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The Influence of Electrical Stimulation *in vitro* on Protein Synthesis and other Metabolic Parameters of Rat Extensor Digitorum Longus Muscle

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1. Apparatus is described in which rat extensor digitorum longus muscle can be incubated in buffer under conditions of light tension and be subject to contractures induced by electrical stimulation *in vitro*. Under these conditions the tissue retains its weight, its content of potassium and size of the extracellular space at values similar to those *in vivo*. 2. Though uptake of glucose was enhanced on addition of insulin, there was little increase in glucose consumption on stimulation. Breakdown of glycogen and enhancement of lactate output were found on stimulation. 3. Incorporation into protein of several labelled amino acids was diminished during stimulation. Accumulation of [^{14}C]leucine was enhanced whereas that of glycine was decreased. 4. There were no very consistent changes in the content of free unlabelled amino acids during incubation with or without stimulation. Comparison of actual amino acid concentrations in tissue and incubation mixture with accumulation of ^{14}C-labelled amino acid indicated that full equilibration of the cell pool of amino amino acids with the medium is slow. 5. Substantial oxidation of several ^{14}C-labelled acids was observed. 6. The ATP content of the tissue declined a little during incubation and somewhat faster after a period of stimulation. 7. The results are discussed in relation to the way in which exercise can induce muscle hypertrophy.

Muscular hypertrophy occurs in response to exercise and is accompanied by an increase in the protein content (Lawrie, 1953; Helander, 1961). Histological examination after exercise reveals the presence of enlarged fibres (Goldspink, 1964). Denervation of skeletal muscle, on the other hand, is usually followed by muscular atrophy together with a decrease in the content of contractile protein (Stewart, 1962), but these changes can be partially prevented by daily electrical stimulation of the denervated tissue. Hypertrophy due to exercise can occur in both hypophysectomized and diabetic rats; it is thus not dependent on the presence of growth hormone or insulin (Goldberg, 1967*a*, 1968).

Muscular hypertrophy can also be brought about by other means, e.g. tenotomy of the synergistic muscles, which results in a 30% increase in the weight of rat soleus muscle within 4 days (Hamosh, Lesch, Baron & Kaufman, 1967), or constriction of the aorta, which produces hypertrophy of the left ventricle (Moroz, 1967). In both these cases hypertrophy is accompanied by an increase in the RNA content of the microsomal fraction, though apparently the specific activity of the RNA in promoting amino acid incorporation remains unchanged. Similarly, in the post-denervation hypertrophy of rat diaphragm, sucrose-gradient analysis of extracted RNA suggests that the considerable increase in the nucleic acid content is largely of ribosomal RNA (Manchester, Turner & Harris, 1970). Increasing the fluid load of the perfused heart also increases the rate of protein synthesis (Schreiber, Oratz & Rothschild, 1966), but it seems likely that at least in this instance the availability of oxygen to the tissue is a significant factor (Schreiber, Evans, Oratz & Rothschild, 1967).

With the isolated frog sartorius muscle Kendrick-Jones & Perry (1967) observed that electrical stimulation led to a gradual increase in incorporation of radioactive leucine into muscle protein. On the other hand Karpatkin & Samuels (1967) found over a shorter period a decrease in the incorporation of leucine into the protein of frog sartorius in response to more rapid stimulation, and Arvill (1967) found a similar decrease on stimulation of incorporation of leucine, valine and proline into the protein of rat levator ani muscle.

The extensor digitorum longus muscle of the rat provides a good example of a mammalian skeletal muscle that can be set up and stimulated electrically *in vitro*. It has the advantage over the intact

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diaphragm and levator ani muscle of being easily dissected in a form suitable for incubation free of extraneous tissue. We describe here apparatus in which the response of this tissue to electrical stimulation can be studied and report on the effects of stimulation on amino acid accumulation and incorporation into protein and on some aspects of glucose utilization.

METHODS

Incubation system

Medium. Muscles were incubated in Krebs–Ringer bicarbonate buffer (Umbreit, Burris & Stauffer, 1959), gassed with O_2+CO_2 (95:5). Unless otherwise specified, the medium contained glucose (2mg/ml), albumin (0.13%) and a trace of silicone antifoam. Where specified, amino acids were added to the medium at the concentrations present in rat plasma. These concentrations were determined by an amino acid analysis of the pooled plasma from six rats of the same type and size as those used as muscle donors, and are given in Table 7 in the column headed 'Initial concentration in medium'. Bovine plasma albumin was obtained from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K., silicone antifoam from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K., and amino acids (AnalaR of chromatography grade) from British Drug Houses Ltd., Poole, Dorset and Sigma (London) Chemical Co. Ltd., London S.W. 6, U.K.

Apparatus. In the earliest experiments muscles were incubated singly in 25ml conical flasks in 1ml of medium. In view of their deterioration under these conditions the tissue was maintained subsequently under light tension and this was achieved by use of the apparatus described in Fig. 1, which was also designed so as to be suitable for stimulation of the tissue. The muscle was tied with cotton by its shorter (knee) tendon to an electrode mounted in the base of a Perspex holder, which was placed in the centre of a water-jacketed tube (internal diam. 0.65cm) and rested on a sinter glass plate. The longer (toe) tendon was tied to shirring elastic, the tension of which was adjusted to keep the muscle at approximately resting length. The plastic holder served also to bring the electrode wires to the incubation medium, the first electrode being in contact with the muscle tendon, the second being in the medium. Fluid placed above the upper sinter plate could be kept up by virtue of the flow of O_2+CO_2 upwards through the apparatus, and, given the cross-section of the tube, 1ml of medium provided sufficient depth to keep the muscle entirely covered. The apparatus was comparatively simple to mount, kept the fluid continuously oxygenated and enabled addition of materials to be made easily during experiments.

Incubation procedure. Muscles were taken from fed 45–50g female rats of a Wistar strain bred in the department. They were killed by cervical fracture followed by decapitation. The extensor digitorum longus muscles were removed and rinsed in ice-cold medium. They were then mounted on the muscle holder and inserted into the incubation vessel as described in Fig. 1. Preliminary experiments indicated that muscles did not gain weight during incubation in this system, the muscles were therefore weighed at the end rather than the beginning of the incubation to minimize handling of the tissue beforehand. Stimulation was applied only to one muscle from each rat, the other acting as the resting control.

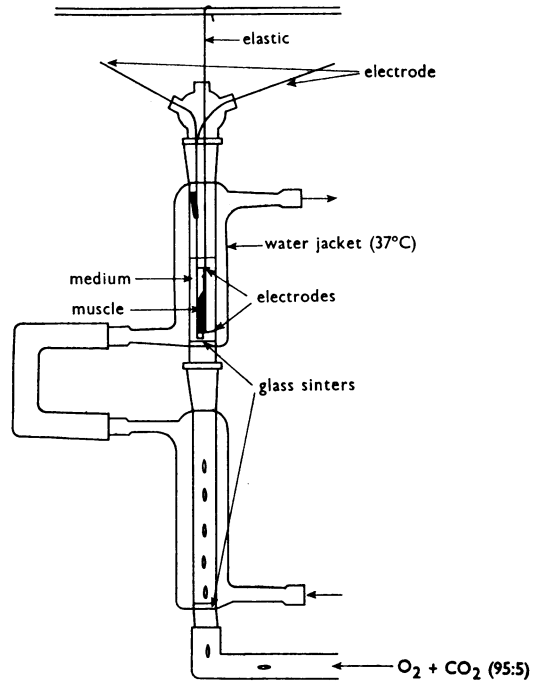

Fig. 1. The apparatus used for incubation of extensor digitorum longus muscle. The muscle is tied by its shorter (knee) tendon to an electrode mounted at the base of a Perspex holder. The longer (toe) tendon is tied to shirring elastic, the tension of which is adjusted to keep the muscle approximately at resting length. The holder is placed in the upper compartment of the vessel in 1ml of incubation medium, which is kept at 37°C by water circulating through the outer jacket. The medium is continuously gassed with O_2+CO_2 (95:5), that is pre-moistened and warmed by passage through water maintained at 37°C in the lower compartment of the vessel. The second electrode is not in contact with the muscle, but is mounted on the Perspex holder below the surface of the medium.

Muscles were stimulated by the passage of impulses of 0.6V of 10ms duration at a frequency of 23 impulses/min. The maximum tension in the muscle resulting from a pulse of 10ms duration appeared to be both greater and longer sustained than the maximal response to a force of 1ms duration, the response being similar to that resulting from a small tetanus brought about by the application of four pulses each of 0.5ms duration over a period of 10ms. Without direct measurement of the electrical changes in the muscle, it was not possible to show whether the apparent summated response was due to repeated contraction of the same fibres or to the reinforcement of the initial response by other fibres being called into play.

Measurement of carbohydrate utilization

Glucose uptake. Glucose uptake was measured as the disappearance of glucose from the medium. The small size of the muscles, together with the low uptake per unit weight, meant that the change in concentration of glucose in the medium was very small, and almost impossible to measure against a background concentration of 2mg/ml. The initial concentration of glucose in the medium for these experiments was therefore decreased to 1mg/ml. Glucose was determined by a modification of the glucose oxidase method in which *o*-tolidine is used as the oxygen acceptor (Marks, 1959).

Lactate. Lactate released into the medium was measured by a spectrophotometric method based on lactate dehydrogenase (EC 1.1.1.27) (Hohorst, 1963).

Glycogen. Muscles were each dissolved in a 1ml portion of hot 40% (w/v) KOH, after which the glycogen was precipitated by the addition of 2.5ml of ethanol in the cold. The glycogen was hydrolysed with 2M-HCl, neutralized with KOH and assayed as glucose with glucose oxidase.

Accumulation and incorporation into protein of radioactive amino acids

Muscles were incubated for 5min to recover from dissection. Radioactivity was then added in a small volume of medium, and, at the same time, electrical stimulation of one muscle of each pair was begun. At the end of the incubation period, each muscle was rapidly rinsed in non-radioactive medium, blotted, weighed and transferred to a glass homogenizer tube containing 1ml of 10% (w/v) trichloroacetic acid. The tubes were heated in a boiling-water bath for 5min then the contents were homogenized with a glass pestle and centrifuged. Samples of the supernatant, containing free amino acids, were taken for scintillation counting and for assay of potassium. The radioactivities of the incubation media were also counted. Radioactivity was measured in a Nuclear–Chicago liquid-scintillation spectrometer, in a scintillation mixture containing 4g of 2,5-bis-(5-*tert.*-butylbenzoxazol-2-yl)thiophen [CIBA (A.R.L.) Ltd., Duxford, Cambridge, U.K.], 80g of naphthalene, 400ml of methyloxitol and toluene to 1 litre. The usual sample sizes were 0.1ml of muscle extract and 0.02 or 0.05ml of medium per 10ml of scintillator. Counting efficiency was determined by the channels-ratio method and was about 63–68%.

The trichloroacetic acid-insoluble (protein) precipitates were prepared for radioactivity counting by a method similar to that of Manchester (1966). The residues from the above treatment were resuspended in fresh 10% (w/v) trichloroacetic acid and heated at 90°C for 15min to remove nucleic acid. The protein was dissolved in 0.4M-NaOH, any insoluble residue being discarded. After reprecipitation with trichloroacetic acid, the precipitates were washed with trichloroacetic acid and then with acetone. The protein samples were finally dissolved in 0.5ml portions of formic acid (98%) and poured on to weighed stainless steel planchets that were gently warmed until the formic acid evaporated. The planchets were then reweighed and the radioactivities counted on a Nuclear–Chicago gas-flow Geiger counter with a thin window. Counts were corrected for self-absorption and efficiency of the counter (about 25%) and incorporation of radioactivity into protein was expressed as d.p.m./mg of protein.

Radioactive amino acids were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. at the following specific radioactivities (Ci/mol): [1-^{14}C]glycine 5, L-[U-^{14}C]leucine 155, L-[U-^{14}C]phenylalanine 244, L-[U-^{14}C]proline 12.6 and α-amino[1-^{14}C]isobutyric acid 44.2.

Analysis of free amino acids in muscle and media

Amino acid analysis was carried out on the supernatants after homogenization at 0°C with 1.2ml of 3% sulphosalicylic acid of groups of eight similarly-treated muscles. Norleucine (0.1μmol) was added to each sample before homogenization as an internal standard. Samples of the eight media corresponding to the muscles in each group were mixed and deproteinized with 3 vol. of 3% sulphosalicylic acid. The samples were analysed on a Technicon Amino Acid AutoAnalyzer (20h system). Samples were kept below 0°C before loading. The column was at room temperature during loading, then heated to 45°C at the commencement of the run. The temperature was only increased to 60°C after glutamine had emerged from the column. This procedure minimizes the breakdown of glutamine, that gives falsely high values for glutamate (D. L. Bloxam, unpublished work).

Determination of adenine nucleotides

Preparation of samples. Muscles after dissection or incubation were rapidly plunged into liquid nitrogen, weighed and crushed in a small Teflon percussion mortar cooled in solid CO_2. The powdered muscle was transferred to a glass tube containing 1ml of ice-cold 5% (v/v) $HClO_4$ and homogenized immediately with a glass pestle cooled in solid CO_2. The mixture froze during homogenization and was allowed to thaw slowly at 0°C. After centrifugation, 0.7ml of the supernatant was transferred to a cooled tube and neutralized to pH7.6 with a mixture of 5M-KOH, 1M-potassium acetate and 0.25M-triethanolamine hydrochloride. The precipitated perchlorate was removed by centrifugation in the cold, and the supernatants were stored at −30°C until analysed.

Assays. ATP was measured by an enzymic method based on phosphoglycerate kinase, by using the fluorimetric procedure of Greengard (1963) with slight modifications. Samples (two for each extract, one 30μl and one 50μl) were added to cuvettes containing 0.1μmol of NADH, 0.384μmol of D-3-phosphoglyceric acid (sodium salt), 0.6μmol of EDTA, 9μmol of $MgSO_4$, 30μmol of cysteine and 27μmol of triethanolamine buffer, pH7.6, final vol. 3ml. The decrease in fluorescence was followed at room temperature in an Eppendorf fluorimeter with a recording attachment after the addition of 50μg of glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) and 10μg of phosphoglycerate kinase (EC 2.7.2.3). The former enzyme was added first to remove any diphosphoglycerate present in the sample.

ADP and AMP were assayed fluorimetrically by the method described by Adam (1963), the quantities of some

reagents being altered to accommodate the small quantities of ADP and AMP and the use of fluorimetric rather than spectrophotometric measurement. Samples of extract (usually two different volumes of each, between 0.1 and 0.4 ml) were added to cuvettes containing 60 nmol of NADH, 420 nmol of phosphoenolpyruvate (potassium salt), 19.6 μmol of $MgSO_4$, 165 μmol of KCl, 1.2 μmol of EDTA and 63 nmol of ATP. The volume in the cuvette was made up to 3 ml with 0.05 M-triethanolamine buffer, pH 7.6. The decrease in fluorescence was followed after the addition of 25 μg of lactate dehydrogenase, and 50 μg of pyruvate kinase (EC 2.7.1.40), the former being added first to remove any pyruvate present. After removal of the ADP, the AMP was measured by the addition of 25 μg of myokinase (EC 2.7.4.3).

All enzymes, substrates and coenzymes used in these assays were obtained from the Boehringer Corp. (London) Ltd., London W.5., U.K., except ATP, which came from Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan.

Amino acid oxidation

Oxidation of amino acids was measured by the method of Manchester (1965). Muscles were incubated at 37°C with shaking in the main compartments of Marie flasks, which were sealed with skirted rubber bungs. Incubation was for 90 min in 1 ml of Krebs–Ringer bicarbonate buffer containing glucose (2 mg/ml), amino acids at plasma concentrations and albumin (0.13%). Oxidation was stopped by injection of 1 ml of 2 M-HCl into the main compartment. At the same time 0.3 ml of 1 M-Hyamine hydroxide in methanol was injected into the centre well to collect the CO_2. After 2 h, the Hyamine, with methanol washings, was transferred to vials containing 10 ml of a scintillation mixture of 4% 2,5-bis-(5-*tert.*-butylbenzoxazol-2-yl)thiophen in toluene and the radioactivity counted.

Potassium content

The potassium content of the tissue was measured with an EEL flame photometer on diluted samples (1 in 20) of the trichloroacetic acid supernatants prepared from the muscles after incubation. The potassium content of freshly dissected muscle from rats killed in the usual way was found to be the same as that in muscles removed from rats under Nembutal anaesthesia.

RESULTS AND DISCUSSION

Condition of the preparation

The primary object of the present work was to study the influence of exercise as induced by electrical stimulation on the rate of protein synthesis in the isolated extensor digitorum longus muscle. Since the rate of protein synthesis in isolated tissues is very sensitive to deterioration in condition, it was thought desirable to study, in conjunction with the experiments on amino acid metabolism, the behaviour of the tissue in terms of its change of weight, extracellular space and potassium content and some aspects of its carbohydrate metabolism under the conditions employed for studying protein synthesis.

Changes in weight of the tissue during incubation. Muscles were originally incubated singly in 1 ml of medium contained in sealed 25 ml conical flasks. Under these conditions the muscles became swollen, and gained up to 20% of their initial weight. This swelling was unaltered by the presence of glucose, insulin or albumin in the medium, but could be halved by continuous gassing of the flasks throughout the incubation. A similar increase was found by Fitch & Shields (1966) who incubated extensor digitorum longus muscles in open beakers. We found that the muscle did not increase in weight when incubated in the apparatus shown in Fig. 1. This was possibly due in part to better oxygenation in this system, but another factor was the maintenance of the muscles at resting length by the application of light tension. [Previous reports indicate that this is beneficial to isolated muscle (Weiss, 1933; Harris, 1954; Geffen, 1964).] Hider, Fern & London (1969) also found that the increase in weight of the extensor digitorum longus muscle resulting from incubation in sealed flasks was substantially decreased by stretching the muscle. Buresova, Gutmann & Klicpera (1969) have shown that stretch results in an increased rate of incorporation of amino acids into the protein of the extensor digitorum longus and levator ani muscles, possibly as a result of the increase in oxygen consumption (Feng, 1932) or facilitation of amino acid penetration.

Retention of potassium by the tissue. Potassium is rapidly lost from damaged or anoxic tissue. Fig. 2 shows that when incubated in the apparatus

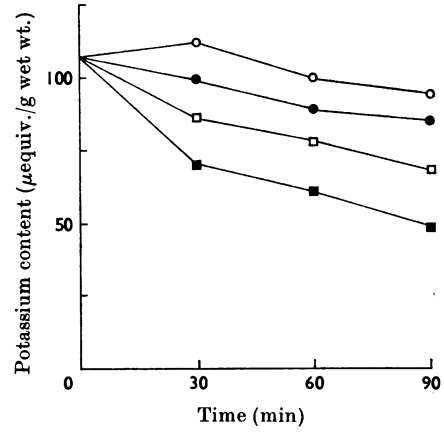

Fig. 2. Effect of stimulation and the presence of albumin on the potassium content of rat extensor digitorum longus muscle. ○, Resting muscle with albumin added; ●, stimulated, with albumin; □, resting, without albumin; ■, stimulated, without albumin.

Table 1. *Effect of incubation and stimulation on the water content and the extracellular space of rat extensor digitorum longus muscle*

Each value is the mean ± S.E.M. of the number of observations shown in parentheses. [^{14}C]Sorbitol, where present, was added at a concentration of 0.1 μCi/ml. No unlabelled sorbitol was added.

	Incubation period (min)	Resting muscle	Stimulated muscle
Water content (% wet wt. of muscle)	0	77.6 ± 0.9 (6)	
	60	79.8 ± 0.3* (6)	80.8 ± 0.2† (6)
Sorbitol space (ml/100g wet wt.)	30	18.8 ± 1.3 (4)	18.1 ± 1.9 (4)
	60	17.9 ± 0.3 (3)	20.7 ± 0.3 (3)
	90	20.2 (2)	20.0 (2)

* Significantly greater than unincubated muscle ($P < 0.05$).
† Significantly greater than resting muscle ($P = 0.02$).

of Fig. 1 the extensor digitorum longus muscle lost some potassium. This was appreciable in the absence of added protein, but minimal when albumin was present. A slightly greater loss of potassium was observed in the stimulated muscle. Addition of protein also improved the contractility of the tissue, a phenomenon previously noted by Zachariah (1961) for the perfused heart. Armstrong & Knoebel (1966) and Creese (1968) believe that albumin complexes traces of heavy metals found even in highly purified reagents. Addition of cytidine (1 mM) was not found to have any beneficial effect (see Zierler, 1959).

During this study, the concentration of potassium in the muscles at the end of the incubation period was measured routinely as an indication of the condition of the preparation. Muscles containing less than 70 μmol/g wet wt. were discarded as in poor condition.

Extracellular space and water content. The water content of both the resting and stimulated muscle rose slightly during incubation and the increase was marginally greater for the latter (Table 1). Stimulation had no effect on the extracellular space of the muscle, as measured by the penetration of radioactive sorbitol, and was similar to that of the intact preparations of diaphragm (Kipnis & Parrish, 1965; Grossman & Manchester, 1966) and levator ani muscle (Arvill & Ahren, 1966). It was considerably smaller than the sucrose space found on incubation of the larger plantaris muscle by Law (1967), who suggested that the plantaris muscle became hypoxic during incubation *in vitro*. Indeed, we found a much larger extracellular space (about 30% of wet wt.) when we incubated muscles weighing more than about 30 mg. This change was accompanied by poor contractility and an abnormally large decrease in ATP concentration. [Bombara & Bergamini (1968) found a similarly large extracellular space, with inulin as marker, in the extensor digitorum longus muscles from rats slightly smaller than those used in our experiments, but these workers incubated muscles in flasks without tension, and in a medium that did not contain albumin.] The extracellular-space measurements thus confirm the conclusions reached above that the best conditions for survival of the extensor digitorum longus and possibly other muscles *in vitro* are incubation under tension in an oxygenated medium containing protein.

Carbohydrate utilization

Table 2 shows that during stimulation the uptake of glucose by digitorum longus muscles was increased only slightly, if at all. This was surprising in view of the large number of reports that work increases the uptake of sugars by muscle (see e.g. Szabo, Mahler & Szabo, 1969). A check that an increase would be detectable and is possible in the present system is provided by the typical result obtained with insulin (Table 2) which increased the uptake of glucose by both resting and stimulated muscles. Stimulation did enhance lactate production, which conversely was not affected by insulin. Enhanced glycogenolysis was presumably the source of the extra lactate formed on stimulation (Table 3). Glycogen thus appears to figure more importantly in the provision of the extra energy required for contraction; a situation similar to that found by Bergstrom & Hultman (1967) and Chapler & Stainsby (1968) for human and dog muscle. According to Jöbsis & Stainsby (1968) the increased lactate production is not an indication of anoxia, since there may be a simultaneous decrease in the degree of NAD^+ reduction, but results from 'overshoot' in the extent of conversion of phosphorylase *b* into phosphorylase *a* consequent to stimulation (Stubbs & Blanchaer, 1965). Extensor digitorum longus muscle has a high proportion of white fibres (Sreter & Woo, 1963) and as such is likely to have a high phosphorylase/hexokinase ratio (Burleigh & Schimke, 1968).

Table 2. *Effect of stimulation and of insulin on the uptake of glucose and the output of lactate by rat isolated extensor digitorum longus muscle*

Each value is the mean ± S.E.M. of the number of observations shown in parentheses. Muscles were incubated for about 90 min in Krebs–Ringer bicarbonate buffer containing 1 mg of glucose/ml but no protein. Insulin was present where indicated at a concentration of 0.1 unit/ml.

Treatment	Uptake of glucose (μmol/h per g wet wt. of muscle)	Output of lactate (μmol/h per g wet wt. of muscle)
Without insulin:		
Resting	10.4 ± 2.2 (10)	19.8 ± 2.9 (9)
Stimulated	11.3 ± 1.8 (10)	23.0 ± 1.9 (9)
With insulin:		
Resting	16.3 ± 1.7 (9)	20.4 ± 2.0 (9)
Stimulated	17.8 ± 1.8 (9)	24.9 ± 2.8 (9)

Table 3. *Effect of stimulation on the content of glycogen in rat digitorum longus muscle*

Each value is the mean ± S.E.M. of ten observations. Muscles were incubated for 60 min in Krebs–Ringer bicarbonate buffer containing 1 mg of glucose/ml, plasma concentrations of amino acids and albumin.

Condition	Glycogen content (μmol of glucose/g wet wt.)	Glycogen broken down (μmol of glucose/g wet wt.
Immediately after removal from rat	19.4 ± 1.1	
After incubation		
Resting	20.1 ± 0.9	−0.7
Stimulated	11.6 ± 1.2	7.8

An enhanced rate of glycogen breakdown could lead to accumulation of glucose 6-phosphate which might be expected to limit glucose phosphorylation and hence uptake.

The lack of effect of insulin on lactate production (Table 2) is surprising in view of other reports of enhancement. However, lactate production by isolated diaphragm in the absence of added glucose is unaffected by insulin, even when lactate production is stimulated by the addition of adrenaline (K. L. Manchester, unpublished work), making it unlikely that in muscle insulin suppresses cyclic AMP formation.

Incorporation of amino acids into protein

Table 4 shows the effect of stimulation on the rate of incorporation of radioactivity from four different amino acids into the protein of digitorum muscles. In all cases the incorporation was less in the stimulated muscle than in its resting control. The extent of this decrease varied between the different amino acids, being greater for proline and glycine than for phenylalanine and leucine. The difference in the individual experiments in Table 4 was statistically significant only for proline, but the decrease was consistent for all the amino acids studied and if the results were pooled with additional observations the decrease resulting from stimulation was significant; e.g. a number of observations were made for glycine where rat serum was added to the medium in place of albumin. Under these conditions incorporation appeared to be greater than when serum was replaced by serum albumin (possibly because serum contains insulin) but the decrease in incorporation during stimulation was similar. Arvill (1967) found that stimulation by insulin of amino acid incorporation was still seen in the stimulated preparation and this would be implicit in the observations with serum versus purified albumin.

Our results confirm the findings of Karpatkin & Samuels (1967) of a decrease in leucine incorporation by frog sartorius muscle during stimulation and the small decrease in incorporation of leucine, proline and valine by rat levator ani muscle under similar conditions (Arvill, 1967). In contrast Kendrick-Jones & Perry (1967) observed an increase in leucine incorporation by frog sartorius muscle during stimulation, but frequency of stimulation was rather less than that used here or in other studies cited and the increase in incorporation became apparent only after stimulation had continued for 4h. We considered the possibility that an increased rate of amino acid incorporation, if occurring, might be more visible in a period of rest following stimulation, but we found no increase in incorporation of leucine or glycine during the 90 min immediately following a period of stimulation.

A diminution in the rate of incorporation during

Table 4. *Effect of stimulation on the incorporation of radioactivity from various amino acids into the protein of rat extensor digitorum longus muscle*

Each value is the mean±S.E.M. of the number of observations shown in parentheses. ^{14}C-labelled amino acids were added to the medium to give the final specific radioactivities shown.

Amino acid	Specific radioactivity in medium (μCi/μmol)	Period of incubation (min)	Incorporation of radioactivity into muscle protein (d.p.m./mg) Resting	Stimulated
Glycine	0.4	90	202±37	123±11 (3)
			266±23	187±19 (3)
Phenylalanine	2.5	60	995±58	864±57 (3)
Leucine	0.9	30	617±74	545±48 (5)
		60	1036±96	944±48 (7)
		90	1642±146	1449±69 (5)
Proline	0.8	30	89±11	58±8 (4)
		60	291±8	216±11* (4)
		90	548±32	282±25* (4)

* Significantly less than resting control ($P<0.01$).

stimulation could have many explanations. We have in the subsequent sections sought to determine the extent to which stimulation affects the entry into the tissue of the labelled amino acids and hence affects their availability for incorporation, and whether stimulation affects the concentrations of adenine nucleotides in the tissue or the steady-state concentrations of free amino acids.

Assuming a specific radioactivity equal to that in the medium the amount of labelled amino acid incorporated into protein is (in nmol/h per g of tissue and assuming each gram of tissue contains 200mg of protein) glycine 28, leucine 110, proline 46 and phenylalanine 33. The rate of protein synthesis is therefore of the order of 50–250 μg/h per g of tissue depending on the proportion of any given amino acid in the protein synthesized. This value is somewhat lower than that observed with rat diaphragm muscle, possibly because white muscle is less active in protein synthesis than red (Goldberg, 1967*b*; Short, 1969), but is almost certainly an underestimate because of dilution of the amino acid on entering the cell and because of reutilization of unlabelled amino acid arising from protein breakdown for protein synthesis (Gan & Jeffay, 1967).

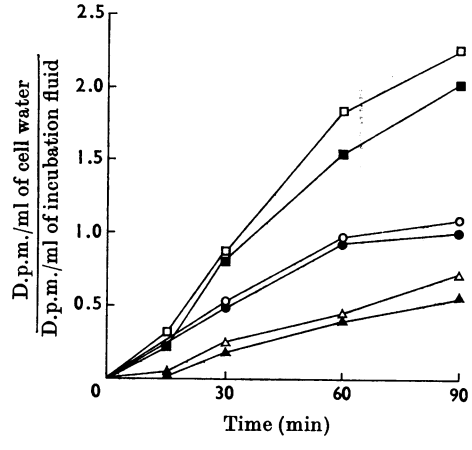

Fig. 3. Effect of stimulation on the distribution of labelled glycine, proline and α-aminoisobutyrate between cell water and medium. □, Glycine, resting muscle; ■, glycine, stimulated; ○, proline, resting muscle; ●, proline, stimulated; △, α-aminoisobutyrate, resting muscle; ▲, α-aminoisobutyrate, stimulated. Cycloheximide (100 μg/ml) was present when accumulation of glycine was measured.

Penetration of radioactive amino acids into muscle

One possible cause of the decrease in incorporation of radioactive amino acids into the protein of stimulated muscle is a decrease in the rate of penetration of the radioactivity into the tissue. The results in Fig. 3 and 4 indicate that this is unlikely, since, whereas stimulation decreases the incorporation of all the amino acids tested, amino acids differ in the effect of stimulation on their penetration into digitorum muscle. Stimulation led to a significant rise in the accumulation of leucine by muscle and a small decrease in the accumulation of proline. The distribution of [^{14}C]phenylalanine was unchanged by stimulation (not shown), giving a value of unity for the ratio of radioactivity in cell water and medium.

In case the effects of stimulation on the accumulation of amino acids could be secondary to effects on incorporation, measurements of the distribution

of radioactive leucine and glycine were carried out in the presence of a concentration of cycloheximide sufficient to block protein synthesis almost completely. Under these conditions, stimulation resulted in a significant decrease in the accumulation of glycine. Cycloheximide did not affect the distribution of leucine in the stimulated muscle, but increased that in the resting control, thus lessening the difference between resting and stimulated muscles (Fig. 4).

Stimulation decreased the accumulation of the non-utilizable amino acid, α-aminoisobutyric acid (Fig. 3). This result, and that with proline, is at variance with those of Arvill (1967), who found that stimulation increased the accumulation by levator ani muscle of all the amino acids studied, namely valine, leucine, proline and aminoisobutyrate. The accumulation of aminoisobutyrate observed in the present work is rather low in comparison with the values reported in the literature for digitorum longus muscle (Bombara & Bergamini, 1968), isolated hemidiaphragm (Manchester & Young, 1960), or levator ani muscle (Arvill, 1967). However, if other amino acids were omitted from the medium, as was so in all these investigations, the cell water/medium ratio was considerably raised, being 1.39 ± 0.09 in the stimulated muscle and 1.53 ± 0.05 in the resting controls after incubation for 90min (six observations). These values are close to those for the intact diaphragm (Manchester & Young, 1960).

The difference in response to stimulation of the accumulation of aminoisobutyrate and glycine on the one hand, and of leucine on the other, parallels that found under the influence of insulin (Manchester, 1970) or denervation hypertrophy (Harris & Manchester, 1966) and is possibly related to the different transport sites supposedly used for uptake (Oxender & Christensen, 1963).

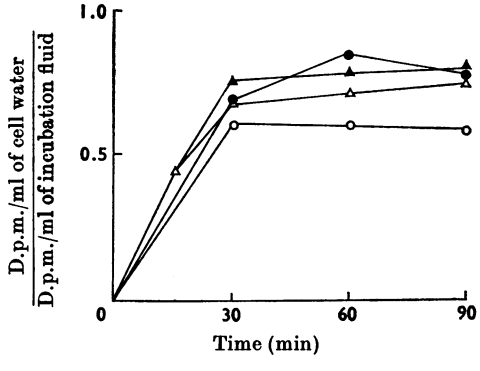

Fig. 4. Effect of stimulation and cycloheximide (100 μg/ml) on the distribution of labelled leucine between cell water and medium. ○, Resting; ●, stimulated; △, resting, with cycloheximide present; ▲, stimulated, with cycloheximide present.

Oxidation of amino acids

Significant degradation of amino acids will lessen their availability for protein synthesis. The capacity of digitorum longus muscle to form $^{14}CO_2$ from [^{14}C]leucine, [^{14}C]proline and [^{14}C]glycine is shown in Table 5. Oxidation of phenylalanine was not detected. As with measurement of incorporation into protein, assessment of precise rates is precluded by lack of knowledge of actual specific radioactivities. Assuming the value for the specific radioactivity of the amino acid in the medium, the rate of oxidation of leucine is appreciably greater than that of glycine or proline (Table 5), but if we use in calculation the specific radioactivity of the intracellular pool [since Rosenberg, Berman & Segal (1963) found for kidney-cortex slices that the rate of oxidation, unlike that of incorporation, followed the specific radioactivity of the intracellular pool] the values become appreciably higher; in fact the rate of glycine oxidation is then greater than that of leucine or proline. On the other hand because of the small size of the free-leucine pool in the tissue

Table 5. *Oxidation of amino acids by the rat extensor digitorum longus muscle*

Incubation was for 90min at 37°C. Each value is the mean ±S.E.M. of the number of observations in parentheses.

Amino acid	Concentration in medium (mM)	Specific radioactivity (Ci/mol)	$^{14}CO_2$ formation (% of added radioactivity per 100mg wet wt.)	Amount oxidized (nmol/100mg wet wt.)		Amount oxidized (% of initial amino acid concn. in muscle)
Leucine	0.115	2.17	1.5 ±0.1 (6)	1.73*	3.60†	34
Proline	0.24	0.46	0.24±0.01 (7)	0.57*	1.10†	2.7
Glycine	0.49	0.82	0.20±0.02 (6)	0.97*	6.93†	1.1

* Based on specific radioactivity of amino acid added to the medium.
† Based on specific radioactivity of amino acid in the tissue (Table 8).

Table 6. *Effect of stimulation on adenine nucleotide concentrations in rat extensor digitorum longus muscle*

Each value is the mean ± S.E.M. of the number of samples indicated.

	Concn. after incubation for (μmol/g wet wt. of muscle)						
		30 min		60 min		90 min	
	Initial	Resting	Stimulated	Resting	Stimulated	Resting	Stimulated
ATP	4.79 ± 0.46	4.12 ± 0.48	3.29 ± 0.29	3.88 ± 0.14	3.93 ± 0.79	3.83 ± 0.38	2.20
ADP	0.80 ± 0.05	0.72 ± 0.06	0.71 ± 0.10	0.68 ± 0.05	0.82 ± 0.10	0.86 ± 0.07	0.66
AMP	0.08 ± 0.01	0.02 ± 0.02	0.04 ± 0.03	0.03 ± 0.01	0.08 ± 0.02	0.05 ± 0.01	0.04
ATP/(ADP + AMP)	5.45 ± 0.38	5.59 ± 0.33	4.57 ± 0.35	5.52 ± 0.53	4.15 ± 1.66	4.30 ± 0.57	3.14
ATP + ADP + AMP	5.67 ± 0.50	4.86 ± 0.54	4.04 ± 0.41	4.59 ± 0.13	4.83 ± 0.75	4.74 ± 0.38	2.90
$\frac{ATP + \frac{1}{2}ADP}{ATP + ADP + AMP}$	0.92 ± 0.01	0.93 ± 0.01	0.91 ± 0.01	0.92 ± 0.01	0.90 ± 0.02	0.90 ± 0.01	0.88
No. of samples	5	4	4	3	3	4	2

the figures suggest that up to 35% of the pool may be degraded every 90 min and the fractional use of the glycine pool will be much lower.

Adenine nucleotides

Since stimulation decreases the incorporation and accumulation of those amino acids that have to enter muscle against a concentration gradient, it is possible that the consumption of extra energy by muscle in the process of contraction leads to limitation of available ATP for amino acid transport and protein synthesis.

We therefore measured the concentrations of ATP, ADP and AMP in the muscle immediately after dissection and after various periods of incubation with or without stimulation (Table 6). The concentrations of all three nucleotides in the tissue were similar to those reported in the literature for skeletal muscle, e.g. in anterior tibialis muscle of rabbits by Imai, Riley & Berne (1964), cat gastrocnemius muscle by Sacks, Murphree & Brown (1966) and rat leg muscle by Marquez-Julio & French (1967). Table 6 shows that, whereas stimulation had little effect on the concentration of ATP in muscle during the first hour, there was considerable depletion during the period between 60 and 90 min. The breakdown of ATP was not accompanied by an equivalent increase in the concentrations of ADP and AMP, so that the total adenine nucleotide content declined. Possibly there was further breakdown to IMP and inosine, as observed in the isolated rat diaphragm (Alertsen, Walaas & Walaas, 1958). The fall in the concentration of ATP, and consequently in the ATP/(ADP + AMP) ratio, could lead to an increased rate of glycolysis, but it is noteworthy that 'energy charge', (ATP + ½ADP)/(ATP + ADP + AMP), which has also been considered as important in metabolic regulation (Atkinson & Walton, 1967), remains remarkably constant in both resting and stimulated muscle.

It is possible that the somewhat sudden fall in ATP concentration in stimulated muscle occurs when utilizable glycogen stores have been depleted (Table 3), since white muscle fibres depend on glycogen to supply the fuel for contraction (Bergstrom & Hultman, 1967) and do not have the fat reserves of red fibres (Cherian, Bokdawala, Vallyathan & George, 1966). A decrease in the quantity of ATP available to support protein synthesis and uphill amino acid transport could well contribute to the decreased incorporation of certain amino acids into the protein of muscle stimulated *in vitro*. It should, however, be noted that the increased incorporation of leucine into the protein of stimulated frog muscle (Kendrick-Jones & Perry, 1967) was accompanied by a decrease in the ATP content of the stimulated muscle (Hajek & Perry, 1967).

Specific radioactivity of amino acids within the muscle

A decreased rate of incorporation might result from enhanced dilution of the specific radioactivity of the labelled amino acid if stimulation were to cause an increase in the rate of protein breakdown in the muscle. We therefore measured the free amino acid content of muscles after incubation, with and without stimulation, and compared the results with values for the fresh tissue (Table 7). After 1 h of incubation with stimulation the concentrations of several amino acids present in high concentrations were somewhat lower, but for the majority the concentration of amino acid in muscle and medium remained fairly constant throughout. Bearing in mind the rate of oxidation of some amino acids (Table 5), it is apparent that proteolysis must occur if only to maintain the free amino acids at their initial concentrations. The decrease in

Table 7. *Content of free amino acids of rat extensor digitorum longus muscle on dissection and after incubation*

Concentrations in muscle are expressed in μmol/100 ml of intracellular water and in media as μmol/100 ml. Eight similarly treated muscles were pooled to give each muscle sample. Portions of the 8 corresponding media after incubation were used to give the medium samples.

	Initial concn.			Concn. after incubation for 60 min					
				Resting			Stimulated		
Amino acid	Muscle	Medium	Muscle/medium ratio	Muscle	Medium	Muscle/medium ratio	Muscle	Medium	Muscle/medium ratio
Taurine	1852	33.7	55.0	1677	34.5	48.6	1224	38.0	32.2
Aspartate	139	2.8	48.9	111	2.7	41.5	64.4	2.1	30.4
Threonine	57.2	14.1	4.1	65.9	13.0	5.1	60.5	17.1	3.6
Serine	—	65.5		623	60.5	10.3	477	67.4	7.1
Glutamate	272	8.5	32.1	277	9.8	28.4	177	9.6	18.5
Proline	60.0	23.8	2.5	108	26.5	4.1	76.4	23.2	3.3
Citrulline	106	8.9	11.9	82.5	9.5	9.3	77.3	8.4	9.2
Glycine	1047	48.5	21.6	1060	45.5	23.3	900	45.1	19.9
Alanine	604	86.0	7.0	573	67.5	8.5	500	84.6	5.9
Valine	19.1	15.4	1.2	20.1	14.8	1.4	23.7	13.6	1.7
Methionine	13.3	5.2	2.6	13.2	5.3	2.5	16.7	4.0	4.2
Isoleucine	11.2	6.4	1.7	10.1	6.1	1.7	11.9	5.7	2.1
Leucine	13.9	11.5	1.2	15.5	11.1	1.4	19.1	9.8	1.9
Tyrosine	20.4	4.1	4.9	15.3	4.0	3.8	10.9	3.9	2.8
Phenylalanine	10.7	4.0	2.7	7.2	3.9	1.9	8.7	3.4	2.6
Ornithine	26.3	7.0	3.8	19.6	6.7	2.9	19.5	6.6	3.0
Lysine	27.7	11.4	2.4	34.7	11.0	3.2	35.3	10.3	3.4
Histidine	105	6.4	16.4	91.6	6.8	13.5	83.7	5.9	14.3
Arginine	39.2	9.1	4.3	25.1	9.0	2.8	29.3	7.7	3.8

Table 8. *Comparison of the distribution ratios for amino acids in rat extensor digitorum longus muscle measured by radioactive and chemical methods*

Radioactive distribution ratios are derived from Figs. 3 and 4. Chemical distribution ratios are from Table 7.

		Radioactive distribution ratio / chemical distribution ratio	
Amino acid	Incubation time (min)	Resting	Stimulated
Leucine	30	0.42	0.44
	60	0.45	0.44
	90	0.48	0.40
Proline	30	0.24	0.27
	60	0.25	0.29
	90	0.48	0.29
Phenylalanine	60	0.51	0.37

glutamate and aspartate concentrations might particularly result from oxidation.

The values in Table 7 agree well with those for rat skeletal muscle found by Ryan & Carver (1963). They also confirm the observations of Scharff & Wool (1965) that muscle retains the concentrations of amino acids found *in vivo* when incubated in medium containing normal plasma concentrations. On the chromatograms of muscle extracts, a peak was obtained in the position given in this system by carnosine corresponding to a concentration of about 200 μmol/100 ml of tissue water. It never appeared in the incubation medium. Its concentration was not altered by stimulation for 30 min, but it fell slightly after stimulation for 60 or 90 min.

A determination of the specific radioactivity of an amino acid in the total intracellular pool of muscle can be made from the distribution of a radioactive amino acid between muscle and medium compared with its chemical distribution ratio. These values are given in Table 8. It can be seen that in no case has the specific radioactivity of the amino acid in the cell reached that in the medium. The corresponding values for glycine can be determined by comparing the radioactive distribution in the presence of cycloheximide (Fig. 3) with the chemical distribution in its absence (Table 7). This gives a value for the intracellular specific radioactivity of glycine after 90 min incubation of only 10% of that in the medium in stimulated muscle, and 14% in the resting control. Somewhat similar findings were made by

Table 9. *Comparison between the proportional changes brought about by stimulation in the specific radioactivity of intracellular amino acids and in their incorporation into the protein of rat extensor digitorum longus muscle*

Amino acid	Incubation time (min)	Specific radioactivity of amino acid in cell water of stimulated muscle (% of resting control)	Incorporation into protein of stimulated muscle (% of resting control)
Leucine	30	105	88
	60	98	91
	90	83	88
Proline	30	113	65
	60	116	74
	90	60	51
Phenylalanine	60	73	87
Glycine	90	71*	61

* Measured in the presence of cycloheximide (100 μg/ml).

Hider *et al.* (1969). Such calculations, however, do not take into account the possibility of compartmentation of the cell pool, but the extent of this is impossible to assess.

The factors determining the extent of labelling of the cell pool are necessarily complex by virtue of the flux of amino acids across the cell membrane and into and out of protein as well as movement into pathways of degradation. As pointed out by Gan & Jeffay (1967) and Waterlow & Stephen (1968), as long as the protein of a tissue constitutes a pool of unlabelled amino acid the turnover of tissue protein prevents the rise of the intracellular specific radioactivity to that pertaining in the extracellular fluid even *in vivo*. The normal steady-state size of the intracellular pool for any given amino acid will also be an additional factor affecting how rapidly equilibration with the label occurs.

In Table 9 the incorporation of each amino acid into the protein of the stimulated muscle is expressed as a percentage of the incorporation in the resting controls. The specific radioactivity of each amino acid in the cell water of the muscle is treated in the same way, so that the proportional changes in incorporation can be compared directly with those in specific radioactivity. It can be seen that for the most part changes in the intracellular specific radioactivity of the free amino acids do not account for the decreases in incorporation observed. Thus we can say that irrespective of whether the amino acids going into protein are principally those just entering the tissue or from the intracellular pool the incorporation values suggest a decrease in the rate of protein synthesis during stimulation. With our preparation it seems likely that there is ultimately deterioration in condition after 90 min or so of stimulation, which prejudices immediate enhancement of protein synthesis. Though observations of NADH fluorescence (Jöbsis & Stainsby, 1968) suggest that muscle does not go anoxic during exercise *in vivo*, there is evidence that the phosphate potential in the cytoplasm may indeed decrease (Hultman, Bergström & McLennan Anderson, 1967). It seems unlikely therefore that protein synthesis will be enhanced during exercise *in vivo*, but rather the reverse, and the increase in protein formation after exercise is more probably a compensatory response to an initial decline than a result of the exercise *per se*.

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