BBA 75824

# α-AMINOISOBUTYRIC ACID UPTAKE BY CULTURED BEATING HEART CELLS

P. DUNAND*, B. BLONDEL, L. GIRARDIER AND B. JEANRENAUD

*Institut de Biochimie clinique, Institut de Physiologie and Institut d'Histologie et d'Embryologie, Geneva (Switzerland)*

(Received August 2nd, 1971)

SUMMARY

1. The uptake of the amino acid analog, α-aminoisobutyric acid (AIB) by cultured beating rat heart cells has been investigated, taking advantage of a culture technique enabling to obtain 80–90 % myoblasts.

2. AIB was found to be transported and accumulated within the myoblasts by a process requiring energy, $K^+$ and $Na^+$, as evidenced by the marked drop in AIB uptake observed in myoblasts incubated with oligomycin, or in the absence of $Na^+$ or $K^+$ in the medium.

3. The observed decrease in net AIB uptake brought about by the lack of either $Na^+$ or $K^+$ in the medium appears, however, to be related more to drastic changes in ionic gradients than to a modulation of the $Na^+$–$K^+$ pump activity, as the myoblasts were very insensitive to ouabain.

4. Glycolysis is likely to be of considerable importance for optimal AIB accumulation by the myoblasts. Thus, iodoacetate markedly inhibited this process; glucose completely overcame the inhibitory effect of oligomycin on net AIB uptake, an action of glucose that could be prevented by further addition of 2-deoxyglucose.

5. When added alone to the incubation medium, insulin failed to alter AIB uptake. However, under conditions in which the energy supply was limited (*e.g.* cells treated with oligomycin) and AIB accumulation consequently decreased, the hormone brought the uptake of the amino acid back towards the normal level provided glucose (not pyruvate) was present in the medium. This suggests that insulin acts on the amino acid concentrative process mainly via its well-substantiated stimulatory effect on the transmembrane transport of glucose.

## INTRODUCTION

Following the first report of HARARY AND FAIRLEY[1] on cultured beating heart cells, several studies have been carried out with such a preparation[2–6]. However, these cultures, derived from trypsinized hearts, have been shown to consist of a mixture of myoblastic and mesenchymal cells, and to contain a greatly varying

Abbreviations: AIB, α-aminoisobutyric acid.

* Address reprint requests to: P. Dunand, Institut de Biochimie clinique, Sentier de la Roseraie, 1211 Geneva 4, Switzerland.

percentage of the latter cell type[7-9], thus making it difficult to assess the metabolic behavior of the myoblasts *per se*. The method of cultured heart cells of BLONDEL *et al.*[10] used in the present experiments is based, as others reported elsewhere[11], upon the differential speed of attachment of the different cellular species to the Petri plates, and makes it possible to obtain a culture that contains less than 20 % mesenchymal cells after 3, and even after 7 days of culture. This is in contrast with the observation of up to 50 % non-myoblastic elements in cultures that are not processed through such a "selection procedure"[10]. The experiments to be reported here represent the first of a series in which an attempt is being made to analyse metabolic activity and electrophysiology of such beating cultured heart cells. These experiments deal mostly with the uptake of the non-metabolizable analog α-aminoisobutyric acid (AIB) by the cultured heart cells. The ionic fluxes and content[12], as well as the electrogenesis of these myoblastic cells[13] have been studied separately, and the results reported elsewhere.

## MATERIALS AND METHODS

2–3-day-old male and female newborn rats derived from a Wistar stock bred at the Institut de Biochimie clinique (Geneva, Switzerland) were used throughout these studies. They were sacrificed by decapitation, and their heart quickly removed and cultured in Petri plates following exactly the method of BLONDEL *et al.*[10]. This method permitted to eliminate the greatest portion of the mesenchymal cells, and to obtain a cell population of 80–90 % myoblastic cells, a percentage that remained constant for about a week. After 3 days of culture, the cells were confluent, monolayered, and had a spontaneous contractile activity of 200–300 beats per min. This contractile activity, which was greater than those previously reported[4,14], was regularly controlled during the experiments, and so was the morphological appearance of the cells, using an inversed phase contrast microscope (Zeiss, Oberkochen, West-Germany).

The experiments were performed as follows. The plates containing the cultured cells were used as such for the actual incubation. The culture medium was first removed by washing the plates 4 times with 5 ml warm (37.5°) Krebs–Ringer bicarbonate (or phosphate in a few experiments) medium containing 1 g per 100 ml dialyzed human albumin. The incubation started upon addition of 5 ml Krebs–Ringer albumin medium (37.5°) containing 1 mM α-amino [1-$^{14}$C]isobutyric acid, and other substrates, hormone or agent, as indicated for each experiment. The plates were then incubated at pH 7.4 and 37.5°. At the end of the incubation, the medium was removed and the cells were washed 4 times by adding to the Petri plates 5 ml of cold (4°) Krebs–Ringer bicarbonate (or phosphate) buffer without albumin. The washing medium was quickly swirled in the plates, then discarded. At the end of four such washes, only trace amounts of the initial radioactivity was still present in the medium. Following those washes, each plate was punched with a mechanical hand puncher. This produced 6–7 discs covered with the previously incubated cardiac cells. The discs were dropped into plastic counting vials to which 10 ml of a BRAY[15] liquid scintillation solution with 3 g per 100 ml Cab-o-Sil (Packard Instrument) were immediately added. The plastic discs dissolved overnight in this solution, the cellular residues being then dispersed by shaking the vials with a Vortex apparatus (Scientific

Industries, Inc., Springfield, Mass., U.S.A.). The radioactivity contained in the cell preparation was then counted in a Model 3002 Packard liquid scintillation spectrometer. The labelled AIB still present in the extracellular space and/or adsorbed on the cell layers was accounted for, in each experiment, by running the following controls: plates with cultured cardiac cells were treated exactly as described above, then incubated for 15 sec in a cold (4°) incubation medium containing the same amount of labelled AIB, and other substrate(s) or agent(s) used for each particular experiment. The plates were then washed as previously described, punched, and the discs so obtained counted for their radioactive content. These control values, which were remarkably constant within any one experiment, were substracted from all subsequent values obtained with the incubated cardiac cells. For each experiment, two additional plates containing cardiac cells were washed, and punched as indicated above, although they were not added with labelled AIB. The cells present on these particular discs were placed in separate glass vials, added with 1 ml bidistilled water and 0.2 ml 1 M NaOH, shaken for 30 min at 55°, and their protein content was then determined according to the method of LOWRY[16]. This served as a reference measurement to express the metabolic activity of the incubated cardiac cells, as nmoles AIB taken up per mg protein. This method of measuring the protein content of the cells appeared to be reproducible as this content did not vary more than 10 % from one punched disc to another, even when taken from different Petri plates. The results presented in this study are thus the mean (± S.E.) of values obtained with at least two plates, *i.e.* 12–14 individual values from 12–14 punched discs.

Krebs–Ringer bicarbonate buffer was of the following composition: NaCl, 118 mM; KCl, 5.72 mM; $MgSO_4$, 1.19 mM; $KH_2PO_4$, 1.19 mM; $CaCl_2$, 2.45 mM; $NaHCO_3$, 26.0 mM. Krebs–Ringer phosphate buffer was: NaCl, 141 mM; KCl, 5.9 mM; $MgSO_4$, 1.2 mM; $KH_2PO_4$, 0.1 mM; $CaCl_2$, 2.45 mM; $NaH_2PO_4$, 1.2 mM. When the concentrations of $Na^+$ or $K^+$ in the incubation medium were altered, $Na^+$ was replaced by choline, and $K^+$ by $Na^+$.

All organic and inorganic reagents were purchased from E. Merck (Darmstadt, Germany). Human serum albumin was obtained from the Swiss Red Cross (Berne, Switzerland) and was used following a 24-h dialysis against bidistilled water. Ouabain (Strophantin G) was a product of Merck, oligomycin of Sigma Chemical (St. Louis, Mo., U.S.A.). α-amino [1-$^{14}$C]isobutyric acid was secured from the Radiochemical Centre (Amersham, Buckinghamshire, England). Pork insulin (10 times recrystallized) was a gift of the Novo Laboratories (Copenhagen, Denmark).

## RESULTS

### *Effect of albumin on cell activity*

As mentioned in MATERIALS AND METHODS, albumin (1 g per 100 ml) was added to all incubation buffers. Indeed, when albumin was omitted (be it in a bicarbonate or a phosphate buffer) irregular beating activity followed by the cessation of it, as well as swelling and detachment of cells from the bottom of the plates was observed. Lowering the temperature to 20° or adding pyruvate (5 mM) to the medium partly prevented these phenomena. In all cases, however, 60 min incubation without albumin led to cellular death. It should be mentioned, however, that when a medium without albumin was continuously renewed, as in the case of electrophysiological

studies reported elsewhere[13], the addition of sodium pyruvate (5 mM) was sufficient to maintain the cells in a normal morphological and electrical state for at least 8 h.

*Effect of buffers on cell activity*

Since several previous experiments dealing in particular with electrophysiological measurements have been performed with Krebs–Ringer phosphate buffer[13], it was of interest to evaluate whether this or the Krebs–Ringer bicarbonate buffer was best suited for AIB uptake experiments. The results of such experiments are summarized in Fig. 1 and indicate a higher net uptake of AIB in bicarbonate buffer. One should mention that, whatever the buffer or the temperature used, the beating activity remained regular.

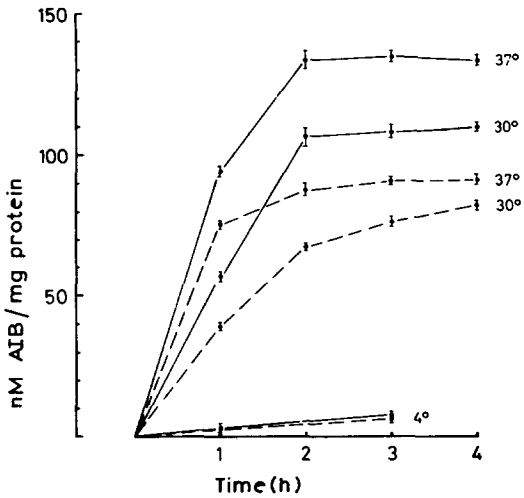


Fig. 1. Effect of temperature on AIB uptake by cultured beating heart cells incubated in different buffers. Incubation in 5 ml Krebs–Ringer bicarbonate (———) or phosphate (– – – –) buffer containing 1.0 g per 100 ml albumin, for 1–4 h in the presence of 1 mM α-amino [1-$^{14}$C]isobutyric acid. No energy-yielding substrate added. Each point is the mean of 12 values ± S.E.

TABLE I

EFFECT OF AGING ON AIB UPTAKE BY CULTURED BEATING HEART CELLS

Cultured cells derived from the same population were used after 3, 5, 7 and 10 days of growth. Incubations were carried out in 5 ml Krebs–Ringer bicarbonate buffer containing 1.0 g per 100 ml albumin. Cells first preincubated for 60 min in the absence of substrate, then further incubated for 60 min following addition of 1 mM α-amino [1-$^{14}$C]isobutyric acid. Results are expressed as nmoles AIB taken up per mg protein. Each figure is the mean of 13–14 values ± S.E. Calculation of AIB in/AIB out, see text.

| *Days of culture* | *Total protein content of the tested cells* | *Net α-amino [1-$^{14}$C]-isobutyric acid uptake* | *Calculated AIB in/AIB out* |
|---|---|---|---|
| 3 | 66.4 ± 5.4 | 150.2 ± 4.5 | 22.1 |
| 5 | 71.7 ± 7.8 | 298.3 ± 5.6 | 43.9 |
| 7 | 94.1 ± 7.3 | 221.0 ± 4.4 | 32.5 |
| 10 | 142.5 ± 11.2 | 51.0 ± 2.8 | 7.5 |

All subsequent experiments were carried out in Krebs–Ringer bicarbonate buffer.

*Effect of aging on cell activity*

In another series of experiments, net AIB uptake was measured in cells cultured for 3, 5, 7 or 10 days. The results of these experiments are summarized in Table I, and show that the highest AIB uptake was observed in cells in their fifth or seventh day of growth. Despite this fact all subsequent experiments were carried out with 3-day old cultured cells because these cells had the best and most stable myoblastic to non-myoblastic ratio[10], and permitted excellent morphological control as they were almost monolayered.

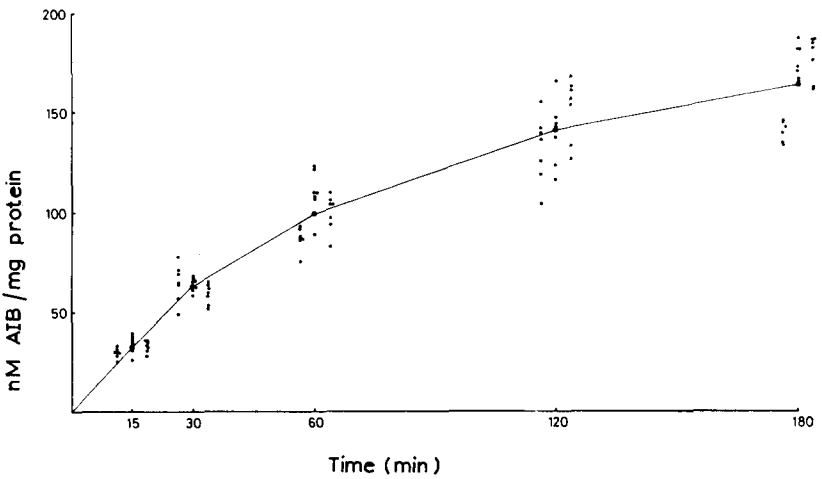


Fig. 2. Time curve of AIB uptake by cultured beating heart cells. Incubation in 5 ml Krebs–Ringer bicarbonate buffer containing 1·0 g per 100 ml albumin and 1 mM α-amino [1-$^{14}$C]isobutyric acid. No energy-yielding substrate added. Each point represents one individual value.

*Basic characteristics of AIB uptake*

Fig. 2 illustrates the time course of AIB uptake by beating myoblasts in Krebs–Ringer bicarbonate buffer. Throughout this experiment, the beating activity of the cells remained between 200–300 beats per min despite the fact that no energy-yielding substrate was added to the incubation medium. As can be seen, after an initial rapid phase, the uptake of AIB slowed down to become near maximum at 180 min, although steady state levels of uptake were not reached before 3 h of incubation. Although net uptake of AIB only is illustrated in Fig. 2, it could be calculated that such an uptake does actually represent an accumulation against a concentration gradient. The calculation was based on results summarized in Fig. 3. In these experiments, the resting potential of cardiac cells was determined as a function of extracellular $K^+$. It was observed that for $K^+$ concentrations in the medium higher than 6 mM, the slope of the depolarization followed very closely the theoretical Nernst equation for a $K^+$ diffusion potential in our temperature conditions. Extrapolating the curve to zero potential gave the internal $K^+$ concentration, which was found to be 140 mM. On the other hand, the chemical measurement of cellular $K^+$ indicated

that this cation amounted to 0.95 nmole/g protein. This value, together with that of the internal $K^+$ concentration (140 mM) allowed the estimation of the cell water content which was calculated to be 6.8 $\mu$l/mg protein. Using this latter value it could be further estimated that the highest intracellular AIB concentration in 3-day-old cultured cells incubated for 180 min (Fig. 2) was about 24.5 mmoles/l cell water. Since

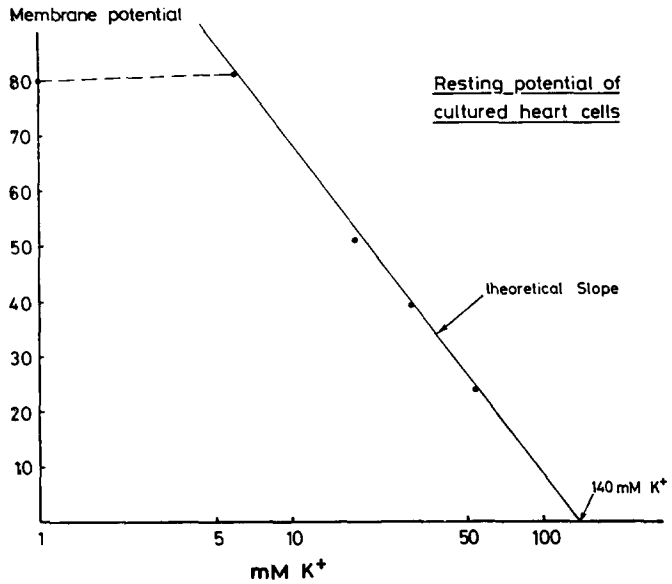


Fig. 3. Resting potential of cultured beating heart cells. Experiment carried out in Krebs–Ringer phosphate buffer without albumin, with 10 mM sodium pyruvate. Flow through experiment with continuous renewal of the medium, according to HYDE *et al.*[12].

in these experiments, AIB was used at a concentration of 1 mM, and since this compound has been previously shown not to be metabolized[17,18], it can be concluded that the analog had accumulated about 25 times, presumably by an energy-requiring process. The experiments shown in Fig. 2 were carried out in the absence of added substrate, thus further indicating that beating myoblasts do contain endogenous energy stores that enable considerable AIB accumulation during a 3-h incubation. Actually, such high AIB accumulation could be maintained for as long as 5 h without adding substrate in the medium, and was not increased by the further addition of glucose, fructose or pyruvate to the incubation medium.

The energy dependency of AIB uptake, previously suggested by the marked inhibitory effect of cooling (Fig. 1) is further illustrated in Table II. As can be seen, the presence of increasing concentrations of oligomycin, 2,4-dinitrophenol or iodoacetate eventually resulted in a marked decrease in the uptake of the amino acid. When intracellular AIB concentration was estimated, on the basis of the above-mentioned intracellular water content of 6.8 $\mu$l/mg protein, it was observed that even at the highest concentrations of oligomycin or dinitrophenol used, some accumulation of AIB still occurred (*i.e.* intracellular AIB concentration was 6 or 2 times that of the extracellular AIB, respectively). In addition, although all the concentrations of oligomycin tested did not result in an inhibition of net AIB uptake, all stopped

TABLE II

EFFECT OF VARIOUS INHIBITORS ON AIB UPTAKE BY CULTURED BEATING HEART CELLS

Incubations were carried out in 5 ml Krebs–Ringer bicarbonate buffer containing 1.0 g per 100 ml albumin. Cells preincubated first for 60 min in the presence of the inhibitor (without substrate), then further incubated for 60 min under the same conditions following addition of 1 mM α-amino [1-$^{14}$C]isobutyric acid. Results are expressed as nmoles AIB taken up per mg protein. Each figure is the mean of 12–14 values ± S.E.

| *Inhibitor* | *Concentration* | *Net α-amino [1-$^{14}$C]isobutyric acid uptake* |
|---|---|---|
| Oligomycin | 0 μg/ml | 120.5 ± 1.8 |
| | 0.001 μg/ml | 114.7*** ± 2.5 |
| | 0.01 μg/ml | 113.9** ± 2.5 |
| | 0.1 μg/ml | 58.9* ± 1.4 |
| | 0.5 μg/ml | 41.0* ± 2.9 |
| | 1.0 μg/ml | 39.0* ± 1.3 |
| | 2.0 μg/ml | 44.2* ± 1.0 |
| | 5.0 μg/ml | 40.0* ± 1.9 |
| 2,4-dinitrophenol | 0 M | 124.0 ± 2.4 |
| | $10^{-6}$ M | 126.6 ± 1.6 |
| | $10^{-5}$ M | 127.0 ± 3.1 |
| | $10^{-4}$ M | 113.5 ± 9.2 |
| | $10^{-3}$ M | 15.0* ± 0.9 |
| Iodoacetate | 0 | 124.0 ± 2.4 |
| | $10^{-7}$ M | 84.3* ± 7.4 |
| | $10^{-6}$ M | 108.1* ± 3.7 |
| | $10^{-5}$ M | 93.1* ± 7.1 |
| | $10^{-4}$ M | 57.0* ± 2.6 |
| | $10^{-3}$ M | 0.9* ± 0.13 |

* Difference from controls: $P < 0.001$.
** Difference from controls: $P < 0.01$.
*** Difference from controls: $P < 0.025$; other groups: not significant.

the beating activity of the myoblasts. This effect was almost immediate, with a small lag period (1–10 sec) that depended upon the concentration of oligomycin (*i.e.* the higher the concentration, the shorter the lag period). This observation was attributed to a direct effect of oligomycin on the electrogenesis of the cells, as will be discussed below. In contrast, the effect of oligomycin on AIB uptake was not instantaneous, and preincubation of the cells in the presence of oligomycin prior to the addition of labelled AIB was needed to clearly evidence the inhibition. Dinitrophenol did not alter the morphological aspect of the cells but, at the highest concentrations used, reduced beating activity without altering its regularity. Iodoacetate at very high concentrations ($10^{-3}$ M) completely abolished AIB accumulation (Table II). Under these conditions, however, the calculated intracellular AIB concentration was found to be 10 times lower than that theoretically expected, had simple diffusion of AIB occurred. This finding could actually be explained by the morphological examination of these cells which cease to beat, and presented severe structural damages when exposed to such high iodoacetate concentration. When used at lower concentrations ($10^{-4}$ M), iodoacetate was markedly inhibitory without inducing apparent changes in the morphological aspect or contractile activity of the cells. The experiments depicted in

Table II thus indicate that the concentration process leading to AIB accumulation is dependent upon a normally operating oxidative metabolism. Moreover, the results obtained with iodoacetate also suggest that, under normal conditions, endogenous stores are being metabolized via the glycolytic pathway, thereby producing a sizable part of the total ATP needed.

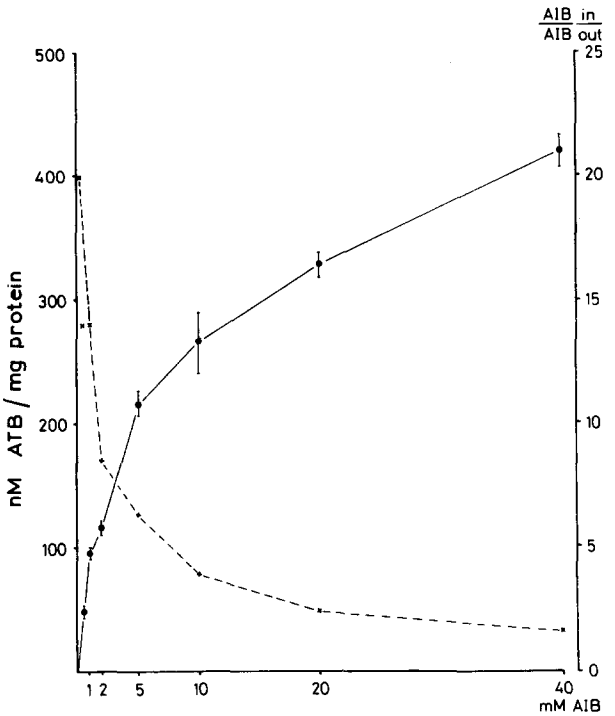


Fig. 4. Relationship between AIB uptake by cultured beating heart cells, and the extracellular concentrations of the analog. Incubation in 5 ml Krebs–Ringer bicarbonate buffer containing 1.0 g per 100 ml albumin and α-amino [1-$^{14}$C]isobutyric acid as indicated. No energy-yielding substrate added. $Na^+$ concn. reduced by 1/2 the amino acid concentrations. AIB uptake ———; AIB in/AIB out – – – –. Each point is the mean of 12 values ± S.E.

As illustrated in Fig. 4, the concentration of AIB in the incubation medium was raised from 1 to 40 mM, the uptake of the amino acid was progressively increased. Up to about 10 mM, the uptake increased hyperbolically with the extracellular concentration of the analog but did not reach a maximum. Instead, at concentrations greater than 10 mM AIB net uptake continued to increase in a linear fashion suggesting that, as for other tissues[18–21], this process represents a complex phenomenon that could comprise at least two components, *i.e.* an active transport system, and a linear process rather similar to passive diffusion. Fig. 4 also shows that the greatest capacity of the cells to accumulate AIB was observed at extracellular AIB concentrations ranging between 0.25 and 1.0 mM.

Since beating cardiac cells transported and accumulated AIB by an energy-requiring process it was of interest to investigate the influence of other amino acids on the uptake of the analog. These experiments are summarized in Table III. The addition of one of several neutral amino acids such as methionine, alanine, serine,

glycine, proline, leucine, phenylalanine or valine to cells incubated with labelled AIB reduced the uptake of the latter, the most potent inhibitors being alanine, methionine and serine. These observations suggest the existence of a competition between different amino acids for a same transport system.

TABLE III

EFFECT OF NEUTRAL AMINO ACID ON AIB UPTAKE BY CULTURED BEATING HEART CELLS

Incubations were carried out for 60 min in 5 ml Krebs–Ringer bicarbonate buffer containing 1.0 g per 100 ml albumin, 1 mM α-amino [1-$^{14}$C]isobutyric acid with or without 20 mM unlabelled amino acid as indicated. Results are expressed as nmoles AIB taken up per mg protein. Each figure is the mean of 29 (controls) or 14 (addition of unlabelled acid) values ± S.E. Difference from controls for all groups: $P < 0.001$.

| *Addition of unlabelled amino acid* | *Net α-amino [1-$^{14}$C]isobutyric acid uptake* |
|---|---|
| 0 | 102.3 ± 2.8 |
| Methionine | 3.1 ± 0.1 |
| Alanine | 7.0 ± 0.9 |
| Serine | 9.9 ± 0.3 |
| Glycine | 31.8 ± 1.4 |
| Proline | 32.5 ± 1.0 |
| Valine | 48.7 ± 1.3 |
| Leucine | 51.3 ± 1.8 |
| Phenylalanine | 62.9 ± 1.1 |

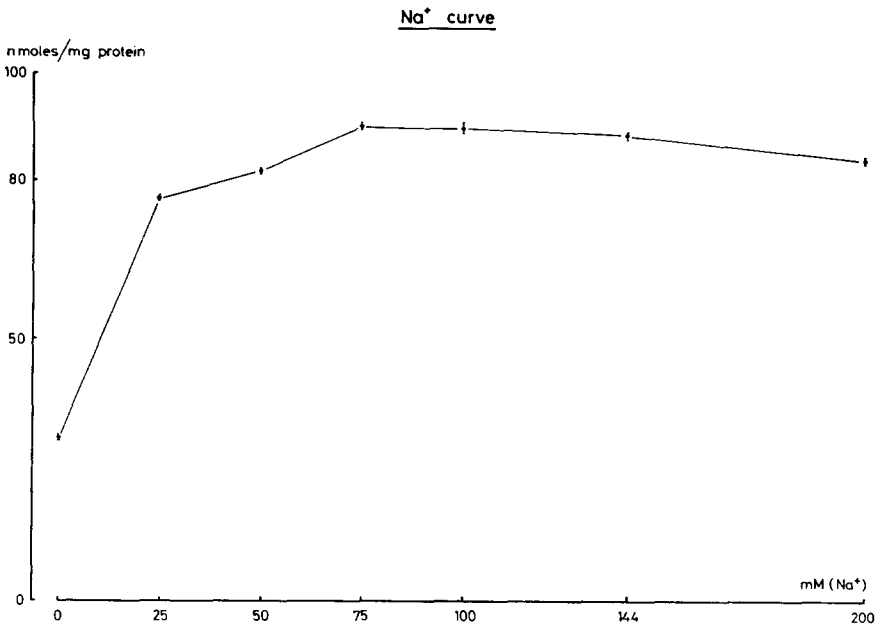


Fig. 5. Effect of sodium on α-aminoisobutyric acid uptake by cultured beating heart cells. Incubation in 5 ml Krebs–Ringer bicarbonate buffer containing 1.0 g per 100 ml albumin and 1 mM α-amino [1-$^{14}$C]isobutyric acid. No energy-yielding substrate added. Incubation time: 1 h. $Na^+$ replaced by choline. Each point is the mean of 12 values ± S.E.

*Ionic requirement for AIB uptake*

Experiments done in many different tissues have previously shown that amino acid uptake is greatly influenced by the ionic environment, by $Na^+$ and $K^+$ in particular[18-21]. The influence of these two cations on the beating heart cells was therefore investigated by measuring net AIB uptake as a function of increasing concentrations of either of them in the presence of the physiological concentration of the other. As illustrated in Fig. 5, it was observed that maximal AIB uptake occurred when sodium concentration was 75 mM or higher. $K^+$ had a biphasic effect, as shown in Fig. 6.

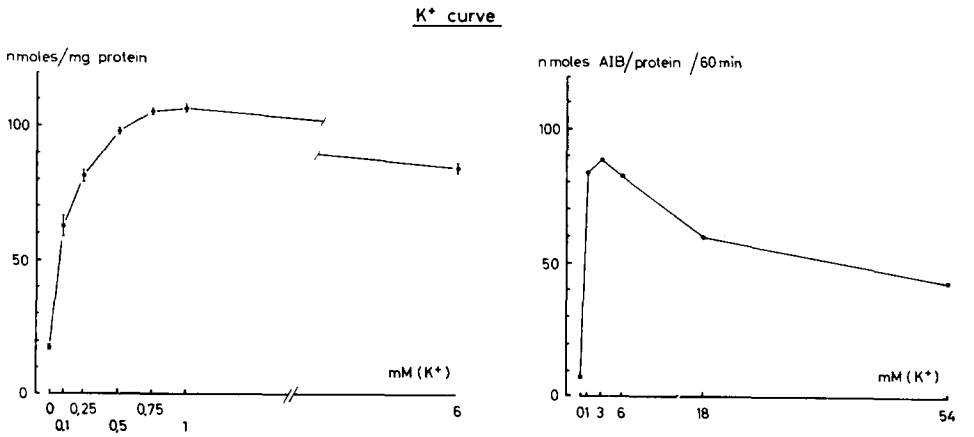


Fig. 6. Effect of potassium on α-aminoisobutyric acid uptake by cultured beating heart cells. Incubation in 5 ml Krebs–Ringer bicarbonate buffer containing 1.0 g per 100 ml albumin and 1 mM α-amino [1-$^{14}$C]isobutyric acid. No energy-yielding substrate added. Incubation time 1 h. Osmolarity kept constant by adjusting $Na^+$ concn. Each point is the mean of 12 values ± S.E.

Maximal AIB uptake occurred when the concentration of the cation was around 6 mM. Below and above this concentration, AIB uptake was less, the lowest value being observed when $K^+$ was completely removed from the incubation medium. It should be mentioned that the beating activity of the myoblasts remained normal at all $K^+$ concentrations except at 0.75 mM and below. When these latter $K^+$ concentrations were used, beating activity became irregular, asynchronous and some quivering was observed. In addition, although the microscopic appearance was still normal, many cells detached from the bottom of the culture plates. The microscopic appearance of myoblasts incubated in the absence of $Na^+$ in the medium was normal, and cells did not detach. Some quivering was observed at 25–50 mM $Na^+$ concentration; above 50 mM the beating activity was normal (200 beats per min); it was increased (250–300 beats per min) by raising $Na^+$ concentration from 75 to 200 mM but was regular and synchronous.

Using the cellular water content of 6.8 μl/mg protein mentioned above, it was possible to estimate the intracellular concentration of AIB obtained during incubation of myoblasts in a $Na^+$- or a $K^+$-free medium. After 60 min of incubation with 1 mM AIB, the calculated intracellular AIB concentration of cells incubated in the absence of sodium was 4.4 mmoles/l cell water. In $K^+$-free medium, the AIB taken up by the myoblasts amounted, depending upon the experiments, to 1.1–2.6 mmoles/l cell water. Thus, in the absence of either one of these cations, the process of AIB accumu-

lation was either very low without being nil or was, as in the case of some $K^+$-free experiments, completely suppressed. Under those conditions, the AIB measured in the cells was likely to be related only to passive diffusion of the amino acid across the cell membrane.

These experiments suggested the possible existence of a coupling between the $Na^+$ pump and the uptake of AIB. In an attempt to substantiate such a coupling, experiments were carried out in which cells were incubated in the absence or in the presence of ouabain. However, as shown in Table IV, the presence of ouabain resulted in a decrease in the AIB uptake only when used at very high concentration ($10^{-3}$ M), while lower concentrations were totally uneffective.

TABLE IV

EFFECT OF OUABAIN ON AIB UPTAKE BY CULTURED BEATING HEART CELLS

Incubations were carried out in 5 ml Krebs–Ringer bicarbonate buffer containing 1.0 g per 100 ml albumin. Cells preincubated first for 60 min in the presence of ouabain (without substrate), then further incubated for 60 min under the same conditions following addition of 1 mM α-amino [1-$^{14}$C]isobutyric acid. Results are expressed as nmoles AIB taken up per mg protein. Each figure is the mean of 12–14 values ± S.E. Difference from controls: $P < 0.001$; other groups: not significant.

| *Ouabain* (*M*) | *Net α-amino* [1-$^{14}$C]*isobutyric acid uptake* |
|---|---|
| 0 | 89.1 ± 3.0 |
| $10^{-7}$ | 90.6 ± 2.4 |
| $10^{-6}$ | 104.2 ± 2.5 |
| $10^{-5}$ | 100.2 ± 3.7 |
| $10^{-4}$ | 95.2 ± 3.4 |
| $10^{-3}$ | 15.0 ± 1.3 |

*Glycolysis and AIB uptake*

Previous experiments (Table II) suggested that the energy required for AIB accumulation in myoblastic cells were partly derived from glycolysis. In an attempt to delineate this problem further, experiments were carried out using cells treated with oligomycin (viewed mainly as a mitochondrial inhibitor of ATP production), and with or without various energy-yielding substrates. Table V shows that there was a significant decrease in AIB accumulation upon addition of oligomycin to an incubation medium containing no substrate. Furthermore, although the addition of 0.1 mM glucose to oligomycin-treated cells brought about little or no change at all in AIB uptake (Tables VA and B), the observed decrease in the uptake of the analog induced by oligomycin was completely overcome by the addition of 1.0–10 mM glucose in the incubation medium. This was likely to be due to the fact that such concentrations of glucose had allowed sufficient uptake of this substrate by the cells, as well as adequate glucose-6-phosphate metabolism and ATP production *via* the glycolytic pathway. When a substrate such as pyruvate was added to oligomycin-treated cells (Table VC) no prevention of the oligomycin effect was observed, as could be expected. Other substrates, *i.e.* fructose or glycerol, were also unable to prevent the inhibitory action of oligomycin on AIB accumulation, presumably because they could not be adequately metabolized by the cells (Table VC). The suggestion that the

TABLE V

EFFECT OF OLIGOMYCIN ON AIB UPTAKE BY CULTURED BEATING HEART CELLS: ITS PREVENTION BY GLUCOSE

Incubations were carried out in 5 ml Krebs–Ringer bicarbonate buffer containing 1.0 g per 100 ml albumin. Cells preincubated first for 60 min in the presence of oligomycin (1 μg/ml) and unlabelled substrate as indicated, then further incubated for 60 min with the same compounds and 1 mM α-amino [1-$^{14}$C]isobutyric acid. Results are expressed as nmoles AIB taken up per mg protein. Each figure is the mean of 10–14 values ± S.E. $A_1$ *versus* $A_2$: $P < 0.001$; $A_2$ *versus* $A_3$, $A_4$: $P < 0.001$; $B_1$ *versus* $B_2$: $P < 0.001$; $B_2$ *versus* $B_3$: not significant; $B_2$ *versus* $B_4$, $B_5$, $B_6$: $P < 0.001$; $C_1$ *versus* $C_2$: $P < 0.001$; $C_2$ *versus* $C_3$: $P < 0.001$; $C_2$ *versus* $C_4$, $C_5$, $C_6$: not significant.

| | *Oligomycin* | *Unlabelled substrate* | *Net α-amino [1-$^{14}$C]isobutyric acid uptake* |
|---|---|---|---|
| A. 1 | 0 | 0 | 128.5 ± 3.6 |
| 2 | + | 0 | 54.6 ± 5.0 |
| 3 | + | Glucose (0.1 mM) | 73.5 ± 3.9 |
| 4 | + | Glucose (0.25 mM) | 107.0 ± 3.6 |
| B. 1 | 0 | 0 | 80.8 ± 4.2 |
| 2 | + | 0 | 51.6 ± 2.9 |
| 3 | + | Glucose (0.1 mM) | 53.0 ± 2.1 |
| 4 | + | Glucose (1.0 mM) | 100.8 ± 5.5 |
| 5 | + | Glucose (5.0 mM) | 93.2 ± 3.0 |
| 6 | + | Glucose (10.0 mM) | 112.4 ± 2.2 |
| C. 1 | 0 | 0 | 110.0 ± 4.2 |
| 2 | + | 0 | 52.8 ± 3.5 |
| 3 | + | Glucose (5 mM) | 115.4 ± 3.8 |
| 4 | + | Fructose (5 mM) | 35.5 ± 2.5 |
| 5 | + | Pyruvate (5 mM) | 43.6 ± 4.3 |
| 6 | + | Glycerol (5 mM) | 32.3 ± 3.0 |

TABLE VI

EFFECT OF OLIGOMYCIN, GLUCOSE, AND 2-DEOXYGLUCOSE ON AIB UPTAKE BY CULTURED BEATING HEART CELLS

Incubations were carried out in 5 ml Krebs–Ringer bicarbonate buffer containing 1.0 g per 100 ml albumin. Cells preincubated first for 60 min in the presence of oligomycin (1 μg/ml), glucose, 2-deoxyglucose as indicated; then further incubated for 60 min under the same conditions following addition of 1 mM α-amino [1-$^{14}$C]isobutyric acid. Results are expressed as nmoles AIB taken up per mg protein. Each figure is the mean of 12–14 values ± S.E. $A_1$ *versus* $A_2$: $P < 0.001$; $A_3$ *versus* $A_4$: $P < 0.001$; $B_1$ *versus* $B_2$: $P < 0.001$; $B_3$ *versus* $B_4$: $P < 0.001$.

| | *Oligomycin* | *Glucose (mM)* | *2-deoxy-glucose (mM)* | *Net α-amino [1-$^{14}$C]isobutyric acid uptake* |
|---|---|---|---|---|
| A. 1 | 0 | 0 | 0 | 128.5 ± 3.6 |
| 2 | + | 0 | 0 | 54.6 ± 5.0 |
| 3 | + | 1 | 0 | 144.7 ± 4.5 |
| 4 | + | 1 | 2.5 | 76.3 ± 7.9 |
| B. 1 | 0 | 0 | 0 | 110.0 ± 4.2 |
| 2 | + | 0 | 0 | 52.8 ± 3.5 |
| 3 | + | 5 | 0 | 115.4 ± 3.8 |
| 4 | + | 5 | 10 | 32.6 ± 1.2 |

prevention of the inhibitory effect of oligomycin on AIB uptake by glucose was related to the metabolism of the hexose via the glycolytic pathway was strenghtened by the experiments summarized in Table VI. It is evident, from these experiments, that glucose (1 or 5 mM) completely prevented the decrease in AIB uptake produced by oligomycin. Furthermore, the presumed inhibition of glucose metabolism following the addition of 2-deoxyglucose to the incubation medium coincided with a partial or complete disappearance of the glucose effect on the oligomycin-treated cells.

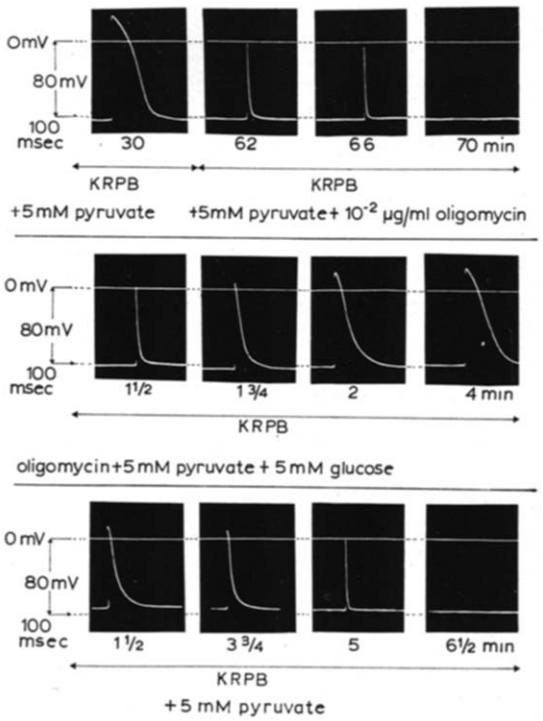


Fig. 7. Effect of oligomycin on electrogenesis in cultured beating heart cells. Measurement of electrical activity done according to HYDE *et al.*[12]. Experimental procedure as in Fig. 3. Top row: electrical activity before and after addition of oligomycin ($10^{-2}$ µg/ml): progressive narrowing of the action potential and partial disappearance of the overshoot in the presence of the agent. Middle row: disappearance of oligomycin-induced changes upon addition of glucose. Bottom row: reappearance of oligomycin effects (which binds irreversibly to cells) upon removal of glucose from the medium by washing the cells with normal buffer. KRPB, Krebs–Ringer phosphate buffer.

The presence of glucose not only restored the capacity of the oligomycin-treated cells to accumulate AIB, it also normalized the change in the action potential observed in the presence of this inhibitor, as illustrated in Fig. 7. As can be seen, oligomycin produced a marked change in the electrical activity of myoblasts. Thus, following the addition of this inhibitor to the incubation medium, the action potential of the cells became shorter, and its shape was markedly modified, taking the appearance of a thin, single spike. A few minutes later, the action potential completely disappeared, although the resting potential was unaffected. In the absence of glucose, this effect of oligomycin persisted, and no spontaneous recovery was observed. In

contrast, as soon as glucose was added to the system, the action potential was restored towards normal, and the beating activity that had been stopped by oligomycin resumed in a normal fashion.

*Effect of insulin on AIB uptake*

The above-mentioned experiments suggested that glucose had probably an important role in furnishing the energy required for optimal AIB accumulation. It was therefore of interest to test the effect of insulin on this process. The results of such experiments are summarized in Table VII. First, although not indicated in the table, insulin was completely ineffective in stimulating AIB uptake when added alone to cells incubated, either in the absence or in the presence of substrate (glucose, pyruvate). Second, diminution in AIB uptake brought about by oligomycin could not be prevented by pyruvate added alone or together with insulin (Table VIIA). Third, a small concentration of glucose (0.1 mM) that was not or little effective by itself in preventing the decrease in AIB uptake produced by oligomycin became effective, or more effective, when added together with insulin (Table VII, B and C). Fourth, when glucose at higher concentrations (1 or 5 mM) was added to oligomycin-

TABLE VII

EFFECT OF OLIGOMYCIN, GLUCOSE, PYRUVATE AND INSULIN, SINGLY OR COMBINED, ON AIB UPTAKE BY CULTURED BEATING HEART CELLS

Incubations were carried out in 5 ml Krebs–Ringer bicarbonate buffer containing 1.0 g per 100 ml albumin. Cells preincubated first for 60 min in the presence of oligomycin (1 $\mu$g/ml), glucose, pyruvate (5 mM) or insulin (1 mU/ml) as indicated: then further incubated for 60 min under the same conditions following addition of 1 mM $\alpha$-amino [1-$^{14}$C]isobutyric acid. Results are expressed as nmoles AIB taken up per mg protein. Each figure is the mean of 14 values $\pm$ S.E. $A_1$ *versus* $A_2$, $A_4$, $A_5$: $P < 0.001$; $A_1$ *versus* $A_3$: not significant. $B_1$ *versus* $B_2$, $B_3$: $P < 0.001$; $B_2$ *versus* $B_3$: not significant; $B_2$ *versus* $B_4$, $B_5$, $B_6$: $P < 0.001$. $C_1$ *versus* $C_2$: $P < 0.001$; $C_2$ *versus* $C_3$: $P < 0.002$; $C_2$ *versus* $C_4$, $C_5$, $C_6$, $C_7$, $C_8$: $P < 0.001$.

| | *Exp.* | *Oligomycin* | *Glucose (mM)* | *Pyruvate* | *Insulin* | *Net $\alpha$-amino [1-$^{14}$C]isobutyric acid uptake* |
|---|---|---|---|---|---|---|
| A. | 1 | o | o | o | o | 170.1 $\pm$ 6.2 |
| | 2 | + | o | o | o | 16.0 $\pm$ 1.4 |
| | 3 | o | o | + | o | 188.0 $\pm$ 7.5 |
| | 4 | + | o | + | o | 19.1 $\pm$ 1.9 |
| | 5 | + | o | + | + | 19.4 $\pm$ 2.1 |
| B. | 1 | o | o | o | o | 81.3 $\pm$ 1.1 |
| | 2 | + | o | o | o | 29.2 $\pm$ 0.8 |
| | 3 | + | 0.1 | o | o | 27.3 $\pm$ 1.1 |
| | 4 | + | 0.1 | o | + | 63.3 $\pm$ 2.6 |
| | 5 | + | 1.0 | o | o | 74.8 $\pm$ 0.9 |
| | 6 | + | 1.0 | o | + | 74.9 $\pm$ 1.0 |
| C. | 1 | o | o | o | o | 115.8 $\pm$ 2.4 |
| | 2 | + | o | o | o | 38.7 $\pm$ 2.4 |
| | 3 | + | 0.1 | o | o | 62.6 $\pm$ 3.6 |
| | 4 | + | 0.1 | o | + | 98.5 $\pm$ 8.0 |
| | 5 | + | 1.0 | o | o | 121.2 $\pm$ 3.0 |
| | 6 | + | 1.0 | o | + | 126.7 $\pm$ 3.5 |
| | 7 | + | 5.0 | o | o | 126.2 $\pm$ 3.1 |
| | 8 | + | 5.0 | o | + | 129.3 $\pm$ 4.0 |

treated cells, the inhibitory effect of oligomycin was completely suppressed, and insulin did not modify this effect any further.

## DISCUSSION

The present experiments have been carried out with cultured rat cardiac cells, taking advantage of a technique[10] which permits to obtain a cell population consisting mostly (80–90%) in beating myoblasts.

When such cells were incubated in a closed *in vitro* system, it was found that the presence of albumin in the medium was of prime importance. Thus, albumin prevented an early necrosis of the myoblasts, and made it possible to prolong the duration of the incubations up to several hours without alteration in the contractile activity or in the morphological appearance of the cells. When, as shown in a few experiments, albumin was omitted from the incubation medium, the latter had to be continuously renewed in order to maintain the myoblasts in a satisfactory functional and morphological state. The mechanism(s) by which albumin brings about such an improvement in the survival of the incubated cells is not known but may be related to the adsorption of some toxic metabolites.

When comparing cells cultured for one to seven days, it was found that 3-day-old cultures were best suited for *in vitro* investigation, as they showed no trend at all towards an increase in their non-myoblastic elements, and were almost monolayered, thus allowing excellent control of their microscopical appearance and beating activity.

These studies demonstrate that cultured heart cells take up AIB from the incubation medium, and accumulate it against a concentration gradient (Figs. 2 and 4). This concentration process is an energy-requiring one (Fig. 1, Table II) resembling that described for other tissues[17–21], and appears to be a complex phenomenon that is consistent with the existence of at least two components: a saturable process, and a process similar to passive diffusion (Fig. 4). Several amino acids are able to decrease net AIB uptake by the cultured cells, thus suggesting the existence of a competition between different amino acids for the same transport system. Of additional interest is the observation that the net movement of AIB into the cardiac cells is dependent upon the presence of both $Na^+$ and $K^+$. Thus, the absence of either one of the cations in the incubation medium was found to completely prevent or to greatly decrease AIB accumulation within the myoblasts (Figs. 5 and 6). This suggested the existence of a coupling between the $Na^+$ pump and the process of AIB uptake. However, experiments carried out in the presence of ouabain indicate that such a coupling is unlikely to have any significance: myoblasts were extremely insensitive to ouabain, and only when used at very high concentrations ($10^{-3}$ M) could the cardioside induce a significant decrease in AIB accumulation (Table IV). This indicates that the observed inhibitory effects of $K^+$ lack or of high ouabain concentrations on net AIB uptake are more probably related to extreme changes in ionic gradients than to an actual modulation of the ($Na^+$-$K^+$)pump activity.

Glycolysis appears, from these experiments, to be of considerable importance for optimal AIB accumulation by the myoblasts. Thus iodoacetate ($10^{-4}$ M) was found to markedly inhibit net AIB uptake by these cells (Table II). Furthermore, the inhibition of AIB uptake by oligomycin was completely overcome upon addition of glucose (1–5 mM) to the oligomycin-treated cells. This clearly supports the concept

that, under those conditions, glucose had entered the cells and provided them with energy via metabolic pathways that were unaffected by oligomycin, *i.e.* presumably via the Embden–Meyerhof pathway. Consonant with this interpretation is the finding that the normalization of AIB uptake by glucose when added to oligomycin-treated cells is prevented by further addition of 2-deoxyglucose (Table VI). The observation that the inhibitory effect of oligomycin on AIB accumulation by myoblasts incubated without substrate was not immediate but required 20–60 min to occur also suggests the likely importance of glycolysis from endogenous stores in providing the necessary energy. On the contrary, the rapid oligomycin effect in stopping beating activity of the cells, even at concentrations that were ineffective in decreasing AIB uptake (Table IV, Fig. 7), is consonant with an action of this inhibitor on the electrogenesis *per se*, without any alteration in energy metabolism.

Insulin alone failed to modify net uptake of AIB by the myoblasts. On the contrary, the only stimulatory effect of insulin was seen under conditions in which the energy supply was limited, *i.e.* during the presumed inhibition of mitochondrial ATP production by oligomycin. Under those conditions, insulin could restore AIB uptake towards normal when glucose (0.1 mM), but not pyruvate, was the added energy-yielding substrate (Table VII). However, again in oligomycin-treated cells, when glucose concentration in the incubation medium was high enough (1–5 mM), the oligomycin-induced drop in AIB uptake was completely prevented, and the addition of insulin did not modify it any further (Table VII). This suggests that the hormone is exerting its effect by increasing the supply of intracellular glucose-6-phosphate via the glycolytic pathway. Since no additive effect of maximal concentrations of glucose and of insulin upon AIB uptake was observed in oligomycin-treated cells, it is unlikely that they act by different mechanisms in restoring AIB uptake towards normal (Table VII). This, together with the lack of effect of insulin on AIB uptake when added alone in the incubation medium, suggest that insulin does not "activate" an amino acid transport system *per se*, but probably regulates AIB accumulation almost exclusively via its well-known stimulatory action on the transmembrane transport of glucose. In this respect, the effect of insulin on AIB uptake by myoblasts appears to closely resemble that previously reported for other energy-requiring processes in the isolated fat cells[23–25].

ACKNOWLEDGEMENTS

The authors are greatly indebted to Miss Marylène Moinat for her very expert and dedicated technical assistance. This study was possible thanks to the Grant No. 4848.3 of the Fonds National Suisse pour la Recherche Scientifique, Berne, Switzerland, and to a grant-in-aid from Nestlé-Alimentana S.A., Vevey, Switzerland.

REFERENCES

1 I. HARARY AND B. FARLEY, *Exp. Cell Res.*, 29 (1963) 451.
2 H. KURAMITSU AND I. HARARY, *Biochim. Biophys. Acta*, 86 (1964) 65.
3 A. FUJIMATO AND I. HARARY, *Biochim. Biophys. Acta*, 86 (1964) 74.
4 I. HARARY AND E. C. SLATER, *Biochim. Biophys. Acta*, 99 (1965) 227.
5 M. W. SERAYDARIAN, I. HARARY AND E. SATO, *Biochim. Biophys. Acta*, 162 (1968) 414.
6 I. G. F. GILBERT AND J. M. RADLEY, *Biochim. Biophys. Acta*, 82 (1964) 618.
7 G. E. MARK AND F. F. STRASSER, *Exp. Cell Res.*, 44 (1966) 217.

8 G. E. MARK, J. D. HACKNEY AND F. F. STRASSER, in R. D. TANZ, F. KAVALER AND J. ROBERTS, *Factors influencing myocardial contractibility*, Academic Press, New York, 1967, p. 301.
9 R. L. DE HAAN, *Dev. Biol.*, 16 (1967) 216.
10 B. BLONDEL, I. ROIJEN AND J. P. CHENEVAL, *Experientia*, 27 (1971) 356.
11 D. YAFFE, *Proc. Natl. Acad. Sci. U.S.*, 61 (1968) 477.
12 J. P. CHENEVAL, A. HYDE AND L. GIRARDIER, in preparation.
13 A. HYDE, J. P. CHENEVAL, B. BLONDEL AND L. GIRARDIER, in preparation.
14 I. HARARY AND B. FARLEY, *Exp. Cell Res.*, 29 (1963) 466.
15 G. A. BRAY, *Anal. Biochem.*, 1 (1960) 279.
16 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
17 H. M. GOODMAN, *Am. J. Physiol.*, 211 (1966) 815.
18 M. TOUABI AND B. JEANRENAUD, *Biochim. Biophys. Acta*, 173 (1969) 128.
19 D. M. KIPNIS AND J. E. PARRISH, *Fed. Proc.*, 24 (1965) 1051.
20 J. P. ROSENBUSCH, B. FLANAGAN AND G. N. NICHOLS, *Biochim. Biophys. Acta*, 135 (1967) 732.
21 J. A. SCHAFER AND J. A. JACQUEZ, *Biochim. Biophys. Acta*, 135 (1967) 1083.
22 A. E. RENOLD, in E. F. PFEIFFER, *Handbuch des Diabetes Mellitus*, J. F. Lehmanns Verlag, Munich, Vol. 1, 1969, p. 553.
23 J. D. VASSALLI AND B. JEANRENAUD, *Biochim. Biophys. Acta*, 202 (1970) 477.
24 M. TOUABI AND B. JEANRENAUD, *Biochim. Biophys. Acta*, 202 (1970) 486.
25 B. JEANRENAUD, D. HEPP AND A. E. RENOLD, in B. JEANRENAUD AND D. HEPP, *Adipose Tissue Regulation and Metabolic Functions, Suppl. No. 2, Horm. Metab Res.*, 1970, p. 76.