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Lysosome membrane permeability to amines

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Abstract

The permeability of rat liver lysosomes to xenobiotic organic compounds possessing nitrogen functions was investigated, using an osmotic-protection methodology. It was first shown that rat liver lysosomes are stable for at least one hour when incubated in 250 mM sucrose within the pH range 5 to 9. Primary and tertiary amines with pK_a values within this pH range, and with differing numbers of aliphatic hydroxy or ether groups, were chosen for study and their permeability investigated at a range of pH values. The results indicate that uncharged amines can cross the lysosome membrane, and that the permeability of such molecules can be predicted from their total hydrogen-bonding capacity. The notional hydrogen-bonding capacity of an uncharged tri-substituted nitrogen with no attached hydrogen atom, as in pyridine or in a tertiary aliphatic amine, is deduced to be approximately 1, and that of an uncharged primary amine approximately 2. A hydrogen-bonding capacity of at least 11 is deduced for cationic nitrogen, implying that most if not all molecules containing a charged nitrogen atom cannot cross the lysosome membrane by passive diffusion. The implications for lysosome physiology and pharmacology are discussed. © 1997 Elsevier Science B.V.

Keywords: Lysosome membrane; Amine (organic)

1. Introduction

The lysosome membrane constitutes a physiologically important barrier between the lysosome matrix and the surrounding cytoplasm. The membrane's impermeability to macromolecules ensures the retention within the organelle of both the lysosomal enzymes and their substrates. By contrast, the products of lysosomal metabolism must leave the lysosome. Most of these metabolites are thought to cross the membrane by transport on substrate-specific porters, although a contribution from passive diffusion is likely in a few cases (see [1] for a review).

Passive diffusion is certain to be the only mechanism available for most non-physiological molecules to cross the lysosome membrane. Several current approaches to targeted drug delivery require the drug to cross the lysosome membrane in order to reach the subcellular site where its action is desired [2]. It will be valueless to target a drug efficiently to some cell type if the drug remains trapped in the lysosomes. An ability to predict how well a given xenobiotic structure will cross the lysosome membrane is therefore of potential practical utility.

In our first study addressing this topic [3], we tested some 40 organic non-electrolytes containing aliphatic hydroxy, ether, hemiacetal and ester moi-

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eties. The results showed that the rate of passive diffusion of solutes across the rat liver lysosome membrane is not significantly influenced by their molecular weight. However there was a strong inverse correlation between rate of penetration and a substance's notional hydrogen-bonding capacity, a parameter calculated from the number and nature of the functional groups the molecule possesses. The ability of these non-electrolytes to cross the lysosome membrane appeared to be wholly predictable from chemical structure.

Solute permeabilities across biological membranes are commonly correlated with oil–water partition coefficients. This approach is of limited value for many molecules of biochemical interest, since they are too hydrophilic for their partition coefficients to be determined with accuracy. Stein [4] was the first to point out the high degree of correlation between oil–water partition coefficient and notional hydrogen-bonding capacity, and to demonstrate the ability of the latter parameter to predict solute permeabilities across a variety of biological membranes.

Stein [4] proposed theoretically derived numerical values for the hydrogen-bonding capacity of several common functional groups found in organic molecules. Subsequently Diamond and Wright [5] slightly modified these values in respect of some functional groups; we used Stein's values and these modifications in our study [3] on the permeability of the lysosome membrane to organic non-electrolytes. A subsequent study of the permeability of rat kidney lysosomes to seven low (<100) molecular weight non-electrolytes [6] revealed a good correlation between measured permeability for 6 of the 7 solutes and their partition coefficients in various oil–water systems. Using the permeability data from that publication and Stein's [4] values for functional group hydrogen-bonding capacities, a good correlation can be demonstrated between these two parameters (Fig. 1). The correlation coefficient (including all 7 compounds) is 0.88, greater than that reported [6] for three out of the four sets of partition coefficients.

Since drug moieties possess many functional groups other than the three oxygen-containing functions previously [3] studied, it is of value to obtain direct evidence on the ability of substances with such groups to cross the lysosome membrane. Our ultimate aim is to make the permeability of any organic molecule across the lysosome membrane reliably predictable from its structural formula. In the work reported in the present paper we have extended our previous studies to embrace organic molecules containing nitrogen atoms that have or can acquire a positive charge.

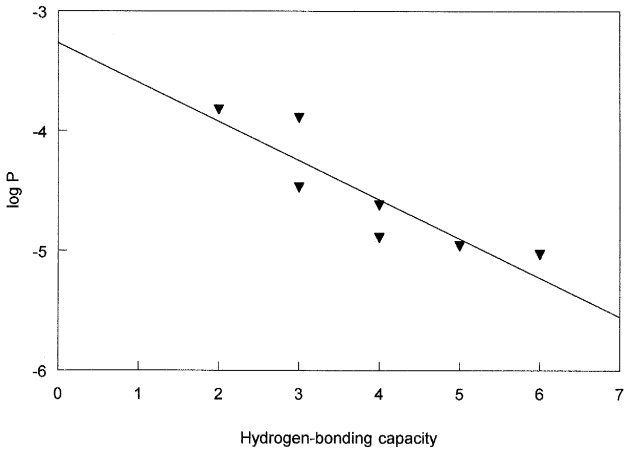


Fig. 1. Relationship between the logarithm of the measured permeability of rat kidney lysosomes and the notional hydrogen-bonding capacity of solutes. Points on the graph are, from left to right, for butanol, butyramide (upper point), acetamide (lower point), propylene glycol (upper point), ethanolamine (lower point), urea and glycerol. Permeability data are from Piqueras et al. [6]; hydrogen-bonding capacities are calculated from Table 3.2 of Stein [4].

The permeability of the lysosome membrane to organic amines has attracted much attention in the past. This is because of an early observation that cells exposed to certain weak bases, such as chloroquine, accumulate them to high concentration in the lysosomes. The phenomenon was explained as resulting from the pH difference between the lysosome matrix and the cytoplasm and from the differential permeability of the lysosome membrane to the unprotonated and protonated form of amines. The early data and the underlying theory were discussed in detail by de Duve et al. [7]. Briefly, amines enter the lysosome by crossing the membrane from the cytoplasmic side in the uncharged form. In the more acidic environment of the lysosome the amine acquires a proton, thus generating the much less permeable cationic form. The law of mass action predicts a lysosome:cytosol concentration ratio with maximum value equal to the antilogarithm of the pH difference. Ohkuma and Poole [8] studied a large number of organic weak

bases for their ability to accumulate in and vacuolize macrophage lysosomes. With a few exceptions, their results were broadly consistent with theory. Their data additionally provided an early indication that the presence of multiple hydroxy groups on an aliphatic amine decreases its permeability. They found that triethanolamine and Tris fail to vacuolize lysosomes, despite having pK_a values above neutrality, and concluded that this is because ''the compounds are relatively hydrophilic even in their neutral forms and probably would not permeate easily through membranes''.

Stein [4] assigned notional hydrogen-bonding capacities of 2 to the uncharged nitrogen of primary amines and 1 to that of secondary amines. It is implicit in these assignments that the uncharged nitrogen of tertiary amines has zero hydrogen-bonding capacity. These values may each be one unit too low. In theory any uncharged aliphatic amine can accept a hydrogen bond, by virtue of the nitrogen's unshared electron pair, while the hydrogen atoms of primary and secondary amines can additionally donate two or one hydrogen bonds respectively [5].

We have previously studied the ability of some charged but electrically neutral molecules to cross the rat liver lysosome membrane. Our data [9] on ω-amino-aliphatic acids and dipeptides indicated that the combination of a protonated primary amine group and an anionic carboxylate group renders a molecule rather impermeant. It was proposed by Ginsburg and Stein [10] that the combination of these two charged groups contributes a hydrogen-bonding capacity of 11, and it has been tentatively inferred [11] from their data that the protonated amino group may account for 8 or 9 of this total.

The present investigation is a systematic study of the permeability of the lysosome membrane to charged and uncharged nitrogenous compounds. The principal aims were to discover whether permeability can be explained in terms of hydrogen-bonding capacity and, if so, to determine which of the theory-derived numerical assignments [4,5] are correct. As in our previous reports [3,9] we investigated the permeability of lysosomes by making use of the osmotic properties of these organelles. This indirect approach avoids the impracticable custom synthesis of a multitude of labeled molecules. We believe it also has an advantage over methods that demand the preparation of highly purified lysosomes, since the latter represent a very small and likely unrepresentative fraction of the entire lysosome population. Furthermore, if due caution is used, the results can be interpreted with more confidence than might be expected of an essentially indirect method of approach.

In order to reach reliable conclusions about the effect of different functional groups on permeability, one should study as many compounds as possible. In our work on non-electrolytes [3] we tested over 40. In the present work we used fewer, as each had to be tested at several pH values in order to evaluate the effect of protonation on permeability. We chose only non-physiological compounds, so as to minimize any influence from the metabolite porters that are present in the lysosome membrane.

2. Materials and methods

All chemicals were from Aldrich or Sigma.

Solutions of the test compounds were prepared at 250 mM in water. The pH was adjusted by adding 250 mM HCl to achieve the desired value. This method ensures that the total osmolarity of every solution was 250 mOsm/l. The amount of HCl added varied from compound to compound, according to the pK_a and with the target pH. It is possible to calculate, for each solution, the fraction of the total osmolarity due to the amine and that due to chloride.

A lysosome-rich subcellular fraction was prepared from the liver of an overnight-starved rat. The liver was pushed through a sieve and the pulp homogenized in 10 ml of ice-cold 250 mM sucrose per gram, followed by centrifugation at 4°C and $1100 \times g$ for 10 min. The pellet was discarded and 20 ml of the supernate centrifuged at 4°C and $22\,500 \times g$ for 10 min. The resulting pellet was gently resuspended in 1 ml of ice-cold 250 mM sucrose.

To test the ability of various salts to afford osmotic protection to lysosomes, 200 μl of the resuspended lysosome-rich pellet was added to 3.8 ml of test solution at 25°C. This diluted suspension was maintained at 25°C and 50- or 100-μl samples taken at 0, 30 and 60 min.

The integrity of the lysosomes in these samples was measured by determining the free activity of *N*-acetyl-β-glucosaminidase at 25°C. The assay mix-

ture contained the substrate 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide (2.5 mM) and sucrose (250 mM) in 200 mM sodium citrate–HCl buffer pH 5.0. Incubation was for 2 or 5 min and was terminated by the addition of 5 volumes of sodium carbonate (1 M). Liberated 4-methylumbelliferone was measured by determining the fluorescence of a 400-μl sample in the well-plate attachment of a Perkin Elmer LS 50B luminescence spectrometer (λ_{ex} 360 nm; λ_{em} 448 nm). In controls, substrate was added after the sodium carbonate. The free activity was expressed as a percentage of the total activity of *N*-acetyl-β-glucosaminidase, as measured in simultaneous parallel assays in the presence of Triton X-100 (0.2%).

3. Results

Table 1 shows the *N*-acetylglucosaminidase free activity of rat liver lysosomes incubated at 25°C in 5 mM potassium phosphate buffers containing 250 mM sucrose. Lysosomes were stable for at least 60 min when the pH was within the range 5.0 to 9.0. At pH 4 or 10 the lysosomes began to break, despite the protective effect of the 250 mM sucrose, and similar results were seen at pH 4.5 and 9.5. This experiment identifies the range of pH within which osmotic protection experiments can be used to indicate lysosome membrane permeability. It was shown that lysosomes break immediately when suspended in 5 mM potassium phosphate buffer without sucrose (results not shown).

Typical aliphatic amines have pK_a above 10, and consequently exist almost entirely in the protonated form within the pH range 5–9. In order to study the permeability of lysosomes to amines in their unprotonated form, we required substances with much lower pK_a. We identified three groups of molecules suitable for the purpose of this project: some substituted pyridines, some *N*-alkyl-morpholines and some hydroxylated alkylamines. The pyridines have pK_a values between 4 and 6, the *N*-alkyl-morpholines values around 7, and the alkylamines values between 6 and 10, so that these molecules change from the unprotonated to the protonated form within the pH range feasible for this investigation. Moreover, within each of these three groups, compounds are commercially available containing different numbers of aliphatic hydroxy groups, which we and others have shown substantially decrease a molecule's ability to cross biomembranes. Thus, as in our study of non-electrolytes [3], we aimed to distinguish between the effects of two parameters, in this case pK_a and hydrogen-bonding capacity, on a molecule's permeability. Table 2 shows the structures, pK_a values and notional hydrogen-bonding capacities of the compounds studied.

Table 1
Free activity of *N*-acetyl-β-D-hexosaminidase in rat liver lysosomes pre-incubated in 250 mM sucrose buffered with 5 mM potassium phosphate

pH of the buffered sucrose solution [a]	Number of experiments	Free activity (% of total activity)		
		0 min	30 min	60 min
3.0	5	13±5	23±4	32±10
4.0	4	6±1	13±2	22±6
4.5	3	8±5	13±7	21±7
5.0	4	7±1	9±2	12±2
6.0	4	6±2	10±2	8±1
7.0	3	7±1	8±1	11±3
8.0	3	6±1	8±1	9±1
9.0	3	6±1	8±0	10+1
9.5	3	9±5	13±3	21±2
10.0	3	6±2	12±4	29±6

[a] The solutions also contained valinomycin (2.5 μg per ml) to enhance K^+ permeability.

A lysosome-rich fraction from rat liver was diluted into the buffered sucrose at 4°C, and then incubated at 25°C. Samples were removed for assay of *N*-acetyl-β-D-glucosaminidase at the indicated times. Free enzyme activity is expressed as a percentage of the total activity measured in the presence of Triton X-100 (0.2%). Values shown are the mean±SD for the number of experiments shown.

Table 3 shows the results of incubating rat liver lysosomes in 250 mOsm/l solutions of each of the eight bases at pH values within the range 5–9. Sev-

[a] pK_a values were determined by titration. They confirm published values where shown as available [12,13].
[b] Excluding any contribution from the nitrogen function.
[c] Hydrogen-bonding capacities are calculated by reckoning 2.0 for each C-O-H group and 0.8 for each C–O–C. These values are those assigned in Ref. [3].

Table 2
Structures, pK_a values and hydrogen-bonding capacities of the compounds studied

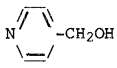
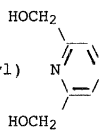
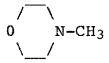
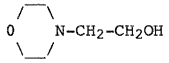
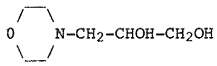
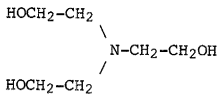
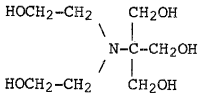
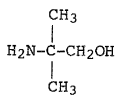
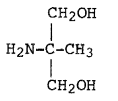
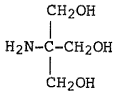
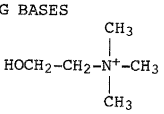
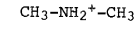
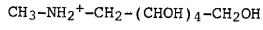
Name	Structure	pK$_a$(25°C)[a]	Hydrogen-bonding capacity[b,c]
PYRIDINES			
4-Methyl		6.0 [12]	0
4-Hydroxymethyl		5.3 [13]	2.0
2,6-Di(hydroxymethyl)		4.3	4.0
MORPHOLINES			
N-Methyl		7.4 [12]	0.8
N-(2-Hydroxyethyl)		6.8	2.8
N-(2,3-Dihydroxypropyl)		6.6	4.8
ALKYLAMINES			
Triethanolamine		7.8 [12]	6.0
Bis-Tris		6.5 [13]	10.0
2-Amino-2-methyl-1-propanol		9.7 [12]	2.0
2-Amino-2-methyl-1,3-propanediol		8.8 [12]	4.0
Tris		8.1 [12]	6.0
QUATERNARY AND STRONG BASES			
Choline		not applicable	2.0
Dimethylamine		10.8 [12]	0
N-Methylglucamine		9.3	10.0

Table 3
Free activity of *N*-acetyl-β-D-glucosaminidase in rat liver lysosomes pre-incubated in 250 mOsm solutions of organic bases

Solute	pH	Number of experiments	Free activity (% of total activity)		
			0 min	30 min	60 min
Pyridines					
4-Methyl	5.0	3	10 ± 4	85 ± 12	94 ± 13
	6.0	3	43 ± 10	94 ± 14	102 ± 21
	7.0	3	110 ± 14	118 ± 3	96 ± 9
4-Hydroxymethyl	5.0	3	17 ± 1	80 ± 3	93 ± 14
	6.0	3	60 ± 5	84 ± 6	91 ± 4
	7.0	3	85 ± 8	94 ± 4	106 ± 7
2,6-Di(hydroxymethyl)	5.0	3	74 ± 9	95 ± 10	94 ± 10
	6.0	3	91 ± 13	86 ± 11	90 ± 2
	7.0	3	93 ± 13	94 ± 9	94 ± 4
Morpholines					
N-Methyl	5.0	3	7 ± 2	50 ± 4	76 ± 12
	6.0	3	11 ± 2	18 ± 5	25 ± 4
	7.0	3	45 ± 9	66 ± 6	62 ± 6
	8.0	3	102 ± 13	89 ± 10	110 ± 4
N-2-Hydroxyethyl	5.0	3	11 ± 6	40 ± 5	79 ± 8
	6.0	3	15 ± 3	25 ± 9	26 ± 6
	7.0	3	72 ± 8	85 ± 4	85 ± 13
	8.0	3	101 ± 5	101 ± 17	103 ± 21
N-2,3-Dihydroxypropyl	5.0	5	10 ± 4	32 ± 8	56 ± 4
	6.0	5	12 ± 1	22 ± 2	23 ± 2
	7.0	4	55 ± 15	85 ± 12	79 ± 10
	8.0	5	86 ± 7	109 ± 10	110 ± 12
Alkylamines					
Triethanolamine	7.0	3	8 ± 2	31 ± 4	41 ± 5
	8.0	3	9 ± 5	103 ± 13	90 ± 10
	9.0	3	22 ± 11	88 ± 8	98 ± 4
Bis-Tris	5.0	6	10 ± 2	13 ± 3	21 ± 6
	6.0	6	10 ± 2	12 ± 2	18 ± 3
	7.0	8	8 ± 5	13 ± 6	19 ± 4
	8.0	6	13 ± 3	16 ± 4	46 ± 15
2-Amino-2-methyl-1-propanol	7.0	3	5 ± 1	27 ± 2	35 ± 1
	8.0	3	11 ± 4	52 ± 2	58 ± 8
	9.0	3	72 ± 8	98 ± 10	100 ± 5
2-Amino-2-methyl-1,3-propanediol	7.0	3	5 ± 1	11 ± 1	20 ± 3
	8.0	3	4 ± 1	71 ± 7	67 ± 7
	9.0	3	7 ± 3	109 ± 12	96 ± 5
Tris	7.0	3	5 ± 4	13 ± 3	13 ± 2
	8.0	3	6 ± 2	55 ± 19	61 ± 17
	9.0	3	1 ± 1	78 ± 3	95 ± 5

A lysosome-rich fraction from rat liver was diluted into the indicated solution, at 4°C, and then incubated at 25°C. Samples were removed for assay of *N*-acetyl-β-D-glucosaminidase at the indicated times. Free enzyme activity is expressed as a percentage of the total activity measured in the presence of Triton X-100 (0.2%). Values shown are the mean ± SD for the number of experiments shown.

eral of the compounds tested caused a degree of inhibition of *N*-acetylglucosaminidase, but this does not affect the validity of the osmotic protection data, as both the free and the total activity were measured in the presence of the same concentration of amine. Inhibition by the hydroxylated alkylamines and morpholines was 40–60% at 30 mM, the maximum concentration encountered in the osmotic protection assays; the three pyridines were not significantly inhibitory.

The results shown in Table 3 indicate that lysosomes break immediately when suspended in some of the test solutions. This finding can arise either because the solution actively disrupts the lysosome membrane, or because the solute enters the lysosomes so quickly that it fails to provide even initial osmotic protection. These alternative explanations can be distinguished by incubating lysosomes in the test solution to which sucrose has been added to a concentration of 250 mM. If the solution now provides initial osmotic protection, the solute is not actively membrane-lytic. This experiment was performed for each of the solutions that gave an initial free activity greater than 20%, as shown in Table 3. In no case was the initial free activity greater than 20% when 250 mM sucrose was also present (data not shown). Moreover, when incubations were continued for 60 min, free activity remained low in most cases. Exceptions were incubations at pH 6 or 7 in sucrose-containing 4-methylpyridine, when free activity reached 100% by 30 min, and in *N*-(2-hydroxyethyl)morpholine and *N*-(2,3-dihydroxypropyl)morpholine at pH 8, where a substantial increase in free activity was seen at 30 min of incubation, and a further rise by 60 min.

Table 4 shows the results of incubating lysosomes in 250 mOsm/l solutions of some quaternary or strong organic base hydrochlorides. These bases have no significant buffering capacity at neutral pH. Choline chloride and dimethylammonium hydrochloride were dissolved in water at 125 mM and the pH measured but not adjusted. *N*-Methylglucamine was dissolved in water, the pH adjusted to approximately 7.0 with HCl and the volume adjusted to give a final concentration of 125 mM. The results obtained with dimethylammonium chloride indicated the necessity to repeat the experiments at pH 7.0. This was done by adding KOH to the 125 mM solution; the amount needed was very small, adding less than 1 mOsm to the osmolarity of the solution.

The complete osmotic protection offered by 125 mM choline or *N*-methylglucamine chloride (Table 4) provides a striking demonstration of the inability of a permeant anion to enter lysosomes when its counter-cation is impermeant.

4. Discussion

4.1. Methodology

As indicated in the introduction, the osmotic protection methodology has two major advantages for

Table 4
Free activity of *N*-acetyl-β-D-glucosaminidase in rat liver lysosomes pre-incubated in solutions of quaternary and strong organic base hydrochlorides

Solution	pH	Number of experiments	Free activity (% of total activity)		
			0 min	30 min	60 min
Choline chloride (125 mM)	4.76	4	5.7 ± 1.7	7.0 ± 1.4	8.0 ± 2.7
Dimethylammonium chloride (125 mM)	5.43	3	8.1 ± 1.9	14.0 ± 3.3	22.2 ± 1.8
	7.00	3	7.8 ± 1.7	19.6 ± 0.6	27.1 ± 3.2
N-Methylglucamine chloride (125 mM)	6.56	4	6.9 ± 1.7	7.5 ± 1.9	8.5 ± 1.0

A lysosome-rich fraction from rat liver was diluted into the indicated solution, at 4°C, and then incubated at 25°C. Samples were removed for assay of *N*-acetyl-β-D-glucosaminidase at the indicated times. Free enzyme activity is expressed as a percentage of the total activity measured in the presence of Triton X-100 (0.2%). Values shown are the mean ± SD for the number of experiments shown.

structure-permeability correlation studies such as this. It demands neither the preparation of a highly purified lysosome fraction nor the availability of the desired compounds in radiolabeled form. It is currently the only practicable method available for testing a large number of xenobiotic amines, such as those studied here. However there are limitations: although the results give a reliable indication of the rank order of permeation rates for the compounds tested, they do not yield absolute rates. Our analysis (Fig. 1) of the recently published permeability data for rat kidney lysosomes [6] indicates however that their permeability to small non-electrolytes decreases approximately two-fold with each additional hydrogen bond.

Another pertinent issue is the relevance of osmotic-protection data to lysosome permeability in situ. Here an analysis [11] of the rather sparse data is encouraging — endocytosed substances that accumulate in lysosomes all have hydrogen-bonding capacity 11.5 or higher. By contrast substances below this threshold seem able to escape into the cytoplasm. Passive diffusion is of course not a threshold phenomenon and every compound will diffuse at some rate. There are as yet no comparative quantitative data available on rates of passive diffusion across the lysosome membrane in situ.

4.2. Effect of pH on lysosome stability

We first showed that rat liver lysosomes suspended in 250 mOsm sucrose show no significant loss of latency at pH values in the range 5 to 9. However, below 5 and above 9, the lysosomal enzyme monitored becomes progressively more available to added impermeant substrate. We have not sought to discover whether the membrane becomes more permeable or begins to break at these extremes of pH, as our purpose was to identify a pH range within which pH alone does not affect the stability of the lysosomes.

4.3. The uncharged nitrogen of an aromatic heterocycle

The three substituted pyridines studied in this investigation have pK_a values between 4 and 6. Thus they exist principally in their uncharged form at pH 7. Table 3 shows that none of the amines give even initial osmotic protection to lysosomes at pH 7.0, indicative of very rapid passage across the lysosome membrane. At pH 6.0, however, results with the three compounds differed — 4-methylpyridine provided a transient osmotic protection, although this was lost after 30 min of incubation; similar but slightly poorer protection was offered by 4-hydroxymethylpyridine; 2,6-di(hydroxymethyl)pyridine provided no initial protection. At pH 5.0 these three amines respectively provided complete, good and poor (albeit some) initial osmotic protection, although in all cases the protection was lost after 30 min incubation. Reference to Table 2 shows that these results correlate with the different pK_a values of the three amines, and are explicable in terms of the pyridines having a lower permeability when the ring nitrogen is protonated.

An alternative explanation of the very high initial free activity seen in many of these experiments is that the pyridine derivative actively disrupts the lysosome membrane, in a similar fashion to detergents such as Triton X-100. This possibility was excluded by repeating experiments with 250 mM sucrose present with the substituted pyridine — in each case and at all pH values, the lysosomes were fully latent at time-zero. In most cases the lysosomes remained intact for the full 60 min of the incubation, although rapid loss of latency was seen with 4-methylpyridine at pH 6.0 and 7.0. This finding does not affect the interpretation of the data.

In our earlier study [3] on non-electrolytes, we found that substances failing to offer even initial osmotic protection to lysosomes, all had a hydrogen-bonding capacity below 7.5. The failure of 2,6-di(hydroxymethyl)pyridine to provide initial protection suggests that the unprotonated ring-nitrogen cannot contribute more than 3.5 units of hydrogen-bonding capacity. The two other pyridines tested have less hydrogen-bonding capacity than 2,6-di(hydroxymethyl)pyridine (Table 2) and their inability to provide osmotic protection is unsurprising.

Even at the most acidic pH tested (5.0), the three pyridines exist significantly in the uncharged form. Therefore the data on these compounds in Table 3 cannot be used to draw any conclusions about the permeability of their protonated forms.

4.4. The uncharged nitrogen of aliphatic tertiary amines

We consider next the three morpholine derivatives tested. Table 2 shows that they have rather similar pK_a values (range 6.6–7.4), but a wider spectrum of hydrogen-bonding capacities (0.8–4.8), reflecting the differing complement of aliphatic hydroxy groups. Table 3 shows that at pH 8.0, when these molecules all exist substantially in the unprotonated form, there is no indication of even initial osmotic protection. That this indicates rapid permeation, and not a detergent-like action, was clear from the initial lysosome stability seen when lysosomes were incubated with the morpholine derivatives in the presence of 250 mM sucrose. Using the argument deployed above in respect of 2,6-di(hydroxymethyl)pyridine, the result with *N*-(2,3-dihydroxypropyl)morpholine indicates that its uncharged tertiary nitrogen contributes no more than 2.7 units of hydrogen-bonding capacity.

As pH is decreased from 8 to 7 and 6, the *N*-alkyl morpholines provide lysosomes with increasing osmotic protection. This result is consistent with a decreasing proportion of amine in the unprotonated form and an increasing proportion in the less permeant charged form. The pH 6 data in Table 3 are similar for all the three morpholines; likewise the pH 7 data. This finding is explicable in terms of pK_a values and hydrogen-bonding capacities (Table 2). With increasing side-chain length, pK_a decreases, thus increasing the proportion of molecules in the uncharged form at any given pH and thus increasing permeability. Concomitantly, the number of hydroxy groups increases, decreasing permeability. Thus, the two effects of the side-chain substituents counteract each other.

The results on osmotic protection by the three morpholines at pH 5.0 were a surprise. At this pH, the amines are principally in the protonated form and therefore were expected to show their lowest permeability. However in all three cases, osmotic protection at pH 5 was poorer than at pH 6. Several possible explanations of this observation can be excluded. First, the results in Table 1, and indeed the data on Bis-Tris (Table 3) show that the pH itself cannot be responsible. Secondly, this is not a membrane-lytic effect: lysosomes incubated in any of the three morpholines in the presence of 250 mM sucrose at pH 5, 6 or 7 remained intact for at least 60 min at all four pH values. A third possible explanation focuses on the composition of the amine solution. As explained above, the solutions were formulated to be 250 mOsm at all pH values: at pH values below the pK_a, a substantial contribution to the osmolarity comes from the counter-anion chloride. Although it had been assumed that little counter-ion would be able to cross the lysosome membrane without its compensating cation, it could be argued that the poorer osmotic protection at pH 5 than 6 reflected the lower concentration of amine. There are strong arguments against this possibility. First, the amine concentration in an iso-osmotic solution changes most significantly around the pK_a value. In a 250 mOsm solution of an amine hydrochloride, the total amine concentration (RNH_2 plus RNH_3^+) is 167 mM at the pK_a, 131 mM at one pH unit below the pK_a and 126 mM at two pH units below. The difference between the osmotic protection data for 4-methylmorpholine (pK_a 7.3) at pH 5.0 and 6.0, as shown in Table 3, is too great to be explicable in terms of decreased total amine concentration. A second argument is to be found in the results with choline chloride (Table 4), where a 125 mM solution gave total osmotic protection even at the low pH of 4.76. At present we have no convincing explanation for the apparently high permeability of the morpholines at pH 5.0.

We turn now to the two tertiary aliphatic amines Bis-Tris and triethanolamine. Lysosomes incubated in Bis-Tris in the presence of 250 mM sucrose remained intact for at least 60 min at all four pH values. Lysosomes suspended in Bis-Tris alone showed only slow loss of latency, even at pH 8.0, when only 3% of Bis-Tris molecules are protonated. Because it can be confidently assumed that the protonated species will be less permeable than the uncharged amine, the permeability of Bis-Tris at pH 8.0 must be attributable to the uncharged amine. The slow loss of latency seen at pH 8.0 is similar to that of non-electrolytes with a hydrogen-bonding capacity between 10 and 11 [3]. The five hydroxy groups in Bis-Tris contribute 10 units of hydrogen-bonding capacity (Table 2), indicating that the tertiary amine nitrogen of Bis-Tris contributes 0–1 units of hydrogen-bonding capacity.

Triethanolamine has a pK_a of 7.8, higher than that of any of the molecules discussed thus far. Pre-

dictably, its permeability is greater at pH values above its pK_a (Table 3), but the most noteworthy result is the initial free activity at pH 9.0, when 94% of the amine is in the free form. The pattern seen at this pH, an initial low free activity, followed by a rapid lysis, is reminiscent of molecules with a total hydrogen-bonding capacity between 7.2 and 8.0 [3]. Since triethanolamine has three aliphatic hydroxy groups, we conclude that its tertiary nitrogen has a hydrogen-bonding capacity between 1 and 2.

Taken together, the data from the pyridines, the morpholines and the tertiary aliphatic amines indicate a value of approximately unity for the hydrogen-bonding capacity of an uncharged tri-substituted nitrogen atom.

4.5. The uncharged nitrogen of aliphatic primary amines

In the amines discussed thus far the nitrogen atom has had no directly attached hydrogen atoms. Secondary amines, which have one such hydrogen, are stronger bases than their cognate primary or tertiary amines. As shown in Table 2, the pK_a of dimethylamine is 10.8. We were unable to identify any secondary amine with a low enough pK_a to be useful for the present study.

Although most primary amines are also strong bases, tri-hydroxylated *t*-butylamine (Tris) has a pK_a of 8.1. This compound was therefore investigated at pH 7, 8 and 9. For comparison the mono- and di-hydroxylated *t*-butylamines, 2-amino-2-methyl-1-propanol and 2-amino-2-methyl-1,3-propanediol, were also included in the study, notwithstanding their high pK_a values (see Table 2).

2-Amino-2-methyl-1-propanol has a hydrogen-bonding capacity of only 2.0 in addition to the contribution of the primary amine moiety. At pH 9.0, when 83% is in the protonated form, this amine fails to afford lysosomes even initial osmotic protection, indicative of a rapid penetration of the unprotonated form. This is a clear indication that an uncharged primary amine moiety cannot possess a hydrogen-bonding capacity in excess of about 5. With decreasing pH 2-amino-2-methyl-1-propanol provides good osmotic protection, indicative of a hydrogen-bonding capacity of at least 9 for the $-NH_3^+$ group.

2-Amino-2-methyl-1,3-propanediol at pH 9.0 affords lysosomes initial protection, but this is entirely lost by 30 min of incubation, a pattern typical of substances of hydrogen-bonding capacity about 8 [3]. A rather similar pattern is seen at pH 8.0. Since this molecule is substantially (39% and 86% respectively) in protonated form at pH 9.0 and 8.0, and since its two hydroxy groups contribute 4.0 units of hydrogen-bonding capacity, the uncharged $-NH_2$ group cannot contribute more than 4 units. Decreasing the pH to 7.0 leads to a marked increase in osmotic protection by 2-amino-2-methyl-1,3-propanediol, again indicative of the greater hydrogen-bonding capacity of the $-NH_3^+$ group.

Turning finally to Tris, we see patterns similar to those described above for 2-amino-2-methyl-1,3-propanediol. Tris at pH 9.0 is 89% uncharged, and even at pH 8.0 is 44% uncharged, so that the permeability seen at these pH values represents that of the uncharged amine. Comparison with earlier data [3] indicates that the uncharged $-NH_2$ group contributes approximately 2 units of hydrogen-bonding capacity.

4.6. Charged nitrogen atoms

Although the data of Table 3 show that the weak bases are all much less permeable at pH values below their pK_a, several of the cationic forms are apparently significantly permeant. However, because even at low pH values a fraction of the amine exists as the free base, it is possible that the cationic form is wholly impermeant and that permeation at low pH is due to the small fraction of unprotonated amine. In order to throw light on this question, we studied the permeation of some organic amines that cannot exist in unionized form or whose pK_a values are so high that an explanation in terms of free base penetration is implausible. Choline is a quaternary amine that bears a permanent positive charge, and dimethylamine is a strong base of pK_a 10.8. *N*-Methylglucamine is a polyhydroxylated derivative of dimethylamine.

Table 4 shows that 125 mM choline chloride is as good an osmotic protector as sucrose or mannitol [3], even at the low pH of 4.8. This finding indicates that the combination of a charged nitrogen and one aliphatic hydroxy equates to a hydrogen-bonding ca-

pacity of at least 12. Thus the charged nitrogen must contribute at least 10 units of hydrogen-bonding capacity. *N*-Methylglucamine chloride (125 mM) likewise gave full osmotic protection, a result consistent with its possessing a hydrogen-bonding capacity of approximately 10 in addition to that conferred by the protonated nitrogen. The strong secondary amine dimethylammonium chloride, also at 125 mM, gave a degree of osmotic protection typical of a substance with hydrogen-bonding capacity of about 11 [3]. Since this was observed at pH 5.43, over 5 pH units lower than its pK_a, it is difficult to attribute the permeation to free base. This result can be explained in two ways. Either the charged nitrogen contributes a hydrogen-bonding capacity of about 11, or its permeation is carrier-mediated.

4.7. General conclusions and implications for lysosome physiology and pharmacology

Two overall conclusions can be drawn from this work. The first is that non-physiological organic compounds containing an uncharged nitrogen atom can diffuse across the lysosome membrane, and that the effect of such groups is predictable in terms of hydrogen-bonding capacity, as was previously shown to be the case for some oxygen-containing functions. The second conclusion concerns the hydrogen-bonding capacities of nitrogen atoms in organic structures. We propose the following assignments: 1 for an uncharged nitrogen with no attached hydrogen, as in a tertiary aliphatic amine or an aromatic structure such as pyridine; 2 for an uncharged nitrogen with two attached hydrogen atoms, as in a primary amine. These values are broadly consistent with theory [4,5]. We do not propose a value for an uncharged nitrogen with one attached hydrogen, as in a secondary amine, since our data do not include any such compounds. However the matter is largely academic in the context of permeability through biological membranes in situ, owing to the high pK_a of secondary amines.

Many investigators take it as axiomatic that the possession of a positively charged nitrogen function makes a molecule incapable of diffusion through biological membranes, and that any observed permeation must be due to the uncharged moiety or to the presence of a carrier or channel, such as the organic cation/proton exchanger recently described [14] in rat liver lysosomes. Our data are consistent with this interpretation. However, they do not exclude the possibility that passive diffusion of a molecule such as dimethylamine could still occur. This diffusion may be too slow to be observable in most experimental systems, but may yet have physiological and pharmacological relevance to the penetration across small organelles such as the lysosome, in which the surface:volume ratio is very high compared with, for example, that of the whole cell. We note that Ohkuma and Poole [8] also envisioned some charged nitrogenous bases passively traversing lysosome membranes because they are ‘‘lipophilic even in their protonated forms’’. We conclude that the hydrogen-bonding capacity of a cationic nitrogen is at least 11.

The results we report are relevant to two issues, one physiological and one pharmacological. As discussed elsewhere [1], there are some products of the lysosomal catabolism of macromolecules that bear positive charges and for which no carrier has been identified in the lysosome membrane. Sphingosine is one such metabolite. It is a primary amine with two aliphatic hydroxy groups, and so can be assigned a total hydrogen-bonding capacity of 6 in the uncharged form and at least 15 in the charged form. At the pH of the lysosome interior, the latter form must predominate and it is unclear whether or how sphingosine can escape into the cytoplasm.

In the context of drug delivery, the results we report make it possible to compute the total hydrogen-bonding capacity of a wider range of structures than hitherto. As explained elsewhere [11], lysosomes in situ are effectively impermeable to molecules of hydrogen-bonding capacity greater than about 11.5. Many xenobiotics contain nitrogen atoms that are present in aromatic or heterocyclic structures and have low pK_a values, such that a significant fraction is uncharged at lysosomal pH. Their ability to cross the lysosome membrane will depend chiefly on the other functional groups they contain. By contrast all or most chemical moieties containing a charged nitrogen will not diffuse across the lysosome membrane at a significant rate. Here the lysosomal organic cation–proton exchanger [14] is potentially of great significance in achieving what diffusion cannot. It will be valuable to know more about the substrate specificity of this system.

Acknowledgements

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References

[1] J.B. Lloyd, in: J.B. Lloyd, R.W. Mason (Eds.), Biology of the Lysosome, Subcellular Biochemistry, Vol. 27, Chapter 11, Plenum Press, New York, 1996, pp. 361–386.
[2] R. Duncan, Anti-Cancer Drugs 3 (1992) 175–210.
[3] G.P. Iveson, S.J. Bird, J.B. Lloyd, Biochem. J. 261 (1989) 451–456.
[4] W.D. Stein, The Movement of Molecules across Cell Membranes, Academic Press, New York, 1967.
[5] J.M. Diamond, E.M. Wright, Annu. Rev. Physiol. 31 (1969) 581–646.
[6] A.-I. Piqueras, M. Somers, T.G. Hammond, K. Strange, H.W. Harris, M. Gawryl, M.L. Zeidel, Am. J. Physiol. 266 (1994) C121–133.
[7] C. De Duve, T. de Barsy, B. Poole, A. Trouet, P. Tulkens, F. van Hoof, Biochem. Pharmacol. 23 (1974) 2495–2531.
[8] S. Ohkuma, B. Poole, J. Cell Biol. 90 (1981) 656–664.
[9] S.J. Bird, J.B. Lloyd, Biochim. Biophys. Acta 1024 (1990) 267–270.
[10] H. Ginsburg, W.D. Stein, J. Membr. Biol. 96 (1987) 1–10.
[11] J.B. Lloyd, in: J.G. Thoene (Ed.), Pathophysiology of Lysosomal Transport, Chapter 7, CRC Press, Boca Raton, 1992, pp. 295–308.
[12] D.D. Perrin, Dissociation Constants of Organic Bases in Aqueous Solution, Butterworths, London, 1965.
[13] D.D. Perrin, Dissociation Constants of Organic Bases in Aqueous Solution: Supplement 1972, Butterworths, London, 1972.
[14] R.H. Moseley, R.W. Van Dyke, Am. J. Physiol. 268 (1995) G480–G486.