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ORGANIC ION TRANSPORT DURING SEVEN DECADES

THE AMINO ACIDS

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Contents

This review derives from a presentation to the Physiological Society of London in a Symposium on the Transport of Amino Acids arranged by Professor D.L. Yudilevich at Queen Elizabeth College, University of London.

I. Introduction: amino acid transport is ion transport

My title is intended to suggest that the dynamic subject of ion transport had an earlier beginning as *organic* ion transport than as inorganic ion transport. Furthermore, an unfortunate poverty in analogs for the usual inorganic ions has led to extra difficulties in differentiating their transport systems and other means of transmembrane flows. I refer to analogs that can be recognized for transport in the chemical and geometric detail I shall be reviewing here for amino acid transport.

In 1913, Van Slyke and Meyer [1] found the hepatic level of the amino acids increased in 30 min by 81 mM in the dog, out of all proportion to the 30 mM reached for the blood level on infusing casein hydrolysate. In the same decade, through the insights of E.Q. Adams [2] and of Niels Bjerrum [3], the amino acids came to be understood to be ions, even those that are dipolar and hence without net charge. Not only the anionic amino acids such as glutamate, and the cationic amino acids such as lysine, but also the ordinary dipolar ones, need to be understood as ions in interpreting their transit of membranes. As Bjerrum said, the latter are ammonium salts, a character which the term *zwitterion* should never cease to evoke. Unfortunately this character remains less than universally understood.

Some of our own analyses [4], made much later than those of Van Slyke, confirmed that large gradients may be generated between the liver and the plasma, whether as an average among the amino acids or for two especially abundant dipolar ions we selected for analysis, glycine and glutamine. Glycine averaged 33-times as concentrated in the cell water of the liver as in the plasma, 8-times as concentrated in the muscle cell water. The amino acids other than these two were found in the guinea pig to be as an average about 16-times as concentrated in the liver, 5-times as high in muscle, as in the plasma. In placing organic ion transport historically ahead of inorganic, Van Slyke and Meyer may have considered that the amino acids maintain higher chemical potentials in the various cells than outside, although quite properly they retained the cautious reservation that these apparently free ions might instead be bound [1]. It was another quarter century after Van Slyke's remarkable observation that the principal inorganic ions were understood to be subject to rapid pumping into or out of cells against gradients, rather than more or less permanently sealed into place. My object here will be to show the importance of ion charge for membrane transport, a consideration that makes it worthwhile to retain meticulously the charge signs on the symbols for both organic and inorganic ions to stress the key feature of charge that these substances have in common.

It is remarkable that the finding of Van Slyke and Meyer as it describes amino acid metabolism still seems to have gone largely unrecognized and still often ignored. Should we still consider it a premature observation? Does our picture of the regulation of interorgan nutrient flows [5] still have a place only for enzymatic steps, none for transport steps? I predict that by the time membrane functions in biology are fully understood, greatly increased importance will accrue to Van Slyke's contribution.

Within reasonable physiological pH ranges, the amino acids occur to only tiny degrees as a lipid-soluble species, for example as $RCH(NH_2)COOH$, about 4 parts per million. Evidence is so far not available that a charge transfer occurs within the membrane to generate reversibly a high-energy species of this sort. Occasionally it has been proposed that a ubiquitous, low-specificity, background rate of amino acid migration into and out of cells occurs by diffusion because of the predictable lipid solubility of this uncharged species. That species is simply too rare, however, to account for the unsaturable flux. Some years ago we designed two isomeric amino acid analogs so as to maximize the difference in the proportions of the two present in the uncharged form. By comparing their transport we confirmed once more a degree of dependence for the nonsaturable fluxes on amino acid structure, a finding that establishes again that diffusion is ordinarily an unfortunate designation for these fluxes, even though a diffusion component undoubtedly contributes occasionally. Furthermore, the isomer with the higher proportion present in the totally uncharged form did not show the faster non-saturable influx into the Ehrlich cell [6]. Evidence is still lacking, then, that all the

charges on the amino acid molecule are ever eliminated in the course of transport. Others have pointed to differences in the isoelectric points of amino acids, and hence in the proportions present in the zwitterionic form. The latter differences prove on calculation too small, however, to provide a plausible origin of observed differences in a diffusion-like transmembrane passage. For example, α- and β-alanine should differ by only about 0.03% in the proportions present as the zwitterion. Given that the amino acids are ions, we should probably not ignore a priori the possibility of passage by selective ion channels, as well as by specific mediations asymmetric as to direction, however narrow a definition we accord the term *ion channel.*

Ordinarily, mediated transport, usually concentrative inward, overwhelms the apparently non-saturable migration of amino acids across the plasma membrane. I will concern myself here with these mediated processes, usually demonstrable by their undergoing saturation with increasing levels of the transport substrate along a rectangular hyperbola. Historically the first stage of experimental discrimination among the mediating agencies showed that different facilities serve for the uptake of zwitterionic, for the anionic, and for the cationic amino acids. One still notes a tendency to suppose that cells are readily permeable to 'neutral' amino acids (say glutamine) and not to those of net charge (say glutamate), whereas actually all three pass through on the same basis: unless a suitable mediating system is present, little permeation will be observed, whether the ion has a net charge or not. I urge that we speak of zwitterionic amino acids, less we mislead ourselves with the dubious synonym, neutral. Furthermore, the consequences of avoiding the charge signs are more insidious for the zwitterions than for the anionic and cationic amino acids, and perhaps than for inorganic ions.

II. Zwitterionic transport. Heterogeneous mediation

November 1983 was the 20th anniversary of a paper by Oxender and myself [7] in which we showed that, even though competition is almost universal among the zwitterionic amino acids, at least two quite different agencies serve in parallel for their mediated passage, with a heavily overlapping selectivity among them. We subsequently displayed our evidence by three correlation diagrams [8]; that is, the extents to which leucine and phenylalanine uptakes by the Ehrlich cell were inhibited by various amino acids were well correlated, and between inhibition of glycine and alanine uptakes the correlation was rather good; between inhibition of leucine and glycine uptakes, however, correlation was negligible. It was soon necessary to add two more transport agencies to our list. Table I summarizes the list of generally occurring amino acid transport systems characterized as to homogeneity [5], as of early 1984.

IIA. On the strategy of discriminating systems

Our strategy has been to use amino acid analogs designed to emphasize the differences in molecular recognition shown by each transport system; at their best such analogs serve specifically to measure transport by a single mediating agency. Fig. 1 shows that if such paired analogs are well selected, they may eliminate first one and then the other component of transport of the test amino acid. Furthermore, the circumstance that the partition between the components is scarcely changed by applying the inhibitory analogs in reversed order can serve to show that the second component is not an artifact of saturating the first and thereby altering the trans-membrane potential or depleting other energy storage. This strategy also requires that the half-saturating concentration for each analog be evaluated, i.e., in Fig. 1 the K_m for uptake be determined for each under conditions producing homogeneity of transport, and the K_i be determined for each as an inhibitor under corresponding conditions. Unless these parameters are consistent, the indications remain ambiguous. This test is occasionally ignored.

IIB. Relations among transport systems for zwitterionic amino acids

Among the agencies we recognize as serving for zwitterionic amino acids (Table I), we note some that are rather alike. For example, the widely encountered System Gly looks rather like a variant

TABLE I

SUMMARY OF SOME KNOWN TRANSPORT SYSTEMS IN TISSUES AND CELLS OF HIGHER ANIMALS

Further literature citations pertinent to this table may be found in Table I of Ref. 5. See text for some other details, especially Section VII on terminology.

Systems for Zwitterionic amino acids		
Na^+-dependent systems [a]		
System Gly	Widespread. Glycine and sarcosine.	Tolerance of *N*-methyl group or other group on α-amino N.
System A	Ubiquitous. Serves for most zwitterionic amino acids. Variants (A_2 etc.) known.	
System ASC	Ubiquitous, with varying scope. Excludes N-methyl amino acids, but may include prolines. Protonated system accepts certain anionic amino acids	
System N	Glutamine, asparagine, histidine. Characterized so far only in hepatocytes and hepatoma cells.	
β-System	β-Alanine, taurine, 4-aminobutyric acid	
Na^+-independent systems		
System L	Ubiquitous. Prefers amino acids with branched and apolar side chains. Bicyclic amino acids as model substrates. High exchanging activity. Heterogeneity should be excluded in each occurrence.	
System L_1	Refers to a distinct low-K_m component developing on extended incubation of hepatocyte monolayers.	
System T	Human red blood cells; distinct affinity pattern preferring benzenoid amino acids.	
Na^+-independent systems for cationic amino acids		
(Systems specific to arginine or to ornithine are known. Evidence for a system sharing cystine transport: see text.)		
System y^+	Ubiquitous; fails to discriminate between arginine and lysine homologs. Level initially low in cultured rat hepatocyte (see Ly_1 and Ly_2 suggested for intestinal mucosa)	
Systems for anionic amino acids (capital X proposed where system is Na^+-dependent, Ref. 45)		
System X^-_{AG}	Na^+-dependent, similar reactivity with aspartate and glutamate. As described in this review, wide occurrence, including neuronal, with provocative relations to recognition of anionic neurotransmittory amino acids.	
System X^-_A	Na^+-dependent; aspartate and its analogs; largely excludes glutamate and longer analogs. In characterized occurrences, e.g., in hepatocytes and erythrocytes, proves identical with System ASC.	
System x^-_G	Glutamate and its analogs, largely excluding aspartate and shorter analogs. So far, only Na^+-independent versions known, one highly specific system in endogenous neuronal vesicles, another which transports cystine and glutamate as described herein, and hence designated x^-_C.	

[a] Several other systems under this heading have been proposed for intestinal brush-border absorption [5a,40], mainly on the basis that each is not identical with other systems rather than on a demonstration of its homogeneous mediation. Even with detailed kinetic evidence for homogeneity, it must be admitted that supposedly homogeneous transport components (e.g., L) have subsequently shown heterogeneity.

of System A, narrowed sharply to limit it to glycine and sarcosine. Both of these two Na^+-dependent systems tolerate an *N*-methyl group on the amino acid; they are highly concentrative and show similar handicaps on lowering the pH even from 7.4 to 7.0, and to 6.5. Na^+-dependent System N shows a similar pH sensitivity, and all three are stimulated when cells are starved for their amino acid substrates. Another Na^+-dependent system ASC, at first glance seems similar, but we shall see below that it is really quite different. Co-transport with Na^+ means that even amino acids without net charge may pass the osmotic barrier in (bound) cationic forms. Finally, Na^+-independent System L transport is also quite unlike the other transports, obviously as to its substrate preferences and its energization, and unlike Systems A and Gly in an especially intense participation in *trans* stimulation, i.e., in the exchange of its amino acid substrates across the membrane. Na^+-independent transport of zwitterionic amino acids inhibitable by the norbornane amino acid, which we embrace under the term System L, has functions quite unlike the steeply concentrative Na^+-dependent systems. By exchange among amino acids, this component allows the energy represented by a gradient of a given amino acid to be redistributed in the form of gradients for a variety of amino

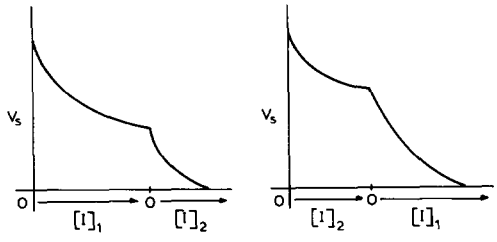


Fig. 1. Idealized representation of use of analogs for discriminating two independent routes of transport of a test substrate. Each analog eliminates competitively an independent component of uptake. See text for discussion.

acids. In this process System L is caused to run backward; for several of its substrates, cellular uptake typically occurs by System A or another Na^+-dependent system, and exodus by System L [9]. For other amino acids, the intersystem cooperation may take the reverse sequence.

IIC. Importance of system ASC

System ASC also is concentrative and shows a good deal of *trans* stimulation or exchange across the plasma membranes (for example in the fibroblast, see Fig. 4 in Ref. 9a). This system may well be the most conspicuous uphill system in the cells of the intact animal and in cultured cell lines, their growth in amino acid-rich environments having led to substantial repression of System A. System ASC appears to be unaccompanied by System A in various red blood cells. After stimulation of a reticulocytosis in the rabbit, System ASC activity persists in the erythrocyte population some days longer than does the reticulocyte morphology [10]. In man, System ASC activity persists in the mature erythrocyte, according to two independent observations [11,12]. As I shall discuss below, System ASC has recently revealed an increasing ability to transport anionic amino acids instead of zwitterionic amino acids as the pH is brought to values below 6. Prior application of System ASC substrates at physiological concentrations to the 'i' surface of pigeon red blood cell vesicles, along with ATP, GTP, P_i, and other trace cellular constituents, stimulates the subsequent ATP-dependent Ca^{2+} transport. The amino acids are required for the stimulation by P_i. A signaling role has therefore been suggested for System ASC transport in metabolic regulation [13]. These considerations do not support the idea that the several transport systems are physiologically equivalent ways of moving the total list of amino acids. For transport systems largely specific to a single amino acid, e.g., System Gly for glycine and System N for glutamine, asparagine, and histidine, unique biological functions are implied because of special physiological roles played by these amino acids. We also note special roles for amino acid transport in neurotransmission, in mitochondrial function, in lysosomal function, and so on. In short, it is an oversimplification to say nature developed several systems because there are several zwitterionic amino acids: the explanation is more complex. As a price for the convenience of abbreviations, we find ourselves needing to contest occasional popular misinterpretations that System A serves exclusively for one group of amino acids, System L for another, and especially that System ASC serves only for alanine, serine and cysteine. In fact, the homologs containing one more carbon atom than these three amino acids are frequently more reactive with System ASC, and the scope of System ASC has proved somewhat variable [14].

III. Why do amino acid transport systems tend to ignore a third or fourth charged group on the amino acid molecule?

IIIA. Tripolar amino acids received for transport as dipolar

The transport systems serving for ordinary zwitterionic amino acids can also serve to some extent for diamino acids and dicarboxylic amino acids. If the pH has been appropriately altered, this capability is an obvious one, as we may see for histidine transport in the Ehrlich cell in Fig. 2 [15]. As the pH is lowered, smaller and smaller quantities of the amino acid are present to be recognized as simply a zwitterionic amino acid; hence, curves similar to alkalimetric titration curves are obtained. We see that effect in the right-hand curve in the declining transport of histidine by neutral System L as it becomes more and more completely protonated, and in the left-hand curve in the increasing transport by the cationic system. In this case the cross-over point for the two curves is not

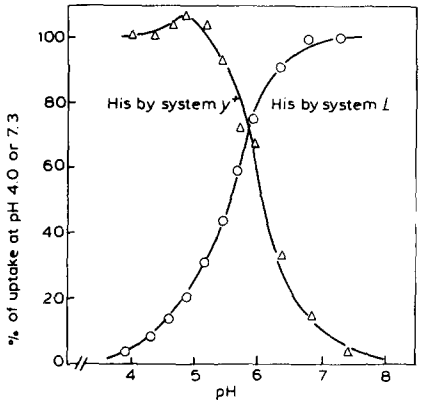


Fig. 2. Changes in the rates of histidine uptake by the Ehrlich cell via System L (●) and System y^+ (○) as the charge on the molecule is modified with the pH. Adapted from Ref. 15

far removed from the pK_2' for the imidazole group as observed on titration in free solution. To get System L to conduct glutamic acid across the plasma membrane we must in contrast lower the pH, usually to pH 5 or less, so that we have in fact substantial concentrations of the zwitterion, glutamic acid ($pK_2' = 4.1$), even though the glutamate anion may still dominate [16]. We show elsewhere curves corresponding to those of Fig. 2 for the uptake of glutamic acid and some of its analogs by System L of the Ehrlich cell (Fig. 2 in Ref. 17). In this case, we see no crossing curve for transport as the anion, glutamate, because in that cell we have rather low activity for specific transport of this species. Furthermore, the displacement of the positions of these curves from the pK' values applying for titration in free solution becomes unmistakable, and greater for some than for other analogs. We take this displacement to arise from a relative stabilization of the transported form (here the protonated species) through the energy of its selective binding for transport.

A diamino acid may likewise behave as a zwitterion at neutral pH because the distal amino group is recognized in its unprotonated form. At first glance this would seem an obvious consequence of a low value for pK_2'. In the homologous series, lysine, ornithine, 2,4-diaminobutyric acid, and 2,3-diaminopropionic acid, the values for pK_2' take the declining order 9.2, 8.7, 8.24 and 6.7. Hence the shortest molecule in this series should be present 83% as a zwitterion, and only 17% as a tripolar cation. For the longest molecule, lysine, these proportions should be less than 2% and more than 98%. In either case, a functional selection of the zwitterionic component might as for histidine allow appreciable transport in that form.

IIIB. Altered sequence of dissociation of diamino acids

The transport of these diamino acids occurs, then, rather more by the zwitterionic systems than would be expected from their titration curves. Furthermore, the zwitterion formed by a diamino acid in free solution will be mainly the α,ω-zwitterion, not the α,α-zwitterion required by Systems A, L, etc. This standard prediction we confirmed some years ago by proton magnetic resonance analyses of the titration of 2,4-diaminobutyric acid, as illustrated in Fig. 3. The familiar zwitterion transport systems do not accept other than α,α-

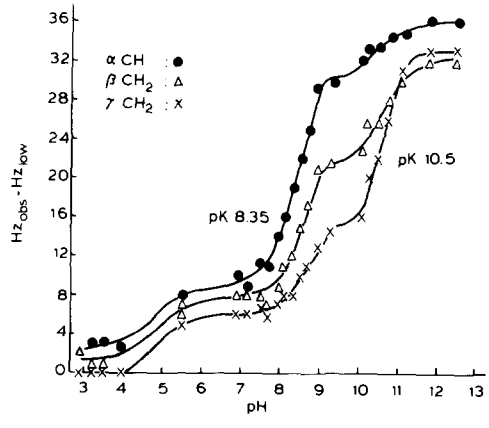


Fig. 3. Assignment of the dissociation sequence of L-α,γ-diaminobutyric acid by NMR spectroscopy. Measurements made with a Varian T-60 instrument in ^2H_2O, titrating a 0.7 solution with NaO^2H and ^2HCl. The pH values were obtained by plotting $\Delta pH/\Delta Hz$ against ΔpH. The proton attached to the alpha carbon shows the larger chemical shift with a pK' of 8.35, whereas the protons attached to the gamma carbon show their larger chemical shift with a pK' of 10.5. The pK' values obtained by Albert [64] by acid-base titration (c-0.2 M) were 8.24 and 10.44. The present results are consistent with standard chemical logic that the ionization takes predominantly the pathway via the α,ω-zwitterion A. Exact assignment of the relative contribution of the minor alternative pathway is prevented because we do not know to what degree the smaller chemical shift seen in each titration curve is due to the minor alternative pathway of dissociation, and to what degree this signal arises instead from the ionization of the other, nearby amino group. Experiment by Daniel Schwass in this laboratory, 1975.

zwitterions, as we may illustrate by our customary use of ϵ-aminocaproic acid as an inert buffer in such experiments [16]. Furthermore, the diamino acids with pK_2' in the range of 8.0 to 8.5 show unusually strong accumulation by Systems A or L. Hence, we conclude that the transport receptor sites act to reverse the sequence of protonation or deprotonation of the two amino groups from the sequence seen in free solutions. A predictable consequence of the stabilization of the ϵ-amino group in its deprotonated form will be the stabilization of the α-amino group in its protonated form; hence the reversal is not totally unexpected. Accordingly we conclude that the transport systems cause the formation of the needed, α,α-zwitterionic structure, and not the predicted and often untransportable, α,ω-zwitterion.

It is a predictable consequence of the uptake of cationic amino acids by a zwitterionic transport system that their steady-state accumulation would be intensified by the usual orientation of the transmembrane potential. The intense accumulation of the scarcely cationic 2,3-diaminopropionate ($pK_2' = 6.7$) may, however, have further implications, as we have discussed elsewhere [18,19].

IIIC. Possible utility of tripolar amino acids for discriminating among Na^+-independent mediating systems for zwitterionic amino acids

In the meantime, evidence has been obtained that the transport activity attributed to System L, namely the Na^+-independent transport inhibited by the 2-aminonorbornane-2-carboxylic acid isomer called BCH, includes in some cases two or more somewhat dissimilar components. In the human red blood cell, a component especially responsive to tryptophan and phenylalanine has been detected, although scarcely responsive to leucine and histidine [20], and a different variant System L increases in activity while the rat hepatocyte is held in primary culture [21]. We have extended the list of amino acids to which tryptophan migration into the human red blood cell scarcely responds by inhibition, to include these usual System L substrates: methionine, 4-amino-1-methylpiperidine-4-carboxylic acid, valine, thialysine (Fig. 4), glutamine, threonine, *p*-azidophenylalanine and β-(dimethylamino)alanine (azaleucine). Uptake by this route is also low in sensitivity to BCH and BCO [22]. By instead testing Na^+-independent leucine transport, Vadgama, in my laboratory, notes a different unfamiliar component of leucine uptake also scarcely inhibited by these amino acids, or by histidine, but highly sensitive to inhibition by BCH and BCO (see Fig. 4), as well as leucine (Vadgama, J.V., unpublished data).

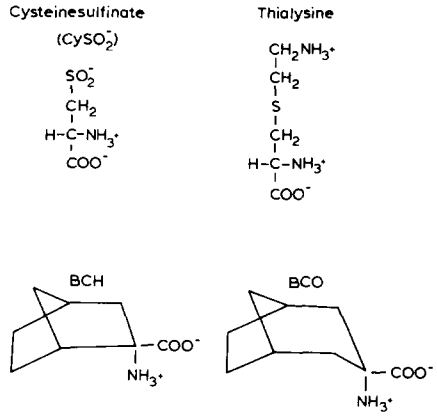


Fig. 4. Some pertinent model amino acids. Cysteinsulfinate (β-sulfanyl-L-alanine), aspartate analog as well as a cysteine metabolite; *S*-aminoethylcysteine or thialysine; BCH, b(−)-2-amino*endo*bicyclo[2,2,1]heptane-2-carboxylic acid; BCO, 3-amino*endo*bicyclo[3,2,1]octane-3-carboxylic acid [22].

These results point to the usefulness of the wide range of System L substrates heretofore identified, for the further description of unidentified components of System L transport. I should stress that such heterogeneity has emerged where Na^+-independent uptake had previously appeared homogeneous by simpler kinetic tests. Clearly, obtaining a rectangular hyperbola for transport rate versus substrate concentration is not a sufficient proof of homogeneity of catalysis. Weissbach and Kilberg have informed us of somewhat similar indications of further heterogeneity in Na^+-independent amino acid uptake by the rat hepatocyte in primary culture, although in our hands uptake by the hepatoma cell HTC so far has not revealed heterogeneity [23].

Until such heterogeneity is fully resolved for various cells, we are entitled to question whether the special intensity of accumulation of certain diamino acids by System L pertains to a special component of that system, and hence whether

such diamino acids might serve to discriminate that component. The model amino acid BCH has now been improved upon [22] to gain a different advantage by introducing one more methylene group into the larger alicyclic ring, to yield 3-amino*endo*bicyclo[3.2.1]octane-3-carboxylic acid (BCO; see Figure 4). The molecule is thereby rendered symmetrical to make unnecessary a stereochemical resolution during the preparation. Furthermore, BCO may be seen as a leucine analog, not an isoleucine analog, whichever direction we proceed from the alpha carbon around the ring system.

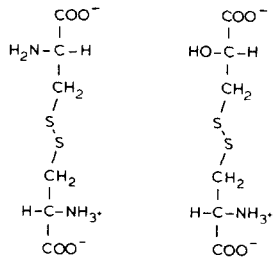


Fig. 6. Structure of cystine anion compared with that of the product of its monodeamination, β-mercaptolactatecysteine disulfide. Both alpha carbon atoms have in each case the L-configuration.

IV. Tetrapolar amino acids seen for transport as tripolar

What we have considered so far in this discussion and still seek to interpret is that tripolar amino acids can react for transport as dipolar, i.e., zwitterionic molecules, and not necessarily as the particular α,ω-zwitterion one would expect. I want now to add another striking case, that of the tetrapolar amino acid, cystine, which can be transported as a tripolar molecule, analogous to and competing with the anionic amino acid, glutamate. The formal demonstration of this mode of cystine transport as an anion was made for a transport system shared by cystine and glutamate [24] in various cells cultured in monolayer [24,25]. The

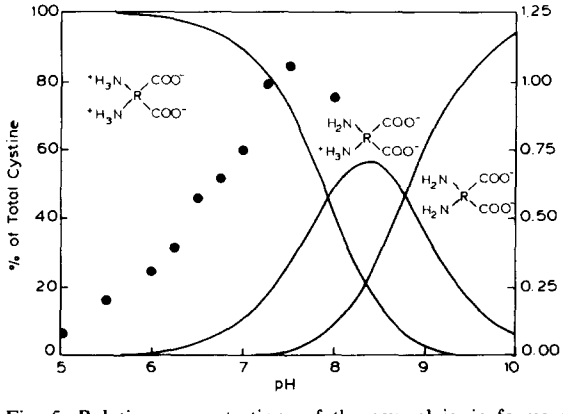


Fig. 5. Relative concentrations of the several ionic forms of cystine calculated as a function of pH, using pK_3' and pK_4' values of 7.94 and 8.79. The points at the left and the scale at the right show for comparison the rates of glutamate-inhibitable cystine uptake in nanomol/mg protein per min. Reproduced with permission from Ref. 25.

pH dependence of this interaction (Fig. 5) shows that the cystine molecule needs to be deprotonated to enter into competition with glutamate. Note also in Fig. 5 that the loss of glutamate-sensitive cysteine uptake with falling pH occurs along a curve displaced by more than a pH unit from the position predicted by the acid-base titration of cystine in free solution. Again, we attribute to the energy of binding of the tripolar ion at the binding site the increased ease of deprotonating the tetrapolar species for transport. This discrepancy led us to seek reinforcement of the evidence for selection of the anionic form of cystine for transport. When we replace one of the amino groups of cystine with a hydroxyl group, to lock the structure into the tripolar ion in the β-mercaptolactate-cysteine mixed disulfide (Fig. 6), we extend its uptake and its inhibitory action on glutamate uptake to much lower pH values. This behavior is what we should predict for that new tripolar amino acid.

So we have here a transport system in which one of the amino groups of cystine is seen not in the charged state we tend to expect above pH 7, but instead as an uncharged group which can be ignored by the receptor site through selective binding, hence stabilization of the amino acid as its unprotonated anion. Let me recall the hypothesis of Dent and Rose [26]. We may suppose that these authors were perhaps unintentionally predicting a converse of the same process to explain the pattern of amino acid losses of cystinuria, namely that one of the carboxyl groups of cystine is received by the transport system defective in that disease in its less probable protonated form. That is, cysteine would be received as a cationic tripolar transport analog

of lysine rather than of the anionic glutamate. It is theoretically possible but inherently unlikely that these two modes of cystine transport could coexist in the same membrane, to cause parallel transport both as a tripolar anion and as a tripolar cation, presumably leading to two pH optima for cystine transport. The preliminary study of specific cysteine release from lysosomes suggests reception for transport there also as a tripolar cation, as for the renal tubule a lysine-like species, but with the added feature of a necessary recognition of one of the sulfur atoms [27]. Here we are offered a surprising new physiological problem. This recognition of a sulfur atom is signaled by an inhibitory action of *S*-2-aminoethylcysteine (Fig. 4), and of other related analogs but not by sulfur-free analogs, on [^{35}S]- for [^{32}S]cystine exchange between the exterior and interior of the lysosome [27]. A separate transport system exchanges lysine for lysine analogs across the lysosomal membrane of cultured fibroblasts, whether cystinotic or normal; cysteine-cysteamine mixed disulfide may escape by that route in cysteamine therapy [28]. The pattern of transport recognition of cystine as a cation is one that should favor its efflux over its influx at the usual pH gradient between cytoplasm and lysosomal interior, the opposite polarity expected for the plasma membrane with recognition of cystine as an anion. Because the inborn defect in this transport system in cystinosis apparently does not influence everywhere the distribution of cystine, we need not be surprised to find the lysosomal transport system for cystine unlike the systems so far described for the plasma membrane. Apparently lysosomes have a considerable set of amino acid transport systems, and the view current until recently that lysosomes have holes large enough for smaller but not larger amino acids to pass appears obsolete. Other organelles, namely mitochondria, a glutamate-accumulating neuronal vesicle [29], and now perhaps the epithelial brush border [30,31], extend the difference between the systems found in endogenous vesicles from those of the plasma membrane. Such differences probably would allow separate functional and regulatory responses. For the study of cystine transport, identification of a stable analog such as *S*-(2-aminoethyl)cysteine [28], not cleavable into two parts by a sulfhydryl-disulfide exchange reaction, should provide an important investigative advantage. It is not yet clear whether the competition between cystine and diaminopimelate in *E. coli* [32] and in the kidney really means that cystine is in some cases transported as a tetrapolar ion, i.e., as the double zwitterion we tend to expect. Diaminopimelate administration causes lysinuria in the rat [33], suggesting its transport, like that of cystine, as a tripolar cation.

V. Redressing of charge discrepancies with the sodium ion

We have proceeded so far in the confidence that the decisive determinant of which mediating system will serve for transport of an amino acid is the state of charge in which the amino acid can be seen by the pertinent receptor site. Over a decade ago we noted that the charge difference between zwitterionic (dipolar) amino acids and cationic (tripolar) amino acids can, however, also be made up selectively by the sodium ion, and to some degree by its close analogs such as lithium. Thus a charge imbalance is made up in another way than by selection between a protonated and an unprotonated species. We may say that this effect has been observed both as a subtraction and as an addition of Na^+. For zwitterionic System ASC, Na^+ is ordinarily essential as the co-substrate, but cationic amino acids such as arginine or lysine can react with this system in a Na^+-independent manner. The evidence persuades us that the positively-charged group on the side-chain of such amino acids serves at the recognition site as a surrogate for the sodium ion. Even more interesting is the ability of various neutral amino acids (homoserine is a good example) to serve in a Na^+-dependent fashion as surrogate substrates in the Na^+-independent system for cationic amino acids, which we call y^+ (formerly Ly^+). Fig. 7 shows a recent demonstration of the effect, specifically by the Na^+-dependent inhibition of Na^+-independent arginine uptake into cultured fibroblasts by either of three zwitterionic neutral amino acids [34]. Fig. 8 visualizes in two dimensions how the cationic amino acid and the zwitterionic amino acid plus Na^+ may alternatively be recognized by a given transport receptor. One might be tempted to suppose that these interactions mean that Sys-

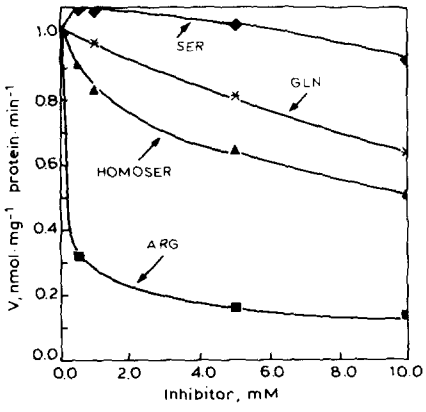
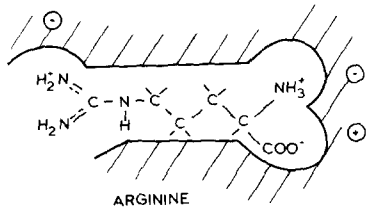


Fig. 7. Zwitterionic amino acid inhibition of arginine uptake in cultured human fibroblasts. Uptake from 0.1 mM [^{3}H]arginine was measured during 30 s at pH 7.4 and 37 °C. When the Na^+ of modified Earle's medium was replaced with choline, the inhibitory action of the three zwitterions became negligible, although that by excess arginine was unchanged. Reproduced with permission from Ref. 65.

tem ASC and y^+ are actually one and the same agency, serving alternatively for zwitterionic amino acids plus sodium ion, or for tripolar cations



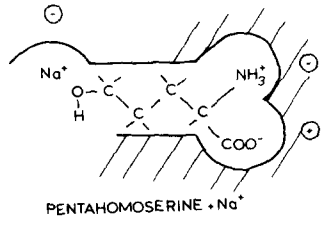


Fig. 8. Representation of mode by which an ω-hydroxy amino acid might be recognized by a receptor site normally recognizing a cationic amino acid. The sodium ion is seen as a surrogate for the cationic group on the amino acid side chain. The critical character of the location of the hydroxyl group is explained here by the sharing of orbital electrons by Na^+ and O. The transport of the normal substrate is Na^+-independent. A different transport system appears to recognize arginine and homoarginine, but not ornithine, lysine, or N^5-methylhomoarginine, by H bonding to the N5 hydrogen of the guanidinium group [41].

without Na^+. We have, however, refuted this idea in detail (see p. 237 of Ref 35); indeed, the surrogate actions just described are weak enough to make one wonder how much physiologic importance they have, although they obviously carry mechanistic significance. Interactions between lysine and zwitterionic amino acids are also seen for intestinal transport.

VA. Decisive effects of the position of unrequired side-chain hydroxyl, sulfhydryl or other functional groups. Implication for intestinal amino acid transport

In both of these cases, the presence of an omega hydroxyl or a sulfhydryl group on the side-chain strongly enhances the cooperativity with Na^+. Furthermore, the acceptable distance between this group and the two functional groups on the alpha carbon is characteristic of the transport system, i.e., whether the hydroxyl group is borne on carbon 3, carbon 4 or carbon 5. We may say that this test reveals a 'hot spot' in the amino acid chain, pointing to the relative positions taken by Na^+ and the amino acid in the transport complexes. Differences in the position of the 'hot spot' provides one more basis for discriminating transport systems. An even closer localization of the Na^+ in the transport complex was obtained by exploiting the restraint imposed on the orientation of the hydroxyl group in the isomeric hydroxyprolines. The observation of a 'hot spot' for a terminal hydroxyl group on carbon 5 at a principal Na^+-dependent amino acid transporting agency of the rabbit ileum as measured by methionine influx (undoubtedly at the brush border side of the epithelial cells) probably points not to an identity of that system with either Systems ASC or y^+, but to a major dissimilarity of the system to System A. The latter system fails to show any 'hot spot' of the sort described here (Fig. 5 in Ref. 36). Cultured LLC-pK_1 cells, from the pig kidney, express Systems A, ASC and L at their basolateral surface; since these cells apparently do not express the principal Na^+-dependent system otherwise present at the brush-border surface [30], we may infer that the latter system probably is not genetically identical with the ASC component at the basolateral surface. The failure of the LLC-pK_1 cells in monolayer to

express at least one renal transport system at the brush-border side has led to a paradoxical net transport in vitro in the reversed direction from that corresponding to its main physiological function in vivo [37]. The evidence for renal tubular cells may suggest that the intestinal brush border also has transport systems unlike those of most plasma membranes. Nevertheless, System L, or variants thereof, appears to be present at both poles of the jejunal mucosal cells, and System y^+ has been reported for the basolateral pole [38]. Both of these systems and system ASC have been described for the basolateral surface of the cat salivary epithelium in situ by a paired tracer perfusion technique [39]. Differences in gene expression do not of course mean that one or more brush-border systems may be totally unlike known transport systems. We do find mutual inhibitory action between threonine and aspartate for uptake by jejunal segments of the rat, but do not consider our evidence sufficient yet to establish that the Na^+-dependent brush-border system has the trait of interconvertibility associated elsewhere with System ASC, as described in the following section. The exceptionally wide range of the Na^+-dependent brush-border transport activity of neutral amino acids also discourages a close identification with System ASC, and is likely to make difficult a decision as to how many transport systems it includes. Substantial differences of intestinal brush-border systems among species [40] may intensify this problem. Standards of their kinetic discrimination present another problem.

When a side-chain group is observed to enhance transport reactivity of a zwitterionic amino acid, that group is generally found to provide an added basis for chirality of the amino acid, even if the group is not essential to acceptance for transport. Although I seek to focus particularly in this paper on receptor recognition of chemical groups through their ability to assume a charge, I should of course avoid an impression that this ability is the sole basis of recognition. Cationic amino acid transport systems may achieve specificity to arginine on a basis other than localization of charge, namely by specific recognition of the N-5 hydrogen atom of that amino acid (Fig. 8), apparently by H bonding to it [41]. Replacement of the H atom on N-5 with a methyl group terminates transport; furthermore, serine homologs like that shown in Fig. 8 inhibit the specific arginine transport. Apparently in this case the guanidinium group supplies two points of recognition, although only one cationic charge.

VI. Titration of the receptor site itself

We come finally to the case where the transport system itself, not the substrate, is the target for redressing of charge to accommodate alternatively dipolar and tripolar amino acids. Makowske, Vadgama and myself have now identified such a case, that of the ubiquitous Na^+-dependent transport System ASC, for which amino acids of two quite different states of charge compete in a wide range of cells. The zwitterionic threonine and the unambiguously anionic cysteate or cysteinesulfinate illustrate the contrasting substrates, although the first of these needs to be understood as representing several of the zwitterionic amino acids, and the latter two may be joined in biological function by aspartate, the latter however, a somewhat inferior substrate for the same system. Even for glutamic acid, a small component of uptake by the Ehrlich cell at pH 4.3 was identified earlier with System ASC (Ref. 16; see Fig. IV therein), although the state of charge in which glutamic acid is accepted is not yet established. Not only do these unlike amino acids compete kinetically for uptake by the hepatocyte and by hepatoma cells in monolayer; they also undergo *trans*-acceleration, one by the other, of movement across the plasma membrane [12,42]. Furthermore, the limitation placed on the length of the molecules is much the same for zwitterionic and for anionic substrates. What is especially significant is that the side-chain anionic group of cysteinesulfinate (and also of cysteate) has a pK' so low (namely 1.5) that one cannot readily believe that it could be received in its protonated form at pH values as high as 6. Nor is a change in the state of protonation of threonine at all likely in the range pH 5 to 6.5. Instead, we believe that the crossed sigmoid curves of Fig. 9, showing the uptake rates of these two amino acids as functions of pH, arise from a reversible titration of the carrier, to produce two distinct receptor sites at equilibrium. When $CySO_2^-$-for-threonine exchange is catalyzed, a net H^+ cotransport oc-

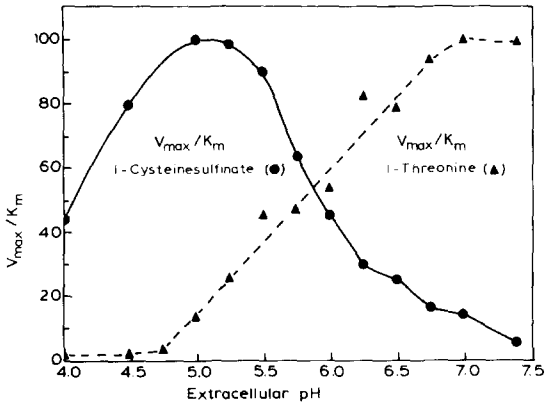


Fig. 9. pH Dependence of the Na^+-dependent transport of threonine (▲) and cysteinesulfinate (●) into the hepatoma cell HTC. The Na^+-dependent rates are expressed in terms of V_{max}/K_m, i.e., the rate constant applying at low concentrations, setting the highest rate obtained at 100. The kinetic parameters are based on data of Makowske and Christensen [42], consistent with transport in each case by a single, homogeneous mediator. Note that the two curves cross at about pH 5.8.

curs. Fig. 10 shows the scheme for the interconversion. The case has a precedent in the red cell chloride exchanger, whereby its acceptance of the sulfate ion is enhanced by carrier protonation [43]. The present case appears, however, to produce a cleaner discrimination between the paired substrates of discrepant charge, and, as may be characteristic of organic ion transport, also recognizes the substrates in far greater molecular detail.

Can we find special meaning in the ability of System ASC to serve not only for zwitterions but to some degree also for anionic amino acids plus H^+, and to some degree for cationic amino acids without Na^+? Might this system, alone or along with System L, have allowed transport of a sufficient range of amino acids for primitive life? As already mentioned, intestinal absorption shows

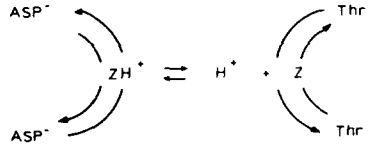


Fig. 10. Scheme for interconvertibility of transport systems ASC and x^-_A of hepatocytes and other cells. How Asp^- and Thr are seen (a) competing for uptake; (b) showing trans-membrane exchange. Z, a receptor site for zwitterionic amino acids.

traits that have suggested to us dominance by an ASC-like mediator (although not ASC per se), based on the similarity in the position of the 'hot spot' in the amino acid side-chain (see Fig. 5 in Ref. 42). The more highly regulated Systems A, N and Gly might conceivably have been added later in the evolutionary scale. Furthermore, despite the indications of functional differences among the systems, we find no indication of a totally different mode of construction of any of them; for example no substantial evidence for a role for an enzymatically catalyzed group translocation, e.g., a γ-glutamyl transpeptidation. For these organic ions, as well as for inorganic ions, no need for their net metabolic modification in the course of transport has yet been shown.

VII. Terminology: towards a simple set of symbols

The transport of aspartate by a Na^+-dependent hepatocyte system not transporting glutamate significantly [25,44] was the initiating observation for the discovery of the dual service of System ASC. Operating in parallel is another Na^+-dependent system transporting both aspartate and glutamate in competition; also the already mentioned Na^+-independent system transporting glutamate and cystine. We had already committed ourselves to the abbreviation y^+ (earlier, Ly^+) for a system requiring a positive charge on the amino acid side chain, and x^- for any system requiring a negative charge on the side chain [25]. Fig. 10 illustrates that this symbolism holds in reserve the letter z for systems transporting zwitterionic substrates. To deal with the three types of x^- system so far distinguished for anionic amino acids, we proposed to use x^-_A where the system selects aspartate and analogs of that shorter chain length, to use x^-_G where the system selects glutamate and analogs of that or greater chain length and is almost unreactive with aspartate, cysteate and cysteinesulfinate, and to use x^-_{AG} for the conspicuous system which is nearly as reactive with glutamate as with aspartate. The results for a series of analogs of varying separation of the α- and the distal functional groups supports the importance of this distance in these discriminations [42]. After conferring with Professor Bannai [24], we introduced the further term x^-_C for the Na^+-independent system for which

glutamate and cystine compete. This cystine-accepting system may be considered generically to be of the x_G^- type, a term that can also logically be applied to the glutamate-specific system characterized by Naito and Ueda [29] for an endogenous vesicle of bovine brain. Other interested persons have been invited to comment on the x_A^-, x_{AG}^-, x_G^-, x_C^- scheme, including Drs. Ellory, Gazzola, Guidotti, Kilberg, Lajtha, Sacktor, Smith and Young; we note use of this terminology in the recent paper of Dall'Asta et al. [45] for the corresponding systems in human skin fibroblasts. That paper uses the capital X for the Na^+-dependent systems, and a lower-case x for the Na^+-independent, a further detail whose optional use we hope proves not to overload the scheme, as we proceed under a collective provisional approach. With the discovery that System x_A^- as described for the rat hepatocyte is a manifestation of System ASC, we propose to reserve unqualified use of the term x_A^- for possible occurrences of an aspartate-specific system unrelated to System ASC. In a survey of the occurrences of System ASC, including human red blood cells, pigeon red blood cells, Ehrlich cells, and the Chinese hamster ovary cell line, we have so far found no case where lowering the pH to 5.5 fails to intensify the inhibition of threonine transport by cysteinesulfinate [12,46]. Hence we will take the designation System ASC to embrace the capacity for anionic amino acid transport added on carrier protonation.

VIII. The apparently ubiquitous system X_{AG}^-

Let me turn then to the widely distributed X_{AG}^- system, characterized in detail for the rat hepatocyte. This Na^+-dependent system appears to have also been described for dog erythrocytes [47] and for skin fibroblasts [44,45]. Its V_{max} is sharply increased in a family of dogs with low [Na^+], high [K^+] and a high corresponding ATPase in their red blood cells [48,49]. The so-called glutamate system of the renal tubule also shows traits apparently justifying the term System X_{AG}^- for it also [50]. The kinetic parameters for Na^+-dependent aspartate and glutamate uptake reported for neurones [51] are highly similar to those for the hepatocyte and hepatoma cells. We have called attention elsewhere [52] to a considerable series of similarities in the recognition chemistry among anionic amino acid analogs for neuronal transmittory effects and for the X_{AG}^- system as we have described it for the liver [42]. These include in particular the acceptance of D-aspartate quite as well as L-aspartate, even though D-glutamate is poorly accepted. We point out this anomaly does not justify the doubt that has been expressed about the biological chirality of the corresponding recognition site for neurotransmission [52]. We were able to show through the use of 3-aminoglutaric acid that components of the hepatic X_{AG}^- site are oriented in space with enough latitude to prevent good discrimination between the respective positions in space of a formate and an acetate group attached to the alpha carbon to constitute a dicarboxylic amino acid, thus accounting for the anomalous defect in stereospecificity. This loose fit allows D-aspartate to bind backwards with respect to the position taken by L-aspartate, although a propionate side chain is discriminated sharply enough from formate or acetate to minimize the acceptance of an inverted D-glutamate molecule in place of L-glutamate [44,52,53], with one possible biological exception [54]. In some biological occurrences, the site prefers D- somewhat over L-aspartate, perhaps not a surprising consequence of the low sophistication of recognition. The anomalous stereoselectivity has also been observed in a cultured cerebellar nerve cell line [53] and for the renal tubular system [50]. It will be interesting to observe whether System X_{AG}^- occurs in some instances without this stereoselective anomaly.

IX. Comment on regulation of amino acid transport

Ultimately, the detailed transport recognition of structural features of the amino acids, including their different states and distributions of charge, determines which have their flows modulated independently by membrane transport, and which appear to present problems of collective or overlapping regulation. In the developing drive to model and redesign catalytic domains of proteins, attention will obviously go to those catalyzing transport dynamics. Discrimination of routes of transport becomes vital to the study of transport regulation. It has been emphasized that Na^+-dependent System A is especially responsive to regulation, both

through a cellular adaptation to the amino acid economy and as a response to humoral messengers from other cells; but this is not the only system under physiologic regulation. The Na^+-dependent Systems Gly, N, and the Na^+-independent transport system for cysteine and glutamate [45] are also under adaptive regulation. Furthermore, it appears likely that the other zwitterionic amino acid systems are under regulation [55,56], although by different agencies and on different principles. So far regulation of transport by recruitment of mediator molecules held in reserve within the cell has, I believe, not been proved for amino acids. For certain transport systems in which a narrow range of amino acid selectivity is shown, special roles of that molecular selectivity and under regulation suggest themselves: e.g., regulation of System N might direct certain physiological flows of glutamine, for example, to the kidney (cf. Refs. 57–59) or across the blood-brain barrier to allow subsequent System L exchange for other amino acids. The regulation of System Gly could modulate flows of glycine; the regulation of System y^+, movements of arginine and ornithine between the liver and the rest of the organism. Interpretation of the physiological interrelations between wider-range systems such as A, ASC, and L promises to provide more stubborn problems.

White and I [61] recently observed for hepatoma cell line HTC that regulation of System A by the supply of amino acid substrate ('adaptive regulation', Ref. 60) works somewhat equally on influx and efflux. A precise equality would render adaptive regulation futile if these fluxes are permitted to come to equilibrium, an event which seems in many cases unlikely. The regulation is kept effective in important instances because transport remains rate limiting to the metabolism of a given amino acid for a given tissue, e.g., for alanine utilization by the liver. A coordinate regulation of catabolism may be necessary to keep transport rate limiting to alanine utilization [62,63]. Similar problems in the regulation of interorgan flows [5] now seem accessible.

X. Summary

The amino acids are ions of various charge combinations, and one can argue that historically they were the first ions for which the ongoing problem of membrane transport was presented; also that among transported ions these may undergo a highly detailed molecular recognition. Furthermore, the distribution of charge on the amino acid molecule determines by what route or routes it is conducted across the biological membrane, with what directional and structural specificity, and therefore what regulation is imposed, and where. Cases where a presumably charged chemical group behaves as if it were somehow absent from the amino acid have been observed to fall into several categories:

(1) Straightforward cases where the pH has been low enough or high enough to remove the charge by protonation or deprotonation, even in free solution.

(2) Cases where that protonation or deprotonation is facilitated at the binding site, and perhaps by the total transport process. The cystine molecule can apparently thus be rendered either a tripolar anion or a tripolar cation for transport.

(3) Cases where an otherwise co-transported Na^+ is omitted to redress charge, or where a Na^+ serves as a surrogate for a missing charged group on the amino acid molecule.

(4) A case where the protonation occurs reversibly at the receptor site rather than on the amino acid molecule.

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