A Reassessment of Decreased Amino Acid Accumulation by Ehrlich Ascites Tumor Cells in the Presence of Metabolic Inhibitors

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ABSTRACT This study was undertaken to examine the mechanism by which metabolic inhibition reduces amino acid active transport in Ehrlich ascites tumor cells. At 37°C the metabolic inhibitor combination 0.1 mM 2,4-dinitrophenol (DNP) + 10 mM 2-deoxy-D-glucose (DOG) reduced the cell ATP concentration to 0.10-0.15 mM in less than 5 min. This inhibition was associated with a 20.6% ± 6.4% (SD) decrease in the initial influx of α-aminoisobutyric acid (AIB), and a two- to fourfold increase in the unidirectional efflux. These effects could be dissociated from changes in cell Na+ or K+ concentrations. Cells incubated to the steady state in 1.0-1.5 mM AIB showed an increased steady-state flux in the presence of DNP + DOG. Steady-state fluxes were consistent with trans-inhibition of AIB influx and trans-stimulation of efflux in control cells, but trans-stimulation of both fluxes in inhibited cells. In spite of the reduction of the cell ATP concentration to <0.15 mM and greatly reduced transmembrane concentration gradients of Na+ and K+, cells incubated to the steady state in the presence of the inhibitors still established an AIB distribution ratio 13.8 ± 2.6. The results are interpreted to indicate that a component of the reduction of AIB transport produced by metabolic inhibition is attributable to other actions in addition to the reduction of cation concentration gradients. Reduction of cell ATP alone is not responsible for the effects of metabolic inhibition, and both the transmembrane voltage and direct coupling to substrate oxidation via plasma-membrane-bound enzymes must be considered as possible energy sources for amino acid active transport.

INTRODUCTION

Many of the earliest investigations of amino acid transport in Ehrlich mouse ascites tumor cells demonstrated that the active accumulation was diminished by anoxia or any of several metabolic inhibitors (1-5). This effect was initially attributed to a requirement for high-energy phosphate compounds in the active transport process. More recent investigations, however, have attributed the energy supply for the active amino acid transport to the concentration gradients of Na+ and possibly K+ existing across the cell membrane (6, 7). Particularly convincing in this regard are the experiments of Eddy et al. (7, 8) in which amino acids were shown to be actively transported into Ehrlich cells which had been
depleted of ATP by NaCN but had cation gradients which were transiently normal in direction. If cation concentration gradients are the only energy source for active amino acid transport, the effects of metabolic inhibitors would be referable solely to the dissipation of these gradients, rather than being directly referable to the depletion of high-energy phosphates available to the transport mechanism.

In support of the latter view, several investigators have reported that metabolic inhibitors seldom reduce amino acid distribution ratios to unity (1, 2, 4). Furthermore, the effect of metabolic inhibitors is not immediate and requires at least 5–10 min (with 0.1 mM 2,4-dinitrophenol) to manifest itself (2). Riggs et al. (6) associated this delayed action with the rate and extent of ion gradient dissipation occurring with the inhibition.

If the hypothesis is correct, and virtually all active amino acid transport in these cells is coupled solely to the ion gradients, then it might be expected that the primary cause of the inhibited accumulation would be an increased efflux of amino acid due to increased cell Na⁺ concentrations (9). On the other hand, unless there were a trans effect of increased cell Na⁺, metabolic inhibitors might be expected to have little effect on the amino acid influx, since extracellular cation concentrations would remain unaltered (9). In fact, almost all early studies in the Ehrlich ascites tumor cell showed that metabolic inhibition resulted in a decrease in amino acid influx with no change in the efflux (2–5, 10). These results are in direct contrast to those of Chez et al. (11) for amino acid transport in the rabbit ileum. These investigators found that metabolic inhibitors and ouabain acted primarily to increase the efflux of amino acid from the mucosal brush border with no change in the influx across this membrane. However, unidirectional flux studies in the Ehrlich cell were often conducted under circumstances which tended to underestimate the efflux (see the discussion of this problem in reference 10) and without measurements of cellular cation concentrations. Therefore, these results are difficult to interpret in light of the ion gradient hypothesis. The more recent experiments of Johnstone (12), using the metabolic inhibitor rotenone under conditions where more accurate effluxes could be measured, showed that this inhibitor not only reduced the initial uptake rate of glycine but also increased its efflux. Furthermore, these changes in uptake (but not efflux) were shown to be independent of intracellular cation concentration changes but coincident with decreased cell ATP levels (12).

The present investigations were undertaken to examine the effect of metabolic inhibition on the unidirectional fluxes of α-aminoisobutyric acid (AIB) under conditions in which cell ATP levels were reduced more rapidly than was possible with rotenone (12), and in which the possible concomitant effects of changes in cell Na⁺ and K⁺ concentrations were controlled by other means. Furthermore, since Jacquez (13) has convincingly demonstrated the importance of examining trans concentration effects on amino acid influx and efflux, the steady-state flux was examined in the presence and absence of metabolic inhibition.

The results demonstrate that the combination of 0.1 mM 2,4-dinitrophenol plus 10 mM 2-deoxy-D-glucose decreased cellular ATP levels to 0.10–0.15 mM in less than 5 min. Associated with this metabolic inhibition there was a 20%
decrease in the unidirectional AIB influx which could be dissociated from an increase in the cell Na⁺ concentration. Far more striking, there was a two- to four-fold increase in the unidirectional AIB efflux from inhibited cells which could be dissociated from changes in the intracellular Na⁺ concentration. In the steady state, metabolic inhibition increased the unidirectional flux by more than two-fold. These findings were interpreted to indicate that metabolic inhibition increased the amount of intracellular carrier available to mediate AIB efflux and thereby promoted accelerative exchange diffusion (14). However, these changes in amino acid transport did not appear to be related to changes in cell ATP levels produced by the metabolic inhibition or to changes in transmembrane cation concentration gradients.

**MATERIALS AND METHODS**

The general methods used in this series of experiments were the same as those described previously (15). The tumor was of the Ehrlich-Letré line (hyperdiploid), and was maintained in this laboratory by weekly intraperitoneal injection. Tumors were used for experiments 7–10 days after injection and were not grossly hemorrhagic. In most experiments, cells were suspended in a Krebs-Ringer phosphate buffer (referred to as Na-KRP) containing: 130 mM NaCl, 10 mM Na₂HPO₄/NaH₂PO₄, 8 mM KCl, 2.0 mM CaCl₂, and 1.5 mM MgSO₄. In some experiments either all or 100 mM of the NaCl was replaced by choline chloride. These solutions contained all other components in concentrations identical to those in Na-KRP except that sodium phosphates were replaced by the corresponding potassium salts. The latter two solutions are referred to as choline-KRP (130 mM choline chloride, 0 mM NaCl) and low NaKRP (90 mM choline chloride, 40 mM NaCl). All the buffer solutions contained 1% bovine serum albumin (Fraction V, Sigma Chemical Co., St. Louis, Mo.), and were adjusted to pH 7.4 with a small amount of 1 N HCl. Their osmolality was 290 mosmol 1⁻¹.

**Initial Uptake Studies**

Initial uptakes were measured by mixing a cell suspension with Na-KRP containing varying concentrations of ³H-AIB (New England Nuclear, Boston, Mass.; and ICN, Inc., Irvine, Calif.) in an incubation flask with attached side arm (described previously [15]). After the desired incubation period, the uptake was stopped by plunging the side arms containing the suspension into an ice-water-salt mixture at -1° to -3°C. The side arms were then centrifuged for 10 min at 4°C, 3,000 rpm, in the HL-8 head of a Sorvall RC3 centrifuge (DuPont Instruments, Sorvall Operations, Newtown, Conn.). Pellet and supernatant solutions were handled as described previously. Uptake rates are given in terms of micromoles/gram dry weight⁻¹ minute⁻¹.

**Efflux Studies**

Christensen and Handlogten (10) have pointed out that efflux experiments for a highly concentrative transport mechanism, such as that for AIB in Ehrlich cells, must be carefully controlled to prevent any significant underestimate of efflux rates due to re-entry of amino acid. One must ensure that extracellular amino acid concentrations at zero time are negligible and that large suspending volumes are used. With this in mind, we employed very dilute suspensions for our efflux studies. In all cases, the ratio of extracellular volume to pellet wet weight was at least 500:1 and usually ~750:1.

Suspensions of cells were incubated with various ³H-AIB concentrations for 30 min at 37°C in order to obtain a desired intracellular AIB concentration. The suspension was then centrifuged and washed twice with ice-cold Na-KRP in a volume ratio of 200:1.
(medium to packed cells). The cells were resuspended in low Na-KRP at 37°C at a volume ratio greater than 500:1. In order to obtain pellet samples from the necessarily large volumes of dilute suspension, we employed specially constructed centrifuge tubes. Lucite adaptors were designed to fit snugly in the bottoms of 50-ml polycarbonate centrifuge tubes. The lucite adaptors were center-drilled to accommodate specially cut 10 mm OD x 15-mm glass sample tubes which could be inserted and removed rapidly from the whole assembly. Before each experiment the necessary number of centrifuge tube assemblies containing prepared inner glass sample tubes were chilled to 0-4°C. At appropriate times, samples were taken from the dilute incubating suspension by quickly pouring ~30 ml of the suspension into the 50 ml centrifuge tube assemblies. The assemblies were then rapidly centrifuged at 3,000 rpm, 4°C, in a Sorvall Model RC-3 centrifuge fitted with an HL-8 swinging bucket head. The entire pellet was deposited in the small glass tube, which was removed for weight determinations, pellet lyophilization, and extraction. Samples of the supernatant fluid were saved for analysis.

The dilute suspensions of cells preloaded with AIB were shaken at 100 cycles s⁻¹ in a water bath at 37°C. Care was taken to ensure that the suspension depth allowed adequate aeration of the cells. Duplicate or triplicate samples were taken from control and inhibited suspensions as described about 5 min after beginning the final 37°C incubation. One or more sets of samples were taken at 5-min intervals thereafter. The efflux rate was calculated from the difference in cell AIB content (µmol g dry wt⁻¹) between successive samples divided by the 5-min efflux duration.

Although we will provide evidence below that the efflux process may not be described in terms of a first-order rate process, we have calculated efflux coefficients (kₑ, µmol g⁻¹ min⁻¹ mM⁻¹) from the efflux divided by the arithmetic mean of the intracellular AIB concentrations at the beginning and end of each efflux period. It should be recognized that these efflux coefficients are not constants but rather depend on the intracellular AIB concentration; as such they are used for purposes of comparison only. Similarly, also for comparison, we have computed influx coefficients (kᵢᵣ) for uptake studies. Again these coefficients are dependent on the extracellular AIB concentration, and they should not be equated with permeabilities.

Steady-State Fluxes

In order to obtain cells with steady-state distributions of AIB, we employed a method adapted from that of Jacquez (15). Cell suspensions were incubated with 10 mM ³H-AIB at 37°C for 30 min. The cells were then centrifuged and resuspended in 1 mM ³H-AIB made from the identical stock solution and therefore having an identical specific activity. The cells were incubated for 70 additional min in the 1 mM solution. The existence of a steady state in all cases was confirmed by taking duplicate samples at 60 and 70 min after beginning the incubation in 1 mM ³H-AIB. All experiments in which the intracellular concentration was not the same (±2%) at 60 and 70 min were discarded. At 70 min 4 ml of the suspension were pipetted into each of several incubation flasks. The side arms of the flasks contained 0.1 ml of a solution of ¹⁴C-AIB (New England Nuclear, sp act 5.1 mCi/mmol) which gave a final tracer concentration of 0.1 µCi ml⁻¹. The side arm solution was mixed with the cell suspensions and incubated for 50 s or 1 min at 37°C. The side arms were then handled as described for influx studies, and pellet extracts and supernates were counted for ³H and ¹⁴C-AIB activity. The intracellular AIB concentration in these samples provided another check for the existence of a true steady state. Since the system was in the steady state, the ¹⁴C-AIB uptake was equal to the AIB efflux over the same period, and thus both an efflux and an influx coefficient could be calculated and compared. Steady-state AIB concentration gradients were also measured in experiments
in which the initial incubation in 10 mM AIB was omitted, and the cells were incubated directly in 1 mM AIB for 90 min.

**Estimation of Trapped Volume**

In those cases where only $^3$H-AIB was used, extracellular volume in the pellet was estimated by adding 0.05 μCi $[^14]$C-sorbitol or $[^14]$C-mannitol (both from New England Nuclear) to each chilled cell suspension before centrifugation. After this addition, the side arms were mixed thoroughly and then centrifuged. The $^{14}$C activity in the pellet extract provided an accurate measure of the trapped volume. When both $^{14}$C and $^3$H-AIB were used, extracellular pellet volume was estimated by its predetermined relation to the pellet wet weight. This relationship is remarkably constant among cell suspensions, as shown previously (15).

**Determination of Cellular ATP Concentrations**

In those experiments in which cellular ATP concentrations were measured, the cell pellets were frozen and lyophilized immediately after centrifugation. They were then extracted with 3 ml of ice-cold 5% trichloroacetic acid for 1 h at 0°C. The resulting suspension was centrifuged at 0°C, and 0.25 ml of the supernate was neutralized with 2.25 ml of ice-cold 10 mM NaH$_2$PO$_4$ + 4 mM MgSO$_4$ (pH 7.4). These extracts were kept at 0°C for short periods of time or frozen when necessary. Pellet extracts were analyzed for ATP according to the method of Stanley and Williams (16).

**Biochemicals**

Most biochemicals were obtained in purest available form from Sigma Chemical Co. $\alpha$-Aminosobutyric acid (A grade) was obtained from Calbiochem (Los Angeles, Calif.).

**Statistics**

In each experiment, each analysis was made at least in duplicate and often in triplicate or quadruplicate. Results from individual experiments represent the mean of such determinations with the standard deviation appended when pertinent. In most cases the mean results of several experiments were averaged. Whenever possible experimental comparisons were made by using the same cell suspension in a single experiment. In these cases, differences in parameters are expressed as mean paired differences with the standard deviation appended and probability level determined from the Student t-test by comparison to zero.

Data from efflux experiments were fitted to various nonlinear equations by using an iterative Marquardt least-squares technique programmed as a part of the Statistical Analysis System (17) and run on the IBM 370-155 computer system of the Division of Biophysical Sciences, University of Alabama in Birmingham.

**RESULTS**

The results of these experiments will be considered in four parts: (a) the general effects of the metabolic inhibitors dinitrophenol and deoxyglucose on intracellular ATP and ion concentrations, cell water content, and cellular viability; (b) the effect of metabolic inhibition on AIB influx; (c) the effect of metabolic inhibition on AIB efflux; and (d) the effect of metabolic inhibition on the unidirectional steady-state AIB flux.

Table I shows the effects of the metabolic inhibitors 2,4-dinitrophenol (DNP) and 2-deoxyglucose (DOG) on intracellular ATP concentrations at 37°C in Na-
KRP medium. After 5 or 30 min of incubation in the absence of the metabolic inhibitors, the intracellular ATP concentration ranged from 1.8 to 3.0 mM in these and several additional experiments reported below. Incubation with 0.1 mM DNP alone for 5 min diminished intracellular ATP only to approximately 50% of control levels. It is reasonable to assume that during this period when further ATP synthesis is uncoupled from the electron transport chain, cell ATP stores are reduced by ongoing metabolism but not at a rate sufficient to deplete them completely in 5 min. (It should be noted that in all but one experiment the extracellular medium contained no glucose; the sole metabolic substrates were endogenous cell lipids and amino acids [18].) When 10 mM DOG was added to the extracellular medium along with 0.1 mM DNP, cell ATP fell to 0.1 mM within 5 min and remained at this level even after 30 min of incubation (Table I). A considerably smaller decrease in cellular ATP levels was noted when 5.5 mM glucose was added to the medium along with the DNP and DOG. The combination of 0.1 mM DNP and 10 mM DOG was used in all subsequent experiments to reduce cellular ATP levels rapidly with minimal cellular damage, and is referred to simply as metabolic inhibition in the subsequent text.

Fig. 1 illustrates the time course of intracellular concentrations of Na+ and K+ and the number of cells staining with trypan blue during incubation with 0.1 mM DNP + 10 mM DOG. After the addition of the metabolic inhibitors, cell Na+ rose and K+ fell until intracellular concentrations of the two cations were nearly identical at 90-120 min. In other experiments (e.g., Table II below), the addition of 2 mM AIB 5 min after the inhibitors caused the cation concentrations to change more rapidly in the initial phase, but the same final distribution was reached at 60-120 min.

Changes in cell volume were assessed by the ratio of grams intracellular water to grams dry weight, which we refer to in the text as the swelling index. In all of the initial uptake experiments reported below the control swelling index (after 6 min of incubation in Na-KRP) was 3.203 ± 0.237, and in the presence of 0.1 mM DNP + 10 mM DOG for the same time interval the swelling index was 3.092 ± 0.266 (mean paired difference, -0.111 ± 0.089, P < 0.02). For 90-100-min incubations (see steady-state experiments below), the control swelling index was

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**Table I**

**EFFECT OF METABOLIC INHIBITION ON INTRACELLULAR ATP LEVELS**

| Additions to the medium | Duration of incubation | [ATP] mM |
|-------------------------|------------------------|---------|
| Control                 | 5                      | 2.90±0.51 (3) |
| 0.1 mM DNP              | 5                      | 1.44±0.11 (3) |
| 0.1 mM DNP + 10 mM DOG | 5                      | 0.11±0.04 (3) |
| 0.1 mM DNP + 10 mM DOG + 5.5 mM Glucose | 5 | 1.02±0.15 (3) |
| Control                 | 30                     | 1.78±0.41 (2) |
| 0.1 mM DNP + 10 mM DOG | 30                     | 0.10±0.02 (2) |

The cells were incubated for the times indicated at 37°C in either normal Na-KRP (control), or in the same buffer containing the indicated additions. The numbers in parentheses indicate the number of individual experiments.
3.648 ± 0.312; the inhibited was 3.303 ± 0.280 (mean paired difference, −0.345 ± 0.122, \( P < 0.001 \)). Thus, metabolic inhibition was associated with a slight but significant decrease in cell volume with time of exposure in the range of 6–100 min. However, even at 100 min the cell volume was changed by less than 10% compared to controls.

One abiding concern with metabolic inhibition experiments is the possibility that one is dealing with cells which no longer have an effective permeability barrier. To counter this possibility, we measured the percentage of cells staining with trypan blue after varying times of incubation with the metabolic inhibitors. As shown in Fig. 1, although there was initially a slight rise in the percentage of cells stained with time, it plateaued within 30 min at relatively low levels. Even lower percentages of cells were found to stain with nigrosin according to the test for viability described by Kaltenback et al. (19). Thus the contribution of “dead” cells to the total population was slight even after 2 h of incubation with the inhibitors. Again, the same results were obtained with 2 mM AIB present in the extracellular medium.

**Effect of Metabolic Inhibition on the Initial Uptake Rate of AIB**

As shown in Table II, cells which were incubated for 5 min with 0.1 mM DNP +

![Graph showing intracellular concentrations of Na⁺ and K⁺, and cellular viability.](image)
10 mM DOG had ~20% lower AIB 1-min uptake rates than control cells. We wish to consider this observation, previously demonstrated by numerous investigators for other amino acids in the Ehrlich ascites cell (2-5, 9), in the context of the following questions. First, are the 1-min uptakes in fact representative of the effect of the inhibitors on the true unidirectional influx? Second, Table II shows that, concomitant with the reduced AIB uptakes, the cell Na⁺ concentration measured at the end of the 1-min uptake period was significantly increased and the K⁺ concentration was decreased. Is the reduced AIB uptake observed the consequence of reduced transmembrane Na⁺ and K⁺ concentration gradients, or of a reduced intracellular ATP concentration? Finally, might a generalized decrease in membrane permeability account for the reduced AIB uptake in the presence of metabolic inhibitors?

In measuring the rate of uptake of an amino acid such as AIB in the Ehrlich ascites tumor cell one is faced with the problem that amino acid accumulation in

| Sustaining medium | [Na⁺] | [K⁺] | [AIB] | [H⁺-AIB Uptake] | [Na⁺] | [K⁺] |
|-------------------|-------|------|-------|----------------|-------|------|
| Na-KRP 1.0-1.9    | 13.37±4.35 | 10.60±3.94 | 2.76±0.86 | 146.9±9.9 |
| Mean paired difference | (8; P<0.001) | (8; P<0.001) | (8; P<0.001) | (8; P<0.001) |
| Na-KRP 1.0-1.9    | 14.46±1.83 | 14.18±2.24 | 0.28±1.25 | 143.0±21.2 |
| Mean paired difference | (6; P>0.5) | (6; P<0.001) | (6; P<0.001) | (6; P<0.001) |
| Na-KRP 1.0-1.2    | 12.40±4.24 | 12.51±2.28 | 0.21±1.59 | 146.9±9.9 |
| Mean paired difference | (4; P>0.5) | (4; P<0.001) | (4; P<0.001) | (4; P<0.001) |
| Na-KRP + 10⁻⁵ M valinomycin | 12.40±4.24 | 12.51±2.28 | 0.21±1.59 | 146.9±9.9 |
| Mean paired difference | (4; P>0.5) | (4; P<0.001) | (4; P<0.001) | (4; P<0.001) |

The cells were first incubated in the solution indicated for 5 min at 37°C. After this incubation, AIB was added to the bathing solution at the concentration indicated and the cells were incubated for 1 additional min. The ion concentrations were those measured at the end of this 1-min influx period. The extracellular Na⁺ concentration averaged 40.7 meq/liter in low Na-KRP and 2.4 meq/liter in choline-KRP. The numbers in parentheses give the number of individual experiments and the significance of the mean paired difference compared to zero.
this system is so rapid that the intracellular AIB concentration (C_i) exceeds the extracellular concentration (C_e) within the 1-min uptake period. Thus, although the results with inhibitors were identical over 15- and 30-s uptake periods, the distribution ratio (C_i/C_e) was greater than unity within either incubation period. In order to circumvent this difficulty we adopted a method of extrapolation of initial uptake data described by Jacquez (13). In this method 30-, 60-, 90-, and 120-s uptakes of ^3H-AIB were measured concurrently on the same cell suspension with and without metabolic inhibitors. The resulting plot of cell AIB content vs. time was then fitted with a quadratic by the method of least squares. The result of one such experiment is shown in Fig. 2. This quadratic relating intracellular micromoles gram^-1 to time was solved for the slope at the origin. This slope was then taken to be the best approximation to the true initial rate of uptake. As seen in Fig. 2, this initial rate was decreased by metabolic inhibition. In five such experiments, the fitted initial uptake rate (µmoles g^-1 min^-1) from 1.5 mM AIB was 13.65 ± 1.76 under control conditions and 10.30 ± 1.00 with

1 It is reasonable by analogy with enzyme kinetics to assume that the time course of uptake might be initially S-shaped. In this fitting procedure, it was assumed that the initial convex upward portion was of sufficiently short duration so as not to complicate the analysis.
metabolic inhibition \((P < 0.001)\). The same results were obtained when the data were fitted with a logarithmic function. Thus, comparison with Table II shows that exit of accumulated amino acid was not a significant factor in attenuating l-min flux measurements, and these uptake rates are a reliable index of the initial unidirectional influx. This conclusion is supported by the nearly linear uptake observed in the 1st min (Fig. 2) and the relatively low efflux coefficients reported below.

In order to test the effect of changes in intracellular cation composition independently of metabolic inhibition, we also examined the effect of ouabain on the initial AIB uptake rate. As shown in Table II (second group), a 5-min incubation period with 0.1 mM ouabain followed by a 1-min AIB uptake period, also in the presence of ouabain, increased cell \(\text{Na}^+\) and decreased cell \(\text{K}^+\) to almost exactly the same extent observed with the metabolic inhibitors (Table II). However, in spite of the reduction in both \(\text{Na}^+\) and \(\text{K}^+\) transmembrane concentration gradients, the 1-min uptake of AIB was unaffected. These results suggested that changes in factors other than intracellular cation concentrations were responsible for the decrease in the initial AIB uptake rate. The most likely factor was the decrease in intracellular ATP produced by the metabolic inhibition. However, when the cells were first incubated with \(10^{-5}\) M valinomycin, the intracellular ATP concentration was reduced from 2.66 \pm 0.60 to 0.15 \pm 0.05 mM \((P < 0.001, \text{four experiments})\), cell \(\text{Na}^+\) rose by 28 mM, and cell \(\text{K}^+\) fell by 24 mM, but the 1-min uptake of AIB was unaltered (Table II, third group). In other words, valinomycin had effects on intracellular ATP and cation concentrations which were comparable to those observed with DNP + DOG, but this agent did not affect the initial AIB influx.

We also considered the possibility that the intracellular \(\text{Na}^+\) concentration determined from total cell extracts did not reflect the actual cytoplasmic \(\text{Na}^+\) concentration due to nuclear sequestration of \(\text{Na}^+\) as demonstrated in the Ehrlich ascites tumor cell by Pietrzyk and Heinz (20). Since these authors showed that the cytoplasmic \(\text{Na}^+\) concentration was identical to that computed from the overall cellular \(\text{Na}^+\) concentration when the extracellular \(\text{Na}^+\) concentration was below 40–45 mM (20), we examined the effect of metabolic inhibition on AIB uptake from low Na-KRP. As shown in the fourth group of results in Table II, metabolic inhibition in low Na-KRP had little effect on the intracellular \(\text{Na}^+\) concentration but reduced the 1-min uptake of AIB by 20%, the same reduction as observed in regular Na-KRP. Thus, a difference in their effects on the true cytoplasmic \(\text{Na}^+\) concentration does not seem to be a likely explanation for the differences in the effects of metabolic inhibition, ouabain and valinomycin on AIB uptake.

Another consideration which is often raised in connection with metabolic inhibition experiments is the possibility that the inhibitors directly or indirectly change the permeability properties of the membrane. Table II also presents the effects of metabolic inhibitors on AIB uptake from choline-KRP medium. In these experiments the metabolic inhibitors had no effect on the 1-min AIB uptake. We interpret this finding to indicate that the inhibitors did not act on a mode of AIB uptake other than the \(\text{Na}^+\)-dependent one. Finally, 0.1 mM DNP + 10 mM DOG had no effect on the 1-min uptake of four nonelectrolytes
examined in separate single experiments: thiourea, glycerol, nicotinamide, and erythritol. Thus, the results demonstrate that metabolic inhibition reduces the initial influx of AIB, and that this reduction is not solely the consequence of altered intracellular Na⁺, K⁺, or ATP concentrations or of changes in membrane permeability unrelated to Na⁺-dependent AIB entry.

**Efflux Studies**

As discussed under Materials and Methods, the primary problem in obtaining reliable efflux data is to prevent significant re-entry of effluxed amino acid. We attempted to avert this problem by the use of very large suspending volumes. Second, we ascertained that the efflux coefficient measured in the periods 0-5 min, 5-10 min, and 10-15 min did not differ, and in most experiments the reported efflux rate was taken as the average efflux in two or three periods. Fig. 3 presents the results of 36 such efflux experiments. In each experiment, efflux was measured in both the presence and the absence of metabolic inhibitors. All such efflux experiments were conducted in low Na-KRP medium. The low extracellular Na⁺ concentration (35-45 meq/liter) prevented any significant rise in the intracellular Na⁺ concentration of inhibited cells during the course of the efflux incubation. Due to the technical difficulties attendant to such experiments, there is scatter in the data. Nevertheless, in every experiment the efflux was significantly higher for inhibited than for control cells.

As can be readily seen Fig. 3, the data points show saturation of the efflux at high intracellular AIB concentrations. The data were fit by a nonlinear fitting program to a form of the Michaelis-Menten equation:

\[ J_e = \frac{J_e^{\text{max}} C_i}{K_m + C_i} \]  

where: \( J_e \) is the efflux (\( \mu \text{mol g}^{-1} \text{ min}^{-1} \)), \( K_m \) is the apparent Michaelis-Menten constant (mM), and \( C_i \) is the mean intracellular AIB concentration. The results of fitting to this equation gave high correlations to the data for both control (0.96) and inhibited cells (0.98). Residuals of the plotting were significantly lower for the Michaelis-Menten fit than for linear, logarithmic, exponential, or power function fitting. Table III gives the best-fitting parameters, \( J_e^{\text{max}} \) and \( K_m \), with their asymptotic standard errors. The parameter analysis shows that the \( J_e^{\text{max}} \) was significantly higher for inhibited compared to control cells, whereas there was no significant difference between \( K_m \) values in the two groups. However, the standard errors for the \( K_m \) parameter were too high to make this point conclusive.

We also attempted to fit both control and inhibitor data sets to a modified Michaelis-Menten equation:

\[ J_e = \frac{J_e^{\text{max}} C_i}{K_m + C_i} + K_D C_i \]  

where: \( K_D \) is an efflux constant representing a nonsaturable, presumably diffusional exit route. Iterative nonlinear fitting of this equation converged rapidly, but the 95% confidence limits for the three parameters \( J_e^{\text{max}} \), \( K_m \), and \( K_D \) were too large to give any reason to select Eq. (2).
Since the efflux incubations were conducted in low Na-KRP media in which the extracellular Na\(^+\) concentration (35–45 meq/liter) approximated the initial intracellular Na\(^+\) concentration, there was little difference in the intracellular Na\(^+\) concentration between control and inhibited cells. To ascertain that an increase in cell Na\(^+\) was not responsible for the increase in AIB efflux seen with metabolic inhibition, the paired experiments shown in Fig. 3 were divided into two groups: one group in which there was less than a 5 meq/liter difference in the cell Na\(^+\) concentration between control and inhibited groups, and one group in which this difference was greater than 10 meq/liter. The mean paired differences in the efflux coefficient and effluxes for both groups and the entire paired
set are given in Table IV. Note that although there was no significant difference in the average change in the efflux, the difference in the efflux coefficient was greater for cells having the greater change in intracellular Na\(^+\) concentration. However, the changes in both the efflux coefficient and efflux were significant with \(P < 0.001\) for each group of paired experiments.

Four additional experiments were conducted in which effluxes into low Na-KRP or into low Na-KRP containing 0.1 mM ouabain were compared. In each experiment the intracellular AIB concentration was 8–10 mM. The efflux was 0.647 ± 0.063 into low Na-KRP and was unaltered by 5–25 min of incubation with ouabain (mean paired difference, −0.045 ± 0.136).

**Steady-State Experiments**

Cells incubated to the steady state in 1.0–1.5 mM AIB developed steady-state intracellular AIB concentrations of ~45 mM in control cells and ~19 mM in cells treated with either DNP + DOG or ouabain. The 1-min uptakes of \(^{14}\)C-AIB at the steady state are given in Table V. In control cells the 1-min uptake of AIB at
the steady state was significantly lower than the initial 1-min uptake (see Table II). However, in the presence of DNP + DOG the 1-min steady-state AIB uptake was increased by more than twofold (Table V) compared to control uptakes in the steady-state or initial uptakes (Table II). Metabolic inhibition also increased the AIB efflux coefficient.

As shown in the bottom half of Table V, treatment with ouabain also increased both the steady-state 1-min AIB uptake and the efflux coefficient, but not to the same extent as metabolic inhibition. Thus, even though intracellular cation concentrations were altered equally by DNP + DOG and ouabain, the former inhibition produced a significantly greater effect on the steady-state AIB flux.

In addition to the increase in the steady-state flux, and influx and efflux coefficients seen in the presence of metabolic inhibition (Table V), yet another aspect of these steady-state studies is particularly interesting. In the presence of metabolic inhibition over a 100-min period, the cells are still able to develop an

| Table V | EFFECT OF METABOLIC INHIBITION AND OUABAIN ON STEADY-STATE 14C-AIB UPTAKE AND RATE COEFFICIENTS |
|---------|-------------------------------------------------------------------------------------------------|
| β14C-AIB uptake | kν | kξ | Na+ | K+ |
| μmol g⁻¹ min⁻¹ | μmol g⁻¹ min⁻¹ | mmol L⁻¹ | meq L⁻¹ | meq L⁻¹ |
| Control | 11.82±2.36 | 8.22±1.98 | 0.35±0.06 | 60±8 | 138±11 |
| 0.1 mM DNP + 10 mM DOG | 25.00±8.99 | 15.05±7.03 | 1.28±0.38 | 154±5 | 91±17 |
| Mean paired difference | 13.20±7.33 | 6.82±5.93 | 1.05±0.35 | 63±6 | -46±21 |
| (6; P<0.01) | (6; P<0.05) | (6; P<0.001) | (6; P<0.001) | (6; P<0.001) |
| Control | 7.19±1.22 | 5.08±0.66 | 0.16±0.02 | 39±11 | 132±13 |
| 0.1 mM ouabain | 12.52±4.67 | 6.40±1.55 | 0.70±0.13 | 101±16 | 110±20 |
| Mean paired difference | 5.33±3.03 | 1.32±0.93 | 0.54±0.13 | 63±18 | -23±15 |
| (4; P>0.10) | (4; P>0.10) | (4; P<0.01) | (4; P<0.01) | (4; P<0.01) |

Cell suspensions were incubated in 10 mM 3H-AIB for 30 min at 37°C, and then for 60 min in 1.0-1.5 mM 3H-AIB, in either Na-KRP (control) or in Na-KRP containing the indicated inhibitors. After the second incubation the presence of a true steady state was checked as described in Materials and Methods. 14C-AIB was added to the extracellular solution in a trace amount and a 1-min uptake was measured. The average intracellular AIB concentration was 45.0±5.1 mM in controls, 19.4±3.7 in DNP + DOG-treated cells, and 19.0±5.1 in ouabain-treated cells.

AIB distribution ratio (Cν/Cθ) of 13.8 (average extracellular concentration for the experiments in Table V, 1.4; intracellular concentration, 19.4). Thus, there was net accumulation of AIB against a considerable concentration gradient in spite of the facts that cell ATP levels were reduced to about 0.1 mM and that the intracellular Na⁺ concentration approached that of the suspending medium.

In order to confirm that the cellular AIB concentration was actually monotonically rising during a period when the intracellular ATP concentration was already reduced and the intracellular Na⁺ concentration was already high, we repeated these experiments in the following way. Cells were first incubated for 30 min at 37°C in Na-KRP containing DNP + DOG. After this incubation, intracellular Na⁺ had risen to 85 mM. 3H-AIB was then added at 2 mM to the medium and the time course of AIB uptake was measured. As seen in the upper portion of Fig. 4, AIB was accumulated to a distribution ratio of greater than 8 in the presence of the metabolic inhibitors. AIB accumulation was also observed in low Na-KRP in the presence of metabolic inhibitors, but in this case the distribution ratio achieved was only 2.7.
DISCUSSION

As shown in Fig. 1, metabolic inhibition with 0.1 mM 2,4-dinitrophenol in combination with 10 mM 2-deoxy-d-glucose results in a loss of cell K⁺ and a gain of Na⁺ until the cellular Na⁺ concentration approaches the K⁺ concentration after ~100 min. Yet there is no evidence of any substantial cell disruption, as assessed by the number of cells staining with trypan blue or nigrosin. The dissipation of the transmembrane cation concentration gradients may be attrib-

![Graphical representation of the data showing the time course of ³H-AIB uptake from an extracellular concentration of 2 mM. Top, Cells were first incubated for 30 min in either Na-KRP (control) or Na-KRP + 0.1 mM DNP + 10 mM DOG at 37°C, then 2 mM ³H-AIB was added at time = 0 and the distribution ratio was followed for 90 min. Bottom, Procedures were the same as described above except that low Na-KRP was substituted for Na-KRP.](image)

uted to the rapid diminution of cellular ATP concentrations produced by DNP + DOG (Table I). 2,4-Dinitrophenol is a classical uncoupler of oxidative phosphorylation (21); however, at a concentration of 0.1 mM, the cell ATP concentration was only reduced to one-half its normal level after 5 min. The addition of 10 mM 2-deoxy-d-glucose to the 0.1 mM DNP in the medium reduced the cell ATP level to 0.1-0.15 mM within 5 min. The effect of DOG in reducing ATP levels is attributed to the phosphorylation of the sugar analog without subsequent metabolic breakdown (22, 23).

In the presence of 0.1 mM DNP + 10 mM DOG, the 1-min uptake of AIB
from extracellular concentrations of 1.0–1.9 mM was reduced by 20% (Table II). Extrapolation of 30–120-s uptake data by the method of Jacquez (13) indicates that the 1-min uptakes were reliable indices of the true unidirectional influxes (with C_i = 0, Fig. 2). The decreased AIB uptake in the presence of inhibitors does not appear to be due to a gross increase or decrease in nonmediated transport on the basis of three findings: (a) the uptakes of four nonelectrolytes whose transport is not mediated are unaltered by metabolic inhibition; (b) the Na⁺-independent moiety of AIB accumulation is not changed by metabolic inhibition (Table II). Although a proportional change in Na⁺-independent uptake cannot be ruled out, it cannot be the sole basis for the change in the AIB uptake observed in the presence of Na⁺; (c) the AIB efflux coefficient (computed from Table III), although increased by metabolic inhibition, nevertheless remains far less than the influx coefficient (computed from Table II) at concentrations of 1–1.9 mM.

Metabolic inhibition over the 5-min incubation period also increases cell Na⁺ and decreases cell K⁺ in addition to decreasing the AIB initial influx (Table II). However, the reduced influx of AIB does not appear to be due to changes in intracellular cation concentrations, because 0.1 mM ouabain, which produces almost identical changes, has no effect on AIB uptake (Table II). Although ouabain has been demonstrated to be an inhibitor of amino acid transport in many systems, including the Ehrlich cell (9, 24), Bittner and Heinz (24) demonstrated that at lower concentrations and short incubation times ouabain had no effect on amino acid accumulation. Similarly, for amino acid transport in the small intestine, Chez et al. (11) demonstrated that ouabain reduced mucosa to serosa amino acid flux only if added to the serosal side of the epithelium and well in advance of the measurement of amino acid flux.

A potential complication in the analysis of the above data is the fact that cytoplasmic concentrations of Na⁺ are lower than estimated from the whole cell average due to nuclear sequestration (20). On the basis of this finding, Heinz (25) has suggested that redistribution of Na⁺ between the nucleus and cytoplasm during metabolic inhibition could potentially decrease the transmembrane Na⁺ concentration gradient to a much greater extent than would be calculated from total cellular Na⁺ concentrations. Thus the true cytoplasmic Na⁺ concentration might be quite different in metabolically inhibited and ouabain-treated cells (Table II). However, when additional experiments were conducted with metabolic inhibitors in low Na-KRP (extracellular Na⁺ concentration 40 mM), the results in Table II show that DNP + DOG again produced a 20% decrease in the initial AIB uptake with no change in the total cellular Na⁺ concentration. Since Pietrzyk and Heinz (20, 26) have demonstrated that at extracellular Na⁺ concentrations of 40–45 mM there is no nuclear Na⁺ sequestration and the total cellular Na⁺ concentration is equivalent to the cytoplasmic concentration, these results demonstrate that the inhibition of the initial AIB uptake rate occurs independently of changes in the cytoplasmic Na⁺ concentration.

After observing this inhibition of the initial uptake rate, which was independent of any change in the transmembrane Na⁺ concentration gradient, we at first felt that the effect must be directly related to the observed decrease in the intracellular ATP concentration produced by the metabolic inhibition. How-
ever, this hypothesis was contradicted by other experiments with the ionophore valinomycin. As shown in Table II, 10^{-5} M valinomycin elevated the intracellular Na^+ concentration and decreased the intracellular ATP concentration to nearly the same extent as DNP + DOG, but valinomycin produced no change in the initial AIB uptake rate. This finding supports the lack of coupling between cellular ATP hydrolysis and AIB influx observed by Geck et al. (27). The results with valinomycin are also discussed below in relation to the transmembrane voltage as a driving force for AIB accumulation.

The results of the efflux studies shown in Fig. 3 demonstrate two interesting properties of AIB efflux. First, the efflux in both the presence and the absence of metabolic inhibition exhibited saturation at high intracellular amino acid concentrations. Second, metabolic inhibition with DNP + DOG increased the AIB efflux by two to four times the control. The saturation of amino acid efflux in agreement with other recent efflux measurements for AIB and N-methyl-AIB using large volume dilutions (10, 13), and shows that the efflux cannot be described in terms of a single exponential. In contrast, other investigators (2, 12) have found no change in the half-time for glycine efflux with 10-fold changes in intracellular concentrations. Other than the fact that these measurements were made with glycine, the reason for the discrepancy is not clear. For purposes of comparison with other efflux data given in terms of first-order rate constants, the approximate "rate constant" was calculated at two intracellular concentrations from the kinetic constants given in Table III. At an intracellular AIB concentration of 5 mM the "rate constant" would be 0.014 min^{-1} for control cells and 0.029 min^{-1} for inhibited cells; at a concentration of 40 mM, the rate constants would be 0.005 min^{-1} (control) and 0.0084 min^{-1} (inhibited). Since the efflux studies were analyzed in terms of Eq. (1), no term was included for nonmediated exit; however, the data were compatible with a diffusional permeability on the order of 0.05 \mu mol g^{-1} min^{-1} mM^{-1} as determined by Jacquez (13) and also observed in this laboratory for influx experiments (28).

As shown in Fig. 3, when cells which had been loaded with AIB were subsequently incubated with DNP + DOG for 5-15 min, there was a striking increase in the AIB efflux. In terms of the kinetic constants, inhibition significantly increased the $J_{e}^{max}$ and decreased the $K_{m}$, but the latter effect was not statistically significant. Although Jacquez (13) has stressed the problems in obtaining accurate unidirectional efflux data, and although there was considerable scatter in the experimental points, the results indicate that reliable trends in effluxes can be established with large numbers of experiments. However, the kinetic constants calculated from nonlinear fitting of the efflux data have considerable standard errors, especially the value of $K_{m}$. Therefore, caution must be taken in the interpretation of differences between kinetic constants even with the large number of experiments conducted in these studies.

Since the efflux experiments shown in Fig. 3 were conducted in low Na-KRP, the cytoplasmic Na^+ concentration could be determined from the total cellular Na^+ concentration (cf. above, and references 20 and 26). The intracellular Na^+ concentration rose little with metabolic inhibition (Table IV), due also to the low extracellular Na^+ concentration in these experiments. Thus the observed increase in AIB efflux with metabolic inhibition could not be attributed to any
change in the transmembrane Na⁺ concentration gradient. The possibility that an increase in intracellular Na⁺ without metabolic inhibition could also increase the efflux was not specifically tested. However, in a smaller number of experiments (not shown), in which efflux into normal Na-KRP was measured, metabolic inhibition resulted in an increase in the intracellular Na⁺ concentration and an even greater increase in the efflux than seen in Fig. 3.

Most early studies of amino acid efflux showed no change in the rate with metabolic inhibition (2, 3, 5); however, in each of these studies the number of experiments was very few, and large suspending volumes were not used. Johnstone (12) has also suggested that the length of exposure to the metabolic inhibitors might not have been sufficient to produce an effect on the efflux. Using long periods of exposure to rotenone, Johnstone (12) reported a significant increase in the efflux of glycine which she attributed to the diminution of cell ATP levels. In similar experiments in which the efflux of AIB into large suspending volumes was measured in the presence and absence of DNP, Christensen and Handlogten (10) could demonstrate no effect of metabolic inhibition; however, the effect was examined only in a single experiment, and after an unstated period of incubation with DNP.

In view of the above effects of metabolic inhibition on influxes (Table II) and effluxes (Tables III and IV), the reduced accumulation of AIB during metabolic inhibition may be attributed both to a decrease in the initial uptake and an increase in the efflux, although the latter appears to be the greater effect. This conclusion was also reached by Eddy (8) for Ehrlich ascites tumor cells, and by Chez et al. (11) for the rabbit ileum in which metabolic inhibition produced an increase in cell to mucosal solution amino acid efflux. The fact that both influx and efflux of the amino acid are affected by the metabolic inhibition is not an unexpected result. As stressed by Christensen (29), if one considers that reversal of the uptake step may mediate efflux of the amino acid, then uncoupling of an active transport system could be expected to involve changes in both the influx and efflux. However, due to the potential importance of trans effects of amino acids on influx and efflux, the change in AIB accumulation must also be examined in terms of the unidirectional flux coefficients in the steady state.

Under control conditions the ¹⁴C-AIB uptake in the steady state was slightly less than that observed when the intracellular AIB concentration was zero (Table V); that is, there was an apparent trans inhibition of AIB influx by intracellular AIB. On the other hand, the AIB efflux under control conditions at an intracellular concentration of 45.6 mM is predicted to be 1.46 µmol g⁻¹ min⁻¹ from the kinetic constants given in Table III, whereas the observed steady-state flux was eight times this value (Table V). This apparent trans inhibition of AIB influx by intracellular AIB and trans stimulation of efflux by extracellular AIB has also been observed by Jacquez (13, 30) under similar control conditions.

With metabolic inhibition the steady-state AIB distribution ratio decreased to ~40% of the control value, and the ¹⁴C-AIB flux more than doubled to 25 µmol g⁻¹ min⁻¹ (Table V). Thus, metabolic inhibition was associated with an apparent conversion of the trans inhibition of influx observed under control conditions to a trans stimulation. Similarly, the predicted unidirectional efflux, calculated from the kinetic parameters in Table III, was 2.19 µmol g⁻¹ min⁻¹ or less than
one-tenth the steady-state flux, indicating substantial trans stimulation of efflux during metabolic inhibition. These changes in influx and efflux kinetics in the steady state could well have been the result of increased cell Na\(^+\) and/or decreased K\(^+\) concentrations produced by metabolic inhibition. Because of the necessity of incubating to the steady state over a 90-min period, these changes in cellular cation concentrations (Table V) could not be prevented. However, additional experiments in Table V show that when the intracellular Na\(^+\) concentration was increased by 0.1 mM ouabain, the steady-state AIB flux was also somewhat greater than the initial influx, but this increase was much less than observed with metabolic inhibition and was not statistically significant.

The results presented to this point demonstrate that metabolic inhibition produces a decrease in the initial AIB influx and an increase in AIB efflux which occur independently of changes in cell Na\(^+\). In the steady state, metabolic inhibition produces an increase in the unidirectional fluxes. Under control conditions, AIB influx is inhibited by the presence of intracellular AIB (cf. above and references 13 and 30), whereas the trans inhibition is converted to a trans stimulation by metabolic inhibitors. Viewed for convenience in terms of a carrier mechanism, the action of metabolic inhibitors may involve a decrease in the coupling of an energy source which operates normally to reduce efflux of AIB via the carrier by returning it to the exterior of the membrane in an altered form (see reference 14). Thus, increased availability of the carrier for efflux during metabolic inhibition could increase the influx of AIB by accelerative exchange diffusion (14), whereas, in the absence of appreciable intracellular AIB, return of the free carrier and thus the initial influx of AIB would be reduced by metabolic inhibition. Although the metabolic inhibitors reduce the extent of AIB accumulation, the results of Table V and Fig. 4 also illustrate that these cells were able to develop steady-state AIB distribution ratios of 9–14 during the continuous presence of metabolic inhibitors even though intracellular ATP levels were in the range 0.10–0.15 mM and both Na\(^+\) and K\(^+\) concentration gradients were substantially reduced. Other investigators have also reported the development and maintenance of amino acid distribution ratios significantly greater than 1.0 in the presence of metabolic inhibitors (1, 2, 4–6). These results present two important and possibly distinct questions: first, if AIB influx is reduced independently of changes in either cell Na\(^+\) or ATP levels, what is the mechanism by which metabolic inhibition exerts this effect? Second, what is the energy source for the steady state in the presence of metabolic inhibitors? Both of these questions are considered below with respect to other possible energy sources.

The most often cited possibility for an additional driving force for amino acid accumulation is the transmembrane voltage (31–33). If Na\(^+\) and amino acid are cotransported electrogenically, then the transmembrane voltage would be expected to contribute to the energy available to amino acid accumulation. In support of this hypothesis, Gibb and Eddy (31) and Reid et al. (32) observed that amino acid uptake was stimulated by valinomycin. They attributed this increase to a hyperpolarization of the transmembrane voltage produced by a presumed increase in K\(^+\) conductance in the presence of valinomycin (31, 32). These results are supported by our present observations with valinomycin (Table II) which
showed no change in AIB uptake in the presence of $10^{-5}$ M valinomycin despite reduced cell ATP and increased cell Na$^+$ concentrations. This result might be expected if the hyperpolarization of the membrane produced by valinomycin offset the reduced transmembrane Na$^+$ concentration gradient. If one uses the same argument, the decreased AIB uptake observed with metabolic inhibition in the absence of any change in the intracellular Na$^+$ concentration (Table II, low Na-KRP experiments) could be explained if DNP + DOG resulted in a depolarization of the transmembrane voltage. For example, as suggested by Gibb and Eddy (31) and Heinz et al. (33), the decrease in amino acid uptake with metabolic inhibition might occur as a result of inhibition of an electrogenic cation pump mechanism. However, our results with ouabain do not support this hypothesis. Although ouabain was present at a concentration in excess of that required to inhibit active Na$^+$ pumping (0.1 mM), neither the initial uptake nor the unidirectional efflux of AIB was affected by ouabain. Similarly, Schafir and Heinz (15) found that even in the presence of ouabain the energy requirement for active AIB accumulation exceeded the energy available in the combined Na$^+$ and K$^+$ electrochemical potential gradients. Thus if the metabolic inhibitors acted to depolarize the transmembrane voltage, the origin of this voltage must be other than electrogenic Na$^+$ pumping.

Any complete assessment of the role of the transmembrane voltage in amino acid accumulation depends upon both direct measurements of this voltage and the demonstration of the electrogenic nature of the amino acid accumulation throughout the time course of uptake. Unfortunately, direct measurements of the transmembrane voltage by microelectrodes remain suspect due to the rapid decay of the observed voltages and the likelihood of high-conductance electrical leaks induced by the procedure. The Cl$^-$ equilibrium potential is also not a good index of the transmembrane voltage since it is probable that the half-time for Cl$^-$ equilibration is much longer than previously assumed (33). More recently several investigators have employed lipid soluble cations such as tetraphenylphosphonium (33), dibenzylidimethylammonium (34), and the cyanine dye 3,3-dipropylthiodicarbocyanine (DiS-C$_2$-[5]; see reference 35) to estimate transmembrane voltages, and Laris et al. (35) using DiS-C$_2$-[5] have demonstrated an apparent depolarization of this voltage during the uptake of amino acids, supporting the electrogenic nature of the transport. However, measurements of the membrane voltage using these agents must be regarded with caution. All of these probe molecules must certainly distribute across the membranes of cellular organelles in accord with their transmembrane voltages. Accordingly, an apparent depolarization of the membrane voltage with metabolic inhibition, as observed by Laris et al. (35) with rotenone, could be attributed to changes in the mitochondrial membrane voltage. Certainly the demonstration of the nuclear sequestration of Na$^+$ and Cl$^-$ should introduce an additional note of caution into the interpretation of the distribution of these agents. Thus none of the available methods of approximating the transmembrane voltage appear to be completely reliable, especially when changes in mitochondrial membrane voltages may occur concomitantly with changes in the plasma membrane voltage.

Nevertheless it is instructive to consider the magnitude of the transmembrane voltage which would be required to account for the steady-state AIB accumula-
tions developed during metabolic inhibition (Fig. 4). If an Na⁺ to AIB stoichiometry of 1:1 is assumed, then by following the analysis of Jacquez and Schafer (36; Eq. [6]), the required transmembrane voltage to maintain the steady-state AIB concentration gradient would be −53 mV in Na-KRP and −30 mV in low Na-KRP. These voltages are in the range of maximum transmembrane voltages assessed by the cationic probes under control conditions (33, 35); however, it must be recognized that if a decrease in the transmembrane voltage is postulated to account for the effect of metabolic inhibition on AIB influxes and effluxes, then the transmembrane voltage must be even greater than −53 mV in the control cells. Furthermore, the required transmembrane voltages above were calculated on the basis of the assumption of a 100% efficiency of coupling between Na⁺ and amino acid entry. Finally, the assumption of a 1:1 stoichiometry between Na⁺ and AIB may be incorrect, especially when the intracellular AIB concentration becomes appreciable. Heinz and Geck (37) have approximated an overall efficiency of coupling of about 0.6, and Christensen et al. (38) have shown that Ehrlich cells demonstrate net AIB uptake with little accompanying Na⁺ uptake if they are previously incubated with AIB. Thus although transmembrane voltages may be sufficiently large to explain the maintenance of an AIB gradient during metabolic inhibition, such a coupling appears to require inordinately high efficiencies of coupling between amino acid and Na⁺ fluxes whereas this coupling may in fact be negligible during net accumulation (38).

Another potential energy source which might be coupled to the active accumulation of amino acids is either a transmembrane or, as suggested by Christensen (39), an intramembrane H⁺ electrochemical potential gradient. However, since Poole et al. (40) observed that DNP abolishes the transmembrane H⁺ gradient in Ehrlich ascites cells, one would suspect that any intramembrane H⁺ gradient would also be dissipated. Therefore, the persistence of such H⁺ electrochemical potential gradients as an explanation for the maintenance of an AIB distribution ratio greater than 1.0 during metabolic inhibition seems unlikely.

Although AIB is not found to exchange readily with other amino acids (15), the effect of the endogenous amino acid pool on the present results must be considered. Unfortunately, even with extensive cold shock treatment and incubation with other amino acids, the endogenous amino acid pool in these cells can only be reduced to about 40% of its initial concentration (15, 38). Therefore, the effects of DNP + DOG on the initial influx of AIB could involve a change in the trans effect of the endogenous amino acid pool on the influx process, although this possibility seems unlikely. However, since both the efflux and steady-state incubation experiments involved prior incubation of the cells with AIB in large suspending volumes and at least one change of suspending medium, transmembrane concentration gradients of any exchangeable endogenous amino acids should have been dissipated in these experiments.

A final possible source for the energy required to maintain the observed steady-state distribution ratio of AIB (Cᵣ/Cₒ = 13.7) in the presence of metabolic inhibitors is the small residual ATP concentration (0.1–0.15 mM). In spite of prolonged treatment with metabolic inhibitors, this steady-state ATP concentration could not be reduced further even though the assay method could have reliably detected cell concentrations as low as 0.01 mM. Lin and Johnstone (41)
have shown that in the mouse pancreas, in which amino acid transport exhibits a
marked dependence on cell ATP levels, half-maximal rates of glycine uptake
were produced at a cell ATP concentration of 0.5 mM. They stated further that
"measurable transport activity would be obtained at concentrations as low as 0.1
mM." The possibility that such residual activity could develop and maintain the
presently observed steady-state gradients needs further examination.

In summary, the effect of metabolic inhibition on AIB accumulation in
Ehrlich ascites tumor cells may be dissociated from changes in cell Na\(^+\) and K\(^+\)
concentrations, and the effect of metabolic inhibition on the initial influx of AIB
may also be dissociated from the decrease in intracellular ATP. Furthermore,
even during metabolic inhibition, AIB was accumulated to a significant trans-
membrane concentration gradient. It is possible that the effects of the metabolic
inhibitors may be attributed to a reduction of the transmembrane voltage; how-
ever, the steady-state AIB accumulation during metabolic inhibition would
require the persistence of a considerable voltage. Thus, other energy sources
should be considered in attempting to explain the effects of metabolic inhibition.
It is possible that the cell membrane may contain enzyme systems capable of a
direct coupling of substrate oxidation to the active transport of amino acids.
Although there are no data currently available to support such a hypothesis,
both this energetic coupling as well as the transmembrane voltage should be
considered in further investigations.

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