The accumulation of amino acids by mouse ascites-tumour cells

Dependence on but lack of equilibrium with the sodium-ion electrochemical gradient

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1. The fluorescent dye 3,3'-dipropylloxadicarbocyanine was used to show that the tumour cells absorbed 2-aminoisobutyrate, glycine, L-leucine and L-isoleucine and certain other amino acids electrogenically. The K_m values with respect to amino acid concentration ($[A]_o$), obtained from the fluorescence assays, varied through the above series from 0.8 to 26 mM, with V_{max} fairly constant. 2. Similar K_m values described the uptake of the ^{14}C -labelled amino acids in five instances where this was measured. 3. Each amino acid lowered the membrane potential (E) by 10–20 mV when its cellular concentration ($[A]_i$) had reached a steady value and $[A]_o$ was 10 mM. In these experiments energy metabolism was maintained by glycolysis, 2,4-dinitrophenol was present and cellular respiration was inhibited. The corresponding net flow of amino acid through the Na^+ symport was deduced by making use of the fact that the depolarization an amino acid initially caused was roughly proportional to the net influx of amino acid itself. 4. The steady-state depolarization was attributed to the presence of a leak pathway for the amino acid with a rate coefficient P_A . As assayed in the absence of Na^+ , P_A was about 5-fold larger for isoleucine than for glycine. 5. Direct estimates of V_{max}/P_A were similar to those inferred from the extent of depolarization in the steady state and $[A]_i$. 6. A mathematical model was used to predict $[A]_i/[A]_o$ in terms of the measured values of $[Na]_o$, $[Na]_i$, E , K_m and V_{max}/P_A . The predicted and observed values agreed fairly well when $[A]_o$ was 1 mM or 10 mM. 7. $[A]_i/[A]_o$ varied from about 2.5 for 10 mM-isoleucine to 30 for 1 mM-2-aminoisobutyrate when $\Delta\mu_{Na}$, expressed as a ratio, was ostensibly in the range 19–43. 8. The concentration of 2-aminoisobutyrate from a 0.1 mM solution in the presence or absence of ouabain was consistent with the model, whereas the concentration of isoleucine from a 0.1 mM solution exceeded the predicted values 2–5-fold. 9. The tumour cells concentrated 2-amino-bicyclo[2,2,1]heptane-2-carboxylic acid by a non-electrogenic mechanism, with which isoleucine may also interact.

That mammalian cells and tissue concentrate certain amino acids at the expense of the Na^+ electrochemical gradient acting across the plasma-membrane now seems very likely (Johnstone, 1979a; Ullrich, 1979), but whether this is the only mechanism involved is not known (Christensen, 1979; Griffith *et al.*, 1979). In the present work we have attempted to assess the extent to which mouse ascites-tumour cells use the Na^+ gradient to concentrate glycine, 2-aminoisobutyrate, L-leucine and L-isoleucine. When these cells are incubated with a specific amino acid, at 37°C in an appropriate Ringer solution, the ratio of the cellular amino acid

concentration to the corresponding extracellular concentration ($[A]_i/[A]_o$) reaches a steady value after about 30 min. Qualitatively, a plausible explanation is that one or more pumps, represented by the Na^+ -dependent A system of amino acid transport and the Na^+ -independent L system (Christensen, 1979), concentrate the amino acids to an extent governed by their leakage out of the tumour cells through a separate route (Heinz & Mariani, 1957; Morville *et al.*, 1973; Jacquez, 1975; Philo & Eddy, 1978b; Eddy *et al.*, 1979, 1980). A net influx of Na^+ through the Na^+ symport would therefore be expected to occur in the steady state of amino acid

distribution unless other factors intervened. This current of Na^+ would tend to depolarize the tumour cells (Philo & Eddy, 1978a), as we found in preliminary work with L-leucine or glycine in which a carbocyanine dye was used to assay the membrane potential (Eddy *et al.*, 1979, 1980). We have now studied this phenomenon further by using several other amino acids, which differ in their apparent affinity for the Na^+ symport as compared with the putative leak pathway. The work bears on a claim that loading mouse ascites-tumour cells with certain amino acids can uncouple their subsequent uptake into the cells from the uptake of Na^+ itself (Garcia-Sancho *et al.*, 1977).

Materials and methods

These followed the general procedures described in Morville *et al.* (1973) and Philo & Eddy (1978a). The mouse ascites-tumour cells were collected, washed and incubated for 30 min at 37°C in the standard Ringer solution. This contained 155 mM- Na^+ , 8 mM- K^+ , 131 mM- Cl^- , 16 mM-orthophosphate and 1.2 mM- MgSO_4 . The cell preparation was then collected and washed again with a similar Ringer solution and stored at 0°C for up to 40 min.

Fluorescence changes caused by amino acid addition

A portion (about 13 mg dry wt.) of the above preparation was incubated at 37°C in the standard Ringer solution (40 ml) containing 60 μM -2,4-dinitrophenol, oligomycin (0.24 μg), antimycin (40 μg) and glucose (80 mg). At intervals samples (4 ml) of the cell suspension were added to the stirred cuvette of an EEL-Corning 244 fluorimeter (Corning Medical, Halstead, Essex, U.K.). The temperature was maintained at 37°C . The membrane potential was assayed by recording at 620 nm the fluorescence excited at 577 nm and 579 nm in the presence of 3,3'-dipropylxadicarbocyanine iodide (Philo & Eddy, 1978a). The fluorescence decreased to a steady value, usually reached within 2 min of adding the dye. Addition of an amino acid that enters with Na^+ , dissolved in the standard Ringer solution, caused the fluorescence to increase to a maximum within a further 2 min (Fig. 1). The fluorescence change (ΔF) was extrapolated to the time the amino acid was added (Philo & Eddy, 1978a). An extensive series of controls showed that, whereas addition of up to about 0.4 ml of the standard Ringer solution had a negligible effect on the fluorescence of the system, larger volumes lowered the fluorescence by 1–2 (arbitrary units). The effects of relatively large amounts of leucine or isoleucine, determination of which necessitated the addition of up to 0.8 ml of a 0.15 M solution, were computed relative to the fluorescence of appropriate controls. The validity of this procedure was checked by determining ΔF due

to the presence of leucine in a further series of assays in which the tumour cells were added to a cuvette already containing a solution of the dye and the amino acid. The two procedures led to very similar estimates of the K_m and ΔF_{max} for leucine.

Amino acid accumulation and fluorescence assays in the steady state

The tumour cells (13 mg dry wt.) suspended in the standard Ringer solution (40 ml) containing glucose and the metabolic inhibitors were incubated in the above manner either in the presence of 1 mM- or 10 mM-amino acid or in its absence. After 30, 60 or 90 min the carbocyanine dye (1 $\mu\text{g}/\text{ml}$ of cell suspension) was added and the fluorescence of a portion of the suspension was assayed 4–5 min later (Philo & Eddy, 1978a). The magnitude of the fluorescence signal assayed under these standard conditions was converted into an equivalent membrane potential by means of the calibration curve illustrated in Fig. 2 of Philo & Eddy (1978a). The respective cellular and extracellular contents of water, Na^+ , K^+ and ^{14}C maintained in the steady state of amino acid distribution were assayed by collecting the tumour cells from the larger portion of the cell suspension that was not assayed in the fluorimeter (Philo & Eddy, 1978a,b). Manipulation of the cell suspension was conducted in polypropylene centrifuge tubes of 15 ml capacity (MSE Scientific Instruments, Crawley, Sussex, U.K.). No significant change in the pH of the cell suspension occurred during 60 min fermentation of glucose under these conditions.

Determination of P_A

The procedure was based on that used by Morville *et al.* (1973), but the tumour cells were not first hypo-osmotically stressed. The cell preparations that had been incubated in the standard Ringer solution for 30 min were collected and washed twice with a K^+ /Ringer solution containing 163 mM- K^+ and no Na^+ . The cells (1 ml) were collected and suspended in a similar solution (25 ml) for 15 min at 37°C . They were again collected and resuspended (1 ml packed cells) in the K^+ /Ringer solution (3 ml). A portion (2 ml) of this suspension was mixed with K^+ /Ringer solution (8 ml) containing the selected amino acid (10–80 mM) labelled with ^{14}C (1–4 μCi). The suspension was kept at 37°C , usually for up to 20 min, samples (1 ml) being withdrawn at intervals and mixed with K^+ /Ringer solution (9 ml) at 0°C . The tumour cells in each sample were collected by centrifugation and mixed with 10 mM- HNO_3 (1.5 ml). The ^{14}C content of a portion (1 ml) of the extract was assayed and, after correction for the small amount of extracellular ^{14}C present, led to an estimate of the cellular ^{14}C content. The water content of the cells was determined in

parallel incubations lacking ^{14}C , portions (2 ml) of the cell suspension being taken at 1, 7 and 15 min. These were put into weighed centrifuge tubes, mixed with [^3H]inulin (0.5 μCi) and the cellular and extracellular H_2O contents were determined (Morville *et al.*, 1973). The magnitude of P_A was determined by plotting $\ln([A]_i - [A]_o)$ against the incubation time to obtain a first-order rate coefficient, which was multiplied by an appropriate factor representing the mean cellular water content and dry weight.

The efflux rate coefficient was also determined in a number of instances. The tumour cells were incubated in turn in the standard Ringer solution for 20 min and in the K^+ /Ringer solution for 25 min, and then, after being collected and washed in a similar solution, the cell preparation (25 mg dry wt.) was loaded with, for instance, isoleucine. To do this it was first kept at 37°C for 15 min in K^+ /Ringer solution (10 ml) containing 10 mM-L- ^{14}C isoleucine (1–2 μCi). The tumour cells were then collected and washed at 0°C with K^+ /Ringer (2 $\times$ 10 ml) and stored (10 mg dry wt./ml) in a similar solution at 0°C . To start the assay, several portions (0.25 ml) of the above cell suspension were mixed with K^+ /Ringer solution (9.75 ml) at 37°C and incubated for different selected intervals of up to 15 min. The cell suspension (10 ml) was cooled to 0°C , and the tumour cells were collected in each instance and their ^{14}C contents determined (Morville *et al.*, 1973). Other incubations were used to determine the cellular water content at the start and at the end of the period in which the efflux of the amino acid was observed.

Chromatography

We studied the chromatographic behaviour of the ^{14}C -labelled material extracted from cell preparations (10 mg dry wt.) incubated for 30 min at 37°C in the standard Ringer solution containing glucose, the metabolic inhibitors (see above) and 0.1 mM- ^{14}C isoleucine (1.25 μCi). The incubated tumour cells were mixed with aq. 70% (v/v) ethanol (2.5 ml) for 2 h at 22°C . The cellular debris was separated by centrifugation at 1500 g for 10 min and washed with a further portion of aqueous ethanol (2.5 ml). The ethanolic extracts were combined and a portion (50 μl) was assayed for ^{14}C . The cellular debris was suspended in water (1 ml) and likewise assayed. The ethanolic extracts were evaporated to dryness and dissolved in water (0.5 ml), a portion (20 μl) of which was assayed for ^{14}C . Other portions (100 μl) were subjected to chromatographic analysis. This was done on paper (Whatman no. 1) either (1) by development for 8 h with butan-1-ol/acetic acid/water (50:11:25, by vol.) or (2) by development for 18 h with phenol/water (4:1, w/v). The dried paper strips were scanned in a Nuclear-

Chicago Actigraph III (Nuclear-Chicago Corp., Searle Analytic, Des Plaines, IL, U.S.A.) to locate areas associated with ^{14}C . Only one component was detected, and this had the same R_F value as authentic [^{14}C]isoleucine, namely 0.56–0.60 in the former solvent and 0.81–0.83 in the latter solvent. In other instances t.l.c. of the extract (10 μl) was performed on Eastman Chromagram cellulose sheets (type 13254; Kodak, Kirkby, Liverpool, U.K.), with butan-1-ol/pyridine/water (1:1:1, by vol.) as solvent. The developed sheets were clipped to X-ray film (Eastman Kodak Co., Rochester, NY, U.S.A.) for 1 week. One component was detected in the cell extract, and this migrated with [^{14}C]isoleucine, R_F 0.66.

Chemicals

3,3'-Dipropylxadicarbocyanine iodide was the preparation described in Philo & Eddy (1978a). ^3H -labelled inulin and the labelled amino acids were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Oligomycin and antimycin were obtained from Sigma Chemical Co., Kingston upon Thames, Surrey, U.K. We thank Dr. H. N. Christensen for generous gifts of 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid containing 96% of the *b* isomer and 4% of the *a* isomer, including two preparations labelled with ^{14}C (0.4 mCi/mmol).

Mathematical methods

These were based on those of Philo & Eddy (1978a,b). Let the amino acid A, Na^+ and the carrier C form the ternary complex ANaC, exhibiting the dissociation constant K . This complex bears a net charge of +1, whereas the unloaded carrier bears no net charge. The translocation of ANaC and of C are the rate-limiting steps. Let the inward and outward translocation of C be characterized by the rate coefficient k' , that of ANaC being xk' in the absence of an electric field. The membrane potential (E) might affect carrier kinetics simply by changing the activation energy determining the transition rate constants for charged complexes (Jacquez, 1972; Hall *et al.*, 1973; Geck & Heinz, 1976; Heinz & Geck, 1978). According to this transition-rate treatment, the inward and outward translocation of ANaC can be written as $\theta xk'$ and $\theta^{-1}xk'$, respectively, where $\theta = e^{-zEF/RT}$. The resultant equations are somewhat simpler than those used by Morville *et al.* (1973) and Philo & Eddy (1978a) to describe the effect of E on the velocity of amino acid uptake in terms of Goldman's constant-field equation, although we stress that both treatments are physically valid. We then rewrite eqn. (6) of Philo & Eddy (1978a) in the above terminology, obtaining an expression for the net rate of amino acid uptake

in terms of the carrier parameters, $[A]_o$, $[A]_i$, $[Na]_o$, $[Na]_i$, E and the total carrier concentration $[C]_t$. When $[A]_i = 0$ we find that the K_m with respect to $[A]_o$ is given by:

$$K_m = \frac{2K}{[Na]_o(x\theta + 1)} \quad (1)$$

The corresponding V_{max} is:

$$V_{max} = \frac{[C]_t k' x \theta}{(x\theta + 1)} \quad (2)$$

The expression for the net rate of amino acid uptake through the Na^+ symport is then equated with the rate of leakage of the amino acid, $P_A[A]_i - [A]_o$, in order to define $[A]_i$ in the steady state of amino acid distribution [eqn. (9) of Philo & Eddy (1978a)]. The resultant quadratic expression is:

$$a[A]_i^2 + b[A]_i + c = 0 \quad (3)$$

where:

$$a = \frac{[Na]_i x}{\theta} + [Na]_i + \frac{[Na]_o[A]_o[Na]_i x}{K\theta} + \frac{[Na]_o[A]_o[Na]_i x \theta}{K} \quad (4)$$

$$b = \frac{[C]_t k' x [Na]_i}{P_A \theta} + [Na]_o[A]_o x \theta + [Na]_o[A]_o + 2K - \frac{[Na]_i[A]_o x}{\theta} - [Na]_i[A]_o - \frac{[A]_o^2[Na]_o[Na]_i x}{K\theta} - \frac{[A]_o^2[Na]_o[Na]_i x \theta}{K} \quad (5)$$

$$c = - \left(\frac{[C]_t k' x \theta [Na]_o[A]_o}{P_A} + [A]_o^2[Na]_o x \theta + [Na]_o[A]_o^2 + 2K[A]_o \right) \quad (6)$$

The predicted value of $[A]_i$ in terms of the other variables is therefore the positive real root of eqn. (3) computed as $[(b^2 - 4ac)^{1/2} - b]/2a$. (1) θ is defined by E , the membrane potential. (2) The measured value of K_m for a given amino acid defines K provided that x is known (see below). E typically averaged 45 mV in these assays. (3) The measured value of V_{max} defines $[C]_t k'$ provided that x is known. (4) The parameters a , b and c defined by eqns. (4)–(6) were then computed, for a given value of x . (5) We observed from the above relations that the influx of amino acid was independent of $[A]_i$ when $x = \theta$. Moreover there is some experimental support for the view that, in the steady state of glycine distribution, glycine influx is independent of $[glycine]_i$ (Johnstone, 1979b). As θ is typically about 3, we computed $[A]_i$ at three selected values of x , namely 1, as used by Philo & Eddy (1978b), 3 or 10.

Results

Membrane depolarization caused by different amino acids

Preliminary work had indicated that the addition of 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid, subsequently referred to as the norbornane amino acid, caused no increase in the fluorescence of the suspension of the mouse ascites-tumour cells under the standard conditions used to assay the membrane potential by means of the carbocyanine dye (Eddy *et al.*, 1979). The compound is a specific substrate of the L system of amino acid transport (Christensen, 1979). In the present work a series of eight such

Fig. 1. Increase in fluorescence intensity (depolarization) after the addition of glycine, L-isoleucine or the norbornane amino acid to a cell suspension in the standard Ringer solution containing glucose, the mitochondrial inhibitors and the carbocyanine dye at 37°C

The fluorescence was assayed as described in the Materials and methods section. Traces (a) and (b) show respectively how the fluorescence intensity varied immediately after the addition of 0.1 ml of 0.4 M-glycine or 0.2 ml of 0.2 M-isoleucine to the tumour-cell suspension (4 ml) at 37°C. Trace (c) shows what happened when 0.4 ml of 200 mM-2-aminobicyclo[2,2,1]heptane-2-carboxylic acid was added. Trace (d) is a control in which 0.4 ml of the standard Ringer solution was added.

assays gave results like that illustrated in trace (c) of Fig. 1 when the amino acid was added to a final concentration of about 0.5 mM, 1 mM, 5 mM or 20 mM. No significant change in fluorescence occurred relative to the fluorescence of controls to which the standard Ringer solution was added (Fig. 1, trace d; see the Materials and methods section). The subsequent addition of 1 mM-glycine (Philo & Eddy, 1978a) or 10 mM-L-tryptophan (Eddy *et al.*, 1979) nevertheless caused a definite increase in fluorescence (results not shown), so that the tumour cells evidently remained intact in the presence of the norbornane derivative. We conclude that the norbornane amino acid was not absorbed by an electrogenic mechanism. It was nevertheless concentrated about 4.5-fold during 60 min in each of two assays made with a 1 mM solution in the standard Ringer solution containing glucose.

The above observations contrast with the behaviour of L-isoleucine and glycine (Fig. 1, traces b and a). Philo & Eddy (1978a) found that the increase of fluorescence intensity (ΔF) caused by the addition of a given concentration ($[A]_0$) of glycine or methionine roughly fitted a rectangular-hyperbolic relationship, with an apparent K_m rather similar to that found by assaying the initial rate (v) of amino acid uptake as a function of $[A]_0$ under similar but not identical conditions. Laris *et al.* (1976) made similar observations with glycine. Table 1 sum-

marizes a series of further measurements of the concentration-dependence of the fluorescence change caused by glycine, L-leucine, 2-aminoisobutyrate, L-isoleucine and L-methionine. The maximum response to L-isoleucine required relatively large concentrations of the amino acid (Fig. 2). Morville *et al.* (1973) measured the rate of uptake of L-leucine or L-phenylalanine as a function of $[A]_0$ and found that K_m was 8.7 ± 0.9 mM (S.E.M.; $n = 3$) and 8.4 mM respectively. The fluorescence assays corresponded to 5.3 ± 0.8 mM (Table 1) and 4.1 ± 0.8 mM (S.E.M.; $n = 3$). The mean of two similar estimates of the K_m for 2-aminoisobutyrate uptake was 1.25 mM, whereas the fluorescence assays gave 0.84 mM (Table 1). The comparison can be extended to the values of V_{max} , representing the amino acid influx on the one hand, and the maximum value of ΔF on the other hand (ΔF_{max}). (1) Thus V_{max} for 2-aminoisobutyrate was about 43 nmol/min per mg, whereas the corresponding values for glycine (Philo & Eddy, 1978b), leucine, methionine and phenylalanine (Morville *et al.*, 1973) were in the range 32–46 nmol/min per mg. (2) ΔF_{max} for phenylalanine was 19 ± 2 (S.E.M.; $n = 3$) arbitrary units and therefore similar to the values for the other amino acids (Table 1), despite the large differences in K_m shown through the series. Inui & Christensen (1966) also demonstrated that various neutral amino acids were absorbed, through the A transport system of the mouse ascites-tumour cells, at about the same maximum rate, although the corresponding parameter K_m varied from 0.3 mM to 40 mM through the amino acid series.

Relation between ΔF and the Na^+ -dependent flow of amino acid (v)

Because the fluorescence assays and the corresponding tracer assays gave similar K_m values, in each instance, for glycine, leucine, methionine, phenylalanine and 2-aminoisobutyrate, we suggest that amino acid uptake occurred mainly through the Na^+ symport and that:

$$v/V_{max} \simeq \Delta F/\Delta F_{max}. \tag{7}$$

Table 1. K_m and ΔF_{max} values and their standard errors deduced for five amino acids by expressing the fluorescence increase (ΔF) as a hyperbolic function of the amino acid concentration ($[A]_0$) added in assays like those shown in Fig. 2

The system was assayed on n occasions.

Amino acid	n	K_m (mM)	ΔF_{max} (arbitrary units)
Glycine	3	0.83 ± 0.07	27.3 ± 0.6
Methionine	4	0.69 ± 0.10	21.6 ± 1.3
Leucine	13	5.3 ± 0.8	22.3 ± 1.1
2-Aminoisobutyrate	10	0.84 ± 0.08	24.5 ± 0.9
Isoleucine	5	25.7 ± 1.8	20.1 ± 0.8

Fig. 2. Increase in fluorescence intensity caused by selected concentrations of L-isoleucine

The assays were performed in the standard Ringer solution containing glucose and the metabolic inhibitors at 37°C. The increase in fluorescence intensity was assayed as illustrated in traces (a) and (b) of Fig. 1, and the magnitude of the increase was extrapolated to the time the addition was made. Observations were made at five selected amino acid concentrations, on at least five occasions, and on 19 occasions at 9 mM-isoleucine. The mean values $\pm$ S.E.M. are represented.

both factors being $[A]_o/([A]_o + K_m)$ for a given amino acid. The magnitude of ΔF thus provides a measure of v , the Na^+ current through the symport coupled to the initial uptake of the amino acid. As ΔF reflects the relative magnitudes of the ionic permeabilities of the system (Philo & Eddy, 1978b), it seemed possible that the magnitude of ΔF in the steady state of amino acid absorption could be used to estimate the net flow of amino acid through the symport under these conditions. This flow equals L , where:

$$L = P_A([A]_i - [A]_o) \quad (8)$$

and P_A is the first-order rate coefficient describing the Na^+ -independent leak pathway (Philo & Eddy, 1978b). Hence, taking eqn. (7) as an equality:

$$P_A \cdot ([A]_i - [A]_o) = \frac{\Delta F}{\Delta F_{\max}} \cdot V_{\max} \quad (9)$$

for the steady state. We use this relationship below to estimate $V_{\max}/P_A$ in terms of known values of $[A]_i$, $[A]_o$ and $\Delta F_{\max}$, making the crucial further assumption that eqn. (7) describes both the initial effect of the amino acid on the system and the corresponding steady-state behaviour.

Magnitude of ΔF in the steady state

Table 2 shows that, when the mouse ascites-tumour cells were incubated with a 10 mM solution of one of seven amino acids, for a period of 30–90 min, the

Table 2. Increase in fluorescence intensity (depolarization), relative to controls lacking the amino acid, caused by prolonged incubation of the tumour-cell preparation with a 10 mM solution of a selected amino acid

The controls were incubated in parallel with the cell suspension in the standard Ringer solution containing the amino acid at 37°C. The preparations also contained glucose and the metabolic inhibitors (see the Materials and methods section). A portion of each suspension was mixed with the carbocyanine dye at 30, 60 or 90 min and the membrane potential was assayed 4–5 min later. The fluorescence increase (ΔF) was computed as (fluorescence of the sample containing the amino acid) – (fluorescence of the control incubated for the same time interval). As no significant change in ΔF with time was observed, all the measurements with a given amino acid were pooled to give mean values $\pm$ S.E.M. for the stated number of observations (n).

	n	ΔF (arbitrary units)
Glycine	35	7.67 ± 0.51
Leucine	23	7.07 ± 0.46
2-Aminoisobutyrate	12	4.58 ± 0.39
Norbornane amino acid	15	0.2 ± 0.70
Isoleucine	18	6.25 ± 0.57
Alanine	9	5.16 ± 0.42
Methionine	6	8.75 ± 0.51
Tryptophan	12	8.35 ± 0.65

fluorescence of the system in the standard assay was consistently larger than was exhibited by controls lacking the amino acid. The magnitude of the increase with a given amino acid was roughly the same whether the assays were done at 30, 60 or 90 min. When 1 mM-glycine, -leucine or -methionine (Philo & Eddy, 1978a) was present ΔF approached zero, being 0.7 ± 1.8 (S.E.M.; $n = 5$) for glycine and 1.5 ± 2.1 (S.E.M.; $n = 11$) for leucine. The norbornane amino acid, alone among the amino acids, influenced neither the steady-state membrane potential significantly (Table 2) nor its initial value (Fig. 1).

A 10 mM solution of L-tryptophan was found to increase the fluorescence of a solution of the carbocyanine dye by 2.36 ± 0.30 (S.E.M.; $n = 7$) units both (1) in the absence of the tumour cells and (2) to a similar extent in their presence when the Ringer solution contained K^+ instead of Na^+ . The estimate of ΔF for tryptophan given in Table 2 was obtained by subtracting 2.36 from the observed value found when the tumour cells were incubated with tryptophan. None of the other amino acids produced such artifacts.

Comparison of the initial and steady-state values of ΔF

The addition of 10 mM-glycine, -leucine, -methionine or -2-aminobutyrate led to an increase in fluorescence at least 3 times that observed in the steady state of amino acid distribution, normally reached in about 30 min. The explanation appears to be that in the steady state the continuing influx of the amino acid with Na^+ was neutralized to a greater or smaller extent by the simultaneous efflux of Na^+ through the symport. Our model assumes that this Na^+ was accompanied by an equivalent amount of amino acid, an assumption consistent with the Na^+ -dependence of methionine (Morville *et al.*, 1973) and glycine efflux (Johnstone, 1978). It is noteworthy that the addition of 10 mM-isoleucine increased the fluorescence of the cell suspension in the standard assay by 5.4 ± 0.6 (S.E.M.; $n = 19$), whereas the corresponding value given in Table 2 for the steady state is roughly the same, namely 6.25 ± 0.57 (S.E.M.; $n = 18$). In this instance the initial current of Na^+ through the amino acid carrier was therefore probably maintained in the steady state.

Associated changes in the cellular contents of Na^+ , K^+ and H_2O

In agreement with earlier work (Christensen & Riggs, 1952; Jacquez, 1975), the extensive accumulation of glycine lowered cellular $[\text{K}^+]$ ($P < 0.001$) and caused the tumour cells to swell ($P < 0.001$) (series 5, Table 3). Unlike Christensen & Riggs (1952), we observed no significant increase in

Table 3. *Difference in cellular water content, $[Na]_i$, $[K]_i$ and membrane potential (E) maintained in the steady-state distribution of a selected amino acid as a result of increasing its extracellular concentration from 1 mM to 10 mM*

Except for the norbornane derivative, which required 60 min, the amino acid distribution appeared to reach a steady state in about 30 min, in the standard Ringer solution containing glucose and the metabolic inhibitors. ΔH_2O ($\pm$ S.E.M.) was computed as the increase in cellular water/g dry wt. of tumour cells in a 10 mM as compared with a 1 mM solution of the chosen amino acid. $\Delta[Na]_i$, the increase in $[Na]_i$ was similarly computed. $-\Delta[K]_i$ is the mean fall in $[K]_i$ due to $[A]_o$ being raised, whereas ΔE is the corresponding depolarization (compare Table 2). The stated additions of sucrose were intended to limit water uptake occurring in the presence of the amino acid. In series 5, the above parameters for 10 mM-glycine are compared with those for 1 mM-glycine (control). For series 6, sucrose was added with 10 mM-glycine but not to the control, whereas for series 7 a control with sucrose was compared with one without sucrose.

Series	Amino acid	No. of observations	ΔH_2O (ml of cell water/ g dry wt.)	$\Delta[Na]_i$	$-\Delta[K]_i$	ΔE (mV)
1	Leucine	4	0.51 ± 0.21	-4.8 ± 5.9	15.4 ± 16.3	13.8 ± 1.5
2	Isoleucine	3	0.23 ± 0.15	0.0 ± 2.3	19.3 ± 3.3	8.2 ± 3.9
3	2-Aminoisobutyrate	3	0.52 ± 0.50	3.7 ± 9.0	31.9 ± 9.2	11.9 ± 0.4
4	Norbornane amino acid	2	-0.04 ± 0.23	5.4 ± 6.6	-9.80 ± 12.4	7.8 ± 5.0
5	Glycine	8	1.11 ± 0.12	7.0 ± 5.2	37.2 ± 5.5	20.9 ± 3.5
6	Glycine + 80 mM-sucrose	4	0.04 ± 0.13	11.6 ± 8.1	2.8 ± 12.0	27.9 ± 2.0
7	Control + 30 mM-sucrose	4	-0.4 ± 0.24	10.5 ± 8.6	-31.7 ± 11.2	1.8 ± 3.4

cellular $[Na^+]$ under these conditions. Similar behaviour of K^+ ($P < 0.05$) was observed with isoleucine or 2-aminobutyrate, but probably not with the norbornane derivative.

Despite the scatter of the results, it might be argued on the basis of the observations in series 1–5 of Table 3 that the depletion of cellular $[K^+]$ and the lowering of the membrane potential were due to the uptake of water rather than the uptake of the amino acid. We therefore restricted the swelling of the tumour cells in the presence of 10 mM-glycine by the addition of 80 mM-sucrose. The controls in this instance contained 1 mM-glycine (series 6) and included some (series 7) in which the presence of sucrose was shown not to affect the membrane potential significantly. The addition of 80 mM-sucrose during the fluorescence assay likewise had no immediate effect on the fluorescence of the cell suspension. It will be observed that the presence of 80 mM-sucrose virtually stopped the extra uptake of water caused by 10 mM-glycine, but without significantly changing the depolarization it produced. The apparent depolarization was evidently not simply due to an effect of cellular swelling on the signal produced by the fluorescent probe. No significant depletion of the cellular K^+ content occurred in the presence of sucrose and 10 mM-glycine (series 6 versus series 5). This behaviour appears to be related to the fact that raising the osmotic pressure, by the addition of 30 mM-sucrose, to the controls containing 1 mM-glycine tended to increase cellular $[K^+]$, again without affecting the apparent membrane potential. Clearly, the depolarization caused by the amino acid was not

necessarily accompanied by a decrease in cellular $[K^+]$. The maintenance of $[K]_i$ in series 6, despite the fall in membrane potential, requires either that the passive permeability to K^+ was lowered in the presence of sucrose and glycine or that the uptake of K^+ accelerated.

Two distinct estimates of V_{max}/P_A for glycine, 2-aminoisobutyrate, leucine and isoleucine

(1) V_{max}/P_A was first estimated on the basis of eqn. (9) from a knowledge of the magnitudes of ΔF (Table 2) and $[A]_i$ maintained when the system reached a steady state in the presence of 10 mM-2-aminoisobutyrate, -L-leucine or -L-isoleucine (Table 5). $[A]_i/[A]_o$ for 10 mM-glycine was 10.60 ± 0.49 (S.E.M.; $n = 21$). ΔF_{max} was assumed to be 23, the weighted mean of the values shown in Table 1.

(2) V_{max}/P_A was also estimated directly from the separate parameters. P_A was determined as the first-order rate constant describing the equilibration of the added amino acid with the tumour cells suspended in the K^+ /Ringer solution (see the Materials and methods section). The observations were usually made within 15 min of adding the amino acid to the cells (Fig. 3). By this time the cellular concentration of L-leucine was typically about 37 mM when the extracellular concentration was about 44 mM. After a further 15 min the corresponding values, in this same experiment, were 41 mM and 44 mM, in general agreement with the notion that the cellular and extracellular concentrations were tending to the same value. Fig. 3 shows that the relevant value of P_A for leucine was

Fig. 3. Determination of P_A , the leak coefficient defining the Na^+ -independent component of amino acid uptake. The cellular uptake of leucine (●) from a 45 mM solution in the K^+ /Ringer solution, or of 2-aminoisobutyrate (○) from a 55 mM solution, was assayed at various intervals (see the Materials and methods section). The above amino acid concentrations are roughly equal to the value of $([A]_i - [A]_o)$ established by the tumour cells when $[A]_o$ was 10 mM (Table 5). The amino acid uptake, when represented as a first-order process in which $\ln \{ [A]_0 / ([A]_0 - [A]_i) \}$ increased linearly with time, defined a rate constant (min^{-1}) that was multiplied by the cellular water content to obtain P_A in units of l/min per kg dry wt. (Table 5).

several times larger than that for 2-aminoisobutyrate. The amino acid concentrations studied in this connection were similar to the respective magnitude of $([A]_i - [A]_o)$ maintained by the tumour cells in the steady state reached in the standard Ringer solution (Table 5). The same value of $V_{\max}$, of 40 nmol/min per mg dry wt. (see above) was used for all four amino acids.

Table 4 shows that the two estimates of $V_{\max}/P_A$, obtained in the above manner for a specific amino acid, were sufficiently similar to encourage the view that eqn. (9) roughly describes the flow of electric charge through the system in the steady state.

In one set of assays with the Ehrlich tumour cells Jacquez (1975) found that $V_{\max}$ for 2-aminoisobutyrate was 0.041 mmol/min per g cellular dry wt. and P_A was 0.056 ml/min per g. Thus $V_{\max}/P_A$ was 0.73 mol/l, a value similar to the corresponding

Table 4. Comparison of the respective values of $V_{\max}/P_A$ deduced (1) from ΔF and $[A]_i$ by applying eqn. (9) to the steady state of amino acid distribution and (2) from the tracer fluxes observed in initial-rate measurements

Amino acid	$V_{\max}/P_A$ (mol/l)	
	Fluorescence assays	Rate measurements
Glycine	0.29	0.38
2-Aminoisobutyrate	0.28	0.59
Leucine	0.14	0.10
Isoleucine	0.05	0.08

estimate of 0.59 for our tumour cell line given in Table 4.

Effect of K^+

The initial rate of uptake of 10 mM-glycine or 10 mM-leucine from a Ringer solution lacking Na^+ and containing 20 mM- K^+ and 140 mM-choline chloride corresponded to P_A values of 0.284 ± 0.058 (S.E.M.; $n = 3$) and 0.578 ± 0.068 (S.E.M.; $n = 4$) respectively, values similar to those observed in the K^+ /Ringer solution.

Net efflux of isoleucine

Two similar estimates of the rate of efflux of isoleucine in the presence of the K^+ /Ringer solution corresponded to a mean value of P_A of 0.4941/min per kg, which is not significantly different from the influx coefficient of 0.525 ± 0.043 (S.E.M.; $n = 3$) determined under similar conditions. We conclude that the exchange of extracellular isoleucine with endogenous amino acids was not an important factor in these estimates.

The efflux coefficient for isoleucine was also determined with cell preparations that had been suspended in 163 mM- Na^+ without K^+ , in the presence of glucose, the metabolic inhibitors and 0.3 mM-ouabain for 50 min to dissipate the Na^+ gradient. The mean of two similar estimates of P_A , assayed in the above circumstances, was 0.451/min per kg, with the cellular isoleucine concentration initially about 1 mM. Thus isoleucine efflux was not apparently stimulated by the presence of Na^+ under these conditions, a circumstance consistent with the K_m of 26 mM (Table 1) found in the standard Ringer solution.

Amino acid metabolism

It seemed possible that leucine or isoleucine might be metabolized under the conditions in which the amino acid distribution was determined, although previous work in these laboratories had shown that glycine and 2-aminoisobutyrate were not significantly metabolized during such assays (Morville

et al., 1973). The tumour cells (10 mg dry wt.) were accordingly incubated in the standard Ringer solution (40 ml) containing glucose, antimycin, oligomycin and 2,4-dinitrophenol (Philo & Eddy, 1978a) and 0.1 mM- ^{14}C isoleucine (1.25 μCi). Similar preparations also contained 0.3 mM-ouabain and no added K^+ . No significant change in the ^{14}C content of the cell suspension occurred during 30 min incubation at 37°C , at which time the tumour cells were separated by centrifugation and extracted with aq. 70% (v/v) ethanol (2.5 ml) for 2 h. About 95% of the ^{14}C label associated with the cellular pellet dissolved and, after concentration, was found to migrate as a single component at the same rate as ^{14}C isoleucine during chromatography in three separation systems (see the Materials and methods section). We conclude that no significant metabolism of isoleucine occurred under these specific conditions, in which respiration was prevented and energy metabolism depended on glycolysis. Similar observations were made with cell preparations incubated with 1 mM solutions of leucine.

Predicted values of $[A]_i/[A]_o$

The two-state carrier model described in the Mathematical methods section was used to predict the magnitude of $[A]_i/[A]_o$, in the presence and absence of ouabain, on the basis of the following. (1) The second of the above estimates of the respective V_{max}/P_A was assumed. (2) $[\text{Na}]_i$ in the absence of ouabain was assumed to be the mean of the observed values 44 ± 1.9 mM (S.E.M.; $n = 34$); $[\text{Na}]_i$ in the presence of ouabain was 244 ± 9 mM (S.E.M.; $n = 7$). (3) The membrane potential E was set at the appropriate values shown in Table 5. (4) The values used for the apparent K_m for each amino acid are those shown in Table 1. The computations were done by using three values of the parameter x defined in the Mathematical methods section, and with an activity coefficient for Na^+ of either 1 or 25/44 to take account of the observations made by Philo & Eddy (1978b). Table 5 shows that the predicted values of the amino acid gradient for glycine, 2-aminoisobutyrate and leucine agreed fairly well with the observed values, especially when the smaller activity coefficient for Na^+ was employed. The accumulation of isoleucine from a 1 mM or 10 mM solution was roughly consistent with the model, whereas its accumulation from a 0.1 mM solution, both in the presence and in the absence of ouabain, was much larger than was predicted. The model is of course irrelevant to the behaviour of the norbornane amino acid, which was absorbed by an electrically silent mechanism (Fig. 1), in agreement with other work (Kilberg & Christensen, 1980).

Further observations with 0.1 mM-isoleucine

(1) Preincubation of the tumour cells with 0.3 mM-ouabain in a Ringer solution containing

163 mM- Na^+ , glucose and the metabolic inhibitors for 50 min lowered $\Delta\mu_{\text{Na}}$ to 1.8 ± 0.2 (S.E.M.; $n = 3$). The tumour cells were then resuspended in a similar solution containing 0.1 mM- ^{14}C isoleucine at 37°C . During the next 10 min a 10-fold amino acid gradient formed. A similar isoleucine gradient formed when the cells were incubated with ouabain for 50 min and were then transferred to a Ringer solution containing 30 mM- K^+ , 133 mM- Na^+ , 0.3 mM-ouabain and 10 μg of valinomycin/ml, together with 0.1 mM- ^{14}C isoleucine, glucose and the metabolic inhibitors. $\Delta\mu_{\text{Na}}$ was lowered in this way to 1.4 ± 0.4 (S.E.M.; $n = 4$). It is unlikely that either a residual Na^+ gradient or an electroneutral coupled movement of both Na^+ and K^+ drove the system, because $[\text{K}]_i/[\text{K}]_o$ was about 2.6 and $[\text{Na}]_o/[\text{Na}]_i$ was about 0.87 under these conditions. (2) The isoleucine gradient formed from a 0.1 mM solution in the presence of 123 mM-choline chloride, 40 mM- K^+ , glucose and the metabolic inhibitors was 16.7 ± 1.6 (S.E.M.; $n = 3$) after 60 min incubation at 37°C . Parallel assays in the presence of 123 mM- Na^+ and 40 mM- K^+ gave a value of 16.1 ± 2.7 (S.E.M.; $n = 3$). The above observations indicate that Na^+ was probably not involved in the concentration of isoleucine from these dilute solutions. We do not understand why ouabain nevertheless partially inhibited the concentration of both isoleucine and the norbornane amino acid (Table 5).

Comparison with estimates of $\Delta\mu_{\text{Na}}$ and $[A]_i/[A]_o$ in the literature

We follow the usual practice of assuming that the ionic activity coefficients of Na^+ in the cellular and extracellular phases are both 1 and that the cellular phase behaves as a single compartment. (1) Jacquez (1975) observed that when the distribution of 2-aminoisobutyrate reached a steady state, in which $[A]_i/[A]_o$ was about 45 at low amino acid concentrations, $[\text{Na}]_o/[\text{Na}]_i$ was about 7 for the Ehrlich strain of mouse ascites-tumour cells maintained in a conventional Ringer solution. The membrane potential was not determined. The different strain of mouse ascites-tumour cells used in the present investigation produced a similar gradient of 2-aminoisobutyrate from a 0.1 mM solution, and $[\text{Na}]_o/[\text{Na}]_i$ from about 3 to 4.6. (2) Similarly $[\text{Na}]_o/[\text{Na}]_i$ was found to be about 7.8 and $[A]_i/[A]_o$ about 27 in the study made by Henius & Laris (1979) with enucleated preparations of the Ehrlich cell line. $\Delta\mu_{\text{Na}}$ corresponded to a ratio of about 19. (3) The observations reported in the present paper and those made by Philo & Eddy (1978b) show that $\Delta\mu_{\text{Na}}$ varied from 18 to 42 during glycolysis in the standard Ringer solution. (4) Hoffmann *et al.* (1979), who used the Ehrlich tumour cells, reported observations showing that $[\text{Na}]_o/[\text{Na}]_i$ was about 5.0, whereas $\Delta\mu_{\text{Na}}$ was

Table 5. *Amino acid gradient ($[A]_i/[A]_o$) as a function of $[A]_o$ in the presence and in the absence of ouabain, showing the relation to the Na^+ electrochemical gradient ($\Delta\mu_{\text{Na}}$ expressed as a ratio)*

The values of E , $\Delta\mu_{\text{Na}}$ and $[A]_i/[A]_o$ maintained in the steady state of amino acid distribution were determined after incubation of the cell suspension (13 mg dry wt.) in the standard Ringer solution (40 ml) containing 155 mM- Na^+ , 8 mM- K^+ or, when 0.3 mM-ouabain was present, in a similar solution containing 163 mM- Na^+ . The system also contained 60 μM -2,4-dinitrophenol, as well as 6 ng of oligomycin, 1 μg of antimycin and 2 mg of glucose per ml of cell suspension. Incubation with the indicated concentration of ^{14}C -labelled amino acid was continued for 30 min (60 min when 0.3 mM-ouabain or the norbornane amino acid was present), during which time the amino acid distribution reached a steady state. The distribution of ^{14}C label, the respective magnitudes of the membrane potential (E) and of $\Delta\mu_{\text{Na}}$ and their standard errors were determined as described in the Materials and methods section. The leak coefficient P_A was assayed as illustrated in Fig. 3. The predicted values of $[A]_i/[A]_o$ were obtained from eqn. (3) of the Mathematical Methods section as explained in the text. The activity coefficient (a) of cellular Na^+ was assumed to be either 1 or 25/44. The latter fraction corresponds to an assumed value of 25 mM for $[\text{Na}]_i$ in the steady state maintained in the standard Ringer solution lacking ouabain. The carrier parameter x of eqns. (1) and (2) was varied from 1 to 10. The predictions thus led to a range of values of the accumulation ratio in each instance, the extremes of which are given where these differed by more than 0.1. The mean values of $[A]_i/[A]_o$ observed when $[A]_o$ was 1 mM as opposed to 10 mM were compared by means of the t test for small samples. P was <0.001 , except for leucine, where $P < 0.025$ on a one-tailed test. A similar analysis of the effect of raising $[A]_o$ on $\Delta\mu_{\text{Na}}$ showed that these differences were not significant ($P < 0.2$). Table 2 shows, however, that E itself was significantly lowered ($P < 0.001$). In the same group of observations, when the values of $\Delta\mu_{\text{Na}}$ and $[A]_i/[A]_o$ obtained with a particular concentration of a selected amino acid were compared, the two parameters differed significantly ($P < 0.025$; $P < 0.05$ for 1 mM-glycine), with one exception, 1 mM-2-aminoisobutyrate ($P < 0.3$).

	$[A]_o$ (mM)	No. of observations	$-E$ (mV)	$\Delta\mu_{\text{Na}}$ (as ratio)	Observed gradients $[A]_i/[A]_o$	Predicted amino acid gradient $[A]_i/[A]_o$		P_A (l/min per kg)
						$a = 1.0$	$a = 25/44$	
Glycine	1.0	4	60.7 ± 6.6	41.8 ± 6.6	26.2 ± 0.83	19.6–24.6	28.0–36.7	0.146 ± 0.015 (3)
	10.0	4	48.2 ± 6.6	25.3 ± 4.2	9.15 ± 0.34	4.4–7.4	5.7–9.8	0.106 ± 0.008 (3)
2-Amino-isobutyrate	0.1	2	61.4 ± 2.8	45.3 ± 0.6	$55.3 \pm 3.70^*$	32.7–33.1	54.7–55.8	0.084
	1.0	3	57.1 ± 5.7	43.3 ± 22.9	30.0 ± 1.35	21.1–24.8	31.8–39.0	0.084
	10.0	3	45.2 ± 6.1	18.5 ± 4.1	6.50 ± 0.26	5.1–8.5	6.8–11.7	0.068 ± 0.005 (3)
	0.1 + 0.3 mM-ouabain	4	26.4 ± 6.0	1.9 ± 0.4	3.30 ± 0.41	1.8	3.1	0.084
Leucine	1.0	4	57.5 ± 6.4	37.8 ± 10.7	8.85 ± 1.17	9.4–10.0	11.2–11.7	0.529
	10.0	4	43.7 ± 6.5	21.9 ± 4.3	5.45 ± 0.59	3.7–4.6	4.4–5.4	0.395 ± 0.049 (3)
Isoleucine	0.1	3	58.5 ± 4.3	33.1 ± 9.2	16.8 ± 0.38	4.3	4.5	0.52
	1.0	3	57.1 ± 3.8	27.2 ± 6.3	4.40 ± 0.06	4.0	4.2	0.52
	10.0	3	48.9 ± 1.9	19.1 ± 0.6	2.33 ± 0.19	2.7–2.8	2.9–3.0	0.525 ± 0.045 (3)
	0.1 + 0.3 mM-ouabain	3	27.6 ± 1.0	1.8 ± 0.2	9.17 ± 0.63	1.5	1.9	0.52
Norbornane amino acid	0.1	3	50.0 ± 4.3	24.7 ± 3.2	22.3 ± 2.03			
	1.0	2	48.5 ± 4.3	18.4 ± 3.4	4.50 ± 0.40			
	10.0	3	43.3 ± 6.0	12.4 ± 1.9	1.47 ± 0.03			
	0.1 + 0.3 mM-ouabain	3	13.3 ± 7.8	1.3 ± 0.5	10.0 ± 1.39			

* Mean of three assays at 30 min and two at 60 min.

about 13.6. We conclude from the above evidence that our distinct tumour cell line and the various Ehrlich strains maintain rather similar electrochemical gradients of Na^+ across the plasmalemma and produce similar 2-aminoisobutyrate gradients.

Discussion

Relation between $\Delta\mu_{\text{Na}}$ and $[A]_i/[A]_o$

Philo & Eddy (1978b) observed that the magnitude of $[A]_i/[A]_o$ for L-methionine in the steady

state slightly exceeded the corresponding ratio for the Na^+ electrochemical gradient of about 26 when $[A]_o$ was 1 mM. Henius & Laris (1979) reached a similar conclusion about 2-aminoisobutyrate. In the present work $\Delta\mu_{\text{Na}}$ represented a factor of about 27–43 when $[A]_o$ was 1 mM. The amino acid distribution of 26–30 reached in 1 mM-glycine or -2-aminoisobutyrate was therefore apparently near equilibrium with the Na^+ gradient, whereas the distribution ratio of leucine or isoleucine of about 9 or 4 respectively was significantly smaller than the equilibrium value.

When $[A]_0$ was 10 mM the mean $\Delta\mu_{Na}$ observed for each amino acid tended to be smaller than in the presence of 1 mM amino acid, and the amino acid gradient was much smaller, by a factor of at least 2, than the Na^+ gradient itself (Table 5). The system thus behaves as though the flow of amino acid was partially uncoupled from the flow of Na^+ at the larger amino acid concentration.

(1) *Model 1. Pump with leak.* If the uptake of amino acid through the symport is in fact strictly coupled to the uptake of 1 equiv. of Na^+ (Philo & Eddy, 1978b), the failure to reach the thermodynamic equilibrium could arise from the presence of a separate leak pathway for the amino acid outside the Na^+ symport. This is the pathway defined by the parameter P_A . Such an arrangement means that a net current of Na^+ ions through the symport would necessarily be produced in the steady state of amino acid distribution. We suggest that the partial electrical depolarization of the tumour cells detected after incubation of the tumour cells for 30–90 min in the presence of 10 mM solutions of various amino acids arises in that way (Table 2). This interpretation is supported by the approximate analysis based on eqn. (9). Despite the several assumptions involved in relating the net flow of amino acid through the Na^+ symport to ΔF , the fluorescence assays and the tracer assays led to rather similar estimates of V_{max}/P_A for a given amino acid (Table 4). One assumption underlying this use of eqn. (9) is that the permeability coefficients and other ionic parameters determining the magnitude of ΔF (Philo & Eddy, 1978b) were unchanged during the approach to the steady state. Clearly this assumption does not hold exactly, if only because cellular $[Na^+]$ and $[K^+]$ changed to some extent. However, we suggest that such changes were sufficiently small as not to invalidate the use of eqn. (9) in the steady state.

Christensen *et al.* (1973) and Garcia-Sancho *et al.* (1977), on the basis of studies with $^{22}Na^+$, have proposed that loading the mouse ascites-tumour cells with certain amino acids, including 2-aminoisobutyrate, uncouples the net uptake of Na^+ from the net uptake of 2-aminoisobutyrate itself, which nevertheless remains Na^+ -dependent. Some of the conditions these workers used were similar to those attained in the steady state of 2-aminoisobutyrate distribution. The observations in Table 2 indicate, in contradiction to the above work with $^{22}Na^+$, that the amino acid symport retained its electrogenic character in the steady state.

(2) *Model 2. Pump with slip.* The uptake of Na^+ and of the amino acid through the symport itself might alternatively be supposed not to be strictly coupled on a 1:1 basis, in the sense that the coupling between the two flows was regulated by a special mechanism built into the symport. Morville *et al.*

(1973) and Heinz (1974) use the term internal leaks in this connection, to contrast them with the external leaks represented by P_A of Model 1. Although such a 'slip' model might in principle give rise to the observed depolarization and the inferred current of Na^+ (Eddy, 1980), the present work is difficult to explain in that way. Thus the amino acid flow represented by P_A , assayed in the absence of Na^+ , would be expected on the basis of Model 2 to be more or less inhibited in the presence of Na^+ (Eddy, 1968). The observations in Table 4 show that in fact V_{max}/P_A , determined from ΔF in the steady state maintained in the standard Ringer solution, was quite similar to the ratio determined by tracer studies of the amino acid uptake in which P_A was estimated in the absence of Na^+ . Furthermore, our estimates of P_A determined in the absence of Na^+ appear to be too small, on the basis of Model 2, effectively to uncouple the Na^+ symport in the presence of Na^+ . We therefore prefer Model 1 to Model 2. Model 1 is also generally consistent with the work of Jacquez (1975) and Inui & Christensen (1966), who have shown that the influx of 2-aminoisobutyrate in the presence of Na^+ is made up of two additive components, with characteristics similar to those given in Table 5 for glycine and 2-aminoisobutyrate. Inui & Christensen (1966) resolved the uptake of L-methionine, L-leucine and other substrates of both the Na^+ -dependent A transport system and the Na^+ -independent L transport system into three components. Two of these correspond to the A and L systems respectively, whereas the third is a carrier-mediated 'leak' exhibited at relatively high amino acid concentrations (Christensen & Handlogten, 1977). Further work appears to be needed to clarify the relationship between the estimates of P_A for leucine and isoleucine shown in Table 5 and the L system of amino acid transport. However, our observations show that the tumour cells concentrated each of the four amino acids from a 1 mM or 10 mM solution, under the conditions studied in the present work, to an extent roughly consistent with the process being coupled to the Na^+ gradient as its exclusive energy source. Thus leucine and isoleucine, which are certainly substrates of the L system (Christensen, 1979), were apparently not concentrated from a 1 mM or 10 mM solution to a greater extent than the A transport system was expected to maintain. Philo & Eddy (1978b) likewise found that 1 mM-methionine was concentrated to the extent predicted on the basis of the magnitude of $\Delta\mu_{Na}$ provided that the apparent value of the latter was multiplied by a factor of about 1.8. Ohsawa *et al.* (1980) have suggested different interpretations of this behaviour, including the possibility that the factor of 1.8 arises because methionine efflux occurs in part through the L system, which is itself energized by metabolism and is not therefore a

simple leak process. We stress that the extent of concentration of 2-aminoisobutyrate from dilute solutions also apparently exceeded the values expected on the Na^+ -gradient hypothesis unless cellular $[\text{Na}^+]$ was assumed to be smaller than its apparent value (Table 5). Henius & Laris (1979) reached a similar conclusion. We suggest that the magnitude of the discrepancies involved, both with 2-aminoisobutyrate and methionine, can be explained in terms of the effective activity of cellular Na^+ , rather than by postulating a novel supplementary source of energy for the A system (see Ohsawa *et al.*, 1980). In contrast, the accumulation of isoleucine from relatively dilute solutions, in the physiological range, clearly exceeded the range of magnitudes the A system of amino acid transport was expected to produce. We suggest that, as with the norbornane amino acid (Ohsawa *et al.*, 1980), the L system was involved. The mechanism of this process has not been established (Ohsawa *et al.*, 1980).

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