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α-AMINOISOBUTYRIC ACID UPTAKE *IN VITRO* BY THE RAT EXTENSOR DIGITORUM LONGUS MUSCLE AFTER DENERVATION AND TENOTOMY*

GIUSEPPE BOMBARA AND ETTORE BERGAMINI

Institute of General Pathology, University of Messina, Messina and Institute of General Pathology, University of Siena, Siena (Italy)

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SUMMARY

1. α-Aminoisobutyric acid uptake *in vitro* in the rat extensor digitorum longus muscle was studied shortly after denervation and tenotomy.

2. An increase in the intracellular concentration of this amino acid 48 and 72 h after nerve excision and 72 h after tenotomy, in comparison with the contralateral control muscle, was observed.

3. Stimulation of the active transport of α-aminoisobutyric acid was apparent. The increased uptake depends only on an increased penetration rate of the amino acid into the fibers, as no modifications of the efflux rate were observed. Furthermore, anoxia and 2,4-dinitrophenol inhibited increased uptake, as did inhibitors of glycolysis (NaF and iodoacetic acid) to a lesser extent.

4. Concurrently, in the denervated muscle, a parallel increase in O_2 consumption also took place.

5. Ouabain inhibited completely the active transport of α-aminoisobutyric acid in the normal muscle and suppressed almost completely the increased uptake of the denervated muscle.

6. The significance of these results is discussed.

INTRODUCTION

The process of muscle atrophy, which can be observed after denervation, tenotomy, disuse and other pathological conditions, is characterized by a progressive decrease of the protein content of muscle, in which reduction of contractile and soluble proteins of the muscle fibers occurs.

In principle, a decrease of muscle proteins could be due to an increased rate of protein hydrolysis or to a decreased rate of protein synthesis. Evidence in support of the former mechanism is supplied by ultrastructural data[2] which show that an early degeneration process takes place in the fibers after denervation. This is accompanied and followed by another process by which peripheral filaments are lost from the myofibrils. Evidence of an increase in lysosomes during atrophy comes from

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both ultrastructural observations and biochemical determinations of lysosomal enzymes[3,4]. Concerning the second possible mechanism, that of a decrease of proteosynthesis, the situation is far from clear. In rabbits, *in vivo*, a decrease in the rate of incorporation of amino acids into proteins has indeed been found by some authors[5,6], but by other authors no modification[7,8] or even an increase was recorded[9,10]. On the other hand, *in vitro*, an increase of valine incorporation into the proteins of myofibrillar supernatant has been shown[11], as well as an increase of leucine, glycine and alanine incorporation into total proteins of rat extensor digitorum longus muscle[12]. The discrepancies between these results could be related to modifications in the transport of amino acids into the muscle.

We have therefore studied uptake of amino acids *in vitro* in precocious stages of denervation or tenotomy. We used α-aminoisobutyric acid, since unmetabolized[13] amino acid is transferred by the same mechanism as glycine, alanine and proline[14] and, for this reason, it has been widely used in studying amino acids transport.

MATERIALS AND METHODS

Tissue

Wistar–Glaxo albino rats bred in this department's colony, weighing 30–45 g, were used. Extensor digitorum longus muscle which, according to various authors, is suitable for incubation *in vitro* because of its low volume/surface ratio was used. Denervation was performed by excising about 1 cm of sciatic nerve at the level of the coxofemoral joint; the proximal tendon of the muscle was cut. In general muscle from the contralateral, unoperated limb served as a control. When the effects of metabolic inhibitors were to be tested, both hind limbs were operated and a comparison was made with the contralateral muscle incubated under basal conditions (aerobiosis without inhibitors).

Materials

α-[1-^{14}C]Aminoisobutyric acid (spec. act. 14.6 mC/mmole) was supplied by the Radiochemical Centre (Amersham, Great Britain); α-aminoisobutyric acid, ouabain, iodoacetic acid, diethanolamine and inulin by The British Drug House Ltd. (Poole, Great Britain); 2,4-dinitrophenol and NaF by Merck (Darmstadt, Germany).

Incubation conditions

Muscles were excised from the animals under Nembutal anesthesia (5 mg/100 g body weight) by cutting the tendons, taking great care not to damage the fibers. The muscles were gently blotted on filter paper and weighed on a torsion balance.

Incubation was performed in Warburg flasks, containing 2 ml medium, with agitation, at 38°. At the end of the incubation, the muscles were rinsed with 3 ml cold Ringer solution without inulin and α-aminoisobutyric acid. Incubation medium was a Krebs–Ringer bicarbonate–glucose medium (pH 7.4) composed of: NaCl, 117 mM; KCl, 3.5 mM; $CaCl_2$, 2.5 mM; KH_2PO_4, 1.2 mM; $MgSO_4$, 1.2 mM; $NaHCO_3$, 28 mM; glucose, 10 mM; gas O_2–CO_2 (95:5); inulin 0.75 % (w/v); α-[1-^{14}C]aminoisobutyric acid, 0.034 mM (0.50 μC/ml). In some experiments amino acid concentration was increased by adding unlabeled α-aminoisobutyric acid. Anaerobiosis was obtained by equilibrating Ringer with N_2–CO_2 (95:5), with yellow phosphorus in

the central well. Metabolic inhibitors were used at the concentrations given in Table I.

In order to study the efflux rate of α-aminoisobutyric acid from muscle, extensor digitorum longus muscle, preloaded with the amino acid by incubation in medium containing α-[1-^{14}C]aminoisobutyric acid (0.034 mM), at 38° for 2 h, was transferred to new medium with the same composition but without any α-aminoisobutyric acid or with only unlabeled α-aminoisobutyric acid, and incubated at 38° for varying periods. Radioactivity in the medium and residual radioactivity in the muscle were determined.

O_2 consumption

O_2 consumption was determined by Warburg's method. Muscles were incubated in a Krebs–Ringer phosphate medium containing 7 mM $NaHCO_3$ and equilibrated with O_2–CO_2 (97.5:2.5) (see Table II). Dickens and Simer flasks were used, in the presence of 4 M diethanolamine[15].

α-Aminoisobutyric acid and inulin determination

α-Aminoisobutyric acid was extracted by boiling water as described by DIEHL[16]. Aliquots of 0.75 ml of the extract were dried on stainless-steel disks under an infrared lamp and counted in a gas-flow counter (FD 1, Tracerlab) with an error of less than 2 %. Aliquots were used for determining inulin content according to the method of ROE, EPSTEIN AND GOLDSTEIN[17]. Inulin concentration and radioactivity were determined also for incubation medium.

α-Aminoisobutyric acid concentration ratio determination

α-Aminoisobutyric acid uptake in muscle is expressed as the concentration ratio:

$$\frac{\text{counts/min per ml intracellular fluid}}{\text{counts/min per ml incubation medium}}$$

Intracellular water was calculated from a wet weight/dry weight ratio of 5:1 and by correcting this value (total muscle water) for extracellular space on the basis of inulin distribution corrected for the average fructose content of muscle. Counts/min per ml intracellular fluid was, therefore, determined as follows:

$$\frac{\text{total counts of extract/min} - (\text{medium counts/min per ml}) \times (\text{ml inulin space})}{\text{ml tissue water} - \text{ml inulin space}}$$

Sometimes intracellular α-aminoisobutyric acid content was expressed as μmoles/ml intracellular fluid as follows:

$$\mu\text{moles/ml intracellular water} = \frac{\text{counts/min per ml intracellular water}}{\begin{array}{l}\alpha\text{-aminoisobutyric acid specific activity} \\ \text{in incubation medium as counts/min per } \mu\text{mole}\end{array}}$$

RESULTS

α-Aminoisobutyric acid uptake in muscle following denervation and tenotomy

An increase of the concentration ratio (Fig. 1) in denervated muscle is already evident on the second day (28 %) and becomes more marked on the third day after operation (60 %). After tenotomy α-aminoisobutyric acid uptake increases later and the increase is less (35 % on the third day).

Fig. 2 shows that the curve of α-aminoisobutyric acid uptake *versus* incubation time is similar in normal and denervated muscle. In both cases the concentration ratio increases at a constant rate reaching a nearly maximum value within 120 min, then the uptake rate decreases and the concentration ratio becomes nearly constant.

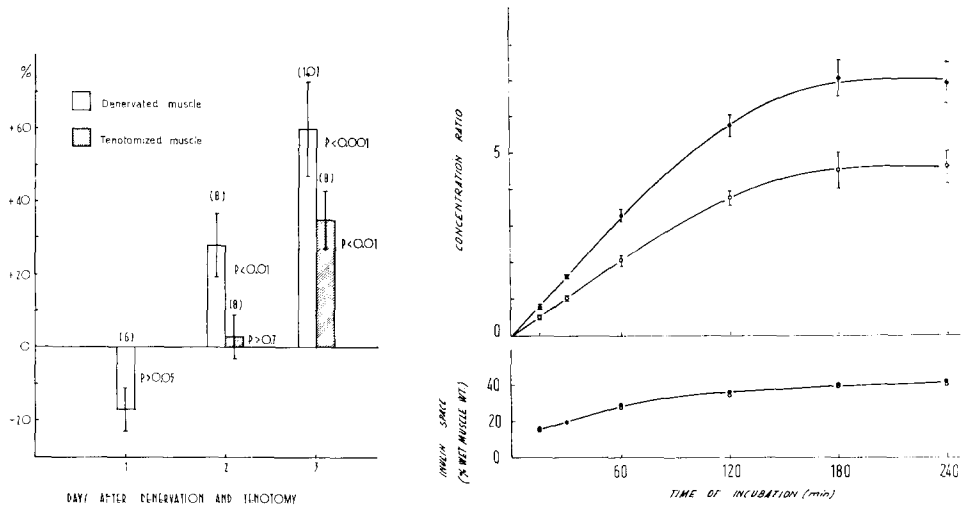

Fig. 1. α-[1-^{14}C]Aminoisobutyric acid concentration ratio of denervated and tenotomized muscles. Values are reported as % of contralateral unoperated muscles. The number of cases are in parentheses. Vertical bars represent S.E.M.

Fig. 2. α-[1-^{14}C]Aminoisobutyric acid uptake *in vitro* of control and denervated (72 h) muscle as a function of incubation time. Experiments were performed in Krebs–Ringer bicarbonate–glucose medium, under aerobiosis at 38°. Each point represents the mean of 8 cases. Vertical bars represent S.E.M. ●—●, denervated muscle; ○—○, contralateral unoperated muscle.

In denervated muscle, however, the concentration ratio is always significantly greater than in the control (in the range $P < 0.01$–0.001)*, even when, after 30 min of incubation, amino acid transport is not yet taking place against a concentration gradient. No difference is evident, as regards inulin space, between normal and denervated muscle. The increased uptake of α-aminoisobutyric acid in the denervated muscle is therefore evident during early incubation and then slows down, as in the controls. This suggests that it is not the consequence of modifications caused by the incubation in the pathological muscle, but depends on the same mechanism which underlies amino acid transport under normal conditions.

As α-aminoisobutyric acid does not undergo metabolism, its intracellular concentration depends only on the balance between influx and efflux rates. The efflux rate was determined (Fig. 3) and it shows no difference between normal and denervated muscle. It is evident, therefore, that the increased α-aminoisobutyric acid concentration in denervated muscle depends only on an increased rate of uptake of the amino acid.

* Statistical significance always regards the average of differences between every pair of values.

Effect of α-aminoisobutyric acid extracellular concentration

In order to describe α-aminoisobutyric acid transport in the muscle in terms of Michaelis–Menten kinetics[18], the uptake rates of denervated and normal muscle were determined in the presence of different α-aminoisobutyric acid concentrations in the medium. In Fig. 4 the results plotted by the double-reciprocal method of LINEWEAVER AND BURK[19] are presented. These data reveal that in the denervated muscle the maximum rate (v_{max}) at which the amino acid is accumulated is greater than in the control, K_m remaining unaltered (K_m, 2.76 mM).

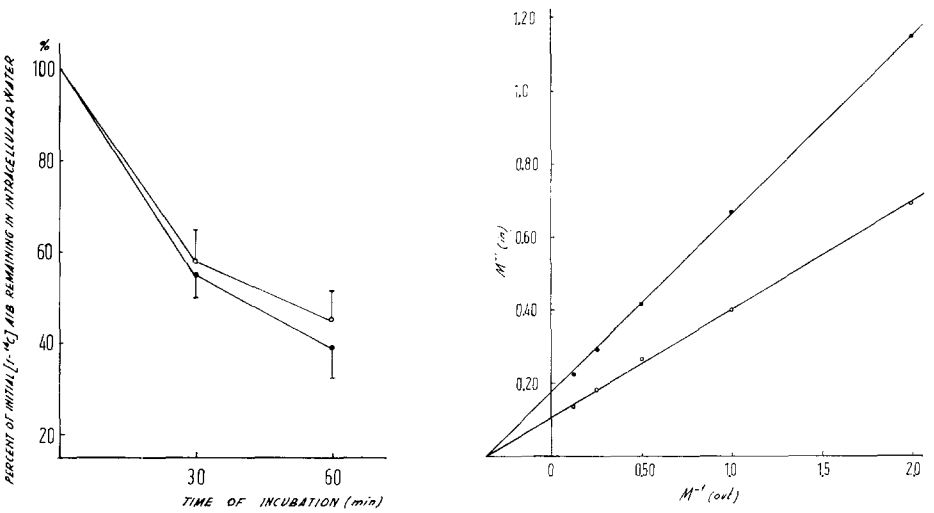

Fig. 3. α-Aminoisobutyric acid (AIB) efflux from denervated muscle. Experiments were performed as follows: muscles, preincubated in the presence 0.034 mM α-[1-^{14}C]aminoisobutyric acid in Krebs–Ringer bicarbonate–glucose medium for 2 h, were transferred to medium of the same composition but containing only unlabeled α-aminoisobutyric acid (1 mM), incubated at 38° for 30 min and then transferred and incubated again for 30 min. Values are the mean of 6 determinations. Vertical bars represent S.E.M. ●—●, denervated muscle; ○—○, contralateral unoperated muscle.

Fig. 4. α-Aminoisobutyric acid uptake in denervated muscle as a function of the amino acid extracellular concentration. Incubation in Krebs–Ringer bicarbonate–glucose medium under aerobiosis for 60 min at 38° in the presence of α-[1-^{14}C]aminoisobutyric acid (0.034 mM) and different amounts of unlabeled α-aminoisobutyric acid. The reciprocals of the external concentrations are plotted against the reciprocals of the intracellular concentrations, according to LINEWEAVER AND BURK. Each point is the mean value of 8 cases. ●—●, denervated muscle; ○—○, contralateral unoperated muscle.

It is evident, therefore, that the increased uptake of α-aminoisobutyric acid in denervated muscle does not depend on altered affinity of the amino acid for its transport mechanism. This may be the result of a real increase of the number of carriers or of a shortening of the transport time through the membrane.

Effect of anaerobiosis and metabolic inhibitors

In order to investigate the relationship between the increased α-aminoisobutyric acid uptake by denervated muscle and some aspects of muscle metabolism, the effects of anaerobiosis and metabolic inhibitors (2,4-dinitrophenol, NaF and iodoacetic acid) were studied (Table I).

TABLE I

EFFECT OF ANAEROBIOSIS AND OF SOME METABOLIC INHIBITORS ON *in vitro* UPTAKE OF α-[1-^{14}C]AMINOISOBUTYRIC ACID IN NORMAL AND OPERATED MUSCLE

Animals were denervated or tenotomized in both limbs 72 h earlier. Incubation in Krebs–Ringer bicarbonate–glucose medium for 60 min at 38°. The means ± S.E.M. of concentration ratio are reported. The number of cases is in parentheses.

Animals		*Aerobiosis*	*Anaerobiosis*	*Difference*	*t*	*P*
Normal	(9)	2.22 ± 0.18	1.94 ± 0.11	0.27 ± 0.14	1.92	> 0.05
Denervated	(9)	3.41 ± 0.20	1.98 ± 0.15	1.43 ± 0.30	4.76	< 0.01
Tenotomized	(9)	3.27 ± 0.22	2.68 ± 0.22	0.59 ± 0.22	2.66	< 0.05
		Without 2,4-dinitrophenol	*With 2,4-dinitrophenol (0.27 mM)*			
Normal	(6)	1.66 ± 0.10	1.39 ± 0.05	0.27 ± 0.10	2.70	< 0.05
Denervated	(6)	2.68 ± 0.12	1.54 ± 0.24	1.14 ± 0.20	5.70	< 0.01
		Without NaF	*With NaF (10 mM)*			
Normal	(6)	2.69 ± 0.42	1.79 ± 0.22	0.89 ± 0.32	2.78	< 0.05
Denervated	(6)	3.48 ± 0.24	2.38 ± 0.31	1.10 ± 0.40	2.70	< 0.05
		Without iodoacetic acid	*With iodoacetic acid (0.5 mM)*			
Normal	(5)	2.00 ± 0.14	1.57 ± 0.14	0.43 ± 0.11	3.90	< 0.02
Denervated	(5)	3.71 ± 0.33	2.40 ± 0.36	1.30 ± 0.36	3.61	< 0.05
			With iodoacetic acid (0.5 mM)			
Contralateral unoperated muscle	(5)		1.19 ± 0.10			
Denervated muscle (72 h)	(5)		0.97 ± 0.07			

During incubation in anaerobiosis α-aminoisobutyric acid transport in normal muscle is not significantly depressed. On the contrary, the concentration ratio of denervated muscle is decreased (−42 %), within the range of that of normal muscle. Less marked effects are observed in tenotomized muscle, where the decrease amounts to −18 %. Uncoupling oxidative phosphorylation has effects similar to those of anaerobic incubation and decreases slightly, but significantly, the α-aminoisobutyric acid concentration ratio in normal muscle.

The effects of two inhibitors of glycolysis were also studied. A significant decrease in the α-aminoisobutyric acid concentration ratio was observed both in the normal and the denervated muscle.

Blocking both aerobic metabolism (by anaerobiosis) and glycolysis (by iodoacetic acid) results in a complete inhibition of α-aminoisobutyric acid concentration in normal and denervated muscle.

Comparison between O_2 uptake and α-aminoisobutyric acid transport

In order to compare more strictly the relationship of tissue respiration and α-aminoisobutyric acid uptake in the same experiment, Q_{O_2} and α-aminoisobutyric acid concentration ratios were measured. Since no concentration of α-aminoisobutyric acid was apparent in the Krebs–Ringer phosphate medium, the experiments were performed in Krebs–Ringer phosphate–bicarbonate (see Table II). They showed that a parallel increase of both O_2 consumption and α-aminoisobutyric acid concentration ratio took place in the denervated muscle.

TABLE II

O_2 CONSUMPTION AND α-[1-^{14}C]AMINOISOBUTYRIC ACID UPTAKE IN DENERVATED MUSCLE

Determinations in Ringer phosphate–bicarbonate medium were performed in Dickens and Simer flasks. These contained, in the main cavity, 3 muscles and 3 ml incubation medium (pH 7.4) whose final composition was: NaCl, 121 mM; KCl, 12.8 mM; $CaCl_2$, 1.83 mM; $MgSO_4$, 0.65 mM; sodium phosphate buffer, 5 mM; $NaHCO_3$, 7 mM; glucose, 10 mM; inulin 0.75% (w/v); unlabeled α-aminoisobutyric acid, 0.034 mM. In central well were 3 ml diethanolamine buffer (4 mM). Equilibrating time: 15 min. Incubation temperature: 38°. Gas O_2–CO_2 (97.5:2.5). α-Aminoisobutyric acid uptake in muscle was determined by using 0.034 mM α-[1-^{14}C]aminoisobutyric acid. Average values of Q_{O_2} (μl O_2/mg dry weight per h) and concentration ratio are reported; the number of cases is in parentheses.

		Contralateral unoperated muscle	*Denervated muscle (72 h)*	*Difference*	*t*	*P*
Q_{O_2}	(4)	4.09 ± 0.33	5.20 ± 0.39	−1.11 ± 0.26	4.25	< 0.05
Concn. ratio	(5)	2.13 ± 0.22	3.06 ± 0.22	−0.93 ± 0.31	3.00	< 0.05

TABLE III

OUABAIN EFFECT ON α-[1-^{14}C]AMINOISOBUTYRIC ACID UPTAKE IN DENERVATED MUSCLE

Denervation had been produced in both limbs 72 h earlier. Incubation in Krebs–Ringer bicarbonate–glucose medium for 60 min at 38°. The means (5 cases) ± S.E.M. of concentration ratio are reported.

Animals	*Concn. (mM)*	*Without ouabain*	*With ouabain*	*Difference*	*t*	*P*
Normal	0.5	2.64 ± 0.28	1.56 ± 0.14	1.07 ± 0.26	4.11	< 0.02
Denervated	0.5	3.40 ± 0.14	1.82 ± 0.11	1.57 ± 0.20	7.85	< 0.01
Normal	1	1.90 ± 0.10	0.92 ± 0.04	0.97 ± 0.11	8.81	< 0.001
Denervated	1	2.17 ± 0.15	1.42 ± 0.13	0.75 ± 0.23	3.26	< 0.05

Effect of ouabain

It has been demonstrated that ouabain impairs active transport of amino acids. This effect has been interpreted as due to the inhibitory effect on Na^+ transport[20,21], but a direct action on transport mechanisms is also possible, as a dissociation of these two phenomena was sometimes observed[22,23]. It was interesting, therefore, to study to what extent ouabain could inhibit the extra uptake of α-aminoisobutyric acid in the denervated muscle.

Muscles were therefore incubated in the presence of ouabain (0.5 mM), a concentration which is effective in inhibiting Na^+ active transport in extensor digitorum

longus muscle[24]. As is shown in Table III ouabain decreases α-aminoisobutyric acid active transport to the same extent in both normal and denervated muscle. A greater concentration of ouabain (1 mM) blocks α-aminoisobutyric acid active transport completely in extensor digitorum longus muscle, as was shown for diaphragm[23], and markedly decreases the concentration ratio of denervated muscle. The residual active transport in denervated muscle suggests that extra uptake of α-aminoisobutyric acid is not completely ouabain dependent.

DISCUSSION

The more marked increase in the concentration ratio of α-aminoisobutyric acid following denervation than after tenotomy, probably depends on a stimulation of basal active transport, as it appears that: (1) α-aminoisobutyric acid concentration ratio is a function of incubation time and quite similar in normal and denervated muscle, with differences only in the amount of transported amino acid; (2) the greater α-aminoisobutyric acid quantity accumulated is determined by its concentration in the external medium according to Michaelis–Menten kinetics; this means that active carrier systems are involved; (3) the extra uptake of α-aminoisobutyric acid depends only on a greater penetration rate into the fibers; (4) it is inhibited by incubating muscles under unfavorable conditions (anaerobiosis, uncoupling oxidative phosphorylation, inhibition of glycolysis). It depends, therefore, on energy production by the fibers.

As regards the influence of muscular metabolism we can say that energy used for stimulating active transport is supplied both by glycolysis and oxidations, but the latter are probably more important for denervated muscle extra uptake than anaerobiosis and 2,4-dinitrophenol completely blocks this phenomenon. Determinations of O_2 consumption under the conditions where amino acid transport was studied, show a good relationship between tissue respiration and amino acid transport. An increased O_2 consumption following denervation and in other kinds of muscular atrophy was already reported[25,26]. This effect is more marked in white than in red muscle. Indeed, under normal conditions the white muscles have a predominantly anaerobic metabolism while the red muscles show a high activity of oxidative enzymes[27]; following denervation, biochemical and functional differences seem to vanish, and O_2 consumption of white muscle increases reaching the values found in red muscle[28].

It is of some interest to discuss the possible mechanisms by which the increase of α-aminoisobutyric acid uptake takes place in the first days following denervation. In the last few years it has been shown that asymmetric distribution of Na^+ and K^+ through cellular membrane can influence active transport of some organic solutes such as hexoses and amino acids. The importance of Na^+ has been demonstrated in several biological systems by the strict correlation found between amino acid levels in intracellular fluid and cation concentration in the incubation medium[20–23,29] and amino acid uptake seems linked to Na^+ entry into the cell or K^+ extrusion[30]. These Na^+–K^+-dependent transport mechanisms, effective for some neutral amino acids (α-aminoisobutyric acid, glycine, alanine, proline)[14,31], are probably due to an increase of the diffusion rate of monovalent cations. The amino acid uptake in cut diaphragm preparations is greater than in intact diaphragm preparations incubated *in vitro*[32,33].

Likewise, α-aminoisobutyric acid extra uptake in denervated muscle may depend on the lack of balance between these cations, since an increase in intracellular Na^+ and a simultaneous decrease of K^+ concentration were observed under the same experimental conditions *in vitro*[24]. Furthermore, according to this theory, the marked decrease of the α-aminoisobutyric acid concentration ratio in denervated muscle incubated in the presence of ouabain may be explained by the impairment of the efficiency of Na^+ active transport, although some authors[22,23] have demonstrated a direct action of glycoside on amino acid active transport. However, it was found[23,34] that α-aminoisobutyric acid influx is modified by Na^+ through an effect of the cation on the K_m of the carrier system, v_{max} remaining unaltered; in our experiments the K_m value was equal in both denervated and normal muscle. This is, therefore, in contrast to the theory of α-aminoisobutyric acid extra uptake dependence on Na^+ movement across the cellular membrane in denervated muscle.

Another point has to be discussed here, concerning the correlations between amino acid transport across the cellular membrane and proteosynthesis, which have not yet been extensively studied. An increased incorporation rate of leucine, alanine and glycine was described in the total proteins of extensor digitorum longus muscle in the first days following denervation[12]. In addition a parallel increase of α-aminoisobutyric acid transport and amino acid incorporation into proteins was demonstrated in isolated rat diaphragm during the transitory hypertrophy following phrenicectomy[35,36]. There is, therefore, a strict correlation between amino acid transport and incorporation. We suggest that increased α-aminoisobutyric acid uptake may be explained by the following mechanisms: (a) a stimulation of amino acid incorporation rate into proteins with a subsequent increase of transport rate; (b) a stimulation of amino acid transport rate with a subsequent increase of incorporation rate into proteins; (c) an independent and simultaneous stimulation of both penetration and incorporation rate into proteins as previously described for insulin action on isolated diaphragm[37].

Similar observations were made in muscular dystrophy induced by avitaminosis E, where increased O_2 consumption, increased amino acid incorporation rates *in vivo* and *in vitro* and increased active transport were also observed[16,38–41]. It is under discussion[41] whether the increased amino acid active transport in this pathological condition is related to a direct stimulation at the membrane level or the increased rate of incorporation into proteins.

It is interesting to note, however, that extra uptake of amino acids has been shown in many pathological muscle conditions (denervation, tenotomy, avitaminosis E-induced muscular dystrophy, cut diaphragm). This may suggest that the mechanisms of stimulation of amino acid uptake are similar in all these conditions. As changes in permeability are a common reaction of muscle to many injuries[42], it is likely that these modifications are responsible, in every pathological muscle condition, for the increase of amino acid uptake, without excluding, however, the possibility of an earlier stimulation at the level of proteosynthesis.

Our results, obtained *in vitro*, do not agree with the data of DREYFUS[43] obtained on rabbit muscles *in vivo*. This author showed a slight decrease of the α-aminoisobutyric acid concentration ratio in gastrocnemius muscle (which is mainly composed of white fibers) and a more marked decrease in red muscle (soleus) 24 h after denervation. Our experiments[44] in rat muscles *in vivo* have shown, on the contrary,

an increased α-aminoisobutyric acid uptake in extensor digitorum longus muscle, and a decrease in soleus. However, a greater α-aminoisobutyric acid uptake has recently been demonstrated[45] in rabbit gastrocnemius and extensor digitorum longus muscle *in vivo*, 7–21 days after denervation, and in muscular dystrophy.

These results show differences which may be related to the type of muscle examined (red or white fibers); the stimulation of α-aminoisobutyric acid transport seems to take place only in white muscles. It is of the greatest importance, therefore, to consider on what kind of fibers experiments are performed. It should also be noted that the phenomenon, occurring *in vivo* and *in vitro*, does not depend on the increased blood flow which follows denervation[46], as has already been demonstrated for dystrophic muscle[45].

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