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Lysosome membrane permeability to anions

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Abstract

The permeability of rat liver lysosomes to some inorganic and aliphatic organic anions was investigated, using an osmotic-protection methodology. Lysosomes were incubated at 25°C in 250 mOsm solutions of potassium salts of the anions, in the presence of valinomycin, and the latency of lysosomal hexosaminidase measured at intervals. Lysosomes suspended in 250 mM sucrose at 25°C were stable for up to 4 h. When suspended in 250 mOsm solutions of potassium salts of inorganic acids, latency was lost at rates indicating anion permeance decreasing in the order thiocyanate, nitrate and iodide > bromide > chloride > sulfate. This rank order does not correspond with the anion selectivity of any known anion transporter, and is closer to that of the lyotropic series. Results with the potassium salts of aliphatic organic acids indicate little correlation between permeation and hydrocarbon chain length, although formate was more rapidly permeant than acetate and its higher homologs. By contrast, oxalate was less permeable than other dicarboxylic acids. The presence of one or more hydroxy groups decreased permeance. A correlation between permeance and the acid's lowest pK_a suggested that penetration was due principally to the entry of the undissociated acid, but there is evidence that the (much more abundant) singly charged anionic form is also significantly permeant.

Keywords: Lysosome membrane; Anion, inorganic; Anion, organic

1. Introduction

Lysosomes are the major intracellular site where biopolymers are digested. The products of this continuous catabolic activity are chiefly monomers, e.g. amino acids and monosaccharides, which the cell can utilize for either biosynthesis or further degradation. The pathways and products of lysosomal metabolism have recently been reviewed [1–4]. In order for the digestion products to participate in further metabolism, they must cross the lysosome membrane, either on substrate-specific porters, through channels, or by passive diffusion. The carriers present in the lysosome membrane were comprehensively reviewed in 1992 [5], and the probable mode of translocation of each of the major metabolites was recently discussed [6].

The ability of xenobiotics to cross the lysosome membrane is also of interest. One current approach to tissue-targeted drug delivery is to generate a drug–macromolecule conjugate that can only enter cells by endocytosis [7]. Release of the drug from its carrier occurs in the lysosomes, followed by efflux into the cytoplasm. This rationale demands drug penetration

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across the lysosome membrane. Because of the substrate specificity of porters and channels, most xenobiotics capable of crossing the lysosome membrane probably do so by passive diffusion.

Studies of solute translocation across the lysosome membrane commonly use one of two methodologies. Each has advantages and also limitations. In the first lysosomes are incubated in the presence of a radiolabeled solute and its uptake with time measured. In a variant of this method lysosomes are preloaded with the radiolabeled solute and its efflux with time monitored. Advantages of this approach are that flux is measured directly and quantitatively. Disadvantages are that the compounds investigated are limited to those available in radiolabeled form; other compounds can be studied as potential competitive inhibitors of the carrier-mediated uptake of a radiolabeled compound, but this approach cannot be applied when passive diffusion is the mechanism responsible. A second approach makes use of the osmotic properties of lysosomes: its advantage is that it can study any compound that is reasonably water-soluble; its disadvantage is that it yields only semi-quantitative data on rates of permeation. The data yielded by these two approaches have been extensively discussed [6,8].

Using the osmotic-protection methodology we have studied the permeability of rat liver lysosomes to non-electrolytes [9], to some bipolar, but electrically neutral, molecules [10], and to molecules containing protonatable nitrogen-containing functions [11]. By concentrating largely on xenobiotics, we interpret our data as indicating the ability of solutes to cross the lysosome membrane by passive diffusion. This conclusion is reinforced by our repeated observations of a strong inverse correlation between a solute's apparent rate of penetration and its hydrogen-bonding capacity, a theory-derived indicator of the difficulty the solute will experience in moving from an aqueous to a lipid environment.

In the present report, we focus attention on organic anions, and specifically on compounds containing one or more carboxylate functions. Our aim has been to determine whether these anions can penetrate across the lysosome membrane and, if so, to consider the mechanism(s) by which this occurs. Use of the osmotic-protection methodology has permitted us to study some homologous series of organic anions.

Many years ago [12], the osmotic protection technique was used to investigate the permeability of rat liver lysosomes to some (mostly) inorganic anions. In order to provide a baseline comparison with earlier data, we used our methods first to study lysosome permeability to some inorganic anions.

2. Materials and methods

All chemicals were from Aldrich or Sigma.

Each substance to be tested for permeance was dissolved in water at a concentration that would give a 250 mOsm solution. Thus solutions of potassium (or sodium) salts of monobasic acids were 125 mM, of dibasic acids 83.3 mM, and of tribasic acids 62.5 mM. The solutions were prepared from the salts, if commercially available, or from the free acid and potassium (or sodium) hydroxide. All solutions were adjusted to the desired pH with KOH (or NaOH for sodium salts) or HCl, but not otherwise buffered.

A lysosome-rich fraction of rat liver was prepared from an overnight-starved rat, as recently described [11], and 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 mixed with 3.8 ml of test solution at 25°C. This diluted suspension was maintained at 25°C and samples withdrawn at timed intervals. Lysosome integrity in these samples was measured by determining the percentage free activity of *N*-acetylhexosaminidase at 25°C, as previously described [11].

3. Results

Table 1 illustrates the behavior of rat liver lysosomes when incubated at 25°C in various solutions for periods up to 1 h. All the solutions afforded the lysosomes an initial osmotic protection, as indicated by the low percentage free activity at time zero. No increase in free activity within a 60-min period was seen when lysosomes were incubated in 250 mM sucrose but, when incubated in a 125 mM solution of potassium or sodium thiocyanate, there was a major loss of latency. The presence of valinomycin in-

Table 1
Free activity of *N*-acetyl-β-D-hexosaminidase in rat liver lysosomes pre-incubated at pH 7.0 in solutions of sucrose, KCNS and NaCNS

Solution		Free hexosaminidase (% of total activity)			
		0 min	30 min	60 min	
Sucrose	250 mM	5 ± 2	8 ± 1	8 ± 2	(9)
KCNS	125 mM	7 ± 3	34 ± 3	60 ± 3	
KCNS/Vm[a]	125 mM	9 ± 1	69 ± 14	78 ± 6	
NaCNS	125 mM	6 ± 0	18 ± 1	34 ± 3	(4)
NaCNS/Vm[a]	125 mM	5 ± 1	20 ± 1	46 ± 5	(3)
KCNS/Vm[a]/sucrose	125 mM/250 mM	7 ± 1	9 ± 1	9 ± 0	

A lysosome-rich fraction from rat liver was diluted into the indicated solution 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 ± S.D. for five experiments, except where a different number is shown in parentheses.
[a]Vm indicates the presence in the incubation mixture of valinomycin at 2.5 μg/ml.

creased the rate of loss of latency in KCNS and to a lesser extent in NaCNS. Lysosome rupture in KCNS did not occur if 250 mM sucrose was also present.

Table 2 shows the results of incubating lysosomes at 25°C in iso-osmotic solutions of salts of some inorganic acids. Valinomycin was included in all incubations with potassium salts. The rate of loss of latency of hexosaminidase was greatest in KI, followed by KNO_3, KBr, KCl and NaI, in that order. No loss of latency was seen in 83.3 mM K_2SO_4, or with any of the salts if 250 mM sucrose was also present (data not shown).

Table 3 shows the percent free activity of hexosaminidase of lysosomes incubated at 25°C in valinomycin-containing solutions of potassium salts of 14 organic acids. In these experiments, the incubation period was extended to 4 h, with sampling at 0, 30, 60, 120 and 240 min. This was done because preliminary experiments had shown little loss of latency in the first hour with most of the salts. For comparative purposes similar extended incubations were conducted with four non-electrolytes: xylitol, mannitol, perseitol and sucrose. These data are shown in Table 4. It is clear that lysosomes are stable in 250 mM sucrose for 4 h. The loss of latency evident in 250 mM mannitol by 2 h and in perseitol by 4 h must therefore reflect solute penetration, albeit at a rate that does not lead to significant loss of latency within 1 h.

Since the potassium salts of monobasic aliphatic acids appear to penetrate the lysosome membrane to a considerable extent over a 4-h incubation (Table 3), the question arises as to whether the anion or the free acid is the penetrant species. Table 5 shows the results of incubating lysosomes in 125 mM potassium formate or potassium propionate at a range of pH values. Similar experiments with potassium oxalate were performed for comparison.

Table 2
Free activity of *N*-acetyl-β-D-hexosaminidase in rat liver lysosomes pre-incubated at pH 7.0 in solutions of potassium and sodium salts of inorganic acids

Solution		Free hexosaminidase (% of total activity)			
		0 min	30 min	60 min	
Sucrose	250 mM	7 ± 3	9 ± 3	10 ± 3	(10)
KI	125 mM[a]	10 ± 5	58 ± 8	67 ± 6	
KNO_3	125 mM[a]	11 ± 3	47 ± 8	64 ± 8	(5)
KBr	125 mM[a]	7 ± 2	21 ± 5	35 ± 7	
KCl	125 mM[a]	10 ± 1	16 ± 4	24 ± 6	(5)
NaI	125 mM	10 ± 3	12 ± 1	18 ± 3	
K_2SO_4	83.3 mM[a]	5 ± 2	8 ± 2	8 ± 2	(3)

A lysosome-rich fraction from rat liver was diluted into the indicated solution 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 ± S.D. for four experiments except where a different number is shown in parentheses.
[a]Indicates the presence in the incubation mixture of valinomycin at 2.5 μg/ml.

Table 3
Free activity of *N*-acetyl-β-D-hexosaminidase in rat liver lysosomes pre-incubated at pH 7.0 in solutions of potassium salts of aliphatic organic acids

Salt (pK_a values)[a]	Free hexosaminidase (% of total activity)				
	0 min	30 min	60 min	120 min	240 min
Monobasic acid salts (125 mM)					
Formate (3.7)	10 ± 2 (3)	15 ± 2 (3)	26 ± 3 (3)	38 ± 6 (3)	57 ± 3 (3)
Acetate (4.8)	9 ± 1 (5)	14 ± 2 (5)	18 ± 4 (5)	27 ± 4 (3)	51 ± 7 (3)
Propionate (4.9)	9 ± 1 (3)	12 ± 3 (3)	15 ± 3 (3)	33 ± 3 (3)	60 ± 5 (3)
n-Butyrate (4.8)	9 ± 1 (6)	10 ± 1 (4)	12 ± 1 (4)	30 ± 5 (4)	59 ± 2 (4)
Glycolate (3.7)	9 ± 1 (7)	11 ± 1 (4)	13 ± 3 (4)	25 ± 5 (5)	50 ± 6 (5)
D-Gluconate (3.6)	7 ± 2 (3)	9 ± 3 (3)	13 ± 3 (3)	11 ± 2 (3)	24 ± 4 (3)
Dibasic Acid salts (83.3 mM)					
Oxalate (1.3, 3.6)	7 ± 2 (10)	8 ± 1 (9)	9 ± 1 (10)	9 ± 1 (6)	13 ± 1 (5)
Malonate (2.8, 5.7)	6 ± 0 (3)	7 ± 1 (3)	8 ± 1 (3)	13 ± 1 (3)	36 ± 3 (3)
Succinate (4.2, 5.6)	7 ± 1 (6)	8 ± 2 (5)	9 ± 1 (6)	13 ± 1 (6)	28 ± 4 (6)
Glutarate (4.3, 5.5)	8 ± 1 (4)	8 ± 1 (4)	10 ± 1 (4)	15 ± 4 (4)	27 ± 7 (3)
Malate (3.5, 5.1)	8 ± 1 (3)	9 ± 1 (3)	9 ± 1 (3)	11 ± 1 (3)	21 ± 4 (3)
L-Tartrate (3.0, 4.4)	7 ± 1 (3)	9 ± 1 (3)	8 ± 1 (3)	9 ± 2 (3)	14 ± 1 (3)
Tribasic acid salts (62.5 mM)					
Tricarballylate (3.5, 4.5, 5.9)[b]	11 ± 1 (4)	12 ± 0 (3)	17 ± 2 (5)	17 ± 2 (4)	29 ± 3 (4)
Citrate (2.8, 4.3, 5.7)	9 ± 1 (3)	10 ± 0 (3)	10 ± 1 (3)	10 ± 1 (3)	13 ± 2 (3)

A lysosome-rich fraction from rat liver was diluted into the indicated solution (containing valinomycin, 2.5 μg/ml) 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 ± S.D. for the number of experiments shown in parentheses.

[a] pK_a values refer to aqueous solutions at 25°C, except where indicated. The principal sources of pK_a values are [13] and [14]. Where minor discrepancies were encountered, a mean value is quoted. Other sources consulted are [15–17].

[b] pK_a values refer to aqueous solution at 20°C.

4. Discussion

4.1. Permeability to potassium and thiocyanate

Lysosomes are stable for at least 60 min when incubated at 25°C in 250 mM sucrose, but break immediately when incubated in 250 mM sucrose containing a chaotropic substance, such as the detergent Triton X-100 [9–12]. In the present experiments, we first showed that lysosomes break rapidly when incubated in iso-osmolar (125 mM) KCNS. This latter result could indicate either that KCNS crosses the lysosome membrane rapidly, thus eliminating its ability to provide osmotic protection, or that it actively breaks the membrane. The second of these possibilities was excluded by incubating lysosomes with both KCNS and sucrose present: here the lysosomes were as stable as in 250 mM sucrose alone, demonstrating that KCNS does not directly disrupt the lysosome membrane. The latter conclusion differs from that of Casey et al. [12], who incubated rat liver lysosomes at 37°C in a solution containing 160 mM KCNS and either 70 or 135 mM sucrose. Observing a rapid release of hexosaminidase, they postulated a direct chaotropic effect of thiocyanate on the lysosome membrane. However this explanation appears unnecessary, as 135 mM sucrose is insufficient to afford osmotic protection to lysosomes in the absence of another non-penetrant solute.

If a salt, such as KCNS, crosses the lysosome membrane, both cation and anion must be penetrant and, in order to maintain electrical neutrality, both must enter at the same rate. This rate will be determined by the permeance of the less permeable ion. Our finding, in agreement with Casey et al. [12], that the presence of valinomycin markedly increased the rate of hexosaminidase release in KCNS, suggests that the membrane's permeability to CNS^- exceeds its basal permeability to K^+. We conclude, with Harikumar and Reeves [18], that lysosomes exhibit "a low, but significant, permeability to K^+".

Lysosome rupture in 125 mM NaCNS was mark-

edly slower than in 125 mM KCNS, from which it is deduced that Na^+ penetrates the membrane more slowly than either K^+ or CNS^-. Unexpectedly, in view of its strong preference for K^+ over Na^+, valinomycin appeared to stimulate lysosome rupture in NaCNS. This result presumably reflects the ionophore's lack of absolute cation-specificity.

4.2. Permeability to inorganic anions

Having demonstrated the lysosome membrane's high permeability to K^+ in the presence of valinomycin, it was possible to examine the ability of other potassium salts to afford osmotic protection to lysosomes and infer from the results the lysosome membrane's permeability to the constituent anion. If the observed rate of lysosome rupture is less than that seen in KCNS, it can be confidently assumed that the counter-anion is the rate-determining species.

Table 2 shows that in the presence of valinomycin, 125 mM KI or KNO_3 provide a similar pattern of osmotic protection to 125 mM KCNS, suggesting that permeability to iodide and nitrate approximates that to thiocyanate. The data also suggest that the permeance of the halides is iodide > bromide > chloride, and that sulfate is effectively non-permeant. These conclusions are compatible with data from several other laboratories. Casey et al. [12], using similar methods to ours, reported the following order of permeance: thiocyanate > iodide > acetate > chloride, bicarbonate and phosphate > sulfate. A subsequent study, using quite different methodology, indicated anion permeability of rat kidney lysosomes declining: thiocyanate > nitrate > chloride > phosphate [18]. Finally, van Dyke [19], also using a different experimental approach, concluded that rat liver lysosomes are more permeable to nitrate than to chloride.

We found that lysosomes incubated in 125 mM NaI were much more stable than in 125 mM KI, a result demonstrating the rate-limiting effect of a poorly permeant counter-ion.

4.3. Permeability to organic anions

Table 3 shows that, with few exceptions, potassium salts of the organic acids tested provide good osmotic protection for the first hour of incubation. Substantial differences between the acids begin to appear however when the incubations are extended to 4 h. In order to interpret these findings, it was necessary to determine whether lysosomes could remain stable in any solute for as long as 4 h. Table 4 shows that lysosomes incubated in 250 mM sucrose are indeed stable. Also that two other non-electrolytes with lower hydrogen-bonding capacity than sucrose, mannitol and perseitol, offer substantially less protection over the longer period. This result indicates that the behavior of lysosomes over a 4-h incubation can reliably indicate the relative permeances of molecules of low intrinsic permeance.

As explained above, all the solutions of organic acid salts used in this study were iso-osmotic with 250 mM sucrose. In the case of the polybasic acids, the total osmolarity is due principally to the potassium content. Consider, for example, 62.5 mM po-

Table 4
Free activity of *N*-acetyl-β-D-hexosaminidase in rat liver lysosomes pre-incubated at pH 7.0 in 250 mM solutions of non-electrolytes

Substance	Hydrogen-bonding capacity[a]	Free hexosaminidase (% of total activity)				
		0 min	30 min	60 min	120 min	240 min
Xylitol	10.0	4 ± 1 (3)	26 ± 4 (3)	56 ± 5 (3)	69 ± 1 (3)	79 ± 6 (3)
Mannitol	12.0	3 ± 1 (5)	8 ± 1 (4)	9 ± 2 (5)	17 ± 3 (5)	32 ± 7 (5)
Perseitol	14.0	4 ± 4 (3)	7 ± 4 (3)	12 ± 5 (3)	12 ± 3 (3)	21 ± 2 (3)
Sucrose	18.4	5 ± 2 (9)	7 ± 3 (10)	7 ± 2 (10)	7 ± 3 (8)	12 ± 2 (8)

A lysosome-rich fraction from rat liver was diluted into the indicated solution, 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 ± S.D. for the number of experiments shown in parentheses.

[a]Hydrogen-bonding capacities are taken from [9]. Each hydroxy group in a molecule is given a value of 2.0 and each glycosidic moiety a value of 0.8.

tassium citrate. This solution is iso-osmotic with 250 mM sucrose or 125 mM KCNS, but K^+ comprises 187.5 of its 250 mOsm. If this K^+ was to enter the lysosomes, it would be sufficient to induce substantial lysis within an hour. It is very striking, therefore, that the lysosomes are stable for so long in this solution. Clearly, a permeant cation cannot enter lysosomes in osmotically significant amount unless it is accompanied by its counter-ion. According to theory, this is because an electrogenic ion flux quickly generates an intolerable electrochemical potential.

The salts investigated represent a number of homologous series. Among the non-hydroxylated species, the monocarboxylic acids appear substantially more permeant than the dicarboxylic acids. There appears to be little effect of hydrocarbon chain length within either of these series, although formate, the smallest monocarboxylate, appears to be more rapidly permeant. By contrast oxalate, the smallest dicarboxylate, is anomalously impermeant. In most cases substitution with hydroxy groups lowers permeance. Thus permeance decreases in the order succinate > malate > tartrate, and citrate is less permeable than tricarballylate. Not surprisingly, gluconate, with its five hydroxy groups, is the least permeant of the monocarboxylic acids. However, there is no apparent difference between the permeabilities of acetate and glycolate.

4.4. Mechanism of anion permeation

4.4.1. Inorganic anions

Chloride conduction across the lysosome and endosome membranes is seen as an important functional adjunct to the electrogenic proton pump that maintains the internal acidity of these organelles [20,21]. However, the mechanism(s) by which chloride ions cross the lysosome membrane remain unclear. Anion transporters for sulfate and phosphate have been identified in the lysosome membrane. The sulfate porter [22–24] shares a number of properties with the Band 3 chloride porter, and at pH 5.0 chloride *trans*-stimulates sulfate uptake [22]; it is proposed that the porter is responsible for sulfate efflux from the lysosome, following release from glycosaminoglycans and sulfatides, and functions as a sulfate, proton:chloride antiport. The phosphate transporter is apparently unaffected by chloride (154 mM) [25]. A nucleotide-activated chloride channel has been described in the liver lysosome membrane [26], but its role is not yet clear.

The experiments we report, and those of other investigators, indicate that the lysosome membrane is permeable to chloride and other inorganic anions. Moreover, as discussed above, there is a remarkable concordance in the data on the relative permeance of these ions: the rank order appears to be thiocyanate, nitrate and iodide > bromide > chloride > sulfate. This order differs from the halide selectivity of both the major CLC family of chloride channels and the CFTR channel, resembling more that of the GABA- and glycine-gated channels [20]. The order also differs from the efficacy of inorganic ions to serve as the counter-ion for the Band 3 chloride transporter of the erythrocyte: the rate with thiocyanate is less than 10% of that with chloride [27].

The rank order of inorganic anion permeances across the lysosome membrane correlates better with the so-called lyotropic series, which reflects purely physicochemical phenomena (the stability of the ion's hydration shell [28]), than to the order of preference of any known anion carrier or channel. Casey et al. [12] were the first to point out this correlation, interpreting it as evidence that anion translocation takes place by passive diffusion. And indeed inorganic anions do cross artificial lipid bilayers, such as those of liposomes [29], with rates much greater than those of inorganic cations.

It appears unlikely, however, that passive diffusion alone could be an adequate mechanism for the conduction of physiological anions across the lysosome membrane in situ, when anion concentrations are much lower. In a recent report [30], the H^+-ATPase from liver lysosomes was reconstituted into proteoliposomes. Chloride, bromide, nitrate and thiocyanate all promoted ATP-driven acidification in intact lysosomes (by charge compensation), but they were ineffective in the reconstituted liposomes. This result indicates that anion uptake into the proteoliposomes, which of course lack anion channels and porters, is too slow to keep pace with proton pumping. This suggests that the entry of certain anions into lysosomes in situ is unlikely to occur by unaided passive diffusion. Certainly sulfate, which appears to be in-

capable of passive diffusion, has a specific carrier in the lysosome membrane.

We conclude this sub-section by noting that the anion concentrations used in our experiments are much higher than typical K_m values of anion porters. For example, the sulfate porter in the lysosome membrane has a K_m of 160 μM [22]. As pointed out many years ago in the context of lysosomal monosaccharide transport [31], porters are ineffective mediators of net solute translocation at concentrations greatly above their K_m. Thus in osmotic-protection experiments the solute concentrations will minimize any contribution from substrate porters [8].

4.4.2. Organic anions

In considering the data for organic anions (Table 3), we first note that in most cases little loss of latency is apparent within the first hour of incubation, indicating a generally low level of permeation. However significant latency loss does occur during a further period of incubation. That this reflects permeation, and not an intrinsic instability over a 4-h incubation, is indicated by the stability of lysosomes incubated for 4 h in 250 mM sucrose (Table 4). The other generalization that can be made from the data in Table 3 is that the monovalent anions are apparently more permeant than the polybasic anions. This despite the fact that the anion concentration is highest (125 mM) for the monobasic anions than for the polybasic anions (83.3 or 62.5 mM).

In contrast to the inorganic salts, the organic salts tested are those of weak acids. It is, therefore, necessary to ask whether the penetrant species is not the anion, but the free acid. In discussing his data on the permeation of benzoate and some of its derivatives across phospholipid bilayers, Gutknecht [32] concluded that the anionic form of salicylic acid was truly permeable, but that its permeance was seven orders of magnitude lower than that of the free acid. Thus at pH 7, despite the concentration of salicylate being four orders of magnitude greater than that of salicylic acid (pK_a 3.0), the permeability observed is almost entirely due to the penetration of the free acid.

If the data shown in Table 3 reflect the penetration of free acid and not of anion, there should be a correspondence between a molecule's rate of penetration and its lowest pK_a. Fig. 1 explores this relationship, taking the mean percentage free activity at 240 min as an approximate indicator of permeance. Although the correlation is far from perfect, there is an indication that permeance decreases with the fraction of the molecules that exist in unionized form: the higher the lowest pK_a, the higher the permeability at pH 7. The anomalously rapid permeation of formate, as compared with its higher homologs (Table 3), finds a parallel in the results of Walter and Gutknecht [33] on the permeation of (unionized) monocarboxylic acids across lipid bilayers.

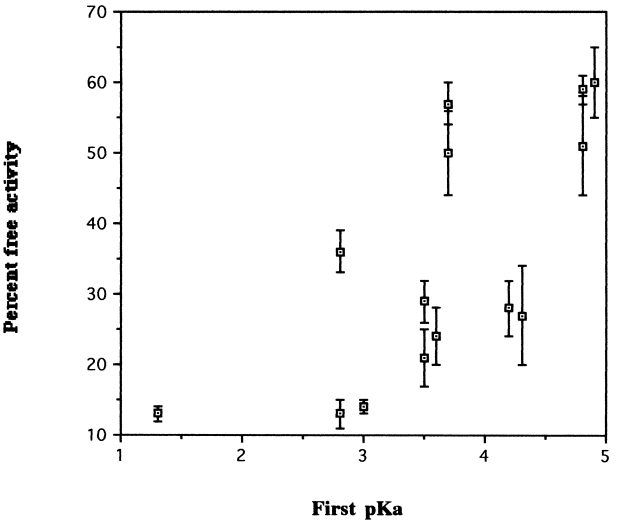

Fig. 1. Relationship between the measured percentage free activity at 240 min and the pK_a of the first ionization. The data relate to the mono-, di- and tri-carboxylic acids, and are taken from Table 3.

Closer inspection of Fig. 1 makes it clear that the poor correlation is in large part due to the consistently higher permeance of the monocarboxylic acids as compared with the dicarboxylic and tricarboxylic acids. If either group is graphed alone, the correlation markedly improves (graphs not shown). The simplest explanation is that singly charged anions have a quantitatively significant intrinsic permeance. On this hypothesis, permeation at pH 7 is due both to the few molecules of high permeance (the free acid) and the many molecules of low permeance (the mono-anion). If this is the case, the singly charged form of the dicarboxylic acids will also be permeant, and the permeation of these anions should correlate with the pK_a of their first protonation. Fig. 2 shows that this is indeed the case.

Table 5 provides some further evidence on this

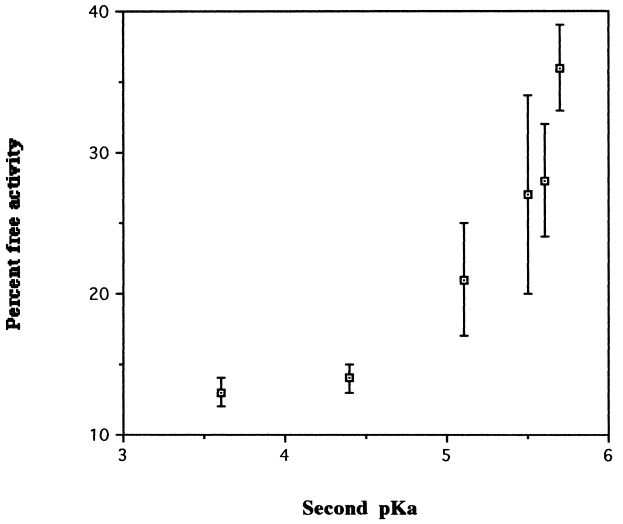

Fig. 2. Relationship between the measured percentage free activity at 240 min and the pK_a of the second ionization. The data relate to the dicarboxylic acids, and are taken from Table 3.

question. Because propionic acid has a pK_a of 4.9, the fraction in the anionic form, only 1% at pH 7, is 10% at pH 6. Table 5 shows that the osmotic protection provided by 125 mM potassium propionate was substantially less at pH 6 than at pH 7. This suggests that the permeation of non-ionized propionic acid contributes significantly to the overall permeation at pH 6. Permeation at pH 8 was similar to that at pH 7, suggesting that at either pH it is principally due to the movement of anion across the membrane. Consistent with this interpretation, the osmotic protection provided by potassium formate (pK_a 3.6) or potassium oxalate (pK_a 1.3 and 4.3) is not pH-sensitive within the range 6–8.

Finally, we compare the permeance of a monocarboxylic acid anion such as propionate with those of the non-electrolytes reported in Table 4 and [9]. It would appear that the carboxylate anion confers a hydrogen-bonding capacity equivalent to that conferred by 5 or 6 aliphatic hydroxy groups. As a working hypothesis we propose that the carboxylate anion has a notional hydrogen-bonding capacity of 11. In our study of amines [11], we concluded that the presence of a charged nitrogen atom contributes at least 11 units of hydrogen-bonding capacity.

If the carboxylate anion is assigned a hydrogen-bonding capacity of 11, the total hydrogen-bonding capacity of gluconate is 21, greater than that of sucrose. The permeance of D-gluconate (Table 3) is, therefore, unexpectedly high. This could reflect the activity of a high-K_m gluconate transporter in the rat liver lysosome membrane. Although substrates for the neutral-sugar porter in this membrane have high K_m values, in the range 25–75 mM [34], it does not recognize D-gluconate [35]. By contrast D-gluconate is known to be an inhibitor of the porter for acidic monosaccharides [36], and therefore may be a substrate. This porter's K_i for D-gluconate is reported as 260 μM [36].

We conclude that the presence of one or more

Table 5
Free activity of *N*-acetyl-β-D-hexosaminidase in rat liver lysosomes pre-incubated at pH 6, 7 and 8 in solutions of potassium formate, oxalate and propionate

Solution	pH	Free hexosaminidase (% of total activity)			
		0 min	30 min	60 min	
Formate (125 mM)	6	9 ± 1	20 ± 4	33 ± 4	(5)
	7	9 ± 2	21 ± 4	38 ± 5	(6)
	8	10 ± 3	25 ± 5	46 ± 6	(4)
Propionate (125 mM)	6	9 ± 2	28 ± 4	48 ± 4	
	7	6 ± 3	10 ± 5	21 ± 4	
	8	5 ± 3	10 ± 3	25 ± 1	
Oxalate (83.3 mM)	6	5 ± 1	9 ± 2	9 ± 2	
	7	4 ± 4	10 ± 2	11 ± 3	
	8	7 ± 1	11 ± 2	12 ± 4	

A lysosome-rich fraction from rat liver was diluted into the indicated solution and then incubated at 25°C in the presence of valinomycin, 2.5 μg/ml. 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 ± S.D. for three experiments except where a different number is shown in parentheses.

carboxylate moieties in a molecule decreases permeance across the lysosome membrane very sharply, the effect of each carboxylate approximating to that of five or six alcohol-hydroxy groups. Because carboxylates are weak acids, and because a free acid is much more permeant than its anion, passage of the non-ionized species can contribute significantly to overall penetration, particularly at pH values that do not greatly exceed the lowest pK_a. Nevertheless, some molecules containing carboxylate moieties appear to be significantly permeant in their singly charged anionic form. Because the surface:volume ratio of an organelle is extremely high, as compared with this ratio for a cell, such low intrinsic permeances can be physiologically relevant to anion movement into and out of the lysosome. In subsequent studies, we hope to discover whether organic mono-anions cross the lysosome membrane by passive diffusion or by means of porters and channels. Finally, we conclude that molecular species containing two or more ionized carboxylates are effectively non-permeant.

Taken together with our earlier findings on the effects of other substituent groups on the permeation of organic molecules across the lysosome membrane [9–11], the data we report here make it increasingly possible to predict from its chemical structure the ability of a given xenobiotic to exit the lysosome. This information will be useful in identifying candidate drugs for delivery in the form of a conjugate that is endocytosed and then degraded in the lysosomes of the target cell.

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