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Differences between Resting and Insulin-Stimulated Amino Acid Transport in Frog Skeletal Muscle

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Summary. We have compared some features of the resting and the insulin-stimulated uptake of α-aminoisobutyrate (AIB) in frog skeletal muscle. We found a substantial difference between the two processes, namely, that resting AIB uptake is Na-independent while the insulin-stimulated fraction of the AIB uptake is Na-dependent.

Since the amino acid transport systems in frog skeletal muscle are poorly characterized, we have also surveyed some of their properties. One of the most interesting findings of this survey is that both the uptake and efflux of AIB are inhibited by low concentrations of PCMBS (parachloro-mercury-benzene sulfonic acid 5×10^{-5} M). In contrast, the carrier mediated transport of basic amino acids is neither inhibited by this mercurial agent nor accelerated by insulin.

The action of PCMBS strongly suggests the presence of a critical sulfhydryl group in the amino acid carrier system utilized by AIB. This group is exposed to the outside solution since PCMBS penetrates cell membranes poorly, and in addition its inhibitory actions were reverted by agents that do not penetrate the cell membrane like albumin or glutathione.

Insulin stimulates the transport of amino acids across the membranes of a variety of cells including those of skeletal muscle (Kipnis & Noall, 1958; Riggs & Mackirahan, 1973; Narahara & Holloszy, 1974). The simplest explanation for this stimulation is that the turnover rate of the transport sites is increased. However there is evidence that suggests that other mechanisms may exist. Thus, in many cell types the insulin induced stimulation of amino acid transport is dependent on the synthesis of proteins (Goldfine, Gardner & Neville, 1972; Elsas *et al.*, 1975). This activity could lead to the formation of new transport sites; however, it is also possible that only the synthesis of additional enzymes that generate energy for the transport process is involved. A different possibility is

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that insulin unmasks latent transport sites, as has been shown for the stimulation of alkali cation transport (Grinstein & Erlij, 1974). In an attempt to distinguish among these mechanisms we have determined whether there are any differences between the characteristics of amino acid transport of control and insulin treated muscles.

Since the movement of amino acids is a Na-dependent process in many cells, we defined the relationship between the requirements for this cation in control and insulin treated muscles. In addition, because information on amino acid transport in muscle is scarce, we have studied the kinetics of the amino acid transport system and explored some of its molecular features. In most experiments we measured the fluxes of the nonmetabolizable amino acid α-aminoisobutyrate (AIB), and in additional experiments we surveyed some of the basic features of the transport of other amino acids.

Our results show that resting AIB uptake is unaffected by eliminating outside Na^+, while the insulin induced increase in AIB uptake is dependent on the presence of this cation in the bathing solution.

We also present evidence that strongly supports the view that one of the transport systems involved in the movements of neutral amino acids has an SH group accessible from the outer solution, while the system involved in the uptake of basic amino acids has no critical SH group facing the outer medium. A preliminary account of these results has been published (Grinstein & Erlij, 1976).

Materials and Methods

Paired sartorii dissected from the same frog (*Rana pipiens*) were used to provide control and test muscles. For efflux determinations the muscles were first incubated in small Erlenmeyer flasks containing 4 ml of the labeled amino acid solution for known periods of time. Immediately after loading, the muscles were soaked in a series of tubes containing 3 ml of nonradioactive solution for carefully timed intervals. At the end of the experiment the muscles were weighed and prepared for counting by dissolving them in 0.3 ml of Nuclear Chicago Solvent. The aqueous effluents were mixed with 10 ml modified Bray solution, while a toluene mixture was added to the dissolved muscles. The samples were then counted in a Packard Scintillation Counter and quenching was corrected by the External Standard Channels Ratio Method. By adding up in reverse order the corrected counts of the effluents to the radioactivity remaining in the muscle at the end of the experiment, it was possible to determine the radioactivity present in the muscle at the mid-time of each collecting period. These values were plotted against time in a semilogarithmic scale, as illustrated in Fig. 1. As shown in this figure, an initial period of rapid radioactivity loss was followed by a second phase in which the radioactivity decreased less steeply. Graphical analysis of these results showed that the efflux process is made up of the sum of two exponentially decaying components. This analysis, taken together with previous measurements of isotope

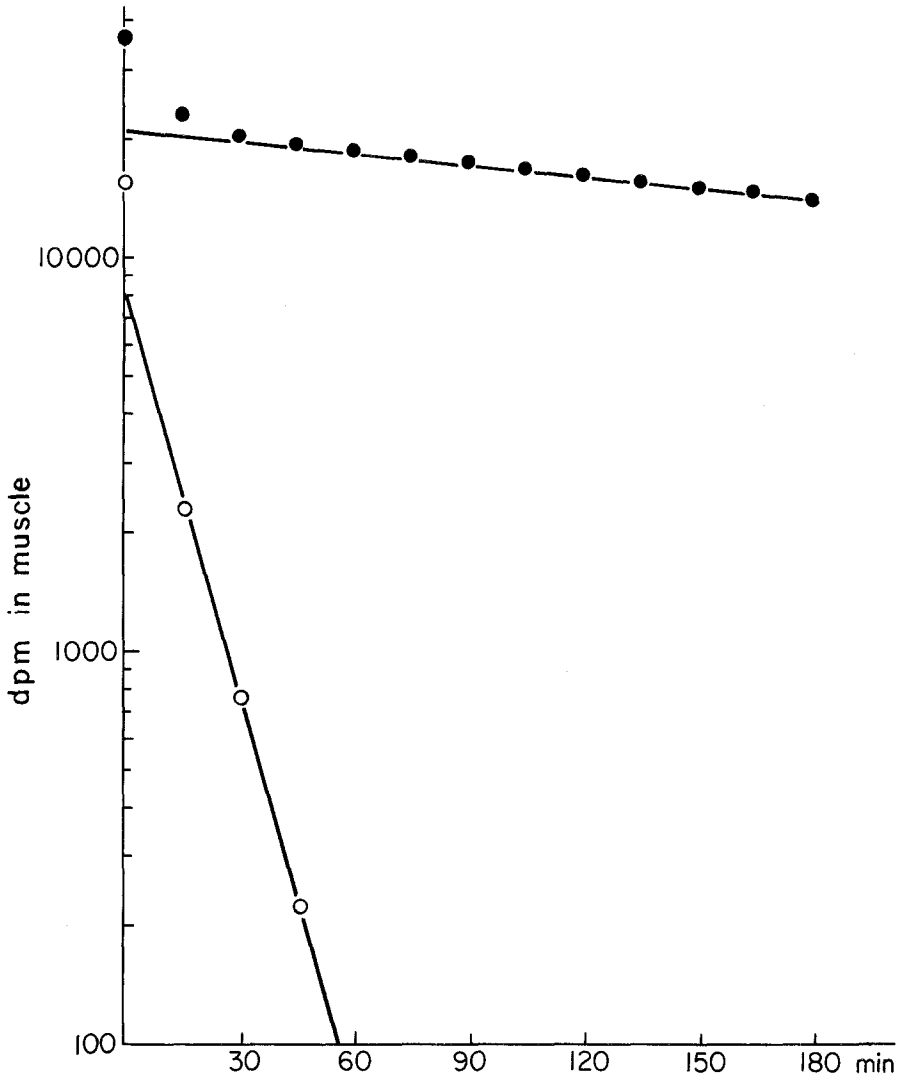

Fig. 1. Washout of AIB from a frog sartorius muscle. The ordinate is the total amount of radioactivity remaining in the muscle at any time. The open circles were calculated by subtracting the slow exponential from the total curve (filled circles). Only one intracellular compartment was discernible after 3 hr washout. The sum of the extrapolated intercepts do not add up to the total radioactivity. This is probably due to the radioactive material adhering to the surface of the muscle, which is washed in the first efflux period

washout from skeletal muscle (*see* Harris, 1963), clearly indicates that the fast component corresponds to the washout of the labeled amino acid from the extracellular space, whereas the slow component represents efflux from the intracellular compartment. The uptake of amino acids was determined by two methods that provided identical results. One is based on the graphical analysis of the washout curves. According to this analysis the extrapolated intercept of the slow component corresponds to the amount of intracellular amino acids at the end of the incubation in the loading solution.

In other experiments, the muscles were blotted, weighed and solubilized for counting immediately after withdrawing them from the radioactive solution. The amount of radioactivity present in the extracellular space was deducted from the total radioactivity. A value of 0.13 ml/g for the extracellular space was used in these calculations. This value was derived from the intercept of the fast component and is in agreement with previous determinations carried out elsewhere (Desmedt, 1953; Adrian & Slayman, 1966) as well as in our own laboratory (Erlij & Grinstein, 1976).

Although it could be argued that some effects on the uptake of amino acids may have been overlooked by an inaccurate estimation of the extracellular space, there are some findings that suggest that this is a remote possibility. First, the rate of efflux of amino acids from the muscle is very low ($K = 0.002\ min^{-1}$) and stable, allowing for a straightforward determination of the intercept. Second, in two groups of experiments the measurements were carried out after incubating the muscles in the labelled solution for 24 hr (*see* p. 15 and Table 3); under these circumstances, the concentration of label inside the muscle more than doubled the concentration in the extracellular medium; while the size of the extracellular space remained constant, contributing a very small fraction – less than 6% – of the total counts in the sample.

Except when otherwise indicated, uptake rates were determined in experiments lasting one hr. This incubation period was selected because, in agreement with Narahara & Holloszy (1974), we found that the entry of AIB and arginine is linear for periods up to two hr. Steady state amino acid accumulation was determined after incubating the muscles for at least 24 hr in the labeled solution, since Narahara & Holloszy (1974) demonstrated that such long periods are required to approach equilibrium.

In the experiments in which efflux was studied it was expressed as a rate constant, i.e., the fraction of the radioactivity lost per unit time.

When either the uptake or the efflux from paired sartorii incubated under the same conditions were compared, the observed differences never exceeded 5%.

The standard Na-Ringer's had the following composition (in mmoles/liter): NaCl 115, KCl 2.5, $CaCl_2$ 2.0, Tris Cl 3.0, pH 7.4.

Na-free Ringer's were prepared by isosmotic substitution of all NaCl by either Tris Cl, Choline Cl or LiCl. Potassium-Ringer's had the following composition: K_2SO_4 95, $CaSO_4$ 9.0, K_2HPO_4 1.08, KH_2PO_4 0.43. In muscles incubated for 24 hr the basic Na-Ringer's had the following composition: NaCl 115, $CaCl_2$ 2.0, Tris Cl 3.0, K_2HPO_4 0.43, $MgSO_4$ 1.0, glucose 5.0. To avoid damage caused by bacterial growth, 50 μg/ml streptomycin and 50 U/ml penicillin were added to this medium. Sodium-free solutions for long incubations were prepared by isosmotically replacing all NaCl by Tris Cl, LiCl or choline Cl.

The temperature was kept constant by means of a thermoregulated water jacket. Unless otherwise indicated, the temperature for both the uptake and efflux experiments was 19 °C.

The concentration of sodium in nominally sodium-free solutions was checked by flame-photometry using a Unicam SP-90 photometer. Intracellular water content was determined as described by Adrian & Slayman (1966).

The purity of some of the radioactive compounds was determined by thin layer chromatography (TLC). For this purpose, samples of radioactive AIB and arginine were chromatographed on aluminum cellulose sheets (Merck, Darmstadt) using butanol/acetic acid/water (60:15:25) as the solvent.

The concentration of insulin used for both efflux and uptake experiments was always 250 mU/ml. This concentration has been reported to produce a maximal stimulation of alkali-cation movements in frog sartorius muscles (Erlij & Grinstein, 1976).

^{14}C-AIB, ^{14}C-Arginine, ^{14}C-Serine, ^{14}C-Proline and ^{3}H-Phenylalanine were obtained from Amersham-Searle. Unlabelled amino acids, crystalline bovine insulin, cycloheximide, *p*-chloromercuribenzene sulfonic acid (PCMBS), N-ethyl maleimide, iodoacetic acid, pyri-

doxal phosphate, trinitrobenzene sulphonic acid, reduced glutathione, dithiotreitol and bovine albumin were purchased from Sigma Chemical Co. All other substances were reagent grade.

The abbreviations used for the amino acids are those approved by the International Union of Pure and Applied Chemistry.

All the values are given as the mean ± one standard error. Straight lines were fitted by the least squares method.

Results

The Rate of Entry of AIB and External Sodium

Fig. 2 shows the relationship between AIB concentration in the medium and its rate of entry into muscle cells. In agreement with previous observations (Akedo & Christensen, 1962; Narahara & Holloszy, 1974) the shape of the curve suggests the presence of at least two components: one readily saturable and a diffusion-like linear component which is apparent only at high substrate concentrations. The dashed line in Fig. 2, representing the estimated diffusion-like linear component, was drawn by passing a straight line through the origin and parallel to a line fitted by the least squares method to the points with the four highest concentrations of AIB.

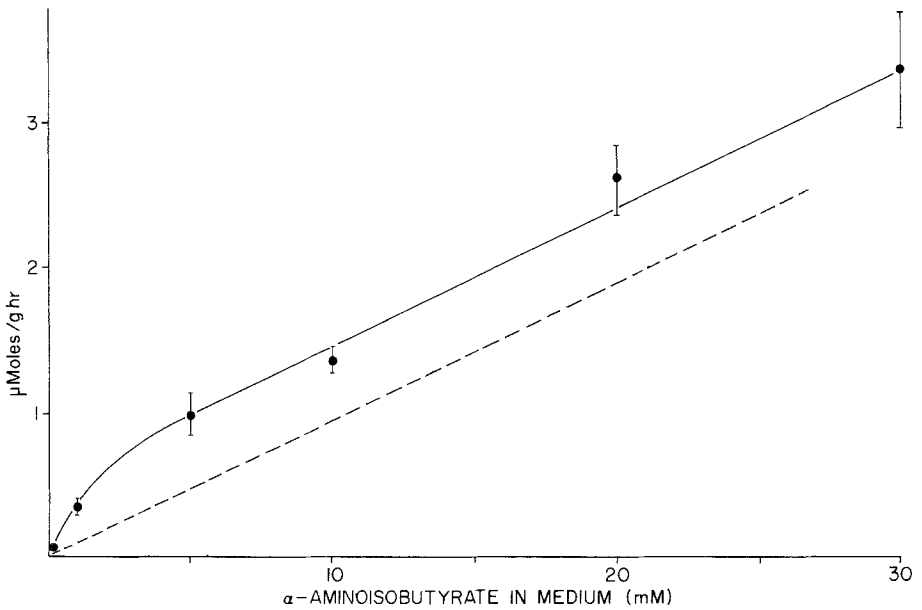

Fig. 2. The initial rate of entry of AIB was measured as a function of AIB concentration. The dashed straight line representing the contribution of the postulated diffusion–like component to the overall uptake rate was drawn parallel to the terminal portion of the original curve. The bars represent ± one SE

Table 1. Effects of Na-Free Solutions on the Initial Rate of AIB Uptake[a]

Substituent	μmoles/g/h	n	*P*
Control	0.0200±0.0034	8	N.S.[b]
Tris	0.0231±0.0042	8	
Control	0.0306±0.0070	6	N.S.[b]
Lithium	0.0322±0.0063	6	
Control	0.0365±0.0023	4	N.S.[b]
Choline	0.0399±0.0018	4	
Control	0.0318±0.0026	5	N.S.[b]
K_2SO_4	0.0304±0.0020	5	

[a] Paired muscles were used throughout. $[AIB]_0 = 0.1$ mM.
[b] N.S. = Non significant $p > 0.05$.

In Table 1 are shown the effects of sodium-free solutions on AIB uptake measured after 1-hr incubation in the labeled amino acid. Lithium, Tris, choline and potassium were used to substitute for Na^+. When the highly permeant K^+ was used as a substitute, sulfate was used as the counterion to prevent cell swelling. In each group, the AIB uptake of control muscles immersed in Na-Ringer's is compared with the uptake of the paired experimental muscle immersed in Na-free solutions. The rate of amino acid uptake was not significantly affected by any of the four solutions in which sodium had been eliminated. As a control, the Na^+ concentration in the Na-free solutions was determined by flame photometry at the end of each experiment; the concentration of Na^+ was always less than 70 μM.

The lack of effects of Na-free solutions on AIB uptake was also observed in experiments in which paired muscles were incubated in 0.1 mM AIB for 24 hr either in Na^+-containing or Na^+-free Tris solutions. At the end of this period, the intracellular AIB concentration was 0.23±0.02 mmole/liter cell water (n=8) in the muscles equilibrated in Na-Ringer's and 0.23±0.01 mmole/liter cell water in the case of Tris-Ringer's. At the end of the experiment the nominally Na-free solution contained about 80 μM Na^+.

The Effects of Insulin

Tables 2 and 3 summarize the data of experiments carried out to determine the effects of insulin on AIB uptake and its dependence on

Table 2. Sodium Dependence of the Effects of Insulin on the Rate of AIB Uptake

	Preincubation time[a] (hours)	μmoles/g/h	n	*P*
Sodium		0.0292 ± 0.008	6	≧0.05
Sodium + insulin	2	0.0352 ± 0.010	6	
Tris		0.0186 ± 0.003	4	>0.05 N.S.
Tris + insulin	2	0.0192 ± 0.004	4	
Sodium		0.0285 ± 0.003	8	<0.01
Sodium + insulin	6	0.0426 ± 0.004	8	
Tris		0.0471 ± 0.009	4	>0.05 N.S.
Tris + insulin	6	0.0496 ± 0.012	4	

[a] Preincubation time refers to the period spent by the muscles in insulin containing media before the initiation of the uptake determinations.

Table 3. Sodium Dependence of the Insulin Induced Accumulation of AIB

Solution	mM	n	*P*
Sodium	0.259 ± 0.03	8	<0.01
Sodium + insulin	0.453 ± 0.05	8	
Tris	0.246 ± 0.02	7	
Tris + insulin	0.248 ± 0.02	7	N.S.
Lithium	0.218 ± 0.01	4	
Lithium + insulin	0.232 ± 0.03	4	N.S.
Choline	0.278 ± 0.04	4	
Choline + insulin	0.268 ± 0.04	4	N.S.

Muscles were incubated with 0.1 mM AIB and insulin for 24 hr in control and sodium free solutions. Values refer to concentration of amino acid in mmoles/liter cell water.

outside sodium. Several points emerge from the results in Table 2. First, the increase in the rate of AIB uptake caused by insulin is dependent on the period of preincubation in insulin; the increase in uptake was barely significant when the muscles were incubated with insulin for 2 hr before transferring them to the solution containing labelled AIB, while in muscles which were preincubated for 6 hr, the hormone produced a clearcut increase in AIB influx. Second, the insulin-induced increase in AIB transport was absent when the experiments were carried out in Na-free solutions. The final point to be shown by the data in Table 2 is that there is a large variability among the values of resting AIB uptake in muscles isolated from different batches of frogs. As described above,

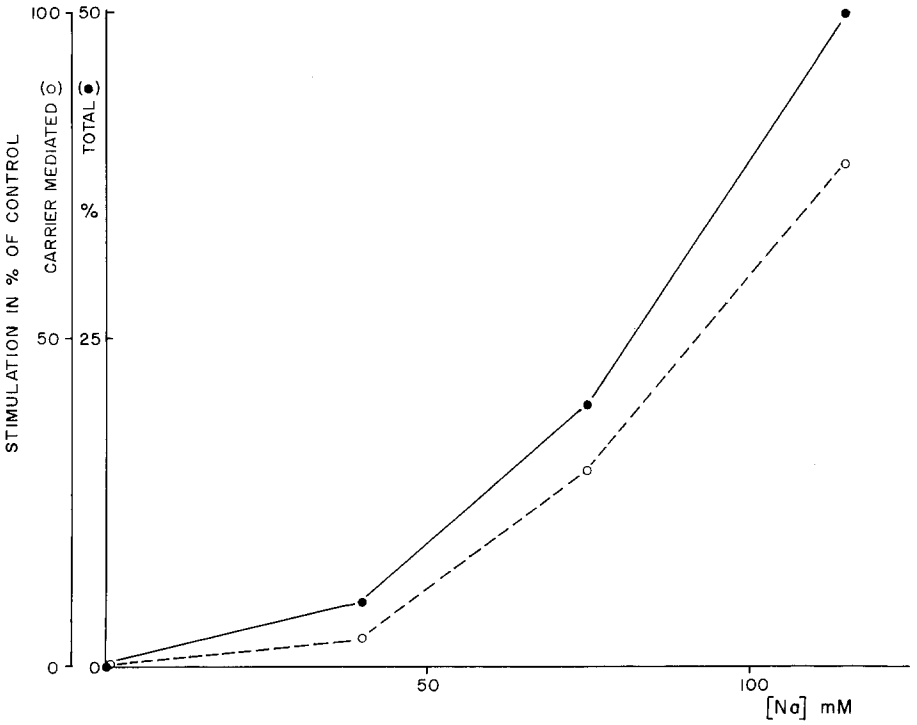

Fig. 3. Sodium dependence of the insulin-induced increase in AIB uptake. Abscissa: sodium concentration in medium. Ordinate: the increase in AIB uptake is expressed both as the percentual increase in total AIB uptake (filled circles, solid line) and as the percent of the saturable uptake component (open circles, dashed line) calculated as in Fig. 6*A*

no effects of substituting sodium by Tris were detected when paired muscles were used (Table 1). However, if determinations made in different batches of frogs are compared, (Table 2) erroneous conclusions can be reached since large differences were observed not only between the Na-containing and Na-free groups, but also among the Na-free groups themselves.

The Na-dependence of the stimulation by insulin is also observed in the data of Table 3, where it is shown that the amount of AIB that equilibrated after 24 hr with the muscle water was enhanced by insulin only when the incubation medium contained sodium.

Fig. 3 summarizes the results of experiments in which the relationship between outside sodium concentration and insulin stimulation of AIB uptake was determined in muscles that had been preincubated for 6 hr with the hormone. The simplest expectation derived from the mass action law is that the relationship between outside sodium and amino acid uptake should follow a rectangular hyperbola with a linear start in the lower

range of sodium concentrations. The results in Fig. 3 deviate from such expectation in two respects: First, no saturation is apparent within the concentration range studied; this can be attributed to a low affinity of the transport sites for Na^+. Second, the results show a marked tendency to curve upwards; this shape suggests that a cooperative action of several Na ions is necessary for amino acid uptake. When the results in Fig. 3 were replotted using double logarithmic coordinates, the slope of the resulting line suggested that two sodium ions were required to activate each site. However, this value is a rough approximation since only a few Na^+ concentrations were tested.

Another feature of the insulin-stimulated AIB uptake that we explored was its sensitivity to inhibition by leucine, proline and glycine (10 mM). These amino acids respectively inhibited 50, 20 and 80% of the AIB uptake in insulin-treated muscles. This inhibition was nearly the same as that observed in control muscles (*see* Table 4) implying that the amino acid selectivity of the carrier is similar in the resting and the stimulated conditions.

To test whether the sodium-dependence of insulin stimulation of AIB transport is related to the activity of the sodium pump, we measured the effects of ouabain (5×10^{-5} M) on AIB transport. Incubation with the glycoside for periods of 90 min did not affect either resting or insulin-stimulated AIB uptake.

In another group of experiments we measured the effect of insulin on AIB efflux. In one type of assay, insulin was added to the efflux solution and the rate of exit of AIB was measured for 1 hr. In other assays the experimental muscle was preincubated for 4 hr in a medium containing insulin and then loaded for 1 hr in AIB solution containing the hormone and finally the efflux was followed in an insulin-containing medium. The control muscle was subjected to the same experimental sequence but using insulin-free solutions throughout. The rate constant for AIB efflux was unaffected by insulin in either type of assay.

PCMBS and Other Inhibitors

An unexpected feature of the resting AIB uptake, namely, its independence of outside [Na], suggested that it was desirable to carry out a survey of the general features of the movements of aminoacids in frog skeletal muscle.

As a part of this survey we measured the effects of a number of protein reagents as inhibitors of the fluxes of AIB. We found PCMBS

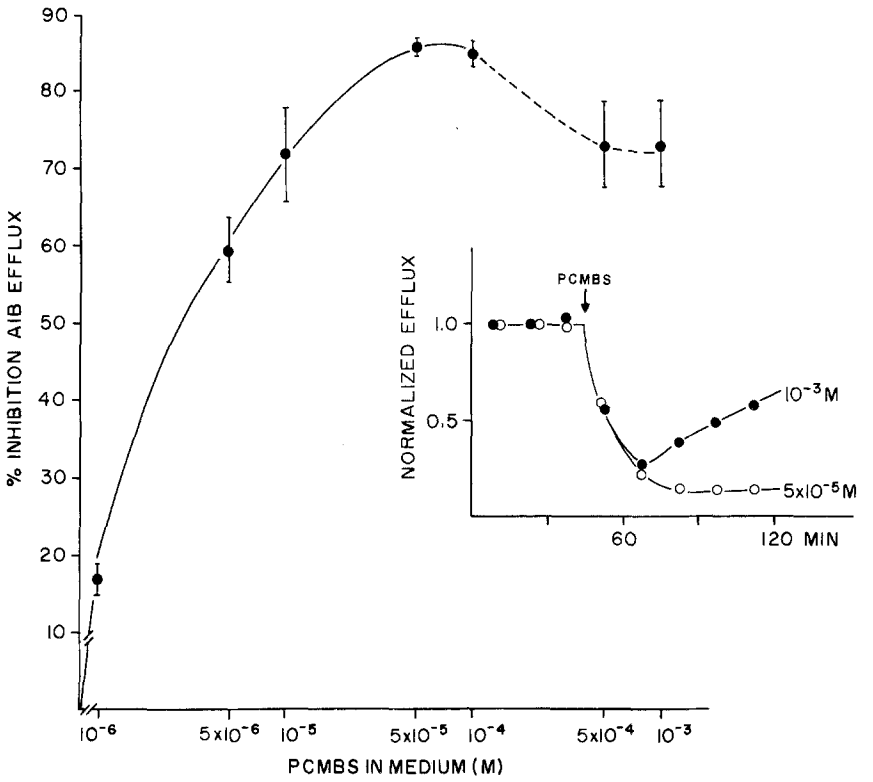

Fig. 4. The rate of efflux of ^{14}C-AIB from frog sartorius muscles was measured as a function of PCMBS concentration in the bathing fluid. Ordinate: maximal percentual inhibition of AIB efflux. The bars represent ± one SE. The inset shows two individual experiments in which we studied the effects of 10^{-3} M (filled circles) and 5×10^{-5} M (open circles) PCMBS on the efflux of AIB from paired sartorii. Ordinate: normalized efflux

to be the most effective agent among those tested. The relationship between PCMBS concentration and inhibition of AIB efflux is plotted in Fig. 4. Half inhibition of the efflux was reached with concentrations of about 2×10^{-6} M and maximum inhibition – about 85% of the total efflux – with 5×10^{-5} M. Since the remaining 15% of the efflux is only slightly affected by changing the temperature or by the addition of AIB to the external medium, it is likely that this fraction of the efflux is not carrier-mediated. The inset in Fig. 4 shows two individual experiments that illustrate the effects of high PCMBS concentrations. When 5×10^{-5} M PCMBS was added to one muscle, AIB efflux was reduced to a new steady low level, while the addition of 10^{-3} M to the paired muscle caused a transient reduction which was rapidly followed by an increased efflux.

The finding that the inhibitory effects on amino acid efflux are reduced

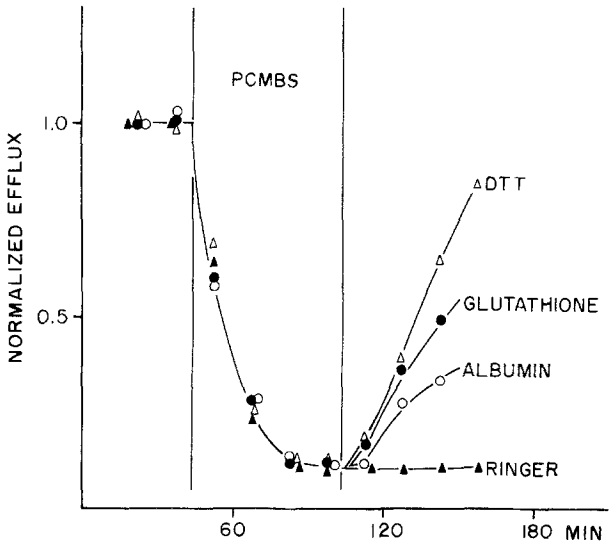

Fig. 5. The effects of several reducing agents on AIB efflux from PCMBS poisoned muscles. After a control period 5×10^{-5} M PCMBS was added to the bathing solution. One hour later PCMBS was removed and the external solution was replaced by Ringer's (filled triangles) or Ringer's containing 10^{-4} M of either dithiotreitol (open triangles), reduced glutathione (filled circles) or bovine albumin (open circles). Ordinate: normalized AIB efflux

when PCMBS concentration is increased beyond a certain level, is similar to observations on the effects of PCMB on the ouabain-insensitive Na^+ efflux (Erlij & Leblanc, 1971). As suggested by the experiment in the inset of Fig. 4, it seems likely that higher concentrations of these mercurial agents induce unspecific leaks in the membrane through which intracellular solutes can leave the cytoplasm.

Fig. 5 shows that the inhibitory effect of PCMBS on AIB efflux can be reverted by a number of reagents. In these experiments AIB efflux was first measured in Ringer's solution and then followed for 1 hr in a solution containing 5×10^{-5} M PCMBS. Finally the muscles were transferred to solutions containing either inhibitor-free Ringer's or 10^{-4} M of albumin, reduced glutathione or dithiotreitol. It is clearly shown that in the presence of all these agents the reduced efflux increased towards the initial level. Dithiothreitol rapidly restored the efflux while albumin was only slowly effective.

A clearcut inhibitory effect of PCMBS was also observed when amino acid uptake was measured. Table 4 shows the determinations of the effects of 5×10^{-5} M PCMBS on the uptake measured at different concentrations of AIB. Although the absolute reduction of external transport increased as the concentration of AIB was increased, the percentage inhibition de-

Table 4. Effects of PCMBS on the Uptake of α-Aminoisobutyrate

AIB concentration	Uptake rate in μmole/g/hr			
	Control	PCMBS treated	PCMBS sensitive	% Inhibition
10^{-4} M	0.0371 ±0.0034	0.0177 ±0.0012	0.0194	60
10^{-3} M	0.245 ±0.0407	0.156 ±0.0190	0.0890	37
5×10^{-3} M	0.590 ±0.0680	0.340 ±0.0370	0.250	42
10^{-2} M	1.260 ±0.100	0.981 ±0.070	0.279	22
2×10^{-2} M	2.600 ±0.310	2.100 ±0.300	0.500	19
3×10^{-2} M	3.830 ±0.380	3.300 ±0.290	0.530	14

Four muscles were used for each determination. Values are means ± one standard error. PCMBS concentration was 5×10^{-5} M.

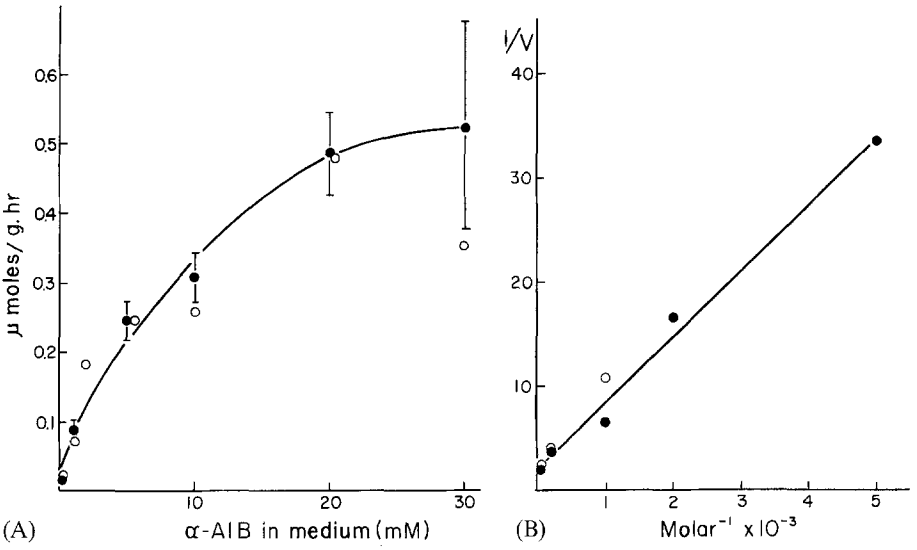

Fig. 6. (*A*): Comparison of the PCMBS sensitive influx of AIB with the saturable component of entry, calculated by subtracting the linear component from the total uptake in Fig. 2. (*B*): The same data are plotted in a double reciprocal form

creased. Such behavior would be expected if the system were composed of a saturating plus a linear diffusion-like component, and provided that only the saturating fraction of the uptake were inhibitor-sensitive. Fig. 6*A* shows a comparison between the PCMBS-sensitive AIB influx and the saturating component of the uptake calculated by subtracting the linear diffusion-like component from the total AIB uptake in Fig. 2. If allowance is made for the scatter inherent to data obtained from different animals, the agreement between the two groups of data is remarkable. Fig. 6*B*

shows a double reciprocal plot of these data; the apparent K_m was 2.87 mM and $V_{max} = 0.444$ μmole/g/hr.

Several other sulfhydryl group reagents were assayed as inhibitors of AIB efflux. Ethacrynic acid (10^{-3} M) inhibited 20 to 60% of the efflux, but lower concentrations were completely ineffective. Addition of 10^{-4} M of either N-ethyl maleimide or iodoacetate had barely detectable effects on amino acid transport, whereas millimolar concentrations of these agents produced an increased efflux, presumably as a consequence of membrane breakdown. The amino reagents trinitrobenzene-sulfonate and pyridoxal phosphate had no effect on AIB efflux when added to the bathing solution at 1 mM.

Competition and Countertransport

In Table 5 are summarized the effects of several amino acids on the uptake of AIB. Except for the basic amino acids arginine and lysine, all the other amino acids tested had significant inhibitory effects on the uptake of AIB. Greater effects were observed with the aliphatic amino acids; the least effective ones were the dicarboxylic and basic amino acids.

Fig. 7*A* illustrates the effects of adding several amino acids to the bathing medium on the efflux of AIB from frog sartorius muscles. After measuring the resting efflux for three periods of 15 min each, the washout was followed in solutions containing 10 mM of the selected amino acid. The average rate constant for resting AIB efflux was 0.0021 ± 0.0001 per

Table 5. Effects of Several Amino Acids on AIB Fluxes in Frog Skeletal Muscle

Amino acid	Uptake			Efflux
	Control	+Inhibitor	% Inhibition	% Stimulation
AIB	–	–	–	216
Gly	0.0321 ± 0.006	0.0045 ± 0.0001 (4)	75	66
Leu	0.0335 ± 0.006	0.0171 ± 0.004 (7)	49	108
Phe	0.0438 ± 0.004	0.0227 ± 0.003 (3)	48	70
Ser	0.0275 ± 0.003	0.0139 ± 0.002 (3)	49	44
Pro	0.0312 ± 0.004	0.0267 ± 0.004 (7)	14	8
Glu	0.0294 ± 0.005	0.0260 ± 0.004 (5)	12	3
Arg	0.0219 ± 0.001	0.0230 ± 0.003 (3)	0	–
Lys	0.0456 ± 0.002	0.0468 ± 0.004 (3)	0	3

Paired sartorii were used for all the uptake measurements. Numbers in parentheses refer to the number of assays performed for each amino acid.

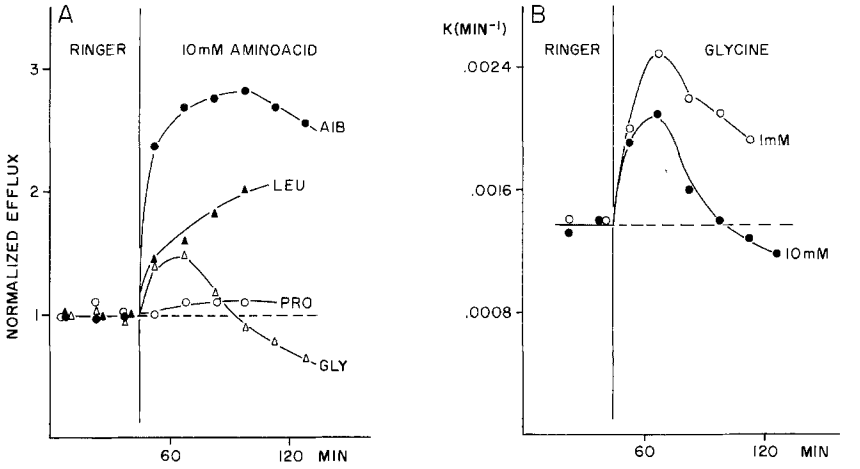

Fig. 7. (*A*): Effect of several nonradioactive amino acids on the efflux of ^{14}C-AIB from frog sartorii. After 45 min, 10 mM of either AIB, leucine, proline or glycine were added to the bathing solution. Ordinate: normalized efflux. (*B*): Effect of the addition of 1 and 10 mM glycine on the efflux of ^{14}C-AIB from paired sartorii. Ordinate: rate of AIB efflux, in min^{-1}

min (n = 38). Most of the amino acids tested produced a rapid increase in AIB efflux when added to the external solution. The sequence of effectivity for the stimulation of efflux (summarized in the last column of Table 5) is nearly the same as that observed for inhibition of uptake. The most notable exception was glycine which, in spite of being most effective for inhibiting uptake, produced a relatively small increase in efflux.

Fig. 7 *A* and *B* show a particular feature of the effects of glycine on ^{14}C-AIB efflux which may provide a clue to explain its poor ability to induce counterflow. The increase in AIB efflux caused by the addition of 10 mM glycine rapidly decayed falling below the control level. A possible explanation for this effect is to assume that glycine rapidly penetrates the muscle cells and competes with ^{14}C-AIB for the efflux sites on the inside surface of the membrane, thus reducing ^{14}C-AIB efflux. The experiment in Fig. 3 *B* supports this explanation; when only 1 mM glycine was added to the outside solution the resulting increase in AIB efflux was greater and declined less steeply than when 10 mM were used, since a smaller amount of glycine is expected to enter the muscle cells when 1 mM of the amino acid is used.

Temperature

Fig. 8 *A* shows that reducing temperature to 4 °C markedly depressed resting efflux AIB and completely abolished the AIB stimulated counter-

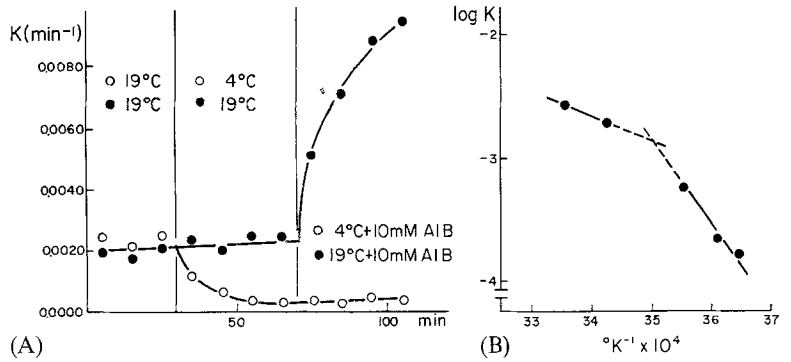

Fig. 8. (*A*): The effects of low temperature on AIB efflux from frog skeletal muscle. First, efflux was measured in both muscles at 10 °C. After 45 min, the experimental muscle (open circles) was cooled to 4 °C, while the control muscle (filled circles) was kept at 19 °C. In the final period 10 mM nonradioactive AIB was added to the external solution. (*B*): Arrhenius plot obtained from measurements of the rate of efflux of AIB from frog sartorii at different temperatures

flow. Fig. 8*B* shows an Arrhenius plot of AIB efflux. Two regions of the curve can be discerned: one at high temperatures that corresponds to an activation energy of 9.5 kcal per mole, another observed at temperatures below 12 °C corresponding to an activation energy of 32 kcal per mole.

Movement of Basic Amino Acids

It was shown in Table 5 that AIB fluxes were not modified by the addition of basic amino acids. This finding suggests that, in line with observations in other tissues (Beyer *et al.*, 1947; Kamin & Handler, 1951), basic and neutral amino acids move through different carrier systems across the muscle membrane.

We have found that the relationship between arginine uptake and concentration can be described, as was the case for AIB, by the sum of two processes: a linear diffusion-like component with a rate constant $k_d = 0.155$/hr and a saturating component (apparent $K_m = 1.01$ mM, $V_{max} = 0.794$ μmole/g/hr). Addition of cationic amino acids caused a marked inhibition of arginine uptake while addition of AIB had no effect (*see* Table 6, first column). In agreement with these findings are the results on efflux; as shown in Fig. 9*A* the addition of AIB to the external solution had a minimal effect on the efflux of arginine, while other dibasic amino acids such as lysine, histidine and arginine itself greatly stimulated ^{14}C-arginine efflux. In contrast to the effect of PCMBS on the uptake of AIB, the mercurial agent did not modify the rate of uptake of arginine

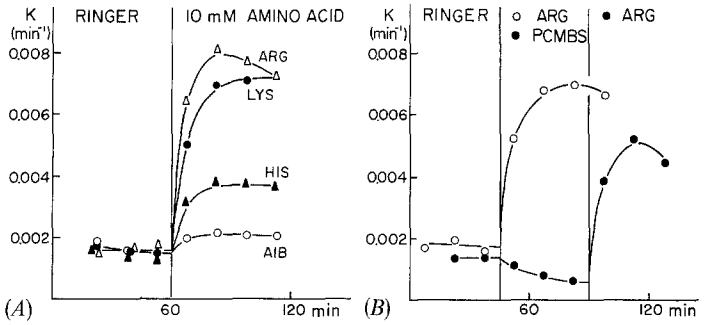

Fig. 9. (*A*): Effect of several nonradioactive amino acids on the efflux of ^{14}C-arginine from frog sartorii. After a control period 10 mM of either AIB, histidine, lysine or arginine were added to the bathing solution. (*B*): The effect of PCMBS on resting and arginine-stimulated ^{14}C-arginine efflux from sartorius muscles. After 45 min, nonradioactive arginine (10 mM) was added to the control muscle (open circles), while PCMBS (10 mM) was added to the experimental muscle (filled circles). In the final period 10 mM nonradioactive arginine was added to the experimental muscle

Table 6. The Effects of Several Variables on the Uptake of Labelled Amino Acids

	Arginine		Serine		Glutamate		Phenyl alanine		Proline	
Control uptake	0.0904 ± 0.0047		0.1917 ± 0.0098		0.0110 ± 0.0011		0.1299 ± 0.0088		0.0226 ± 0.0022	
PCMBS		0%		72%		35%		32%		9%
Competition	Lys	81%	Thr	83%	Asp	35%	Try	38%	Aze	35%
	His	67%	Phe	65%			Tyr	32%	Gly	12%
	AIB	0%	AIB	9%				–		–
Na-dependent		0%		32%		–				
Insulin		0%		35%		0%		–		–

The uptakes from solutions containing 0.1 mM of the amino acids listed at the head of each column were measured under the experimental conditions listed in the first column. The control rates are expressed as μmoles/g/hr. All the comparisons were carried out between paired muscles. Competition was tested by adding 10 mM of the unlabeled analogue. Inhibition by analogues, PCMBS (10^{-4} M) and Na-free solutions is expressed as the percentual decrease of the control flux. Insulin stimulation was measured after 4 hr preincubation with the hormone. The data refer to the percentual increase above the control level. At least four determinations were performed for each condition. Aze = Azetidine carboxylic acid.

(Table 6). More complicated results, however, were found when the effects of PCMBS were measured on arginine efflux. Fig. 9*B* illustrates a typical experiment; first the resting level was determined, then the control muscle was transferred to a medium containing 10 mM arginine; this treatment resulted in a large increase in the efflux of radioactivity. When the paired muscle was treated with PCMBS we found, unexpectedly, that the mercurial reduced the efflux to 0.50 times the control level. Such an observation

is contrary to the simpler predictions derived from the finding that PCMBS had no effect on arginine uptake. This observation became even more intriguing when we found that – as shown in the last part of Fig. 9*B* – PCMBS did not block the arginine-induced enhancement of the efflux. These results are in contrast to the findings on the AIB transport system where PCMBS inhibited both the resting efflux and the counterflow induced by external addition of AIB.

The presence of two components of efflux of radioactivity from muscles loaded with ^{14}C-arginine – one observed in resting muscles and sensitive to PCMBS, the other PCMBS-insensitive and induced by external arginine – brings forth the question of whether the labeled compound leaving the muscles is indeed arginine. To check this point we collected the radioactivity leached from six muscles after loading with ^{14}C-arginine and washing the extracellular space. When this effluent was analyzed by TLC we found that more than 95% of the radioactivity comigrated with arginine.

This finding implies that arginine efflux indeed occurs through two different paths. Counterflow takes place through a path analogous to that used for the uptake, since both are PCMBS insensitive, while resting efflux occurs partially through a different route.

Transport of Other Amino Acids

Table 6 summarizes results of the experiments in which we surveyed some features of the uptake of several amino acids. In the first place it is interesting that, except for arginine, the uptake of all the other amino acids was significantly inhibited by PCMBS (10^{-4} M). In the latter cases the magnitude of the inhibition produced by PCMBS and that caused by a 100-fold larger concentration of the most closely related amino acids roughly coincide. Thus, in the case of serine, PCMBS abolished 72% of the uptake while threonine caused an inhibition of 83%. Similar findings were made in the cases of glutamate and phenylalanine.

Another interesting feature shown in Table 6 is that about one third of the uptake of serine was dependent on external Na.

Discussion

We will consider in turn, three properties of the amino acid transport system that emerge from the results described here: first, the system used for AIB uptake in resting muscle is independent of external sodium; second, the insulin induced AIB uptake is sodium dependent; third, there

is an SH reactive site in the AIB transport system facing the outside solution which is not present in the system that transports dibasic amino acids.

Although there are several examples of Na-independent amino acid carrier systems, our findings on the Na-independence of AIB uptake were somewhat unexpected in view of the conclusion of Narahara & Holloszy (1974) who claimed that AIB uptake in frog sartorius muscle is sodium dependent. Their conclusion is based on the comparison of data obtained in muscles isolated from different batches of frogs. Since—as observed by Narahara & Holloszy and by ourselves—amino acid uptake can differ up to about 100% when muscles from different batches of frogs are studied, the comparison is reliable only in experiments employing paired muscles. Moreover, as we found no detectable effects of sodium lack when four different cations were used to substitute for sodium, the notion that AIB fluxes in frog skeletal muscle occur through a sodium independent system seems well substantiated.

After 24 hr of incubation the final intracellular concentration of AIB exceeded that in the outside solution. It is unlikely that the energy derived from ion gradients is the source for AIB accumulation, since similar equilibrium concentrations were found in the absence of Na^+, and under conditions that abolish membrane potential such as substitution of Na^+ by K^+.

Two sources remain as likely candidates for the supply of energy necessary for accumulation: it could be derived either from energy generated by metabolic processes or be provided by the pool of soluble amino acids in the muscle cytoplasm via an exchange process.

Perhaps the most interesting finding of this investigation is that the resting and the insulin-induced AIB uptake systems can be clearly distinguished; the former is not Na^+-dependent while the latter is. This finding constitutes probably the most drastic qualitative difference between resting and insulin-stimulated amino acid transport systems described so far. Several explanations can be suggested to explain the observed differences between resting and insulin-stimulated transport: one possibility is that under the action of insulin the preexisting components of the AIB transport system acquire a Na^+ site which when occupied can accelerate the turnover rate of the carrier. The Na requirement of the modified sites could be either obligatory—i.e., no transport would occur in the absence of Na—or only a requisite to increase the turnover rate of the carrier above the resting level. If in the presence of insulin the amino acid carrier became obligatorily Na-dependent, AIB uptake in

Na^+-free solutions should be faster in the absence than in the presence of insulin; obviously, this prediction was not fulfilled.

Another possibility is that insulin induces the appearance of additional transport sites which, in contrast to the resting sites, are Na^+-dependent. An increase in the number of amino acid transport sites could be due either to synthesis of new carrier sites or to the unmasking of a population of inactive sites already existing in the membrane. As a test to decide between these possibilities we measured the effects of 100 μg/ml cycloheximide on the amino acid uptake system. We could not detect any effects of the inhibitor on the insulin stimulated AIB uptake. Even though the doses of cycloheximide used in our experiments have been shown to be sufficient to block protein synthesis in other amphibian tissues (Fanestil & Edelman, 1966), we hesitate to completely rule out the possible synthesis of new sites because of the long latency involved in the stimulation of amino acid transport, and the fact that other authors have found an inhibitory effect of cycloheximide on insulin-induced amino acid transport (Goldfine *et al.*, 1972; Elsas *et al.*, 1975). Nevertheless, our findings with cycloheximide, added to the fact that protein synthesis is not a universal requirement for stimulation by insulin of amino acid transport (Elsas, Albrecht & Rosenberg, 1968; Elsas, McDonell & Rosenberg, 1971), indicate that activation of preexisting sites is a distinct possibility.

Although so far we are unable to provide a detailed description of the mechanism by which the insulin-induced movements of AIB occur, it is clear that the increase in transport rate is not simply due to an increased turnover rate of the unmodified carrier, but rather that a more drastic change in the conformation of the transport system is involved. Moreover such drastic changes in membrane carriers may underlie many of the effects of insulin on membrane components; indeed when we explored the effects of insulin on the (Na^+-K^+) ATPase, the data were best explained by proposing that insulin unmasks latent Na^+-K^+ ATPase sites (Grinstein & Erlij, 1974).

The experiments, in which the effects of several reagents were determined, showed that PCMBS is a very effective inhibitor of amino acid fluxes; such an effect of SH reagents on amino acid fluxes had been already observed in other cells (Hare, 1975; Sterling, 1975) but the effective concentrations of the inhibitor were larger than those employed here.

The action of PCMBS – a reagent that poorly penetrates the cell membrane (Rothstein, 1970) – and its reversal by substances that very likely do not penetrate the cell membrane, such as albumin and reduced glutathione, imply that a critical SH group of the carrier system faces the outside

solution. Moreover, since the uptake of basic amino acids was unaffected by PCMBS, these results identify a specific molecular difference between different amino acid transport systems. Finally, the elimination by PCMBS of the saturable component while the linear component was nearly unaffected provides direct support for the notion that the saturable and linear components of amino acid uptake represent different processes.

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