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Amino Acid Absorption by Mouse Ascites-Tumour Cells Depleted of both Endogenous Amino Acids and Adenosine Triphosphate

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1. Despite the depletion of both their content of exchangeable endogenous amino acids and reserves of ATP, starved hypo-osmotically shocked preparations of the tumour cells accumulated relatively large amounts of ^{14}C-labelled 2-aminoisobutyrate, L-alanine, glycine, L-leucine, L-methionine, L-phenylalanine and L-serine, against their respective concentration gradients, by a process apparently driven by the spontaneous flow of Na^+ ions into the cellular phase. Dependent on (*a*) which compound was used, (*b*) its concentration and (*c*) the direction of the Na^+ ion gradient, the peak value of the ratio of the cellular to extracellular amino acid concentration varied from about 0.4 to 7. 2. The extent to which ATP increased the ratio was defined for L-methionine. 3. Chemical analysis of the cellular amino acid content showed that this increased in parallel with the absorption of ^{14}C. 4. The accumulation of L-methionine and of glycine, against their own concentration gradients, continued in the presence of either 0.3mM-ouabain or 10μg of oligomycin/ml. Thus the sodium pump was probably not involved in the process when ATP was lacking. 5. L-Leucine caused 0.72 ± 0.12 (S.E.M.; 6) extra equivalents of Na^+ to enter the shocked starved tumour cells in parallel with the uptake of leucine itself. Only a small loss of K^+ was induced. 6. The influx and efflux of L-methionine in preparations depleted of ATP were both markedly accelerated by the presence of Na^+ ions. 7. The observations provide further examples of the application of the ion-gradient hypothesis, according to which Na^+ ions act as co-substrates of the amino acid pump. The quantitative importance of parallel Na^+-independent systems was studied with a new mathematical model.

When the energy metabolism of mouse ascites-tumour cells is inhibited and the cells are first depleted of Na^+ and then suspended in Ringer solution containing 1mM-[^{14}C]glycine, they accumulate the labelled amino acid against its own concentration gradient despite the apparent absence of ATP (Eddy, 1968*b*). Current controversies about the roles of Na^+ and K^+ in the system include the question (Eddy, 1968*b*; Whittam & Wheeler, 1970) of whether (1) the glycine simply exchanges with the various amino acids that are normally present as an endogenous pool (Christensen *et al.*, 1952; Johnstone & Scholefield, 1965*a*), or whether (2) the potential energy of the alkali-cation gradients drives the amino acid 'uphill'. The second explanation seemed more likely because (1) the extent to which the glycine was concentrated varied systematically with the magnitude of the alkali-cation gradients (Eddy, 1968*b*); (2) the absorption of glycine displaced Na^+ ions into the tumour cells (Eddy, 1968*a*, 1969).

It is also important to know how Na^+ and K^+ interact with the flow of other amino acids into the tumour cells, if conclusions of general significance are to be reached. The present work was therefore undertaken to strengthen the arguments based on the behaviour of glycine by showing that six other amino acids could be accumulated, under the influence of the ionic gradients, in conditions where exchange with the endogenous pool was not involved. For this purpose we used preparations of the tumour cells that had received a controlled hypo-osmotic shock (Oxender, 1965) that removed most of their endogenous amino acid content. The expected displacement of Na^+ into the tumour cells during the absorption of leucine was also shown to occur. The extent to which the provision of ATP affected the accumulation of L-methionine was studied as a function of the methionine concentration, and proved to be similar to the behaviour of glycine (Eddy, 1968*b*). A new mathematical analysis of the phenomenon was attempted which may reconcile the basic postulates of the ion-gradient hypothesis with those of the widely accepted pump–leak hypothesis (Johnstone & Scholefield, 1965*b*). Finally, the suggestion that the sodium pump may be involved in the process whereby the

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Na^+ gradient drives the accumulation of amino acids (Kimmich, 1970) has been examined by using preparations containing inhibitors of the sodium pump.

Materials and Methods

These were in general as described by Eddy & Hogg (1969). Suspensions of the tumour cells in the standard Ringer solution lacking glucose were first shaken in open conical flasks for 15min at 37°C. The Ringer solution contained 155mequiv. of Na^+/l, 8mequiv. of K^+/l, 1.2mM-$MgSO_4$ and 16mM-sodium phosphate buffer, pH7.4. The cells (1vol.) were centrifuged and then suspended for 10min at 37°C in a hypo-osmotic solution (10–15vol.) prepared by mixing water with an equal volume of potassium–Ringer solution. The latter contained 159mequiv. of K^+/l and the other constituents mentioned above, except that it contained no Na^+ ions. The tumour cells were next collected by centrifugation, washed once with the iso-osmotic potassium–Ringer solution (10vol.) and then suspended for 20–30min at 37°C in the potassium–Ringer solution (10–15vol.). When the energy metabolism was to be depleted, 2mM-NaCN was included in the potassium–Ringer solution at that stage, the suspension being kept in a stoppered flask for 25min. The presence of 10mM-2-deoxyglucose for the last 10min helped to lower the ATP content of the system.

Shock treatment of the tumour cells in a Ringer solution containing Na^+ instead of K^+ displaced the amino acid pool as effectively as the above procedure. It resulted in a marked increase in the cellular content of Na^+, which would have been inconvenient in some of the experiments described below. Shock treatment of the tumour cells at 0°C displaced only about one-sixth as much ninhydrin-reactive material from the endogenous pool.

Chemical assays

In the work summarized in Table 3 a given sample of tumour cells (about 200mg dry wt.) was either (1) kept at 100°C for 10min with 3% (w/v) sulphosalicylic acid or (2) mixed with 90% (v/v) ethanol and kept at room temperature for at least 2h (Eddy *et al.*, 1967). The acid extracts were preserved in the frozen state and their amino acid content was assayed (1) in terms of ^{14}C (see below) and (2) with an EEL amino acid analyser (Mark I; Evans Electroselenium Ltd., Halstead, Essex, U.K.) operated in accordance with the maker's instructions. A known amount of norleucine was added to each sample to calibrate the system. The two-column procedure for use with physiological fluids was employed (Spackman *et al.*, 1958). Additionally, (3), the total amino acid content of either the acidic or the ethanolic extracts was determined with a ninhydrin reagent (Eddy, 1968*a*) and expressed in terms of a glycine standard.

Amino acid distribution

Method 1 was the small-sample technique of Eddy (1968*a*). Method 2 was based on the large-sample technique of Eddy (1968*a*). One series of samples was taken from a given cell suspension to determine the dry weight of cellular material present. Each sample (5ml) in a second series was chilled to 0°C and mixed with [^{3}H]inulin (0.2μCi/ml), which served as an extracellular marker. The tumour cells (25–40mg dry wt.) were separated by centrifugation, weighed and then mixed with 10ml of 0.01M-HNO_3. Cellular debris was separated by centrifugation after about 2h at 25°C. The extract was assayed for Na^+ and K^+ by flame photometry, and for ^{14}C and ^3H in a liquid-scintillation spectrometer (Packard Tri-Carb model 3310). The extracellular phase was similarly assayed. The scintillation fluid contained toluene (0.7 litre), Triton X-100 (0.3 litre), 2,5-diphenyloxazole (3g) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (100mg). Sample quenching was assessed by the channels-ratio method.

Influx and efflux rate coefficients. The shocked cell preparations suspended in the potassium–Ringer solution (about 6mg dry wt. of cells/ml of suspension) were put with an appropriate concentration (1–5mM) of the selected amino acid (0.03μCi/ml) for up to 40min at 37°C. Samples (5ml) were withdrawn at various intervals, chilled and processed as described above. During about 30min the system appeared to reach a steady state in which the cellular and extracellular concentrations of the amino acid were approximately equal. The influx rate coefficients were accordingly determined by plotting the natural logarithm of the difference between the cellular and extracellular amino acid concentrations against time. Amino acid efflux in the absence of Na^+ was studied after the preparation had been loaded with the amino acid for 30min. The cells were separated by centrifugation and then transferred to a similar volume of fresh potassium–Ringer solution at 37°C. Four samples taken during the next 8min were processed by Method 2.

When the efflux of L-methionine was to be studied in the presence of various concentrations of Na^+ and K^+ ions, the tumour cells (1vol.) were first loaded with the selected concentration of L-methionine at 37°C and then washed with 40vol. of a similar Ringer solution at 0°C to remove surplus amino acid. To initiate amino acid efflux the preparation was then mixed (1–2mg of cells/ml) with an appropriate Ringer solution at 37°C. Three samples (1ml each) were taken during the next 4min and processed by Method 1. The cellular amino acid content decreased logarithmically with time for at least the first 3min of the observations.

Chemicals

Triton X-100 and oligomycin were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. 2,5-Diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)-benzene were both from Packard Instruments Ltd., Wembley, Middlesex, U.K. [^{3}H]Inulin (code TRA 324) and the other radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The following compounds were uniformly labelled with ^{14}C; L-alanine, L-arginine, L-aspartate, L-leucine, L-lysine, L-phenylalanine. Other compounds used were; 2-amino[1-^{14}C]isobutyrate, [1-^{14}C]glycine, L-[*Me*-^{14}C]methionine, L-[3-^{14}C]serine.

Kinetic analysis

Method

Pump–leak models. Johnstone & Scholefield (1965*b*) summarize the arguments showing that in the steady state the ratio of the cellular ($[A]_2$) to extracellular ($[A]_1$) concentrations of a given amino acid becomes smaller when $[A]_1$ is raised because the amino acid pump functions in parallel with an independent leak mechanism. Let the amino acid be absorbed through the pump at a rate of:

$$v_{in} = V_{max.} \frac{[A]_1}{K_m + [A]_1} \quad (1)$$

where $V_{max.}$ and K_m have the conventional meanings. Let the net rate of amino acid efflux through the leak be:

$$v_{out} = k([A]_2 - [A]_1) \quad (2)$$

where k is a first-order rate constant.
In the steady state:

$$([A]_2 - [A]_1)^{-1} = \left(\frac{k \cdot K_m}{V_{max.}}\right)[A]_1^{-1} + \frac{k}{V_{max.}} \quad (3)$$

We examine below the form that eqn. (3) takes when the pump is driven by the Na^+ ion and K^+ ion gradients.

Ion-gradient hypothesis. The influx of the amino acid (A) on the carrier (E) involves a ternary complex (E Na A) and is supposed to be tightly coupled to the absorption of one equivalent of Na^+, the return of the carrier being partially neutralized by the efflux of K^+ (as EK) to an extent defined by a parameter θ (Eddy, 1968*a*).

Recent work has indicated that the effect of a membrane potential (V) on the movement of the various carrier species is important under certain conditions (Eddy, 1972; Gibb & Eddy, 1972). The equation defining the amino acid distribution then becomes:

$$\frac{[A]_2}{[A]_1} = \frac{[Na^+]_1}{[Na^+]_2} \cdot \frac{P' + \theta[K^+]_2}{P + \theta[K^+]_1} \quad (4)$$

where

$$P' = \frac{VF/RT}{1 - e^{-VF/RT}} \quad \text{and} \quad P = \frac{VF/RT}{e^{VF/RT} - 1}$$

Here F, R and T have the conventional significance and the constant-field assumption is used to predict the effect of the membrane potential on the inwards and outwards movements of the various carrier species differing in net charge (Britton, 1965; Katz, 1966). The derivation of eqn. (4) is based on the assumption (1) that the carrier has the same inherent properties whether it is situated in the inner or in the outer phase of the cell membrane. We found the problem of computing the effect of the leak pathways on the properties of such a system intractable, however, unless we ignored the effect of the membrane potential. Thus an additional assumption made for that purpose was (2) that $P = P' = 1$ (Eddy, 1968*a*).

Na^+-dependent amino acid pump with separate leak pathway. Eqn. (1) of Eddy & Hogg (1969) describes the initial rate of uptake (v) of the amino acid as a function of $[A]_1$, and of the respective values of $[Na^+]$ and $[K^+]$ in the two phases, when the amino acid is confined to phase 1 and assumptions (1) and (2) above again apply. When $[A]_2$ is not zero, we find that:

$$v = \frac{ck^{na}}{k_1 k_4}\left(\frac{[Na^+]_1 [A]_1 \alpha}{\beta} - \frac{[Na^+]_2 [A]_2}{\beta}\right) \quad (5)$$

where

$$\alpha = \frac{\frac{k^n[Na^+]_2[A]_2}{k^c k_1 k_4} + \frac{k^k[K^+]_2}{k^c k_2} + 1}{\frac{k^n[Na^+]_1[A]_1}{k^c k_1 k_4} + \frac{k^k[K^+]_1}{k^c k_2} + 1}$$

$$\beta = \alpha\left(1 + \frac{[Na^+]_1}{k_1} + \frac{[K^+]_1}{k_2} + \frac{[A]_1}{k_3} + \frac{[Na^+]_1[A]_1}{k_1 k_4} + \frac{[K^+]_1[A]_1}{k_2 k_6}\right) +$$

$$\left(1 + \frac{[Na^+]_2}{k_1} + \frac{[K^+]_2}{k_2} + \frac{[A]_2}{k_3} + \frac{[Na^+]_2[A]_2}{k_1 k_4} + \frac{[K^+]_2[A]_2}{k_2 k_6}\right)$$

The symbol c for the carrier concentration replaces e used in eqn. (1) of Eddy & Hogg (1969) and k^{na} replaces k^{ns}.

The magnitudes of the various parameters k_1–k_6 were estimated by Eddy *et al.* (1967) and Eddy & Hogg (1969) for the glycine-transport system. They were respectively 37.4mM, 76mM, 6.4mM, 3.5mM, 21mM and 92mM. Also $k^k/k^c = k_2\theta$, where an initial estimate of θ was made by applying eqn. (4) (with $P = P' = 1$) to the appropriate experimental observations (cf. Eddy, 1972). Further, the actual magnitude of v at one particular value of $[A]_1$, when $[A]_2$ is zero and the ionic conditions are defined, can be determined by experiment and provides an estimate of ck^{na}.

The effect of the leak pathway is contained in the simultaneous solution of eqns. (5) and (2). The parameter k in eqn. (2) was estimated by observing the magnitude of v in the absence of Na^+. The equations were solved by keeping $[A]_1$ constant and varying $[A]_2$, the values of v consistent with eqn. (5) being plotted against selected values of $[A]_2-[A]_1$. The values of v defined by eqn. (2) were similarly plotted. The intersections between the two series of lines represented the required solutions.

Results

Properties of the hypo-osmotic cell suspension

The hypo-osmotic shock treatment at 37°C (see the Materials and Methods section) lowered the amino acid content of the tumour cells by more than 80%. Three similar preparations examined in detail initially contained, per mg dry wt. of cells, an average of 50.5nmol altogether of the various neutral amino acids (threonine, serine, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, tryptophan, phenylalanine, histidine), 25.0nmol altogether of aspartic acid and glutamic acid and 3.3nmol altogether of ornithine, lysine and arginine, as well as 55nmol of taurine. The corresponding values after the shock treatment were 7.6, 4.0, 1.5 and 5nmol/mg dry wt. of cells. The cellular content of acid-soluble material, reacting with the ninhydrin reagent and computed as glycine, likewise fell by about 60% from 222±5 (S.E.M.; 8) to 72±3 (S.E.M.; 29) nmol/mg dry wt. of cells. The water content of the shocked preparations suspended in the iso-osmotic potassium–Ringer solution was 6–9μl/mg dry wt. of cells. It returned to values in the normal range (3–4μl/mg) when the tumour cells were transferred to the standard sodium–Ringer solution at 37°C. The amino acid pool was apparently not then replenished from endogenous sources.

Amino acid accumulation by the shocked preparations

During respiration. Table 1 shows that various amino acids were each accumulated by the shocked cell preparations up to characteristic concentrations that were almost as large as those found in the unshocked cell preparations used as controls. Lowering the concentration of extracellular K^+ to about 2mequiv./l and also including 0.1mM-ouabain in the

Table 1. *Accumulation of various amino acids by the respiring preparations of shocked and unshocked tumour cells*

The preparations (3mg dry wt./ml of suspension) were shaken in open flasks for 30–40min at 37°C either (1) in the standard Ringer solution lacking glucose and containing 150mequiv. of Na^+/l, 8mequiv. of K^+/l and a 1mM solution of the selected amino acid labelled with ^{14}C, or (2) in a similar solution in which the K^+ was replaced by Na^+ and 0.1mM-ouabain was present. The ratio of the cellular to extracellular amino acid concentrations was determined (Method 1). Average values ±S.E.M. are shown, with the number of preparations assayed in parentheses. A dash signifies that the system was not examined.

	Concentration ratio		
	Shocked preparations	Unshocked preparations	
Amino acid	No ouabain	No ouabain	Plus ouabain
2-Aminoisobutyric acid	—	31.6±3.8 (5)	2.1±0.5 (2)
L-Alanine	18.4±1.2 (4)	21.3±1.3 (8)	1.2±0.4 (3)
L-Arginine	—	2.5 (3)	2.0±0.2 (3)
L-Aspartate	—	0.34 (2)	0.09 (2)
Glycine	19.5±1.4 (19)	24.1±1.3 (29)	2.8±0.4 (3)
L-Leucine	4.2±0.3 (14)	5.5±0.3 (15)	1.3±0.2 (4)
L-Lysine	—	3.2±0.6 (6)	2.0±0.2 (3)
L-Methionine	15.3±0.7 (4)	18.4±0.8 (7)	2.8±0.1 (2)
L-Phenylalanine	11.0±0.7 (13)	14.4±1.1 (14)	2.6±0.1 (3)
L-Serine	27.4±2.6 (4)	24.9±2.2 (7)	1.3±0.4 (3)

Table 2. *Effect of the hypo-osmotic shock treatment on the accumulation of various amino acids in the presence of 2 mM-NaCN with or without 10 mM-2-deoxyglucose*

The shocked preparations were starved to deplete them of ATP as described in the Materials and Methods section. The unshocked preparations were similarly starved in the iso-osmotic potassium–Ringer solution containing the metabolic inhibitors. Each preparation was then transferred to the standard sodium–Ringer solution at 37°C containing 2mM-NaCN and a 1mM solution of the selected amino acid, with or without deoxyglucose. A series of samples was taken to ascertain the maximum value of the ratio of the cellular to extracellular concentrations of the amino acid (Method 1). This was reached in 5–20min. Several independent determinations of these ratios were averaged. A dash indicates that the system was not examined.

	Concentration ratio		
	Shocked preparations		
Amino acid	Cyanide	Cyanide+ deoxyglucose	Unshocked preparations Cyanide+deoxyglucose
2-Aminoisobutyric acid	—	7.2±0.05 (2)	5.6±0.8 (4)
L-Alanine	7.6±0.4 (5)	6.3±1.6 (2)	5.4±2.8 (2)
L-Arginine	—	—	1.1±0.1 (2)
Glycine	4.6±0.3 (7)	3.8±0.3 (11)	5.0±0.4 (3)
L-Leucine	3.7±0.1 (3)	2.3±0.2 (4)	2.6±0.1 (6)
L-Methionine	6.7±0.4 (6)	5.8±0.5 (6)	5.1±0.3 (5)
L-Phenylalanine	4.4±0.4 (3)	3.1±0.2 (6)	3.4±0.2 (7)
L-Serine	7.3±0.5 (5)	5.8±0.8 (5)	3.0±0.3 (3)

system inhibited the absorption of the amino acids and inhibited the sodium pump so that cellular [Na^+] was increased to at least 120mequiv./l. The cellular concentration of Na^+ in the steady state reached in the presence of 1mM-leucine was 50–70mequiv./l and was similar to that found in the presence of glycine. Thus the driving forces inherent in the concentration gradients of Na^+ and K^+ were apparently the same for both the amino acids, although the glycine gradient was four to five times as large as the leucine gradient.

Energy metabolism restricted. After being starved, both the shocked cell preparations and, in other assays, the unshocked cell preparations were kept for up to 30min with the metabolic inhibitors in a 1mM solution of one of the amino acids listed in Table 2. Whereas the Ringer solution contained 150mequiv. of Na^+/l and about 10mequiv. of K^+/l, cellular [Na^+] was initially less than 20mequiv./l in these assays. The ATP content of the shocked preparations was 0.23±0.03 (S.E.M.; 5) nmol/mg dry wt. of cells when both metabolic inhibitors were present. The endogenous rate of glycolysis was 0.56±0.02 (S.E.M.; 2)nmol of lactate/min per mg. The rate increased to 9.3±0.6 (2)nmol/min per mg when glucose replaced deoxyglucose. The cellular ATP content then increased to 4.0±0.6 (3)nmol/mg. Eddy & Hogg (1969) found both similar amounts of ATP and similar rates of glycolysis in unshocked cell preparations of the ascites tumour.

The ratio of the cellular to extracellular concentrations of the selected amino acid reached the maximum values shown in Table 2. Despite the apparent lack of both energy metabolism and of potentially exchangeable endogenous amino acids, the shocked cells accumulated relatively large amounts of various amino acids against their own concentration gradients. Still larger amounts accumulated during respiration (Table 1). The hypo-osmotic-shock treatment had no consistent effect on the maximum accumulation ratio observed with a given amino acid, nor did the use of deoxyglucose (Table 2).

Dependence on the Na^+ ion gradient. In the assays illustrated in Table 2 the sodium pump was not working and the accumulation of the amino acids leucine and phenylalanine reached its maximum values at a time when the Na^+ ion gradient ($[Na^+]_1/[Na^+]_2$) was smaller than with, for example, glycine or methionine. The lower part of Fig. 1 illustrates the marked correlation that was observed between the magnitude of the Na^+ ion gradient and the respective gradients ($[A]_2/[A]_1$) of L-methionine, L-leucine and glycine that developed in the osmotically shocked cells depleted of ATP. The differences between the amino acids were then less apparent. The relationship between the ionic gradient and the amino acid gradient was roughly linear when $[Na^+]_1/[Na^+]_2$ was greater than about 0.4 ($P<0.001$, for leucine and methionine). A regression analysis gave the following values for the coefficients in the equation $\log([A]_2/[A]_1) = a\log([Na^+]_1/[Na^+]_2)+b$. The coefficient a (±S.E.M.) was 1.27±0.08 for methionine and 0.95±0.21 for

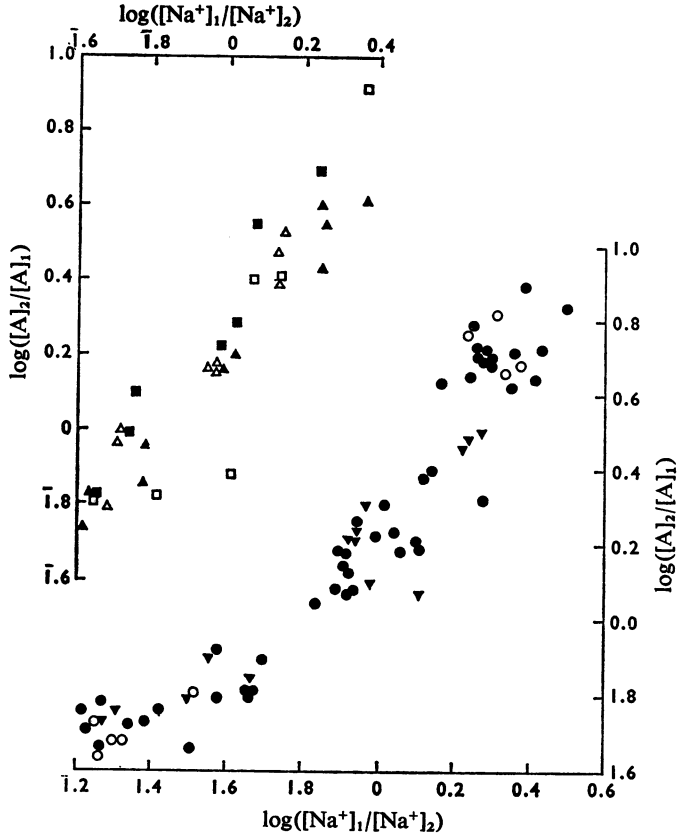

Fig. 1. *Variation of the amino acid accumulation ratio* ($[A]_2/[A]_1$), *in the starved cell preparations, with the magnitude of the* Na^+ *ion gradient* ($[Na^+]_1/[Na^+]_2$)

The procedure described in the legend to Table 2 was used to deplete the cellular ATP content. Cellular $[Na^+]$ ($[Na^+]_2$) was varied during the preliminary starvation, in the presence of both the metabolic inhibitors, by the replacement of extracellular Na^+ by K^+. The preparations were then put in a Ringer solution containing a 1mM solution of the selected amino acid, 2mM-NaCN and 10mM-2-deoxyglucose. The Na^+ content of the Ringer solution ($[Na^+]_1$) ranged from 25 to 150mequiv./l, $[Na^+]_1+[K^+]_1$ being constant at 160mequiv./l. The observations in the lower part of the figure were made with hypo-osmotically shocked cell preparations assayed by Method 2, those in the upper part with unshocked cell preparations assayed by Method 1. The various symbols represent observations made with different amino acids. The number of observations is shown in parentheses. Shocked cells; ● (47), L-methionine; ○ (9), glycine; ▼ (14), L-leucine; unshocked cells; ▲ (10), L-phenylalanine; △ (9), L-serine; □ (6), L-alanine; ■ (8), 2-aminoisobutyrate.

leucine. Whereas only the former differed significantly from 1 ($P<0.001$), the two mean values were not significantly different ($P>0.05$). The coefficient b (±S.E.M.) was 0.24±0.02 for methionine and 0.22± 0.04 for leucine. Thus $[A]_2/[A]_1$ for both compounds was about 1.6 and significantly greater than 1 ($P<0.001$) when the cellular and extracellular concentrations of Na^+ were apparently equal. A recent study of the behaviour of L-methionine in hyperosmotic preparations of the tumour cells (Gibb & Eddy, 1972), in which $[K^+]_1$ was 150mequiv./l and $[Na^+]_1$ was 37mequiv./l, suggests that the factor of 1.6 may be related to the Donnan membrane potential.

The upper part of Fig. 1 shows how four other amino acids responded to variations in the Na^+ ion gradient when the cellular preparations were depleted of ATP, but not shocked. The correlation with the amino acid gradient was highly significant in each case ($P<0.001$). The corresponding values of a (±S.E.M.) were: 1.38±0.12 for 2-aminoisobutyrate

($P<0.02$); 1.57 ± 0.37 for L-alanine ($P=0.2$); 1.25 ± 0.14 for L-serine ($P=0.1$); 1.19 ± 0.07 for L-phenylalanine ($P<0.05$), where the various values of P show whether $1-a$ differed significantly from zero. Unpublished work by M. Reid with L-methionine and L-leucine and the unshocked cellular preparations gave similar results to those obtained with the shocked preparations.

Effects of oligomycin and ouabain. In three experiments the shocked tumour cells were depleted of ATP and allowed to accumulate L-methionine from a 1mM solution in the standard sodium–Ringer solution containing 2mM-NaCN and, except in one experiment, 10mM-deoxyglucose. The maximum ratio (±S.E.M.) of the cellular to extracellular amino acid concentrations was reached at about 5min and was 7.71 ± 0.95. It was 7.79 ± 0.44 when 10μg of oligomycin/ml was present and 7.07 ± 0.49 when 0.3mM-ouabain was present, a concentration that we have shown significantly retarded the extrusion of Na^+ ions during energy metabolism (see also Maizels *et al.*, 1958). The ratio $[Na^+]_1/[Na^+]_2$ was near 3 in each case. Similar observations with ouabain were made (1) in two experiments with glycine in which accumulation ratios of about 5 and 6 were achieved with the shocked tumour cells in the presence of CN^- and (2) in two experiments with methionine and the unshocked cell preparations containing both CN^- and deoxyglucose, in which $[Na^+]_1/[Na^+]_2$ was near 1.4 and the amino acid gradient was between 3 and 4 in both the presence and the absence of ouabain. Because ouabain and oligomycin each had only a small effect on amino acid transport under these conditions, it seems unlikely that the sodium pump was involved in the process.

Composition of the amino acid pool during amino acid absorption

Both the absorption of leucine in the presence of CN^- (Fig. 2), and of glycine in the presence of both CN^- (Fig. 3) and deoxyglucose, depended on the presence of Na^+ and resulted in an increase in the amount of material assayed with the ninhydrin reagent that was approximately equivalent to the amount of ^{14}C absorbed. In these assays the amino acids were concentrated to an approximately 4mM solution in the cellular phase. Analogous observations were made with L-methionine (Eddy, 1972), L-phenylalanine, L-serine and L-alanine, some of which are summarized in Table 3. A net uptake of amino acid occurred in each instance, rather than an exchange with other constituents of the cellular pool. In the course of the work we confirmed that during respiration the amino acids glycine, alanine and phenylalanine accumulated in solution in the cellular phase largely without undergoing chemical transformation. A similar conclusion applies to methionine and glycine in the presence of 2mM-NaCN (Table 3).

Rate of amino acid absorption by the shocked cells

Dependence on Na^+ and K^+. The uptake of leucine, phenylalanine and methionine from a 1mM solution in the standard Ringer solution was in each instance lowered when (1) extracellular $[Na^+]$ was lowered and (2) cellular K^+ was replaced by Na^+ during the preliminary treatment of cells (Table 4). Table 4 further shows that when extracellular $[Na^+]$ was 30mequiv./l, the presence of 120mequiv. of K^+/l retarded the uptake of each amino acid. Similar observations were made with glycine (Eddy & Hogg, 1969). During respiration of the tumour cells, whereas methionine was absorbed several times faster than either leucine or phenylalanine from a 1mM solution

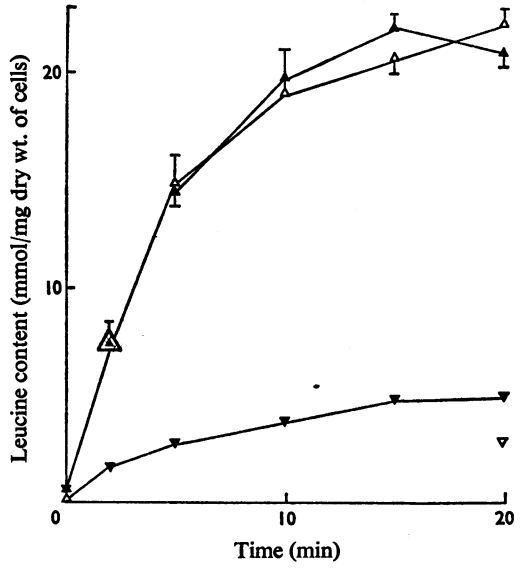

Fig. 2. *Increase with time in the cellular contents of ^{14}C and of ninhydrin-reactive material during the absorption of [^{14}C]leucine by the shocked preparation of tumour cells*

Energy metabolism was restricted by the presence of 2mM-KCN. The mean values (±S.E.M.) of three independent assays by the chemical method (△) and with ^{14}C (▲) are shown. The Ringer solution contained 150mequiv. of Na^+/l, about 10mequiv. of K^+/l and 1mM-leucine. Likewise two series of assays were carried out by using the potassium–Ringer solution (158mequiv. of K^+/l) and the radioisotopic (▼) and chemical (▽) methods. For further details see the Materials and Methods section.

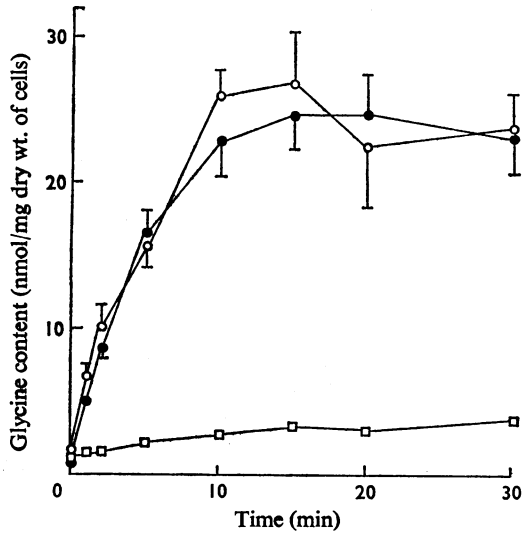

Fig. 3. *Increase with time in the cellular contents of ^{14}C and of ninhydrin-reactive material during the absorption of [^{14}C]glycine by the shocked preparation of tumour cells*

Energy metabolism was restricted by the presence of 2mM-KCN and 5mM-2-deoxyglucose. A 1mM solution of glycine was used. Both the cellular ^{14}C content (●) and the cellular ninhydrin-reactive material (○) were assayed in six independent experiments, in which the uptake of glycine occurred from a solution containing 150mequiv. of Na^+/l with about 10mequiv. of K^+/l; the mean value ±S.E.M. is shown. Two other series (□) involved the assay of [^{14}C]glycine absorbed from the potassium–Ringer solution. For further details see the Materials and Methods section.

in the presence of 150mequiv. of Na^+/l, these differences became smaller at larger amino acid concentrations. Thus after the subtraction of the rate of uptake in the absence of Na^+ (Table 5), the rate in the presence of Na^+ exhibited a K_m value (±S.E.M.) with respect to Phe of 8.4±0.7mM (2) and a $V_{max.}$ of 33±8nmol/min per mg. The corresponding values for leucine were 8.7±0.9mM (3) and 46±12nmol/min per mg, and for methionine 0.96±0.1mM (2) and 41±1nmol/min per mg.

The first-order rate constants characterizing the influx and efflux of glycine, leucine, phenylalanine and methionine, occurring in cell preparations deprived of Na^+, were quite similar (Table 5).

Dependence on ATP. Table 6 shows that restricting energy metabolism lowered the rate of uptake of methionine principally by lowering $V_{max.}$ by about 25%. Glycine behaved similarly (Eddy & Hogg, 1969). Potashner & Johnstone (1971) found that the K_m value with respect to Met, rather than $V_{max.}$, was so affected. They used a different strain of tumour cells, at 25°C, in rather different physiological circumstances.

Methionine influx and efflux compared. Work on the absorption of L-leucine (Hoare, 1972) and of various carbohydrates (Geck, 1971) by human erythrocytes and the absorption of glycine by pigeon erythrocytes (Vidaver & Shepherd, 1968) has indicated that the entry and efflux of each of these substrates may differ kinetically. In our system, just as the influx of methionine was faster when the Ringer solution contained Na^+ ions, so the efflux of methionine from cellular preparations containing both 2mM-KCN and 5mM-deoxyglucose became much faster when the cellular concentration of Na^+ was raised (Fig. 4). The kinetic parameters inferred from the observations shown in Fig. 4 are listed in Table 6. It is very striking that in the series of assays in which the respective cellular and extracellular concentrations of Na^+ and of K^+ were roughly the same, the K_m value with respect to Met was at least 10 times larger for the efflux of methionine than for its influx. A similar conclusion applies to the results shown in Fig. 5, in which the methionine entered a phase containing less than 50mequiv. of Na^+/l from one containing more than 100mequiv. of Na^+/l.

We wish to stress that the marked differences between the entry and efflux kinetics that are illustrated in Table 6 apparently did not represent a major source of free energy for concentrating the amino acid (see Fig. 1, where $[Na^+]_1 = [Na^+]_2$).

Variation of the methionine gradient with [Met]$_1$

Dependence on energy metabolism. The extracellular concentration of methionine ($[Met]_1$) was varied from 0.25 to 9mM under conditions where the ionic gradients acting across the plasmalemma were roughly constant. By analogy with eqn. (3) of the kinetic analysis section, the observed values of $([Met]_2-[Met]_1)^{-1}$ were plotted against $[Met]_1^{-1}$. Fig. 6 shows that these two variables were linearly related in both the presence and the absence of the inhibitors of energy metabolism. Whereas the concentration gradients with respect to $[Na^+]$ and $[K^+]$ were roughly the same in either situation, the maximum value of $[Met]_2-[Met]_1$ was about 40mM during respiration and 9mM when cyanide and deoxyglucose were both present. Thus the respiring cells formed the larger gradients of amino acid concentration. The value of $-[Met]_1$ where $([Met]_2-[Met]_1)^{-1}$ was zero was 1.3mM (Fig. 6) and was therefore roughly equal to the K_m of 1mM for methionine absorption with respect to $[Met]_1$ (Table 6). This result is also consistent with eqn. (3) and with the literature (Johnstone & Scholefield, 1965*b*), although we prefer a different model to the one on which that equation is based (see below).

Table 3. *Comparison of the chemical and radioisotopic assays of amino acid absorption*

The amount of ^{14}C extracted from the cellular amino acid pool (see the Materials and Methods section) was compared with (1) the increase in the total amount of ninhydrin-positive material present in the extract and, in single experiments, with (2) the total amount of the specific amino acid that was detected in the extract by chromatographic analysis (see the Materials and Methods section). Mean values ±S.E.M. are shown, except when a single experiment was performed. We observed no significant changes in the amino acid composition of the extracts other than with respect to the amino acid that was being absorbed. In the assays illustrated the tumour preparations were collected for analysis 20–30min after being put with a 1mM solution of the ^{14}C-labelled amino acid under the conditions described in Tables 1 and 2. Various samples collected in the period up to 20min behaved similarly.

		Amino acid absorption (nmol/mg dry wt. of cells)		
	Amino acid supplied	^{14}C-labelled amino acid	Ninhydrin assay of whole extract	Chromatographic analysis of extract
Respiring shocked cell preparations	Glycine	50.8±1.5 (8)	51.3±2.9 (8)	—
	L-Alanine	53.7±3.1 (3)	53.7±2.2 (3)	—
	L-Phenylalanine	33.3±0.7 (8)	32.7±1.9 (8)	—
	L-Phenylalanine	32.1	37.3	28.1
	L-Alanine	36.7	41.7	33.9
	Glycine	48.9	48.3	45.0
Shocked cell preparations with 2mM-NaCN	Glycine	24.7±2.4 (6)	26.9±3.5 (6)	—
	L-Phenylalanine	24.9±1.7 (3)	24.6±1.5 (3)	—
	L-Leucine	21.9±0.5 (3)	20.5±0.6 (3)	—
	L-Serine	38.9±3.1 (3)	37.0±4.4 (3)	—
	L-Alanine	41.2±1.5 (3)	39.0±2.0 (3)	—
	L-Methionine	36.7±1.8 (3)	35.9±1.9 (3)	—
	Glycine	16.8	17.1	16.2
	L-Methionine	28.8	34.7	28.4

Pump–leak hypothesis. According to the simple version of the ion-gradient hypothesis represented in eqn. (4) of the kinetic analysis section, $([Met]_2-[Met]_1)^{-1}$ would be directly proportional to $[Met]_1^{-1}$, when $[Na^+]$, $[K^+]$, V and θ were fixed and the amino acid only crossed the cell membrane through the Na^+-dependent pump. These two quantities are also directly proportional to one another (Eddy, 1968*a*) when the movements of Na^+ and the amino acid through the pump itself are not tightly coupled. There would then be leaks situated, as it were, within the pump itself. Since the observations in Fig. 6 conform to eqn. (3) rather than to eqn. (4), however, the implication seemed to be that, at larger values of $[Met]_1$, $[Met]_2/[Met]_1$ became smaller owing to the presence of a leak pathway for the amino acid that was independent of Na^+ and lay outside the amino acid pump. Table 5 indicates that these pathways might correspond to a rate constant of the order of $0.05\,min^{-1}$.

Fig. 6 shows that such a model predicts a linear dependence of $([A]_2-[A]_1)^{-1}$ of at least the correct order of magnitude. Thus the leak pathway, which in earlier work (Eddy, 1968*b*) had seemed quantitatively unimportant, in fact significantly lowered the ratio $[A]_2/[A]_1$. This happened especially (1) when $[A]_1$ was large and (2) when the affinity of the amino acid for the sodium-dependent carrier was relatively small. Rather unexpectedly, the mathematical model reproduced the experimental finding with methionine that the negative value of $[A]_1$ where $([A]_2-[A]_1)^{-1}$ was zero was equal within a factor of 2 to the K_m value of the transport system with respect to A_1. The following example serves to illustrate these conclusions.

When $[Na^+]_1/[Na^+]_2$ was 150/40, $[K^+]_2/[K^+]_1$ was 150/10 and θ for glycine was set at 0.04, $[A]_2/[A]_1$ was 18.8 according to eqn. (4), whereas it was 12.8 when $[A]_1$ was 1mM and the leak pathway was introduced. Thus the leak lowered the apparent value of θ from 0.04 to 0.021. The K_m value with respect to A_1 was computed to be 3.0mM for glycine whereas the negative value of $[A]_1$ where $([A]_2-[A]_1)^{-1}$ was zero was computed at 3.6mM.

Effect of leucine on the cellular contents of Na^+ and K^+

Exposure of the shocked preparations of the tumour cells for up to 5min to a 10mM solution of L-leucine, in the presence of the standard sodium–Ringer solution and 2mM-NaCN, raised the cellular

Table 4. *Initial rate of uptake of various amino acids by the shocked cell preparations as a function of the ionic composition of the cells and the Ringer solution*

The rate of uptake of a given amino acid from a 1 mM solution was assayed (Eddy & Hogg, 1969) by using a Ringer solution of the composition specified. The Na^+ was replaced either by K^+ or by choline ions. The preparations were first shocked and then equilibrated (see the Materials and Methods section), either in the potassium–Ringer solution or in the standard sodium–Ringer solution, with or without 2mM-KCN and 10mM-2-deoxyglucose. Cellular [Na^+] became less than 10mequiv./l of cell water as a result of the former treatment. It increased to about 150mequiv./l after the latter treatment. When the two metabolic inhibitors were used they were also present in the test with the amino acid. A dash signifies that the system was not studied.

Preliminary treatment in	Concn. of ions in Ringer solution containing the amino acid (mequiv./l) [Na^+]	[K^+]	Initial rate of uptake of the amino acid (±S.E.M.) (nmol/min per mg) Glycine	L-Methionine	L-Phenylalanine	L-Leucine
Potassium–Ringer solution	0	160	0.44±0.13 (4)	0.36±0.05 (7)	0.51±0.07 (7)	0.51±0.06 (5)
Potassium–Ringer solution*	30	5	—	22.1 ±1.3 (2)	2.28±0.44 (5)	1.74±0.38 (5)
Potassium–Ringer solution	30	130	3.2 (1)	11.5 ±1.4 (2)	1.24±0.14 (5)	1.15±0.30 (5)
Sodium–Ringer solution	150	8	2.8 (1)	7.3 ±0.7 (3)	1.7 ±0.1 (2)	2.6 ±0.0 (2)
Sodium–Ringer solution+the metabolic inhibitors	150	8	—	3.6 ±0.7 (2)	—	—
Potassium–Ringer solution	145	13	16.6±1.3 (2)	28.5 ±3.8 (3)	5.9 ±1.0 (4)	4.6 ±0.8 (3)
Potassium–Ringer solution+the metabolic inhibitors	145	13	—	15.4 ±1.1 (6)	—	—

* Choline was present.

Table 5. *First-order rate constants characterizing the influx and efflux of* L-*phenylalanine,* L-*methionine,* L-*leucine and glycine in the absence of* Na^+ *ions*

The observations were made with shocked cell preparations suspended at 37°C in the potassium–Ringer solution, without the metabolic inhibitors (see the Materials and Methods section). The amino acid concentration specified is the initial value in the Ringer solution when the influx was observed and is the initial cellular concentration when efflux was observed. The rate constants were obtained from the slope of the line representing the natural logarithm of the difference between the cellular and extracellular concentrations of methionine as a function of time, and are means ±S.E.M. with the number of determinations in parentheses.

Amino acid	Net flux	Concn. of amino acid (mM)	Rate constant (min^{-1})
L-Phenylalanine	Influx	1	0.125±0.016 (3)
	Influx	5	0.038±0.007 (2)
	Efflux	2.9	0.042±0.005 (2)
L-Methionine	Influx	1	0.083±0.006 (7)
	Influx	5	0.056±0.004 (2)
	Efflux	3.6	0.068 (1)
Glycine	Influx	1	0.091±0.001 (2)
	Influx	5	0.047±0.006 (2)
	Efflux	3.5	0.045 (2)
L-Leucine	Influx	5	0.053±0.002 (2)
	Efflux	3.9	0.076±0.004 (2)

Table 6. *Effects of varying* $[Na^+]$ *and* $[K^+]$ *on the influx and efflux kinetics observed with* L-*methionine*

The tumour cells were first shocked in the potassium–Ringer solution, the ionic composition of the cellular phase then being manipulated by equilibrating the cells in an appropriate Ringer solution. The latter also contained 2mM-NaCN and 5mM-2-deoxyglucose as shown. L-Methionine (1–30mM) was present when the cells were to be loaded with methionine for the study of the efflux kinetics. The preparations were next transferred to a Ringer solution of the stated composition containing the metabolic inhibitors and with or without methionine (0.3–12mM) dependent on whether the influx or the efflux of the amino acid was being observed (see the Materials and Methods section). The results are further illustrated in Figs. 4 and 5. The K_m and $V_{max.}$ values for the efflux of methionine are rough estimates based on the pooled observations from three to five experiments ±S.E.M. The influx kinetics from a given experiment were analysed separately and averaged as shown. When the cellular ion contents varied considerably during the flux measurements the range of values observed is stated.

		Ion concn. (mequiv./l)					
		Extracellular		Cellular			
Metabolic inhibitors	Net flux	$[Na^+]$	$[K^+]$	$[Na^+]$	$[K^+]$	K_m for methionine (mM)	$V_{max.}$ (nmol/min per mg)
+	Influx	86	75	80–90	125	1.65±0.54 (4)	11.4±3.3 (4)
+	Efflux	86	75	80–90	125	25	40
+	Influx	150	10	10–50	170–120	0.94±0.07 (4)	27.4±1.8 (4)
+	Efflux	145	16	45–65	150–130	25	30
+	Efflux	31	133	150–100	45–100	25	58
−	Influx	150	10	10–60	170–120	0.96±0.10 (2)	41±1 (2)

content of Na^+ and slightly lowered that of K^+. The extra Na^+ absorbed with the leucine increased roughly in parallel with the amount of leucine absorbed ($P<0.01$). The induced loss of K^+ was not so correlated ($P>0.1$). The stoicheiometry was computed from the mean ionic displacements observed in the samples taken at 4–5min, when the cellular and extracellular concentrations of leucine were almost equal (Table 7). The mean value of $\Delta Na/\Delta Leu$ was smaller, though not significantly so, than that of

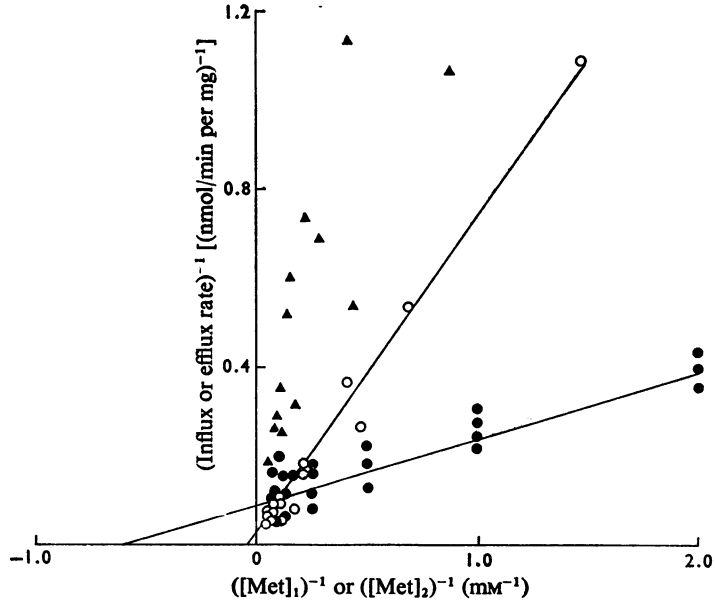

Fig. 4. *Contrast between the influx of* L-*methionine as a function of the extracellular methionine concentration* ([*Met*]$_1$) *and the dependence of methionine efflux on the cellular methionine concentration* ([*Met*]$_2$)

Methionine influx (●) occurred from a Ringer solution containing 86mequiv. of Na^+/l, 75mequiv. of K^+/l, 2mM-KCN and 5mM-2-deoxyglucose. Methionine efflux (○) occurred into a similar Ringer solution. The cellular phase contained 80–90mequiv. of Na^+/l of cellular water and about 125mequiv. of K^+/l in both series of assays. The slower rate of efflux observed in the potassium–Ringer solution lacking Na^+ is also illustrated (▲). Cellular [Na^+] was then below 10mequiv./l and cellular [K^+] was about 190mequiv./l. For further details see the Materials and Methods section.

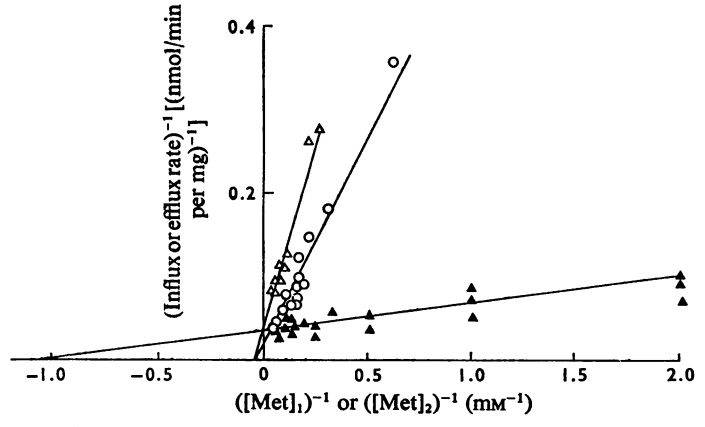

Fig. 5. *Further comparison of the efflux and influx of methionine as a function of the methionine concentration in the cellular* ([*Met*]$_2$) *or extracellular* ([*Met*]$_1$) *phases respectively*

Conditions were as for Fig. 4, but with a somewhat larger concentration of Na^+ (>100mequiv./l) in the phase that the methionine was leaving, and a lower concentration of Na^+ (<50mequiv./l) in the one that it was entering. ▲, Influx occurred from a Ringer solution containing 150mequiv. of Na^+/l, 10mequiv. of K^+/l, 2mM-NaCN and 5mM-2-deoxyglucose; cellular [Na^+] varied from 10 to 50mequiv./l; ○, efflux occurred from the cellular phase containing 150–110 mequiv. of Na^+/l into a Ringer solution containing 31mequiv. of Na^+/l, 133mequiv. of K^+/l plus the metabolic inhibitors; △, efflux occurred from the cellular phase containing 45–65mequiv. of Na^+/l into 145mequiv. of Na^+/l, with 16mequiv. of K^+/l, plus the metabolic inhibitors.

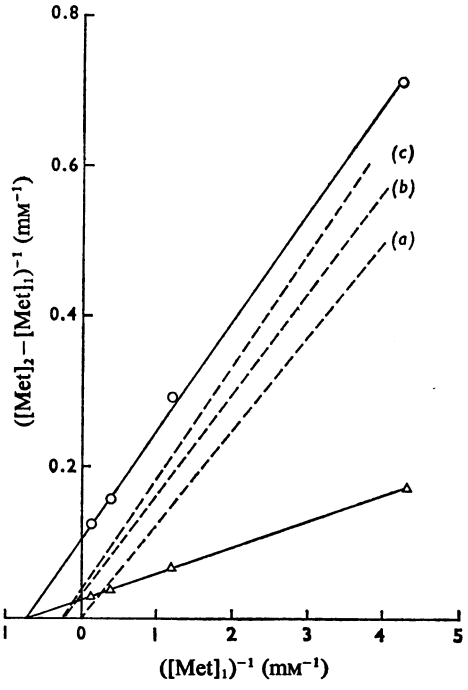

Fig. 6. *Dependence on energy metabolism of the relationship between the reciprocal of the extracellular methionine concentration* $([Met]_1)^{-1}$ *and the reciprocal of the difference between the cellular and extracellular methionine concentrations* $([Met]_2-[Met]_1)^{-1}$

Mean values from two independent series of assays are shown. The shocked preparations of the tumour cells were suspended in the sodium–Ringer solution (150mequiv. of Na^+/l; 10mequiv. of K^+/l) with 0.3, 1, 3 or 9mM-L-methionine (0.1μCi/ml) in the presence (○) or in the absence (△) of 2mM-NaCN and 5mM-2-deoxyglucose. Assays based on Method 2 showed that the maximum amount of methionine had accumulated in the cells by about 15min in the presence of the metabolic inhibitors and by about 20min in their absence. In their presence the sodium gradient ($[Na^+]_1/[Na^+]_2$) varied with $[Met]_1$ from 1.7 to 1.9. It varied from 1.5 to 2.2 in their absence. The mathematical model described in the text was used to compute the positions of the three discontinuous lines. Line (*a*) is based on eqn. (4) with $P=P'=1$, the supposition being that the amino acid enters the cells only in conjunction with Na^+. Line (*b*) shows how the accumulation of the amino acid, produced by the same ionic gradients ($[Na^+]_1/[Na^+]_2=150/70$; $[K^+]_1=10$, $[K^+]_2=120$mequiv./l; $\theta=0.04$), would be lowered when a Na^+-independent leak pathway ($k=0.05\,min^{-1}$) operated in parallel with the Na^+-dependent mechanism. Discontinuous line (*c*) is based on a faster leak pathway ($k=0.1\,min^{-1}$). The computations used the carrier parameters describing the behaviour of glycine (Eddy & Hogg, 1969), as those for methionine have not been determined.

Table 7. *Changes in the cellular contents of* Na^+, K^+ *and water accompanying the absorption of* L-*leucine in the presence of* 2mM-*NaCN*

The shocked cell preparations absorbed the specified amount (ΔLeu) of L-leucine during 4–5min at 37°C spent in the presence of a 10mM solution of the amino acid in the standard sodium–Ringer solution. The changes listed (ΔNa^+, ΔK^+, Δwater) are increases (±S.E.M.) relative to the controls from which the amino acid was omitted. The results of six independent assays were averaged. Eddy (1968*a*) describes the procedure used.

ΔNa^+ (nequiv./mg dry wt. of cells)	57.7±10.4
ΔK^+ (nequiv./mg)	−17.5±4.6
ΔLeu (nmol/mg)	79.2±2.1
ΔWater (μl/ml of cellular water)	54.1±11.2
$\Delta Na^+/\Delta Leu$	0.718±0.118
$\Delta K^+/\Delta Leu$	−0.224±0.058

0.89±0.11 (5) found for glycine with unshocked cellular preparations (Eddy, 1968*a*). Leucine also displaced less K^+ ($P<0.01$) than did glycine. The values of $\Delta Na/\Delta Leu$ might be corrected for the amount of amino acid absorbed without Na^+ through pathways functioning in parallel with the carrier system, a quantity not greater than about 10nmol of leucine/mg dry wt. (Table 5). The mean value of $\Delta Na/\Delta Leu$ would then be 0.84. Although the present evidence is thus consistent with a model in which 1equiv. of Na^+ is absorbed through the amino acid carrier with both L-leucine and glycine, the scatter of the observations means that a smaller coupling ratio for leucine in particular is not excluded.

The extra amount of water absorbed in the presence of leucine exceeded, by the equivalent of 9.92±1.85 (S.E.M.; 6)mosmol of solute/ml of cellular water, the amount expected on the basis of the observed movements of Na^+, K^+ and leucine itself. The above quantity roughly equals ($P>0.5$) the fraction of the Na^+ ions (6.83±2.03mosmol) that was not neutralized by the efflux of K^+. This would be consistent with electroneutrality being maintained by the uptake of Cl^- ions.

Concentration of amino acids by preparations lacking Na^+ *ions*

Potashner & Johnstone (1970) showed that during 60min at 25°C the Ehrlich ascites tumour concentrated L-methionine about twofold from a 2mM

solution in iso-osmotic choline chloride solution lacking Na^+ ions. Because the process required ATP, these workers suggested that the presence of ATP was a more significant factor than the magnitude of the Na^+ ion gradient in driving the amino acid pump. Our ascites strain behaved similarly at 37°C in a Ringer solution containing 1.2mM-$MgSO_4$, 140mM-choline chloride and 20mequiv. of K^+/l with 8mM-potassium phosphate buffer, pH7.4. The ratio of the cellular to extracellular concentration of amino acid at 40–45min was 3.29±0.16 (S.E.M.; 3) when the Ringer solution contained 0.3mM-methionine. It was 2.02±0.10 (2) with 1mM- and 1.65±0.10 (2) with 3mM-methionine. The extent to which methionine was concentrated from a 1mM solution was lowered to 0.84±0.02 (2) in the presence of 2mM-NaCN. It was lowered to 1.13±0.04 (6) when the choline was replaced by an equivalent of KCl. Similar observations were made with 2-aminoisobutyrate. An accumulation ratio of 2.94±0.01 (2) was observed with a 0.3mM solution of 2-aminoisobutyrate and one of 2.31±0.22 (3) with a 1mM solution. A possible function for the ionic gradient of K^+ across the cell membrane seems to be indicated, therefore, for amino acid transport under these conditions. Thus the fact that 'uphill' movements of the amino acids take place in the absence of Na^+ may not be incompatible with the gradient hypothesis.

Discussion

The present observations show very clearly that, having been depleted of both ATP and most of their endogenous amino acid content, the shocked tumour cells accumulated relatively large amounts of various amino acids. The argument applies to L-alanine, 2-aminoisobutyrate, glycine, L-leucine, L-methionine, L-phenylalanine and L-serine. We have shown that the increase in the cellular pool of amino acids was roughly equivalent to the amount of ^{14}C absorbed. Hence the process did not depend on exchange diffusion. The amino acid was absorbed without being chemically modified in the four examples studied, so that factor can also be excluded. The combined evidence also makes it unlikely that the amino acid pumps in these preparations were driven by residual energy metabolism. (1) The cellular ATP content, as we confirmed in the present work with shocked cells, was relatively small (Eddy & Hogg, 1969). (2) Both respiration and glycolysis of endogenous substrates were greatly impaired (Eddy *et al.*, 1967). Whittam & Wheeler (1970) have suggested that the known rapidity of glycolysis in tumour cells in general means that ATP was likely to have been available from that source in our preparations. Since an appropriate nutrient reserve is, however, required for that purpose, glycolysis may have been restricted both in these and our earlier preparations (Eddy *et al.*, 1967) by the lack of such compounds, as indeed is indicated by the rapid formation of lactate that followed the addition of glucose. (3) Various metabolic inhibitors, including F^- ions and 2-deoxyglucose that might have further restricted endogenous glycolysis, had virtually no effect on the accumulation of glycine in the presence of CN^- (Eddy *et al.*, 1967; Eddy & Hogg, 1969). Table 2 summarizes various observations with deoxyglucose that extend that conclusion to the other amino acids. (4) Johnstone (1972) has suggested that ATP formed by the reversal of the reactions of the sodium pump might drive the amino acid pump in these preparations. The various arguments considered below in relation to the lack of effect of ouabain and oligomycin on the accumulation of methionine and glycine by the depleted preparations make that proposal unacceptable, however. We suggest therefore that ATP was not implicated in the working of the amino acid pump under these specific conditions.

Role of the ionic gradients

All the available evidence seems consistent with the view (Eddy, 1968*b*) that the amino acid pump, in the preparations depleted of ATP, was driven by the energy inherent in the alkali-cation gradients acting between the cellular and extracellular phases. There are two important considerations. (1) The ion-gradient hypothesis is based on the notion that Na^+ and perhaps K^+ not merely associate with the amino acid carrier, but may be translocated through that carrier down their respective gradients of chemical potential. The accelerated uptake of Na^+ that occurred when leucine was absorbed by the shocked cellular preparations depleted of ATP (Table 7) is thus specially significant. It parallels the induced flow of Na^+ accompanying the absorption of glycine by unshocked cellular preparations held in similar conditions (Eddy, 1968*a*, 1969). In contrast, the relative efflux of K^+ during the absorption of leucine was both small and less than that which occurred in the assays with glycine (Eddy, 1968*a*). Hence, despite the observations in Table 4 showing that the presence of K^+ decisively lowered the rate of uptake of leucine, the contribution of the K^+ ion gradient to the forces driving the leucine pump under these conditions was probably small. We are quite unable to accept the suggestion (Schultz & Curran, 1970) that the gradient of K^+ might directly contribute to the energy requirement of the amino acid pump in the absence of a coupled movement of K^+ through the system (see Eddy & Hogg, 1969).

The contribution of the gradient of K^+ to the forces driving the accumulation of both methionine and glycine is in any case small in the presence of CN^- (Eddy, 1972). It might even be zero if a correction were applied for the effect of the membrane potential

(Gibb & Eddy, 1972), although further work is required before firm conclusions can be drawn. Accordingly, it seems possible that the somewhat smaller accumulation ratios observed with leucine, compared with methionine or glycine, when $[Na^+]_1/[Na^+]_2$ was near 2 in the preparations depleted of ATP (Fig. 1), reflect the relative importance in these cases of the 'external leaks' that we discussed in connexion with Fig. 6.

(2) The second consideration in favour of the view that the ionic gradients drive the amino acid pumps in the preparations depleted of ATP is that the magnitude and direction of the amino acid gradient varied systematically with the magnitude of the gradient of Na^+ in the tests with a series of seven amino acids (Fig. 1). Only one of these had previously been studied in detail (Eddy, 1968*b*).

Ouabain and the role of ATP

In one model that has been critically examined in the literature, the amino acid enters the tumour cells with 1 equiv. of Na^+, 1 equiv. of K^+ meanwhile leaving them. When a steady state is reached $[A]_2[Na^+]_2[K^+]_1/[A]_1[Na^+]_1[K^+]_2 \leqslant 1$ (Eddy, 1968*a*; Jacquez & Schafer, 1969). Observations made with 2-aminoisobutyrate (Schafer & Heinz, 1971), with methionine (Gibb & Eddy, 1972) and with glycine (Johnstone, 1972) were, however, incompatible with that hypothesis especially when $[Na^+]_2$, $[K^+]_2$ and $[K^+]_1$ were all relatively large and the ratio $[K^+]_2/[K^+]_1$ was relatively small, for the value of the above function of $[A]_2/[A]_1$ then varied from 3 to 10 during energy metabolism. Such a model, or one in which the influx of the amino acid was only partially coupled to the efflux of K^+, nevertheless adequately described the way $[A]_2/[A]_1$ varied with the ionic gradients in various other circumstances (Jacquez & Schafer, 1969; Reid & Eddy, 1971). Therefore it may be premature to reject altogether the notion that the K^+ ion gradient may be coupled to the amino acid pump, even if, as Gibb & Eddy (1972) suggest, little coupling occurred in the above situation where $[Na^+]_2$ was large.

When $[Na^+]_2$ was large and $[K^+]_2/[K^+]_1$ was nearly 1, ouabain lowered the value of the above function from a number greater than 4.7 to about 2.5, as did both the depletion of the cellular ATP content and the presence of valinomycin (Gibb & Eddy, 1972; Schafer & Heinz, 1971). Comparison with the present observations leads to the view that ouabain restricted amino acid absorption only during energy metabolism.

The behaviour of the cellular preparations depleted of ATP, in which $[K^+]_2/[K^+]_1$ was large, was also specially significant in that valinomycin then increased, by up to threefold, the absorption of various amino acids (Gibb & Eddy, 1972). The methionine gradient, for instance, reached values in the physiological range. Such behaviour strongly suggests: (1) that the coupling of the amino acid flux to the efflux of K^+ was small in the absence of valinomycin. (2) At least when ATP was lacking, the coupling between the Na^+ ion gradient and the amino acid gradient appears to have been sufficiently close to provide a relatively efficient means of concentrating the amino acid. It is precisely the latter point that has recently been questioned (Schafer & Heinz, 1971; Johnstone, 1972). (3) The response to the ionophore indicates that the membrane potential may be an important factor governing the uptake of the amino acids.

To explain the behaviour with ouabain during energy metabolism, Gibb & Eddy (1972) proposed that, when $[Na^+]_2$ was relatively large, the electrogenic extrusion of Na^+ through the sodium pump elevated the membrane potential, above the values that previous workers have considered in connexion with this problem (cf. Jacquez & Schafer, 1969). The presence of ouabain in these circumstances would be expected therefore to retard amino acid absorption by lowering the membrane potential, quite apart from any subsequent effects due to changes in $[Na^+]_2$. Ouabain would thus inhibit the system only when ATP was available, as indeed the present observations indicate. The acceleration of glycine efflux that Eddy *et al.* (1967) detected, either in the presence of ouabain or when $[K^+]_1$ was lowered, might also be explained in terms of changes in the membrane potential.

The provision of ATP increased the rate of uptake of glycine (Eddy & Hogg, 1969) and, as Table 6 indicates, that of methionine as well (Potashner & Johnstone, 1971). In circumstances where the Na^+ ion gradient was roughly constant, energy metabolism also increased the maximum accumulation ratio for glycine about threefold (Eddy, 1968*b*), that for L-methionine three- to four-fold (Fig. 6) and that for L-leucine about twofold (M. Reid, unpublished work). Examination of the results in Tables 1 and 2 suggests that the various neutral amino acids are qualitatively similar in that respect. It is not clear at present whether these latter effects can be attributed to changes in the membrane potential or whether, as suggested by Reid & Eddy (1971), either ATP or a related compound regulates the coupling between the flows of Na^+ and K^+ and the flow of the amino acid.

A different view of these problems has been proposed by Kimmich (1970), who worked with intestinal epithelial cells. In his scheme the amino acid pumps were driven directly by the hydrolysis of ATP, some of the energized components involved being shared with the sodium pump. When ATP was in short supply the flow of Na^+ and K^+ back through the sodium pump was supposed to generate these same components, which might then be used to drive the amino acid pump. Kimmich (1970) emphasized that

whereas his model reproduced certain aspects of the behaviour of the depleted mouse tumour cells that were consistent with the ion-gradient hypothesis (Eddy, 1968*b*), the scheme used ATP rather than the ionic gradients to drive the amino acid pump in physiological circumstances. Johnstone (1972) has recently made a similar suggestion in which ATP itself was the compound shared with the sodium pump. The functions that Kimmich (1970) and Johnstone (1972) envisage for the sodium pump in the absorption of the amino acids by our depleted preparations are inconsistent, however, (1) with the present observations showing that both ouabain and oligomycin failed to inhibit the absorption of glycine and methionine in the presence of CN^- and deoxyglucose. They are also inconsistent with: (2) the evidence for the role of Na^+ as a co-substrate of the glycine pump in the presence of both 0.1mM-ouabain and 2mM-NaCN (Eddy, 1969); (3) the symmetry properties of the sodium pump (Eddy & Nowacki, 1971); (4) the fact that low concentrations of K^+ that activate the sodium pump fail to inhibit the amino acid pump when ATP is in short supply (Eddy & Hogg, 1969).

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