher in similar fractions from control muscle. Studies with muscle slices showed a markedly greater rate in synthesis of structural proteins during atrophy.

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