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THE CHARACTERISATION OF TWO PARTIALLY PURIFIED SYSTEMS FOR Na^+-DEPENDENT AMINO ACID TRANSPORT

COLIN WATTS and KENNETH P. WHEELER *

School of Biological Sciences, University of Sussex, Falmer, Brighton BN1 9QG (U.K.)

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Summary

Two systems mediating the transport of amino acids were studied in vesicles derived from protein-depleted membranes of pigeon erythrocytes. One system (ASC system) catalysed the Na^+-dependent exchange of small neutral amino acids, such as alanine, serine and cysteine. The other system, also Na^+-dependent, mediated the active transport of glycine. The ASC and glycine systems were distinguished by the sensitivity of the latter to the anion present, by the former's requirement for an exchangeable amino acid and by the inability of alanine to inhibit the transport of glycine. Preliminary results indicated that the influx of glycine was electrically silent. The only major integral protein retained in the vesicles was the band 3 protein, but that could not be unequivocally identified as the transporter.

Introduction

In recent years the emphasis of research into membrane transport phenomena has changed from one where kinetic parameters were measured in whole cells to one where the major effort has been to try to identify the molecular structures involved. Progress in this field, though slow, has been made possible by the development of the use of vesicles for transport measurements [1,2], the controlled use of detergents for solubilising membranes [3], the use of liposomes in the reconstitution of membrane proteins [4,5] and the development of gel-electrophoretic procedures to monitor the polypeptide composition of membrane preparations.

* To whom correspondence should be addressed.

The identification of transport proteins is made particularly difficult when transport is achieved without an associated cleavage of chemical bonds that might be used as a marker during purification. In this case, the progress of purification can be monitored only by replacing or conserving the putative carrier protein in a bilayer separating two aqueous compartments. A demonstration of transport activity is then required before any purification or partial purification can be claimed. Reconstitution of the transport process under study is, of course, also highly desirable when purification of the component is achievable by other means; for instance, by using its strong affinity for a labelled solute [6], its intrinsic enzymic activity [7] or its ability to bind specifically a labelled inhibitor [8].

A particularly interesting class of transport processes is that in which concentrative solute movements are achieved as a result of coupling to an independently generated cation gradient [9]. Na^+-dependent transport processes of this sort are usually thought of in connection with the epithelial cells of the intestine and kidney where they are clearly of great importance. In fact, Na^+-dependent amino acid transport also occurs in the Ehrlich ascites tumour cell and in the avian erythrocyte and is more easily studied in these free cells. Though no Na^+-dependent solute transfer systems have been defined in molecular terms, we have recently reported the partial purification of an Na^+-dependent system for the exchange of neutral amino acids in the pigeon erythrocyte membrane [10]. In this report, we confirm and extend our study of the exchanging system and also show that the preparation retains a separate system for the active transport of glycine, well studied in whole cells by Vidaver and his collaborators [11–16].

In the absence of either specific inhibitors of these systems or any associated enzymic activity, our approach has been to isolate intact transport systems in vesicles derived from pigeon erythrocyte membranes depleted of most membrane proteins. In the following paper [17] we show that, compared with human erythrocyte membranes, rather more disruptive conditions were required to remove proteins and generate vesicles from pigeon erythrocyte membranes; this is shown to be due to a different mode of association of peripheral proteins, including spectrin, with the pigeon erythrocyte membrane.

Methods

Pigeon erythrocyte membrane vesicles were prepared from purified membranes depleted of several proteins, as described previously [10,18] and in the following paper [17]. Membranes were purified more rapidly by using an MSE 18 centrifuge during the steps prior to the homogenisation of nucleated ghosts [18]. Unless stated otherwise in the text, the membranous pellets obtained after treatment with EDTA at pH 11.0 were resuspended by homogenisation in approx. 0.4 ml of 140 mM choline chloride, 2.0 mM $MgSO_4$ and 20 mM Tris-HCl (pH 7.5 at 22°C) plus, for alanine transport measurements, 5 mM L-alanine.

Vesicles were used as soon as they had been warmed to 37°C; the bath-sonication and extended incubation steps described previously [10] were

omitted as they did not alter the transport activity. Incubation media for influx measurements consisted of the choline chloride medium described above, or similar solutions in which the choline chloride had been replaced by a sodium or potassium salt, plus either ^{14}C-labelled alanine (final concentration 0.12 mM) or ^{14}C-labelled glycine (final concentration 0.5 mM), both at 1.0 μCi/μmol. For alanine influx, experiments were initiated by the addition of 10 μl of vesicles (about 2 μg lipid P) to 0.4 ml of incubation medium, whereas glycine transport was measured by adding 15—20 μl of vesicles (about 3—4 μg lipid P) to 0.2 ml of incubation medium; this enhanced the effects observed.

Termination of transport, filtration of the vesicles, measurement of lipid phosphorus and assay of radioactivity were carried out as previously described [10]. Deviations from the concentrations and volumes given above are noted in the text for specific experiments. Results representative of two or more experiments are shown except for those in Fig. 3, which are from a single experiment.

Results

Estimation of the volume enclosed by the vesicles

It was desirable to know the volume trapped by a given amount of membrane material for two reasons. Firstly, it enables the calculation of intravesicular solute concentrations where this differed from that outside the vesicles (see below) and, secondly, it enabled the amount of radioactivity required to detect a transport process to be estimated. Indeed, it indicated that a simple equilibration of solute with the enclosed space cannot be detected when the amounts of radioactivity and vesicles that may be used, per time-point, are limited. In order to obtain an estimation of the volume trapped by a given amount of vesicles and retained on filters of 0.45 μm pore-size, we performed the following experiment.

Three vesicle pellets were homogenised separately in a buffer containing 10 mM NaCl, 10 mM glycine, 10 mM sucrose, 20 mM Tris-HCl (pH 7.5 at 4°C), 2 mM $MgSO_4$, 130 mM choline chloride, 0.1 mM NaN_3 and (a) 2.5 μCi/ml [^{14}C]sucrose; (b) 2.5 μCi/ml [^{14}C]glycine; (c) 2.5 μCi/ml ^{22}NaCl. Thus, each of the three suspensions carried a different marker for internal space. The size of this space was estimated for each of the three populations by quenching and filtering small portions of suspension in either the buffer described above or distilled water. The filters were then assayed for radioactivity [10]. It was assumed that trapped radioactivity in an osmotically sensitive space was released by the swelling and bursting of the vesicles quenched and washed in water. Specific trapped space was therefore measured thus:

$$\text{Specific trapped space } (\mu\text{l}/\mu\text{g lipid P}) = (C_b - C_w) \cdot V_t/C_t \cdot L$$

where C_b and C_w denote radioactivity (cpm) for samples quenched and washed in buffer and water, respectively. C_t denotes the total radioactivity (cpm) in the sample taken for quenching and washing. V_t denotes the volume of the

sample taken for quenching and L denotes μg lipid phosphorus (P_i) therein. Duplicate measurements were made for C_b, C_w and C_t; triplicate estimates of L were obtained. Specific trapped volumes obtained were (a) 0.095 μl/μg lipid P (sucrose as marker), (b) 0.103 μl/μg lipid P (glycine as marker) and (c) 0.085 μl/μg lipid P (Na^+ as marker). The experiment was also used to test the response of the membrane filters to increasing amounts of lipid. A linear relationship of slope, 0.1 μl/μg lipid P, was observed between the amount of lipid applied to the filters and the apparent trapped volume retained by them, measured using sucrose as a marker as described above (data not shown). This linearity was observed up to at least 4 μg lipid P per filter, though filtration times were rather slow when this amount of lipid was used. Generally, 2 μg lipid P were used per filter for transport measurements.

The operation of the exchanging system in vesicles

Earlier results showed that a transport system mediating the Na^+-dependent exchange of L-alanine was conserved in the vesicles [10]. We noted a number of observations that distinguished solute transport from mere binding of solute to the vesicles; for example, the striking dependency of alanine accumulation on an internal pool of alanine. Na^+-dependent exchange of L-alanine has been observed in whole cells [19,20] and in resealed pigeon erythrocyte ghosts (Wheeler, K.P., unpublished observations). The system, described as the ASC system [21], is specific for amino acids with linear aliphatic or hydroxy aliphatic side chains such as alanine, serine and cysteine. Fig. 1 shows that this specificity was retained in the vesicle preparation; uptake of alanine was completely inhibited by the presence of unlabelled serine but unaffected by the same concentration of unabelled lysine (1 mM). The influx of L-alanine

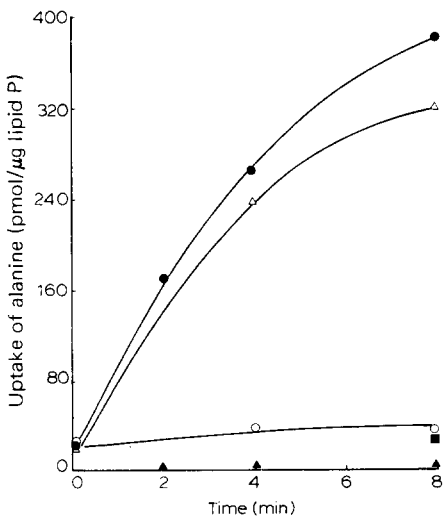

Fig. 1. The specificity of the system mediating Na^+-dependent L-alanine uptake. Vesicles were prepared in the presence of L-alanine and the uptake of 0.12 mM L-alanine was measured from Na^+-containing medium (see Methods) alone (●), plus 1 mM L-lysine (△) or plus 1 mM L-serine (○). The uptake after 8 min from choline-containing medium is shown (■) together with the uptake from Na^+-containing medium by vesicles prepared in the absence of L-alanine (▲).

invariably reached a plateau after about 10 min under the assay conditions used (Ref. 10 and Fig. 1). It was of some interest to determine what the internal concentration of alanine was at this point. Was it still 5 mM, indicating that a tightly coupled one-for-one exchange had taken place, with no uncoupled leakage of alanine? Or was the uncoupled leakage of the vesicles considerable, so that after 10 min the internal concentration of alanine equalled that in the bathing medium, i.e., 0.12 mM? The latter value assumes that the concentration in the much larger external volume was not significantly altered by this leakage. In fact, we were able to answer the question posed above and deduced that, for the most part, alanine movement across the vesicle membrane is confined to exchange on the carrier. Consider the following simple argument: since we know the amount of alanine accumulated by the vesicles after 10 min (400 pmol/μg lipid P for a good preparation, Fig. 1), we may calculate limiting values for the volume enclosing that amount, from the limiting values for intravesicular concentration discussed above. Thus, since volume = mass/concentration, then the volume enclosed per μg lipid P = $4 \cdot 10^{-10}$ mol/$1.2 \cdot 10^{-4}$ M = 3.2 μl; or, volume = $4 \cdot 10^{-10}$ mol/$5 \cdot 10^{-3}$ M = 0.08 μl. Since only 10 μl of vesicle suspension were used in the assay it was, therefore, inconceivable that as much as 32% of this volume was actually trapped within the vesicles themselves. We were sure then, that the concentration inside the vesicles was not 0.12 mM at the time when the plateau was reached and therefore that a concentration gradient persisted at this point. But what was the concentration of alanine inside the vesicles at this point? The estimate of 0.08 μl/μg lipid P obtained by assuming that, after 10 min, exchange accounted for all alanine movement, is in quite good agreement with the values of about 0.1 μl/μg lipid P obtained as described in the previous section. Using the latter estimate of volume, we obtain a value for the concentration inside the vesicles at the plateau point of $4 \cdot 10^{-10}$ mol/$1 \cdot 10^{-7}$ l = $4 \cdot 10^{-3}$ M, i.e., 4 mM. Thus, although some leakage had occurred, a 33-fold concentration gradient, higher inside, persisted.

This result demonstrated that a tightly coupled exchange of L-alanine had occurred between an internal non-radioactive pool at 5 mM and a radio-

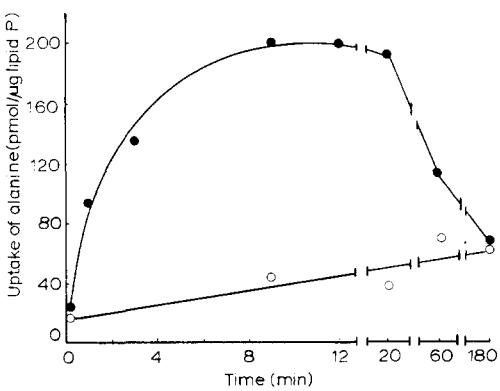

Fig. 2. An extended time course for the movement of L-alanine. Uptakes at the times shown were measured in the presence of NaCl (●) or choline chloride (○).

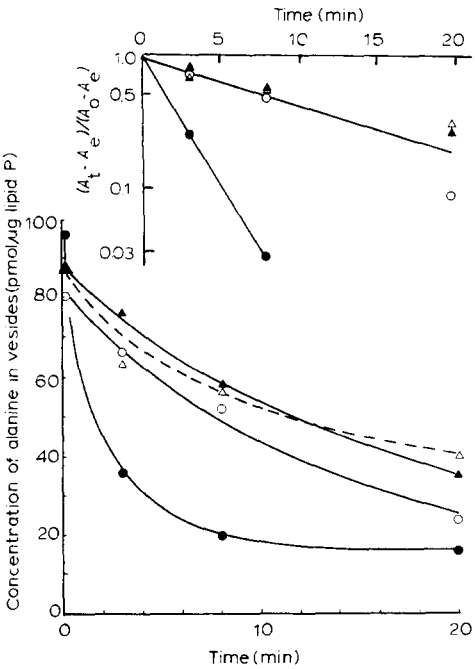

Fig. 3. Efflux from vesicles previously loaded with 1 mM ^{14}C-labelled L-alanine and choline chloride buffer. Vesicles were diluted 40-fold into choline (○, △) or NaCl buffer (●, ▲) in the presence (●, ○) or absence (▲, △) of 1 mM unlabelled L-alanine. The ordinate scale refers to the specific activity of L-alanine inside the vesicles at time zero. Inset: the same data as in the main figure are plotted semilogarithimically. A_o, A_e and A_t refer to radioactivity present initially, at equilibrium and at intermediate times equal to t, respectively.

active external pool at 0.12 mM. It was therefore predicted that after equilibration of the labelled amino acid between the two pools, a slow efflux from the vesicles down the concentration gradient should occur. This is clearly shown in Fig. 2; after 3 h the uptake in the presence of sodium had fallen to that observed in the absence of sodium.

The efflux of labelled L-alanine from preloaded whole cells is drastically increased by the presence of alanine in an Na^+-containing bathing medium [19]. The data presented above indicated that a similar stimulation of alanine efflux by external alanine should be observed in vesicles. To test this, an EDTA-extracted pellet was homogenised in 140 mM choline chloride medium containing 1 mM [^{14}C]alanine at a specific activity of approx. 7 μCi/μmol. 10-μl portions were diluted into 0.4 ml of various iso-osmotic media and the radioactivity remaining inside the vesicles after increasing periods of incubation was estimated by the usual quenching and filtration assay. The data in Fig. 3 demonstrate that maximal rates of efflux (half time 1.5 min) were obtained when both sodium (140 mM) and alanine (1 mM) were present in the incubation medium. The other conditions of incubation, where either sodium or alanine or both were absent, produced a much slower rate of efflux (half time, 9 min). It should be noted that, in this experiment, alanine was never completely absent from the external face of the vesicles; as a result of the

dilution of the vesicles containing alanine, its concentration at this external face under 'minus' alanine conditions was 0.025 mM. Efflux apparently obeyed first-order kinetics under the conditions of this experiment (Fig. 3, inset).

Conditions for the demonstration of glycine transport activity

The system discussed above, although sodium-dependent, is not involved in the transport of alanine against its electrochemical concentration gradient, that is to say, it is not involved in active transport. On the other hand, glycine transport is mediated by a concentrative system resulting in a net accumulation of this amino acid. This system, which has been extensively investigated by Vidaver and his collaborators [11—16], provided one of the first direct demonstrations of the general validity of the Na^+ gradient hypothesis for the active transport of sugars and amino acids [22]. We were particularly interested to see if this system had been retained in the vesicle preparation; some early observations, using vesicles free of internal amino acids but otherwise prepared as described for assays of alanine transport, indicated that Na^+-stimulated glycine transport was occurring. However, the effect was rather feeble and in this situation the inevitable scattering of experimental points obscured the differences which needed to be demonstrated before one could conclude that active transport by a distinct Na^+-dependent system was occurring. After testing different conditions it was found that, by preparing the vesicles in buffers of low ionic strenght, the initial rate of glycine uptake could be considerably enhanced. The composition of these solutions (see Methods) was the same as those used for testing alanine transport activity, except that the chloride salt was replaced by mannitol or sucrose and Tris-Hepes replaced Tris-HCl as a buffer. Buffers of similar composition have been used to prepare vesicles derived from rat intestinal epithelium [23,24], rat liver [25] and several other tissues. Those studies showed that sodium-stimulated active transport of sugars and amino acids was electrogenic and was enhanced under conditions where a membrane potential, negative inside, was generated. Such a potential is apparently generated when vesicles made in solutions containing sugars and buffered with Tris-Hepes are diluted into buffers containing NaSCN. Separation of charge occurs because SCN^- is rather lipid-soluble and therefore permeates membranes more quickly than does sodium. Although these conditions were effective for demonstrating active glycine uptake in vesicles derived from pigeon erythrocyte membranes, we have no evidence to suggest that this was due to stimulation of a specifically electrogenic transfer of glycine (see below). Observations with the phase-contrast microscope showed clearly that these vesicles aggregated much faster in buffers of high ionic strength [26]. It is believed that vesicles prepared and stored in buffers of relatively high ionic strength show poor glycine uptakes because aggregation reduces the surface area and therefore carrier sites available to mediate transport [26]. An enhancement of alanine uptake by the (surely electroneutral) exchanging system as a result of the preparation of vesicles in a sugar- or sugar/alcohol-based buffer was observed (data not shown), giving support to this interpretation.

Active transport of glycine in pigeon erythrocyte membrane vesicles

Uptake measurements were made as described in Methods. The vesicles were added to 0.2 ml of incubation medium and quenching was usually carried out with isotonic NaCl medium. Fig. 4 demonstrates that glycine uptake was observed in vesicles derived from pigeon erythrocyte membranes. Uptake occurred irrespective of whether or not there was an exchangeable amino acid inside the vesicles. This of course was not the case for alanine [10]. The influx of glycine was practically non-existent in the absence of sodium and was greater in the presence of NaCl than NaSCN (Fig. 4). This observation, also made by Imler and Vidaver [15] using intact pigeon erythrocytes, contrasts with reports on other Na^+-dependent systems which showed that when SCN^- replaced Cl^- as the counterion, an enhancement of uptake was observed [23–25]. An uptake followed by a slower efflux or 'overshoot' was observed with either anion in the presence of sodium, indicating that active transport of glycine had occurred (Fig. 4). It should be noted that this overshoot, indicative of active transport, was quite different from the apparent overshoot observed for the alanine-exchanging system (Fig. 2). It may be recalled that that was caused simply by the concentration gradient imposed at the beginning of the experiment and the subsequent 'labelling' of the more concentrated internal pool by the exchanging system.

Consideration of the estimate of internal volume obtained above (0.1 μl/μg of lipid P) led us to expect that about 46 pmol of glycine would be enclosed per μg lipid P, at final equilibrium under the conditions of the experiment, i.e., a final volume of 0.22 ml and a glycine concentration of 0.5 mM. Examination of Fig. 4 reveals that this was in fact approximately the uptake observed after 1 h when KSCN or choline chloride replaced the sodium salts. With Na^+ present, a 5- or 3-fold accumulation had therefore taken place, depending on whether Cl^- or SCN^-, respectively, was the counteranion.

It might be argued that the glycine influx observed in Fig. 4 simply reflected a time course of swelling followed by shrinking or bursting of the vesicles. The strict requirement for sodium, the enhancement observed with Cl^-, and

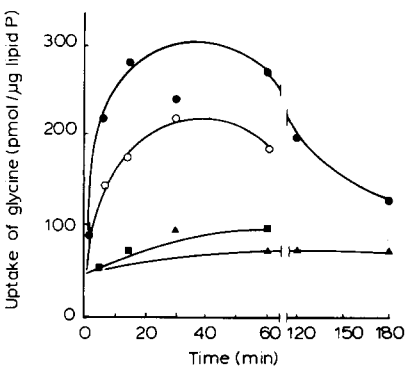

Fig. 4. The uptake of glycine by pigeon erythrocyte membrane vesicles prepared in 0.28 M mannitol, 2 mM $MgSO_4$, 20 mM Tris-Hepes (pH 7.5 at 22°C). Assays were started by the addition of 10–20 μl of vesicles to 0.2 ml of isoosmotic buffer containing Na^+, K^+, or choline salts, NaSCN (○), NaCl (●), KSCN (■), choline chloride (▲), plus 0.5 mM labelled glycine.

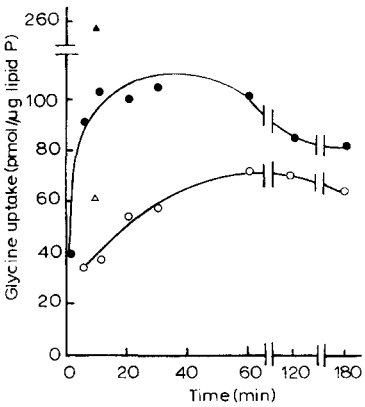

Fig. 5. The osmotic sensitivity of glycine uptake. Vesicles, made in a mannitol-containing buffer (see legend to Fig. 4) were assayed for transport activity by incubation in media containing an iso-osmotic salt as usual (NaCl, ▲; choline chloride, △) or the same plus an equivalent osmotic concentration of mannitol (NaCl, ●; choline chloride, ○).

the failure of alanine to show a similar influx under these conditions all signified that the accumulation observed was not simply due to swelling. To eliminate this possibility, an experiment was performed where the mannitol concentration inside the vesicles was exactly matched by an equal concentration of mannitol in the assay medium, in addtion to the NaCl normally present. The osmotic activity outside was therefore twice that present inside. A rapid initial shrinkage of the vesicles was expected under these conditions and the reduction in glycine influx observed reflected this (Fig. 5). Nevertheless, not only was there still a pronounced Na^+ dependency but also a small overshoot was still detectable, confirming that an active transport system for glycine was retained in the vesicles (Fig. 5).

Glycine transport is mediated by a system distinguishable from the ASC system

It was of considerable importance to establish that the movement of glycine into vesicles was effected by a system operationally distinct from that concerned with exchange. The term 'system' can only be used rather loosely at the present time as no Na^+-dependent solute-transfer systems have been characterised in molecular terms. Nevertheless, a variety of criteria [21] may be used to demonstrate that, in general, separate pathways mediate the movement of different classes of amino acids in animal cells. This is not to say, of course, that one amino acid may not react with more than one transport system. This clearly occurs in the case of methionine entry into Ehrlich ascites cells [27] and it is also clear that glycine may react, under certain conditions, with the exchanging system of the pigeon erythrocyte membrane [12]. Three pieces of evidence are offered here to show that, as in whole cells [15,19], nearly all the uptake of glycine is confined to its own specific pathway.

(1) The results in Fig. 4 show that glycine, unlike alanine [10], does not require an exchangeable amino acid for its entry.

(2) The data shown in Fig. 6 demonstrate that the nature of the anion in the assay medium affects glycine uptake quite markedly, but not alanine

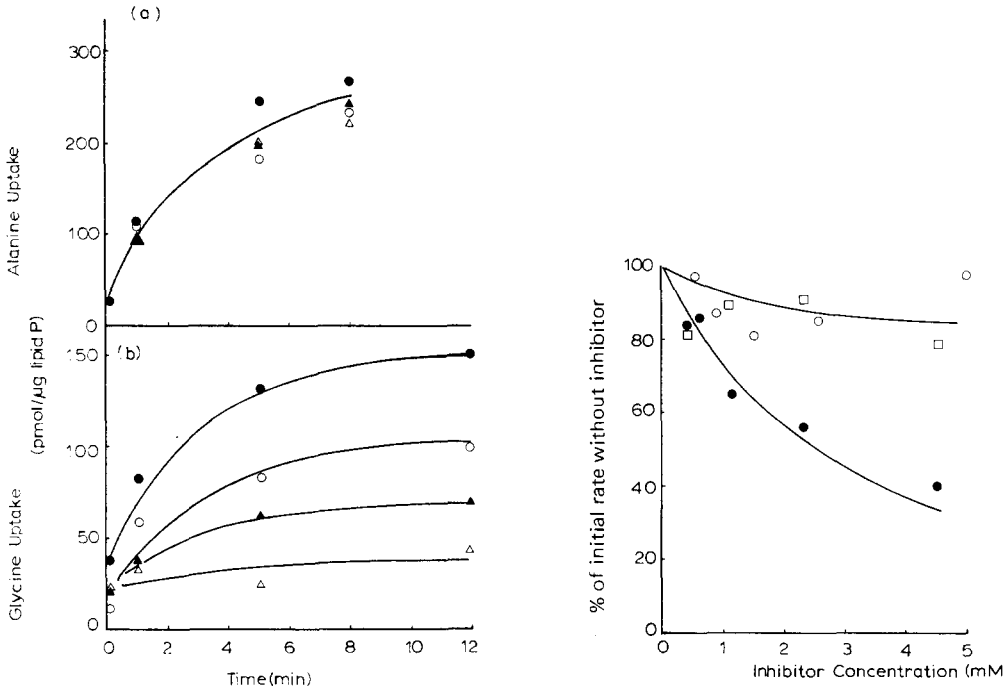

Fig. 6. Sensitivity of (a) alanine and (b) glycine uptake to anions. Vesicles were prepared in a buffer containing 140 mM mannitol, 70 mM KCl, 2 mM $MgSO_4$, 20 mM Tris-HCl (pH 7.5 at 22°C) plus 5 mM L-alanine for the data shown in (a). Uptake of ^{14}C-labelled alanine or glycine was measured from media containing 140 mM NaCl (●), NaSCN (○), $NaNO_3$ (▲) or sodium acetate (△), plus $MgSO_4$ and Tris buffer as listed above.

Fig. 7. Interactions between glycine and alanine and the systems that mediate their uptake. The effect of increasing concentrations of glycine on the initial rate of uptake of 0.35 mM L-alanine is shown (●) together with the effect of increasing concentrations of alanine on the initial rate of uptake of 0.22 mM glycine into vesicles containing (○) or not containing (□) 5 mM L-alanine.

uptake. This distinction and the order of activation ($Cl^- > SCN^- > NO_3^- >$ acetate) of the glycine system, closely reflect the results of Imler and Vidaver [15], whose examination of the effect of anions on the Michaelis parameters for glycine entry in whole pigeon erythrocytes led them to conclude that there was an anion-combining site on the glycine carrier that is filled prior to glycine binding. Because of the high permeability of the membrane to Cl^- and other anions, a direct demonstration of anion cotransport with glycine and sodium could not be made [15]. The same problem exists for vesicles.

(3) The most convincing evidence for the existence of two distinct pathways is shown in Fig. 7. Although high concentrations of glycine inhibited alanine entry by the exchanging system, high concentrations of alanine did not interfere with glycine entry whether or not alanine was present inside the vesicles. The data in Fig. 7 were collected by measuring the initial rate of glycine or alanine entry (influx after 4 min) in the presence of increasing concentrations of the other amino acid, i.e., glycine or alanine. Vesicles were prepared in a sucrose-based buffer containing 5 mM L-alanine. The same vesicle preparation was used to test the uptake of both amino acids. As labelled substrates for

uptake, glycine and alanine were present at 0.2 mM and 0.35 mM, respectively. As inhibitors of uptake they were present in the incubation media prior to the addition of vesicles at the following concentrations: 5.0, 2.5, 1.25, 0.63 and 0.32 mM. With alanine present inside the vesicles, some will always bathe the external face of the vesicles even when no extra is added as an inhibitor, because it is present in the solution in which the vesicles were prepared.

The experiment showed that even under conditions where the exchanging system was fully saturated with alanine (5 mM), such that its concentration throughout the system was 23-fold greater than that of glycine, the glycine influx was 80–100% of that observed in the absence of added alanine (Fig. 7). This provided a striking demonstration of the autonomy of the glycine-transport system. The small inhibition of glycine uptake by alanine sometimes detected may have been due to the weak interaction of the former amino acid with the exchanging system. The experiment confirmed that at higher concentrations, glycine interacts quite strongly with the exchanging system [19] and produced a 60% inhibition of alanine uptake when present at a 13-fold excess (Fig. 7).

Is glycine influx electrogenic?

This question has already been touched on in an earlier section. Experiments on whole pigeon erythrocytes have led Vidaver and his collaborators to suggest that two Na^+ and an anion (preferably Cl^-) are involved in a binding complex and become translocated with glycine [15]. They consider that the carrier itself is charged and that the only step involving charge movement is the reorientation of the empty carrier [16].

Some efforts were made to try to answer the question of whether glycine influx into vesicles was electrically silent or electrogenic. Vesicles were prepared in a buffer containing 140 mM mannitol, 70 mM KCl, 2.0 mM $MgSO_4$, 20 mM Tris-Hepes (pH 7.5 at 22°C) and uptake of glycine was tested in the

TABLE I

EFFECT OF IONIC STRENGTH AND LACK OF EFFECT OF VALINOMYCIN ON THE UPTAKE OF GLYCINE

Vesicles were prepared and stored as described in the text, either in (a) 280 mM mannitol, 2 mM $MgSO_4$, 20 mM Tris-Hepes (pH 7.5 at 22°C); or in (b) 140 mM mannitol, 70 mM KCl, 2 mM $MgSO_4$, 20 mM Tris-Hepes (pH 7.5 at 22°C). Uptake of glycine by the vesicles was measured by incubation in Na^+ medium (140 mM NaCl plus $MgSO_4$ and buffer as above) or in choline medium (140 mM choline chloride plus $MgSO_4$ and buffer). The valinomycin was added as a ethanolic solution to give a final concentration of 10 μM and 1% ethanol.

Conditions		Incubation time (min)	Uptake of glycine (pmol/μg lipid P)	
			—valinomycin	+valinomycin
(a)	Na^+ medium	10	308	—
	Na^+ medium + ethanol	10	288	—
	Choline medium	10	70	—
(b)	Na^+ medium	2	108	116
	Na^+ medium	10	172	164
	Choline medium	10	34	44

presence and the absence of 10 μM valinomycin. The results in Table I show that there was no effect on glycine uptake after 2 or 10 min. The experiment was repeated and the same result obtained. It was expected that the increase in permeability to K^+ caused by addition of valinomycin would result in the setting up of a K^+-diffusion potential, negative inside, thereby restraining further rapid K^+ efflux. Such a state of affairs should accelerate the translocation, in an inward direction, of a complex carrying a net positive charge; or, should accelerate the reorientation of an empty carrier bearing a net negative charge. An increase in the initial rate of uptake of amino acid will only be observed, however, if these translocation steps are rate-limiting. In fact, there are now many reports in the literature where Na^+-dependent uptakes of amino acids and sugars have been accelerated by K^+ and valinomycin used in this way [28–30]. All these groups found, in contrast to the results presented above (Figs. 4 and 6), that in the systems they studied, SCN^- produced an Na^+-dependent solute influx greater than that observed when Cl^- was the counter anion. This, as discussed in an earlier section, was interpreted as being due to the high permeability of the SCN^- species causing an acceleration of electrogenic influx.

It is not possible from the results in Fig. 6 and Table I to conclude definitely that Na^+-dependent glycine transport in the pigeon erythrocyte is electrically silent, though the results are compatible with this conclusion. Experiments where polarisation or depolarisation is assumed to occur, as a result of the supposed induction of differential ion permeabilities, may only be useful when positive effects are observed. When there is no effect on transport, we feel that a change in the membrane potential as a result of these manipulations should be directly demonstrated rather than assumed; only then can a lack of effect on Na^+-stimulated solute transport to be taken as evidence of electroneutrality. We intend to use a carbocyanine dye, the fluorescence of which is sensitive to the membrane potential [31,32], to investigate the question of the movement of charge during glycine transport across the pigeon erythrocyte membrane.

Discussion

Some points of interest are left unanswered by the data presented in this paper. For example, it is not yet clear to us whether or not Na^+ is required at the inner face of the vesicles for the operation of the exchanging sytem. Although the system was active in vesicles prepared in the absence of Na^+, the permeability of the vesicles to Na^+ at 37°C may be sufficiently great to ensure the rapid attainment of activating concentrations inside. Unfortunately, the measurement of Na^+ influx into vesicles is hampered by the limitations of the filtration technique and the difficulty of detecting a simple equilibration of externally added solute (Na^+) with a small intravesicular volume. Thus, attempts to measure alanine-stimulated transport of Na^+ have so far been largely unsuccessful. An exchange of Na^+ as well as amino acids certainly appears to occur in the whole cell; the ASC system substrates in an Na^+ containing bathing medium produced an acceleration of ^{22}Na efflux from cells preloaded with $^{22}NaCl$ [20] independently of the normal active extrusion of

sodium. By maintaining the Na^+ gradient, exchange of Na^+ ensures the conservation of metabolic energy. The ASC system may have become modified to this end, during the course of evolution, perhaps having been at one time concerned with the net transport of amino acids [19].

Another question that remains to be answered is that of the orientation of the vesicles — are they predominantly right-side-out, inside-out, or a mixture of both forms? These various possibilities also suggest another interpretation of the observed differences in behaviour between vesicles prepared in salt solutions and those prepared in mannitol or sucrose. If these two different conditions somehow determine which way the membrane tends to be oriented in the sealed vesicles, then the observed differences in behaviour could reflect the two different orientations. For example, both the tendency to aggregate and the low uptake of glycine shown by vesicles prepared in salt solutions could reflect a predominantly inside-out population of vesicles, because the stripping of proteins from the cytoplasmic surface of the membrane might expose hydrophobic regions and the glycine transport system is known to be asymmetric in pigeon erythrocyte ghosts [33]. Clarification of the orientation of the vesicles is, therefore, an important objective for future investigation.

The observations presented in a previous [10] and the following [17] paper show that the only major proteins retained in the vesicles are the band 2 and band 3 proteins. Since band 2, by analogy with human erythrocytes, is almost certainly confined to the cytoplasmic surface of the membrane, it is unlikely to mediate the transport of the amino acids. Also it seems highly likely that, as in the human erythrocyte [34], the major part of the band 3 region is the anion carrier for pigeon erythrocytes. (Certainly, the chicken erythrocyte membrane resembles that of the human cell in mediating extremely rapid exchange of Cl^- and HCO_3^- [35].) However, at present, we have little idea of the number of sites involved in the amino acid transport processes. If there are only relatively few copies of the protein(s) involved per cell, then it is possible that these are band 3 proteins. On the other hand, it is also possible that minor integral proteins which do not stain clearly on the gels are retained in the vesicles and such components could equally well be responsible for Na^+-dependent transport of amino acids in the pigeon erythrocyte.

It seems that a conclusive identification of the components responsible for the processes described in this paper will be achieved only by their complete reconstitution, following detergent solubilisation and fractionation of the vesicle components. It will be of particular interest to see to what extent the two Na^+-dependent systems described co-purify. Although the data demonstrate that different pathways are used for the exchange of alanine and the active transport of glycine, this does not rule out the possibility that the same polypeptide chain is involved. If it could be shown that there are more transport functions present in these vesicles than there are proteins, then one might be justified in suggesting some sort of multifunctional behaviour among these proteins. It might be energetically advantageous for a cell to synthesis one protein carrying on separate domains different transport system for solutes which the cell needs to be able to transport at similar rates. It would of course make no sense for the red cell, for example, to link the anion carrier (10^6

copies/cell) and the ($Na^+ + K^+$)-dependent ATPase (10^2—10^3 copies/cell) in this manner; a cell producing equal numbers of these systems, whatever that number is, would clearly be inefficient, in terms either of production or of performance.

Na^+-dependent transport of amino acids is a major concern of a variety of cells in both avian and mammalian tissues, though we cannot be sure of its importance to the avian erythrocyte. The systems in these cells, now partially purified in membrane vesicles, show similar specificities to those found in different cells and different species. It is very likely that the molecular basis of transport, coupled to a cation gradient, has been conserved, at least throughout the animal world. Hence, the study of the proteins and mechanisms involved in such processes in the pigeon erythrocyte will shed light on the process in general.

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