EFFECT OF SUBSTRATE STRUCTURE ON COUPLING RATIO FOR NA+-DEPENDENT TRANSPORT OF AMINO ACIDS*

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SUMMARY

We have measured the coupling ratio, *i.e.* the ratio of the augmentation of Na+ entry due to the presence of a substrate amino acid, to the augmentation of the entry of that amino acid due to the presence of Na+, for a characteristic transport system studied in the pigeon red blood cell. The value of this ratio has been shown to be approximately independent of the interval of observation and of the concentrations of the two cosubstrates. It does, however, depend strongly on the structure of the amino acid, varying from about 0.2 for proline to about 4 for threonine and 5 for cysteine. The coupling ratio both for entry and for exodus also depends on the composition of the internal pool of substrate amino acids. The presence of a hydroxyl group on carbons 3 or 4 produces sharp increases in the coupling ratio. A sulfhydryl or carboxamide group on carbon 3 appears to have a similar effect. A model consistent with these features is presented, which indicates that the presence of a hydroxyl group probably acts by disproportionately accelerating the release, displacement or replacement of Na+ from the transport complex at the interior surface of the membrane.

INTRODUCTION

The ratio of the augmentation of Na+ influx into a cell due to the presence of an amino acid, to the augmentation of amino acid influx due to the presence of Na+, might be expected to reveal the composition of a transport complex into which these two components are joined as cosubstrates. In the case of the transport system for glycine in the pigeon red blood cell, VIDAVER[2] observed a flux ratio of 1.53 ± 0.45, roughly in agreement with this observation that the rate of glycine uptake depends approximately on the square of the Na+ concentration. WHEELER AND CHRISTENSEN[3] observed in contrast a flux ratio for Na+ and alanine of 2.5 in a different transport system in the same cell, even though alanine uptake shows a first-order dependence on the Na+ concentration. An inconsistency between the two criteria in the opposite direction was seen for β-alanine transport. These contradictions have led us to examine the coupling ratio for several substrates of the system serving for alanine and numerous other amino acids, which we refer to as System ASC[4]. We find that the coupling

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* A preliminary report has been published[1].

ratio depends on the structure of the amino acid substrate, ranging from about 0.2 for proline to about 5 for cysteine. These coupling ratios appear to be independent of the concentrations of the cosubstrates and of the interval of observation. A principal structural factor increasing the value of the ratio is the presence of a polar group, *e.g.* a hydroxyl or sulfhydryl group, on carbon 3 or 4.

METHODS

Pigeon red cells were collected and handled essentially as described earlier[3,4]. Uptake of $^{22}Na^+$ and ^{14}C- or ^{3}H-labeled amino acids from 15 mM Tris–HCl buffered medium (pH 7.4) was measured at 37° in a shaking waterbath in air. Choline chloride replaced NaCl to give desired Na⁻ concentrations; amino acids when present at more than 5 mM replaced choline chloride or NaCl isoosmotically. Incubation intervals were initiated by adding 0.2 to 0.5-ml portions of heavy suspensions of washed cells (about 100- to 200-mg cells) to 5- or 10-ml portions of medium containing the labeled cosubstrates, and terminated by adding the resultant suspensions to 2 vol. of ice-cold, amino acid-free, Na⁺-free medium, followed by brief, rapid centrifugation; a second washing of the cells with similar cold medium followed. The weighed, packed cells were extracted with 5 % trichloroacetic acid solution, and the extracts as well as the diluted supernatant solution were assayed simultaneously for Na⁻ and ^{3}H or ^{14}C by liquid-scintillation counting, using separate channels, and correcting for the influence of $^{22}Na^+$ on the counts for the β emissions. After correcting for entrapped extracellular water determined by parallel experiments, the entry rates of the cosubstrates were calculated.

Dithiothreitol was added at 10 mM just before the test when cysteine transport was under study. This agent decreased somewhat the amino acid-independent uptake of Na⁺. Although the rate of coupled cysteine uptake was not modified significantly by varying the dithiothreitol concentration, the cysteine-dependent entry of Na⁺ decreased strongly at levels of dithiothreitol below 10 mM and moderately at levels above 20 mM. The decrease on lowering the dithiothreitol was taken to arise from incomplete maintenance of cysteine in the reduced form.

For investigating possible linkage of a K⁺ flux to amino acid uptake, experiments with ^{42}K disintegrations were counted for both the cellular and the extracellular phases, in a series of five 2-min counts during an 11-min interval. The group of samples was then recounted four times. In this way losses of ^{42}K by decay applied equally to all samples in an experiment, so that no special correction had to be calculated. Substrate amino acids labeled with ^{3}H (proline, alanine, threonine and asparagine) or ^{14}C (serine) were counted only after ^{42}K decay had eliminated any measurable radioactivity from that source.

In determining the coupling ratio between Na⁺ and amino acids for exodus, ouabain was also introduced at 10^{-4} M. In this determination we could not expect to measure corrections for exodus of Na⁺ in the absence of internal amino acid substrates, or for exodus of an amino acid in the absence of internal Na⁺. Since the exchange process in question appears to require the presence of both cosubstrates on both sides of the membrane, we corrected the observed Na⁺ exodus for an appreciable rate seen when the suspending medium contained Na⁺ but lacked any amino acid. We might conversely have corrected the observed exodus of the labeled amino acid for the rate

seen when the suspending medium contained the unlabeled amino acid but no Na^+. This rate was insignificant. On the basis of evidence that the exodus of amino acids into Na^+-containing medium lacking any substrate amino acid has a significance unrelated to System ASC[5], we deducted that small component instead, to obtain the rate used in calculating the coupling ratio for exodus.

RESULTS

Development of a strategy for measuring the coupling ratio is illustrated in Fig. 1. The results taken together reveal no consistent trend of the ratio to change with the interval of observation. We had anticipated that we might be able to improve on a simple use of the shortest time compatible with reasonable precision (usually 4 min) by extrapolating to time zero to eliminate negative contributions of back fluxes to the observed rates of cosubstrate uptake. Fig. 1 shows instead that within the range of our observations the coupling ratios appear independent of time. Accordingly, reflux appears to be limited largely if not entirely to the coupled route.

The lowest 6 lines of Fig. 1 likewise show no consistent tendency for the coupling ratio to change with alanine concentration. This test was made with each of the amino acids examined. Similarly, Na^+ concentrations from 2 to 140 mequiv./l were tested without finding distinct effects on the coupling ratio. Plotting together of the data
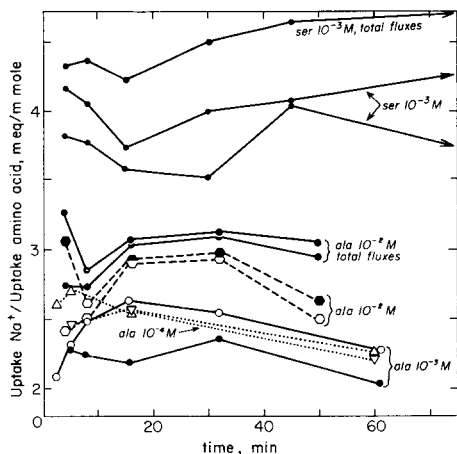

Fig. 1. Relation of coupling ratio to interval of observation for serine and alanine; also to amino acid concentration, for alanine; variability of results. Each of the bracketed pair of lines represents a single flask from which samples were taken serially during incubation at 37°, it and its partner and two other flasks representing the same sample of blood. In the third flask in each group, Na^+ had been replaced by choline to provide a measure of Na^+-independent amino acid uptake, which did not exceed 6% of the total uptake. The fourth flask contained no added amino acid and measured the amino acid-independent uptake of $^{22}Na^+$. This represented less than 6% of the total Na^+ uptake in the experiment with serine, which was made at $[Na^+]$ = 0.01 equiv/l; it represented typically 1/4 to 1/3 of the Na^+ uptake at $[Ala]$ = 10^{-2} or 10^{-3} M, and 50–66% of the Na^+ uptake at $[Ala]$ = 10^{-4} M, $[Na^+]$ having been set at 0.14 equiv/l for the alanine experiments. The amino acid-independent Na^+ uptake was approximately linear with time, although the fraction it represented of total uptake rose at the lowest and highest time intervals. All fluxes shown have been corrected for the independent fraction except where the label *total fluxes* is used. The uppermost line represents the average of observations with paired flasks. Note that the baseline represents a coupling ratio of 1.8. The upper 3 lines point at unplotted values obtained at 86 min.

of such experiments yielded values for K_m and v_{max} for the two cosubstrates as illustrated in Fig. 2 (cf. refs. 5,6). The values obtained for K_m for the amino acid and Na⁻ were indistinguishable whether Na⁺ or amino acid uptake was plotted as a function of amino acid concentration. When K_m for Na⁺ was high, the coupling ratio tended, in an imperfect relation, to be low. The ratio of the respective v_{max} values was similar to the coupling ratios obtained at lower concentrations (Table I). With amino acids
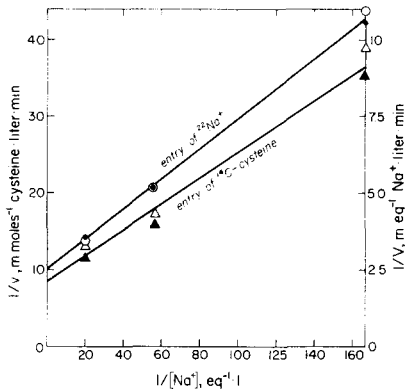

Fig. 2. Double-reciprocal plot of coupled uptakes of ²²Na⁺ and [¹⁴C]cysteine as functions of the Na⁺ concentration at 10⁻⁴ M cysteine. Uptake was observed during 4 min at 37°. Choline replaced Na⁺ to obtain Na⁺ levels of 6, 18 and 50 mequiv/l. Dithiothreitol was present at 0.01 M. Uptake of cysteine occurring in the absence of Na⁺ (3.2 μmoles/l·min) and Na⁺ uptake occurring in the absence of cysteine (5–54 μequiv/l·min, depending on [Na⁺]) was deducted from observed uptakes to obtain the coupled uptakes. The lines correspond to values of v_{max} for cysteine entry = 118 μmoles/l·min and for Na⁺ entry = 394 μequiv/l·min, both applying only for [cysteine] = 10⁻⁴ M and infinitely high [Na⁺]. The mean values of the coupling ratio for all observations of this figure was 3.4, the same ratio calculated from the two v_{max} values. This value lies at the lower limit of those observed for cysteine (Table I). Converse experiments at [Na⁺] = 0.05 equiv/l and using variable cysteine levels gave coupling ratios in the same range. This figure serves to illustrate numerous determinations of the coupling ratios from maximal velocities under similar conditions, as summarized in Table I.

TABLE I

SUMMARY OF AVERAGE COUPLING RATIOS FOR ENTRY OBSERVED FOR SEVERAL AMINO ACIDS

The term v_{max} represents the derived rate for uptake of the indicated cosubstrate at an infinite concentration of one and a known concentration of the other cosubstrate. Abbreviation: a.a., amino acid.

	Coupling ratio	
	$\Delta v_{Na^+}/\Delta v_{a.a.}$ at various concentrations ± S.D.	$v_{max\,(Na^+)}/v_{max\,(a.a.)}$
Alanine	2.52 ± 0.32	2.41
Serine	3.94 ± 0.52	3.74
Threonine	4.50 ± 0.46	4.66
Proline	0.22 ± 0.08	0.25
Hydroxyproline	3.16 ± 0.20	3.13
Asparagine	1.66 ± 0.28	1.84
Cysteine*	4.5	3.4–6

* In the presence of 10 mM dithiothreitol.

of low reactivity, *e.g.* proline, the independence of the coupling ratio of cosubstrate concentrations could be examined over only a very narrow range, and accuracy was rather poor even then. In some cases time intervals as long as 25 min were used for such poor substrates as glutamine.

Fig. 1 also shows that the coupling ratio for serine (in this case determined for $[Na^+] = 0.010$ mequiv/l) is much higher than that for alanine. The corrections made for the uptake of each cosubstrate in the absence of the other cannot to an important degree account for the difference. (See also Table V below for some values for uncoupled uptake.) The excellent precision obtained with serine (in Fig. 1, the standard deviation is 0.11) enhances the significance of the apparent time-independence of the coupling ratio of that amino acid. Table I summarizes our best mean values for the coupling ratios of several amino acids, based on numerous estimates as indicated above.

A possible explanation for the variation in the coupling ratio between Na^+ and amino acids was that K^+ uptake might partially replace Na^+ uptake in those cases where the coupling ratio is low. Therefore experiments were made in which $^{42}K^+$ was used to compare the change in K^+ uptake associated with the augmentation in amino acid uptake produced by increasing the initial Na^+ level of the medium from 0 to 0.140 mequiv/l. Table II shows that K^+ entered the cells about 50 % faster in the absence of Na^+ than in its presence. The presence of any one of the 5 test amino acids produced no characteristic or significant increase in K^+ entry, whether Na^+ was present or not; hence there was no K^+ flux that could be associated with either the Na^+-independent or the Na^+-dependent influx of the amino acids. To increase the sensitivity of our search for a linked flux, 10^{-4} M ouabain was added to reduce sharply the entry of K^+. Again, no quantitatively important alanine-dependent flux could be seen (Table II).

WHEELER AND CHRISTENSEN[3] showed that the exodus of Na^+ from the pigeon red blood cell was also accelerated by the presence of external alanine, and that entry of Na^+ during alanine uptake led to no measurable change in cellular Na^+. It was also shown that various substrates of System ASC endogenously present in

TABLE II

TEST FOR CHANGE IN K^+ ENTRY IN ASSOCIATION WITH Na^+-DEPENDENT UPTAKE OF AMINO ACIDS

Entry of K^+ or the amino acid is recorded in μmoles per kg cell water·min.

Amino acid in suspension medium	K^+ entry			Incremental amino acid entry
	Na^+ absent	$[Na^+] = 140$ mequiv/l	Increment	
None	375	258	—117	—
None*	78*	45*	— 33*	—
Proline, 5 mM	366	258	—108	5
Asparagine, 2 mM	398	268	—130	88
Alanine, 1 mM	354	257	— 97	124
Alanine, 10 mM	388	242	—146	95
Alanine, 10 mM*	72*	43*	— 29*	89*
Serine, 0.5 mM	381	242	—139	84
Threonine, 0.5 mM	375	262	—113	61

* Ouabain, 10^{-4} M, present.

the cell suffered losses during alanine uptake that might well be quantitatively equal to the alanine taken up. Accordingly, those authors proposed that the event under study is a balanced exchange of external amino acid *plus* Na^+ for internal amino acid *plus* Na^+ (ref. 3). To test this proposition further we introduced $^{22}Na^+$ and a ^{14}C- or ^3H-labeled amino acid into the cell, and estimated the coupling ratio between the two for exodus; *e.g.* the ratio between the augmentations of the two exodus rates produced by adding each of 5 different substrate amino acids (unlabeled) to the medium. Ouabain was present at 10^{-4} M to diminish unrelated Na^+ exodus. Table III shows for illustration a protocol for such an experiment, and Table IV shows values obtained in such experiments with ten combinations of internal and external test

TABLE III

ILLUSTRATIVE DATA FOR INFLUENCE OF STRUCTURE OF THE EXCHANGING AMINO ACIDS ON THE VALUE OF THE APPARENT COUPLING RATIO FOR EXODUS, $\Delta v_{Na^+}/\Delta v_{a.a.}$

The cells were first brought to apparent concentrations of 5.27 mmoles of [^3H]alanine and 15.6 mequiv $^{22}Na^+$ per kg pellet water, by incubation 2.5 h in the usual medium, $^{22}Na^+ = 0.140$ equiv/l, containing 0.010 M [^3H]alanine. Another portion of cells was similarly brought to 2.12 mM and 7.9 mequiv/l, respectively, in apparent internal [^3H]threonine and $^{22}Na^+$ concentrations. After washing, each of these two samples of cells was further divided into four portions. Two of these were introduced into fresh medium, 50–100 ml per g cells, [Na^+] = 0.140 equiv/l, containing 10^{-4} M ouabain and either 10 mM proline or 0.5 mM threonine, as indicated. Simultaneously two other portions were incubated in 0.5 mM serine or alanine, or in 2 mM asparagine, the results not being shown here. The decline in cellular content of the radioactive isotopes, relative to the suspending fluid, were then observed, and converted to μmoles per kg pellet water·min. Flux ratios were calculated as described in the text.

Amino acid, concn. (mmoles/kg water) in		Augmentation in exodus rate (μmoles/kg pellet water·min) of		Coupling ratio
Cells	Medium	Amino acid	Na+	
[^3H]Ala, 5.27*	Pro, 10	50.1, 47.0	69.4, 56.8	1.4, 1.2
	Thr, 0.5	123, 134	390, 404	3.2, 3.0
[^3H]Thr, 2.12**	Pro, 10	8.4, 7.6	27.0–21.2	3.2, 2.7
	Thr, 0.5	48.2, 47.4	207, 202	4.3, 4.3

* External Na^+, 140 mequiv/ml.
** External Na^+, 50 mequiv/ml.

TABLE IV

ESTIMATES FOR COUPLING RATIO FOR EXODUS FOR PAIRS OF EXCHANGING AMINO ACIDS, DETERMINED AS ILLUSTRATED IN TABLE III

Where a range is given, four results are included.

Entering amino acid	Coupling ratio	
	Ala exiting	Thr exiting
Pro	1.2–1.6	2.7, 3.2
Asn	1.3, 1.6	2.4, 2.9
Ala	2.2–2.6	3.8
Ser	2.2–2.5	3.7, 3.7
Thr	2.5–3.2	4.3, 4.3

amino acids. These results show that exodus is also characterized by structure-determined coupling ratios, both of the exchanging amino acids apparently participating in the determination of the ratio.

We noted that the coupling ratios were considerably more reproducible for a given sample of red blood cells than they were from cell lot to lot. The experiments of Table V show that the rates of uptake both of Na^+ and of a substrate amino acid could readily be modified by modifying the internal amino acid content of the cells. Two 10-ml portions of cells were incubated for 4 or 5 h in media containing 25 mM concentrations, respectively, of two amino acids with highly dissimilar coupling ratios. Table V shows that prior loading with proline or asparagine sharply reduced the subsequent uptake rate for asparagine, serine or threonine, relative to that seen after threonine loading. Although this difference also applied to the linked uptake of Na^+,

TABLE V

EFFECT OF PRIOR LOADING OF CELLS WITH A SUBSTRATE AMINO ACID ON THE SUBSEQUENT RELATIVE UPTAKE RATES FOR Na^+ AND AN AMINO ACID

Illustrative results. The cells were loaded with the amino acid indicated in the second column by incubating 4–5.25 h in a 25 mM solution of it, in the same medium used later during the uptake experiment, except that glucose was present at 0.1 %. After washing the cells twice with the same medium, uptake of $^{22}Na^+$ and a ^{3}H-labeled amino acid was observed from the usual medium in which all but 16 mequiv/l (Expt. 3) or 50 mequiv/l (Expt. 4) of the Na^+ had been replaced by choline. Uptake was observed as usual during 5 min (Expt. 4) or 10 min (Expt. 3). The entry rates are expressed in mequiv. or mmoles per l of cells in 1 min. "Independent" entry is that occurring for one cosubstrate in the absence of the other. The omitted figures for independent Na^+ entry did not differ significantly from those provided.

Uptake of	Cells loaded with	Independent entry		Coupled entry		
		Na^+	Amino acid	Na^+	Amino acid	Coupling ratio
Expt. 3						
2.5 mM Asn	Asn	25	11.2	54	64	0.85
		23	11.6	49	64	0.76
2.5 mM Asn	Thr	26	11.7	57	87	0.65
		28	13.0	50	82	0.61
0.5 mM Thr	Asn	25	7.2	191	34	5.7
		23	7.4	190	29	6.5
0.5 mM Thr	Thr	26	6.6	244	78	3.1
		28	8.1	236	79	3.0
Expt. 4						
0.5 mM Ser	Pro	27	3.6	177	44	4.0
			3.2	156	40	4.0
0.5 mM Ser	Thr		4.0	368	240	1.53
			3.6	418	234	1.22
0.5 mM Thr	Pro	27	4.9	296	19.2	15.4
			4.9	238	18.5	12.8
0.5 mM Thr	Thr		6.3	484	117	4.2
			6.0	467	103	4.6

the effect on amino acid entry was substantially the larger one so that the coupling ratio was significantly increased by elevated internal proline or asparagine and decreased by elevated internal threonine. These changes could be verified by comparing the results with those for cells with unmodified internal amino acid content.

DISCUSSION

The foregoing results show for both aspects of the exchange of external amino acid *plus* Na^+ for internal amino acid *plus* Na^+ that the apparent coupling ratios for System ASC vary with the structure of the external test amino acid. Further, they indicate that the structure of the internal amino acid also plays a role in determining the value of the ratio.

We have assumed in our calculations that any migration of Na^+ into and out of the cells in the absence of an external amino acid is unrelated to the process in question; also that any entry of amino acid in the absence of external Na^+ is unrelated, and that any exodus of amino acid in the absence of an external amino acid, even if Na^+ is present externally, is likewise unrelated. Omission of the corrections implicit in these assumptions does not modify our conclusion that the values of the coupling ratio for entry depend on the structure of the amino acid, but are practically independent of the interval of observation and of the concentration of the two cosubstrates. Omission of the assumptions with regard to exodus likewise does not modify the conclusion that the coupling ratios for exit change when the amino acid composition of the cell is changed.

Aside from the usually minor roles played by these migrations occurring in the absence of one of the cosubstrates in one of the phases, we have more direct evidence for the validity of the exclusion of these fluxes from consideration. The uptake of substrate amino acids observed in the absence of Na^+ is essentially a linear function of amino acid concentration[3] (*cf.* ref. 2). It is not inhibited by methionine or arginine[5] although these amino acids show a Na^+-independent inhibition of the Na^+-dependent component of serine uptake[5,7]. Conversely, the uptake of Na^+ retained in the absence of ASC substrates is not inhibited by arginine[5]. Furthermore, Li^+ does not inhibit the component of serine uptake retained in the absence of Na^+, even though Li^+ is an inhibitor competitive with Na^+ for the linked uptake of serine and Na^+ (ref. 5). The latter point is important in justifying the special way used here for correcting the rate of exodus of an amino acid for a minor component occurring in the absence of an external amino acid. If the combination, substrate amino acid *plus* Na^+ from inside the cell, could exchange by System ASC for external Na^+ alone, Li^+ should be able to inhibit that process by virtue of its competitive action with Na^+. No such effect is seen[5].

No procedure has been available to us by which a single substrate amino acid can be presented in pure state at the interior surface of the plasma membrane. In two experiments resembling that shown in Table II, the composition of the cell extract after uptake of a labeled amino acid was examined for us with the amino acid analyzer by our associate, Andrea M. Cullen. The specific activity and concentration of accumulated alanine was found to be very nearly that calculated from ^3H uptake, and very little ^3H was present except in the alanine peak. After threonine accumulation over 95 % of ^3H was found in the threonine peak, and a few per cent in the glycine peak.

The threonine concentration by ninhydrin was essentially as calculated from ^{3}H uptake.

A less satisfactory aspect of these experiments is, however, that ASC substrates other than the labeled amino acid were still present in the cell. After uptake of alanine during 1.5 h from a 5 mM solution, the aggregate molar concentration of serine, asparagine and glutamine (the first two being good substrates for System ASC) was approximately equal to that of alanine. The situation should be more favorable for the experiments of Tables III and V, where 2.5 h or more were allowed for uptake of alanine from a 10 or 25 mM solution. After accumulation for 3 h of [^{3}H]threonine from a 10 mM solution, cellular alanine was barely detectable, but asparagine and glutamine were present at an aggregate concentration half that of threonine. Under these circumstances a hypothetical value for the specific activity applying for a weighted summation of all effective internal substrates for System ASC would be somewhat smaller than the value we used for the labeled amino acid itself. Hence we cannot validly call the coupling ratios of Table III precise and characteristic values for the labeled internal amino acid; they may be considerably exaggerated from the values that would apply if all amino acid exodus by exchange through System ASC could be taken into account. What we can assert confidently is that their relative magnitudes show that the composition of the mixture of internal substrate amino acids plays a part in determining the apparent coupling ratio for exodus.

When we examine Table I, we may note that the principal structural feature of the substrate amino acid causing high values for the coupling ratio is the presence of a hydroxyl or sulfhydryl group on carbon 3 or 4 (or a carboxamide group on carbon 3). Subsequent to completion of the present study this feature has been observed to yield other indications of strongly increased substrate activity and strong interaction between the amino acid and the sodium ion[5,7]. For example, introducing a 4-*trans* hydroxy group on the proline molecule decreases its K_i for the inhibition of serine uptake to one-seventh at [Na$^+$] = 25 mM and to one-eleventh at [Na$^+$] = 118 mM. At the same time there is an enhancement in the response of the amino acid flux to the presence of Na$^+$ (ref. 5). The results of Table I show that the converse response of Na$^+$ flux to the presence of the imino acid is enhanced much more strongly, the migration of Na$^+$ being increased about 15 times as much as that of the imino acid. At the same time the ability of the external substrate to stimulate exodus of previously accumulated alanine is also strongly intensified, in both the pigeon red blood cell and the rabbit reticulocyte. Interestingly, if the hydroxyl group is presented in the *cis* orientation, these effects are in the opposite direction[5]. These findings have led us to propose that when a properly positioned hydroxyl, sulfhydryl or carboxamide group is present, the sodium ion assists in bonding between the receptor site and the amino acid[5,8,9]. The extensive evidence for this proposal is summarized elsewhere[5].

The possible participation of a second or third sodium ion per amino acid molecule in the reaction with System ASC[3,5,6] is not supported by any direct kinetic evidence. Accordingly we propose the model of Fig. 3 to account for coupling ratios of less than one or as high as four or five. Since Na$^+$ and the amino acid enter the cell at different rates in their linked uptake, a different step must then limit the rates of uptake of each of the two cosubstrates. Hence the translocation step shared among the three processes of Fig. 3 cannot generally be rate-limiting. Instead, one of the

subsequent steps producing release and replacement of one or both of the cosubstrates must limit the rate of uptake of that substrate. Presumably, the same steps at the surface of the membrane also limit the rates of exodus.

Fig. 3. Scheme to account for differential rates of exchange of Na^+ and an amino acid substrate by System ASC. Only the steps at the internal surface of the membrane are illustrated here. E. L. Thomas participated both by discussion and by kinetic experiments[5] in the development of this scheme. According to this model, when the coupling constant is increased from 0.2 to 3 by introducing a 4-*trans* hydroxyl group into proline, process 2 is decreased from a contribution of between 80 and 83 % in the total effective inward reorientations of the ternary complex represented here, to a contribution between zero and 25 %. Simultaneously, process 1 is increased from a contribution of between zero and 17 % to one of 67 to 75 %. Changes in the contribution of process 3 to the total can only cause variations of the changes in processes 1 and 2 within the stated ranges. Accordingly, the hydroxyl group has caused a shift from process 2 to process 1 as the preferred process. Since the rate of amino acid entry is simultaneously increased, this shift must arise from an even greater acceleration of process 1 than of process 2.

Under the proposed model the value of the coupling constants for entry will not depend on the external levels of the two cosubstrates, but will be determined by the relative rates of release or displacement of $*Na^+$ and *S from the ternary complex, or by the rates of reassociation, whichever is in each case rate-limiting. The latter rates will depend on the internal concentrations of the two cosubstrates, and as shown in Table V, on the composition of the internal pool of amino acids. Under initial-rate conditions internal composition will be essentially constant, so that the only variable affecting the rates will be the structure of *S.

It was an expected consequence of our model that the coupling ratios for entry would be changed by modifications of the internal content of substrate amino acids. In the experiments illustrated in Table V, the composition of the internal pool had been changed even more sharply than in Table III, although the composition was not determined and its homogeneity is not known. Comparisons between accumulations of threonine on the one hand, and on the other hand, of amino acids showing lower coupling ratios, indicate that the principal difference was an acceleration of the entry of the external amino acid by the former and a slowing by the latter. Effects on Na^+ entry were in the same direction but smaller. It is not possible to decide whether

this effect arises because threonine displaces or because it replaces the entering amino acid from the transport complex faster than does proline or asparagine.

How then does the introduction of a hydroxyl group increase the coupling ratio? Accepting that the coupling ratio for entry during proline uptake in exchange for endogenous amino acids is 0.2. and that during uptake of hydroxyproline it is 3, we can readily show within our model that a change of this magnitude can occur only by extensive replacement of Process 2 by Process 1 (Fig. 3). Since introduction of the hydroxyl group at the same time greatly accelerates uptake of the substrate amino acid, the change in the coupling ratio must be produced by an enhanced replacement of Na^+ rather than by a retarded replacement of the amino acid.

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