Characteristics of α -aminoisobutyric acid transport in rat skeletal muscles

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1. α -Aminoisobutyric acid (AIB) transport into the intracellular compartment of extensor digitorum longus and soleus muscles was measured (*in vitro*) after allowance for the equilibration of the amino acid in the extracellular space. The latter was determined with three markers, [^{14}C]inulin, ^{60}Co -EDTA and [^3H]mannitol. Net transport of AIB was subsequently divided into its two components, i.e. influx and efflux. Rates of influx were measured as the intracellular accumulation of [^{14}C]AIB after a short incubation (5 min), and efflux was measured as the release of AIB with time (up to maximum of 50 min) from muscles that had previously been preloaded with AIB. This intracellular efflux was resolved into two phases, which probably represent two separate components of exit. 2. The influence of extracellular Na^+ on the transport of this neutral amino acid (representing the A system) was investigated. Na^+ depletion resulted in lower accumulations of AIB, the effects becoming more pronounced with progressive depletions of external Na^+ . These changes arose from an inhibition of AIB influx, concomitant with an enhancement of its efflux. In contrast, all components of tyrosine transport (representing the L system) were unaffected by lowering external Na^+ concentrations. 3. The net accumulation of AIB was also suppressed by cortisol. This inhibitory effect was, however, Na^+ -dependent and resulted solely from the steroid's enhancement of AIB efflux, the hormone being without effect on AIB influx.

Over the past two decades considerable interest has been directed towards characterizing systems of amino acid transport across the plasma membranes of several tissues. For obvious reasons the small intestine (Wiseman, 1974; King *et al.*, 1981; Munck, 1981) and kidney (Hammerman & Sacktor, 1978; Mircheff *et al.*, 1982) have been intensively studied. The use of ascites-tumour cells (see, e.g., Christensen, 1975, 1977; Hacking & Eddy, 1981) has also been largely responsible for our present concepts of amino acid transport. The latter studies have shown that cells may possess several amino acid transport systems, including A, L and ASC systems, with which they carry neutral amino acids into the cytosol. These systems are quite distinct from other systems used to transport acidic or basic amino acids into the same cells. For example, in the wide range of tissues studied the A and ASC transport systems are believed to be Na^+ -

dependent, whereas the L system is independent of Na^+ (Schultz & Curran, 1970; Guidotti *et al.*, 1978). The absence of such information concerning amino acid influx and efflux in skeletal muscle has prompted the present study.

Characterization of amino acid transport in skeletal muscle is important because the musculature contains the largest reserve of the body's amino acids, both existing free and covalently bound in protein. The movement of amino acids between the plasma and muscle is therefore important both in normal everyday homeostatic regulation of plasma nutrients and in times of stress. In stress, amino acids can be released from muscle (Felig, 1975) to provide substrates for hepatic gluconeogenesis and for essential protein synthesis in other tissues. Hence, in addition to its well-recognized contractile function, the musculature plays an essential role in the body's metabolism. Understanding the precise effects of glucocorticoids on amino acid transport is also of importance because of the muscle wasting and concomitant weakness which characterize diseases

Abbreviations used: AIB, α -aminoisobutyric acid; EDL, extensor digitorum longus.

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such as Cushing's syndrome, or prolonged clinical use of corticosteroids. Despite this, amino acid transport in skeletal muscle has received surprisingly scant attention, and in consequence little is known about its hormonal or metabolic regulation. In the few previous studies performed on isolated skeletal muscles, amino acid transport has been studied over long incubation periods (Kipnis & Parrish, 1965; Goldberg & Goodman, 1969), so that the transport measured was simply net transfer, i.e. the net result of both influx and efflux components of transport. For example, it has been shown that the net accumulation of AIB in diaphragm *in vitro* was inhibited by corticosteroids (Kostyo, 1965). As a result of that work, it has been

was added to 5 ml of scintillation cocktail (Patterson & Greene, 1965) and the [^{14}C]AIB was measured in a Packard scintillation counter by using an external standard for quench correction.

The acid-soluble [^{14}C]AIB measured in this way was derived from both the intracellular and extracellular compartments of the muscle. Hence to determine the AIB component originating solely from the intracellular fluid, appropriate allowances had to be made for both the amino acid located within the extracellular space and the non-aqueous volume of the muscle. The percentage water content was therefore measured in both EDL and soleus muscles and found to be $84 \pm 1\%$. Hence

$$[^{14}\text{C}]\text{AIB in } 1 \mu\text{l of intracellular water} = \left(\text{d.p.m./mg of muscle} - \text{d.p.m./}\mu\text{l of medium} \times \frac{E}{100} \right) \left/ \left(0.84 - \frac{E}{100} \right) \right.$$

assumed that the glucocorticoids act by suppressing the influx of amino acids into muscle. This, however, does not appear to be the case (see below).

In the present study we have attempted to characterize the AIB-transport system in skeletal muscle by examining the effects of cortisol on all components of AIB transport, in both the presence and the absence of extracellular Na^+ .

Methods

All experiments involved the use of young growing female rats (CD strain, 40–50 g) obtained from Charles River (U.K.), Manston, Kent, U.K. Animals were killed by cervical dislocation and the appropriate muscles rapidly dissected out and placed in oxygenated (O_2/CO_2 , 19:1) Krebs-Ringer bicarbonate buffer (KRB; DeLuca & Cohen, 1964).

Measurements of AIB influx and net accumulation

Individual muscles (range 15–20 mg) were incubated in 3 ml of KRB at 37°C for either 7 or 120 min to measure influx or net accumulation, respectively. The incubation buffer was supplemented with glucose (10 mM), chloramphenicol (9 μM) and AIB (1 mM) containing 0.3 μCi of 2-amino[1- ^{14}C]isobutyric acid (sp. radioactivity 50 Ci/mol; Amersham International, Amersham, Bucks., U.K.). After incubation the muscles were rinsed in ice-cold KRB to remove adherent radioactivity, weighed and rapidly frozen in liquid N_2 . Muscles were then homogenized in 3 ml of 0.2 M- HClO_4 and centrifuged at 2000 g for 10 min to separate the precipitated macromolecules from the acid supernatant, which contained the accumulated AIB. A sample (0.5 ml) of this acid-soluble supernatant

where E is the extracellular space expressed as a percentage of the muscle water. This was determined with various extracellular-space markers (see below). From this, a distribution ratio (D.R.) of the radioactivity between the intracellular and extracellular fluids was calculated:

$$\text{D.R.} = \frac{\text{d.p.m./}\mu\text{l of intracellular water}}{\text{d.p.m./}\mu\text{l of incubation medium}}$$

The AIB accumulated (pmol/ μl of muscle water) was then calculated by multiplying the D.R. (based on [^{14}C]AIB only) by the concentration of AIB in the incubation medium.

The rate of AIB influx was calculated similarly, except that the amount of the intracellular amino acid (pmol) was divided by time. The incubation time actually used to measure influx was 7 min, the first 2 min allowing for equilibration of the extracellular space with the incubation medium (see below). True intracellular accumulation arising from influx was therefore measured over 5 min only.

Measurement of AIB efflux

Intact isolated EDL or soleus muscles were preloaded with [^{14}C]AIB by incubating them for 120 min in flasks containing oxygenated (O_2/CO_2 , 19:1) KRB (3 ml), glucose (10 mM), AIB (1 mM) and [^{14}C]AIB (3.6 μCi). At the end of this loading period efflux was measured, each muscle being transferred to a new flask containing medium (1 ml) identical in composition with the loading medium, except for the omission of AIB.

Regardless of how efflux was ultimately expressed, correction had to be made for the release of AIB from the preloaded extracellular space. Since approx. 80% of the extracellular-space marker [^{14}C]inulin was washed out within 5 min of

incubation (see below), zero time for commencing measurements of true intracellular efflux was taken as 5 min of incubation. After transfer of each muscle (six in all) to new media, samples (40 μ l) of this medium were withdrawn at various time intervals during efflux. Each sample of the medium was mixed with scintillation cocktail (5 ml) and the [14 C]AIB released from each muscle determined with time. Allowance was always made for the decreasing volume into which the AIB was released. At the end of efflux, when all appropriate samples of the medium had been taken, each muscle was rapidly frozen in liquid N₂ and weighed. The [14 C]AIB remaining in the muscle was determined in a manner identical with that described for influx measurements.

Where possible, AIB efflux was expressed as the AIB released per μ l of intracellular water per min. However, a direct comparison of the AIB released from control and experimental muscles was not always meaningful, because preloading conditions were unavoidably different and hence muscles contained different concentrations of AIB at zero time of efflux (see below). For example, in the presence of cortisol, the preloading of AIB was significantly decreased because net accumulation is inhibited by cortisol's action on muscle (see below). In such instances efflux had to be expressed as a fraction of the preloaded AIB which was subsequently released with time, i.e.

$$\frac{\text{pmol of AIB released}/\mu\text{l of intracellular water per min}}{\text{pmol of AIB accumulated}/\mu\text{l of intracellular water at } t_0}$$

where t_0 is zero time of efflux.

Results

Time course of AIB accumulation in skeletal muscle

To measure the transport of AIB into the sarcoplasm of skeletal muscle, allowance had first to be made for the proportion of the amino acid present within the extracellular volume of the tissue. The extracellular space of the EDL muscle was measured with three markers of different molecular weights, i.e. [14 C]inulin, ^{60}Co -EDTA and [^3H]mannitol. Ideally, such markers should mimic the movements of AIB by rapidly and homogeneously distributing themselves throughout the entire extracellular space. In contrast, they should not gain access to the intracellular compartment. In the EDL muscle extracellular-space measurements increased rapidly over the first 1 min, before reaching a plateau from 2 min onwards. This trend was the same for all the three markers used and indicated a rapid equilibration

between the medium and the extracellular space. Since the three very different markers gave values of 23–26%, in excellent agreement, a mean extracellular value of 24% of muscle volume was subsequently used. The extracellular space of the soleus muscle was similarly measured and found to be 31% with both [14 C]inulin and [^3H]mannitol.

Initially the net transfer of AIB with time was measured in EDL, soleus and hemidiaphragm muscles (Fig. 1a). Over the first 10 min there was a rapid and fairly linear increase in AIB accumulation in all three muscles. Thereafter the distribution ratio increased more slowly to values greater than unity. When expressed as a rate, net transfer into all tissues decreased with time (Fig. 1b). However, a reasonably constant rate of AIB accumulation was observed over the first 10 min of incubation [Fig. 1b (inset) represents a separate experiment to define this period more fully]; thereafter it fell rapidly (Fig. 1b). This suggests that AIB influx, i.e. unidirectional transport, predominates over the first 10 min only. As the intracellular accumulation of AIB increases with time, efflux from the muscle probably becomes increasingly prominent, leading to a decrease in the rate of net transfer (Fig. 1b). Hence, although the distribution ratio continues to increase (Fig. 1a), the net rate falls (Fig. 1b). From our data (Fig. 1b) it appears that in earlier studies (by other workers) where transport was measured after 30 min or more, only

net transfer was actually measured, thereby indicating nothing of the respective contributions of influx and efflux. Appropriate experimental conditions had therefore to be found to separate influx and efflux for independent characterization.

Very thin diaphragm preparations were initially included in our studies to confirm that the slightly thicker leg muscles did not prohibit the uptake of nutrients and AIB into any fibres, particularly those located at the centre of the tissue. The broadly similar transport of AIB (Figs. 1a and 1b) into all three muscles validates the use of the leg muscles. Indeed, such leg muscles should represent a more suitable preparation for studying amino acid transport *in vitro*, since they can essentially be isolated as intact tissues; isolation of the diaphragm inevitably involves cutting many of its fibres.

Measurement of the rate of efflux

Efflux was measured as the release of preloaded AIB from the muscle fibres, via the extracellular

Fig. 1. Time course of AIB transport in isolated skeletal muscles

EDL (●), soleus (○) and hemidiaphragm (▲) muscles were incubated at 37°C for increasing periods of time in KRB, containing glucose (10 mM), AIB (1 mM) and [^{14}C]AIB (0.3 μCi). The net accumulation of AIB in each muscle was subsequently determined (see the Methods section). Results are expressed in (a) as distribution ratios, representing the ratio of radioactivity in the intracellular medium to that in the incubation medium, and in (b) as the rate of accumulation as a function of time. The inset represents separate experiments designed to measure the rates in more detail between 0 and 14 min. Each value is the mean $\pm$ S.E.M. for six separate EDL, soleus or hemidiaphragm preparations.

space into the surrounding incubation medium. However, the initial appearance of [^{14}C]AIB in the medium does not originate in the intracellular fluid, but comes from the preloaded extracellular space. Hence allowance has to be made for the wash-out of the extracellular space. To assess the rapidity with which the extracellular space empties under these conditions *in vitro*, EDL and soleus muscles were preloaded for 10 min with the extracellular marker [^{14}C]inulin; this time was more than sufficient for the marker to equilibrate

Fig. 2. [^{14}C]Inulin wash-out from the extracellular space of EDL and soleus muscles

EDL (●) and soleus (○) muscles were preloaded with [^{14}C]inulin by incubation for 10 min at 37°C in KRB containing glucose (10 mM) and 1.3 μCi of inulin (sp. radioactivity 5 Ci/mol). Muscles were then transferred to flasks containing fresh KRB, and the time-related release of [^{14}C]inulin from the extracellular space was determined by its appearance in the surrounding medium. Results are expressed as the percentage of the total preloaded inulin (i.e. 100%) remaining in each muscle with time. Each value is the mean $\pm$ S.E.M. for at least five muscles.

between the medium and the extracellular space (see above). Muscles were then transferred to fresh medium minus inulin, and the loss of the extracellular-space marker was measured with time (Fig. 2). Most of the preloaded inulin was rapidly washed out of the EDL muscle, with 55% and 80% of the total being released after 1 and 5 min respectively. Thereafter, the remaining inulin appeared to be released very slowly. The soleus behaved similarly, with 75% of the loaded inulin appearing in the medium after 5 min (Fig. 2). Since most of the material in the extracellular space had been washed out by 5 min, this incubation time was always taken to represent the commencement of true intracellular efflux. The intracellular release of AIB was then studied as a function of time. EDL muscles were preloaded with AIB (1 mM) for 120 min, and then transferred to identical fresh media (without AIB) from which the released AIB was measured over the ensuing 180 min. A desaturation curve was subsequently constructed by determining the amount of [^{14}C]AIB remaining in the EDL muscle at each sampling time (Fig. 3a). This was calculated by adding in reverse order the radioactivity released into the medium at each sampling time to that remaining in the tissue at the

Fig. 3. AIB efflux from EDL muscle

EDL muscles were loaded with AIB by preincubating them for 2 h at 37°C in KRB containing AIB (1 mM) and [¹⁴C]AIB (3.6 μ Ci). Each muscle was then transferred to fresh media, and the release of [¹⁴C]AIB from it was determined in 40 μ l samples of the medium taken at short intervals over a period of 180 min. The amount of AIB remaining in each muscle at each sampling time was calculated by adding, in reverse order, the radioactivity released over each sampling period to that remaining in the tissue at the end of the unloading period (i.e. 180 min). The AIB initially released from the extracellular space only (i.e. first 5 min of incubation) was subtracted throughout. Results are expressed as: (a) a desaturation curve showing the AIB (pmol) remaining in the muscle as a function of time; (b) a semi-logarithmic plot of the data of the desaturation curve. The amount of AIB remaining in the muscle at any time, t , during efflux was resolved into two exponential components defined by $P_t = A \exp(-\lambda_1 t) + B \exp(-\lambda_2 t)$, as derived from computer analysis (Provencher, 1976). After 40 min, the AIB remaining in the muscle approximated to a straight line (●) as described by $B \exp(-\lambda_2 t)$, where B is the amount of AIB present in the muscle's intracellular fluid at the commencement of this second phase of intracellular efflux. This line was extrapolated to zero time and its ordinates were subtracted from measurements before 40 min. A second straight line (Δ) can be fitted to these values according to the expression $A \exp(-\lambda_1 t)$, where A is the amount of AIB present in the muscle at the commencement of this first phase of efflux.

Fig. 4. Effects of external Na⁺ on transport of AIB, cycloleucine and tyrosine

EDL muscles were preincubated for 30 min in the presence of various concentrations of external Na⁺ (0–145 mM). They were then transferred to fresh media containing 0.015 mM-AIB (Δ ; 50 Ci/mol), 0.17 mM-cycloleucine ($\circ$; 1-aminocyclopentane-1-[¹⁴C]carboxylic acid; 50 Ci/mol), 0.07 mM-tyrosine ($\bullet$; 509 Ci/mol). The amino acids were obtained from Sigma and Amersham International. These concentrations are roughly equivalent to those of the natural amino acids, α -aminobutyrate, leucine and tyrosine, which occurs in the plasma. The net accumulation of each amino acid was measured after 2 h and each value represents the mean $\pm$ S.E.M. for six EDL muscles.

end of the total unloading period (i.e. 180 min). The number of exponential components that could be fitted to this curve was determined (Provencher, 1976). Two components were found, as shown in a semi-logarithmic plot (Fig. 3b). Since these two components probably represent separate phases or modes of intracellular efflux (both distinct from extracellular efflux), suitable incubation times (e.g. 15–20 min and 45–50 min) were subsequently chosen to measure each phase of efflux.

Effects of external Na⁺ depletion on the transport of neutral amino acids in skeletal muscle

The net accumulations of AIB (A system), cycloleucine (A and L systems) and tyrosine (L system) were measured separately in EDL muscles (Fig. 4). Muscles were preincubated for 30 min in either KRB or in the same buffer that had been progressively depleted of Na⁺, osmolar concentrations of choline salts being added to substitute for the sodium salts. This preincubation period allowed for any equilibration of a Na⁺ gradient across the sarcolemma (after partial replacement

of Na^+) before measurements of transport were made. Each muscle was then transferred to fresh medium (with Na^+ replaced or otherwise) now containing one of the amino acids in question, and its transport was measured over 2 h. Decreasing the external Na^+ caused a significant ($P < 0.025$; Fig. 4) decrease in AIB accumulation, with the effect becoming more pronounced with each progressive Na^+ depletion. In the complete absence of Na^+ , the distribution ratio of AIB was less than 1.0 (i.e. 0.77). That is, AIB was no longer being accumulated in the muscle against a concentration gradient, suggesting that active transport was no longer occurring. Although these experiments clearly point to the Na^+ -dependence of this A system, some transport, presumably carrier-mediated diffusion, still occurs in the absence of Na^+ (Fig. 4). In marked contrast, accumulation of the natural amino acid tyrosine (L system) was completely unaffected by all decreases in the concentrations of external Na^+ , including zero concentration (Fig. 4). This was also true in the presence or absence of cycloheximide, an inhibitor of protein synthesis. Hence, possible complications of intracellular tyrosine utilization cannot explain this observation. The transport of cyclo-leucine (non-metabolized) was significantly ($P < 0.025$) decreased between each step of Na^+

depletion (Fig. 4). Since this particular amino acid is believed to be carried by both A and L systems, the effects of Na^+ depletion were predictably intermediate with respect to the effects on AIB and tyrosine transport.

To determine which component(s) of AIB transport was dependent on Na^+ , the rates of influx and efflux were measured in the presence or complete absence of external Na^+ . AIB influx in the EDL muscle was markedly decreased (24%) by the removal of Na^+ (Table 1). This inhibition of influx correlates with the decrease (62%) in net accumulation of AIB in the absence of external Na^+ (Table 1). In addition to the effect on influx, replacement of extracellular Na^+ with choline induced a marked increase (63%) in the release of AIB during the first phase of intracellular efflux (Table 1). In contrast, AIB release during the second phase of efflux was not significantly (-14% , $P < 0.005$) changed by the removal of Na^+ . Thus this enhancement of efflux, together with the inhibition of influx, results in the decreased net accumulation of AIB in the absence of Na^+ (Table 1). The rates of both influx ($36.1 \pm 2.4 \text{ pmol}/\mu\text{l}$ per min) and efflux ($29.8 \pm 2.1 \text{ pmol}/\mu\text{l}$ per min) for tyrosine were unchanged when Na^+ was replaced by choline salts. These findings are consistent with the lack of

Table 1. *Individual and combined effects of Na^+ and cortisol on AIB transport in the EDL muscle*

For measurements of influx and net transfer, EDL muscles were preincubated for 4 h in KRB in the absence (controls) or presence of cortisol ($1 \mu\text{M}$). In additional experiments, muscles were preincubated in the absence of Na^+ , with or without cortisol. After preincubation, all muscles were transferred to fresh KRB of identical composition but also containing AIB (1 mM) and [^{14}C]AIB ($0.3 \mu\text{Ci}$). Influx was measured after 5 min of incubation and net accumulation after 60 min. For efflux measurements, experimental muscles were preloaded with AIB for 4 h in the presence of cortisol. They were then transferred to normal or Na^+ -free medium, again with or without (controls) cortisol, but all without AIB. The efflux of AIB was measured between 15–20 min (first phase) or 45–50 min (second phase). Efflux is expressed in two different ways; as the rate of AIB release ($\text{pmol}/\mu\text{l}$ per min), or as the fraction of the preloaded amino acid extruded between successive sampling times. Each value is the mean $\pm$ S.E.M. for a minimum of four individual muscles: * $P < 0.001$.

	Influx rate (pmol/ μ l of intracellular water per min)	Efflux rate				Net accumulation D.R.
		(pmol/ μ l of intracellular water per min) during		(fraction of preloaded AIB released/min) during		
		Phase 1 (15–20 min)	Phase 2 (45–50 min)	Phase 1 (15–20 min)	Phase 2 (45–50 min)	
With normal Na ⁺ salts (i.e. 145mM)						
Control	87.1 $\pm$ 3.6	20 $\pm$ 1.9	6.7 $\pm$ 0.1	0.014 $\pm$ 0.001	0.007 $\pm$ 0.0005	2.00 $\pm$ 0.06
+ cortisol	94.2 $\pm$ 4.6	—	—	0.021 $\pm$ 0.002	0.006 $\pm$ 0.0006	1.52 $\pm$ 0.05
Change (%)	+8			+54*	–14	–23*
Choline substituted (i.e. 0mM-Na ⁺)						
Control	63.6 $\pm$ 2.0	33 $\pm$ 3.8	5.7 $\pm$ 0.4	0.022 $\pm$ 0.002	0.006 $\pm$ 0.0004	0.73 $\pm$ 0.02
+ cortisol	68.6 $\pm$ 5.1	—	—	0.025 $\pm$ 0.002	0.006 $\pm$ 0.0005	0.75 $\pm$ 0.05
Change (%)	+7			+12	0	+2

change in the net accumulation of tyrosine in response to Na^+ depletions (Fig. 4). Hence the A and L transport systems for neutral amino acids in skeletal muscle exhibit very different properties with regard to their dependence on external Na^+ .

Effects of cortisol on AIB transport in the presence and absence of external Na^+

In preliminary experiments (Lewis & Goldspink, 1980; Lewis, 1982) it was found that the net accumulation of AIB in skeletal muscle was inhibited by cortisol, at concentrations of $1\text{ }\mu\text{M}$ or higher, after a minimal exposure time (*in vitro*) of 4 h. In the present study the effects of cortisol have been studied in more detail to determine its influence, if any, on influx and efflux, as well as the net transfer of AIB. Since other hormonal actions (e.g. growth hormone and insulin) on AIB transport have been shown (Kostyo, 1964; Riggs & McKirahan, 1973) to be Na^+ -dependent, an involvement of this cation in cortisol's action on AIB was simultaneously sought.

For measurements of net accumulation and influx of AIB, EDL muscles were preincubated in the presence or absence of $1\text{ }\mu\text{M}$ -cortisol for 4 h. After preincubation, the muscles were transferred to incubation media which were identical with that of preincubation but for the inclusion of AIB. For measurements of efflux, cortisol was present in both loading and unloading media of the appropriate groups. For all of these measurements on AIB transport the minimal latency period of 4 h, necessary for the glucocorticoid action on AIB transport to be observed (Lewis, 1982), was fully satisfied. In normal media (145 mM-Na^+) $1\text{ }\mu\text{M}$ -cortisol significantly inhibited the net accumulation of AIB in the EDL muscle (Table 1). However, this inhibition was not achieved by suppressing AIB influx, since this process was not significantly affected by exposure to the steroid. The release of AIB from the muscle's intracellular compartment was significantly enhanced during the first, but not the second, phase of efflux (Table 1). Thus it is probably this steroid-enhanced efflux of AIB which alone accounts for the decreased net accumulation of AIB.

The same measurements of transport were also made in the absence of external Na^+ , where KRB was modified as above (Fig. 4) by replacing Na^+ with osmolar amounts of choline salts. After removal of extracellular Na^+ (Table 1), the glucocorticoid's inhibitory action on AIB transport was lost. That is, the net accumulation in the presence of cortisol was now unchanged. Since influx was not affected by the steroid in the presence of Na^+ , it was not surprising that this situation did not change in the absence of Na^+ .

More important, however, were the effects of cortisol on efflux, since this appears to be the main site of action of the steroid. Once Na^+ had been removed from the medium, the rates of both phases of efflux (after exposure to the hormone) were not significantly different from those of the control muscles. Taken together, these results suggest that the steroid's normal inhibitory action on AIB transport (via efflux) is Na^+ -dependent, the removal of this mainly extracellular ion leading to the loss of the glucocorticoid's influence.

Discussion

Throughout this study the rates of AIB influx and efflux were investigated to determine their respective contributions to the net accumulation of this amino acid in isolated skeletal muscles. This approach differs from most previous studies on muscle (Kostyo, 1965; Goldberg & Goodman, 1969; Cooper & Kohn, 1980), where only the net transport of amino acids was measured, i.e. after incubations of 30 min or more. Clearly considerations of net transport alone are of limited value in characterizing a transport system.

A precise knowledge of the muscle's extracellular space is a necessary prerequisite for accurate measurements of all aspects of AIB transport. This was determined by using three extracellular-space markers, each different in size and structure but all stable and non-toxic. Our values of 24% and 31% for the extracellular components of the EDL and soleus muscles respectively were in close agreement with values of 25–29% reported previously for the EDL (Kernan & MacDermott, 1976) and soleus (Kohn & Clausen, 1971) of the rat and the biceps brachii of the mouse (Goldspink, 1965). However, other studies have reported lower (17%; Harris & Manchester, 1966) and higher (36%; Riggs & McKirahan, 1973) extracellular-space values. This is perhaps not surprising, since such values are known to change with age, sex and species of animal and aspects of experimental design (e.g. muscles stretched or otherwise), and must therefore be measured for each experimental system.

The decreasing rate of net accumulation with time (Fig. 1b) probably represents an increasing predominance of AIB efflux from these muscle preparations *in vitro*, as shown in previous experiments (Lewis & Goldspink, 1982). It is also possible that AIB influx is gradually suppressed (*trans*-inhibition) by the amino acid as it is accumulated intracellularly. Such situations have been described in tumour cells (Jacquez, 1975) and more recently in isolated preparations of the human placenta (Steel *et al.*, 1982). However, if

present in muscle, such *trans*-inhibition is unlikely to occur over the time period (i.e. 5 min) during which we have measured influx, the rate being constant over the first 8 min (Fig. 1b, insert). AIB efflux was only measured after the wash-out of the preloaded amino acid from the extracellular space (Fig. 2). The intracellular release of AIB could then be resolved into two distinct components by Fourier analysis (Provencher, 1976) of the desaturation curve (Fig. 3a). The existence of two separate components of efflux is very unlikely to be attributable to the back-flux of the amino acid once released into the medium, and was supported experimentally. That is, the first phase of release (over approx. 0–35 min) was responsive to both Na^+ depletion and the action of cortisol, whereas the second phase (approx. 35–180 min) was unaffected by either experimental manipulation. The same trend of differential effects on the two phases of efflux was also found when the muscle's supplies of ATP were depleted by various metabolic inhibitors (S. E. M. Lewis & D. F. Goldspink, unpublished work). In addition, the very slow release that we found in the second phase (Fig. 3) is similar to that described by other workers (Cooper & Kohn, 1980) with a different system *in vitro*.

The transport of AIB (A system), but not of tyrosine (L system), into skeletal muscle was shown to be Na^+ -dependent, with progressive depletions of external Na^+ lowering the rates of influx and net transport (Fig. 4 and Table 1). In the complete absence of external Na^+ , AIB accumulation was more markedly inhibited (62%) than was influx (24%), suggesting that efflux might also be a Na^+ -linked process. This was subsequently shown to be the case, the efflux of AIB from the fibres being increased after removal of external Na^+ (Fig. 4 and Table 1). Replacement of Na^+ by choline ions has previously been shown to have no effect on either the resting membrane potential (Kipnis & Parrish, 1965) or extracellular-space volume (Lewis, 1982) of skeletal muscle. Hence, in these Na^+ -substitution experiments, complementary effects on the rates of influx and efflux were observed, with both contributing to the observed decrease in the intracellular accumulation of AIB. The absolute rates of influx and efflux cannot, however, be directly compared, since they were essentially measured under different experimental conditions.

The rates of influx of several, but not all, amino acids has been shown to be linked to the inward movement of extracellular Na^+ in other cell types (Schultz & Curran, 1970; Guidotti *et al.*, 1978). The present study is therefore confirmatory with respect to skeletal muscle. The finding that at least one of the muscle's two phases of AIB efflux was Na^+ -dependent was particularly interesting and

supports the view that amino acid release is a structurally specific carrier-mediated process, similar to that described in other tissues (Christensen, 1977). The Na^+ -dependence of amino acid transport may be explained by a greater affinity between membrane carrier molecules and AIB in the presence of high (extracellular) concentrations of Na^+ , the relatively low concentration then favouring unloading from the carrier. Alternatively, the Na^+ gradient itself may provide the immediate driving force for amino acid influx (Hacking & Eddy, 1981). Since an inward-directed Na^+ gradient should always be re-established in situations where sodium salts were only partially replaced (Kipnis & Parrish, 1965), the decreasing accumulation of AIB under such conditions (Fig. 4) possibly supports the view that the crucial factor is the absolute concentration of Na^+ . In normal physiological situations the high extracellular concentration and/or inward-directed gradient of this ion probably facilitates the influx of A-system amino acids into muscle, but simultaneously suppresses their release from the fibres. Ultimately this would lead to a distribution ratio greater than unity. This can only be viewed as speculative reasoning in the absence of accurate measurements of intracellular as well as extracellular Na^+ concentrations. However, not all of the AIB transport can be linked to Na^+ , because even in the complete absence of this cation AIB was still accumulated (Fig. 4, Table 1) within the isolated muscles, presumably by non-active means, since the distribution ratio was less than 1.

The steroid-induced inhibition of the net accumulation of AIB (Table 1) in skeletal muscle has been assumed to be equivalent to a suppression of AIB influx (Kostyo, 1965). However, when true influx, as distinct from net accumulation (Kostyo, 1965), was measured (i.e. after much shorter incubation), no steroid effect on influx was observed (Table 1). This indicates that the lowering of net accumulation of AIB must be mediated via an enhancement of efflux; this was indeed found to be the case (Table 1). The fact that cortisol preferentially affects efflux and not influx possibly suggests that the carrier molecules participating in influx are different from those used for efflux.

Some workers have hypothesized that glucocorticoid hormones possibly inhibit amino acid transport (i.e. net accumulation; Table 1) by decreasing the number of available carriers (Kostyo, 1965). Steroid-induced inhibitions of muscle protein synthesis have been found only after a minimum of 3 h exposure to corticosteroids (McGrath & Goldspink, 1982). This latency period is therefore similar to that found in transport (Lewis, 1982), and hence the steroid's action on protein synthesis may be a concomitant rather

than a causal relationship with amino acid transport. We now know that the steroid acts not against influx, but rather in facilitating efflux. This appears to represent part of a concerted and generalized inhibition of cellular growth. Within 3–4 h of exposure to adrenal-cortical steroids, muscle DNA synthesis is suppressed (Goldberg & Goldspink, 1975), protein turnover slowed (McGrath & Goldspink, 1982) and AIB efflux accelerated. Thus this accelerated efflux of the muscle's A-system amino acids in response to cortisol complements the hormone's other actions in stimulating gluconeogenesis and essential protein synthesis elsewhere (e.g. synthesis of plasma proteins).

To elucidate glucocorticoid action still further, the influence of cortisol on AIB transport was determined in the absence of extracellular Na^+ . It had previously been shown (Riggs & McKirahan, 1973) that in rat diaphragm an amino acid-transport system stimulated by insulin was completely dependent on the presence of Na^+ . The loss of the usual glucocorticoid effects in Na^+ -free media (Table 1) suggests that the steroid, like insulin, somehow acts on this transport system through Na^+ -sensitive process(es). However, for the glucocorticoid hormone, its action was mediated solely through amino acid efflux and not through both influx and efflux, as appears to be the case for insulin (Guidotti *et al.*, 1978).

The influence of steroids and/or Na^+ on AIB transport in the soleus muscle was also studied throughout (results not shown). Transport in this second muscle type was investigated because these two muscles exhibit different functions within the body. In addition, it has been suggested (Goldberg & Goodman, 1969) that a relationship exists between the degree of contractile activity within a muscle and its degree of susceptibility to circulating hormones, e.g. the more active the muscle (e.g. soleus), the less responsive to steroid action. However, in essence, both muscle types responded almost identically with respect to Na^+ -dependence and steroid action. Similar effects on amino acid transport (measured *in vitro*) were still obtained if the muscles were previously exposed to the hormone while in the intact animal.

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