Specificity and Multiple Forms of β -Galactosidase in the Rat

BY ANNA J. FURTH AND D. ROBINSON

Department of Nutrition, Queen Elizabeth College, London, W. 8

(Received 5 January 1965)

1. Ten rat tissues and organs have been assayed for β -galactosidase with phenyl β -D-galactoside, *p*-nitrophenyl β -D-galactoside, *p*-aminophenyl β -D-galactoside and 4-methylumbelliferyl β -D-galactoside as substrates. 2. The relative activities of these tissues are independent of the mode of assay, and maximum rates of hydrolysis are not greatly affected by the nature of the substrate. 3. Inhibition studies suggest the liver enzyme has no associated β -glucosidase activity. 4. There is no cellular localization of preferential activity towards any of the four substrates in liver, kidney or spleen. 5. Evidence suggesting the non-destructive penetration of liver lysosomal membranes by *p*-nitrophenyl β -D-galactoside is presented. 6. Liver lysosomal β -galactosidase exists in multiple forms that can be separated on DEAE-cellulose, and the enzyme components that are bound to the membrane appear to be similar to those of the lysosome sap. 7. The chromatographic pattern of enzyme excreted in the urine is compared with those from the kidney, intestine, spleen and liver.

The enzyme β -galactosidase occurs in many mammalian organs (Cohen, Tsou, Rutenburg & Seligman, 1952), and a survey of mouse and rat tissues was made by Conchie, Findlay & Levvy (1959), with *o*-nitrophenyl galactoside as substrate, and by Maio & Rickenberg (1960) in mouse organs. Its association with the lysosomal fraction of rat liver was indicated by Sellinger, Beaufay, Jacques, Doyen & de Duve (1960), and subsequently Conchie & Hay (1963) demonstrated the particle-bound nature of the enzyme in other tissues.

Multiple forms of other glycosidases are known (Pettersson, 1963; Berk, Kawaguchi, Zeineh, Ujihara & Searcy, 1963), and Doell & Kretschmer (1962) demonstrated two β -galactosidase components in rabbit and rat intestinal mucosa, one of which hydrolysed *o*-nitrophenyl β -D-galactoside but had little effect on lactose. Thus there is the possibility of error in equating lactase with *o*-nitrophenyl β -galactosidase, and, since previous observations have been usually based on one of a variety of experimental substrates, there is little information to demonstrate either comparability of the various methods or identity of the enzyme from different tissues of the same species. In the present paper we have compared estimations of the β -galactosidase activity of ten rat tissues and organs by four different methods, and examined the possibility of detecting differences in substrate specificity between one organ and another. In addition, the multiple nature of rat β -galactosidase has been demonstrated, and some observations have

been made on the nature of individual components of the liver enzyme.

EXPERIMENTAL

Materials. Phenyl β -D-galactoside and *p*-nitrophenyl β -D-galactoside were made by the method of Glazer & Wulwek (1924). *p*-Aminophenyl β -D-galactoside was prepared from the *p*-nitrophenyl β -D-galactoside by hydrogenation at atmospheric pressure as described by Yariv, Rapport & Graf (1962). 4-Methylumbelliferyl β -D-galactoside was prepared by the method of Robinson (1964).

o-Nitrophenyl β -D-galactoside was purchased from L. Light and Co. Ltd., Colnbrook, Bucks. All salts used for inhibition experiments were A.R. grade, and were chlorides, nitrates or sulphates, these anions having been found to have no inhibitory effect. Aniline (British Drug Houses Ltd., Poole, Dorset) was freshly distilled and used as the hydrochloride. Lactose and galactose were bacteriological grade (Hopkin and Williams Ltd., Chadwell Heath, Essex). Methyl β -D-thiogalactoside, salicin, glucono-(1 $\rightarrow$ 5)-lactone and galactono-(1 $\rightarrow$ 4)-lactone were purchased from L. Light and Co. Ltd.

Tissue preparations. For tissue fractionation studies male Wistar rats 12–16 weeks old were killed by a blow on the head, and organs were rapidly removed into ice-cold 0.25 M-sucrose, weighed and homogenized in a Potter-Elvehjem all-glass homogenizer (diameter clearance 0.3 mm.). The homogenate was filtered through muslin and diluted with the sucrose medium to a suitable concentration for assay.

When preparations of lysosomes were required for detailed study the possibility of mechanical damage during

homogenization was minimized by using a smooth glass homogenizer tube with a Teflon pestle rotated by hand (diameter clearance 0.48 mm). The yield of lysosomal particles was somewhat decreased but the particulate fraction produced had minimal free activity (see below), and activity in the supernatant was also low. Cell fractions were prepared in an MSE High Speed 17 refrigerated centrifuge at 4° in 0.25 M-sucrose. Speeds and duration were calculated to give similar conditions to those used by de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955). A nuclear and debris fraction N (9250 g-min.), a mitochondrial and lysosomal fraction M (370000 g-min.) and an unsedimented supernatant S were obtained. Fraction M was resuspended in 0.25 M-sucrose and sedimented as before, the washings being added to fraction S. For comparison of total activities where cell fractionation was not required the tissues were homogenized in ice-cold water, filtered through muslin and diluted to a suitable concentration.

Preparation of urinary β -galactosidase. Low-molecular-weight material was removed from 10 ml. samples of rat urine by gel filtration through a column (38 cm. $\times$ 2 cm.) of Sephadex G-25 prepared in distilled water. The filtrate obtained before the appearance of electrolyte was freeze-dried and stored at -10°.

Cellulose acetate paper electrophoresis. Approx. 5 μ l. of solution containing 100 μ g. of urinary protein was applied to cellulose acetate paper strips (12 cm. $\times$ 2.5 cm.) (Oxo Ltd., London, S.E. 1), and electrophoresis was carried out horizontally for 2 hr. at 2 mA/strip in 0.07 M-sodium barbital buffer, pH 8.6. The dried strips were stained for protein with Naphthalene Black 10B.

DEAE-cellulose chromatography. Whatman DEAE-cellulose powder DE50 (W. and R. Balston Ltd.) was prepared in 0.01 M-phosphate buffer, pH 6.0, and packed under gravity to give a 10 cm. $\times$ 1 cm. column. Samples of protein (containing up to 10 mg.) were prepared in the same buffer and applied to the columns, which were then developed under a constant head of buffer at a flow rate of 60 ml./hr.; 3 ml. fractions were collected. After 1 hr. an NaCl gradient up to a final concentration of 0.3 M was introduced in the same buffer. The β -galactosidase activity of each fraction was assayed with *p*-nitrophenyl β -D-galactoside and 4-methylumbelliferyl β -D-galactoside.

Assay of β -galactosidase. This was assayed by four methods.

(a) Liberation of phenol from phenyl β -D-galactoside was measured with the Folin-Ciocalteu reagent as follows. The enzyme (1 ml.) was incubated at 37° with 1 ml. of 10 mM-phenyl β -D-galactoside in phosphate-citrate buffer, pH 3.7 (McIlvaine, 1921), for periods up to 3 hr., over which time hydrolysis followed a linear course. The reaction was stopped by adding 1 ml. of Folin-Ciocalteu phenol reagent. The protein precipitate was centrifuged off, 2 ml. of the supernatant added to 2 ml. of 10% (w/v) Na₂CO₃ and the mixture kept at 37° for 20 min., when the blue colour was measured at 770 m μ on a Unicam SP.600 spectrophotometer. Tissue and substrate blanks were run concurrently.

(b) Hydrolysis of *p*-nitrophenyl β -D-galactoside was followed as described by Conchie *et al.* (1959), but m-Na₂CO₃ was used to develop the yellow colour of liberated *p*-nitrophenol, since NaOH caused rapid hydrolysis of the substrate. Incubations, with suitable controls, were

performed at 37° at pH 3.7 for 1 hr., or until 5–35 μ g. of *p*-nitrophenol had been liberated.

(c) *p*-Aminophenyl β -D-galactoside (1 ml. of a 20 mM solution in phosphate-citrate buffer, pH 4.8) was incubated at 37° with 1 ml. of enzyme preparation for 1 hr. or until 5–30 μ g. of *p*-aminophenol had been liberated. The reaction was stopped and deproteinized by adding 1 ml. of 10% (w/v) trichloroacetic acid and centrifuging. Then 2 ml. of the clear supernatant was withdrawn and added to a mixture of 1 ml. of 2 N-Na₂CO₃, 1 ml. of 0.1% phenol and 1 ml. of 0.03% K₃Fe(CN)₆. The mixture was allowed to stand at room temperature for 30 min., and the blue indophenol colour was measured against a tissue + reagent blank at 630 m μ in the Unicam SP.600 spectrophotometer.

(d) 4-Methylumbelliferyl β -D-galactoside (1 ml. of a 2 mM solution in phosphate-citrate buffer, pH 3.7) was incubated at 37° with 1 ml. of enzyme solution for up to 1 hr. or until 0.1–0.6 μ g. of 4-methylumbelliferone had been liberated. The reaction was stopped and fluorescence developed by adding 3 ml. of 0.2 M-glycine-NaOH buffer, pH 10.4, and the fluorescence measured on a Locarte L.F.5 fluorimeter fitted with an L.F.2 filter transmitting 340–380 m μ in the activating beam and with the interference wedge monochromator in the fluorescent light beam set at 440 m μ .

Assay of acid phosphatase. The hydrolysis of sodium β -glycerophosphate (final concn. 0.05 M) at 37° in acetate buffer, pH 5.0, was followed by the colorimetric estimation of the liberated inorganic phosphate (Allen, 1940).

Assay of latent enzyme activity. The latency of β -galactosidase in all fractions was measured by comparing the free activity with the measurable activity after treatment with Triton X-100. Because of the instability of lysosomal particles below pH 4.5 these assays were performed at pH 5.0. Enzyme (1 ml.) in 0.25 M-sucrose was incubated at 37° for 10 min. with 1 ml. of substrate in 0.25 M-sucrose solution in phosphate-citrate buffer, pH 5.0. Assay of the liberated aglycone gave an estimation of the free activity. Total activity was measured by repeating the assay in a substrate medium that also contained 0.2% (w/v) of Triton X-100. The results for free activity were expressed as percentages of total activity after potentiation with Triton X-100. Activities of sucrose homogenates in the presence of Triton X-100 were found to be the same as those for aqueous homogenates of equal weights of the same tissue. The above conditions were also used for free and total acid-phosphatase activity, when the substrate medium contained 0.1 M- β -glycerophosphate in 0.1 M-acetate buffer, pH 5.0.

Michaelis constants. The apparent K_m values were calculated by the double-reciprocal plot method of Lineweaver & Burk (1934).

Assay of chloride. The method of Sanderson (1952) was used.

Assay of protein. This was performed according to the method of Lowry, Roseborough, Farr & Randall (1951).

RESULTS

Activity of rat tissues. A comparison of ten organs or tissues, estimated with four different substrates, is shown in Table 1. For any one substrate the activities are similar to and in the same order as those described by Conchie *et al.*

Table 1. β -Galactosidase activity of rat-tissues assayed by four different methods

The final substrate concentrations used and the pH values (0.15M-phosphate-citrate buffer) at which the incubations were performed are shown in Table 2. The activities are expressed as means $\pm$ s.d., with the numbers of experiments in parentheses.

Tissue	Substrate	β -Galactosidase activity (μ moles of substrate split/mg. wet wt. of tissue/hr.)			
		<i>p</i> -Nitrophenyl β -D-galactoside	<i>p</i> -Aminophenyl β -D-galactoside	Phenyl β -D-galactoside	4-Methylumbelliferyl β -D-galactoside
Epididymis		348 $\pm$ 178 (7)	495 $\pm$ 162 (6)	291 $\pm$ 128 (6)	152 $\pm$ 33 (8)
Kidney		101 $\pm$ 10.0 (6)	137 $\pm$ 14 (6)	66.5 $\pm$ 9.1 (6)	42.4 $\pm$ 23.5 (6)
Spleen		64.1 $\pm$ 18.5 (6)	101 $\pm$ 32 (7)	41.5 $\pm$ 11.7 (7)	48.1 $\pm$ 9.6 (7)
Duodenum		29.1 $\pm$ 4.4 (6)	42.1 $\pm$ 5.8 (6)	20.6 $\pm$ 4.3 (6)	17.2 $\pm$ 3.7 (6)
Liver		21.0 $\pm$ 2.3 (4)	23.3 $\pm$ 5.6 (6)	13.4 $\pm$ 3.7 (5)	11.0 $\pm$ 2.6 (6)
Lung		19.6 $\pm$ 2.6 (4)	30.4 $\pm$ 10.0 (5)	13.1 $\pm$ 3.8 (5)	29.0 $\pm$ 9.0 (6)
Pancreas		11.8 $\pm$ 1.9 (6)	16.6 $\pm$ 4.6 (6)	8.7 $\pm$ 1.9 (5)	9.5 $\pm$ 2.1 (6)
Brain		7.03 $\pm$ 1.70 (6)	10.3 $\pm$ 1.4 (7)	7.7 $\pm$ 1.6 (6)	9.8 $\pm$ 2.9 (7)
Cardiac muscle		3.06 $\pm$ 0.44 (4)	3.68 $\pm$ 0.44 (4)	2.20 $\pm$ 0.52 (3)	4.70 $\pm$ 1.75 (4)
Smooth muscle		1.67 $\pm$ 0.26 (4)	1.92 $\pm$ 0.31 (4)	0.96 $\pm$ 0.03 (4)	2.01 $\pm$ 0.66 (4)

Table 2. Optimum pH, apparent K_m values and concentrations of substrates giving maximum rates for the β -galactosidase activity of rat-liver homogenates

0.15M-Phosphate-citrate buffer was used and other experimental details are given in the text.

Substrate	Optimum pH	Optimum concn. (M)	K_m (M)
<i>p</i> -Nitrophenyl β -D-galactoside	3.6	2×10^{-3}	2.1×10^{-4}
<i>p</i> -Aminophenyl β -D-galactoside	4.5	10×10^{-3}	4.4×10^{-3}
Phenyl β -D-galactoside	3.7	5×10^{-3}	2.6×10^{-3}
4-Methylumbelliferyl β -D-galactoside	3.7	2.5×10^{-4}	2.2×10^{-4}

(1959). Tissues from different animals showed considerable variations in levels of enzyme activity when measured with the same substrate, but when the relative activity towards the four substrates was compared for the tissues of individual animals constant proportions were observed. *p*-Aminophenyl β -D-galactoside is the most rapidly hydrolysed of the four substrates but has the lowest affinity for the enzyme (Table 2). On the other hand, 4-methylumbelliferyl β -D-galactoside is in most cases only hydrolysed at about half the rate of the former substrate, but has a much higher affinity for the enzyme. Though no tissue appears to hydrolyse one of the substrates preferentially to the others, some minor organ differences are detected, as for instance in lung, which hydrolyses 4-methylumbelliferyl β -D-galactoside and *p*-aminophenyl β -D-galactoside at the same rate.

Cellular distribution. Samples of liver, kidney and spleen were fractionated as described above and the β -galactosidase activity of each fraction

was assayed with each of the four substrates. The incubation mixture contained Triton X-100 (0.1%) to potentiate the lysosomal activity fully.

The results (Table 3) indicate that under these conditions each tissue shows a characteristic distribution regardless of the substrate used, and there is no localization of preferential activity towards a particular galactoside. In the liver 70% of the activity was found in the fraction M, which includes the lysosomal fraction described by Sellinger *et al.* (1960), and the 13% activity found here in the supernatant agrees with the combined activities of the microsomal and soluble fractions quoted by those authors. The high nuclear activity may be attributable to unbroken cells. Kidney, and to a smaller extent spleen, displays a good deal of unsedimentable activity, and similar high values were obtained with the latter organ for acid phosphatase (de Duve, 1959). It is possible that macrophages in the spleen and kidney droplets may be damaged by the homogenization technique used, as well as being sedimented in different fractions to some extent.

Nature of liver β -galactosidase. The effect of inhibitors was studied on water homogenates of rat liver, with 4-methylumbelliferyl β -D-galactoside as substrate. The inhibitors were dissolved in buffer solution and adjusted to pH 3.7 if necessary before use. The enzyme is sensitive to Ag^+ , Hg^{2+} and Cu^{2+} , as noted by Conchie & Hay (1959) (Table 4). The effect of tris is pH-dependent, as found by Dahlqvist (1958) for intestinal lactase, and a comparison of some other inhibitors shows a close similarity between the liver and intestinal enzymes. No inhibition was detected with glucose or a glucoside (salicin) at 10mM, and 1.6mM-glucono-(1 $\rightarrow$ 5)-lactone had no effect. No β -glucosidase activity was detected.

Table 3. *Distribution of β -galactosidase in cell fractions obtained by differential centrifugation of liver, kidney and spleen homogenates*

Experimental details are given in the text. The fractions were obtained from homogenates in 0.25 M-sucrose as follows: N, sedimented at 9250g-min.; M, sedimented at 370000g-min.; S, not sedimented at 370000g-min.

Tissue	Fraction	Substrate ...	Percentage of recoverable β -galactosidase activity			
			<i>p</i> -Nitrophenyl β -D-galactoside	<i>p</i> -Aminophenyl β -D-galactoside	Phenyl β -D-galactoside	4-Methylumbelliferyl β -D-galactoside
Kidney	N		18.1	18.2	15.4	14.8
	M		44.1	43.8	50.5	54.2
	S		37.8	38.0	34.1	31.0
Spleen	N		44.8	42.6	44.8	47.4
	M		33.6	36.8	34.6	32.8
	S		21.6	20.6	20.6	19.8
Liver	N		15.7	14.8	15.1	10.1
	M		64.7	73.9	63.5	78.6
	S		19.6	11.3	21.4	11.3

Table 4. *Inhibition of hydrolysis of 4-methylumbelliferyl β -D-galactoside by rat-liver and rat-intestine water homogenates*

The assay procedure is described in the text. Results are expressed as percentage inhibition of controls, similarly incubated.

Inhibitor	Inhibition (%)	
	Liver homogenate	Intestine homogenate
Ag ⁺ (5 μ M)	50	—
Hg ²⁺ (0.5 μ M)	50	—
Cu ²⁺ (5 mM)	50	—
Cd ²⁺ (10 mM)	14	12
Zn ²⁺ (20 mM)	20	—
Ba ²⁺ (10 mM)*	10	—
Iodide (10 mM)	14	10
Aniline (25 mM)	21	—
Tris (0.5 M) at pH 5.7	67	68
Tris (0.6 M) at pH 3.7	30	31
Lactose (25 mM)	43	43
Methyl β -D-thiogalactoside (5 mM)	39	40
Galactose (4 mM)	50	—
Galactono-(1 $\rightarrow$ 4)-lactone (0.4 mM)	50	—
<i>p</i> -Chloromercuribenzoate (0.5 mM)*	76	—

* Assayed on *p*-nitrophenyl β -D-galactoside.

Lysosomal activity. When fraction M was assayed both in the presence and absence of 0.1% Triton X-100 at pH 5, most of the activity was found to be latent, and with 4-methylumbelliferyl β -D-galactoside as substrate only about 8% of the total activity could be estimated in the absence of detergent, a value that agrees well with the acid-

phosphatase value (9.5%) quoted by Sellinger *et al.* (1960).

This amount of free activity apparently depended on the substrate used for assay. The phenomenon was particularly marked with *p*-nitrophenyl β -D-galactoside. A lysosome preparation that had only 8% free activity when assayed with 1 mM-4-methylumbelliferyl β -D-galactoside had 15% free activity towards 5 mM-phenyl β -D-galactoside, 34% with 10 mM-*p*-aminophenyl β -D-galactoside and 37% with 5 mM-*p*-nitrophenyl β -D-galactoside. Conchie & Hay (1963) increased the concentration of *o*-nitrophenyl β -D-galactoside to 30 mM and showed that the free activity could be increased even at substrate concentrations that were inhibitory to the Triton-activated material. This effect was clearly shown with our lysosomal fraction. The Triton-treated preparation showed maximum activity at 2 mM concentration, and above this was inhibited by excess of substrate. Fig. 1 shows that a concentration of 10 mM was required to obtain maximum free activity in sucrose homogenates, and at this substrate concentration about 60% of the activity appeared to be free.

Berthet, Berthet, Appelmans & de Duve (1951) demonstrated penetration of the lysosomal membrane by salts, with 0.5 M-sodium chloride in iso-osmotic sucrose. Resuspension of the particles in salt-free sucrose caused osmotic lysis, the lysosomes now being hyperosmotic. Similar experiments performed with *o*- and *p*-nitrophenyl β -D-galactoside instead of salt were unsuccessful. Two lysosomal suspensions were prepared, one in 0.25 M-sucrose in the usual way, and one in sucrose saturated with *o*- or *p*-nitrophenyl β -D-galactoside. After standing for 15 min. at room temperature the pellets were spun down, resuspended in 0.25 M-sucrose and assayed for acid phosphatase. In each case the

Fig. 1. Variation of lysosomal β -galactosidase activity with concentration of *p*-nitrophenyl β -D-galactoside used as substrate. Assays were performed at 37° at pH 3.7 in 0.25M-sucrose for 10 min. Other experimental details are given in the text. Curve A, in the presence of 0.1% Triton X-100; curve B, in absence of Triton X-100.

free activity was only 19% of the total activity found after adding Triton X-100 (0.1%). Since a saturated solution of the more soluble *p*-nitrophenyl β -D-galactoside is only 65mM the lysosomal membrane may be able to withstand the resulting small difference in osmotic pressure. This was confirmed when the experiment was repeated with up to 90mM-sodium chloride and no lysis occurred.

A further possibility remained that the β -galactosidase of liver lysosomes existed in multiple forms, one of which was readily accessible, perhaps situated in the membrane, and had both a high specificity for *p*-nitrophenyl β -D-galactoside and a high optimum substrate-concentration requirement. Osmotic liberation of latent activity does not necessarily solubilize all the enzyme (Conchie & Hay, 1963; Roy, 1960; Ugazio & Pani, 1963), and some activity may remain firmly bound to the 'ghost' membranes. When lysosomal preparations were homogenized in water and the 'ghosts' sedimented at 900 000g-min., they retained 8–15% of the enzymic activity, which could not be removed by repeated washing with water or dilute salt solutions. This 'bound' β -galactosidase of the lysosome membrane showed optimum activity with 2mM-*p*-nitrophenyl β -D-galactoside and K_m values similar to those of water homogenates of whole liver. Thus the penetration effect of high concentrations of *p*-nitrophenyl β -D-galactoside on the free activity is only evident in the intact lysosome and is not due to a particular enzyme component.

Multiple forms of liver lysosomal β -galactosidase. The identity of the soluble and insoluble fractions of lysosomal galactosidase was investigated further

Fig. 2. DEAE-cellulose chromatography of rat-liver lysosomal β -galactosidase activity. The column (10cm. $\times$ 1cm.) was equilibrated with 0.01M-phosphate buffer, pH 6.0, and eluted with an NaCl gradient (in the same buffer) up to 0.3M. Each 3ml. fraction was assayed with 4-methylumbelliferyl β -D-galactoside. The continuous line represents the activity of the lysosomal preparation and the shaded pattern shows the elution pattern for lysosomal 'ghosts' obtained by osmotic lysis and solubilized with Triton X-100. The broken line represents the chloride concentration.

by chromatography on DEAE-cellulose columns. A sucrose-washed lysosome-rich preparation was homogenized in 0.01M-phosphate buffer, pH 6.0, and the insoluble material was centrifuged (900 000g-min.), washed with buffer and recentrifuged three times. The washings were added to the original lysosome sap, which contained about 90% of the total β -galactosidase activity. A 10ml. sample of this soluble fraction, containing 8mg. of protein, was chromatographed on a DEAE-cellulose column as described above. A component that was apparently not adsorbed was removed before the chloride was introduced, and a further three peaks were found as the chloride concentration was increased (Fig. 2). These fractions were separated in a further experiment, where a stepwise elution was used. After fraction I had been run through the column, fraction II was obtained by elution with 0.015M-sodium chloride and fraction III with 0.06M-sodium chloride. The remaining activity (fraction IV) was removed with 0.2M-sodium chloride, and may be heterogeneous. The total eluate represented 73% of the activity applied to the column, and of this 28, 13, 32 and 27% appeared in fractions I–IV respectively. Fraction I was reapplied to a new column and was again eluted entirely by buffer alone. The absence of further activity on introducing the chloride gradient indicated that fraction I was not simply due to overloading, nor were fractions II–IV artifacts of this.

Table 5. *Distribution and latency of kidney and intestinal β -galactosidase in cell fractions*

Activities were measured against 4-methylumbelliferyl β -D-galactoside both in the absence and presence of Triton X-100. Free activity is expressed as a percentage of the total activity of that given fraction which is measurable in the absence of Triton X-100.

Fraction	Conditions	Kidney		Intestine	
		Percentage of total activity of homogenate	Free activity in each fraction (% of total)	Percentage of total activity of homogenate	Free activity in each fraction (% of total)
N	Sedimentation at 9200g-min.	15	30	—	—
M	Sedimentation at 890000g-min.	62	36	24*	94
P	Agglutination at pH 5	3	85	11	100
S	Supernatant	20	57	65	94

* Fractions N and M combined.

The β -galactosidase activity of the 'ghost' membranes could be solubilized with 0.1% Triton X-100, and when this material was chromatographed in a similar fashion the elution diagram bore a close resemblance to that for the lysosomal sap (Fig. 2), from which it could have been adsorbed. This treatment still left a considerable amount of sedimentable material. When these Triton-washed 'ghosts' were allowed to stand for 15 min. at room temperature with a sample of the soluble lysosomal fraction in water and then centrifuged, they did not adsorb any enzymic activity. This treatment with detergent probably leaves the membranes in a very denatured state because no adsorption was noted when the 'ghosts' were similarly suspended in cellular supernatant.

Cellular distribution of β -galactosidase in kidney. In view of the much higher activity in fraction S of kidney compared with that of liver, the former tissue was re-examined in more detail. By extending the centrifugation time for fraction M to 880000g-min. a further 8% of the total activity could be obtained in the particulate fraction, but increases beyond this to 1200000g-min. produced no further sedimentable activity. Two-thirds of this supernatant activity was latent. Preparation of a microsomal fraction by agglutination at pH 5.2 in 0.1M-acetate buffer and centrifuging yielded less than 3% of the total activity. Very little of this was in a latent form. The final supernatant, however, which contained 20% of the total activity, still had half of its β -galactosidase in a form that required potentiation with Triton X-100. It appears therefore that the particulate enzyme of rat kidney is distributed over a wider range of particle sizes than that of liver, and that these may be more fragile in view of the consistently higher free activities observed (Table 5). As with the liver enzyme hypo-osmotic conditions made all the particulate activity available without completely solubilizing it, and the sediment from frac-

tion M that had been lysed and twice washed in water still retained 30% of the activity of that fraction. In the presence of Triton X-100 almost all of this activity went into solution.

Cellular distribution in intestine. The β -galactosidase activity of cell fractions from rat intestine was estimated with 4-methylumbelliferyl β -D-galactoside. The results obtained (Table 5) are different from those of Doell & Kretchmer (1963), who assayed similar fractions with lactose as substrate and found the lactase activity to be concentrated in the nuclear fraction, for in the present case most of the activity is in the supernatant. These authors remarked on the different distribution of lactase, compared with acid phosphatase, and had previously confirmed (Doell & Kretchmer, 1962) that a second enzyme that was located in the soluble fraction was much more effective on *o*-nitrophenyl galactoside than on lactose.

Urinary β -galactosidase. A small amount of β -galactosidase activity was regularly excreted in rat urine. Freshly voided urines could be stored at -10° for several days without appreciable loss of activity. Pooled urines were passed through a Sephadex G-25 column (38 cm. $\times$ 2 cm. diam.) to remove low-molecular-weight material and freeze-dried as described in the Experimental section. In this way 150 mg. of protein was obtained, representing the excretion of six rats for 5 days. A 1 mg. sample of this material liberated 0.18 μ mole of 4-methylumbelliferone from its galactoside in 1 hr., under the assay conditions described above. Electrophoresis on cellulose acetate paper showed it to be heterogeneous and to contain a good deal of albumin which did not have associated β -galactosidase activity.

The urinary enzyme was chromatographed on a DEAE-cellulose column in the manner described above and the elution pattern compared with that from liver and other tissue homogenates (Fig. 3). More than 50% of the recoverable activity was

Fig. 3. Elution patterns of β -galactosidase of various rat tissues from DEAE-cellulose columns: (a) liver; (b) spleen; (c) intestine; (d) kidney; (e) urine. The conditions were as described for Fig. 2.

present in the first peak, which is rapidly eluted from the column before applying the chloride gradient, and the general pattern bore a close resemblance to that from kidney homogenates.

DISCUSSION

In view of the widespread occurrence of β -galactosidase in animal tissues there may be several functions for this enzyme. Though in the intestine it doubtless has a digestive role, there is no reason to suppose that hydrolysis of lactose is its major function in other tissues. The very high activity recorded in epididymis had been the subject of some discussion (Conchie *et al.* 1959) and may be concerned with mucoid catabolism whereas the spleen enzyme is probably associated, at least in part, with the destruction of the erythrocyte membrane by phagocytic bodies. It is possible that insufficient β -galactosidase activity in this organ may be the cause of the accumulation of partial degradation products of glycolipids, causing lipidoses of the Gaucher type (Phillipart & Menkes, 1964). Our results for lung were obtained from homogenates of the whole unwashed organ and may well be attributable to the alveolar macrophages, since it is known that much of the acid-hydrolase activity is

associated with these scavenger cells (Cohn, Hirsch & Wiener, 1963).

Although β -galactosidase is one of the enzymes associated with lysosomal particles in the liver, in other organs different cell fractions may be major sites of activity, as already noted for intestine, and in this case the apparent distribution depended on the substrate used. Other disaccharidases in small intestine have also been shown to have a different distribution from that of acid phosphatase (Gitzelmann, Davidson & Osinchak, 1964). There have been several other indications in the literature that the commonly used nitrophenyl substrates do not always present a complete or typical picture of enzyme activity. Neil & Horner (1964) found a different intracellular distribution of acid phosphatase in livers of several species, depending on whether *p*-nitrophenyl phosphate or β -glycerophosphate was used as substrate, and Jermyn (1952) examined multiple forms of a fungal β -glucosidase and found that some components were unable to hydrolyse *p*-nitrophenyl glucoside. The possibility of the existence of multiple forms of mammalian glycosidases with differing substrate specificities is also indicated in the competitive-substrate experiments of Conchie & Hay (1959), who found that phenyl α -mannoside did not inhibit the hydrolysis of *p*-nitrophenyl α -mannoside although the hydrolysis of both these substrates by rat tissues could be inhibited by methyl α -mannoside. The rat β -galactosidases examined in the present work, however, show relatively little difference in their maximum rate of hydrolysis of the four substrates used. The liver enzymes were inhibited by galactonolactone and by galactose and lactose, but glucose and gluconolactone had no effect, and there was no detectable β -glucosidase activity, suggesting that the enzyme binds specifically with the galactosyl moiety. In contrast with this Heyworth & Dahlqvist (1962) produced evidence for the hydrolysis of 4-methylumbelliferyl β -glucoside and β -galactoside at the same enzyme site in pig intestine, and Semenza & Auricchio (1962) showed the presence of two lactases in human ileum, both of which were active on cellobiose.

Attempts to demonstrate the multiple nature of rat β -galactosidase by gel electrophoresis and staining with the histochemical reagents of Rutenburg, Rutenburg, Monis, Teague & Seligman (1958) were unsuccessful, owing to the low specific activity of the material, but the elution patterns from DEAE-cellulose columns suggest that it may consist of a series of isoenzymes and that the pattern obtained may be representative of the tissue of origin. It is known that the enzyme from *Escherichia coli* consists of four sub-units (Weber, Sund & Wallenfels, 1963), and that these can be reversibly dissociated to form hybrids (Zipster, 1963).

Unlike the case of acid phosphatase, which appears to have different substrate and inhibitor specificities according to its cellular distribution (Shibko & Tappel, 1963; Neil & Horner, 1964), the present experiments have produced no distinction between the liver lysosomal and unsedimentable β -galactosidase components. Shibko & Tappel (1963) found that a considerable amount of binding of acid phosphatase to the lysosome membrane occurred, and Holtzer, Van Lanker & Swift (1963) found a similar effect with the amylase in zymogen granules. The possibility of different enzymes having specific binding sites was indicated by Ugazio & Pani (1963), who demonstrated differential release of several bound hydrolases from lysosomes by a non-ionic surface-active agent, and by Sawant, Desai & Tappel (1964), who have examined other factors affecting the availability of lysosomal enzymes. On the present evidence the bound β -galactosidase appeared to be identical with that which was easily solubilized after osmotic lysis. The enzyme components removed from the membrane by treatment with Triton X-100 were chromatographically indistinguishable from those of the lysosome sap, and had the same substrate affinities, and it would seem that any molecular variations involved in the various forms do not seriously affect the active site. The fact that resorption of enzyme on to Triton-washed 'ghosts' could be demonstrated neither in the present case nor in that of Ugazio & Pani (1963), who used arylsulphatase A, may well be due to the disruption of the membranes by the treatment involved, for the material was noticeably more difficult to sediment after washing in Triton solutions. The high free activity with *p*-nitrophenyl β -D-galactoside could be due to ease of penetration of the lysosomal membrane by this substrate, a hypothesis supported by its variation with concentration, or to lysis of the membrane. The latter seems unlikely, since the presence of 30 mM *p*-nitrophenyl β -D-galactoside did not increase the ratio of free to total acid-phosphatase activity of a lysosome preparation and it would thus require β -galactosidase and acid phosphatase to be separately located in similar organelles.

Under physiological conditions it appears that the permeability of the lysosomal particle may vary to admit substrate more readily without irreversibly releasing the contained enzymes and producing necrotic conditions (Bitsensky, 1963). Such a non-destructive increase in permeability to certain substrates could explain many of the apparent differential activations previously recorded, as well as the behaviour of the *p*-nitrophenyl β -D-galactoside reported here.

During the course of this work A. J. F. was in receipt of a Department for Scientific and Industrial Research studentship.

REFERENCES

- Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
 Berk, J. E., Kawaguchi, M., Zeineh, R., Ujihara, I. & Searcy, R. (1963). *Science*, **141**, 1182.
 Berthet, J., Berthet, L., Appelmans, F. & de Duve, C. (1951). *Biochem. J.* **50**, 182.
 Bitsensky, L. (1963). In *Ciba Found. Symp.: Lysosomes*, p. 362. Ed. by de Reuck, A. V. S. & Cameron, M. P. London: J. and A. Churchill Ltd.
 Cohen, R. B., Tsou, K.-C., Rutenburg, S. H. & Seligman, A. M. (1952). *J. biol. Chem.* **195**, 239.
 Cohn, Z. A., Hirsch, J. G. & Wiener, E. (1963). In *Ciba Found. Symp.: Lysosomes*, p. 126. Ed. by de Reuck, A. V. S. & Cameron, M. P. London: J. and A. Churchill Ltd.
 Conchie, J., Findlay, J. & Levvy, G. A. (1959). *Biochem. J.* **71**, 318.
 Conchie, J. & Hay, A. J. (1959). *Biochem. J.* **73**, 327.
 Conchie, J. & Hay, A. J. (1963). *Biochem. J.* **87**, 354.
 Dahlqvist, A. (1958). *Acta chem. scand.* **12**, 2012.
 de Duve, C. (1959). In *Subcellular Particles*, p. 128. Ed. by Hayashi, T. New York: The Ronald Press Co.
 de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955). *Biochem. J.* **60**, 605.
 Doell, R. G. & Kretschmer, N. (1962). *Biochim. biophys. Acta*, **62**, 353.
 Doell, R. G. & Kretschmer, N. (1963). *Biochim. biophys. Acta*, **67**, 516.
 Gitzelmann, R., Davidson, E. A. & Osinchak, J. (1964). *Biochim. biophys. Acta*, **85**, 69.
 Glazer, E. & Wulwek, W. (1924). *Biochem. Z.* **145**, 514.
 Heyworth, R. & Dahlqvist, A. (1962). *Biochim. biophys. Acta*, **64**, 182.
 Holtzer, R. L., Van Lanker, J. L. & Swift, H. (1963). *Arch. Biochem. Biophys.* **101**, 439.
 Jermyn, M. A. (1952). *Aust. J. sci. Res. B*, **5**, 433.
 Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
 Lowry, O. H., Roseborough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
 McIlvaine, T. C. (1921). *J. biol. Chem.* **49**, 183.
 Maio, J. J. & Rickenberg, H. V. (1960). *Biochim. biophys. Acta*, **37**, 101.
 Neil, M. W. & Horner, M. W. (1964). *Biochem. J.* **92**, 217.
 Pettersson, G. (1963). *Biochim. biophys. Acta*, **76**, 665.
 Phillipart, M. & Menkes, J. (1964). *Biochem. biophys. Res. Commun.* **15**, 551.
 Robinson, D. (1964). *Comp. Biochem. Physiol.* **12**, 95.
 Roy, A. B. (1960). *Biochem. J.* **77**, 380.
 Rutenburg, A. M., Rutenburg, S. H., Monis, B., Teague, R. & Seligman, A. M. (1958). *J. Histochem. Cytochem.* **6**, 122.
 Sanderson, P. H. (1952). *Biochem. J.* **52**, 502.
 Sawant, P. L., Desai, I. D. & Tappel, A. L. (1964). *Arch. Biochem. Biophys.* **105**, 247.
 Sellinger, O. Z., Beaufay, H., Jacques, P., Doyen, A. & de Duve, C. (1960). *Biochem. J.* **74**, 450.
 Semenza, G. & Auricchio, S. (1962). *Biochim. biophys. Acta*, **65**, 173.
 Shibko, S. & Tappel, A. L. (1963). *Biochim. biophys. Acta*, **73**, 76.
 Ugazio, G. & Pani, P. (1963). *Exp. Cell Res.* **31**, 424.
 Weber, K., Sund, H. & Wallenfels, K. (1963). *Angew. Chem.* **2**, 481.
 Yariv, J., Rapport, M. M. & Graf, L. (1962). *Biochem. J.* **85**, 383.
 Zipster, D. (1963). *J. molec. Biol.* **7**, 113.