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Rabbit β-Glucuronidase

PURIFICATION AND PROPERTIES, AND THE EXISTENCE OF MULTIPLE FORMS

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1. β-Glucuronidase (EC 3.2.1.31) was purified from rabbit liver by a procedure involving autolysis, $(NH_4)_2SO_4$ fractionation, chromatography on DEAE-cellulose and hydroxyapatite, gel filtration, sedimentation in a sucrose gradient, and isoelectric focusing. 2. Electron microscopy revealed ferritin as the major contaminant in later stages of purification and also showed aggregates of enzyme molecules. Particular attention was paid to the removal of ferritin. 3. The purified enzyme was homogeneous in polyacrylamide-gel electrophoresis both in non-dissociating conditions and in the presence of sodium dodecyl sulphate, and in Ouchterlony gel diffusion and immunoelectrophoresis against polyspecific antisera. 4. Sedimentation in sucrose gradients gave a molecular weight of 300000, whereas gel filtration indicated 440000. 5. Subunits of 75000 molecular weight were observed in gel electrophoresis in the presence of sodium dodecyl sulphate and in gel filtration in the presence of urea. 6. The K_m value for *p*-nitrophenyl β-D-glucuronide was 0.6mM, and the enzyme was extremely sensitive to lactone inhibitors. It was also inhibited by Hg^{2+} ions. 7. Multiple forms were observed in the pure enzyme by isoelectric focusing, with pI values of 4.5–5.8. Subunits showed similar heterogeneity. The origin of the multiple forms was investigated in detail, and the possibility of artifact generation largely excluded. Some of the forms of lowest pI disappeared after neuraminidase digestion. The nature of the residual heterogeneity remains to be elucidated.

β-Glucuronidase is an unusual enzyme in that it is found in significant amounts in both lysosomal and microsomal fractions in many cell types (de Duve *et al.*, 1955; Fishman *et al.*, 1967). In bovine liver (Plapp & Cole, 1966, 1967) and in rat liver lysosomes (Stahl & Touster, 1971), it is a glycoprotein with multiple forms. Since glycosylation of proteins mainly occurs in the Golgi apparatus (see Cook, 1973), β-glucuronidase molecules in transit to the lysosomes probably pass through the Golgi apparatus.

Histochemical, morphological and radioautographic evidence supports the hypothesis that primary lysosomes are formed as small vesicles at the periphery of the Golgi apparatus (see Cohn & Fedorko, 1969), but little evidence on the translocation of specific lysosomal enzymes has been presented. However, Kato *et al.* (1970), with mouse kidney, and Van Lancker & Lentz (1970), with rat liver, have studied the incorporation of radioactive precursors into β-glucuronidase and the time-course of appearance of radioactive enzyme in microsomal and lysosomal fractions. Although the enzyme preparations of both groups were probably impure, their results suggested a progressive movement of enzyme from endoplasmic reticulum to lysosomes.

It is now of interest to investigate biochemically the passage of β-glucuronidase through the Golgi apparatus and other microsomal subfractions, and to decide whether there is a population of β-glucuronidase molecules 'intrinsic' to the endoplasmic reticulum as well as one in transit to the lysosomes. This possibility has been suggested by Fishman *et al.* (1967) and they have proposed that intrinsic molecules play a 'structural' (as opposed to enzymic) role. Paigen and co-workers have found a mouse mutant which lacks microsomal β-glucuronidase and yet contains lysosomal enzyme (Ganschow & Paigen, 1967; Paigen, 1971) and this is compatible with the idea that there may be two categories of enzyme in the endoplasmic reticulum. Paigen (1961) has also shown that the subcellular localization is controlled by a gene distinct from the structural gene for β-glucuronidase, and suggests that the amino acid sequence of both the microsomal and the lysosomal enzyme is determined by a single structural gene.

Even if the multiple forms of the enzyme differ primarily in carbohydrate content, as seems likely from this genetic evidence, and from the work of

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Plapp & Cole (1966, 1967), an intrinsic microsomal population might be distinct chemically, in enzymic properties or in biosynthesis, from one in transit to the lysosomes. Thus the first stage of an investigation of the translocation of β-glucuronidase should involve the characterization of the various forms in chemical and enzymological terms.

Multiple forms of β-glucuronidase were demonstrated initially by careful chromatography on DEAE-cellulose (Moore & Lee, 1960; Sadahiro *et al.*, 1965; Plapp & Cole, 1967; Aoshima & Sakurai, 1969; Okochi *et al.*, 1968; Delvin & Gianetto, 1970), and subsequently by gel electrophoresis (Lundin & Allison, 1966). Attempts to demonstrate functional and electrophoretic differences between microsomal and lysosomal enzyme in mouse tissues were initially unsuccessful (Ganschow & Paigen, 1967), but later very slight electrophoretic differences were detected (Ganschow & Paigen, 1968; Ganschow & Bunker, 1970).

Mameli *et al.* (1972) have also claimed to discriminate between the microsomal and lysosomal enzyme of rat liver by polyacrylamide-gel electrophoresis, and recently evidence has been presented for the progressive glycosylation of various lysosomal hydrolases (including β-glucuronidase) during translocation in rat kidney (Goldstone *et al.*, 1973; Goldstone & Koenig, 1973). The data by no means exclude the possibility of an intrinsic microsomal population of β-glucuronidase.

In the present paper, a purification procedure for rabbit liver β-glucuronidase is presented, and the characterization of multiple forms is reported. Since the enzyme from different subcellular fractions was to be studied immunologically (Dean, 1974), the use of lysosomal fractions as starting material (Stahl & Touster, 1971) was unnecessary, and the purification could be performed on a large scale, by using the whole homogenate from many frozen livers.

A preliminary report of part of this work has appeared (Dean, 1973).

Materials and Methods

Materials

Rabbit livers from domesticated animals were supplied by Sainsbury–Spiller Poultry Packing Station, Bury St. Edmunds, Suffolk, U.K. Arquad 2C-50 [a 50% (w/v) solution of crude didodecyl ammonium chloride also containing analogues of longer and shorter chain length in aq. 50% (v/v) propan-2-ol] was from Armour Hess Chemicals Ltd., Leeds LS1 4NR, U.K. Bovine serum albumin (crystallized), ovalbumin (grade V), Fast Garnet GBC salt and potassium saccharate were obtained from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames KT2 7BH, U.K. Coomassie Brilliant Blue R250 was from Imperial Chemical Industries (supplied by G. T. Gurr, High Wycombe, Bucks., U.K.). Hyflo Super-Cel and naphthol-AS-BI-β-D-glucuronide (see below) were purchased from Koch–Light Laboratories Ltd., Colnbrook SL3 0BZ, Bucks., U.K. Sephadex G-200 and G-150, Sepharose 4B and 6B and Blue Dextran were products of Pharmacia (G.B.) Ltd., London W5 5SS, U.K. CM-cellulose (CM-52) and DEAE-cellulose (DE-52) were from Reeve Angel Scientific Ltd., London EC4U 6AY, U.K. Bio-Rex 70 was from Bio-Rad Laboratories, St. Albans, Herts., U.K. Pentachlorophenol was supplied by Ralph N. Emanuel Ltd., Wembley HA0 1PY, Middx., U.K. Bovine haemoglobin was prepared as described by Barrett (1970). Neuraminidase (*Vibrio cholerae*) was prepared by Behringwerke, Marburg, Germany. *p*-Nitrophenyl β-D-glucuronide was obtained from C. F. Boehringer, Mannheim, Germany, and Triton X-100 from Lennig Chemicals, London W.C.1, U.K. Horse spleen ferritin, six times crystallized, was bought from Miles Seravac, Maidenhead, Berks., U.K. The antiserum to rabbit serum proteins was prepared by Dakopatts, Copenhagen, Denmark and purchased from Mercia Diagnostics Ltd., Watford, Herts., U.K.

Methods

Enzyme assays. β-Glucuronidase activity was determined with 2.5mM-*p*-nitrophenyl β-D-glucuronide, 100mM-sodium acetate buffer, pH5.0, in a volume of 1ml. Incubations were at 37°C for up to 2h and were such that less than 10% of the substrate was hydrolysed. The reaction was terminated by the addition of 2ml of 0.2M-glycine adjusted to pH10.4 with 2M-NaOH, and the colour developed was measured at 420nm. Blanks received enzyme only after termination of the reaction. One unit of activity hydrolysed 1μmol of substrate/min under these conditions. Highly purified enzyme solutions were diluted for assay with a solution of bovine serum albumin and Triton X-100 (each at 0.1%) to minimize losses owing to surface adsorption (Bernfield *et al.*, 1957). Values 25% higher than controls without albumin and Triton X-100 were often observed.

Determination of protein. Measurements of E_{280} were usually used, but the method of Lowry *et al.* (1951), with bovine serum albumin as standard, was used where indicated.

Column chromatography. Buffers were made up in glass-distilled water, to which 1g of 1,1,1-trichloro-2-methylpropan-2-ol (Chlorbutol) and 1mg of pentachlorophenol in 9ml of butan-1-ol had been added per litre, as preservatives. pH values were determined at room temperature. Sephadex, Sepharose, DEAE- and CM-cellulose were used according to the manufacturers' recommendations, and run at 4°C with flow rates controlled by peristaltic pumps.

Polyacrylamide-gel electrophoresis and isoelectric focusing. Electrophoresis was performed by the method of Davis (1964) but without sample gels. A Tris–glycine discontinuous buffer system was used; the current was 3mA/tube. In a few experiments, electrophoresis was performed with 100mM-sodium acetate buffers at pH5.0 throughout, but with sample zone at 10mM-sodium acetate, pH5.0.

Isoelectric focusing in polyacrylamide gel was by the method of Barrett (1970) and Ampholines (LKB Instruments Ltd., South Croydon CR8 8YD, Surrey, U.K.) were normally at a final concentration of 1% (w/v).

Preparative isoelectric focusing was performed at 4°C in LKB 8101 columns with pH4–6 Ampholines (at 1%, w/v) in sucrose gradients. Columns were unloaded by gravity and effluents monitored at 280nm.

Staining of gels. Protein was stained with Coomassie Brilliant Blue R250 as described by Barrett (1973).

β-Glucuronidase in gels was detected by incubation of gels in a 100mg/l solution of the glucuronide of naphthol AS-BI (7-bromo-3-hydroxy-2-napth-*O*-anisidide) in 100mM-sodium acetate buffer, pH5.0. The substrate was dissolved in 50mM-$NaHCO_3$ (10–15mg/ml) at 37°C, before dilution with sodium acetate buffer.

Incubations were normally for 20min, but periods up to 3h were used. After incubation, gels were transferred to a solution of Fast Garnet GBC salt (1mg/ml) in glass-distilled water for 10min at room temperature. Purple bands on a pale-yellow background indicated sites of enzyme activity. Coupling was performed after the enzyme reaction because of the considerable inhibition of enzyme activity during simultaneous coupling caused by Fast Garnet GBC, which has been previously noted by Jeffree (1969) and others.

Neuraminidase digestion. Vibrio cholerae neuraminidase (10 units/6mg of partially purified enzyme) was incubated in 50mM-sodium acetate buffer, pH5.5, containing 0.09mg of NaCl/ml and 0.01mg of $CaCl_2$/ml with β-glucuronidase samples, for up to 6h at 37°C; 1 unit releases 1μg of *N*-acetylneuraminic acid from human α_1-acid glycoprotein in the same buffer in 15min. Recoveries of β-glucuronidase activity were always greater than 95%.

Solubilization of crude homogenates. Crude homogenates in water were obtained with an Ultra-Turrax homogenizer, by treatment with five periods of 30s each, with 15s intervals to avoid overheating. Sodium deoxycholate was added to 0.5% and the extract centrifuged at 100000*g* for 1h, in an MSE 65 centrifuge. For isoelectric focusing in a sucrose gradient, the supernatant was dialysed against 1% glycine before use, and the resulting precipitate was removed by centrifugation.

Extracts were also made on occasion with octyl sodium sulphate (at 0.1%, w/v) in place of deoxycholate.

Hydroxyapatite. Hydroxyapatite was prepared by a method used by Dr. J. I. Harris, M.R.C. Laboratory of Molecular Biology, Cambridge (personal communication). $CaHPO_4$, $2H_2O$ was precipitated at pH6.8 (by pumping 2.5 litres of 0.5M-$CaCl_2$ into 3 litres of 0.5M-Na_2HPO_4–NaH_2PO_4, pH6.8) and washed with 12 litres of glass-distilled water. The precipitate was resuspended in glass-distilled water stirred in a fume cupboard and brought to the boil under alkaline conditions, as indicated by phenolphthalein. The pH was controlled by addition of 18M-NH_3, and the suspension was kept simmering, with gentle stirring, for 30 min. The precipitate was cooled and then washed extensively with 5mM-K_2HPO_4–KH_2PO_4, pH6.5, until pH and conductivity were constant.

Ultrafiltration. A Diaflo-50 apparatus fitted with an XM-50 membrane was used (Amicon Ltd., High Wycombe, Bucks., U.K.).

Molecular-weight determinations. For determinations of the molecular weight of the intact enzyme, sucrose gradients from 5 to 20% (w/v) were used as described by Martin & Ames (1961) in 50mM-Tris–HCl buffer, pH7.8, in a 3×23ml swing-out rotor in the MSE 65 centrifuge at 100000*g*. Sedimentations were for 10h, and the gradients were fractionated through a steel tube passed to the bottom, by means of a peristaltic pump. Subunit molecular weights were determined by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Weber & Osborn, 1969).

Electron microscopy. Samples were negatively stained with 1% ammonium molybdate, pH7.0. Collodion-coated grids were floated face down on to the samples for 30s and then transferred to a watch-glass containing negative-staining solution. They were blotted dry and examined in an EM6B GEC-AEI electron microscope.

Immunological methods. Two guinea pigs received intramuscular injections of 250μg of partially purified β-glucuronidase on days 0, 14 and 28. The antigen was in 0.5ml of 50mM-Tris–HCl buffer, pH7.8, and was emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, Mich., U.S.A.). The animals were bled 7 days after the final injection, and both gave antisera which reacted with a number of proteins, including β-glucuronidase.

Double immunodiffusion by the method of Ouchterlony (1967) was done with 1% (w/v) Meath Agarose (from Medical and Biological Instrumentation Ltd., Ashford, Kent, U.K.) in 20mM-KH_2PO_4–Na_2HPO_4 buffer, pH7.0, containing 0.15M-NaCl, or in a buffer containing 21.6mM-barbitone sodium and 21.6 mM-sodium acetate adjusted to pH8.2 with

1M-HCl. Immunoelectrophoresis plates (8cm×8cm) were coated with 1% (w/v) Meath Agarose, in the latter buffer. The antigen well had a capacity of 5μl, and the antigen trough, 50μl. Electrophoresis was at up to 3W/plate for about 1.5h.

Plates were stained for β-glucuronidase activity by the method described for polyacrylamide gels, usually after 24h washing in phosphate-buffered saline [0.80% NaCl, 0.02% KCl, 0.02% KH_2PO_4 and 0.1% Na_2HPO_4 containing 1% (v/v) butanol]. To stain for protein, plates were washed for 48h in the phosphate-buffered saline, washed for 4h in water and then dried. They were immersed for 10min in 0.1% Coomassie Brilliant Blue R250 in the solvent described by Barrett (1973) and destained with the same solvent.

Purification of rabbit liver ferritin The method of Van Kreel *et al.* (1972) was modified. A homogenate (in 50mM-Tris–HCl buffer, pH7.8) was heated to 80°C and cooled, and the solution was clarified by centrifugation. No β-glucuronidase activity survived this heat treatment. The solution was concentrated by ultrafiltration and run on Sepharose 6B in the same buffer. A single ferritin peak was eluted in the position corresponding to the monomer observed in commercial horse spleen ferritin. Purity was checked by electrophoresis.

Results

Purification of β-glucuronidase

A preliminary survey of rabbit organs for β-glucuronidase activity showed that neither male nor female preputial glands were particularly rich sources of the enzyme, unlike those of female rats (Levvy *et al.*, 1958; Ohtsuka & Wakabayashi, 1970). The liver, kidney, spleen, stomach, small and large intestine, skin and serum from one animal were assessed, and liver seemed the most suitable source.

A quantitative summary of three preparations is given in Table 1.

Extraction and autolysis

The initial stages of the purification are those used by Barrett (1973) for cathepsins B1 and D, which exploit the unusual stability of lysosomal enzymes to autolysis (Barrett, 1972). Extraction of 2–3kg of tissue mince was in 2 parts (v/w) of a solution of NaCl (0.17M), butan-1-ol (20ml/l) and disodium EDTA (1mM) by use of a Silverson homogenizer. Arquad 2C-50, in the form of a 20% (v/v) emulsion obtained by warming in water, was added to a final concentration of 1% (v/v), and the mixture adjusted to pH6.5 with 2M-Tris–HCl buffer, pH9.0. After 30min the suspension was centrifuged in an MSE Mistral 6L centrifuge at 1500*g* for 30min at 15°C. The nearly clear supernatant was decanted and the pH adjusted to 4.5 for autolysis, by using 5M-sodium formate buffer, pH2.8. There was a small, consistent increase in total activity during the Arquad precipitation step, possibly owing to partial separation of inhibitory factors from the enzyme molecules.

The autolysis conditions were chosen on the basis of small-scale autolyses run at various pH values and temperatures, and assessed in terms of proteolysis and recovery of β-glucuronidase activity. The optimum conditions were found to be pH4.5 for 16h at 40°C, as used by Barrett (1973). Extensive studies on the use of autolysis in the purification of bovine

Table 1. *Purification of β-glucuronidase from rabbit liver*

The values given are the weighted means of three preparations each starting with 2–2.5kg of tissue, and are expressed per kg of tissue. As explained in the text, the final specific activity, when the Lowry *et al.* (1951) protein assay is used, is 22.3 units/mg. Relative specific activity is defined as specific activity of preparation/homogenate specific activity.

		β-Glucuronidase			
	Protein (g)	Total activity (units)	Specific activity (units/E_{280} unit)	Relative specific activity	Recovery (%)
Homogenate	260	884	0.0034	1	100
Arquad supernatant	80	560	0.0070	2	63
Autolysed extract	78.4	480	0.0061	1.8	54
Celite column $(NH_4)_2SO_4$ fraction	1.64	304	0.1854	54.5	34
DEAE-cellulose	0.272	243	0.8956	263.4	27
Hydroxyapatite	0.072	199	2.76	811.8	22
Sepharose 6B	0.046	141	3.05	897	16
Sedimentation	0.009	138	14.8	4353	15
Isoelectric focusing or	0.008	115	15.0	4412	13
DEAE-cellulose rechromatography	0.006	97	14.9	4382	11

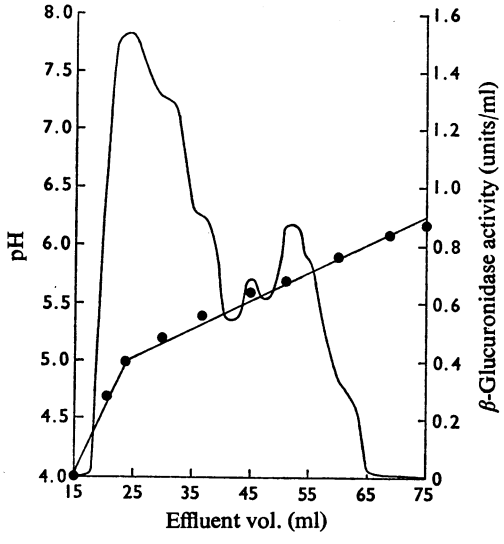


Fig. 1. *Preparative isoelectric focusing of β-glucuronidase*

The distribution of β-glucuronidase activity (—) and pH (●) after focusing in pH4–6 Ampholines is shown.

β-glucuronidase have been published by Plapp & Cole (1966): the rabbit enzyme was not stable to such prolonged autolyses as were used by these authors. The autolysis considerably improved the results of subsequent $(NH_4)_2SO_4$ fractionation.

$(NH_4)_2SO_4$ fractionation

The autolysate was titrated to pH7.8, with 2M-Tris–HCl buffer, pH9.0; then solid $(NH_4)_2SO_4$ was added to 55% saturation, which was required to precipitate all the enzyme at this stage. The mixture was left to stand at 4°C for 30min and the suspension was centrifuged at 1500*g* for 30min at 4°C in the MSE Mistral 6L. The pellets were resuspended in 200ml of 55%-satd. $(NH_4)_2SO_4$ in 50mM-Tris–HCl buffer, pH7.8; then 20g of Hyflo Super-Cel/kg of tissue used was added, and the suspension packed on top of a 40ml bed of Hyflo Super-Cel equilibrated with 55%-satd. $(NH_4)_2SO_4$ in the same buffer (column dimensions 7cm×25cm). The bed was washed with 1 bed vol. of 55%-satd. $(NH_4)_2SO_4$ in the equilibrating buffer and the column was developed with a descending linear gradient of $(NH_4)_2SO_4$ (0.05% satn./ml) from 55% to 10% saturation in the equilibrating buffer as described by King (1972). Fractions containing enzyme (emerging within the range of 40–25% saturation) were combined.

Chromatography on DEAE-cellulose

The product was dialysed against at least 20vol. of 20mM-Tris–HCl buffer, pH7.8, with two changes, and then run on to a column (7cm×25cm, 850cm³ bed vol.) of DEAE-cellulose. The sample was eluted with a linear gradient (2 litres) of 0–0.5M-NaCl in starting buffer. The flow rate was 10ml/h per cm², and the enzyme activity emerged in a single peak.

Hydroxyapatite chromatography

The product from DEAE-cellulose was titrated to pH6.5 with 5M-sodium formate buffer, pH2.8, and applied directly to a column (2.5cm×16cm, 70cm³ bed vol.) of hydroxyapatite, equilibrated in 5mM-K_2HPO_4–KH_2PO_4, pH6.5. In agreement with the theories of Bernardi *et al.* (1972), the enzyme is adsorbed in the presence of NaCl, even up to 3M. After 1 bed vol. of starting buffer, a linear gradient up to 0.5M-K_2HPO_4–KH_2PO_4, pH6.5, was used, at a flow rate of 10ml/h per cm². A single enzyme peak was obtained.

Gel filtration

The enzyme peak was concentrated to a volume of 2.5ml, and transferred to 20mM-Tris–HCl–100mM-NaCl, pH7.8, by ultrafiltration, and gel filtration on Sepharose 6B (column dimensions 1.5cm×90cm, 130cm³ bed volume) was performed in this buffer at a flow rate of 5ml/h per cm². The active fractions were combined and concentrated to 2ml by ultrafiltration.

At this stage the main contaminant was identified as ferritin, by electron microscopy (see Plate 1) and gel electrophoresis and from its behaviour in the chromatographic procedures, and the complete separation of this protein could not be achieved by ion-exchange chromatography or isoelectric focusing, as explained below. Separation of ferritin was achieved by sedimentation in sucrose gradients.

Sedimentation

Samples (0.5ml, 2.5mg) were sedimented as described for molecular-weight determinations by the method of Martin & Ames (1961).

During fractionation of the gradients great care was taken to avoid disturbing the brown layers containing ferritin at the bottom of the tubes. When necessary, the sedimentation was repeated to complete the removal of apoferritin.

After sedimentation, minor impurities were sometimes seen on overloaded electrophoresis gels. These could be removed with very small changes in specific activity by either preparative isoelectric focusing or re-chromatography on DEAE-cellulose.

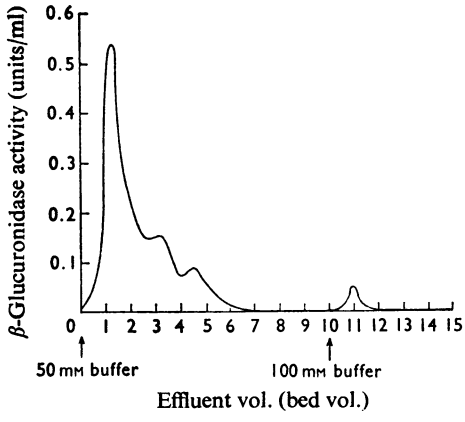


Fig. 2. *Rechromatography on DEAE-cellulose*

An enzyme sample from the sedimentation stage, in 50mM-K_2HPO_4–KH_2PO_4, pH6.5, was loaded on to a 5ml column of DEAE-cellulose equilibrated in that buffer, and eluted with the same buffer. After 10 bed vol., elution was continued with 100mM-K_2HPO_4–KH_2PO_4, pH6.5. The E_{280} exactly followed the activity over the first 10 bed vol.

Isoelectric focusing

A sample dialysed against 1% glycine (pH5.8) was introduced in the light component of the gradient. The column was run for 72h at 4°C, at up to 3W. The distribution of β-glucuronidase after focusing is shown in Fig. 1. The minor impurities removed during focusing had isoelectric points below pH4.5.

The focusing pattern of purified ferritin from the pooled livers was very similar to that reported by Van Kreel *et al.* (1972), and the low-pI form (4.46) was always seen, so that the span of isoelectric points was from 4.46 to 5.2. No dimers were seen in gel filtration. Thus ferritin could not be removed from the β-glucuronidase preparation by these procedures or by ion-exchange chromatography.

Re-chromatography on DEAE-cellulose

β-Glucuronidase samples from the sedimentation procedure could also be freed of impurities by re-chromatography on DEAE-cellulose. A sample dialysed against 50mM-K_2HPO_4–KH_2PO_4, pH6.5, was applied in a small volume to a column (1.0cm × 10cm, 5cm^3 bed volume) equilibrated with the same buffer. After the sample, 10 bed vol., of the buffer were passed and then 5 bed vol. of 100mM buffer at pH6.5. The flow rate was 20ml/h per cm^2. Most enzyme activity emerged in three peaks during the first 7 bed vol. and these peaks were free of the minor impurities which emerged together with small amounts of enzyme during the 100mM step (Fig. 2).

Other purification procedures

Acetone fractionation, used as described by Barrett (1970, 1973) for cathepsins B1 and D, gave rather variable results, often with a very low recovery of β-glucuronidase activity, so this was abandoned in favour of $(NH_4)_2SO_4$ fractionation. Gel filtration on Sephadex G-200 gave slightly poorer purification than on Sepharose 6B, as the enzyme was not clearly separated from the protein emerging at the void volume. An organomercurial–Sepharose used as described by Barrett (1973), but at pH7.8, gave a very low recovery of enzyme in the 10mM-cysteine eluate, suggesting that the enzyme has free thiol groups, but is unstable when bound to the adsorbent. Low recoveries were also obtained on the cation exchangers CM-cellulose and Bio-Rex-70.

The possibility of eluting the enzyme specifically from ion exchangers by means of inhibitors was investigated. Saccharo-1,5-lactone is thought to be responsible for the extremely potent inhibition observed with mixtures of saccharolactones (see review by Levvy & Snaith, 1972). The various lactones including saccharo-1,4-lactone, interconvert freely in solution, but the inhibitory lactone is very unstable, particularly at alkaline pH values. A very inhibitory mixture of lactones is obtained by boiling potassium saccharate at pH 3.5 for 30min. The 1,5-lactone probably adopts the half-chair conformation and thus mimics the transition state of the substrate during the enzyme reaction (Leaback, 1968).

Attempted elution of weakly bound enzyme from DEAE-cellulose by solutions of boiled potassium saccharate was unsuccessful, presumably because the 6-carboxyl group of the inhibitory lactone is still negatively charged when bound to the enzyme, as implied by the results of Wang & Touster (1972*a*, *b*), and the lactone does not induce a conformational change large enough to perturb enzyme binding. It was not practicable to attempt elution from CM-cellulose, on the assumption that the inhibitor increases the negative charge on the protein, because of the low recoveries obtained in CM-cellulose chromatography. Enzyme assays on the eluted pool containing inhibitor were performed after dialysis of the pool against 5M-urea in 50mM-Tris–HCl buffer, pH7.8, which allowed recovery of activity on subsequent dilution in control samples containing enzyme.

Harris *et al.* (1973) have described an affinity-chromatography procedure for β-glucuronidase which uses a lactone inhibitor. The carboxyl group at position 6 of saccharo-1,4-lactone was coupled to the free amino group of a long-arm Sepharose 4B derivative by means of a water-soluble carbodiimide. In spite of the possibility that amidation of the 6-carboxyl group might decrease specificity for β-glucuronidase (Wang & Touster, 1972*a*), which

normally requires a free 6-carboxyl group in its substrates, successful adsorption of the enzyme was claimed when a crude commercial bovine β-glucuronidase preparation was used. However, elution profiles showed that only a small purification was obtained. Samples were run on with very low salt concentrations, and activity was eluted simply by passing 0.1 M-acetic acid. This would be expected to favour the stability of free lactones in solution (Levvy & Snaith, 1972) and to leave bovine β-glucuronidase active (Plapp & Cole, 1966) and thus bound.

A lactone column made as described by Harris *et al.* (1973), but with boiled potassium saccharate, did not adsorb crude rabbit β-glucuronidase in 0.1% octyl sodium sulphate–50 mM-Tris–HCl, (pH 7.8)–0.5 M-NaCl, at which pH the enzyme retained some activity but the free inhibitory lactone would be unstable. Nor did the column adsorb enzyme at pH 5.0 in 0.1% octyl sodium sulphate–100 mM-sodium acetate–0.5 M-NaCl, under which conditions the enzyme was maximally active and the free lactone would be relatively stable. Moreover, the lactone–Sepharose did not inhibit the enzyme when added in various amounts to assay mixtures, thus suggesting that specificity for the enzyme had been lost, or that the long arm had bent in towards the gel matrix (O'Carra *et al.*, 1973), making the lactone inaccessible.

Purity of the enzyme preparation

The product gave a single, slow-moving protein band in polyacrylamide-gel electrophoresis even when grossly overloaded (750 μg of protein/gel). The band possessed enzymic activity. On gels run in the presence of sodium dodecyl sulphate a single band was also obtained. Ferritin molecules were rarely found by electron microscopy.

The immunological purity of the preparation was assessed with both the polyspecific guinea-pig antisera in diffusion and immunoelectrophoresis, and with a commercial antiserum to rabbit serum protein in immunoelectrophoresis. In Ouchterlony gel diffusion on both phosphate and barbitone plates, no contaminants were detected at a wide range of concentrations of antigen and antibodies, both of which were concentrated by ultrafiltration and used in serial dilutions. A single precipitin line staining for β-glucuronidase was usually obtained. In immunoelectrophoresis a single precipitin line containing β-glucuronidase, and moving towards the anode, was observed. On occasion, double or diffuse precipitin lines were seen in Ouchterlony gel diffusion, but they could both be stained for enzyme activity, and were thus identified as line-splitting artifacts, as observed by others (e.g. Weston, 1969).

Properties of the purified enzyme

pH optimum. This was determined in 100 mM-sodium acetate buffers in the range pH 3.5–6.0. A broad peak round pH 5.0 was found. Lower activity is retained up to pH 8.0.

K_m and activation. The K_m value at 37°C in assay buffer (from a double-reciprocal plot) was 0.6 mM for *p*-nitrophenyl β-glucuronide. The enzyme was activated by NaCl, as reported in detail by Wang & Touster (1972*a*,*b*) for the rat liver enzyme, and not inhibited as suggested by Cashman *et al.* (1969), who used rat skin extracts.

Molecular weight, subunits and aggregates

The elution positions of horse spleen ferritin monomer and the enzyme were indistinguishable on Sephadex G-200 and Sepharose 6B, so the best estimate of molecular weight from gel filtration was that of horse ferritin, 440000 (Crichton, 1972). However, values obtained from sedimentation in sucrose gradients, calculated from results by the formula given by Martin & Ames (1961), were near 300000. The standards used were ovalbumin, bovine serum albumin and β-galactosidase from *Escherichia coli*.

Similar discrepancies between gel-filtration and sedimentation results have been reported by Plapp & Cole (1966) for the bovine liver enzyme, and Preiss & Hilz (1971) for that from a mouse mastocytoma. A previous estimation of the molecular weight of rabbit brain β-glucuronidase in gel filtration on Sephadex G-200, by extrapolation from standards, gave a value of 266000 (Jungalwala & Robins, 1968). This supports the possibility that the rabbit liver enzyme may behave anomalously on gel filtration by virtue of a carbohydrate moiety (as discussed by Andrews, 1965). The enzyme from rabbit brain is immunologically identical with that in liver (Dean, 1974).

In gel electrophoresis in the presence of sodium dodecyl sulphate, subunits of approximately 75000 molecular weight were observed. Ovalbumin and bovine serum albumin were used as standards. In gel filtration on Sephadex G-200 in 8 M-urea, subunits were again observed. They were eluted slightly earlier than bovine serum albumin run in 8 M-urea. This indicates a subunit molecular weight in reasonable agreement with that from gel electrophoresis. No stabilization of dimers, as reported by Stahl & Touster (1971), was observed.

Electron microscopy (Plate 1) revealed aggregates of β-glucuronidase molecules. Since the gel filtration, gel electrophoresis and sedimentation experiments gave no indication of the presence of such aggregates in solution, they may have been formed on the electron-microscope grid itself. However, conditions allowing the formation of such aggregates may well exist in some parts of the cell.

Table 2. *Effect of various agents on β-glucuronidase activity*

The enzyme (0.01 unit) was assayed in duplicate under normal conditions except for the presence of the stated concentrations of additives.

Compound	Final concentration (mM)	Inhibition (%)
Potassium saccharate, boiled at pH 3.5 for 30 min (Levvy & Snaith, 1972)	0.001	12
	0.010	77
	0.100	100
$HgCl_2$	0.1	30
	1.0	75
$HgCl_2$ + EDTA	1.0+2.0	3
$HgCl_2$ + cysteine	1.0+1.0	70
	1.0+10.0	0
Sucrose	50	25
	250	28
Urea	5000	65
	8000	98

Specific extinction coefficient

The protein content of purified preparations was determined by the method of Lowry *et al.* (1951). The average E_{280}^{1cm} of solutions containing 1 mg/ml on this basis was 1.5. This may be compared with the value of 1.7 obtained by Plapp & Cole (1966) and with that deduced from the results of Ohishi & Shioya (1971*a*), of 1.5.

Inhibition and stability of the purified enzyme

Complete inhibition was obtained with a 100 μM solution of potassium saccharate boiled at pH 3.5 for 30 min (Levvy & Snaith, 1972). Hg^{2+} ions only inhibited up to 75% at 1 mM, and this inhibition was prevented by 1 mM-EDTA or 10 mM-cysteine. Organomercury compounds such as *p*-chloromercuribenzoate were less effective at similar concentrations. These properties are consistent with the results of Wang & Touster (1972*a*,*b*), Preiss & Hilz (1971) and those of earlier workers such as Fernley (1962). Sucrose at concentrations above 50 mM inhibited up to 25%, as reported by de Duve *et al.* (1955). Table 2 summarizes data on inhibition.

The enzyme was unstable to temperatures above 50°C, when heated in 20 mM-Tris–HCl buffer, pH 7.8, for 15 min, and this is discussed further in the following paper (Dean, 1974). It was completely inactivated by the heat treatment used in the purification of ferritin.

The enzyme was inactivated by even brief exposures to pH values above 10.5 or below 3.0. It was at least partially active in the presence of urea concentrations up to 6 M, and could be reactivated after exposure to 8 M-urea for up to 2 weeks, by simple dilution or by dialysis into buffer. However, if the enzyme was denatured by 6 M-guanidine hydrochloride, 3 M-sodium thiocyanate or 0.1% sodium dodecyl sulphate, activity was not recovered by extensive dialysis into buffer. Presumably careful renaturation via urea may be possible (Weber & Kuter, 1971).

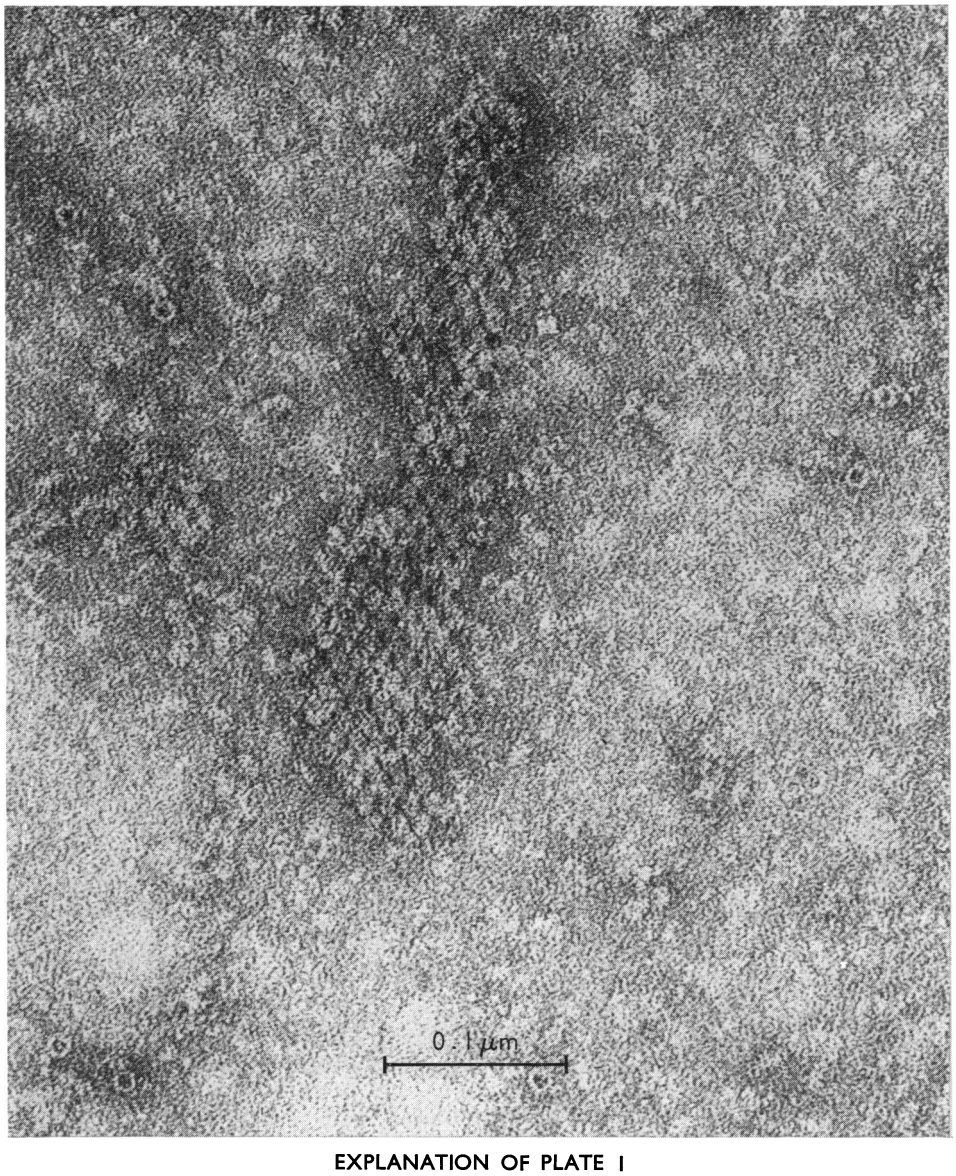
β-Glucuronidase was unstable to freezing, presumably because of subunit dissociation, but was satisfactorily stored in 50% glycerol at −20°C, at neutral pH values. It was much less stable in storage at pH 5.0 than at 7.0 (as reported by Stahl & Touster, 1971). Dilute solutions were 'activated' by bovine serum albumin, as is often found (see review by Wakabayashi, 1970).

Multiple forms

In analytical isoelectric focusing in pH 3–10 Ampholines, gels stained for enzyme consistently gave a large number (at least 12) of sharp lines in a continuous zone. Protein was in the same zone (Plate 2). Similar results on crude enzyme preparations from mouse brain and Ehrlich ascites-tumour cells have been presented by Coutelle (1971). In preparative isoelectric focusing of purified enzymes from rat liver (Stahl & Touster, 1971), rat preputial gland (Ohtsuka & Wakabayashi, 1970) and mouse mastocytoma (Preiss & Hilz, 1971) only single pI values of 5.8, 6.15 and 5.8 respectively were obtained, whereas in rabbit liver multiple peaks were seen, covering a range of pI from 4.5 to 5.5 (Fig. 1). With crude extracts of human liver, Hultberg & Ockerman (1972) obtained isoelectric points of 5.0 and 7.0, but they found considerable variation in extracts from other tissues.

Because of the possibility of artifacts in isoelectric focusing, the nature of the multiple forms was investigated in some detail. The obvious possibility that autolysis produces the multiple forms was excluded by running solubilized extracts from fresh non-autolysed livers. Some 85–95% of the enzyme activity in the homogenates was present in the final solution in 1% glycine. Samples from three separate livers gave similar patterns in gels, and one was run in a sucrose gradient with a result similar to that shown in Fig. 1.

Recoveries of enzyme activity from homogenized gels after focusing were good (80–90%). The pattern was not changed by introducing samples at the top of the gel, in sucrose protected by an Ampholine overlay (rather than polymerizing them in the gels), regardless of which electrode was at the top. It was not affected by changing the Ampholine concentration between 0.5% and 3%, or by running the gels for 1 h to set up the pH gradient (Wrigley, 1971) before applying the samples to the top of the gel. These results make it unlikely that specific Ampholine components were causing binding artifacts.



EXPLANATION OF PLATE 1

Electron microscopy of β-glucuronidase

An aggregate of β-glucuronidase molecules is present in the centre of the field. A few ferritin molecules, which are more circular in outline, are also visible.

EXPLANATION OF PLATE 2

Analytical isoelectric focusing of β-glucuronidase

Partially purified β-glucuronidase preparations were subjected to isoelectric focusing in pH 3–10 Ampholines. The cathode was at the top. The three left-hand gels were stained for β-glucuronidase activity, and the three right-hand gels were stained for protein. The samples in each group were (from left to right) the products from the initial DEAE-cellulose chromatography, the hydroxyapatite chromatography and the gel filtration respectively.

The pattern was unchanged by the inclusion of 1mM-dithiothreitol, which suggests that thiol exchange was not responsible. Enzyme samples denatured in 8M-urea and renatured before running gave unchanged patterns, and sections of the central zone rerun alone, at the enzyme concentrations used in the first focusing, only regenerated the original sections of the central zone. Thus conformational equilibria were probably not involved.

As mentioned in the purification procedure, multiple forms ranged in pI from 4.5 to 5.5 in preparative isoelectric focusing. Samples taken from successive points from such a column correlated well in migration position on 3–10 analytical gels, thus suggesting that the polyacrylamide matrix was not interacting with enzyme components.

Analytical focusing in pH4–6 gels gave rather unsatisfactory results. A continuum of enzyme staining covered nearly the whole of each gel, and bands were not clearly visible. A weak diffuse protein stain was also obtained. This applied even if samples were run for up to 48h at 250V, and the reasons for this were not clear. The pattern on pH3–10 gels after 48h at 250V was similar to that under routine conditions.

The strongest evidence that the multiple forms were genuine came from the observation that three peaks of enzymic activity were generated in the DEAE-cellulose chromatography at pH6.5 described above, and that samples taken from the end of the first peak and successively across the second and third showed a progressive fall in pI values when focused.

Gels focused in the presence of 8M-urea could be stained for activity after immersion in a large volume of buffer to lower the urea concentration, and the patterns obtained were like those of the native protein. Thus probably no particular combinations of subunits were required for activity. As discussed by Ui (1971), these results suggest that there are few buried groups of abnormal pK in the native molecule.

No more than a single band was obtained by routine gel electrophoresis. Presumably size effects are very important in electrophoresis of this protein. Even if gels were over-run so that the enzyme travelled three-quarters of the length of the gel, no band splitting was obtained, nor was it seen in gels containing only 4% polyacrylamide.

In gel electrophoresis at pH5.0, near the isoelectric points of the multiple forms, which might be expected to accentuate charge differences, a slow-moving band was obtained when electrophoresis was either from cathode to anode or vice versa, thus indicating a separation of oppositely charged forms. Clearly separable forms of β-glucuronidase have previously been demonstrated in electrophoresis at acid pH values by Lundin & Allison (1966) with human liver samples on starch gels, by Fondo & Bartalos (1969) with human serum on cellulose acetate at pH4.6, and by Dofuku *et al.* (1971) on starch gels at pH8.0 with mouse liver enzyme.

Because β-glucuronidase was unstable to pH values above 10.5 [even at −20°C in 15% (v/v) glycerol] it was not convenient to investigate the effects of amide groups on electrophoretic mobility (Funakoshi & Deutsch, 1969). But some of the forms of lowest pI were abolished or decreased after neuraminidase digestion, as shown by gel focusing, which suggested that at least some of the multiple forms were due to differences in sialic acid content. Some sialic acid residues may not be sensitive to neuraminidase, so sialic acid variation may well contribute to the remaining heterogeneity.

Evidence presented in the companion paper (Dean, 1974) shows that the multiple forms are immunologically identical and thus implies that the polypeptide chains are very similar. However, only a very small number of non-conservative amino acid substitutions would be required to generate the observed heterogeneity, and in a molecule as large as β-glucuronidase they might be relatively easily accommodated; indeed, the enzyme in a mouse mutant isolated by Dofuku *et al.* (1971) showed a clear electrophoretic difference from the normal enzyme.

Discussion

The purification of β-glucuronidase from bovine liver (Plapp & Cole, 1966), rat liver lysosomes (Stahl & Touster, 1971) and some other sources (Ohtsuka & Wakabayashi, 1970; Preiss & Hilz, 1971) has been described, and a partial purification of the enzyme from rabbit brain has been published (Jungalwala & Robins, 1968). The general characteristics of these enzymes are similar to those now reported.

In the present paper rigorous criteria of purity, including immunochemical criteria, have been applied, and multiple forms have been characterized in detail by the very sensitive technique of isoelectric focusing, which was not in use when Plapp & Cole (1967) performed their elegant chromatographic separations of multiple forms. The possibility of artifact generation in the focusing procedures has been largely discounted by control experiments.

Aggregates of β-glucuronidase molecules were demonstrated in the electron microscope, apparently for the first time. Subunits were demonstrated by a variety of techniques involving dissociation or denaturation, or both.

The molecular weight of the intact rabbit liver enzyme, as determined by gel filtration, is probably anomalously high owing to carbohydrate content, since the molecular weight from sedimentation was only 300000. The latter value agrees well with that of 266000 determined by Jungalwala & Robins (1968) for the rabbit brain enzyme. Since the brain

enzyme is immunologically identical with the liver enzyme (Dean, 1974), the best estimate of molecular weight of the rabbit liver enzyme seems to be 300000. This then leads to the conclusion that the rabbit enzyme is tetrameric, as is the rat enzyme (Stahl & Touster, 1971).

Towards the end of the present work, a paper on the small-scale purification of rabbit liver β-glucuronidase became available (Ohishi & Shioya, 1971*a*). Their rabbit strain gave β-glucuronidase which behaved slightly differently in some respects from that reported here. It was completely precipitated from crude extracts by 35%-satd. $(NH_4)_2SO_4$ whereas 55% was required in the present work. This probably simply reflects the different conditions under which the fractionations were performed. No contamination with ferritin is mentioned; nevertheless Sephadex-gel-filtration parameters, which cannot be precisely determined from the data given, seem similar to those given here, particularly in view of the variation of fractionation range observed with Sephadex (Andrews, 1965). A high activity per g of liver was obtained in the initial extract. A smaller purification factor was achieved, but the final product had higher specific activity than the present enzyme. A lower K_m value was observed, so possibly other kinetic parameters may vary, and produce the various differences. No critical tests of immunological purity were applied, but a specific antiserum against the enzyme was apparently obtained by injection of purified enzyme into a rat (Ohishi & Shioya, 1971*b*). No detailed studies of the multiple forms of the enzyme are reported. The reasons for the discrepancies are not clear, but strain variation may well be responsible. The presence of inactive enzyme in some purified preparations has been demonstrated (Dean, 1974), and this may also contribute to the observed differences.

The affinity-chromatography procedure described by Harris *et al.* (1973), with a lactone–Sepharose column, was unsuccessful when applied to the rabbit liver enzyme. The results suggested that the adsorption reported by these authors was either due to ion-exchange effects, or to hydrophobic interactions (Shaltiel & Er-El, 1973). The use of the substrate *p*-aminophenyl β-D-glucuronide in affinity chromatography, as suggested by Harris *et al.* (1973), seems more promising, but has the disadvantage that hydrolysis of the substrate occurs progressively.

The production of a specific antiserum to rabbit β-glucuronidase is described in the following paper (Dean, 1974). Reasonably efficient affinity chromatography on (anti-β-glucuronidase) immunoglobulin G–Sepharose 4B is also described, as are applications of immunochemical techniques to the study of the possible relationships between microsomal and lysosomal β-glucuronidase mentioned in the introduction.

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