In vivo uptake of [^{14}C]leucine by skeletal muscle ribosomes after injury in rats fed two levels of protein*

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1. After 14 days on a diet containing 5 or 25% casein male rats received a fracture of the left femur. Four hours before they were killed the injured and control rats were injected with [1-^{14}C]leucine; the incorporation of radioactivity into an isolated fraction of skeletal muscle ribosomes was studied 6, 12, 24, 48, 72, 96 and 228 h after injury.

2. The incorporation of [^{14}C]leucine into the ribosome fraction in right thigh muscles dropped to 40% of control values 72 h after fracture in well-nourished rats and after 96 h with diets containing 5 or 25% casein.

3. The specific activity of the trichloroacetic acid-soluble fraction of muscle from injured rats was equal to or higher than that of the controls during the first 72 h but lower at 96 h.

4. These results suggest that a reduced incorporation of amino acids by ribosomes from the right thigh muscle occurred on day 3 after fracture in the group receiving 25% casein but not in the group receiving 5% casein.

5. Muscle RNA and DNA concentrations were not affected by the injury.

6. The relationship between these findings and the loss of muscle N after injury is discussed.

Urinary nitrogen increases after physical injury and may not return to normal for several days in the rat (Campbell & Cuthbertson, 1967) or for several weeks in man (Cuthbertson, 1964). This loss of body N is probably due to a general catabolic effect rather than to damage in local tissues, but the source and mechanism of the loss are still under investigation.

Whenever protein intake is inadequate, the liver, pancreas and intestinal wall lose protein rapidly (Munro, 1964); after injury to protein-deficient rats the catabolic response is reduced or absent (Munro & Chalmers, 1945). While attempting to determine the significance of these phenomena, Calloway, Grossman, Bowman & Calhoun (1955) found that liver protein content does not decrease after burning, and Fleck & Munro (1963) found no change in the liver protein content of injured rats, as compared with controls, 7 days after leg fracture. These workers (Fleck & Munro, 1963) conclude that the protein dissipated after injury must come from other sites, possibly muscle. Cuthbertson (1964) supports this conclusion in reference to man.

Levenson, Braasch, Mueller & Crowley (1959) studied the uptake of [^{15}N]glycine in the various tissues of burned rats at the height of negative N balance, 12 h after intake of the labelled amino acid. They found that incorporation of the label into tissues of burned rats was equal to or greater than that obtained with control animals

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and concluded that, at least for the post-injury period examined, the loss of body N could not be solely the result of a reduced rate of synthesis of a tissue protein. Schønheyder, Heilskov & Olesen (1954) conclude that the loss of N during immobilization results from diminished synthesis, while the catabolic rate remains normal.

The studies reported here evaluate the effect of leg fracture on the incorporation of [^{14}C]leucine by a ribosome fraction of skeletal muscle and the influence of previous dietary protein intake on the metabolic response.

EXPERIMENTAL

Animals

Male rats (CD strain obtained from the Charles River Breeding Laboratories, North Wilmington, Massachusetts, USA) were housed in air-conditioned quarters at about 24°. They were kept in individual metal cages and fed on a commercial chow for 2 days before the experiments began. The rats were then divided into two groups of equal weight, and each group was given one of the two diets. The compositions of the diets are given in Table 1.

Table 1. *Composition (% by weight) of high- and low-protein diets*

Ingredient	High-protein	Low-protein
Casein	25·0	5·0
L-Methionine	0·3	0·06
Dextrin	39·6	52·74
Sucrose	19·3	26·4
Wesson oil	10·0	10·0
Salt mix*	5·0	5·0
Vitamin mix*	0·5	0·5
Choline†	0·3	0·3

* For compositions see Young, Chen & Newberne (1968).
† Added to the diet as a water solution of choline chloride containing 5 g/ml.

Bone fracture

During the operation all rats were under anaesthesia (sodium pentobarbital, 5 mg in physiological saline per 100 g body-weight, given intraperitoneally). An incision of about 0·5 cm was made over the lateral area of the left thigh, and a blunt dissection made down to the femur. The femur was fractured with a haemostat in the mid-diaphysis region with a moderate amount of trauma to the adjacent muscle groups. The skin wound was closed with one stitch, and no wound became septic. Control rats were anaesthetized during the operations on the injured rats.

Preparation of muscle

Rats were killed by decapitation and exsanguination. The skin was then removed from the hind-leg region, and mixed muscle taken and cleaned of visible fatty tissue. Muscle samples from fractured and unfractured legs from the experimental groups were treated separately. Care was taken to avoid the visible haemorrhagic area

in the vicinity of the wound of the fractured thigh. Muscle from rats in each experimental group was pooled and immersed in ice-cold buffer A of the following composition: 0·25 M-KCl, 0·01 M-$MgCl_2$ and 0·01 M-Tris-HCl (pH 7·6). The temperature for all subsequent operations was 4°.

The muscle ribosome fraction was prepared according to a procedure modified from one described previously by Chen & Young (1968). Six grams of pooled muscle were homogenized in 2 vol. of buffer A in a Polytron homogenizer (Kinematica G.m.b.H., Lucerne, Switzerland). The homogenate was then centrifuged in the rotor 30 of a Spinco Model L ultracentrifuge for 15 min at 19600 ***g*** (average), and the supernatant fraction filtered through two layers of nylon cloth (Nitex-230T; Lambert Company, Boston, Massachusetts, USA) wetted with the buffer solution. Sodium deoxycholate and Lubrol WX (I.C.I. Organics, Providence, Rhode Island, USA) were added to bring the final concentrations to 1 and 0·5% (w/v) respectively. The mixture was then shaken vigorously for 40 sec and left standing for 10 min. The ribosomes were precipitated by the addition of 3 vol. of 0·01 M-$MgCl_2$ and 0·01 M-Tris-HCl (pH 7·6) buffer to the detergent-treated supernatant fraction (Chen & Young, 1968). After 15 min of precipitation the tube was centrifuged for 10 min in the rotor 30 at 8700 ***g*** (average). The post-ribosomal supernatant fraction was saved, and the ribosome pellet gently resuspended in a buffer of 0·006 M-KCl, 0·01 M-$MgCl_2$ and 0·01 M-Tris-HCl (pH 7·6) and recentrifuged for 15 min at 8700 ***g*** (average) in the rotor 30. The pellet was finally resuspended in buffer A, and a portion layered over a 5 ml cushion of 0·5 M-sucrose prepared in a medium A and then centrifuged for 1 h at 105000 ***g*** (average) in the rotor 40. The tube was drained, and the pellet washed with 2 ml of buffer A. Protein in the post-ribosomal supernatant fraction was precipitated with cold trichloroacetic acid (TCA). The TCA-soluble fraction was saved for N and radioactivity determinations.

The ribosome pellets were dissolved in 1 N-NaOH, and a 0·5 ml portion was added to an equal volume of hydroxyhyamine. The mixture stood at room temperature for 60 min, after which the pH of the solution was adjusted to 4–6 with 1 N-HCl. Fifteen millilitres of Bray's (1960) solution were then added, and the sample was counted in a Packard liquid scintillation spectrometer (Packard Instrument Company, Downers Grove, Illinois, USA). This counting procedure was also used for the TCA-soluble fraction. Using a [^{14}C]toluene standard, the counting efficiency was determined on each sample.

Chemical analyses of muscle

Samples of muscle were prepared for RNA and DNA analyses by the method of Munro & Fleck (1966), and RNA was estimated by the orcinol reaction. Yeast RNA (Sigma, type XI, purified) was used as the standard. DNA was quantitated according to the diphenylamine method of Giles & Myers (1965). The optimum conditions for muscle DNA hydrolysis were 70° for 45 min in 0·5 N-perchloric acid. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), and N by the Kjeldahl method.

Design of the experiment

A total of eighty-eight rats (mean initial weight, 173 g), divided into two groups of forty-four each, were used in this experiment. One group was given the 5% casein diet, and the other the 25% casein diet, for 14 days before the leg fracture. During the last 6 days of the period before fracture and throughout the remainder of the experiment, rats on the 25% casein diet were pair-fed to those on the 5% casein diet. Food intake remained at prefracture levels (12–19 g/day) during the postfracture period. Fracture of the left femur was performed on twenty-two rats from each group at 09·00 h on the 14th day. Three control and three injured rats from each group were killed 6, 12, 24, 48, 72 and 96 h later, and four rats from each group were killed 228 h (9·5 days) after fracture. [1-^{14}C]Leucine (specific activity 11·5mc/m-mole; 7 μc/100 g body-weight for all groups except those killed 12 h after fracture, which received 3·5 μc/100 g body-weight) was given intraperitoneally 4 h before death. A 4 h period was chosen since the peak specific activity of the muscle ribosome fraction was observed at this time in preliminary studies with weanling and young adult rats, weighing approximately 200 g. The results with young adult rats are given in Table 2 and may be compared with the period of 30 min found for liver by Littlefield, Keller, Gross & Zamecnik (1955).

Table 2. *Specific activity of the muscle ribosome fraction after [^{14}C]leucine injection in young adult rats*

(Male rats (weighing about 200 g each) were given intraperitoneal injections of 7·5 μc [1^{14}-C]leucine/100 g body-weight and killed at the intervals shown. Values are means of duplicate determinations on a pooled sample of muscle prepared from four rats in each group)

Time after injection (h)	Specific activity (dpm/mg protein)
3	1040
4	1500
6	900
8	1100
12	900

RESULTS

The body and liver weights of the control and injured rats given the 5 and 25% casein diets are given in Table 3 for the various times of killing after fracture.

Figs. 1 and 2 show the pattern of incorporation of radioactivity (disintegrations/min (dpm) per mg protein) into the ribosome fractions obtained from the unfractured- and fractured-leg muscle, respectively, throughout the experiment. During the first 48 h after fracture both dietary groups showed similar changes for the unfractured-leg muscle (Fig. 1). At 72 h incorporation of radioactivity in the injured rats given the high-protein diet was only 40% of that in the controls and on the 4th day it was reduced to about 50% of control values in both dietary groups; by the end of the experimental period (9·5 days) incorporation in the unfractured-leg muscle had

Table 3. *Mean body and liver weights at time of death of control and injured rats given high- and low-protein diets*

(In each of the groups studied at 6, 12, 24, 48, 72 and 96 h the mean values are for three rats; in each of the groups studied at 228 h the mean values are for four rats)

	25% casein diet				5% casein diet			
Time after fracture (h)	Control		Fracture		Control		Fracture	
	Body-wt (g)	Liver wt (g)	Body-wt (g)	Liver wt (g)	Body-wt (g)	Liver wt (g)	Body-wt (g)	Liver wt (g)
6	246	8·1	242	8·3	180	5·7	170	5·8
12	243	8·4	237	8·5	181	6·2	174	6·2
24	264	10·5	256	10·8	185	6·9	185	7·4
48	255	9·3	263	9·0	184	7·2	191	7·2
72	260	9·7	253	9·4	191	7·0	197	7·1
96	278	10·4	255	9·0	195	6·6	204	6·3
228	299	11·2	292	10·8	212	7·7	213	8·0

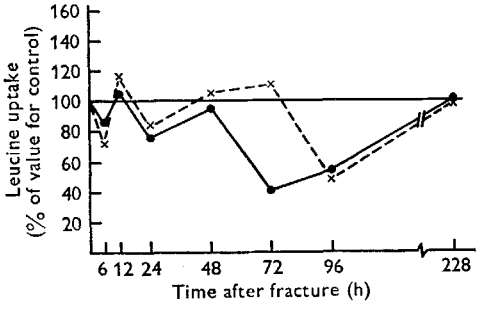

Fig. 1

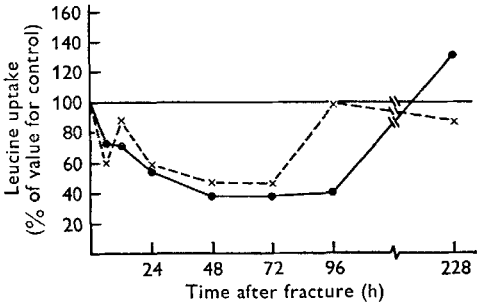

Fig. 2

Fig. 1. Uptake of [^{14}C]leucine in vivo by ribosomes in muscle from the right hind leg after fracture of the left femur in rats given a 5 or 25% casein diet. Values for the injured rats are percentages of values from dietary control groups of rats killed at intervals during the study. Mean values with their standard errors for the 5% casein control groups were 281 ± 48 dpm/mg ribosomal protein, and for the 25% casein control groups, 286 ± 41. ●—●, 25% casein; × - - - ×, 5% casein.

Fig. 2. Uptake of [^{14}C]leucine in vivo by ribosomes in muscle from the fractured thigh. As in Fig. 1, values for the injured rats are percentages of the control values. Further details are also shown in Fig. 1. ●—●, 25% casein; ×- - - -×, 5% casein.

returned to the level observed in the control rats. During the first 72 h after fracture incorporation into the muscle ribosome fraction from the fractured leg was consistently lower than in muscle from controls (Fig. 2). Incorporation returned to control values by the 4th day in rats given the low-protein diet, but remained low until at least 96 h after fracture in well-nourished rats. It had increased to about 30% above the control values in the well-nourished group at the end of this experiment.

The radioactivity in the TCA-soluble fraction of the post-ribosomal supernatant fraction was determined when the rats were killed; this was 4 h after injection of the isotope. The results are shown in Fig. 3 for this fraction of cell sap for muscle obtained from the unfractured leg; Table 4 summarizes the values for muscle obtained

from both legs. During the first 72 h after fracture, the specific activity of the TCA-soluble fraction in the unfractured leg muscle of injured rats was equal to or higher than that of muscle from control rats (Fig. 3).

On the 4th day after fracture the specific activity of the TCA-soluble fraction in the unfractured-leg muscle of injured rats from both dietary groups was reduced

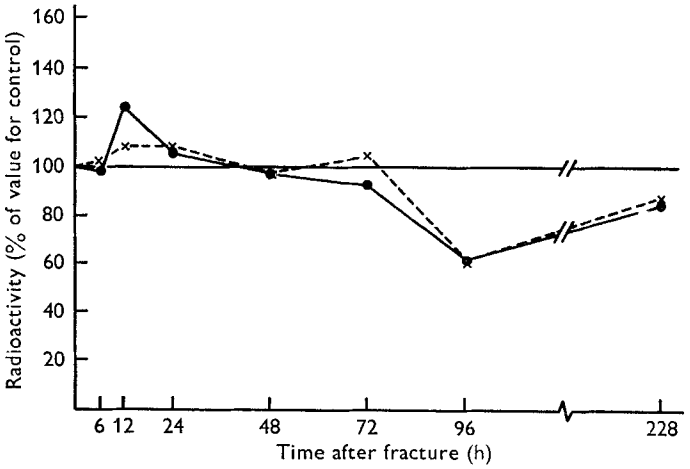

Fig. 3. Radioactivity of the TCA-soluble fraction of muscle cell sap obtained from the right hind-leg muscle after fracture of the left femur in rats given a 5 or 25% casein diet. Values are percentages of values from dietary control groups of rats killed at intervals during the study. These results are summarized in Table 4. ●—●, 25% casein; × - - - ×, 5% casein.

Table 4. *Radioactivity of the TCA-soluble fraction of muscle cell sap in hind-leg muscle from control rats and in fractured and unfractured legs from injured rats fed two levels of protein and killed at various times from 6 to 228 h after fracture*

(Values are given as dpm/mg N. Each is the mean of duplicate determinations on a pooled muscle sample from three rats in each of the 6, 12, 24, 48, 72 and 96 h groups and from four rats in each of the 228 h groups)

Group	6 h	12 h	24 h	48 h	72 h	96 h	228 h
			25% casein diet				
Control	340	170	350	360	340	340	456
Test-unfractured	340	210	370	340	320	210	388
Test-fractured	360	230	380	380	320	190	412
			5% casein diet				
Control	480	223	420	450	430	470	676
Test-unfractured	450	240	470	430	470	280	563
Test-fractured	520	300	520	480	470	590	547

to below that of control rats. The lowered specific activity 4 days after fracture in the ribosome fraction from unfractured thigh muscle, as shown in Fig. 1, may therefore be related to a reduced specific activity of the precursor amino acid pool. If changes in the activity of the TCA-soluble fraction in muscle reflect the changes in the specific activity of the precursor leucine pool, then it appears possible to correct

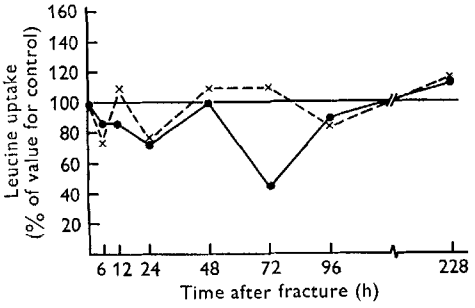

Fig. 4

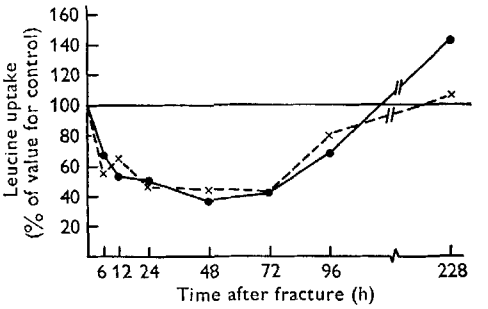

Fig. 5

Fig. 4. Uptake of [^{14}C]leucine by ribosomes in the right hind-leg muscle at intervals after fracture of the left femur in rats given a 5 or 25% casein diet, corrected for changes in the specific activity of the TCA-soluble fraction (see p. 276). Control values are given as (dpm per mg ribosomal protein)/(dpm per mg TCA-soluble N of muscle cell sap). Mean values with their standard errors for the 5% casein control groups during the study were $0{\cdot}68 \pm 0{\cdot}01$, and for the 25% casein control groups, $0{\cdot}91 \pm 0{\cdot}13$. Values for injured rats are percentages of the values from the dietary control groups of rats killed at intervals. ●—●, 25% casein; × - - - ×, 5% casein.

Fig. 5. Uptake of [^{14}C]leucine by ribosomes in muscle from the fractured thigh of rats given a 5 or 25% casein diet, corrected for changes in the specific activity of the TCA-soluble fraction (see p. 276). Values for the injured rats are percentages of the values from the dietary control groups of rats killed at intervals during the study. Further details for the controls are given in Fig. 4. ●—●, 25% casein; × - - - ×, 5% casein.

Table 5. *RNA and DNA concentrations in hind-leg skeletal muscle after leg fracture in rats fed two levels of protein and killed at various times from* 6 *to* 96 *h after fracture*

(RNA and DNA values are given as mg/g wet weight of muscle. Each is based upon duplicate analyses on pooled muscle from three rats in each group)

Group	6 h	24 h	48 h	72 h	96 h
		25% casein diet			
Control					
RNA	2·12	2·10	2·07	2·11	2·14
DNA	0·36	0·35	0·41	0·38	0·35
RNA:DNA	5·9	6·0	5·0	5·6	6·1
Test-unfractured					
RNA	1·98	2·05	2·10	2·10	2·35
DNA	0·35	0·38	0·34	0·38	0·39
RNA:DNA	5·7	5·4	6·2	5·5	6·0
Test-fractured					
RNA	2·09	2·14	2·16	1·74	1·97
DNA	0·40	0·40	0·45	0·36	0·36
RNA:DNA	5·2	5·4	4·8	4·8	5·5
		5% casein diet			
Control					
RNA	1·60	1·61	1·70	1·64	1·64
DNA	0·35	0·34	0·35	0·36	0·33
RNA:DNA	4·6	4·7	4·9	4·6	5·0
Test-unfractured					
RNA	1·43	1·34	1·98	1·61	1·64
DNA	0·29	0·30	0·42	0·34	0·35
RNA:DNA	4·9	4·5	4·7	4·7	4·7
Test-fractured					
RNA	1·63	1·49	1·83	1·62	1·66
DNA	0·37	0·34	0·40	0·37	0·36
RNA:DNA	4·4	4·4	4·6	4·4	4·6

for these changes by dividing the ^{14}C incorporation into the ribosome fraction (expressed as dpm/mg protein in the fraction) by the activity in the TCA-soluble fraction (expressed as dpm/mg TCA-soluble N). The results of these calculations for the unfractured and fractured legs are shown in Figs. 4 and 5, respectively. The results shown in Fig. 4 suggest that a decreased rate of protein synthesis occurred on the 3rd day after fracture in well-nourished rats. Furthermore, on this basis amino acid incorporation by the ribosomes from muscle of injured rats given the low-protein diet did not differ from that in control rats (Fig. 4) on either the 3rd or 4th day after fracture. By 9·5 days after fracture the in vivo ribosomal incorporation of [^{14}C]leucine by injured rats was approximately equal to that of control rats in both dietary groups.

The RNA and DNA concentrations and RNA to DNA ratios of muscle from both dietary groups are summarized in Table 5. Muscle RNA content and RNA to DNA ratio were higher in rats given the 25% casein diet than in those given the 5% casein diet. No consistent effect on muscle RNA concentration in the various groups resulted from fracture of the femur.

DISCUSSION

Because the body N loss after a severe injury often exceeds the amount of N in the liver (Cuthbertson, 1964) and because liver contents of protein and RNA do not fall after injury (Calloway *et al.* 1955; Fleck & Munro, 1963), the increased urinary N appears to be extrahepatic in origin. Muscle contains a major proportion (40–50%) of the total body protein, and Levenson *et al.* (1959) report that the protein content of the muscle decreases after injury and that this decrease accounts mathematically for most of the extra urinary N loss. Candlish & Chandra (1967) found a decreased concentration of protein in thigh muscle 4 days after incision injury to rats. Fleck & Munro (1963) also suggest that muscle may be a major source of the increased urinary N; however, the biochemical events in muscle associated with the loss of body N remain unclear.

Cuthbertson (1964) suggested that an increased excretion of adrenal glucocorticoids after stimulation of the anterior pituitary may result, in part, in an accelerated rate of protein catabolism. However, glucocorticoids reduce amino acid incorporation into muscle protein (de Loecker, 1966; Wool & Weinshelbaum, 1960*a*, *b*); it follows, therefore, that a reduced rate of protein synthesis may occur in muscle tissue after injury. Alterations in the rates of both synthesis and catabolism of muscle protein may contribute to the increased urinary N loss after injury.

Our findings on amino acid incorporation into the muscle ribosome fraction suggest that a reduced rate of protein synthesis occurred in the skeletal muscle of the unfractured leg 3 days after injury in well-nourished rats. These results extend our earlier observations (Young, Chen & Newberne, 1968) and those of Lust (1966) which demonstrate that a reduced rate of muscle protein synthesis in rats occurs during systemic infection, which also causes a loss of body N. In our studies of infection, incorporation of [^{14}C]leucine into muscle ribosomes was also lowest on

the 2nd and 3rd days after infection (Young, Chen & Newberne, 1968). Levenson *et al.* (1959) have reported that ^{15}N incorporation occurs to about the same extent in various tissues from burned and control rats when [^{15}N]glycine is fed at the height of negative N balance. Assuming that the changes in the specific activity of the TCA-soluble fraction are representative of changes in the specific activity of the leucine precursor pool, our results suggest that muscle protein synthesis, as evaluated by the uptake of labelled amino acid by the muscle ribosome fraction, is probably not markedly reduced on the 2nd and 4th days after fracture in well-nourished rats. On days 3 and 15, but not on day 8, Babický, Kolář & Vyhnánek (1966) found a significantly lower uptake of ^{35}S, relative to the healthy controls, by the tail tendon of injured rats irradiated over the right knee.

The reduction of [^{14}C]leucine uptake by the ribosome fraction appears to be greater in muscle from the fractured leg (Fig. 5) than in that from the unfractured leg (Fig. 4). The reduced uptake was maintained during the first 4 days after fracture in both dietary groups (Fig. 5). A marked loss of potassium and gains in sodium and chloride occur in traumatized muscle (Cuthbertson, 1964). The greater inhibition of [^{14}C]-leucine uptake by the ribosomes in muscle of the injured leg compared with that in the opposite leg muscle may be related to a more severe electrolyte change within the muscle cells of the injured leg.

Injury appeared to reduce the uptake of [^{14}C]leucine into the muscle ribosome fraction in the unfractured leg to a greater extent in rats given the 25% casein diet than in rats given the 5% casein diet; this may be related to the lowered catabolic N response observed after injury in rats given the low-protein diet (Calloway *et al.* 1955; Fleck & Munro, 1963). However, the metabolic basis for the differences observed in the two dietary groups and the biochemical events associated with the reduced uptake of leucine by muscle ribosomes after injury require detailed study. The RNA concentration of the unfractured thigh muscle was not affected by the injury in the fractured leg. Because the major proportion of cellular RNA appears to be ribosomal RNA (Blobel & van Potter, 1967; Zak, Rabinowitz & Platt, 1967) our observations suggest that the total ribosome population of muscle was not reduced as a result of injury. However, either the balance between polysomes and the lighter ribosome species or the intrinsic activity of the polyribosomes may have changed after injury. Changes of this nature have been observed in liver (Wunner, Bell & Munro, 1966) and muscle (Young, Chen & Macdonald, 1968) under various dietary conditions.

Our results, as well as those of others, emphasize the complex pattern of tissue reaction to injury and indicate the need for more study of the biochemical events underlying the catabolic body N loss. It does not yet appear possible to identify the extent to which a change in muscle protein synthesis or catabolism accounts for the increased urinary N observed during the post-injury period.

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