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Stimulation of Rat Liver β-Galactosidase Activity by Ions

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1. The *p*-nitrophenyl β-D-galactosidase activity in rat liver homogenates or lysosome-rich fractions was shown to be markedly affected by the ionic composition of the medium. A stimulation of the reaction rate at pH 5 was produced by most of the salts tested, which contained anions such as acetate, SO_4^{2-} and Cl^-, and cations such as Na^+, K^+ and Mg^{2+}. The most pronounced effect was observed with $MgCl_2$. Only potassium glutamate was inhibitory. 2. Five peaks of β-galactosidase activity obtained by DEAE-cellulose chromatography were equally sensitive to changes in the ionic composition of the medium. In the presence of added NaCl, the whole rate–pH curve was displaced towards higher pH values, the optimum being shifted from 2.0–2.5 to 3.5. The stimulation at pH 5.0 appeared to be mainly due to changes in $V_{max.}$, whereas the apparent K_m was slightly modified. 3. Unlike the total, the free β-galactosidase activity remained unchanged or even declined when KCl was added to the reaction medium.

Escherichia coli β-galactosidase (EC 3.2.1.23) is known to be activated by a number of uni- and bivalent cations, among which Na^+, K^+ and Mg^{2+} have been studied more extensively (e.g. Neville & Ling, 1967; Becker & Evans, 1969; Hill & Huber, 1971; Strom *et al.*, 1971; Tenu *et al.*, 1972; Wallenfels & Weil, 1972). However, the influence of ions on mammalian β-galactosidase activities has received little attention (Wallenfels & Weil, 1972). Van Hoof & Hers (1968) mentioned that a shift from 3.6 to 4.5 in the optimum pH of human liver *p*-nitrophenyl β-galactosidase activity occurs when the ionic concentration in the medium is raised. Apparently unrelated to this phenomenon is the requirement for small concentrations of Cl^- or other anions for β-galactosidase activity from the same source to display the maximum hydrolytic rate with different substrates (Ho & O'Brien, 1969; Suzuki & Suzuki, 1974).

In a study on the influence of added ions on the extraction of hydrolase activities from rat liver lysosome-rich fractions (Baccino *et al.*, 1971), *p*-nitrophenyl β-galactosidase activity was noticed to be markedly affected by the ionic composition of the medium. From that original observation stems the work reported in the present paper, in which the influence of added ions on the same enzyme activity has been examined in detail.

Methods and Materials

Male albino rats of a Wistar strain (Morini, S. Polo d'Enza, Italy) weighing about 250 g were used.

Liver homogenates were prepared and fractionated by centrifugation as previously described (Baccino *et al.*, 1971). Soluble extracts were obtained from the light-mitochondrial (lysosome-rich) fraction, suspended in 0.25 M-sucrose, by three freeze–thaw cycles followed by high-speed centrifugation (Baccino *et al.*, 1971). DEAE-cellulose column chromatography was performed as described by Furth & Robinson (1965). β-Galactosidase activity was assayed as described by Sellinger *et al.* (1960), but with the *p*-nitrophenyl derivative as substrate. The usual medium contained 2.5 mM substrate and 35 mM-sodium or -potassium acetate–15 mM-acetic acid buffer, pH 5.0; in the assay for free activity 0.25 M-sucrose was also present. Other details are specified in the Results section.

p-Nitrophenyl β-D-galactopyranoside and sodium *p*-chloromercuribenzoate were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Whatman DE 32 DEAE-cellulose was from H. Reeve Angel and Co. (London E.C.4, U.K.).

Results

Fig. 1 shows how the β-galactosidase activity of rat liver lysosome-rich fractions was modified when salts at increasing concentrations were added to the standard assay mixture. The maximal stimulation was virtually attained at concentrations of added salts of 0.2–0.3 M (see also Fig. 3). The degree of activation varied according to the cationic species added, the highest being observed with $MgCl_2$. It can be easily calculated that, if the added concentrations are expressed in terms of ionic strength rather than molarity, the degree of stimulation by $MgCl_2$ appears to be intermediate between those for KCl and NaCl. Nevertheless, the maximal stimulation by $MgCl_2$ was markedly higher than that afforded by the other salts examined.

p-Nitrophenyl β-D-galactopyranoside has been shown to be hydrolysed by different enzyme forms

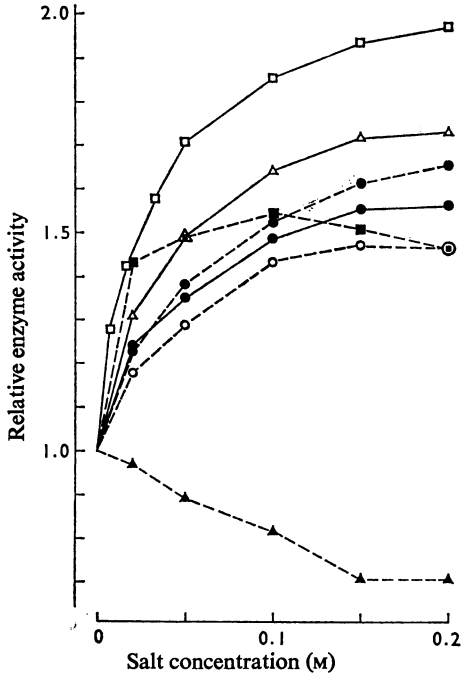

Fig. 1. *Effect of different ionic species on β-galactosidase activity*

On the ordinate, the activity is expressed as a fraction of that observed with the usual reaction medium, containing either 50mM-potassium (----) or 50mM-sodium (——) acetate buffer, pH5.0. The abscissa shows the concentration of the added salts: □, $MgCl_2$; ■, K_2SO_4; △, NaCl; ●, KCl; ○, potassium acetate; ▲, potassium glutamate.

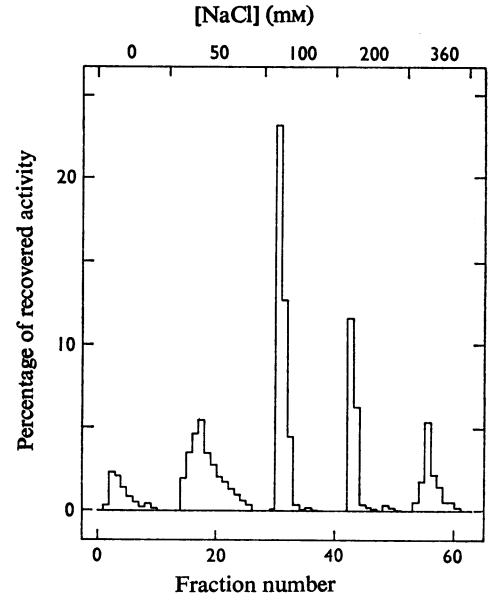

Fig. 2. *DEAE-cellulose chromatography of rat liver β-galactosidase activity*

This shows a representative experiment, where the soluble extract of a lysosome-rich preparation was submitted to stepwise elution (4ml fractions) with NaCl in 10mM-sodium phosphate buffer, pH6.0. The enzyme activity (on the ordinate) is expressed as a percentage of the total activity recovered in the fractions. Recovery was 89% of the activity in the starting preparation. The peaks are numbered I to V in the order of elution.

present in rat tissues (Furth & Robinson, 1965; Robinson *et al.*, 1967; Alpers, 1969; Goldstone *et al.*, 1970; Patel & Tappel, 1970; Miyatake & Suzuki, 1974). Therefore we decided to investigate whether enzyme forms that differed in their sensitivity to the ionic composition of the medium could be separated. For this purpose, soluble extracts were prepared from the light-mitochondrial fraction as described by Baccino *et al.* (1971) and submitted to DEAE-cellulose column chromatography by the method of Furth & Robinson (1965). The β-galactosidase activity of these extracts was then separated into five peaks (Fig. 2). The distribution of activity was, on average, 11, 25, 39, 15 and 10% for peaks I to V respectively. The recovery was approx. 90%, provided that the fractions were collected in the presence of 0.2% (w/v) bovine serum albumin and 2mM-cysteine (final concentrations), otherwise the recovery was much lower (approx. 40%), owing to a loss of activity roughly proportional for all the peaks. The fractions were pooled and dialysed against 2mM-cysteine before further analysis.

Fig. 3 shows that increasing concentrations of acetic acid–sodium acetate buffer, pH 5.0, resulted in a similar stimulation of the β-galactosidase activity for the five chromatographic peaks. On average, a threefold activation was obtained over the range of buffer concentrations used.

The effect of changing the buffer concentration on the substrate–activity curve for one chromatographic peak is illustrated in Fig. 4. Although the double-reciprocal plot shown refers to only one of the chromatographic peaks, quite similar results have been obtained for the others. Varying the buffer concentration resulted in marked changes in $V_{max.}$, whereas the apparent K_m was only slightly affected. Table 1 shows the values for these two parameters as calculated by regression analysis.

The pH–activity curve and the changes caused by adding 0.3M-NaCl were very similar for all the peaks (Fig. 5). After addition of NaCl the profile of the curve was modified and the optimum pH shifted from 2.0–2.5 to 3.5, in such a way that at pH 2.0–2.5 virtually no activity could be measured. The relative

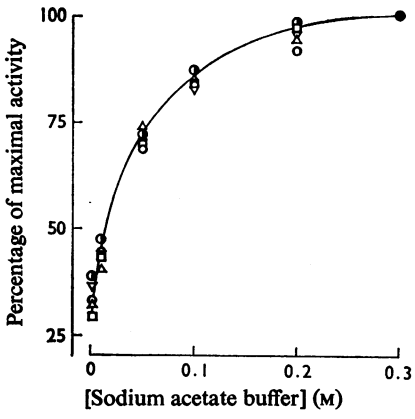

Fig. 3. *Stimulation of β-galactosidase activity by acetate buffer*

The activity of the chromatographic peaks I (△), II (□), III (○) IV (▽) and V ◑, from soluble lysosomal extracts was assayed at increasing concentrations of sodium acetate buffer, pH 5.0 (on the abscissa).

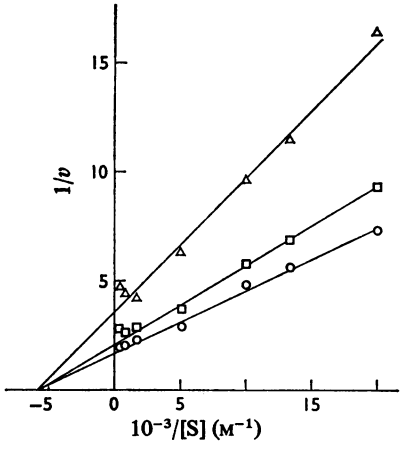

Fig. 4. *Effect of acetate buffer concentration on the rate of hydrolysis of p-nitrophenyl β-galactopyranoside*

Double-reciprocal plot of the reaction rate versus the substrate concentration. The data of this particular experiment were obtained with the chromatographic peak II, in 2.5 (△), 50 (□) or 300 (○) mM-sodium acetate buffer, pH 5.0. v is given in arbitrary units. Slopes, intercepts and apparent K_m values were calculated by regression analysis.

stimulation by NaCl was virtually constant over the pH range from 3.5 to 6.0, which includes the value (5.0) used for most of the present experiments. It should further be noted that the curves in the presence and in the absence of added NaCl intersect at pH 3.2. Since routine assays for β-galactosidase are most

Table 1. *Effect of acetate buffer concentration on apparent K_m and $V_{max.}$ for p-nitrophenyl β-D-galactopyranoside*

Sodium acetate buffer, pH 5.0, was present in the assay at the concentrations indicated. The two parameters for the chromatographic peaks I to V (cf. Fig. 2) have been calculated by regression analysis; the results of a representative experiment are reported. $V_{max.}$ is given in arbitrary units.

Chromatographic peak	Acetate buffer (mM)	K_m (μM)	$V_{max.}$
I	2.5	183	1.00
	50	195	1.99
	300	205	2.77
II	2.5	174	1.00
	50	175	1.99
	300	181	2.76
III	2.5	170	1.00
	50	207	2.00
	300	230	2.61
IV	2.5	89	1.00
	50	135	1.86
	300	196	2.78
V	2.5	186	1.00
	50	188	1.72
	300	210	2.28

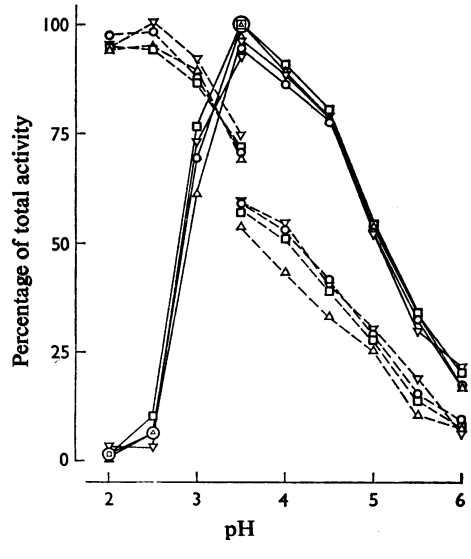

Fig. 5. *β-Galactosidase pH–activity curves*

Chromatographic peaks I (△), II (□), III (○) and IV (▽) from soluble extracts of lysosome-rich preparations (peak V was not eluted in this particular experiment) were assayed with (——) or without (----) added 0.3 M-NaCl. Either 5 mM-glycine adjusted to pH 2.0–3.5 with HCl solution or 5 mM-sodium acetate buffer (pH 3.5–6.0) was used.

often performed at pH 3.0–3.5, this is probably one reason why the activation by ions has been overlooked so far.

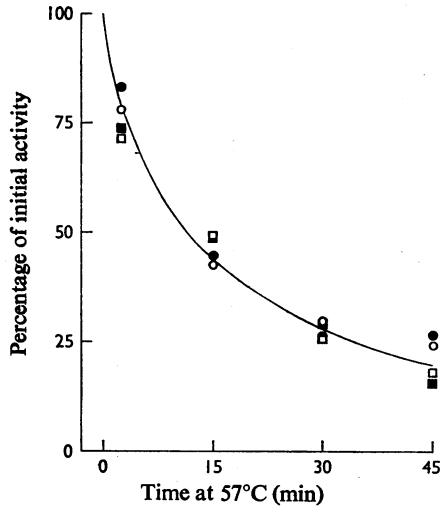

Fig. 6. *Effect of KCl on the heat inactivation of β-galactosidase activity*

Liver homogenates (□, ■) or light-mitochondrial fractions (○, ●) were suspended in water, incubated at 57°C in the presence (●, ■) or absence (○, □) of 0.2M-KCl for the times indicated, and assayed for enzyme activity.

The thermolability of mammalian β-galactosidase activity has been previously investigated (Gatt, 1969; Ho & O'Brien, 1969, 1970; Patel & Tappel, 1970). In the present observations the heat inactivation was not appreciably influenced by the addition of 0.2M-KCl to the preincubation medium (Fig. 6).

p-Chloromercuribenzoate is an inhibitor of mammalian β-galactosidase activity (Furth & Robinson, 1965; Robinson *et al.*, 1967; Patel & Tappel, 1970). At concentrations that caused partial inhibition of the β-galactosidase activity in liver homogenates or lysosome-rich fractions, the increase in activity in the presence of added KCl was markedly higher than that for controls (Table 2). This could be due to the occurrence of enzyme forms differing in both their susceptibility to *p*-chloromercuribenzoate and activation by ions or to the reversal by KCl of the *p*-chloromercuribenzoate inhibition. Indeed a differential sensitivity to this thiol reagent has been observed among chromatographic fractions of rat kidney β-galactosidase activity (Patel & Tappel, 1970). In the present experiments, however, all the fractions virtually exhibited the same degree of susceptibility (Fig. 7).

β-Galactosidase, like most lysosome hydrolases, is largely latent when assayed on subcellular preparations containing well-preserved particles (Baccino & Zuretti, 1975). Unlike total activity (i.e. that measured after complete particle disruption), the free or non-latent activity remained unaffected or even declined on raising the ionic concentration in the medium, thereby causing a progressive decline in the ratio of

Table 2. *Stimulation by KCl of the β-galactosidase activity after inhibition by p-chloromercuribenzoate*

The enzyme preparations, suspended in 0.1% (w/v) Triton X-100, were preincubated for 5min at 37°C with *p*-chloromercuribenzoate and further incubated for 30min for the enzyme assay (1/4 dilution with the reaction mixture). The final concentration of homogenates was 3% (w/v). For light-mitochondrial fractions, 1ml of the preincubation mixture contained the preparation corresponding to 50mg of wet tissue. Enzyme activity is expressed as percentage of the maximum rate. The results are means of six experiments.

	KCl (M)	*p*-Chloromercuribenzoate (μM)	Enzyme activity	% Inhibition	% Increase caused by KCl
Homogenate	0	0	64.9		
	0.1	0	96.6		48.8
	0.2	0	100.0		54.1
	0	200	13.4	82.0	
	0.1	200	32.5	46.2	142.5
	0.2	200	51.9	43.2	287.3
Light-mitochondrial fraction	0	0	62.8		
	0.2	0	100.0		59.2
	0	20	55.1	12.2	
	0.2	20	91.4	6.1	65.9
	0	40	28.9	53.6	
	0.2	40	55.8	44.2	93.1
	0	100	2.8	95.6	
	0.2	100	14.4	85.6	414.3
	0	200	0	100.0	
	0.2	200	1	99.0	

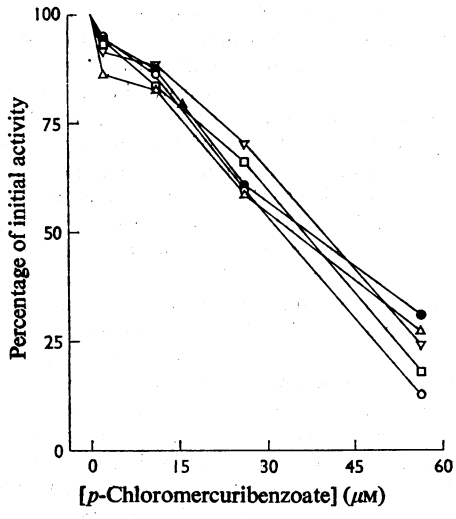

Fig. 7. *Inhibition of β-galactosidase activity by p-chloromercuribenzoate*

The chromatographic peaks I (△), II (□), III (○), IV (▽) and V (●) were incubated for 5 min at 37°C in the presence of the thiol reagent at the concentrations indicated, then assayed for β-galactosidase activity in the presence of 5 mM-sodium acetate buffer, pH 5.0.

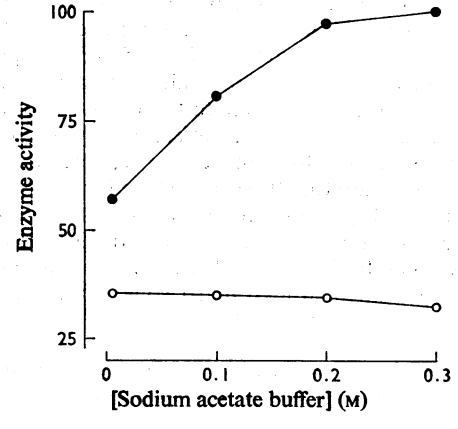

Fig. 8. *Effects of increasing buffer concentration on free and total β-galactosidase activity*

Light-mitochondrial fractions (cf. Baccino *et al.*, 1971) were suspended in 0.25 M-sucrose and assayed for free (○) and total (●) activity in the presence of various concentrations of sodium acetate buffer, pH 5.0 (on the abscissa). The enzyme activity is expressed as percentage of the total activity at 0.3 M buffer; 0.1% (w/v) Triton X-100 was added to the tubes for measurement of total activity.

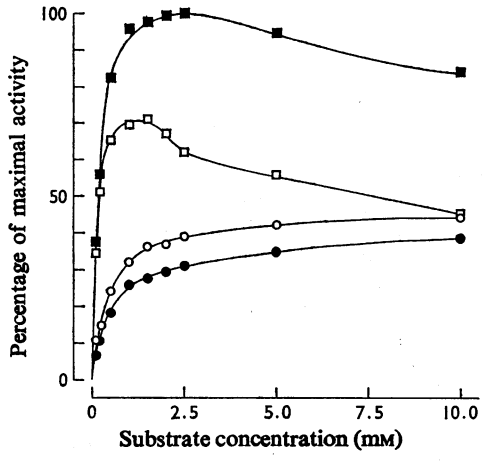

Fig. 9. *Effects of KCl on free and total β-galactosidase activity as a function of substrate concentration*

Light-mitochondrial fractions suspended in 0.25 M-sucrose were assayed for free (○, ●) and total (□, ■) activity in either the presence (●, ■) or the absence (○, □) of added 0.2 M-KCl.

free to total activity (Fig. 8). Since the total activity was affected by substrate inhibition, whereas free activity apparently was not, they became virtually identical at substrate concentrations of 10–20 mM (Fig. 9).

Discussion

Rat liver β-galactosidase activity has been shown to be markedly affected by the ionic composition of the medium. Various salts, differing in both the anionic (acetate, Cl^-, SO_4^{2-}) and cationic (Na^+, K^+, Mg^{2+}) species, resulted in a marked stimulation of the reaction rate at pH 5, $MgCl_2$ proving the most active. The maximum effect was attained at concentrations between 0.2 and 0.3 M, except for K_2SO_4, the stimulatory effect of which declined at concentrations higher than 0.1 M. Among the compounds tested, only potassium glutamate was inhibitory.

The occurrence in the liver of various enzyme forms able to hydrolyse *p*-nitrophenyl β-galactoside is well documented (Furth & Robinson, 1965; Robinson *et al.*, 1967; Alpers, 1969; Goldstone *et al.*, 1970; Patel & Tappel, 1970; Miyatake & Suzuki, 1974). On the other hand our observations, such as the increased degree of activation by ions after partial inhibition with *p*-chloromercuribenzoate or the divergent effect of ions on free and total activity, might suggest the existence of β-galactosidase forms differently affected by ions. Such a possibility was investigated by fractionating the β-galactosidase activity by DEAE-cellulose chromatography. The evidence obtained

does not support the hypothesis above, since the various peaks of β-galactosidase activity were similarly affected by ions. Moreover, no differences could be detected in the β-galactosidase activity associated with the various peaks in terms of apparent K_m, pH–activity relationship and susceptibility to *p*-chloromercuribenzoate.

On examining the effects of added NaCl as a function of pH, the whole velocity–pH profile appeared to be shifted towards higher pH values, both sides of the curve being affected; an activation was observed on the alkaline side (including pH 5.0), an inhibition on the acidic one. The latter effect was particularly marked; at pH 2.0 the activity was maximal with no added NaCl and virtually zero in its presence; in the latter situation the curve approached a bell-shaped profile. In terms of maximal rate of catalysis, however, the activity observed at pH 3.5 in the presence of added NaCl was not different from that at pH 2.0–2.5 in its absence. As observed after addition of NaCl, the activation at pH 5.0 mainly reflected changes in $V_{max.}$, whereas the apparent K_m was but little affected.

The present observations probably have some relevance to the physiology of the intracellular digestive processes. Most lysosomal hydrolases display maximal activity in the acidic range of pH (Barrett, 1972). Nonetheless, the rate–pH profile for β-galactosidase activity at low ionic concentrations was so far displaced towards acid pH values that the occurrence of significant catalysis within the presumably physiological pH range (Mego, 1971; Dingle *et al.*, 1973; Reijngoud & Tager, 1973; Wibo & Poole, 1974) could not be easily accounted for. However, the activity–pH relationship at high ionic concentrations, as reported here, seems to be more representative of the situation under which the hydrolysis occurs *in vivo*. In this connexion, it would be interesting to know whether the hydrolysis of naturally occurring substrates is affected by ions, as is that of *p*-nitrophenyl β-galactoside.

A further implication of our results is that in the assay for β-galactosidase activity the ionic composition of the reaction medium and the effects of the ionic species therein should be controlled. The control might turn out to be particularly critical with such samples as chromatographic eluates, which may differ considerably with respect to ionic composition, or whenever the pH of the assay is removed from the point of equivalence (pH 3.2) between the activity at low and high ionic concentration. To both overcome the influence of ions present in the enzyme preparations and increase the sensitivity of the assay at pH values higher than 3.0, either the final concentration of buffers such as acetic acid–acetate in the reaction mixture should be adjusted to 0.3 M or salts such as NaCl or $MgCl_2$ should be added.

The ratio of free to total hydrolase activity is usually taken as an index of the degree of lysosome integrity (cf. Baccino & Zuretti, 1975). The ratio for β-galactosidase, which usually is higher than that for other hydrolase activities (Baccino & Zuretti, 1975; Zuretti & Baccino, 1976, is now shown to depend on the ionic composition of the medium. In contrast with the stimulation of total activity, free β-galactosidase activity is unaffected or even decreased by 0.2 M-KCl added to the incubation medium, which already contains 0.25 M-sucrose as osmotic protector. This observation might indicate that: (i) *p*-nitrophenyl β-galactopyranoside can partially permeate the lysolemma so that, in the assay for free activity, it is also hydrolysed within particles; (ii) during the incubation at 37°C and pH 5.0, sucrose does not, whereas KCl does, provide (Davidson & Song, 1975) adequate osmotic protection to some lysosome-rich preparations, perhaps rendered somewhat unstable because damaged during the isolation procedure. The first conclusion would also be consistent with other evidence (Baccino & Zuretti, 1975; Zuretti & Baccino, 1976). By contrast, various substrates for lysosomal hydrolases (β-glycerophosphate, *p*-nitrophenyl acetyl-β-D-glucosaminide and phenolphthalein mono-β-glucuronide) have been previously shown to be unable to permeate to an appreciable extent the lysolemma in lysosome preparations, either normal (Baccino & Zuretti, 1975) or damaged *in vitro* (Baccino & Zuretti, 1975) or modified as a result of treatments *in vivo* (ethionine: Zuretti & Baccino, 1975; *Amanita phalloides*: Zuretti & Baccino, 1976.

As to the possibility that sucrose may not always provide complete osmotic protection to lysosomes, it should be mentioned that, in the presence of 0.25 M-sucrose, increasing concentrations of β-glycerophosphate have been found to result in a 'paradoxical' decrease in the ratio of free to total activity for acid phosphatase activity; this effect was particularly evident with partially damaged particle preparations (Baccino & Zuretti, 1975). Therefore ion-containing media at 37°C possibly offer to lysosomes an osmotic protection not only similar to (Ignarro, 1973; Davidson & Song, 1975) but even better than that provided by sucrose alone, at least under some circumstances (cf. Rosenberg & Janoff, 1968).

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