Ionophore-Mediated Coupling between Ion Fluxes and Amino Acid Absorption in Mouse Ascites-Tumour Cells

RESTORATION OF THE PHYSIOLOGICAL GRADIENTS OF METHIONINE BY VALINOMYCIN IN THE ABSENCE OF ADENOSINE TRIPHOSPHATE

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1. Preparations of mouse ascites-tumour cells depleted of ATP and Na^+ ions accumulated L-methionine, in the presence of cyanide and deoxyglucose, from a 1 mM solution containing 80 mequiv. of Na^+ /l and about 5 mequiv. of K^+ /l. Valinomycin increased, from about 4 to 16, the maximum value of the ratio of the cellular to extracellular concentrations of methionine formed under these conditions without markedly affecting the distributions of Na^+ and of K^+ . Similar observations were made with 2-aminoisobutyrate, glycine and L-leucine. Increasing the extracellular concentration of K^+ progressively decreased the accumulation of methionine in the presence of valinomycin. Over the physiological range of ionic gradients, the system behaved as though the absorption of methionine with Na^+ was closely coupled to the electrogenic efflux of K^+ through the ionophore. The process was insensitive to ouabain and so the sodium pump was probably not involved. 2. The amount of methionine accumulated during energy metabolism was similar to the optimal accumulation in the presence of valinomycin when ATP was lacking. It was also similarly affected by increasing the methionine concentration. 3. A mixture of nigericin and tetrachlorosalicylanilide mimicked the action of valinomycin. The anilide derivative inhibited the absorption of 2-aminoisobutyrate in the presence of valinomycin, but not in its absence. 4. Gramicidin inhibited methionine absorption and caused the preparations to absorb Na^+ and lose K^+ . 5. The observations appear to verify the principle underlying the gradient hypothesis by showing that the tumour cells can efficiently couple the electrochemical gradient of Na^+ to the amino acid gradient.

Mouse ascites-tumour cells that are depleted of Na^+ ions, ATP and endogenous amino acids may accumulate various amino acids from an appropriate Ringer solution, against their respective concentration gradients, provided that the extracellular concentration of Na^+ is large. The spontaneous flow of Na^+ into the tumour cells appeared to provide one portion of the energy driving the amino acid pumps under these conditions, the remaining portion being associated either with the related gradient of K^+ ions or with the factors determining the resting membrane potential (Morville *et al.*, 1973). The physiological significance of the above behaviour is complicated by two other circumstances. First, at least 2 or 3 times as much of a given amino acid may accumulate in the tumour cells during energy metabolism as is absorbed by the preparations depleted of ATP in the presence of cyanide and 2-deoxyglucose (Eddy, 1968*b*; Morville *et al.*, 1973). Secondly, the amino acid may become concentrated in the tumour cells during energy metabolism even when the concentration gradients of Na^+ and K^+ acting across the plasma membrane are both relatively small (Schafer

& Heinz, 1971). However, it has been suggested that all the observations can be reconciled with the ion-gradient hypothesis in which the possible role of the membrane potential is taken into account (Gibb & Eddy, 1972). Nevertheless, other workers favour the view that during energy metabolism the hydrolysis of ATP, rather than the flow of the ions, is the main factor driving the amino acid pumps, and that the coupling between these and the ionic gradients, in so far as it occurs at all, is inefficient (Johnstone, 1972; Geck *et al.*, 1972). Indeed, this possibility has prompted the study of the effects of selected amino acids on the hydrolysis of ATP in the presence of fragments of the plasma membrane of Ehrlich ascites-tumour cells (Forte *et al.*, 1973; Colombini & Johnstone, 1973; Ronquist & Christensen, 1973). A model of such an ATPase (adenosine triphosphatase) system has been proposed (Kimmich, 1973), but appears to be inconsistent with certain aspects of the absorption of amino acids by mouse ascites-tumour cells (Morville *et al.*, 1973) and by pigeon erythrocytes (Terry & Vidaver, 1973).

The discovery that the K^+ -selective ionophore

valinomycin markedly stimulated the absorption of various amino acids by the tumour cells, in the virtual absence of ATP, provided a new experimental approach to the problem of testing the gradient hypothesis. Preliminary work suggested that the maximum gradients of amino acid concentration formed in the presence of valinomycin were indeed similar to the maximum values reached during energy metabolism in the absence of the antibiotic (Gibb & Eddy, 1972). Hence it seemed that the coupling between the ionic gradients and the flow of amino acid in these particular circumstances was relatively efficient.

The present work was done to investigate the preparations lacking ATP by further defining the action of valinomycin in terms of its presumed effect on the membrane potential acting across the plasmalemma. We have examined how the amino acid gradients responded to (a) changes in the gradient of K^+ across the plasmalemma, (b) the simultaneous presence of various other metabolic inhibitors and (c) the response to ionophores other than valinomycin. Current ideas about the mechanism of action of various ionophores have been reviewed by Henderson (1971) and by Haydon & Hladky (1972).

Materials and Methods

These were exactly as described by Morville *et al.* (1973). The standard Ringer solution contained 155 mequiv. of Na^+ /litre, 8 mequiv. of K^+ /l, 1.2 mM- $MgSO_4$, 16 mM-sodium phosphate and no glucose. A potassium-Ringer solution contained 159 mequiv. of K^+ /l without added Na^+ ions. Cellular ATP was depleted by incubating the tumour cells in the potassium-Ringer solution (containing 2 mM- $NaCN$) for 25 min at 37°C, 2 mM-2-deoxyglucose being present for the last 10 min. To decrease the amount of K^+ transferred with the tumour cells, these (1 vol.) were then suspended in a Ringer solution (10 vol.) at 0°C containing 83 mequiv. of Na^+ /l, 2 mM- $NaCN$, 80 mM-choline chloride, 1.2 mM- $MgSO_4$ and 16 mM-sodium phosphate. The cells were quickly separated by centrifugation and transferred to an appropriate Ringer at 37°C containing 5 mM-2-deoxyglucose, 2 mM- $NaCN$, the specified amino acid labelled with ^{14}C and the other compounds specified in the text. Samples (5 ml) containing 25–40 mg dry wt. of cells were taken at the stated intervals and treated as described by Morville *et al.* (1973) to assay the cellular contents of Na^+ , K^+ , water and ^{14}C . The values given in the text are means $\pm$ S.E.M. derived from the stated number of independent assays.

Chemicals

Valinomycin, oligomycin and gramicidin (lot 85B-0970) were obtained from Sigma Chemical Co.,

St. Louis, Mo., U.S.A. Dr. Harned of the Research Department, Commercial Solvents Corp., Terre Haute, Indiana, U.S.A., kindly gave us a sample of compound X464, which is identical with nigericin (Henderson, 1971). Dr. W. A. Hamilton of the Department of Biochemistry, University of Aberdeen, Scotland, U.K., gave us a sample of tetrachlorosalicylanilide (Hamilton, 1970). Carbonyl cyanide *m*-chlorophenylhydrazone was obtained from Calbiochem Ltd., London W.1, U.K. Of the above compounds, valinomycin and oligomycin were added to the system in solution in ethanol, whereas the other compounds were dissolved in dimethyl sulphoxide (0.15 ml of solution/30 ml of Ringer). Similar amounts of the solvent were added to the controls, apparently without affecting their behaviour.

Experimental and Results

Methionine accumulation as a function of extracellular $[K^+]$ in the presence of valinomycin

Fig. 1 shows that when extracellular $[K^+]$ ($[K^+]_1$) was 4.5 mequiv./l the absorption of L-methionine from a 1 mM solution was 3–4 times larger in the presence than in the absence of 10 μ g of valinomycin/ml of Ringer. Other work showed that at least 4 μ g of valinomycin/ml of Ringer solution was required to produce the above effect. Fig. 1 also shows that increasing $[K^+]_1$ from 5 mequiv./l, through 20 mequiv./l, to 115 mequiv./l progressively decreased the maximum value of the ratio of the cellular to the extracellular concentrations of methionine ($[Met]_2/[Met]_1$).

In the assays shown in Fig. 2, $[K^+]_1$ was increased from 8 to 68 mequiv./l after a large amount of methionine had been absorbed, the addition of the amino acid being delayed for 15 min in two further preparations that served as controls. The fact that increasing $[K^+]_1$ in this way caused more than 70% of the amino acid to leave the tumour cells further illustrates the dependence of the ratio $[Met]_2/[Met]_1$ on $[K^+]_1$ and $[K^+]_2$, as shown in the behaviour of the control preparations.

Quantitative aspects. We shall suppose that 1 equiv. of Na^+ is absorbed with the amino acid (Eddy, 1968a), that no coupling with K^+ occurs within the amino acid carrier itself (Eddy, 1968b), either in the presence or the absence of the ionophores, and that the methionine gradient is governed simply by the gradient of electrochemical potential of Na^+ (Gibb & Eddy, 1972) acting across the plasmalemma (see Scheme 1). By definition the electrochemical gradient of Na^+ includes terms for (a) the actual concentrations of Na^+ inside and outside the tumour cells and (b) the membrane potential (V). In these circumstances and with the additional assumption that the various activity coefficients each have a

Fig. 1. Effect of valinomycin on the accumulation of methionine from Ringer solutions containing different concentrations of K^+

Subscript 1 denotes the extracellular phase, subscript 2 the cellular phase. The tumour cells were kept at 37°C for 25 min in a Ringer solution that contained 2 mM-NaCN and either 163 mequiv. of Na^+/l (●, ○), or 163 mequiv. of K^+/l (▼, ▲, △). 2-Deoxyglucose (2 mM) was added after 15 min. The preparations were then briefly resuspended at 0°C with 85 mequiv. of Na^+/l , 75 mM-choline chloride and 2 mM-NaCN. They were next transferred to an appropriate Ringer solution at 37°C . About 5 mg dry wt. of tumour cells/ml was used. The solutions contained, together with 2 mM-NaCN, 5 mM-2-deoxyglucose and 1 mM- ^{14}C -methionine, the following concentrations of Na^+ and K^+ : 85 mequiv. of Na^+/l , 5 mequiv. of K^+/l and 70 mM-choline chloride (▲, △); 85 mequiv. of Na^+/l , 22 mequiv. of K^+/l and 55 mM-choline chloride (▼); 45 mequiv. of Na^+/l and 115 mequiv. of K^+/l (○, ●). The closed symbols indicate that $10\mu\text{g}/\text{ml}$ of valinomycin was present, the open symbols that valinomycin was absent. The effect of valinomycin was studied in paired assays, on five occasions with 85 mequiv. of Na^+/l and on two occasions with 45 mequiv. of Na^+/l . Other experimental details are described by Morville *et al.* (1973). When $[\text{Met}]_2/[\text{Met}]_1$ reached a maximum value, $[\text{K}^+]_2/[\text{K}^+]_1$ was respectively about: 15, ▲; 5, ▼; 26, △; 1, both ○ and ●. $[\text{Na}^+]_1/[\text{Na}^+]_2$ was respectively about: 1.2, ▲; 1.0, ▼; 1.5, △; 0.5, ●; 0.4, ○. The relationship between the amino acid gradient and the ionic gradients is further described in Fig. 3.

value of 1, eqn. (4) of Morville *et al.* (1973) leads to the relationship:

$$[\text{Met}]_2/[\text{Met}]_1 \leq e^{-VF/RT} \cdot [\text{Na}^+]_1/[\text{Na}^+]_2 \quad (1)$$

Here F , R , T and the sign of V have their conventional significance and the inequality sign takes into account the possibility that the gradient of Na^+ may be

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Fig. 2. Effect of changing the ionic conditions after methionine had accumulated in the presence of valinomycin

The general procedure was as described in Fig. 1. A preparation of hypo-osmotically shocked tumour cells depleted of ATP and Na^+ (Morville *et al.*, 1973) was added with 1 mM- ^{14}C -methionine to a Ringer containing 2 mM-NaCN, 5 mM-2-deoxyglucose, 154 mequiv. of Na^+/l , 8 mequiv. of K^+/l and $10\mu\text{g}$ of valinomycin/ml. One portion of the cellular preparation was transferred to a similar solution after 15 min (○). Another portion (●) was then transferred to a Ringer containing 68 mequiv. of K^+/l , 94 mequiv. of Na^+/l and the other constituents. In the latter case methionine was expelled from the cells. The addition of methionine was delayed in two other series (△, ▲) in which the tumour cells were initially kept in the Ringer containing 154 mequiv. of Na^+/l for 15 min. One preparation (△) was then transferred to a similar solution with 1 mM- ^{14}C -methionine. The other (▲) was transferred to the Ringer containing 68 mequiv. of K^+/l and 1 mM- ^{14}C -methionine. The value of $[\text{Na}^+]_1/[\text{Na}^+]_2$ was near to 1 at 30 min in each of the four preparations. $[\text{K}^+]_2/[\text{K}^+]_1$ was 8–9 in one pair (○, △) and near 2 in the other pair (●, ▲). The values of $[\text{Met}]_2/[\text{Met}]_1$ are means derived from two independent experiments.

imperfectly coupled to the amino acid gradient. In the presence of valinomycin the value of V might approach the equilibrium potential for K^+ , namely $RT/F(\ln[\text{K}^+]_1/[\text{K}^+]_2)$. Eqn. (1) then becomes:

$$[\text{Met}]_2/[\text{Met}]_1 \leq [\text{Na}^+]_1[\text{K}^+]_2/[\text{Na}^+]_2[\text{K}^+]_1 \quad (2)$$

We shall also use the factor $F = [\text{Met}]_2[\text{Na}^+]_2/[\text{Met}]_1[\text{Na}^+]_1$ in discussing the experimental observations. This is the component of the methionine

gradient not attributable to the gradient of Na^+ concentration in the above sense (Gibb & Eddy, 1972). The presence of valinomycin would thus increase the value of F provided that the ionophore raised the membrane potential.

Fig. 3. Effect of valinomycin on the variation of the maximum amino acid accumulation ratio $[Met]_2/[Met]_1$ with the prevailing sodium ion gradient $[Na^+]_1/[Na^+]_2$ and potassium ion gradient $[K^+]_2/[K^+]_1$.

The tumour cells were depleted of ATP and, in most cases, of Na^+ as well. They were then added to a Ringer solution containing 1 mM- ^{14}C methionine, valinomycin (10 $\mu\text{g}/\text{ml}$), 2 mM-NaCN and 5 mM-deoxyglucose (see Fig. 1). The solution contained 150 mequiv. of Na^+/l , 10 mequiv. of K^+/l in nine assays (▲). In another 44 assays, it contained about 80 mequiv. of Na^+/l , with about 3, 10, 20, 60 or 110 mequiv. of K^+/l (●). In four further assays in which $[Met]_2/[Met]_1$ varied from 0.7 to 3, 40 mequiv. of Na^+/l and 120 mequiv. of K^+/l were used (●). Certain of the above preparations were depleted of endogenous amino acids by means of a hypo-osmotic shock (Morville *et al.*, 1973), a treatment that appeared to change $[Met]_2/[Met]_1$ only in so far as the ionic gradients themselves were changed. Another set of 38 observations, some of which have already been described by Morville *et al.* (1973), were made in the absence of valinomycin. The Ringer solution contained 80 mequiv. of Na^+/l (○), or 150 mequiv. of Na^+/l (Δ), with up to 70 mequiv. of K^+/l . (a) shows how $[Met]_2/[Met]_1$ varied with the product of the two ionic gradients, whereas (b) shows the part ($F = [Met]_2[Na^+]_2/[Met]_1[Na^+]_1$) attributed to factors other than the gradient of Na^+ . The discontinuous lines represent equal ordinates and abscissae.

To test the relationships, the gradient of K^+ was systematically varied over a 30-fold range, in conditions where the gradient of Na^+ varied only about twofold (Fig. 3). One series of 44 observations was made under conditions like those shown in Fig. 1, in which $[Na^+]_1$ was 80 mequiv./l and $[Na^+]_1/[Na^+]_2$ varied from 0.6 to 1.3. A second series of nine observations used 150 mequiv. of Na^+/l and about 10 mequiv. of K^+/l , and the ratio of $[Na^+]_1/[Na^+]_2$ varied from 1 to 2. Fig. 3(a) shows that, in the presence of valinomycin, when the product of the ionic gradients ($[Na^+]_1[K^+]_2/[Na^+]_2[K^+]_1$) was relatively large, the maximum accumulation of methionine approached the 15–20-fold gradients that form during energy metabolism (Morville *et al.*, 1973). Decreasing the ionic gradients progressively decreased the methionine gradient ($r = 0.875$; $n = 48$; $P < 0.001$). When 80 mequiv. of Na^+/l was used the amino acid gradient was in most instances consistent with the above equation. An estimate of the efficiency of the coupling with the ionic gradients was made by plotting the logarithm of the amino acid gradient (y) against the logarithm of the product (x) of the ionic gradients (cf. Eddy, 1968b). The observations (Fig. 3) fitted the equation:

$$y = 0.745 \pm 0.035x + 0.148 \pm 0.034 \quad (r = 0.952, n = 48)$$

Thus the coupling appeared to be about 74% efficient when $[Na^+]_1$ was 80 mequiv./l. A similar analysis of 13 assays with glycine gave the result:

$$y = 0.610 \pm 0.104x + 0.14 \pm 0.08 \quad (r = 0.870, n = 13)$$

Thus an efficiency of about 61% was indicated in that instance.

Similar relationships between the amino acid gradient and the ionic gradients were also observed with various amino acids in the absence of valinomycin (Morville *et al.*, 1973). Fig. 3(a) shows that under these conditions less methionine was absorbed. In 61 such assays, $[Na^+]_1/[Na^+]_2$ varied from about 1 to 2.6 and the ratio F , defined as above, increased by a factor of 3 with $[K^+]_2/[K^+]_1$ ($r = 0.759$; $P < 0.001$). Clearly the ratio $[Met]_2/[Met]_1$ showed a much larger dependence on $[K^+]_2/[K^+]_1$ when valinomycin was present (Fig. 3b).

The tumour cells accumulated relatively more methionine when the Ringer contained 150 mequiv. of Na^+/l (Fig. 3) and the above equation did not then seem to apply (see below).

Effect of methionine concentration: comparison with the respiring system. The above estimates of the efficiency of the coupling between the amino acid flux and the ionic fluxes were made with 1 mM-methionine. The maximum value of $[Met]_2/[Met]_1$ decreased by about 50%, however, when $[Met]_1$ was increased to 5 mM, in circumstances where the product of the ionic gradients was very roughly constant (Table 1). Similar behaviour has been reported before

Table 1. *Methionine accumulation in the presence of valinomycin as a function of extracellular methionine concentration ($[Met]_1$)*

After being starved in the potassium-Ringer solution in the presence of cyanide and deoxyglucose, the preparations were transferred to a Ringer containing 80mequiv. of Na^+/l , about 4mequiv. of K^+/l , 10 μg of valinomycin/ml, the indicated concentration of $L-[^{14}C]$ methionine and the other metabolic inhibitors (see Fig. 1). The mean maximum value ($\pm S.E.M.$) of $[Met]_2/[Met]_1$ was determined with the number of experiments in parentheses. $F = [Met]_2[Na^+]_2/[Met]_1[Na^+]_1$. The ratio $[K^+]_2/[K^+]_1$ is a measure of the gradient of K^+ . Subscript 1 denotes the extracellular phase, subscript 2 the cellular phase.

$[Met]$ (mm)	$[Met]_2/[Met]_1$	F	$[K^+]_2/[K^+]_1$
0.1	29.6	29.6	79
0.1	19.0	17.9	33
0.1	14.2	14.8	31
0.1*	45.0	17.3	23
1.0	12.3 ± 1.2 (18)	12.8 ± 1.3	18 ± 2
2.0	7.61 ± 0.46 (3)	9.3 ± 0.6	23 ± 5
5.0	4.79 ± 0.18 (3)	6.2 ± 0.4	20 ± 4

* Na^+ (150mequiv./l) was used with the hypo-osmotically shocked tumour cells.

and underlies the so-called pump-leak model of these systems (Johnstone & Scholefield, 1965). The model predicts that $([Met]_2 - [Met]_1)^{-1}$ varies linearly with $[Met]_1^{-1}$, in such a way that $[Met]_2 - [Met]_1$ reaches a maximum value when $[Met]_1$ is very large. This can be regarded as the maximum difference in amino acid concentration that the system produces, a quantity that is not to be confused with the ratio $[Met]_2/[Met]_1$. Morville *et al.* (1973) found that whereas the maximum value of $[Met]_2 - [Met]_1$ was about 40mM during respiration in the standard Ringer solution, it was only 9mM when cyanide and deoxyglucose were both present to stop energy metabolism. The observations shown in Table 1,

except for those involving 0.1mM-methionine, were plotted in that form. They corresponded to a maximum value of $[Met]_2 - [Met]_1$ of about 36mM. We conclude that the presence of valinomycin with the other metabolic inhibitors restored the concentration gradient to values similar to those observed during energy metabolism.

The variation of $[Met]_2/[Met]_1$ with $[Met]_1$ itself would be expected if the fraction of the methionine entering the tumour cells with Na^+ ions decreased at the larger values of $[Met]_1$ (Morville *et al.*, 1973).

The above analysis indicated that the behaviour of the tumour cells in the presence of valinomycin and 80mequiv. of Na^+/l was consistent, in general, with a pump-leak model for which $[Met]_2/[Met]_1 < [Na^+]_1/[K^+]_2/[Na^+]_2/[K^+]_1$. However, six of the nine observations made with $[Na^+]_1$ at 150mequiv./l deviated from that pattern, in that the amino acid gradient was actually larger than the product of the ionic gradients (Fig. 3). The assay of the cellular water contents showed that the tumour cells did not contract rapidly, a process that might have resulted in $[Met]_2$ becoming larger than the equation predicted. A further possibility is that our estimates of the average cellular concentrations of both Na^+ and K^+ did not apply to the region of the cell surface in which the amino acid pump functioned (Schafer & Heinz, 1971; Eddy, 1968b). Because the problem has not been resolved, however, the question arises whether the number of Na^+ ions absorbed with each equivalent of methionine can somewhat exceed the value of one that has hitherto seemed most likely for this system (Eddy, 1968a; Schafer & Jacquez, 1967).

Lastly, four assays were done with 0.1mM-methionine. The amount of amino acid absorbed tended to reach a maximum earlier than when a higher methionine concentration was employed. Because in these experiments $[K^+]_2/[K^+]_1$ was then also relatively large, $[Met]_2/[Met]_1$ was correspondingly large (Table 1). A transient 30–40-fold methionine gradient is indeed

Table 2. *Effect of valinomycin on the accumulation of L-methionine, glycine, L-leucine and 2-aminoisobutyrate*

A 1mM solution of the selected amino acid was used in assays like those described in Table 1. The Ringer solution contained either about 80 or 150mequiv. of Na^+/l . The maximum value ($\pm S.E.M.$) of the amino acid accumulation ratio ($[A]_2/[A]_1$) was determined with the number of experiments in parentheses. $F = [A]_2[Na^+]_2/[A]_1[Na^+]_1$.

Amino acid	Valinomycin	$[Na^+]_1$	$[A]_2/[A]_1$	F	$[K^+]_2/[K^+]_1$
L-Methionine	—	80	4.56 ± 0.16 (20)	3.21 ± 0.15	29 ± 2
	+	80	12.3 ± 1.2 (18)	12.8 ± 1.3	18 ± 2
	—	150	5.60 ± 0.3 (8)	2.78 ± 0.14	10 ± 1
	+	150	18.8 ± 1.2 (9)	13.2 ± 1.0	11 ± 1
2-Aminoisobutyrate	—	150	7.2 ± 1.0 (3)	2.5 ± 0.2	15 ± 2
	+	150	22.8 ± 1.8 (5)	10.4 ± 0.7	10 ± 1
L-Leucine	—	80	2.6 ± 0.3 (4)	1.9 ± 0.1	17 ± 4
	+	80	7.6 ± 0.9 (4)	7.1 ± 1.3	10 ± 1
Glycine	—	80	2.1 ± 0.3 (4)	2.7 ± 0.3	27 ± 11
	+	80	4.0 ± 0.9 (4)	5.2 ± 1.0	9 ± 1

larger than the tumour cells normally maintain during energy metabolism.

Other amino acids. Table 2 shows that valinomycin also stimulated the uptake of glycine, L-leucine and 2-aminoisobutyrate. The maximum values of the ratio of the cellular to extracellular amino acid concentration were in each instance near the corresponding value of the ratio $[K^+]_2/[K^+]_1$.

Effect of valinomycin on the cellular contents of Na^+ , K^+ and H_2O . Fig. 4 shows that the presence of valinomycin accelerated both the loss of K^+ from the tumour cells and the absorption of Na^+ . The sum of the amounts of these two ions present/mg dry wt. of cells fell by 0.222 ± 0.018 (21) S.E.M. μ equiv. in the interval from 2 to 20 min when valinomycin was present and by 0.153 ± 0.019 (21) μ equiv. when it was absent. Hence the extra loss of K^+ that valinomycin caused was associated with the uptake of somewhat less than one equivalent of Na^+ ($P = 0.01$). The behaviour of the ions seems qualitatively consistent with the notion that Na^+ ions entered the tumour cells in response to the electrogenic efflux of K^+ ions through the iono-

phore. The volume of the cells fell progressively with time in both systems (Fig. 4).

Valinomycin with fluoride ions, ouabain or oligomycin. The presence of 0.3 mM-ouabain in three experiments, of 20 mM-NaF in two further experiments and of 10 μ g of oligomycin/ml in two other experiments had no significant effects on the extensive accumulation of L-methionine that took place when the value of $[K^+]_2/[K^+]_1$ was near 20 and valinomycin was present. Neither the peripheral sodium pump that these tumour cells contain nor their oligomycin-sensitive mitochondrial ATPase seem therefore to be involved in the process (cf. Kimmich, 1973).

Valinomycin and a presumptive proton conductor. A mixture of valinomycin and a suitable proton conductor would catalyse the exchange of K^+ ions for protons, rather than an electrogenic flow of K^+ ions (Scheme 1). The proton conductor might therefore decrease the amount of methionine and 2-aminoisobutyrate accumulating in the presence of valinomycin. Table 3 shows that of the four uncouplers examined only the tetrachlorosalicylanilide and, to a

Fig. 4. Effect of valinomycin on the changes in the cellular contents of water, Na^+ and K^+ with time

Observations like those shown in Fig. 1 were made in the course of 17 assays in the presence of valinomycin and eight assays in its absence. The Ringer contained in each case 85 mequiv. of Na^+ /l, 70 mM-choline chloride, about 5 mequiv. of K^+ /l, 1 mM- $[^{14}C]$ methionine and the metabolic inhibitors. Mean values $\pm$ S.E.M. are depicted. Cellular Na^+ : Δ , $\blacktriangle$. Cellular K^+ : $\circ$, $\bullet$. Cellular water: ∇ , $\blacktriangledown$. Valinomycin present: $\blacktriangle$, $\bullet$, $\blacktriangledown$. Valinomycin absent: Δ , $\circ$, ∇ .

Table 3. Relations between the prevailing gradients of Na^+ and of K^+ and the maximum concentration gradient of methionine and 2-aminoisobutyrate formed in the presence of valinomycin and various proton conductors

The procedure is outlined in the legend to Fig. 1. The preparations were first starved in the potassium-Ringer solution in the presence of cyanide and deoxyglucose. They were then transferred to a Ringer containing 80 mequiv. of Na^+/l , about 4 mequiv. of K^+/l , 2 mM-NaCN, 5 mM-2-deoxyglucose, either 1 mM-L-methionine or 1 mM-2-aminoisobutyrate, and the selected ionophores. The valinomycin concentration was $4\text{ }\mu\text{g}/\text{ml}$, except in the assays with 0.2 mM-2,4-dinitrophenol, where $10\text{ }\mu\text{g}/\text{ml}$ was used. Subscript 1 denotes the extracellular phase, subscript 2 the cellular phase. The ratio $[\text{A}]_2/[\text{A}]_1$ denotes the amino acid concentration gradient. This was determined on n occasions, the observations being grouped as indicated. Results are means $\pm$ S.E.M.

Ionophore	n	$[\text{A}]_2/[\text{A}]_1$	$[\text{Na}^+]_1/[\text{Na}^+]_2$	$[\text{K}^+]_2/[\text{K}^+]_1$
With methionine				
None	7	4.01 ± 0.10	1.16 ± 0.02	38.0 ± 5.5
0.1 mM-Tetrachlorosalicylanilide	5	4.16 ± 0.26	1.08 ± 0.03	29.8 ± 6.7
20 mM- NaN_3	3	3.99 ± 0.33	1.21 ± 0.03	34.2 ± 2.0
Valinomycin	3	9.65 ± 1.35	0.85 ± 0.07	18.3 ± 3.7
Valinomycin + 0.1 mM-tetrachlorosalicylanilide	3	4.50 ± 0.20	0.94 ± 0.00	20.3 ± 5.4
Valinomycin	2	8.26 ± 0.50	0.95 ± 0.02	16.3 ± 0.1
Valinomycin + 20 mM- NaN_3	2	8.62 ± 1.11	1.23 ± 0.07	28.5 ± 0.1
Valinomycin	2	9.91 ± 1.09	0.83 ± 0.05	20.6 ± 5.0
Valinomycin + 0.1 mM-carbonyl cyanide <i>m</i> -chloro-phenylhydrazone	2	6.78 ± 0.03	0.80 ± 0.02	18.1 ± 2.4
Valinomycin*	2	18.9 ± 0.5	1.16 ± 0.14	38.8 ± 16.7
Valinomycin* + 0.2 mM-2,4-dinitrophenol	2	22.2 ± 1.4	1.27 ± 0.03	38.9 ± 16.1
With 2-aminoisobutyrate				
None	5	4.74 ± 0.22	1.47 ± 0.07	19.3 ± 2.5
0.1 mM-Tetrachlorosalicylanilide	3	5.38 ± 0.55	1.45 ± 0.09	15.2 ± 2.7
Valinomycin	3	12.85 ± 1.10	1.03 ± 0.02	10.3 ± 0.4
Valinomycin + 0.1 mM-tetrachlorosalicylanilide	4	5.63 ± 0.30	1.30 ± 0.07	14.7 ± 0.9

* F^- (20 mequiv./l) also present.

lesser extent, the substituted phenylhydrazone behaved in this way. The efflux of K^+ between 2 and 20 min was 264 ± 23 (7) S.E.M. nequiv./mg dry wt. of cells in the presence of valinomycin, 196 ± 30 (6) when the salicylanilide was also present and 204 ± 22 (21) in the controls. The corresponding values of the amount of Na^+ absorbed were 91 ± 10 , 177 ± 9 and 51 ± 8 . The effects of the proton conductors on the magnitudes of the gradients of Na^+ and K^+ were thus relatively small in the presence of valinomycin (Table 3), and, if the gradient hypothesis is correct, would be expected to have helped rather than hindered the absorption of the amino acids. In the absence of valinomycin the salicylanilide had no significant effect either on the uptake of either amino acid or on the net movements of Na^+ and K^+ .

Nigericin with tetrachlorosalicylanilide

Effect on cellular contents of Na^+ and K^+ . Studies with artificial membranes indicate that nigericin catalyses the exchange of K^+ for either H^+ or Na^+ (Henderson, 1971). Similar behaviour appeared to occur with the mouse tumour cells. Thus, under the conditions described in Fig. 5, the preparations

absorbed 138 ± 24 (5) nequiv. of Na^+/mg dry wt. of cells in the interval from 2 to 20 min, when nigericin was present, whereas the control preparations absorbed 34 ± 5 (7) nequiv./mg. Likewise, the tumour cells in the control preparations lost 132 ± 10 (7) nequiv. of K^+ during the 18 min interval, in comparison with 344 ± 27 (5) nequiv. when nigericin was present. Hence only about half of the efflux of K^+ induced by nigericin was neutralized by the influx of Na^+ . We assume that the other portion exchanged with H^+ . This may be why nigericin raised the water loss during the 18 min from 0.34 ± 0.09 (7) $\mu\text{l}/\text{mg}$ to 1.07 ± 0.23 (5) $\mu\text{g}/\text{mg}$.

The presence of both nigericin and tetrachlorosalicylanilide further increased the absorption of Na^+ to 218 ± 18 (4) nequiv./mg, the loss of K^+ over the same period of 18 min being 303 ± 31 (4) nequiv./mg. The water loss was lowered to 0.25 ± 0.05 (4) $\mu\text{g}/\text{mg}$. In general these observations seem consistent with the notion that, in the presence of both nigericin and tetrachlorosalicylanilide, the influx of protons exchanging with K^+ ions tended to be neutralized by a flow of protons out of the tumour cells (Scheme 1).

Amino acid absorption. As Scheme 1 shows, the above situation means that the two ionophores acting together might mimic the effect of valinomycin

on amino acid absorption. The observations summarized in Table 4 and Fig. 5 were used to test this possibility and showed that: (1) less methionine and aminoisobutyrate accumulated in the presence than in the absence of nigericin. This was at least partly

Fig. 5. Effects of nigericin and tetrachlorosalicylanilide, acting separately and together, on the accumulation of methionine

The assays were carried out as shown in the legend to Fig. 1. The preparations were first starved in the potassium-Ringer containing cyanide and deoxyglucose. They were then transferred to a Ringer containing 80 mequiv. of Na^+ /l, about 5 mequiv. of K^+ /l, 2 mM-NaCN, 5 mM-2-deoxyglucose, 1 mM-L-[^{14}C]methionine. ○, Control; △, 1 μg of nigericin/ml; ●, 0.1 mM-tetrachlorosalicylanilide; ▲, 1 μg of nigericin/ml plus 0.1 mM-tetrachlorosalicylanilide. The observations are means $\pm$ S.E.M. derived from at least four independent sets of assays. Table 4 shows further properties of the system.

because the Na^+ gradient became smaller (Morville *et al.*, 1973). For instance, $[\text{Na}^+]_1/[\text{Na}^+]_2$ was typically near 0.6 after 20 min in the presence of nigericin, in comparison with a value of about 1.0 in the controls. (2) In striking contrast, when both the anilide derivative and nigericin were present, the tumour cells took up more amino acid than when both ionophores were absent. In the former instance, this happened despite the relative lowering of the gradient of Na^+ (Table 4). (3) Tetrachlorosalicylanilide alone scarcely affected the system.

The way in which the amino acid gradient varied with the gradient of K^+ in the presence of the two ionophores is also interesting. On the one hand, Table 4 shows that the mean value of the factor F was 5.3 for aminoisobutyrate when $[\text{K}^+]_2/[\text{K}^+]_1$ was 10, whereas F was 6.3 for methionine when the ionic ratio was 20. On the other hand, Fig. 3(b) shows that, in the assays with methionine and valinomycin, F was between about 6 and 15 over the same range of gradients of K^+ . Valinomycin evidently coupled the amino acid pump to the gradient of K^+ more tightly than nigericin and the salicylanilide achieved together. Nevertheless, the fact that the mixture of the two ionophores behaved in some degree like valinomycin strengthens the argument in favour of the mechanisms postulated in Scheme 1.

Table 4 also shows that, unlike the salicylanilide, 20 mM- NaN_3 had relatively little effect on the accumulation of methionine in the presence of nigericin.

Gramicidin

These compounds were expected to promote the electrogenic flow of Na^+ ions and K^+ ions between the cellular and extracellular phases, with little discrimination between the two ionic species (Henderson *et al.*, 1969). The presence of 1 μg of gramicidin/ml of Ringer solution indeed accelerated the replacement of cellular K^+ ions by Na^+ ions, in assays in

Table 4. Maximum accumulation of methionine and 2-aminoisobutyrate in the presence of nigericin with and without tetrachlorosalicylanilide

The experimental conditions were as described in the legend to Fig. 5. $[\text{A}]_2/[\text{A}]_1$ denotes the maximum ratio of the cellular to the extracellular amino acid concentrations that was achieved (see Fig. 5). Mean values $\pm$ S.E.M. were determined on the basis of n assays. $F = [\text{A}]_2[\text{Na}^+]_2/[\text{A}]_1[\text{Na}^+]_1$. Nigericin (1 μg /ml of Ringer solution) was used.

Ionophores		n	$[\text{A}]_2/[\text{A}]_1$	$[\text{Na}^+]_1/[\text{Na}^+]_2$	F	$[\text{K}^+]_2/[\text{K}^+]_1$
With methionine						
None		7	4.01 ± 0.10	1.16 ± 0.02	3.52 ± 0.09	38.0 ± 5.5
Nigericin		5	2.87 ± 0.14	1.15 ± 0.04	2.58 ± 0.16	33.9 ± 6.3
Nigericin+0.1 mM-tetrachlorosalicylanilide		4	5.56 ± 0.32	0.90 ± 0.06	6.31 ± 0.75	21.0 ± 2.8
Nigericin+20 mM- NaN_3		3	3.25 ± 0.27	0.99 ± 0.08	3.36 ± 0.48	24.3 ± 2.0
With 2-aminoisobutyrate						
None		5	4.74 ± 0.22	1.47 ± 0.07	3.24 ± 0.19	19.3 ± 2.5
Nigericin		4	2.61 ± 0.42	1.09 ± 0.16	2.40 ± 0.22	12.8 ± 3.5
Nigericin+0.1 mM-tetrachlorosalicylanilide		5	5.42 ± 0.75	1.03 ± 0.11	5.62 ± 0.47	10.2 ± 2.7

which 80mequiv. of Na^+ and about 4mequiv. of K^+ /l were used with cyanide and deoxyglucose. The ratio $[\text{Met}]_2/[\text{Met}]_1$ reached a maximum value at 2min of 3.86 ± 0.26 (4), when $[\text{Na}^+]_2$ was about 90mequiv./l of cellular water and $[\text{K}^+]_2$ was about 70mequiv./l. When gramicidin was present some coupling to the gradient of K^+ appeared to have been achieved at 2min. After 30min in the presence of gramicidin the amino acid gradient was smaller [1.15 ± 0.05 (4)]. The value of $[\text{Na}^+]_2$ was then about 106mequiv./l and of $[\text{K}^+]_2$ a mere 13mequiv./l, in comparison with the control values of about 93 mequiv./l and 71mequiv./l respectively. Under the former conditions virtually no energy was derived from the transfer of Na^+ through the amino acid pump, because in the presence of gramicidin the gradient of electrochemical potential of Na^+ across the plasmalemma would decrease to zero. Thus the amino acid gradient would approach 1, as Terry & Vidaver (1973) have shown with preparations of pigeon erythrocytes.

When 4 μg of valinomycin and 1 μg of gramicidin were both present per ml of Ringer, $[\text{Met}]_2/[\text{Met}]_1$ was 3.77 ± 0.58 (3) after 2min and 1.18 ± 0.01 (3) after 30min. The values of $[\text{Na}^+]_2$ and $[\text{K}^+]_2$ were similar to those observed in the presence of gramicidin alone. The failure of valinomycin to stimulate the uptake of methionine when gramicidin was already present seems consistent with the other effects it produced.

Discussion

The first question for discussion is how far the accumulation of the amino acids by the preparations depleted of ATP resembles the behaviour of the system during energy metabolism in the presence of the standard sodium-Ringer solution. Cellular $[\text{Na}^+]$ then ranged from 70mequiv./l (Morville *et al.*, 1973) to 30mequiv./l (Eddy, 1968b). Therefore the value of the product $[\text{Na}^+]_1[\text{K}^+]_2/[\text{Na}^+]_2[\text{K}^+]_1$ was at least 30 when the sodium pump was functioning in these circumstances. However, ionic gradients of that magnitude were only transiently maintained in the cellular preparations depleted of ATP, because these tended to gain Na^+ and lose K^+ (Fig. 4). The changes in the cellular amino acid content (Fig. 1) reflected the movements of Na^+ and K^+ by increasing and then decreasing (Eddy *et al.*, 1967). The maximum size of the amino acid gradient in the presence of valinomycin and 1mM-methionine is noteworthy, especially when $[\text{Na}^+]_1$ was 150mequiv./l, $[\text{Met}]_2/[\text{Met}]_1$ was near 18 when the product of the ionic gradients was about 20 (Fig. 3). Morville *et al.* (1973) observed that, in the presence of 1mM-methionine during energy metabolism, the accumulation ratio was also 18, whereas it was 32 with 2-aminoisobutyrate and 5 with L-leucine. The behaviour of the starved preparations in the presence of 2 or

5mM-methionine and valinomycin (Table 1) likewise resembled the behaviour of the respiring preparations that Morville *et al.* (1973) studied. When the cellular contents of Na^+ and K^+ were in the physiological range, we conclude that the preparations, both containing valinomycin and depleted of ATP, accumulated methionine to roughly the same extent as during energy metabolism. Leucine, 2-aminoisobutyrate and glycine behaved like methionine in preliminary work (Table 2).

A significant piece of evidence in favour of the gradient hypothesis is the marked correlation that was observed between the maximum transient methionine gradient and the magnitudes of the ionic gradients themselves (Fig. 3). This behaviour strongly supports the notion that the coordinated movements of Na^+ and K^+ drove the amino acid pump in the presence of valinomycin. That ATP as such was probably not involved is consistent with (1) the lack of effect of fluoride on the system and (2) the observation of Gibb & Eddy (1972) that valinomycin did not increase the small cellular ATP content of the starved preparations. Reid & Gibb (M. Reid & L. E. Gibb, unpublished work) have since confirmed this result. The different possibility that, in these starved preparations, the amino acid labelled with ^{14}C simply exchanged with endogenous amino acids has been shown to be unlikely (Morville *et al.*, 1973).

The present work thus provides a clearer proof of the principle underlying the ion-gradient hypothesis than has hitherto been available in a Na^+ -dependent system deprived of ATP. It shows that the physiological gradients of amino acid concentration can in fact be reproduced at the expense of the ionic gradients. The point is important because Potashner & Johnstone (1971) and Geck *et al.* (1972) have questioned the validity of the gradient hypothesis on these very grounds.

Valinomycin and the membrane potential

By selectively complexing with K^+ valinomycin increases the permeability of artificial lipid membranes of appropriate composition (Haest *et al.*, 1972) to K^+ specifically (Henderson, 1971; Haydon & Hladky, 1972). The membrane may then behave like a K^+ electrode. The action of valinomycin on the mouse ascites-tumour cells can be rationalized in similar terms, though the actual electrical properties of the surfaces of these cells are conjectural. Our working hypothesis (Scheme 1) is that valinomycin facilitated the passage of K^+ through the plasmalemma in such a way that the flow of Na^+ through the amino acid carrier became electrically coupled to the movement of K^+ with the ionophore (Gibb & Eddy, 1972). In other words the absorption of the amino acid was driven by the gradient of electrochemical potential of Na^+ . The mechanism requires

Scheme 1. *Proposed modes of electrical coupling between the amino acid pump and the flow of ions through the various ionophores*

The influx of the amino acid (A) is accompanied by a parallel stream of Na⁺ into the tumour cells, as indicated by the direction of the arrow (→). A compensating movement of positive charge (or its equivalent) across the plasmalemma, at some possibly distant point, takes place in the opposite direction (←). Seven ways of achieving this are shown. Thus valinomycin (V) catalyses the efflux of K⁺, as does the mixture of nigericin (N) and the proton conductor (P). The gramicidins (G) catalyse the efflux of Na⁺ and K⁺ and would virtually short-circuit the amino acid pump (see text). Electrical coupling between the efflux of Na⁺ through the sodium pump and the influx of Na⁺ through the amino acid carrier is also depicted (Eddy & Gibb, 1972). The interrupted lines represent two processes that are relatively slow in comparison with the fluxes mediated by the ionophores: (1) the efflux of K⁺ and (2) the electrogenic influx of Cl⁻. Two non-electrogenic and therefore ineffectual systems (N, V+P) are also represented. This general scheme contrasts with the earlier view (Eddy, 1968*b*; Reid & Eddy, 1971) that the membrane potential of the tumour cells was always relatively small and that charge neutralization during the uptake of the amino acid involved the efflux of K⁺ through the amino acid pump itself.

that both in the presence and absence of valinomycin the net transfer of charge through the bulk of the membrane, in the form of ions such as Na⁺, H⁺ or Cl⁻, occurs relatively slowly, for otherwise the membrane would be short-circuited. It is significant in this connexion that valinomycin caused extra Na⁺ to be absorbed (Fig. 4) without causing a rapid efflux of KCl.

We suggest that the ability of specific pairs of ionophores to cooperate to produce a certain effect on these transport systems also supports the hypothesis of electrical coupling. Such behaviour makes it less likely that the ionophores combined with the amino acid carrier itself. The following examples are noteworthy (Scheme 1). (1) Tetrachlorosalicylanilide and the chlorophenylhydrazones each decreased the uptake of amino acid in the presence of valinomycin, without affecting the controls (Table 3). However, we do not understand why valinomycin appeared not to cause a rapid exchange of K⁺ for H⁺ in the presence of tetrachlorosalicylanilide (Henderson *et al.*, 1969). (2) Nigericin, which catalyses the non-electrogenic efflux of K⁺, was not equivalent to valinomycin (Glynn, 1967; Henderson *et al.*, 1969). How-

ever, tetrachlorosalicylanilide stimulated amino acid uptake in the presence of nigericin as though the two compounds acting together mimicked the behaviour of valinomycin (Table 4). (3) Valinomycin failed to increase the uptake of methionine in the presence of gramicidin.

The apparent failure of both dinitrophenol and NaN₃ to interact with the amino acid-transport system containing a second ionophore is puzzling, but may simply mean that these compounds failed to catalyse a sufficiently fast conduction of protons through the plasmalemma.

Electrical coupling during energy metabolism

It seems possible that during energy metabolism the membrane potential is governed both by the diffusion of K⁺ and the rate of working of an electrogenic sodium pump (Scheme 1). The striking ability of valinomycin to reproduce some of the effects of ATP on the magnitude of the amino acid gradient, as well as the apparent coupling of the amino acid movements to the gradient of K⁺ that is observed in specific circumstances, can be rationalized in this way

(Morville *et al.*, 1973). It may not therefore be necessary to suppose, as Reid & Eddy (1971) did, that the efflux of K^+ through the amino acid pump is subject to metabolic regulation. This possibility, which Eddy (1968b) also favoured, has not been disproved, however, and needs to be borne in mind. Meanwhile the idea that the membrane potential is the controlling factor seems at present the simplest way of describing the system. We therefore need to know: (1) the actual magnitude of the cellular membrane potential under any particular set of conditions (cf. Okada *et al.*, 1973; Lamb & MacKinnon, 1971; Lassen *et al.*, 1971); (2) details about the related problem of the factors governing the distribution of Cl^- ions and PO_4^{3-} ions across the plasmalemma (Scarpa *et al.*, 1970; Cotterrell & Whittam, 1971); (3) the actions of valinomycin and of ouabain on amino acid absorption (Bittner & Heinz, 1963; Gibb & Eddy, 1972); (4) the magnitude of the increase in the absorption of Na^+ brought about by the presence of a given amino acid, which appeared to be stoichiometrical in one study (Schafer & Jacquez, 1967) and relatively small in another study (Geck *et al.*, 1972).

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