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The Turnover of Hamster Fibroblast Lysosomal β-D-Glucuronidase

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The half-life of hamster fibroblast β-D-glucuronidase (EC 3.2.1.31) was estimated to be 4–5 days by measuring the decay with time of the radioactivity in β-D-glucuronidase isolated from cells grown in the presence of ^{14}C-labelled amino acids. A new affinity-chromatographic procedure for the purification of β-D-glucuronidase is described.

Although lysosomes have been shown to be involved in the degradation of intracellular proteins (Segal *et al.*, 1974; Dean, 1975), little is known about the turnover of the lysosomal enzymes themselves. Total lysosomal protein is degraded with a half-life of between 1 and 4 days (Segal & Dunaway, 1975; Wang & Touster, 1975), whereas the half-lives of individual lysosomal enzymes appear to vary from 3 to 30 days (Goldstone & Koenig, 1974; Warburton & Wynn, 1976*a*). It has been suggested that lysosomal enzymes may be protected from degradation by interaction with their substrates (Van Hoof & Hers, 1972). Rapidly changing conditions within the lysosomes of highly endocytic cells may cause a more rapid denaturation and degradation of the acid hydrolases. Previous studies on the turnover of lysosomal enzymes have been carried out on highly endocytic tissues, such as liver (Wang & Touster, 1975). In the present paper we report studies on the turnover of lysosomal β-D-glucuronidase in a poorly endocytic transformed hamster cell line (Warburton & Wynn, 1976*c*).

Experimental

Cell culture and enzyme extraction

Chinese-hamster CH-23 fibroblasts were cultured and the labelling protocol was carried out as described previously (Warburton & Wynn, 1976*a*). Briefly, cells were grown in the presence of ^{14}C-labelled amino acids (algal protein hydrolysate; The Radiochemical Centre, Amersham, Bucks., U.K.). On reaching confluency, the cells were washed and further incubated in unlabelled medium. Cells were harvested at various times, washed with 0.25M-sucrose and homogenized in 2ml of 20mM-sodium citrate buffer, pH5.0. After addition of Triton X-100 to a final concentration of 0.1%, the homogenate was freed from cellular debris by centrifuging at 100000*g* for 30min.

Preparation of the affinity absorbent for β-D-glucuronidase

β-D-Glucuronidase was purified by a modification of the affinity-chromatographic procedure of Kanfer *et al.* (1973). Benzidine–Sepharose (25ml; prepared by the method of Cuatrecasas, 1970) was suspended in water (45ml) and the pH adjusted to 4.0 with dilute HCl. Glucaro-1,4-lactone [100mg; Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.] was added with stirring. To this mixture was added 1-cyclohexyl-3-(2-morpholinoethyl)carbodi-imide metho-*p*-toluenesulphonate (400mg; Aldrich Chemical Co., Wembley, Middx., U.K.) over 5min. After stirring overnight, the pH was adjusted to 5.0 with dilute HCl. The overnight stirring and pH adjustment were repeated twice. The gel was washed with water (2 litres), resuspended in 50mM-sodium acetate (35ml) and treated with acetic anhydride (2ml) to react with any unsubstituted benzidine. After stirring overnight the gel was washed with water (1 litre). The adsorbent contained 14μmol of glucaro-1,4-lactone/ml of Sepharose as measured by the uronic acid content after hydrolysis by dilute HCl.

Purification of β-D-glucuronidase

The cell extract was applied to a column (6cm × 0.5cm) of glucaro-1,4-lactone–Sepharose and the column was washed with 20mM-sodium citrate buffer, pH5.0, until the column eluate was free from protein. This process was repeated with 0.5M-sodium citrate buffer (pH5.0)/0.1% Triton X-100 as the washing buffer and finally again with 20mM-sodium citrate, pH5.0. β-D-Glucuronidase was eluted from the column with 0.2M-sodium borate, pH8.0, containing 0.1% Triton X-100 and 0.1% bovine serum albumin. Samples of the enzyme solution were assayed for β-D-glucuronidase activity, other glycosidases, protein and for radioactivity as described previously (Warburton & Wynn, 1976*a*,*b*). The rate

constants for the degradation of β-D-glucuronidase were calculated by assuming first-order kinetics.

Results and Discussion

Purification of β-D-glucuronidase

A method which promises to find universal use in the affinity chromatography of glycosidases has been developed by Kanfer *et al.* (1973), in which the corresponding lactone is coupled to Sepharose via a linkage arm. Originally developed for the purification of α- and β-D-galactosidase, this method has been applied to the purification of β-D-glucosidase (Kanfer *et al.*, 1974) and *N*-acetyl-β-D-hexosaminidase (Pokorny & Glaudemans, 1975). The glucono-1,4-lactone–Sepharose adsorbent gave a 550-fold purification of bovine spleen β-D-glucosidase and did not retard other glycosidases (Kanfer *et al.*, 1974). In all cases, the enzyme could be eluted with a pH8 buffer or 1% Triton X-100 at pH5. The ability of Triton X-100 to elute the enzymes may indicate that the binding of the enzyme to the ligand depends to some extent on hydrophobic forces, possibly due to the benzidine side chain. Glucaro-1,4-lactone is a potent inhibitor of hamster fibroblast β-D-glucuronidase, a 10μM solution causing a 100% inhibition of enzyme activity (Levvy, 1952).

The affinity adsorbent prepared by the coupling of glucaro-1,4-lactone appears to be specific for hamster fibroblast β-D-glucuronidase and does not retard other glycosidases such as α-mannosidase, β-galactosidase and *N*-acetylglucosaminidase in the presence of high-ionic-strength buffers (Fig. 1). β-D-Glucuronidase can be eluted from the adsorbent by alkaline buffers. In contrast with similar systems, the enzyme was not eluted by 1% Triton X-100 or by buffers of high ionic strength at low pH. By washing the column with 0.1% Triton X-100/0.5M-sodium citrate, pH5.0, most of the unwanted protein could be eluted. In a typical experiment, a cell homogenate containing 26mg of protein and 4.26μkat of β-D-glucuronidase was applied to a column of the adsorbent. The alkaline sodium borate buffer eluted 34μg of protein and 3.66μkat of β-D-glucuronidase, a yield of 86% and a purification of 650-fold. The purity of the enzyme was checked by chromatographing ^{14}C-labelled β-D-glucuronidase (purified from cells grown in the presence of ^{14}C-labelled amino acids) on DEAE-cellulose, when over 80% of the radioactivity was eluted with the β-D-glucuronidase.

Turnover of β-D-glucuronidase

The half-life of hamster fibroblast β-D-glucuronidase was determined by growing the cells in the presence of ^{14}C-labelled amino acids, followed by a 'chase' period in unlabelled medium. The enzyme was purified from the cells at various time-intervals. The rationale behind this approach, its advantages and

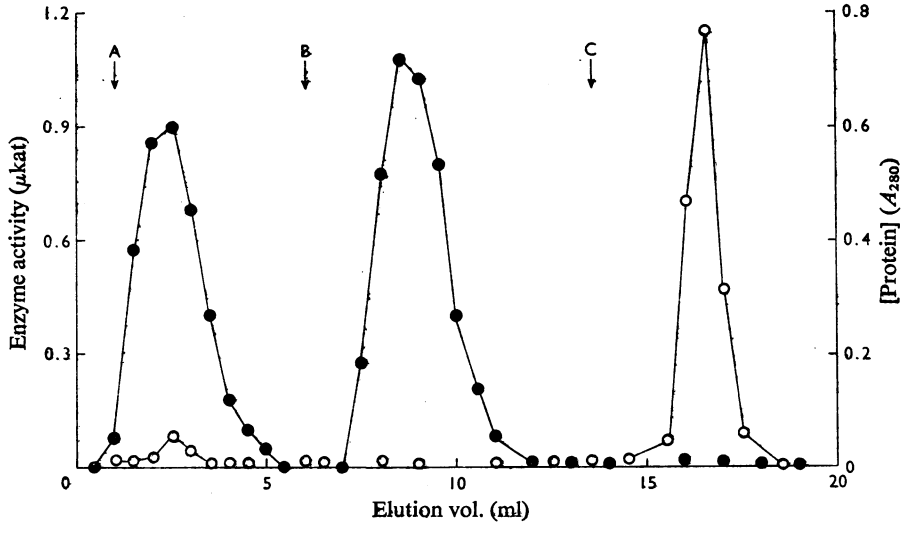

Fig. 1. *Affinity chromatography of β-D-glucuronidase*

A cell homogenate was applied to a column (6cm×0.5cm) of glucaro-1,4-lactone–benzidine–Sepharose. The column was washed with 20mM-sodium citrate buffer, pH5.0 (A), until the eluate was free from protein. This process was repeated with 0.5M-sodium citrate buffer (pH5.0)/0.1% Triton X-100 (B). After further washing with 20mM-sodium citrate buffer, pH5.0, β-D-glucuronidase was eluted with 0.2M-sodium borate buffer, pH8.0, containing 0.1% Triton X-100 and 0.1% bovine serum albumin (C). For further details, see the text. ●, Protein; ○, β-D-glucuronidase.

Table 1. *Rate of degradation of β-D-glucuronidase*
Hamster fibroblasts were grown to confluency in the presence of 5 μCi of ^{14}C-labelled amino acids. The medium was then replaced with unlabelled medium and cells were harvested at the indicated times. β-D-Glucuronidase was isolated from the cells as described in the text, and its radioactivity determined. Results are means ± S.D. for the numbers of experiments indicated in parentheses.

Time (days)	Radioactivity (c.p.m./μkat of enzyme)	Decay constant, k ($days^{-1}$)	Half-life (days)
0	7374 ± 915 (4)	—	—
3	4525 ± 588 (4)	0.163 ± 0.041	4.3
5	3385 ± 317 (3)	0.144 ± 0.006	4.8
8	2457 ± 204 (3)	0.137 ± 0.010	5.0

disadvantages, have been discussed previously (Warburton & Wynn, 1976*a*). The apparent half-life of β-D-glucuronidase, detrmined by this method, is 4–5 days (Table 1). This is similar to the value of 4–6 days obtained for the half-life of mouse liver microsomal and lysosomal β-D-glucuronidase (Smith & Ganschow, 1975). By contrast, rat liver β-D-glucuronidase was found to turn over with a half-life of 30 days (Wang & Touster, 1975), although the labelling procedure used by give a somewhat high value. Hamster fibroblast β-D-glucuronidase is predominantly a lysosomal enzyme (Warburton & Wynn, 1976*b*).

We previously showed that hamster fibroblast arylsulphatase B has a half-life of about 30 days (Warburton & Wynn, 1976*a*). The present results therefore confirm that lysosomal enzymes turn over at heterogeneous rates (Wang & Touster, 1975; Goldstone & Koenig, 1974). Factors which may give rise to the multiplicity of turnover rates include the heterogeneous subcellular distribution of acid hydrolases (Warburton & Wynn, 1976*b*), their size, isoelectric point and susceptibility to proteolytic enzymes (Goldberg & Dice, 1974). The finding that β-D-glucuronidase has a similar rate of degradation in both poorly endocytic fibroblasts and highly endocytic liver suggests that the endocytic activity of a cell is not necessarily a factor that influences the turnover of lysosomal enzymes.

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