Protein degradation in human skeletal muscle tissue: the effect of insulin, leucine, amino acids and ions

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Summary

1. The protein degradation rate of human skeletal muscle was evaluated *in vitro* in isolated fibre bundles from the rectus abdominus muscle by measuring the tyrosine released from muscle tissue proteins. Protein metabolism in this semi-intact preparation was compared with that of the intact extensor digitorum longus muscles from rats under the same experimental conditions.

2. Protein balance was negative in both preparations, but protein synthesis and degradation were two to three times higher in the rat muscles. Tyrosine was released at a constant rate for at least 3 h of incubation independent of whether protein synthesis was inhibited or not. Disintegration of the muscle fibres more than doubled the tyrosine release rate. Human red gastrocnemius muscle showed 37% higher degradation rate compared with the predominantly white rectus abdominus muscle. The half-life of human skeletal muscle protein *in vitro* was estimated to be 20 days when calculated from the rate of tyrosine release.

3. The addition of leucine to the incubation medium decreased the rate of protein degradation, which was further decreased by the addition of other amino acids. Insulin did not influence the protein degradation rate during 2 h of incubation. This did not reflect a lack of sensitivity to insulin of the preparation, since protein synthesis responded to insulin. Calcium (5 mmol/l) stimulated and zinc (0·1 mmol/l) inhibited the protein degradation.

4. This experimental system may be suitable as an additional tool for evaluating protein degradation in human skeletal muscles.

Key words: insulin, leucine, protein degradation, skeletal muscle.

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Introduction

An increasing body of information on the mechanism and the regulation of protein degradation has appeared (Goldberg & Dice, 1974; Schimke & Katunuma, 1975; Neurath & Walsh, 1976). Most of this information has been obtained from the studies of prokaryotic cells and of various experimental animal systems. In studies of the protein turnover in rat diaphragm and leg muscles it has been shown that starvation, branched-chain amino acids and insulin influence the degradation rate of muscle proteins (Buse & Reid, 1975; Fulks, Li & Goldberg, 1975; Li & Goldberg, 1976; Jeffersson, Li & Rannels, 1977). Very little is known about regulation of protein degradation in man. Previously, we reported increased fractional degradation rate of skeletal muscle proteins in cancer patients (Lundholm, Bylund, Holm & Scherstén, 1976). The experimental design in that study was based on prelabelling *in vitro* of proteins, thus giving degradative estimates in highly selective proteins (Lundholm & Scherstén, 1975b). This model may be representative only of proteins that rapidly turn over, and these are mainly of large molecular size, acidic and hydrophobic (Goldberg & Dice, 1974; Schimke, 1974).

In the present work we have applied the tyrosine-release system to determine protein

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degradation (Buse & Reid, 1975; Fulks *et al.*, 1975; Li & Goldberg, 1976) in human skeletal muscle fibres, and compared the results with the rat extensor digitorum longus muscle. The specific aim of the study was to determine if the human muscle preparation also responds to regulatory mechanisms affecting protein degradation; if so then the preparation may be suitable for the study in clinical conditions of where abnormalities of muscle metabolism occur.

Materials and methods

Reagents and isotopes

L-[U-^{14}C]Leucine (250 mCi/mmol) and L-[U-^{14}C]tyrosine (380 mCi/mmol) were from NEN Chemicals, West Germany. Recrystallized insulin was from Novo (Novo lot no. S-23267). A sterile amino acid solution consisting of 19 L-amino acids (asparagine was lacking) at the concentration of 100 times that of human plasma was kindly supplied by AB Astra (Södertälje, Sweden). All reagents were of analytical grade from Sigma Chemical Company, U.S.A.

Muscle biopsies

Biopsies from the rectus abdominus muscle and the calf muscle (gastrocnemius) were taken from 45 metabolically normal patients at operation for varicose veins, uncomplicated gallstone disease or peptic ulcer disease. The mean age of the patients was 57 years (range 35–72 years). The patients were fasted for 10–12 h before the operation and were premedicated and anaesthetized as described previously (Lundholm & Scherstén, 1977). The patients were given sodium chloride solution (150 mmol/l :saline) intravenously for 30–45 min before the muscle biopsy was taken by a standardized procedure (Lundholm & Scherstén, 1977). The patients had given their informed consent. No complications were encountered.

Preparation and incubation procedures

The biopsies were transported to the laboratory, prepared and incubated as previously described (Lundholm, Bylund, Holm, Smeds & Scherstén, 1975) with the exception that for transportation and preparation a buffer solution at 20–22°C was used. Ice-chilled buffer causes irreversible changes in protein metabolism (Lundholm & Scherstén, 1979). When ^{14}C-labelled amino acid incorporation studies were performed 200 mg of muscle fibre bundles was teased from the specimen and incubated for 2 h at 37°C in a medium (4 ml) consisting of Krebs–Ringer bicarbonate buffer solution (pH 7·4) supplemented with glucose (4·4 mmol/l) and with all the amino acids normally present in human plasma but at concentrations in multiples of the normal plasma concentration. The concentrations considered normal for human plasma have been described previously (Lundholm *et al.*, 1975). It was necessary to add tyrosine to a final concentration of 1·1 mmol/l to ensure a constant specific radioactivity of [^{14}C]tyrosine in the incubation medium during 2 h of incubation, irrespective of the concentration of the other amino acids. The concentration of ions and insulin in the incubation media is given in the Tables. In some experiments protein synthesis was estimated by measuring the tyrosine concentration in the incubation medium plus the tissue pool in paired incubates with and without cycloheximide. The difference between the tyrosine concentration in these incubates after incubation reflected the protein synthesis rate. This method is referred to as the non-isotope method.

In some experiments rat muscles were incubated for comparison with the teased human skeletal muscle preparation. The reasons for including animal experiments in this study were the following: initial studies had shown that our semi-intact muscle preparations were in negative nitrogen balance when buffer solutions were supplemented with amino acids, glucose and insulin at physiological levels. This could be due to the overnight fast of the patients before muscle biopsy, to poor integrity of the fibres in our cut-muscle preparation or to the preparation of thin fibre bundles from a biopsy specimen before incubation. Sprague–Dawley rats weighing about 130 g were used. The extensor digitorum longus muscle was chosen since this contains white fibres and thus may be comparable to the human rectus abdominus muscle, which is also a white muscle (Bylund, Holm, Lundholm & Scherstén, 1976). The rats had free access to Purina Chow diet and tap water before the experiments. The rat muscles were taken with care to avoid touching the muscle tissue and the incubation was started immediately. The incubated rat muscles weighed 47·5 ± 1·0 mg (mean ± SEM, n = 12). The measurements of tyrosine incorporation into proteins and tyrosine release from proteins were performed identically with the procedure described for the isolated human muscle fibre bundles. Although Goldberg has reported that the total intracellular pool is more suitable to use in the intact rat muscles (Li, Fulks & Goldberg, 1973), this pool is not a

suitable estimate of the immediate percursor pool for protein synthesis in our cut human-muscle preparation. The incubation media have been shown to be the best available approximations in these conditions *in vitro* (Lundholm & Scherstén, 1975a). Protein synthesis rates in rat muscle experiments were therefore calculated by dividing the specific radioactivity of tyrosine in the muscle proteins by the specific radioactivity in the incubation medium. Proteins were determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine albumin as the standard.

Determination of protein degradation rate

Human muscle fibres (100 mg) were incubated at pH 7·4 for 2 h at 0–3°C and at 37°C as duplicates in Krebs–Ringer bicarbonate buffer solution (3 ml), supplemented with glucose (4·4 mmol/l). In some experiments amino acids without tyrosine were added to the buffer. Cycloheximide was added to the incubation medium (130 μg/ml) to inhibit the recycling of tyrosine from protein degradation to protein synthesis. Net protein degradation rate was measured as the tyrosine release rate from proteins without cycloheximide in the incubation medium. Cycloheximide was selected for routine procedures because puromycin gave a higher blank value. At the end of the incubation the muscle fibres were homogenized in the incubation medium with an Ultraturrax homogenizer at 220 V (Janke and Kunkel, IK Werk, West Germany). Proteins were precipitated with 30% trichloroacetic acid to a final concentration of 10% and the tyrosine concentration was determined in the clear supernatant as described by Waalkes & Udenfriend (1957). The amount of tyrosine determined in this procedure was thus the sum of soluble tyrosine in the incubation medium and that in the incubated tissue pool. The release rate of tyrosine from degraded proteins was the total amount of tyrosine in the incubate at 37°C minus the total amount of tyrosine in the incubate from 0 to 3°C, given as nmol of tyrosine h^{-1} g^{-1} wet weight. This calculation is justified since tyrosine is neither synthesized nor metabolized in skeletal muscle tissue (Fulks *et al.*, 1975). The standard error of the method for determination of tyrosine (SE = $\sqrt{(di.^2/2n)}$, where *di.* is the difference between duplicate measurements) given as a percentage of the mean of all determinations was 1·2% calculated from 50 duplicate determinations at various experimental conditions. The standard error of the method for determination of protein degradation was 4·6%.

Measurements of [^{14}C]leucine incorporation were performed as described above for [^{14}C]-tyrosine, with the exception that the incubation medium contained all amino acids at 10 times the normal human plasma concentration (leucine 1·46 mmol/l).

Statistical methods

Two independent samples were compared by the non-parametric Mann–Whitney *U*-test and matched pairs by Wilcoxon's non-parametric test for matched pairs (Siegel, 1956). Matched pairs were selected by chance and muscle fibres in matched incubates always originated from the same tissue specimen from one individual.

Results

Nitrogen balance, evaluated as net tyrosine balance, was negative under all experimental conditions. This was also true for incubated intact rat muscles obtained from animals eating *ad libitum*. The ratios of tyrosine incorporated from the incubation medium to tyrosine release from proteins were 0·35 ± 0·01 (mean ± SEM) in human muscles as compared with 0·33 ± 0·02 in rat muscles under identical incubation conditions. Protein synthesis (40 ± 2 nmol h^{-1} g^{-1} wet wt., n = 8) and degradation rate (121 ± 3 nmol of tyrosine h^{-1} g^{-1} wet wt., n = 8) were, however, about three times higher in the rat muscles than in human muscles. The degradation rates in rat extensor digitorum longus muscles in the present study were the same as those reported previously by Libby & Goldberg (1978).

Protein balance for human muscles, evaluated as net degradation rate, was significantly improved ($P < 0·001$) in the presence of high amino acid and insulin concentrations in the incubation medium (Table 1). This improvement (53%) was mainly dependent on a significantly increased rate of protein synthesis (66%) while the protein degradation decreased by 20%. Estimating the protein synthesis rate with the non-isotope method gave values of the same magnitude as those obtained from the [^{14}C]-tyrosine-incorporation experiments in incubated human muscles. The non-isotope method for estimating protein synthesis has the advantage that it allows one to measure overall synthesis rates under incubation conditions where steady states in precursor pools are not achieved.

Tyrosine was linearly released from proteins for 3 h of incubation irrespective of whether peptide formation was inhibited or not (Fig. 1). The degradation rate of protein was almost the

TABLE 1. *Influence of amino acids and insulin on the protein balance in incubated rectus abdominus muscle*

The muscle fibres were incubated in Krebs–Ringer bicarbonate buffer solution (pH 7·4) for 2 h at 37°C. The incubation media were supplemented with glucose, amino acids, insulin and cycloheximide (130 μg/ml). Protein synthesis in this experiment was estimated as the difference in tyrosine concentration (incubation medium + intracellular pool) between paired incubates with and without cycloheximide. The validity of this estimation rests on the finding that cycloheximide did not significantly alter the degradation rate. Different incubates were set up with isolated fibres from the same biopsy specimen obtained from four individuals (mean ± SEM, $n = 8$). Significance of differences for all results, $P < 0.01$.

Incubation medium	Net protein degradation	Protein synthesis	Protein degradation in the presence of cycloheximide
	(nmol of tyrosine h^{-1} g^{-1} wet wt.)		
Buffer + glucose (4·4 mmol/l)	34 ± 2	9 ± 2	43 ± 1
Buffer + amino acids at 10 times plasma concn. + insulin (25 units/l)	16 ± 2	15 ± 2	34 ± 1

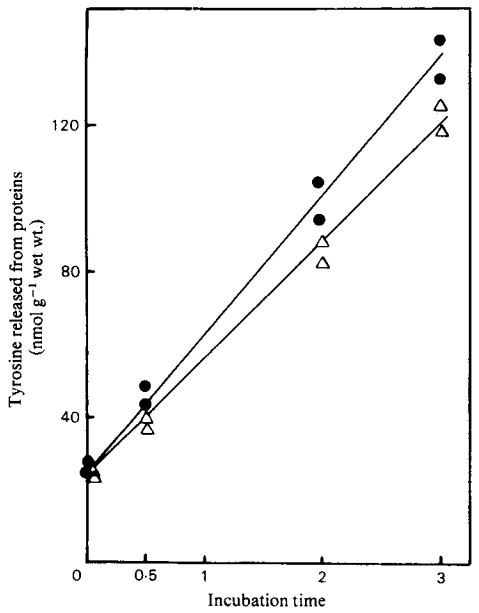

FIG. 1. Time course of tyrosine release from proteins during inhibited (●—●) and uninhibited (△—△) protein synthesis *in vitro*. Cycloheximide (130 μg/ml) was used to inhibit protein synthesis, Human skeletal muscle fibres (rectus abdominus) from one tissue specimen were incubated at 37°C in Krebs–Ringer bicarbonate buffer solution (pH 7·4) supplemented with glucose (4·4 mmol/l).

same if protein synthesis was inhibited by cycloheximide (130 μg/ml) or by puromycin (100 μg/ml) [i.e., 49 ± 2 ($n = 18$) and 47 ± 3 ($n = 18$) nmol of tyrosine h^{-1} g^{-1} wet wt. respectively]. This similarity was seen despite the fact that cycloheximide has been reported to inhibit hepatic protein degradation and gluconeogenesis in the perfused rat liver (Woodside, 1976).

Leucine added to the incubation medium at a concentration corresponding to 10 times that of human plasma (1·46 mmol/l) caused a small (7·5%) but statistically significant decrease in the protein degradation rate ($P < 0.005$; Table 2). This inhibitory effect could be further increased by addition of all other amino acids (except tyrosine). Insulin (25 units) had no effect on the degradation rate.

The degradation rate of protein was higher in calf muscle tissue (gastrocnemius) than in abdominal muscle tissue ($P < 0.01$; Fig. 2). The coefficient of variance was higher for estimated protein synthesis ([^{14}C]leucine incorporation) than for degradation (Fig. 2). The incorporation rate of leucine into proteins was about twice as high as that of tyrosine. This difference is in good agreement with the relative occurrence of these amino acids in human skeletal muscle proteins (Fürst, Josephson & Vinnars, 1972). Calcium ions at physiological concentrations (5×10^{-3} mmol/l) caused a significant increase and zinc ions (10^{-4} mmol/l) a significant decrease of the degradation of protein (Table 3). The degradation rate was not influenced by magnesium ions. Colchicine did not influence the degradation rate but ouabain seemed to cause an increase. Disintegration of the muscle fibres by homogenization caused a marked increase of the degradation rate.

Discussion

In this study the tyrosine-release system was applied to measurements of the protein degra-

TABLE 2. *Influence of amino acids and insulin on the degradation rate of human skeletal muscle proteins in vitro during inhibited protein synthesis*

Muscle fibre bundles from rectus abdominus muscle were incubated for 2 h at 37°C in Krebs–Ringer bicarbonate buffer solution supplemented with glucose and cycloheximide (130 μg/ml). Insulin and all amino acids, without tyrosine, were added at concentrations indicated in the table. All experiments were performed as matched pairs as described in the Materials and methods section. Results are mean ± SEM; $n = 9$. Wilcoxon's test for matched pairs was used for the statistical evaluation: $P < 0{\cdot}005$; *, not significant.

Incubation medium	Degradation rate of muscle proteins (nmol of tyrosine h^{-1} g^{-1} wet wt.)	Decrease of basal value (%)
Buffer with glucose (4·4 mmol/l) plus cycloheximide	40 ± 1·2	
+ Insulin (25 units/l)	40 ± 1·1	0*
+ Leucine (1·46 mmol/l)	37 ± 1·7	7·5
+ Amino acids at plasma concn.	35 ± 1·8	12·5
+ Amino acids at 10 times plasma concn.	32 ± 1·6	20
+ Amino acids at 20 times plasma concn.	27 ± 1·6	32·5

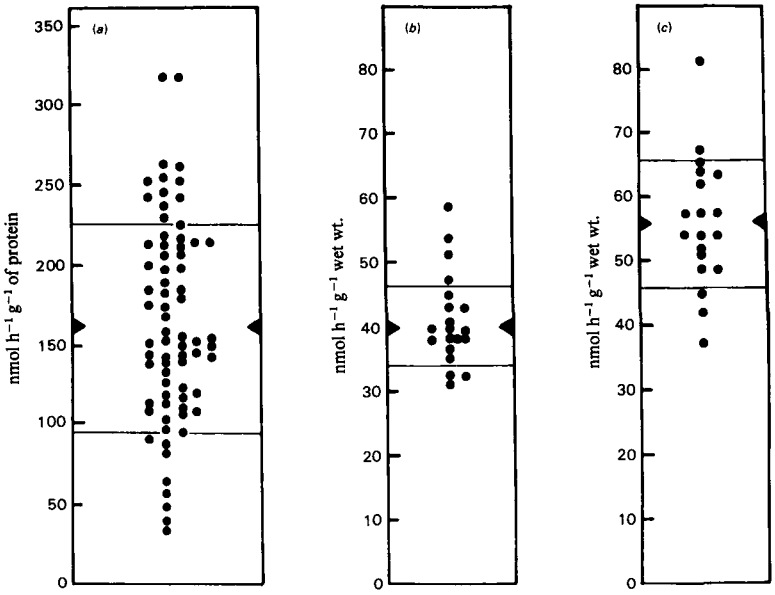

FIG. 2. Rates of leucine incorporation into human rectus abdominus muscle proteins (*a*), and the rates of tyrosine release from the same muscle proteins (*b*), and from calf muscle proteins (*c*) in 111 subjects. Each point represents one individual (mean ± SD).

dation in human skeletal muscles. The results show that leucine, amino acids and ions influenced the protein degradation rate *in vitro*, but insulin had no effect. These results are in agreement with our previous reports, but the experimental methods were different. The previous study was based on the prelabelling *in vitro* of protein and may be representative for proteins with rapid turnover only. However, the tyrosine-release method is probably more suitable, since it is more simple and based on fewer assumptions.The human muscle preparation was compared under identical incubation conditions with the white extensor digitorum longus muscle from rats weighing around 130 g. We do not have any information of the viability of the rat muscle preparation from rats of this size and it cannot be ruled out that muscles from rats weighing around 130 g are less suitable for experiments *in vitro* compared with those from rats weighing 45–85 g. The pronounced negative nitrogen balance in the rat muscles may have related to poor diffusion of oxygen in these larger muscles (47·5 ± 1·0 mg). It is, however, unlikely that this is the

TABLE 3. *Influence of various substances on tyrosine release and on protein synthesis in intact human skeletal muscle proteins during 2 h of incubation and in disintegrated muscle cells*

Muscle fibre bundles from rectus abdominus muscle and disintegrated cells were incubated for 2 h at 37°C in Krebs–Ringer bicarbonate buffer solution (pH 7·4) with glucose (4·4 mmol/l) and various substances at final concentrations as indicated. Protein synthesis was measured by [^{14}C]tyrosine incorporation. All experiments were performed as matched pairs as described in the Materials and methods section. Results are mean ± SEM; n = individuals, given in parentheses. Wilcoxon's test for matched pairs was used for statistical evaluation: $^{*}P < 0{\cdot}001$.

Incubation medium	Tyrosine release during 2 h of incubation (nmol/g wet wt.)	Protein synthesis rate (nmol of tyrosine h^{-1} g^{-1} wet wt.)
Buffer + amino acids at 10 times plasma concn. (tyrosine 1·1 mmol/l)		
Buffer without calcium chloride + cycloheximide (130 μg/ml)	62 ± 3 (8)	15 ± 3 (4)
+ Calcium chloride (5 mmol/l)	95 ± 3 (8)*	16 ± 3 (4)
+ Zinc chloride (0·1 mmol/l)	43 ± 6 (8)*	19 ± 4 (4)
+ Magnesium chloride (30 mmol/l)	60 ± 3 (8)	—
+ Colchicine (0·05 mmol/l)	58 ± 4 (8)	—
+ Ouabain (0·1 mmol/l)	70 ± 3 (4)	—
Buffer with glucose (4·4 mmol/l) and cycloheximide + disintegrated muscle fibres (homogenate)	152 ± 4 (6)*	—

main explanation, since incubated extensor digitorum longus muscles (around 10 mg weight) from mice (22–35 g body wt.) showed exactly the same degree of negative nitrogen balance as our human and rat muscles when the mouse muscles were incubated in a stretched condition (Frayn & Maycock, 1979). The viability of the human muscle preparation has previously been evaluated by measuring potassium gradients in the tissue during the incubation; the incubated fibres maintain high intracellular concentration of potassium during 4 h of incubation (250–275 μmol/g dry wt.) (Lundholm & Scherstén, 1979). The electron microscopic appearances of the fibres, the mitochondria and of the membranes were also normal after 4 h of incubation. Moreover, an initial intracellular oedema disappeared during the incubation (Lundholm *et al.*, 1975). These findings suggest that the fibres were not severely hypoxic.

Protein metabolism was about two to three times higher in the rat than in the human muscles. The patients had been fasted for 10–12 h before the muscle biopsy was taken, whereas the animals were fed *ad libitum*. However, the ratio between protein synthesis and degradation was found to be the same in these muscle preparations suggesting that the 10–12 h period of fasting in the patients was not the main reason for the negative energy balance in human muscles. The human muscles must necessarily be manipulated to a considerable extent before one has a suitable fibre bundle to incubate, whereas the intact rat muscle can be removed without touching the muscle tissue and the incubation can start immediately. Manipulation tends to increase degradation rates so other factors were obviously of greater importance in accounting for the negative nitrogen balance in these preparations. Intact hepatocytes are also in severe negative nitrogen balance when incubated in supplemented buffer solutions, but shift toward a positive nitrogen balance when they are transferred to cell-growing media containing growth factors (Seglen & Solheim, 1977; Seglen, 1978).

The mean degradation rate of proteins as estimated from the release of tyrosine from incubated human muscles in the presence of insulin and amino acids was about 34 nmol h^{-1} g^{-1} wet weight. This degradation rate corresponds to a half-life of approx. 20 days. This figure can be compared with the results reported by Halliday & McKeran (1975) from studies *in vivo* in man. They estimated the half-life of muscle proteins *in vivo* to be about 26 days in five subjects at steady-state conditions. This shows that the degradation rate in our muscle preparation was higher than the degradation rate *in vivo*, owing to as yet unidentified mechanisms. The half-life *in vivo* was about 46 days when estimated from the synthesis rates (15 nmol h^{-1} g^{-1} of muscle). This synthesis value was prob-

ably low, since the patients had fasted for 10–12 h and received saline infusion only before the biopsy was taken. Previously we reported that the synthesis rate was increased by 20% when the patients before biopsy had their insulin secretion stimulated by exogenous glucose infusion preoperatively (Lundholm & Scherstén, 1977).

We are well aware of the risk of misinterpreting quantitative data obtained with this model, but we think it can give valuable qualitative information. Support for this view comes from the finding that leucine and other amino acids regulated the degradation rate of proteins in accordance with previous studies on the intact rat diaphragm (Buse & Reid, 1975; Buse & Weigand, 1977).

On the basis of findings *in vitro* it has been suggested that the branched-chain amino acids exert a regulatory effect on protein turnover in skeletal muscles (Buse & Reid, 1975; Fulks *et al.*, 1975; Buse & Weigand, 1977). In the present study we found that the small inhibitory effect of leucine on protein degradation was more obvious in the presence of other amino acids. Whether this potentiation was just an effect of the other branched-chain amino acids or secondary to changes in energy metabolism, as suggested for isolated hepatocytes (Seglen & Solheim, 1977), cannot be decided. The mechanism for the inhibitory effect of amino acids on the degradation rate of proteins is also unclear, although it may be related to the oxidation of the branched-chain amino acids. It has been reported that infusion of the keto acid analogues of branched-chain amino acids exert a protein sparing effect on a whole-body basis as reflected by decrease in the urinary excretion of urea nitrogen (Sapir & Walser, 1977). Conversely, intra-arterial infusion of the keto analogue of leucine did not influence the release of lysine, tyrosine and phenylalanine from forearm muscles (Pozefsky & Walser, 1977), suggesting an unchanged rate of protein degradation in skeletal muscle.

Insulin did not influence the tyrosine release rate from protein in our short-term experiments (2 h). However, results have been contradictory in studies on normal animals. It seems necessary to use young rats (60–90 g) to get a big response in the degradation rate to insulin (A. L. Goldberg, personal communication). Moreover, protein degradation is affected by insulin only after 2–3 h of perfusion (Jefferson *et al.*, 1977). Thus it seems that insulin does not exert its effect rapidly on protein degradation as it seems to do on protein synthesis in muscles. The reported effect of insulin *in vivo* on human muscle protein metabolism (Pozefsky, Felig, Tobin, Soeldner & Cahill, 1969) may then relate to an increased protein synthesis with a decreased net degradation rate and a decreased fractional release of amino acids. The lack of response of the degradation rate to insulin was not simply due to lack of sensitivity of the human muscle preparation, since protein synthesis responded to the same degree in intact rat skeletal muscle and heart preparations (Fulks *et al.*, 1975; Lundholm & Scherstén, 1977).

The increased degradation rate of muscle proteins in the presence of calcium ions at physiological concentrations in the incubation medium is in line with the recent discovery of Ca^{2+}-activated proteinase(s) in striated muscles (Dayton, Goll, Stromer, Reville, Zeece & Robson, 1975; Stauber, Hedge & Schottelius, 1977). The inhibition of intralysosomal proteolysis in mouse liver and kidney lysosomes by zinc has also been reported (Mego, 1976). Here we found a marked inhibition of protein degradation by addition of zinc to the incubation medium. This inhibitory effect was probably not due to a non-specific effect on cellular viability, since the protein synthesis continued at control levels. It remains unclear whether calcium and zinc are of physiological importance in controlling protein degradation *in vivo*. Our results support the evidence that lysosomes in skeletal muscle cells are part of the sarcotubular system and in some way take part in the degradation of muscle protein (Dayton *et al.*, 1975; Lundholm & Scherstén, 1975b).

The rationale for testing the influence of colchicine on protein degradation in our system related to the role of lysosomes in the degradation of proteins. It has been reported that colchicine inhibits the induction of intracellular degradation of proteins in cultivated macrophages, probably by inhibiting lysosomal fusion and thus rendering ordinary digestible materials indigestible (Pesanti & Axline, 1975). However, we did not find any effect of colchicine on the degradation rate so that degradation may have a different mechanism in human skeletal muscle.

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