Energy Source for Active Transport of \(\alpha\)-Aminoisobutyric Acid in KB Cells*

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SUMMARY

The energy source for concentrative transport of \(\alpha\)-aminoisobutyric acid (AIB) in KB cells is ATP generated during anaerobic glycolysis or oxidative phosphorylation. The dependence of AIB accumulation on the concentration of ATP is described by a rectangular hyperbola from which the ATP concentration supporting half-maximal accumulation of AIB was estimated to be 0.8 mM.

With the aid of inhibitors and uncouplers of oxidative phosphorylation it was shown that energization of AIB transport by ATP does not involve the participation of high energy intermediates which may be generated within the mitochondria during oxidative phosphorylation or from ATP. Evidence was obtained against the function of phosphoenolpyruvate as the immediate energy donor to the transport system.

A kinetic analysis of AIB influx and efflux revealed that ATP and intracellular \(K^+\) act on the step involved in AIB uptake by decreasing the \(K_m\) for AIB influx, whereas oligomycin increases this \(K_m\).

A comparison of the effectiveness of oligomycin, peliomycin, and ossamycin in inhibiting three distinct biochemical systems, namely, oxidative phosphorylation, AIB transport, and the \((Na^+ + K^+)\)-ATPase, showed that all three inhibitors are equally effective in inhibiting oxidative phosphorylation, but peliomycin is the most effective in inhibiting the \((Na^+ + K^+)\)-ATPase. Ossamycin is the least effective in inhibiting the latter and AIB transport.

During the last decade various proposals have been made concerning the energy source of active transport of nonelectrolytes in mammalian systems. According to one of these proposals the energy available from the \(Na^+\) gradient is insufficient and the additional energy could be provided by the \(K^+\) gradient across the plasma membrane.

The validity of this proposal was seriously questioned by subsequent work in which active amino acid transport in various mammalian cells was shown to occur in the absence of \(Na^+\) and \(K^+\) gradients or when the direction of these gradients was reversed. Furthermore, in the absence of metabolic energy the imposed \(Na^+\) and \(K^+\) gradients could support less than 20% of the amino acid accumulation obtained in the presence of metabolic energy.

In experiments in which the \(Na^+\) and \(K^+\) gradients were varied, the concentrations of these cations were also varied from the optimal levels necessary for maximal amino acid accumulation. Therefore, it is not possible to dissociate gradient from concentration effects in those cases in which amino acid transport depends on extracellular \(Na^+\) and \(K^+\) gradients or when the direction of these gradients was reversed. Furthermore, in the absence of metabolic energy the imposed \(Na^+\) and \(K^+\) gradients could support less than 20% of the amino acid accumulation obtained in the presence of metabolic energy.

The above considerations stimulated further research directed towards defining the energy source for active amino acid transport in mammalian cells. Potashner and Johnston (27, 28) were able to show that amino acid accumulation in Ehrlich ascites cells depended on a normal intracellular \(Na^+\) and \(K^+\) (14, 29). From all of the evidence available at present it can be concluded that although the energy from the biochemical potential gradients of \(Na^+\) and \(K^+\), including the transmembrane potential, cannot account for the accumulated amino acid in Ehrlich ascites tumor cells. More recently Geck et al. (21) showed that the maximum efficiency of coupling between the influx of \(Na^+\) and the transport of \(\alpha\)-aminoisobutyric acid (AIB) in ascites cells is only 7% and therefore too low to allow sufficient channelling of energy from the \(Na^+\) and \(K^+\) gradients into amino acid transport.

In experiments in which the \(Na^+\) and \(K^+\) gradients were varied, the concentrations of these cations were also varied from the optimal levels necessary for maximal amino acid accumulation. Therefore, it is not possible to dissociate gradient from concentration effects in those cases in which amino acid transport depends on extracellular \(Na^+\) and \(K^+\) (10, 15, 16, 23-28) and intracellular \(K^+\) (14, 29). From all of the evidence available at present it can be concluded that although the energy from the biochemical potential gradients of \(Na^+\) and \(K^+\) can be used to drive active amino acid transport, a direct coupling between these fluxes can be excluded as the main mechanism of energization of amino acid transport.

The above considerations stimulated further research directed towards defining the energy source for active amino acid uptake in mammalian cells. Potashner and Johnston (27, 28) were able to show that amino acid accumulation in Ehrlich ascites cells depended on a normal intracellular (ATP) rather than on the \(Na^+\) gradient. These results gave support to the idea that cellular ATP could serve as energy source for active amino acid transport, location of \(Na^+\) and \(K^+\) across the plasma membrane; AIB, \(\alpha\)-aminoisobutyric acid; DNP, 2,4-dinitrophenol; PEP, phosphoenolpyruvate; KRB, Krebs-Ringer bicarbonate medium.

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‡ The abbreviations used are: \((Na^+ + K^+)\)-ATPase, the \(Na^+ + K^+\) activated ouabain inhibitable ATPase mediating the transport of and sugars across pigeon erythrocytes and the intestinal epithelium (1-9). However, other investigators working with ascites tumor cells concluded that the energy from the \(Na^+\) gradient is insufficient and the additional energy could be provided by the \(K^+\) gradient across the plasma membrane (10-14). The validity of this proposal was seriously questioned by subsequent work in which active amino acid transport in various mammalian cells was shown to occur in the absence of \(Na^+\) and \(K^+\) gradients or when the direction of these gradients was reversed (15-22). Furthermore, in the absence of metabolic energy the imposed \(Na^+\) and \(K^+\) gradients could support less than 20% of the amino acid accumulation obtained in the presence of metabolic energy (10, 19-21). Schaefer and Heinz (19) concluded that the energy available from the biochemical potential gradients of \(Na^+\) and \(K^+\), including the transmembrane potential, cannot account for the accumulated amino acid in Ehrlich ascites tumor cells. More recently Geck et al. (21) showed that the maximum efficiency of coupling between the influx of \(Na^+\) and the transport of \(\alpha\)-aminoisobutyric acid (AIB) in ascites cells is only 7% and therefore too low to allow sufficient channelling of energy from the \(Na^+\) and \(K^+\) gradients into amino acid transport. During the last decade various proposals have been made concerning the energy source of active transport of nonelectrolytes in mammalian systems. According to one of these proposals the energy available from the \(Na^+\) gradient across the plasma membrane maintained by the function of the \((Na^+ + K^+)\)-ATPase is sufficient for the active transport of amino acids.
but further studies are necessary to establish: (a) the degree of dependence of amino acid accumulation on the concentration of ATP; (b) whether or not the energy of ATP must first be transduced within the mitochondrion to energy-rich intermediates which in turn would drive active amino acid uptake, especially since Van Rossum (30) concluded that the energy which drives the translocation of cations across the plasma membrane of liver cells can be derived directly from high energy intermediates of oxidative phosphorylation; (c) the mechanism by which the energy of ATP is coupled to amino acid transport. In this paper we present the results of our studies bearing on the above aspects of active transport of \( \alpha \)-aminoisobutyric acid in KB cells.

**EXPERIMENTAL PROCEDURE**

**Culture Conditions**—The KB cells (certified line No. 17 of the American Type Culture Collection) were grown in suspension cultures in Eagle’s minimal essential medium containing 10% horse serum (31). The cell density was maintained between 2 \( \times \) 10\(^5\) and 6 \( \times \) 10\(^5\) cells per ml.

**Incubation medium**—The medium used in all of these studies was a modified Krebs-Ringer bicarbonate buffer of the following composition, in mm: KCl, 36.0; NaCl, 7.50; NaH\(_2\)PO\(_4\), 10.0; NaHCO\(_3\), 25.0; MgSO\(_4\), 1.2. The pH was 7.4 by equilibrating with a mixture of 95% \( \text{N}_2 \)-5yo CO*.

**Analytical Methods**—The procedures for determining the cell number, the cell water, the cell viability, and \([^{14}C]\text{AIB}) were described previously (39). Protein was measured by the method of Lowry et al. (40).

**Materials**—Tris-ATP; glucose-6-P dehydrogenase, hexokinase, orsinilin (G-strophanthin), 2,4-dinitrophenol, NADH, NADP, and oligomycin (15% oligomycin A and 85% oligomycin B) were purchased from Sigma. Peliomycin and ossamycin were generous gifts from Dr. Henry Schmitz of Bristol Laboratories, Bristol-Meyers Co., Syracuse, New York. \([^{14}C]\)AIB was purchased from Calbiochem. All other materials were of reagent grade. Oligomycin, pemolycin, and ossamycin were dissolved in absolute ethanol. The (ethanol) in the incubation mixtures resulting from the addition of these compounds was 0.1%. The same amount of ethanol without these compounds was added to the control incubations.

**RESULTS**

**Accumulation of AIB and Steady State Levels of ATP When Metabolic Energy Is Derived from Aerobic or Anaerobic Glycolysis**—Fig. 1 shows the levels of intracellular ATP when the cells are incubated aerobically or with KCN in the presence of 2 mm glucose. It can be seen that inhibition of respiration causes a 25% decrease of the steady state [ATP]. The ability of the cells to accumulate AIB under these conditions is shown in Table I. Aerobic conditions cause a small and comparable reduction of AIB accumulation and the steady state [ATP]. Addition of DNP at concentrations which uncouple mitochondrial oxidative phosphorylation has no effect on either AIB accumulation or the ATP levels. Thus, in the presence of active glycolysis DNP fails to deplete the ATP. As will be shown later DNP can deplete the intracellular ATP when the cells are incubated without glucose.

**AIB Accumulation Driven by Glycolytic ATP When Mitochondrial Respiration and Energy Conservation Are Inhibited**—In order to test whether glycolytically derived ATP can support AIB accumulation without the intervention of a mitochondrial energy-transducing event we studied the effects of oligomycin, peliomycin, and ossamycin on AIB accumulation when the

**Table I**

| Inhibitor       | AIB Accumulation | Intracellular ATP |
|-----------------|------------------|-------------------|
| None            | 100              | 100               |
| NaCN           | 88               | 76                |
| NaCN plus DNP  | 86               | 75                |
| N\(_1\)-          | 80               | 70                |

**Fig. 1** (left). Steady state levels of ATP under conditions of aerobic and anaerobic glycolysis. Cells were incubated at 37° in KRB containing 2.0 mm glucose, with and without 2.0 mm NaCN. The incubations were terminated at the times indicated, and the intracellular ATP was determined. \( \text{O--O} \), with cyanide; \( \Delta--\Delta \), with cyanide.

**Fig. 2** (right). Time course of inhibition by oligomycin of the AIB influx rate. Cells were incubated at 37° in KRB containing 2.0 mm glucose and 2.0 mm NaCN with and without oligomycin (8 \( \mu \)g per ml) for 5, 10, 16, and 37 min. \([^{14}C]\)AIB was then added (0.2 mm), and the incubations were terminated after 1 min. The AIB influx rate is in nanomoles of AIB taken up per 10\(^5\) cells per min. \( \text{O--O} \), without oligomycin; \( \bullet--\bullet \), with oligomycin.
Further studied in terms of its effect on the rate of AIB uptake. Irg per ml, and maximal inhibition by 8 to 10 pg per ml. The results were obtained with peliomycin and ossamycin. Thereafter, in all subsequent studies with these inhibitors the cells were incubated with the inhibitor for 16 min before AIB accumulation was measured. Fig. 3 presents the effects of oligomycin, peliomycin, and ossamycin on AIB accumulation and the steady state [ATP] maintained by anaerobic glycolysis. It is clearly shown that oligomycin and peliomycin inhibit AIB accumulation 80% without causing any decrease in the steady state [ATP]. Both compounds are effective at comparable concentrations. Fifty percent inhibition is produced by 5 to 6 µg per ml, and maximal inhibition by 8 to 10 µg per ml. The effects of ossamycin are less specific and more complex. Much higher concentrations are needed to inhibit AIB accumulation, and in addition to its effect on AIB transport, it causes comparable reduction of the steady state [ATP]. Hence, it is not possible to decide whether the inhibition of AIB accumulation by ossamycin is independent of the reduction of the ATP levels.

The inhibitory action of oligomycin on AIB transport was further studied in terms of its effect on the rate of AIB uptake. Fig. 4 shows the inhibition of the initial influx rate of AIB as a function of oligomycin concentration. Maximal inhibition (88%) is obtained with 10 to 12 µg per ml of oligomycin, similar to the concentration which maximally inhibits AIB accumulation (Fig. 3).

Two interpretations can be considered to explain the results obtained with oligomycin and peliomycin. (a) These compounds could act at the plasma membrane by interacting with the membrane sites involved in AIB transport thereby inhibiting the energization of the transport system by ATP. An analogy to this mode of action is the inhibition by oligomycin of the (Na+ + K+)-ATPase of the plasma membrane (42-45). (b) Alternatively, energization of the AIB transport system by ATP would require energy transduction via the mitochondrial reactions which are inhibited by oligomycin and peliomycin (41, 46-48) and which are involved in the synthesis of ATP during oxidative phosphorylation.

The latter possibility may be evaluated by comparing the concentrations of oligomycin and peliomycin which inhibit oxidative phosphorylation maximally with the concentrations which cause maximal inhibition of AIB accumulation when the intracellular ATP is maintained by anaerobic glycolysis. If, in the presence of normal levels of ATP, AIB accumulation is not inhibited by concentrations of these inhibitors which cause maximal inhibition of oxidative phosphorylation, the second interpretation mentioned above becomes most unlikely if not untenable.

For Determining Concentration of Oligomycin, Peliomycin, and Ossamycin Producing Maximal Inhibition of Oxidative Phosphorylation in Intact Cells—In the absence of glycolysis the steady state [ATP] is maintained by respiration-coupled oxidative phosphorylation. Under these conditions inhibition of oxidative phosphorylation leads to depletion of ATP. From a number of preliminary experiments it was found that the most effective way of depleting the endogenous glycolytic substrates is to incubate the cells with DNP for 35 min. If the DNP is then removed by washing the cells, synthesis of ATP through the mitochondrial oxidative phosphorylation is established, and the steady state [ATP] is restored. A typical experiment is shown in Fig. 5. Exposure of the cells to DNP for 35 min decreases their [ATP] by 80 to 90%. After washing away the DNP the
TABLE II
Effects of various inhibitors on AIB accumulation and ATP levels of cells previously depleted of ATP and glycolytic substrates

Cells were first incubated in KII B without glucose in the presence of 0.3 mM DNP for 35 min in order to deplete the intracellular ATP. At this point the intracellular [ATP] was 0.18 mM. The cells were then centrifuged, washed three times with ice-cold 0.15 M NaCl, and were incubated at 37°C in fresh KII B medium without glucose for 16 min with or without the additions shown. $^{14}$C-AIB (0.2 mM) was then added, and the cells were incubated for an additional 25 min in order to determine the extent of AIB accumulation. Similar incubations with unlabeled AIB were used for the determination of intracellular ATP levels. The measurements of ATP and intracellular AIB were performed at the end of the final incubation period. The "control" represents cells which were processed as described above, except that they were never exposed to any of the inhibitors. In these cells the [ATP] was 1.60 mM and the net accumulation of AIB was 1.30 mM.

| Additions after removal of DNP | AIB accumulation | Intracellular ATP |
|-------------------------------|-----------------|-------------------|
| Control                       | 100             | 100               |
| None                          | 83              | 72                |
| NaCN (2.0 mM)                 | 6               | 4                 |
| DNP (0.3 mM)                  | 6               | 5                 |
| Oligomycin (0.04 μg per ml)   | 7               | 4                 |

ATP is promptly restored, and within 15 min its steady state level is 70 to 75% of that of the control cells. In the latter cells about 20% of the [ATP] is of glycolytic origin since inhibition of respiration by cyanide reduces the [ATP] by 80%. Allowing, therefore, for this 20% fraction of ATP which is of glycolytic origin, the recovery of the steady state [ATP] in the DNP-treated cells represents 90 to 95% of the control value. Since the addition of either cyanide, oligomycin, or DNP at the beginning of the recovery period prevents restoration of normal ATP levels, the energy used to replenish this ATP is of respiratory origin. These results as well as the extent of AIB accumulation after recovery from DNP treatment are shown in Table II. It can be seen that the devised conditions allow AIB accumulation and ATP synthesis to take place entirely from mitochondrial respiratory energy at the expense of endogenous oxidizable substrates.

Lactate was examined as a possible endogenous substrate the oxidation of which furnishes the energy for restoration of the [ATP]. In cells treated with DNP the initial [lactate] following removal of DNP was 1.7 mM, and during the subsequent 20 min of incubation when the cellular ATP is restored to its normal level of 1.2 mM (see Fig. 5) the [lactate] dropped to 1.3 mM. During the same period the [pyruvate] remained unchanged at 7.0 ± 1.5 μM. It is also possible that other endogenous substances such as fatty acids and amino acids could serve as oxidizable substrates. In KB cells incubated in the presence of glucose the steady state [lactate] and [pyruvate] are 7.5 ± 1.4 mM and 70.0 ± 20.0 μM, respectively.

Inhibition of AIB Accumulation and ATP Synthesis by Oligomycin, Peliomycin, and Ossamycin When Cellular ATP Is Generated during Respiration-coupled Oxidative Phosphorylation—Using the conditions described in Table II experiments were carried out with the following objectives in mind: (a) to compare the concentrations of these inhibitors giving maximal inhibition of oxidative phosphorylation with those inhibiting AIB accumulation when the ATP is derived entirely from anaerobic glycolysis (from such a comparison it would appear possible to determine whether or not the energy generated by AIB transport by glycolytic ATP is produced at intramitochondrial energy transduction and generation of energy-rich compounds postulated to be intermediates in the over-all process of oxidative phosphorylation); (b) to establish whether or not the mitochondrial energy-conserving process can drive active AIB transport when ATP synthesis during oxidative phosphorylation is inhibited; (c) to determine the quantitative relationship that may exist between the steady state [ATP] and the magnitude of AIB accumulation.

The results of these experiments are presented in Fig. 6. The concentrations of oligomycin, peliomycin, and ossamycin causing 50% decrease of the [ATP] are 0.012, 0.008, and 0.012 μg per ml, respectively. The corresponding concentrations for 50% inhibition of AIB accumulation are 9.016, 9.008, and 9.018 μg per ml, respectively. Maximal reduction of the [ATP] and inhibition of AIB accumulation are obtained at 0.03 to 0.04 μg per ml with all three inhibitors; addition of as much as 1.0 μg per ml produced no further inhibition. Therefore, this concentration is effecting maximal inhibition of oxidative phosphorylation. The reason why the steady state [ATP] in the fully inhibited cells is maintained at about 0.15 mM could be that the rates of the reactions which utilize ATP decrease considerably when the [ATP] is severely reduced. If the inhibited cells are incubated for periods longer than 60 min the [ATP] drops below 0.05 mM, and after washing away the inhibitors the cells restore their ATP to normal levels (1.2 mM).

The following conclusions can be drawn from the results presented above. (a) AIB accumulation depends on the availability of ATP, and inhibition of ATP synthesis by the above
oxidations via the respiratory chain can be ascribed to the in-
these compounds when the metabolic energy is derived from

d. Respiration

The relationship between the steady state levels of intracellular ATP and AIB. The figure represents the average of the corresponding values of [AIB] and [ATP] presented in Tables I and II, and in Fig. 6. The AIB values shown in this figure represent total intracellular [AIB]. The same data plotted according to the method of Lineweaver and Burk are shown in the inset. In all of these experiments the extracellular [AIB] was 0.2 mM.

Inhibitors results in a concomitant inhibition of AIB accumulation of comparable magnitude. Hence, the energy conserved during electron transport must be converted to ATP before it can energize active AIB transport. (b) The maximal inhibition of AIB accumulation obtained with 0.04 mg per ml of either one of the above inhibitors is the result of inhibition of ATP synthesis rather than a direct effect of these inhibitors on the AIB transport system since under conditions when the ATP is maintained by anaerobic glycolysis no inhibition of AIB accumulation can be detected until the concentration of oligomycin or peliomycin is increased by more than 10-fold (0.5 mg per ml) and that of ossamycin by more than 500-fold (20 mg per ml), while 50% inhibition of AIB accumulation is obtained with 5 to 6 mg of oligomycin or peliomycin and with 105 mg of ossamycin (see Fig. 3). Thus, concentrations of these inhibitors which inhibit maximally AIB accumulation when synthesis of ATP is of respiratory origin exert no effect on AIB accumulation when ATP is maintained by anaerobic glycolysis even when DNP (0.2 mM) is simultaneously present (not shown). Hence the energy of ATP can drive active AIB transport without prior transduction within the mitochondrion to high energy intermediates of oxidative phosphorylation. (c) The inhibition of AIB accumulation by higher concentrations of these inhibitors when ATP is maintained by glycolysis ought to represent an extramitochondrial effect, probably at the plasma membrane.

Relationship between Steady State Levels of ATP and AIB—From the data presented in Tables I and II, and in Fig. 6, the quantitative relationship between the intracellular levels of ATP and AIB was determined as shown in Fig. 7. The curve describing the points is a rectangular hyperbola, and if the data are plotted according to the method of Lineweaver and Burk a straight line is obtained from which the apparent ATP giving half-maximal accumulation of AIB was calculated to be about 0.8 mM. The actual concentration is probably lower than 0.8 mM since an undetermined fraction of the cellular ATP may be compartmentalized and inaccessible to the AIB transport system.

Effects of Oligomycin, Peliomycin, and Ossamycin on Rate of Respiration of KB Cells—The inhibition of ATP synthesis by these compounds when the metabolic energy is derived from oxidations via the respiratory chain can be ascribed to the in-
hibition of the terminal steps of oxidative phosphorylation leading to the synthesis of ATP (41, 46-48). Since similar detailed studies are not available for KB cells it was of interest to study the effect of these inhibitors on the rate of respiration of these cells. If these compounds inhibit mitochondrial ATP synthesis, they should cause an inhibition of respiration which would be relieved by the addition of DNP. These studies showed that when KB cells are incubated in KRB containing 2.0 mM glucose separate addition of oligomycin, peliomycin, and ossamycin (0.04 or 10 mg per ml) causes severe inhibition of respiration. Subsequent addition of DNP not only relieves this inhibition but stimulates the rate of respiration above that obtained by the uninhibited cells. DNP also relieves the inhibition of respiration caused by high concentrations (10 mM) of glucose (49). These results are in agreement with the proposed mode of action of these compounds as inhibitors of ATP synthesis during oxidative phosphorylation, while DNP acts typically as an uncoupler of this process.

Kinetic Parameters of AIB Transport in KB Cells under Various Experimental Conditions—It was shown above that depletion of cellular ATP or inhibition of the cells by oligomycin and peliomycin leads to a loss of active AIB transport. It was earlier shown that depletion of cellular K+ also results in the inability of the cells to concentrate AIB, and the transport system reverts from an active to one of facilitated diffusion (29). Since the loss of active transport can result from either inhibition of the entry or acceleration of the exit of the transported compound, experiments were carried out to determine which kinetic parameter of the AIB transport is affected by the above conditions.

Three types of cell preparations were used in these studies: (a) cells depleted of cellular K+ but containing normal ATP levels. The preparation of these cells by a preliminary incubation at 37°C for 90 min in KRB containing glucose (2.0 mM) but lacking K+ was described earlier (29). Such cells contain 4 to 8 mM K+ and maintain normal levels (2.2 to 2.4 mM) of ATP throughout the subsequent period of the kinetic measurements of AIB transport. The kinetics of AIB transport were studied in the above medium. It was previously shown that AIB transport in KB cells is not affected by omission of extracellular K+ if the cellular K+ is maintained above 30 mM (29). Another portion of the cells was treated as described above, but during the kinetic measurements the incubation medium contained 10 mM K+. These cells served as the “Control”; (b) cells depleted of ATP (the cellular ATP was 0.07 to 0.09 mM) but containing optimal levels (45 to 50 mM) of K+. The conditions for preparing these cells by a preliminary incubation with DNP were described in Table II. The kinetic measurements were carried out in KRB lacking glucose and containing 0.2 mM DNP in order to prevent restoration of cellular ATP. Another portion of the cells treated as described above but allowed to restore normal ATP levels by omitting the DNP during the kinetic measurements served as “Control”; (c) cells containing normal levels of ATP (1.8 to 1.9 mM) and K+ (65 to 75 mM), but incapable of concentrative AIB transport due to inhibition by oligomycin. The preparation of these cells by a preliminary incubation in KRB containing glucose (2.0 mM), cyanide (2.0 mM), and oligomycin (10 mg per ml) was described in Fig. 3. The kinetic measurements were carried out in the same medium. Another portion of the cells treated as above but which was not exposed to oligomycin served as “Control.” The conditions for measuring initial rates of AIB influx and efflux were described in a previous publication (32). For influx
the extracellular concentrations of $[^{14}C]$AIB (specific activity, $4 \times 10^6$ cpm per pmole) were: 0.4, 0.8, 1.6, 3.2, and 6.4 mM. For efflux the concentrations of $[^{14}C]$AIB used to preoad the cells were: 0.63, 2.5, 5.0, and 10.0 mM. Initial flux rates were measured during the 1st min of incubation at 37°, and are expressed in nanomoles of AIB per 10<sup>6</sup> cells per min. The $K_m$ and $V_{max}$ values were derived from Lineweaver-Burk plots of initial flux rates versus the AIB concentrations from which the fluxes emanate. The results of these studies are summarized in Table III. It can be seen that depletion of cellular ATP or of cellular K⁺, as well as inhibition by oligomycin under conditions when the cellular ATP and K⁺ remain normal, lead to an increase of the apparent $K_m$ for AIB influx. Under these conditions the $K_m$ values for influx become similar to those for efflux. None of the other kinetic parameters of AIB transport are changed by the above conditions. Thus, ATP and intracellular K⁺ function by decreasing the apparent $K_m$ for AIB influx. Potashner and Johnstone (28) also reported an increase of the $K_m$ for amino acid influx in Ehrlich ascites cells depleted of ATP. However, no data were given concerning the kinetics of amino acid efflux. Our results offer an explanation for the observation of Heinz (50) that in Ehrlich ascites cells DNP and iodoacetate cause a significant decrease of the influx coefficient of glycine without affecting the efflux coefficient.

### Table III

*Kinetic parameters of AIB entry and exit in KB cells under various experimental conditions*

Three types of cell preparations were used in these studies: 1. cells depleted of cellular ATP and containing optimal levels of K⁺; 2. cells depleted of K⁺ and containing normal levels of ATP; 3. cells containing normal levels of ATP and K⁺ but which lost active AIB transport as a result of inhibition by oligomycin. The preparation of these cells and the conditions used in the kinetic measurements were described in the text. The $K_m$ (millimolar) and $V_{max}$ (nanomoles of AIB per 10<sup>6</sup> cells per min) values were derived from Lineweaver-Burk plots of initial flux rates versus AIB concentrations from which the fluxes emanate.

| Cell preparation          | $K_m$ values (mM) | $V_{max}$ values (nM/min) |
|---------------------------|-------------------|----------------------------|
| 1. ATP-containing         | 1.8, 6.4          | 20.8, 20.0                 |
| ATP-depleted              | 6.6, 6.3          | 19.6, 21.0                 |
| 2. K-containing           | 1.0, 6.0          | 21.0, 19.8                 |
| K-depleted                | 5.9, 6.4          | 20.0, 20.5                 |
| 3. Without oligomycin     | 2.0, 6.8          | 21.8, 20.4                 |
| With oligomycin           | 6.1, 6.5          | 21.0, 21.3                 |

### Table IV

*Relationship between steady state levels of ATP, PEP, and AIB*

In System 1 the cells were incubated in KRB containing glucose (2.0 mM) and KCN (2.0 mM) for 16 min at 37°. $[^{14}C]$AIB (0.2 mM) was then added and the incubations were continued for 25 min for establishing steady state AIB accumulation. At the end of this period the concentrations of ATP, PEP, and AIB were determined. In System 2 the cells were first depleted of ATP and glycolytic intermediates as described in Fig. 5. After washing away the DNP the cells were incubated in KRB without glucose in the presence or absence of oligomycin (0.04 μg per ml) for 16 min at 37°. $[^{14}C]$AIB was then added and the incubations were continued for 25 min for establishing steady state AIB accumulation. At the end of this period the concentrations of ATP, PEP, and AIB were determined. In System 2 the cells were first depleted of ATP and glycolytic intermediates as described in Fig. 5. After washing away the DNP the cells were incubated in KRB without glucose in the presence or absence of oligomycin (0.04 μg per ml) for 16 min at 37°. $[^{14}C]$AIB (0.2 mM) at 37° for 16 min, and the reaction was then initiated by the addition of ATP. The amount of P, released after 15 min was measured colorimetrically. Under these conditions the reaction velocities were linear for 30 min and proportional to the amount of enzyme extract added (0.1 to 0.5 μg of protein per ml). Activities are expressed in micromoles of P, released per mg of protein per 15 min. Solid symbols represent the activity of the ouabain-inhibitable (Na⁺ + K⁺)-ATPase, and open symbols the activity of the ouabain-insensitive ATPase. The concentrations of oligomycin and peliomycin are indicated on the upper scale, while those of osomycin on the lower scale.

| System | Steady state intracellular concentrations |
|--------|----------------------------------------|
|        | ATP (μM) | PEP (μM) | AIB (μM) |
| 1      | 1.7      | 37.0     | 1.3      |
| 2      | 1.3      | 37.0     | 1.0      |
| 3      | 0.10     | 7.0      | 0.13     |

* The values represent net accumulations (intracellular minus extracellular concentrations).*

![Fig. 8. Effects of oligomycin (circles), peliomycin (triangles), and osomycin (squares) on the activities of the ouabain-inhibitable (Na⁺ + K⁺)-ATPase and the ouabain-insensitive ATPase. The activities were assayed using a cell-free particulate fraction (0.3 mg of protein) as described previously (33). The complete assay mixture minus ATP was incubated with or without varying levels of these inhibitors in the presence or absence of ouabain (0.13 mM) at 37° for 16 min, and the reaction was then initiated by the addition of ATP. The amount of P, released after 15 min was measured colorimetrically. Under these conditions the reaction velocities were linear for 30 min and proportional to the amount of enzyme extract added (0.1 to 0.5 μg of protein per ml). Activities are expressed in micromoles of P, released per mg of protein per 15 min. Solid symbols represent the activity of the ouabain-inhibitable (Na⁺ + K⁺)-ATPase, and open symbols the activity of the ouabain-insensitive ATPase. The concentrations of oligomycin and peliomycin) are indicated on the upper scale, while those of osomycin on the lower scale.
glycolysis or respiration-coupled oxidative phosphorylation. Through the judicious use of three inhibitors of oxidative phosphorylation, namely, oligomycin, peliomycin, and ossamycin, it was possible to systematically vary the steady state [ATP] and to show that the extent of AIB accumulation is dependent on the [ATP] in a manner described by a rectangular hyperbola from which the apparent [ATP], which supports half-maximal AIB accumulation, was estimated to be 0.8 mM. All three inhibitors are equally effective in inhibiting ATP synthesis and AIB accumulation when the metabolic energy is of respiratory origin. In these experiments the steady state [ATP] was used as a measure of the relative effectiveness of these agents in inhibiting oxidative phosphorylation and hence ATP synthesis. The possibility that they may affect various cell reactions which utilize ATP in a manner such that the [ATP] may not reflect the rate of its synthesis appears rather unlikely since under conditions of anaerobic glycolysis the [ATP] is not affected by the presence of these compounds at concentrations 1000 times higher than those which abolish oxidative phosphorylation. Furthermore, the inhibitor concentrations causing 50% reduction of the [ATP] are in good agreement with those producing 50% inhibition of ATP synthesis in respiring liver mitochondria (51).

The experiments designed to determine the route by which the energy of ATP is channelled to the AIB transport system have clearly shown that ATP generated during glycolysis serves active AIB transport without prior energy transduction within the mitochondria to energy-rich intermediates of oxidative phosphorylation. Conversely, respiratory energy must be converted to ATP before it can be used in concentative AIB uptake. The possibility that PEP may be the immediate energy donor to the transport system can be excluded since it was shown that AIB accumulation is abolished when the ATP is depleted even though the steady state [PEP] remains unchanged (Table IV). Similarly, on the basis of evidence presented earlier in the introduction, indirect channeling of the ATP energy to the transport system through establishment of cation gradients by the (Na\textsuperscript{+} + K\textsuperscript{+})-ATPase can also be excluded. The reverse situation is more likely since it was shown that this ATPase can catalyze the synthesis of ATP utilizing the energy released from dissipation of the cation gradients (52, 53).

A kinetic analysis of AIB influx and efflux under presteady state conditions revealed that ATP, intracellular K\textsuperscript{+}, and oligomycin act on the entry process of the transport system. The same action was earlier shown for extracellular Na\textsuperscript{+} (26). Thus, depletion of cellular ATP or K\textsuperscript{+}, elimination of extracellular Na\textsuperscript{+}, and inhibition by oligomycin abolish active AIB transport by increasing the K\textsubscript{m} for AIB entry to the same value as the K\textsubscript{m} for exit. Many attempts to induce significant changes of the value of the latter parameter were unsuccessful. These findings indicate that in KB cells the regulation of active AIB transport is accomplished by modulation of the K\textsubscript{m} for AIB entry through changes in the concentration of ATP, intracellular K\textsuperscript{+}, and extracellular Na\textsuperscript{+}. It is not known whether KB cells possess other means of regulating the activity of this transport system.

The abolition of AIB accumulation by higher concentrations of oligomycin and peliomycin when ATP is of glycolytic origin indicates that these agents inhibit AIB transport either by interacting with the carrier sites of the plasma membrane or by inhibiting the energy transfer from ATP to the transport system. It is of interest that another plasma membrane system, the (Na\textsuperscript{+} + K\textsuperscript{+})-ATPase, is inhibited by oligomycin (42-45). Our studies confirm this observation and show that peliomycin also inhibits this carrier system. Compared to oligomycin peliomycin is 5 to 6 times more effective in inhibiting this ATPase,

### Table V

| Inhibitor | Anaerobic glycolysis | Oxidative phosphorylation | (Na\textsuperscript{+} + K\textsuperscript{+})-ATPase |
|-----------|----------------------|---------------------------|---------------------------------------------|
| Oligomycin | 7.5                  | 9.0                       |                                             |
| Peliomycin | 9.6                  | 158.0                     | 145.0                                      |
| Ossamycin  | 158.0                | 0.027                     | 300.0                                      |

* Oligomycin or peliomycin at 30 \(\mu\)g per mg of protein had no effect on the steady state levels of ATP.

depletion of ATP, whereas the [PEP] remains unchanged. These results strongly suggest that PEP is not the immediate energy donor in active AIB transport.

### Inhibition of (Na\textsuperscript{+} + K\textsuperscript{+})-ATPase by Oligomycin, Peliomycin, and Ossamycin

The inhibition by these compounds of active AIB transport by a mechanism not involving mitochondrial oxidative phosphorylation suggests that they may interact with other membrane systems, such as the plasma membrane, causing severe impairment of amino acid transport. Another important transport system of the plasma membrane, the activity of which is essential for the functional integrity of the AIB transport system, is the ouabain-inhibitable (Na\textsuperscript{+} + K\textsuperscript{+})-ATPase (29) which was shown to be inhibitable by oligomycin (42-45). Thus, oligomycin inhibits three distinct cellular processes, namely oxidative phosphorylation, active amino acid transport, and active translocation of Na\textsuperscript{+} and K\textsuperscript{+} across the plasma membrane. It is important therefore to determine whether or not peliomycin and ossamycin which inhibit the first two processes also inhibit this ATPase. The results of these experiments are presented in Table V which also shows the effect of these inhibitors on the ouabain-insensitive ATPase activity. The latter very likely represents the composite action of more than one enzyme. It can be seen that peliomycin is the most effective in inhibiting the (Na\textsuperscript{+} + K\textsuperscript{+})-ATPase, followed by oligomycin and lastly by ossamycin. With regard to their action on the ouabain-insensitive ATPase peliomycin and ossamycin cause some inhibition, while oligomycin produces slight stimulation.

Table V summarizes the concentrations of these compounds which produce 50% reduction of AIB accumulation, the steady state levels of ATP, and the activity of the (Na\textsuperscript{+} + K\textsuperscript{+})-ATPase. The concentrations producing 50% reduction of AIB accumulation and ATP levels when oxidative phosphorylation is the energy source are 6 to 10 times lower than those reported for rat liver mitochondria (51). However, in our studies the actual intracellular concentration of these inhibitors is not known.

### Discussion

Our results show that the energy for active AIB transport in KB cells is derived from ATP generated during either anaerobic glycolysis or respiration-coupled oxidative phosphorylation. Through the judicious use of three inhibitors of oxidative phosphorylation, namely, oligomycin, peliomycin, and ossamycin, it was possible to systematically vary the steady state [ATP] and to show that the extent of AIB accumulation is dependent on the [ATP] in a manner described by a rectangular hyperbola from which the apparent [ATP], which supports half-maximal AIB accumulation, was estimated to be 0.8 mM. All three inhibitors are equally effective in inhibiting ATP synthesis and AIB accumulation when the metabolic energy is of respiratory origin. In these experiments the steady state [ATP] was used as a measure of the relative effectiveness of these agents in inhibiting oxidative phosphorylation and hence ATP synthesis. The possibility that they may affect various cell reactions which utilize ATP in a manner such that the [ATP] may not reflect the rate of its synthesis appears rather unlikely since under conditions of anaerobic glycolysis the [ATP] is not affected by the presence of these compounds at concentrations 1000 times higher than those which abolish oxidative phosphorylation. Furthermore, the inhibitor concentrations causing 50% reduction of the [ATP] are in good agreement with those producing 50% inhibition of ATP synthesis in respiring liver mitochondria (51).

The experiments designed to determine the route by which the energy of ATP is channelled to the AIB transport system have clearly shown that ATP generated during glycolysis drives active AIB transport without prior energy transduction within the mitochondria to energy-rich intermediates of oxidative phosphorylation. Conversely, respiratory energy must be converted to ATP before it can be used in concentative AIB uptake. The possibility that PEP may be the immediate energy donor to the transport system can be excluded since it was shown that AIB accumulation is abolished when the ATP is depleted even though the steady state [PEP] remains unchanged (Table IV). Similarly, on the basis of evidence presented earlier in the introduction, indirect channeling of the ATP energy to the transport system through establishment of cation gradients by the (Na\textsuperscript{+} + K\textsuperscript{+})-ATPase can also be excluded. The reverse situation is more likely since it was shown that this ATPase can catalyze the synthesis of ATP utilizing the energy released from dissipation of the cation gradients (52, 53).

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The abolition of AIB accumulation by higher concentrations of oligomycin and peliomycin when ATP is of glycolytic origin indicates that these agents inhibit AIB transport either by interacting with the carrier sites of the plasma membrane or by inhibiting the energy transfer from ATP to the transport system. It is of interest that another plasma membrane system, the (Na\textsuperscript{+} + K\textsuperscript{+})-ATPase, is inhibited by oligomycin (42-45). Our studies confirm this observation and show that peliomycin also inhibits this carrier system. Compared to oligomycin peliomycin is 5 to 6 times more effective in inhibiting this ATPase,
and the latter is more sensitive to inhibition by peliomycin than
the AIB transport system. In contrast, both carrier systems
are equally sensitive to inhibition by oligomycin. With regard
to ossamycin large concentrations were needed to cause partial
inhibition of the (Na\(^+\) + K\(^+\))-ATPase and AIB accumulation.
Although all three inhibitors are equally effective in inhibiting
oxidative phosphorylation, in agreement with similar findings
with mammalian mitochondria (51, 54, 55), ossamycin appears
to be preferable since it is much more specific than the other
two agents (see Table V).

As a result of the present findings and the development
methods for the preparation of pure plasma membrane vesicles
from KB cells (56) it is now possible to study in greater detail
various aspects of concentrative transport, such as, the specificity
of ATP as energy donor, the mechanism of energy coupling and
its possible regulation by K\(^+\) and Na\(^+\), and the identification of the
membrane component (or components) involved in the
transport process. Some of these aspects are currently being
studied with plasma membrane vesicles.

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