The Locations of Cathepsin Activity and β -Glucuronidase in the Guerin T8 Tumour

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Tumour homogenate fractions, isolated by differential centrifugation, were sub-fractionated by density-gradient centrifugation. Biochemical and electron microscopic analyses revealed that β -glucuronidase and cathepsin activity were associated with a class (possibly two) of lysosomal particles of density greater than those of mitochondria and the endoplasmic reticulum. Lysosomes sedimented by low *g* forces were vacuolar, electron-dense, delineated by a unit membrane and about 0.2 μ m in diameter. β -Glucuronidase was also apparently associated with ribosomes whereas cathepsin was bound in part to the endoplasmic reticulum. Catalase and glucose 6-phosphatase possessed slightly different density-gradient sedimentation profiles.

The locations of acid hydrolases in solid malignant tumours have been little studied in detail. Although much work of this kind has been done with normal tissues, it cannot be assumed that these enzymes occupy the same locations in solid malignant tumours. A detailed examination of their locations in a transplantable rat hepatoma has been reported (Wattiaux & Wattiaux-de Coninck, 1968*a,b*).

The work presented here describes the locations of β -glucuronidase and cathepsin activity (resembling cathepsin D) in a transplantable rat tumour, the Guerin T8 epithelioma. Density-gradient centrifugation was used to sub-fractionate particulate cytoplasmic fractions isolated by differential centrifugation. Biochemical and electron microscopic examinations of isolated gradient fractions were made. Cytochrome oxidase (EC 1.9.3.1), glucose 6-phosphatase (EC 3.1.3.9) and catalase (EC 1.11.1.6) were used as 'tentative' biochemical 'markers' for mitochondria (Baudhuin, Beaufay & de Duve, 1965), endoplasmic reticulum (Tice & Barnett, 1962; Rosen, Kelly & Peters, 1966) and peroxisomes (Baudhuin *et al.* 1965) respectively.

The purified lysosomal preparation isolated here has been used in studies of the ability of the lysosomal enzymes of the tumour to degrade intercellular substance *in vitro* (A. R. Poole, unpublished work). These studies form part of an investigation into the role of these enzymes in invasive tumour growth (Poole, 1970).

EXPERIMENTAL

Tumour and animals. The transplantable Guerin T8 epithelioma was kindly supplied by Dr L. Németh, Onkopathologiae, Kutato Intézet, Budapest, Hungary. It was transplanted on to the spleen (Williams, 1965) of female Sprague–Dawley rats (150–200 g body wt.) supplied by Animal Suppliers Ltd., Welwyn, Herts., U.K. Rats were given drinking water and diet PRM [Dixon and Sons (Ware) Ltd., Elstree, Herts., U.K.] *ad libitum*. Tumours grew into the surrounding tissue and into the peritoneal cavity and were used 3–4 weeks after transplantation when they had reached a weight of 50–70 g. Animals were killed by cervical fracture. Peripheral non-invading and visibly non-necrotic tissue was recovered to a depth of 1–2 mm, after having first removed any loosely applied connective tissue.

Tissue homogenization. Freshly isolated tissue was weighed wet (6–8 g) and moistened with ice-cold 0.25 M-sucrose–1 mM-EDTA (disodium salt) adjusted to pH 7.2 with 1 M-NaOH (medium A). All further operations were then conducted at 2–4°C. Tissue was chopped in two directions at right angles to each other on a McIlwain–Mickle Tissue Chopper (H. Mickle & Co. Ltd., Gomshall, Surrey, U.K.) set at a section thickness of 0.42 mm. The chopped tissue was transferred to the barrel of an Emanuel–Chaikoff homogenizer (Emanuel & Chaikoff, 1957) supplied by Microchemical Specialties Ltd., Berkeley, Calif., U.S.A. The instrument was pre-cooled to about 4°C. Chopped tissue was further moistened with medium A before being passed through the annular orifice (64 μ m wide) by using a hydraulic press, (type 341/3, Apex Construction Ltd., Dartford, Kent, U.K.). The homogenized tissue was collected in about 5 ml of medium A. It was gently manually resuspended in a Potter–Elvehjem homogenizer (Tri-R Instruments Ltd., Jamaica,

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Scheme 1. Differential centrifugation of homogenate: schematic representation. Rotors and tubes used in conjunction with a MSE Superspeed 40 centrifuge were: spins 1 and 2, swing-out rotor, cat. no. 2416, 23ml tubes, cat. no. 59631; spin 3, angle rotor, cat. no. 2409, 9ml tubes, cat. no. 59629. Approximate volumes are indicated in brackets. For further details see the text.

N.Y., U.S.A.), with a clearance of 0.23–0.43mm (difference between pestle diameter and tube inside diameter), with three slow up-and-down cycles. The volume was adjusted to 30ml.

The homogenate was adjusted to pH7.2 with 5M-KOH, from an initial pH of 6.3–6.5. It was filtered through nylon cloth (no. 8 grade, J. Staniar & Co., Manchester, Lancs., U.K.) to remove unbroken cells and any fibrous material. The volume of the filtrate was finally adjusted to 40ml with medium A.

Fractionation of homogenate. (a) Differential centrifugation. Homogenates were initially fractionated by using a method based on that employed to fractionate rat liver homogenates (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955). The freshly isolated homogenate (40ml) was transferred to a 50ml glass centrifuge tube with a conical base and centrifuged in a swing-out position at 750 g_{av} for 10min. The supernatant was carefully removed, with a pipette to just above the pellet and stored. The pellet was manually resuspended with the Potter-Elvehjem homogenizer and the volume was adjusted to 10ml. This was centrifuged as before, the

supernatant was recovered and the washing repeated once more. The resultant pellet was discarded. The original supernatant and washings were combined and further fractionated as shown in Scheme 1. Pellet fractions M and P were each resuspended in 5ml of medium B with the Potter-Elvehjem homogenizer with the pestle rotating at a selected speed of 3500 rev./min., throughout five up-and-down cycles, each lasting about 2s. Medium B contained 0.5M-sucrose, 1mM-EDTA (disodium salt) and was freshly adjusted before use to pH7.2.

(b) Density-gradient centrifugation. The method is based on that used for the separation of rat liver lysosomes from mitochondria and peroxisomes (Beaufay *et al.* 1964). Density gradients were prepared from media B and C. The latter contained 15.3% (w/v) glycogen [Shellfish, type II, Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.], 0.5M-sucrose and 1mM-EDTA (disodium salt). Medium C was prepared by heating glycogen in water in a boiling-water bath for 1h, and cooling to room temperature, before the addition of sucrose and EDTA. The solution was adjusted to pH7.2 with 1M-NaOH, immediately before use.

Nine mixtures of these two solutions were freshly prepared at room temperature: the proportion of medium C increased in steps of 10% (v/v) from 10 to 90%. Individual solutions were well mixed. Gradients were poured by using tubes of equal weight, starting with the mixture containing 90% (v/v) medium C. Portions (1ml) of each of the mixtures (in consecutive order) were carefully layered over each other in 10ml polypropylene tubes (cat. no. 2415, MSE Ltd., Crawley, Sussex, U.K.). Each layer was created by the slow delivery of each mixture on to the wall of the centrifuge tube, just above the meniscus of the liquid already present. Gradients were finally gently stirred by describing, 60 times, a circular path about a vertical axis with a vertically held Pasteur pipette, the tip of which had been sealed. They were then stored in an ice-bath for 1–2h before use.

Of the freshly isolated fraction (M or P) 1ml was gently layered over the top of each gradient. Blank gradients were prepared in exactly the same way, substituting 1ml of medium B per gradient for the homogenate fraction. Tubes were immediately centrifuged in triplicate at $46000g_{av}$ in an MSE Superspeed 40 centrifuge for 2.5h at 2–4°C, in a swing-out rotor (cat. no. 2416) with buckets fitted with adaptors to take the 10ml tubes (cat. no. 59652). After centrifugation the tubes were maintained in an upright position at room temperature, and approx. 1ml volumes were removed starting at the top of the gradient. A calibrated Pasteur pipette, the end of which was upturned, was used. Removal of the subfraction immediately above the pellet was facilitated by the use of a 0.2ml micropipette. The pellet was manually resuspended with a Potter–Elvehjem homogenizer in 3–4ml of medium B. Gradient subfraction volumes were recorded and fractions were stored at –20°C.

Determination of densities of gradient subfractions. The method described by Beaufay *et al.* (1964) was used. This employs a gradient of density formed from *o*-dichlorobenzene and petroleum spirit, boiling range 90%, 80–100°C (both supplied by B.D.H. Ltd., Poole, Dorset, U.K.). Gradients were prepared by using similar techniques to those already described. Gradient subfraction droplets of volume 0.03ml were used and densities were measured at 20°C immediately after calibrating the gradient with aqueous solutions of KBr of known density.

Electron microscopy of gradient sub-fractions. Density-gradient sub-fractions were prepared in triplicate and pooled. To each was added a portion (approx. 3ml) of ice-cold fixative. This was freshly prepared and contained 28mm-sodium barbitone, 28mm-sodium acetate (tri-hydrate) 20mm-HCl, 1% (w/v) osmium tetroxide and 0.5M-sucrose; its pH was 7.3. Mixtures were incubated at 2–4°C for 15min after which they were added to an equal portion of water. Particles were sedimented at $100000g_{av}$ for 60min. The resultant black-brown pellets were transferred to 0.5ml polypropylene tubes (cat. no. A812, Hawksley and Sons Ltd., Lancing, Sussex, U.K.) in which they were dehydrated, and infiltrated with epoxypropane, by using the Microfuge and Micromixer (Beckman Instruments Ltd., Glenrothes, U.K.) for the sedimentation and resuspension, respectively, of particulate material. Pellets were finally embedded in epoxy resin (Fluka A.G. Chemische Fabrik, Buchs S.G., Switzerland). Ultrathin sections were cut at different levels throughout the pellets and stained with uranyl acetate (Watson, 1958) and lead

citrate (Reynolds, 1963). They were examined either in a Siemens Elmiskop I or a Jem-7A electron microscope at accelerating voltages of 60 and 80kV, respectively.

Biochemical assays of gradient subfractions. Protein was determined by the method described by Lowry, Rosebrough, Farr & Randall (1951) with human serum albumin (Calbiochem Ltd., London W.1, U.K.) as a standard. RNA was measured spectrophotometrically (Korner, 1959). β -Glucuronidase (EC 3.2.1.31) was assayed at pH4.5 with phenolphthalein glucuronide (Sigma) as the substrate at a concentration of 0.1mM in the incubation mixture (based on that described by Talalay, Fishman & Huggins, 1946). Density-gradient samples were diluted with water to give 1/20 of the original concentration in the final assay volume. This was done to minimize the inhibitory effect on this enzyme of the glycogen used for gradient preparation. Cathepsin activity was assayed at its optimum pH of 3.7 with haemoglobin [type II bovine, Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.] denatured with acetic acid. The assay is that described by Vaes & Jacques (1965). A 2% (w/v) solution of haemoglobin in 0.1M-acetic acid (final pH3.7) was present in the incubation mixture: a protein determination was made on the trichloroacetic acid-soluble components present at the end of the incubation. This recorded activity was insensitive to 10mM-iodoacetamide and resembles that of cathepsin D (Barrett, 1967). Cathepsin and β -glucuronidase were both assayed over incubation periods of 16h: the initial enzyme concentrations were adjusted so as to ensure a quantitative recording of enzyme activity. To prevent microbial contamination, thiomersal (B.D.H.) was included in the incubation mixture at a concentration of 0.01% (Beaufay *et al.* 1964). Glucose 6-phosphatase was assayed at pH6.5 by the method described by Appelmans, Wattiaux & de Duve (1955); glucose 6-phosphate, disodium salt (hydrate), was obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. P_i was measured as described by Chen, Torribara & Warner (1956). Catalase activity was determined spectrophotometrically at pH7.0 in a final incubation volume of 0.5ml, by the method of Beers & Sizer (1952); the substrate H_2O_2 , 20vol. stock solution, was obtained from B.D.H. Cytochrome oxidase was assayed spectrophotometrically as described by Cooperstein & Lazarow (1951) in a final incubation volume of 0.5ml. Cytochrome C, 90–100%, (B.D.H.) was reduced with sodium dithionite. These spectrophotometric assays were carried out at room temperature (approx. 21°C), and changes in the extinction of the incubation mixture were measured automatically with a Uvispek H.700.308 spectrophotometer (Hilger and Watts Ltd., London N.W.1, U.K.) fitted with a Gilford automatic cuvette positioner.

Cytochrome oxidase was assayed within 24h of isolation because of its lability. Other analyses on pre-frozen samples were completed within 5 days of isolation. All assays were carefully monitored so as to ensure that a quantitative recording of factor concentration was made. Unless otherwise stated, Triton X-100 (0.1%) was included in the incubation mixtures for assays of catalase, cathepsin and β -glucuronidase, to permit the measurement of total activity (Wattiaux & de Duve, 1956). At this concentration, it had no inhibitory effects on the enzyme activities.

RESULTS

Fractions M and P were examined by phase-contrast microscopy before subfractionation and found to be free of nuclei. Both test and blank gradients yielded pellets. The final density gradient was of a concave nature with a range from 1.067 to 1.121. Very little variation in individual sub-fraction densities was encountered in repeated experiments.

Blank and test gradient subfractions of fractions M and P were assayed for RNA (fraction P only since negligible amounts were present in fraction M), protein, cathepsin, β -glucuronidase, catalase, glucose 6-phosphatase and cytochrome oxidase. However, these factors were not detected in blank gradients under the conditions of assay employed for test gradients. The results of test gradient sub-fraction assays are represented in terms of the relative concentration of the material assayed (Beaufay *et al.* 1964). This represents the ratio of the observed concentration to that which would be found if the substance were evenly distributed throughout all the gradient subfractions. Means of duplicate experiments are indicated. Percentage recovery values for gradients of fractions M and P did not differ much from 100%, with the exception of β -glucuronidase (70–80% recovery).

Distributions of protein, RNA and enzymes in gradients of fractions M and P. (a) Fraction M. These results are shown in Fig. 1. Both β -glucuronidase and cathepsin were found to be most concentrated at the bottom of the gradient (density 1.121) and in the pellet, outside the limits of the gradient. The remainder of the β -glucuronidase activity was fairly evenly distributed over the other gradient subfractions. Cathepsin, however, exhibited an additional and reproducible small peak in the relative concentration of activity in sub-fraction 3 (density 1.068). Also, cathepsin activity was more concentrated than that of β -glucuronidase in subfractions 8 and 9. Other enzymes were almost or completely absent from the bottom of the gradient. Cytochrome oxidase was mainly concentrated in the middle of the gradient. Glucose 6-phosphatase was mainly confined to the upper half of the gradient with the highest relative concentration being present in subfraction 3. Catalase had a similar distribution pattern to the glucose 6-phosphatase, but was always most concentrated in subfraction 4 (density 1.072). Protein was most concentrated at the top, middle and bottom of the gradient.

(b) Fraction P. These sedimentation profiles are shown in Fig. 2. Again, cathepsin and β -glucuronidase were located at the bottom of the gradient, although much of their activities was also present in the upper part of the gradient. Moreover,

Fig. 1. Density-gradient subfractionation of fraction M. (a) Glucose 6-phosphatase, (b) catalase, (c) protein, (d) β -glucuronidase, (e) cathepsin, (f) cytochrome oxidase.

the relative concentrations of their activities in the pellet were lower than those found in pellets of fraction M gradients. Relatively little protein and RNA and glucose 6-phosphatase, catalase and cytochrome oxidase activities were present at the bottom of the gradient.

β -glucuronidase and RNA were most concentrated at the top of the gradient in sub-fraction 2 (density 1.065). The majority of the RNA was present in the top 4 sub-fractions of the gradient

Fig. 2. Density-gradient subfractionation of fraction P. (a) Glucose 6-phosphatase, (b) catalase, (c) protein, (d) RNA, (e) β -glucuronidase, (f) cathepsin, (g) cytochrome oxidase.

cathepsin and glucose 6-phosphatase were most concentrated in sub-fraction 3 and the sedimentation profiles of these two components tended to parallel each other in the upper part of the gradient.

Catalase, like glucose 6-phosphatase, had a similar distribution pattern to that recorded in fraction M gradients, with activity again being most concentrated in sub-fraction 4 adjacent to sub-fraction 3 in which the glucose 6-phosphatase concentration was maximal. Cytochrome oxidase was, as before, mainly confined to the central part of the gradient. The sedimentation profile for protein resembled that for RNA with the greatest concentrations being confined to the top four sub-fractions, although their sedimentation profiles in this part of the gradient were significantly different.

The effect of freezing and thawing fraction M before subfractionation. Fraction M was rapidly frozen and thawed eight times in a solid CO_2 -acetone bath and at 37°C , respectively, to examine the nature of the particle-bound β -glucuronidase and cathepsin activities normally concentrated at the bottom of centrifuged fraction M gradients. The results are shown in Fig. 3. After the treatment and centrifugation the activities of these enzymes were most concentrated in the top three gradient subfractions, although β -glucuronidase sedimented less into the gradient than cathepsin. Relatively little activity was present at the bottom of the gradient compared with the controls. The relative concentrations of cathepsin in subfractions 10 and 11 were greater than those for β -glucuronidase.

Electron microscopy of density-gradient subfractions of unfrozen fraction M. Subfractions exhibiting the highest relative gradient concentrations for individual enzymes were examined. The corresponding subfractions in blank gradients were also studied. After fixation and centrifugation of subfractions, black-brown pellets were obtained from test gradient subfractions. With blank gradients it was not possible to recover such compacted pellets.

Subfractions 3 and 4 contained essentially smooth-surfaced vesicular material about $0.02\mu\text{m}$ in diameter. No ribosomes were detected, either free or attached to membranes. Subfractions 6 and 7 contained mainly mitochondria. These were about $0.5\mu\text{m}$ in diameter and contained a poorly stained inter-cristal matrix. Some small poorly stained vesicular material, of the type predominating in subfractions 3 and 4, was also observed.

Subfraction 10 contained essentially electron-dense vesicular material (of about $0.18\mu\text{m}$ in diam.) delimited by a single membrane. No organized internal structure was detected. No mitochondria were observed in this subfraction and poorly stained vesicular material of the type most often observed in subfractions 3 and 4 was absent.

and here the sedimentation profiles of β -glucuronidase and RNA were somewhat parallel. Both

Fig. 3. Density-gradient distributions of β -glucuronidase (a and b) and cathepsin (c and d) from fraction M, before (a and c) and after (b and d) freezing and thawing eight times.

DISCUSSION

In fraction M, β -glucuronidase and cathepsin are bound to the same type of high-density particle. This appears to be distinct from those to which cytochrome oxidase and glucose 6-phosphatase are bound. These acid hydrolase-rich vacuolar particles resemble in morphology and electron density isolated lysosomes of rat liver, which also sediment to the bottom of such gradients (Baudhuin *et al.* 1965), and kidney (Shibko, Pangborn & Tappel, 1965). These particles are also apparently ruptured by repeated freezing and thawing with the production of low-density fragments and probably soluble enzyme that concentrate at the top of the gradients. Freezing and thawing is known to cause lysosome rupture (Shibko *et al.* 1965).

Cytochrome oxidase activity is similarly bound to mitochondria whereas glucose 6-phosphatase activity is bound to vesicular membraneous material of the type noted as being one of the main con-

stituents of isolated microsomal fractions and a product of the effect of the homogenization procedure on the endoplasmic reticulum (Palade & Siekevitz, 1956; Reid, 1967). The absence of ribosomes from these membranes, compared with those *in situ* which are thickly coated with ribosomes (A. R. Poole, unpublished work) may be due to a removal of ribosomes by EDTA, as was observed with normal tissues (Palade & Siekevitz, 1956).

Fraction P analyses reveal that cathepsin and β -glucuronidase are also partly associated with a population of sub-cellular particles of relatively high density (about 1.121), probably of a lysosomal nature in view of the similarity of their sedimentation to fraction M lysosomes and the relative absence of marker enzymes for other organelles. However, since relatively little acid hydrolase activity was detected in the pellet (unlike in fraction M gradients) it would appear that this lysosomal population is of a slightly lower overall density. Since these high-density particles in fraction P are only sedimented by considerable centrifugal forces it is likely that they are smaller than the lysosomes of fraction M. In view of the effect of freezing and thawing on the gradient sedimentations of acid hydrolases of fraction M it would seem very unlikely that these particles are breakdown products of larger lysosomes. Golgi vesicles have been observed in these Guerin T8 tumour cells *in situ* (A. R. Poole, unpublished work) and have a diameter of 0.02–0.04 μ m. These appear to represent primary lysosomes (de Duve & Wattiaux, 1966) and it is possible that these high-density particles of fraction P are Golgi vesicles, whereas those from fraction M mainly represent secondary lysosomes.

The parallel between the sedimentation profiles of cathepsin and glucose 6-phosphatase in the upper part of the gradients of fraction P (and to a lesser extent in fraction M) suggests that cathepsin may be associated with the endoplasmic reticulum, either with the cisternae or with the delimiting membranes. For similar reasons, β -glucuronidase appears to be associated with RNA and protein in the upper regions of gradients of fraction P. Mammalian ribosomes are composed of similar amounts of protein and RNA (Palade & Siekevitz, 1956; Peterman & Hamilton, 1957) and it would seem that this association of β -glucuronidase is with ribosomes. This may be the result of a non-specific adsorption of soluble enzyme on to ribosomes although the enzyme may be present in such sites *in vivo*.

The presence of lysosomal enzymes in a microsomal location has been recognized in a variety of normal tissues (see de Duve & Wattiaux, 1966) and electron microscopic-histochemical examinations of reaction products of enzyme activity have revealed the presence of β -glucuronidase in the membranes

of the endoplasmic reticulum of liver parenchymal cells (Fishman, Goldman & de Lellis, 1967) although the photographic evidence was far from clear. However, as regards the microsomal location of cathepsins in other tissues, little or no information is available; but in liver there appears to be relatively little, if any, cathepsin activity (resembling cathepsin D, as in this tumour) in microsomal fractions isolated by differential centrifugation (de Duve *et al.* 1955).

The sedimentation profiles for glucose 6-phosphatase and catalase are clearly similar but not identical. The maximum relative concentrations of these enzymes were also observed in adjacent glycogen-sucrose-gradient subfractions by Wattiaux & Wattiaux-de Coninck (1968*b*) in a transplantable rat hepatoma. In both this hepatoma (Wattiaux & Wattiaux-de Coninck, 1968*a*) and the Guerin T8 tumour (A. R. Poole, unpublished work) this enzyme exhibits latency but repeated freezing and thawing does not detectably affect its binding to particulate material, yet in rat liver this treatment releases this latent enzyme from peroxisomes (Baudhuin, 1964). If peroxisomes are present in this tumour and the hepatoma they probably represent specialized regions of the endoplasmic reticulum and appear to be organized differently from those in normal liver.

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