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Passive diffusion of non-electrolytes across the lysosome membrane

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An osmotic-protection method has been used to study the permeability of rat liver lysosomes to 43 organic non-electrolytes of formula weights ranging from 62 to 1000. A lysosome-rich centrifugal fraction of rat liver homogenate was resuspended in an unbuffered 0.25 M solution of test solute, pH 7.0, and incubated at 25 °C for 60 min. The free and total activities of 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminidase were measured after incubation for 0, 30 and 60 min. Three patterns of results were seen. In pattern A the percentage free activity remained low throughout the 60 min incubation, indicating little or no solute entry into the lysosomes. In pattern B, the percentage free activity was initially low, but rose substantially during the incubation, indicating solute entry. In pattern C there was not even initial osmotic protection, indicating very rapid solute entry. The rapidity of solute entry into the lysosomes showed no correlation with the formula weight, but a perfect inverse correlation with the hydrogen-bonding capacity of the solutes. The results, which can be used to predict the ability of further compounds to cross the lysosome membrane by unassisted diffusion, are discussed in the context of metabolite and drug release from lysosomes *in vivo*.

INTRODUCTION

The lysosome membrane has recently been shown to contain a number of substrate-specific metabolite porters (for a review see Forster & Lloyd, 1988). The existence of these porters implies, although it does not prove, that they are required for the translocation of the relevant metabolites across the lysosome membrane. Stronger evidence for a physiological role is available in a few cases, where a genetic defect in a carrier leads to impaired efflux of some metabolite from the lysosomes. Thus the intralysosomal accumulation of cystine, sialic acid or vitamin B_{12} occurs when the relevant porter is deficient (see Forster & Lloyd, 1988).

The question arises: can any organic solutes cross the lysosome membrane by passive diffusion? If not, carriers must exist for all the products that arise from the lysosomal catabolism of biopolymers. Another consequence would be a severe limitation on the potential of targeted lysosomotropic drug delivery, since drug efflux from the lysosome is a crucial step in this process (Lloyd *et al.*, 1984), and it is unlikely that carriers exist for many xenobiotics.

There have been extensive studies on the passive diffusion of organic molecules across the plasma membranes of many cell types. Stein (1967) collated an extensive volume of data and found that in many systems the rate of diffusion of substances correlated well with their oil:water partition coefficient, and that both these parameters could be predicted from a rather simple calculation of the molecule's hydrogen-bonding capacity. This calculation assigns a hydrogen-bonding capacity to each functional group (e.g. an aliphatic OH group = 2), and the total for a molecule is a simple sum of the parts. Thus methanol is assigned a hydrogen-bonding capacity of 2, ethylene glycol 4, and glycerol 6. Diamond & Wright (1969), reaching similar conclusions to those of Stein (1967), re-assessed the numerical assignments of hydrogen-bonding capacity. Few changes were proposed: one relevant to the present work is a value of just below unity for ether or ester linkages, contrasting with Stein's (1967) assignments of zero and 0.5 respectively to these functional groups.

In the present paper we report experiments designed to determine the ability of small uncharged molecules to cross the lysosome membrane. This initial study investigates molecules containing only carbon, hydrogen and oxygen, with the oxygen function limited to aliphatic hydroxy, ester and/or ether groups. This class of compound was chosen for two reasons: first, it excludes ionic molecules, which would be expected from studies on plasma membranes to have little diffusional capacity; and secondly, it includes the neutral sugars, which are important products of lysosomal metabolism.

Passive diffusion, in contrast with carrier-mediated mechanisms, cannot translocate physiologically relevant solutes preferentially. If passive diffusion across the lysosome membrane does occur, rates of diffusion will depend wholly on the physico-chemical features of the molecule translocated. Our approach has therefore been to measure the rates of uptake of non-physiological molecules into lysosomes. Since some such molecules might fortuitously be substrates for a metabolite porter in the membrane, we have studied as large a number of solutes as possible. We have also included a few physiological polyols for comparison.

Most of the solutes needed for this investigation are not available in radiolabelled form, thus precluding the use of a direct method to assess rates of uptake into lysosomes. Instead we have used an indirect method that depends on the ability of iso-osmotic solutions of non-permeant solutes to confer osmotic stability on lysosomes (Lloyd, 1969, 1971). This method has a second major advantage over direct measurements of solute uptake: it

does not require that the lysosomes studied should be freed from contaminating organelles, a lengthy procedure that may not leave the lysosome membrane undamaged.

MATERIALS AND METHODS

Materials

The solutes investigated for ability to afford osmotic protection were from Aldrich or Sigma and of the highest grade available. Solutions (0.25 M) in water were adjusted to pH 7.0 with 1 M-NaOH. No buffer was used, in order to minimize any additional osmotic capacity.

Preparation and incubation of lysosomes

Rat liver lysosomes were prepared as previously described (Lloyd, 1969). The lysosome-rich fraction, sedimenting between 1100 *g* (10 min) and 22500 *g* (10 min), was gently resuspended in the appropriate ice-cold test solution (5 ml/0.1 g original pulp wt.). The suspension was then placed in an incubation bath at 25 °C. At 0, 30 and 60 min duplicate samples were withdrawn and assayed for free and total *N*-acetyl-*β*-glucosaminidase (Bird *et al.*, 1987).

RESULTS

There are three reasons why poly(ethylene glycol) oligomers seemed ideal for the first series of experiments. First, they are wholly non-physiological, and therefore inherently unlikely to be substrates for a metabolite porter in the lysosome membrane. Secondly, they are available commercially, in monodisperse preparations up to the hexamer, and with acceptably narrow polydispersity in a graded range of M_r values. Thirdly, every member of the series possesses two terminal hydroxyl groups, differing only in the number of ethylene oxide moieties (Fig. 1).

Table 1 shows the results of incubating lysosomes at 25 °C in 0.25 M solutions of monodisperse poly(ethylene glycol)s. The results of an incubation in 0.25 M-sucrose, a known non-permeant, are also shown. Over 90% of the hexosaminidase activity of lysosomes resuspended in sucrose was unavailable to substrate, indicating that these lysosomes were intact. Incubation in sucrose for up to 60 min did not lead to any significant loss of enzyme latency. In contrast lysosomes resuspended in poly(ethylene glycol) ($n = 1$, 2, 3, 4 or 5) displayed an immediate loss of latency. Hexa(ethylene glycol), however, afforded initial osmotic protection to the lysosomes, although latency was lost by 30 min of incubation.

The rapid loss of latency observed on resuspension of lysosomes in solutions of the low oligomers (Table 1) could be explained in two ways: either the oligomers rupture the lysosomes by a detergent-like effect, or the oligomers cross the lysosome membrane so rapidly that they cannot afford even transient osmotic protection. The results shown in Table 2 demonstrate that the second of these explanations is correct. In this experiment lysosomes were resuspended in solutions containing both 0.25 M-sucrose and 0.25 M-poly(ethylene glycol). These

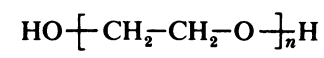

Fig. 1. Chemical structure of poly(ethylene glycol) showing repeating ethylene oxide moieties

Table 1. Free activity of *N*-acetyl-*β*-glucosaminidase in rat liver lysosomes incubated at 25 °C for 0, 30 or 60 min in 0.25 M solutions of poly(ethylene glycol)s

Free activity is expressed as a percentage of the total activity seen in the presence of Triton X-100 (0.2%). Values show are means (±S.D.) for at least three experiments.

Solute	Formula weight	Free activity (%) 0 min	30 min	60 min
Sucrose	342	6±1	7±3	9±2
Ethylene glycol	62	90±8	99±1	93±8
Di(ethylene glycol)	106	76±5	94±3	95±6
Tri(ethylene glycol)	150	94±4	98±3	98±2
Tetra(ethylene glycol)	194	82±11	91±3	93±5
Penta(ethylene glycol)	238	67±15	92±7	79±6
Hexa(ethylene glycol)	282	16±7	84±7	89±5

Table 2. Free activity of *N*-acetyl-*β*-glucosaminidase in rat liver lysosomes incubated at 25 °C for 0, 30 or 60 min in solutions containing both 0.25 M-sucrose and 0.25 M-poly(ethylene glycol)

Free activity is expressed as a percentage of the total activity seen in the presence of Triton X-100 (0.2%). Values shown are means (±S.D.) for at least three experiments.

Solute	Free activity (%) 0 min	30 min	60 min
Sucrose alone	5±2	8±1	9±1
Sucrose and ethylene glycol	4±2	8±2	10±3
Sucrose and di(ethylene glycol)	5±2	6±1	7±1
Sucrose and tri(ethylene glycol)	5±0	7±0	8±0
Sucrose and tetra(ethylene glycol)	6±1	7±1	10±2
Sucrose and penta(ethylene glycol)	6±1	6±3	10±5
Sucrose and hexa(ethylene glycol)	8±1	5±0	7±2

Table 3. Free activity of *N*-acetyl-*β*-glucosaminidase in rat liver lysosomes incubated at 25 °C for 0, 30 or 60 min in 0.25 M solutions of poly(ethylene glycol)s 200–1000

Free activity is expressed as a percentage of the total activity seen in the presence of Triton X-100 (0.2%). Values shown are means (±S.D.) for at least three experiments.

Solute	Free activity (%) 0 min	30 min	60 min
Sucrose	5±4	9±2	10±2
Poly(ethylene glycol) 200	76±8	92±8	85±3
Poly(ethylene glycol) 300	55±8	83±2	82±2
Poly(ethylene glycol) 400	4±1	52±4	62±3
Poly(ethylene glycol) 600	6±1	10±1	17±1
Poly(ethylene glycol) 1000	6±0	6±0	6±0

Table 4. Free activity of *N*-acetyl-β-glucosaminidase in rat liver lysosomes incubated at 25 °C for 0, 30 and 60 min in 0.25 M solutions of various non-electrolytes

Free activity is expressed as a percentage of the total activity seen in the presence of Triton X-100 (0.2%). In calculating hydrogen-bonding capacity, oxygen-containing moieties were assigned values of 2.0 (C–O–H), 0.8 (CO_2–C) or 0.8 (C–O–C) (see the Introduction). Values for free activity shown are means (±S.D.) for at least three experiments.

Solute	H-bonding capacity	Formula weight	Free activity (%) 0 min	30 min	60 min
Carbitol	3.6	134	81±5	87±4	92±5
Ethylene glycol	4.0	62	90±8	99±1	93±8
Cyclohexane-1,4-diol	4.0	116	71±9	92±6	89±3
Hexane-1,6-diol	4.0	118	89±1	95±8	100±0
Cyclohexane-1,4-dimethanol	4.0	144	92±3	90±6	86±6
Di(ethylene glycol)	4.8	106	76±5	94±3	95±6
Di(propylene glycol)	4.8	134	81±3	82±9	94±4
Tri(ethylene glycol)	5.6	150	94±4	98±3	98±2
Dimethyl-D-tartrate	5.6	178	77±7	84±8	85±4
Hexane-1,2,6-triol	6.0	134	58±4	81±5	91±3
Propane-1,1,1-trimethanol	6.0	134	68±3	88±4	93±6
Tetra(ethylene glycol)	6.4	194	82±11	91±3	93±5
Poly(ethylene glycol) 200	6.5*	$200_{average}$	76±8	92±8	85±3
Penta(ethylene glycol)	7.2	238	67±15	92±7	79±6
Isopropylidene-D-mannitol	7.2	262	70±7	88±3	88±2
Erythritol	8.0	122	9±3	75±8	87±4
Pentaerythritol	8.0	136	6±2	74±3	91±10
Octane-1,2,7,8-tetrol	8.0	178	7±1	87±8	90±8
Hexa(ethylene glycol)	8.0	282	16±7	84±7	89±5
Poly(ethylene glycol) 300	8.4*	$300_{average}$	55±8	83±2	82±2
D-Arabinose	8.8	150	6±1	60±6	81±7
D-Lyxose	8.8	150	6±1	63±5	81±7
L-Lyxose	8.8	150	8±3	50±4	69±4
D-Ribose	8.8	150	9±4	73±6	95±3
D-Xylose	8.8	150	5±4	73±4	87±3
2-Deoxy-D-galactose	8.8	164	10±1	57±10	76±6
D-Fucose	8.8	164	12±3	28±1	51±4
L-Rhamnose	8.8	164	7±2	55±10	78±9
3-*O*-Methylglucose	9.6	194	6±2	27±3	47±5
L-Arabitol	10.0	152	10±3	30±7	48±6
Xylitol	10.0	152	9±2	27±5	55±5
Poly(ethylene glycol) 400	10.3*	$400_{average}$	4±1	52±4	62±3
D-Glucose	10.8	180	8±2	39±5	54±2
D-Mannose	10.8	180	9±2	31±5	64±1
D-Sorbose	10.8	180	7±2	21±2	49±8
myo-Inositol	12.0	180	10±3	19±7	36±6
Dulcitol	12.0	182	5±1	9±2	26±4
D-Mannitol	12.0	182	5±2	9±1	12±2
D-Sorbitol	12.0	182	6±2	10±2	23±4
Perseitol	14.0	212	6±4	5±1	10±4
Poly(ethylene glycol) 600	14.0	$600_{average}$	6±1	10±1	17±1
Sucrose	18.4	342	5±4	9±2	10±2
Poly(ethylene glycol) 1000	21.5*	$1000_{average}$	6±0	6±0	6±0

* Calculated from M_r average.

solutions afforded as complete an osmotic protection as did 0.25 M-sucrose alone, demonstrating that the oligomers have no ability to rupture the lysosomes other than by failing to afford osmotic protection.

Table 3 shows how lysosomes behave when resuspended in 0.25 M solutions of poly(ethylene glycol)s of average M_r between 200 and 1000 and with polydispersity of ±5%. Three basic patterns can be observed. When the average M_r was 200 or 300, little or no initial osmotic protection was afforded. When the average M_r was 400, osmotic protection was observed initially, but this was progressively lost on incubation. Poly(ethylene glycol) of average M_r 600 or 1000 offered prolonged osmotic protection, the latter polymer being as effective as sucrose.

The results shown in Tables 1 and 3 indicate clearly that low oligomers of poly(ethylene glycol) can cross the lysosome membrane rapidly. With one minor anomaly, the results also conform to a consistent pattern indicative of decreasing rates of poly(ethylene glycol) entry with increasing molecular size. [The anomaly, the greater initial osmotic protection provided by hexa(ethylene

glycol) (M_r 282) than by the sample of average M_r 300, is probably a consequence of the polydispersity of the latter sample.]

In what way does the increasing degree of polymerization of poly(ethylene glycol) decrease its rate of entry into lysosomes? Is this a consequence of greater molecular size in itself, or does it reflect the greater hydrogen-bonding capacity of larger molecules, resulting from the larger number of ether moieties? This question can only be answered by studying a series of molecules in which the two parameters, M_r and hydrogen-bonding capacity, are independent variables. Table 4 shows the results of this study, which investigated 43 compounds with M_r values ranging from 62 to 1000 and with hydrogen-bonding capacities from 3.6 to 21.5. The data in Table 4 are compiled in increasing hydrogen-bonding capacity, and only secondarily in ascending M_r, because the results indicate so clearly that the former is the major, if not the only, determinant of rate of entry into lysosomes.

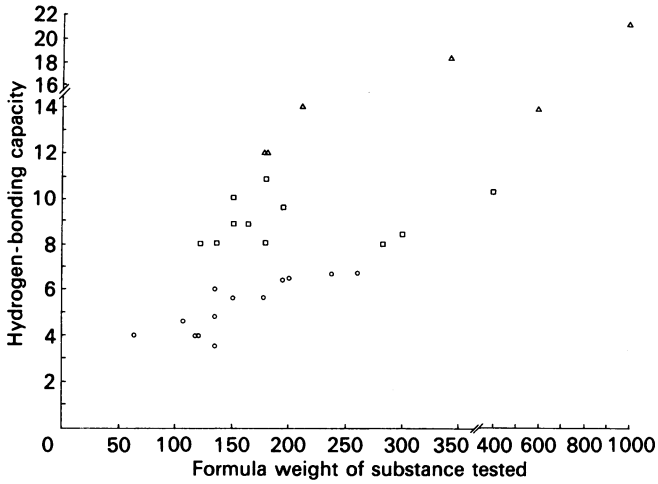

Fig. 2. Matrix showing the formula weight, the hydrogen-bonding capacity and the pattern of osmotic protection provided for all the solutes investigated

Osmotic-protection patterns are as described in Table 5: A (△), B (□) and C (○). Each point on the matrix can be identified by reference to Table 4. Several points represent more than one solute.

DISCUSSION

Methodology

As explained in the Introduction, the osmotic-protection technique was chosen for these studies because there was no feasible alternative. Since the technique has been criticized (Maguire *et al.*, 1988), it is appropriate to consider its strengths and weaknesses before discussing the implications of the data that we report above.

There seems no reason to doubt that the measured rate of loss of latency, observed when lysosomes are suspended in an iso-osmotic solution, reflects the rate of entry of the solute into the lysosomes. The technique has been widely used, without dissent, to study the translocation into lysosomes of sugars (Lloyd, 1969; Docherty *et al.*, 1979; Maguire *et al.*, 1983), amino acids and oligopeptides (Lloyd, 1971; Goldman, 1973; Kooistra *et al.*, 1982), nucleosides (Burton *et al.*, 1975) and inorganic ions (Casey *et al.*, 1978). Nevertheless it is important to acknowledge that the technique cannot be used to calculate absolute or even relative numerical rates of solute entry: only the rank order of entry rates can be confidently inferred. It may be that osmotic-protection studies using a lysosome population of narrow and known size range could yield information on absolute entry rates, but to our knowledge this has not been attempted.

The osmotic-protection technique is also incapable of yielding conclusive evidence concerning the mechanism(s) by which the solutes are entering the lysosomes, since entry by passive diffusion or on a carrier would equally lead to loss of osmotic balance. However, it should be noted that the experimental conditions employed favour solute entry by passive diffusion, where net rate increases linearly with the concentration gradient, but are unfavourable to the net accumulation of solutes by carrier-mediated mechanisms. This is because the external concentration of solute used, 250 mM for non-electrolytes, greatly exceeds the K_m of most membrane porters. As pointed out by Docherty *et al.* (1979), the net rate of solute translocation is very low if the carrier is symmetrical in its kinetics and the solute concentration on both sides is much greater than the K_m. The reported values for the amino acid carriers in the lysosome membrane are in the micromolar range (Forster & Lloyd, 1988); these carriers would therefore be ineffective in the context of an osmotic-protection experiment. In contrast, the putative monosaccharide porter (Maguire *et al.*, 1983) and the recently reported nucleoside porter (Pisoni & Thoene, 1988), whose K_m values are 50 mM (for D-glucose) and 8 mM (for adenosine) respectively, may make a significant contribution to net solute entry, at least at the early stages of an osmotic-protection experiment.

Determinants of rates of passive diffusion

An examination of the data shown in Table 4 clearly identifies three basic patterns. Some solutes provide complete or near-complete osmotic protection for all or much of the 60 min incubation period; this we designate Pattern A. Some solutes provide initial osmotic protection, but the lysosomes rupture extensively during the remainder of the incubation (Pattern B). Yet other solutes fail to provide even initial osmotic protection (Pattern C). In Fig. 2 we show all the compounds tested in this investigation, including the poly(ethylene glycol)s, graphing each in terms of its M_r, its hydrogen-bonding capacity, and its behaviour in the osmotic-protection experiments. It is striking that, without a single exception, the osmotic-protection pattern (A, B or C) is predictable from the hydrogen-bonding capacity. The data can be used to formulate a rule relating these two parameters (Table 5).

It seems inescapable from these results that, in our experiments, the solutes investigated enter lysosomes chiefly, if not wholly, by passive diffusion. The perfect concordance between the hydrogen-bonding capacity and the osmotic-protection pattern is otherwise inexplicable. Moreover, since most of the 43 substances investigated are non-physiological, mechanisms of entry other than diffusion are inherently improbable.

The inverse correlation between diffusion rate and hydrogen-bonding capacity indicates that the determinants of diffusion across the lysosome membrane are similar to those that apply to plasma membranes [see Stein (1967) and Diamond & Wright (1969)]. An analysis

Table 5. Relationship observed between hydrogen-bonding capacity of non-electrolytes and behaviour pattern in osmotic-protection experiments

Hydrogen-bonding capacity	Osmotic-protection pattern
Above 11.5	Pattern A: sustained protection. Free activity remains below 20% for at least 30 min.
7.5–11.5	Pattern B: transient protection. Initial free activity below 20%, but rising to above 20% by 30 min.
Below 7.5	Pattern C: no protection. Initial free activity above 20%.

of the permeability pathways induced in the plasma membrane of human erythrocytes by *Plasmodium falciparum* (Ginsburg & Stein, 1987) indicates the same correlation. Whether the absolute diffusion rates across lysosome membranes are higher or lower than those across other biological membranes cannot be answered at present.

Our results show no apparent correlation between diffusion rate and M_r, within the range of M_r investigated, thus disproving the earlier hypothesis (Cohn & Ehrenreich, 1969; Ehrenreich & Cohn, 1969; Lloyd, 1969, 1971) that an M_r ceiling of around 220 limits passive diffusion across the lysosome membrane. Ginsburg & Stein (1987) similarly found almost no correlation with molecular volume in the *Plasmodium*-induced erythrocyte pathways.

Among the solutes studied were a few substances of physiological importance. It is noteworthy that the rates of latency loss observed with this group of compounds do not differ from those of their non-physiological analogues. Thus the pentoses (hydrogen-bonding capacity 8.8) and the hexoses (10.8) crossed the lysosome membrane no more rapidly than would be predicted from their physico-chemical characteristics. Their entry into lysosomes in these experiments is therefore explicable in terms of passive diffusion alone. This conclusion is further discussed below, with reference to its physiological significance.

In the analysis of our results thus far, we have not distinguished between the different rates of latency loss observed within the group of solutes that display pattern-B behaviour. As noted above, it is unwise to attempt too fine an analysis of osmotic-protection data, but some important features emerge clearly. The first is that, within this sub-group of solutes, the inverse correlation between hydrogen-bonding capacity and rate of solute entry still holds good. Table 4 shows that, as the hydrogen-bonding capacity increases, the rate of latency loss decreases. The second noteworthy feature concerns the few physiological compounds studied. There are no major differences between the rates of latency loss between the four pentoses studied, nor between the three hexoses. This lack of any stereochemical specificity further supports the view that in these experiments sugars penetrate the lysosomes by passive diffusion. Whether the minor differences apparent between the individual pentoses (and hexoses) represent some contribution from a carrier-mediated mechanism cannot be decided without further work using more refined techniques. A similar comment applies to the apparent difference, at 60 min incubation, between mannitol and dulcitol or sorbitol.

Physiological and pharmacological implications

The major flow of metabolites across the lysosome membrane *in vivo* is from lysosome to cytoplasm. By means of this traffic, the monomeric products of the catabolic pathways occurring in lysosomes are made available for further metabolism in the cytosol. The magnitude of the concentration gradient across the lysosome membrane is not known for most metabolites, although measurements have been made for many of the amino acids (Harms *et al.*, 1981). There can be no doubt, however, that this concentration difference will be much lower than 250 mM, the concentration difference initially present in osmotic-protection experiments. Consequently rates of passive diffusion will be much lower *in vivo* than *in vitro* and, as explained above, conditions will be more favourable *in vivo* for a contribution from metabolite porters, for metabolites for which these exist.

These considerations lead to the following conclusions. First, solutes that fail to cross the lysosome membrane in osmotic-protection experiments will be unable to cross by passive diffusion *in vivo*. They will therefore be non-penetrant *in vivo* unless a suitable porter exists in the lysosome membrane. As explained above, a porter with a low K_m might not be an effective net translocator under the conditions of osmotic-protection experiments. We predict that any substance, whether physiological or not, whose hydrogen-bonding capacity is 12.0 or above will be either a non-penetrant *in vivo* or, if a penetrant, will be translocated by a carrier-mediated mechanism. Some molecules are known to be non-penetrant *in vivo*, because they cannot escape from lysosomes if they enter by pinocytosis. Sucrose, dextran and Triton WR-1339 are among the non-electrolytes in this category (Lloyd, 1973); all of these have a hydrogen-bonding capacity in excess of 12.

A second conclusion is that substances whose hydrogen-bonding capacity is below 12 should be capable of diffusion across the lysosome membrane *in vivo*. Substances towards the top of this range may diffuse slowly, and a carrier, where one exists, may greatly increase the overall rate. Substances of hydrogen-bonding capacities below 7.5 should diffuse rapidly *in vivo*, leaving little need for carrier-mediation. Thus it would not be surprising if passive diffusion and membrane porter(s) both contribute to the release of hexoses and pentoses from lysosomes. It is unlikely, however, that glycerol release requires a porter. Ginsburg & Stein (1987) have deduced that the simple neutral amino acids such as glycine have a hydrogen-bonding capacity of 11. This value is just above that of the hexoses, and it is therefore not surprising that the lysosome membrane contains porters for neutral amino acids. The good osmotic protection afforded by 250 mM solutions of the small neutral amino acids (Lloyd, 1971; Kooistra *et al.*, 1982) is explicable in terms of their hydrogen-bonding capacity and the ineffectiveness of low-K_m carriers at these concentrations (see above for amplification of this statement). Those amino acids that contain additional hydrogen-bonding moieties must have values well above 12, and the presence of carriers for acidic, for basic, and for some aromatic amino acids (Forster & Lloyd, 1988) could have been predicted from the data reported here.

The conclusions that we have reached concerning

passive diffusion across the lysosome membrane make it possible to predict whether any substance will be able to cross without a carrier. This will provide focus for the search for further metabolite carriers in the membrane, and will also be valuable to the pharmacologist who aims to target drugs to cells by using the endocytic route. There will be little to be gained by this chemotherapeutic approach if the drug in question has a hydrogen-bonding capacity greater than 12. Fortunately many do not.

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