Heterogeneous Distribution of Glucose 6-Phosphatase in Rat Liver Microsomal Fractions as Shown by Adaptation of a Cytochemical Technique

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(Received 5 December 1972)

1. A novel technique for the subfractionation of rat liver smooth and rough microsomal fractions according to their content of glucose 6-phosphatase is described. This technique, based on the Gomori lead histochemical procedure, involves incubation of smooth and rough microsomal fractions with low concentrations of $\text{Pb}(\text{NO}_3)_2$ and glucose 6-phosphate. Control experiments, in which enzyme was assayed in the presence of various amounts of $\text{Pb}(\text{NO}_3)_2$ or in which microsomal fractions were reisolated after incubation with low concentrations of $\text{Pb}(\text{NO}_3)_2$ and glucose 6-phosphate, showed that lead does not interfere with glucose 6-phosphatase activity. 2. Discontinuous sucrose-density-gradient centrifugation of microsomal fractions which had previously been incubated with various amounts of $\text{Pb}(\text{NO}_3)_2$ and glucose 6-phosphate showed that it is possible to subfractionate both smooth- and rough-microsomal fractions into several bands, owing to a differential modification of the density of the microsomal vesicles by the trapping of lead phosphate within them. 3. When the material in the bands obtained by density-gradient centrifugation of incubated microsomal fractions was assayed for glucose 6-phosphatase activity, it was found that the modification of the density of the microsomal fractions was directly related to their relative enrichment in glucose 6-phosphatase activity. Control experiments, in which microsomal fractions were incubated with $\text{Pb}(\text{NO}_3)_2$ and glucose 6-phosphate and then treated with EDTA, showed that the subfractionation was not due to aggregation of microsomal vesicles, lead and glucose 6-phosphate. Thus the resolution of microsomal preparations into subfractions with different glucose 6-phosphatase activities is interpreted as indicating heterogeneity of glucose 6-phosphatase distribution in the microsomal vesicles. 4. Electron micrographs of both smooth- and rough-microsomal subfractions show deposits of lead phosphate within the microsomal vesicles. The frequency and extent of these deposits correlate with the different amounts of glucose 6-phosphatase activity measured biochemically. 5. The nature of the heterogeneous distribution of glucose 6-phosphatase is discussed and the more general applicability of the technique for studying membrane fractions containing a heterogeneous distribution of phosphatases is indicated.

The endoplasmic reticulum of rat liver parenchymal cells fragments by vesicularization during homogenization (Palade & Siekevitz, 1956), and a proportion of it can be found in the microsomal fraction together with elements of the Golgi apparatus, plasma membrane and other organelles. Several reports have indicated that microsomal fractions are heterogeneous (Dallner & Ernster, 1968; Glaumann & Dallner, 1970; Dallman *et al.*, 1969; Wibo *et al.*, 1971; Svensson *et al.*, 1972), but in all cases the heterogeneity observed depends on subfractionation according to some physical property of the microsomal vesicles, e.g. size or density. From studies in this laboratory of hormone-induced developmental situations, proliferative changes in the endoplasmic reticulum have been shown to be very important in the response of hepatocytes to situations requiring altered or enhanced protein-synthetic activity (Tata, 1967, 1970), and an attempt to subfractionate the

endoplasmic reticulum according to a biochemical parameter was considered to be important.

Glucose 6-phosphatase (EC 3.1.3.9) has been used as a routine as an enzyme marker for hepatic microsomal preparations (de Duve *et al.*, 1955), and has been demonstrated biochemically (Hers *et al.*, 1951) to be present mainly in the microsomal fraction, and cytochemically (Tice & Barnett, 1962; Ericsson, 1966) to be present only in the endoplasmic reticulum and in the outer membrane of the nucleus. Since glucose 6-phosphatase is an inducible enzyme, which is characteristic of the hepatocyte endoplasmic reticulum, we considered it suited to our present studies especially as it is known that the change in the activity of the enzyme is not always synchronized with the change in the amount of endoplasmic reticulum (e.g. after phenobarbital treatment; Orrenius *et al.*, 1965). Further, the enzyme can be demonstrated by a cytochemical technique that

appeared to be suitable for adaptation to biochemical use. The Gomori cytochemical technique (Gomori, 1952) depends on the trapping of the product of enzyme reaction as an insoluble salt. Cobalt and lead have been used to demonstrate phosphatases, since their phosphate salts are highly insoluble. For light microscopy the trapped lead phosphate can be detected by conversion into the black sulphide, and for electron microscopy the lead salt is directly visible owing to its electron opacity. Electron-microscopic cytochemistry has shown that the phosphate released by glucose 6-phosphatase is trapped within the cisternae of the endoplasmic reticulum (Tice & Barnett, 1962; Ericsson, 1966). Leskes *et al.* (1971a,b) made use of the principle that the lead phosphate trapped during the incubation of a microsomal preparation *in vitro* with glucose 6-phosphate and lead would increase the density of the vesicles, thus causing them to sediment much faster during centrifugation. Vesicles devoid of the enzyme should contain no lead phosphate and would thus sediment as unincubated microsomal preparations. They used this principle to demonstrate biochemically the concerted appearance of glucose 6-phosphatase throughout the endoplasmic reticulum of the parenchymal cells of neonatal rat liver. Widnell (1972) used a similar procedure to show that the 5'-nucleotidase activity of rat liver microsomal preparations is at least partially due to the fragments of rough endoplasmic reticulum present in this fraction; here, in contrast to glucose 6-phosphatase, the lead phosphate is localized on the outside of the microsomal vesicles.

In the present paper we describe a biochemical adaptation of the Gomori cytochemical technique which permits a subfractionation of rat liver smooth- and rough-microsomal fractions according to the intrinsic heterogeneous distribution of glucose 6-phosphatase activity. During the development of this technique, Leskes *et al.* (1971b) published their, rather different, procedure for separating microsomal vesicles containing glucose 6-phosphatase from those devoid of such activity. Our method is also of general applicability and can be used, for example, to separate vesicles derived from the endoplasmic reticulum from plasma membrane, Golgi elements and other components of the microsomal fraction as well as separating vesicles containing different amounts of glucose 6-phosphatase activity.

Materials and Methods

Preparation of smooth and rough microsomes

In the present paper microsomes are defined as that fraction of a rat liver homogenate which, after removal of cell debris, nuclei and mitochondria, can be pelleted by centrifugation at 100000g. Male rats (120–140 g) of a Sprague–Dawley strain inbred at this Institute were used throughout. Animals were killed

between 09:00h and 10:00h by cervical dislocation and the livers removed and rinsed in two changes of homogenizing medium (unbuffered 0.35M-sucrose–2.5mM-magnesium acetate). All operations were carried out at 2°C. After the livers had been finely chopped with scissors, they were homogenized in 2.5 vol. of homogenizing medium by eleven up-and-down strokes in a glass–Teflon Potter–Elvehjem homogenizer (radial clearance 0.15–0.20mm when cold) rotating at 800rev./min. The homogenate was filtered through a single layer of nylon Bolting cloth and then centrifuged at 640g for 10min. Although the pellet contains some 40% of the endoplasmic reticulum (J. A. Lewis & J. R. Tata, unpublished work) it was discarded. The supernatant was centrifuged twice at 6000g for 10min and the final supernatant was decanted. After dilution with homogenizing medium to the equivalent of 0.25g of liver/ml, 20ml of suspension was layered over the following discontinuous sucrose density gradients in 35ml Beckman polyallomer tubes; from top to bottom 5.0ml of 1.3M-sucrose–TKM buffer [0.05M-Tris–acetate–0.025M-potassium acetate–0.01M-magnesium acetate (pH 7.6 at 20°C)], 5.0ml of 1.5M-sucrose–TKM buffer and 5.0ml of 2.0M-sucrose–TKM buffer. The gradients were centrifuged at 95000g in the SW 27 rotor of a Beckman L2–65B ultracentrifuge for 1.5h. The bands on top of the 1.3M-sucrose and 2.0M-sucrose layers were collected separately and designated smooth and rough microsomes respectively. The smooth and rough fractions obtained from six such tubes were both diluted to 60.0ml with 0.35M-sucrose–TKM buffer. Then 20.0ml of smooth-microsomal suspension was layered over a cushion of 15.0ml of 1.3M-sucrose–TKM buffer in three Beckman 35ml polyallomer centrifuge tubes. In a similar manner 20.0ml of rough-microsomal suspension was layered over a discontinuous sucrose gradient comprising, from top to bottom, 5.0ml of 1.3M-sucrose–TKM buffer, 5.0ml of 1.5M-sucrose–TKM buffer and 5.0ml of 2.0M-sucrose–TKM buffer in a further three Beckman 35ml polyallomer centrifuge tubes. The gradients were centrifuged as described above. The washed smooth microsomes were then collected as the band on top of the 1.3M-sucrose–TKM buffer layer and the washed rough microsomes were collected from the 2.0/1.5M-sucrose–TKM buffer interface. A portion (0.02ml) of each was assayed directly for protein. The suspensions were diluted with TKM buffer, pH 6.6 at 30°C, to a protein concentration of 1.7mg/ml.

Incubation with Pb(NO₃)₂ and subfractionation of smooth and rough microsomes

Incubations were carried out in 50ml conical flasks in a final volume of 16ml, containing (per ml):

50 μ mol of Tris-acetate buffer, pH 6.6 at 30°C, 25 μ mol of potassium acetate, 10 μ mol of magnesium acetate, between 0.25 and 0.40 mmol of sucrose, 30 μ mol of glucose 6-phosphate (disodium salt), 0.25 μ mol of $\text{Pb}(\text{NO}_3)_2$ (unless otherwise stated) and 0.4 mg of microsomal protein. Control flasks contained no glucose 6-phosphate and incubations were carried out in a final volume of 8.0 ml. The flasks were sealed and incubated for 20 min at 30°C with occasional swirling. The contents were then poured into MSE 20 ml ultracentrifuge tubes previously chilled in ice and the control suspensions were diluted with 8.0 ml of TKM buffer, pH 6.6 at 30°C. The tubes were centrifuged at 105 000g for 1 h in the 8 $\times$ 25 ml rotor of an MSE Superspeed 50 ultracentrifuge and the pellets resuspended in 2.0 ml of 0.35 M-sucrose-TKM buffer in a hand-operated glass homogenizer. Control incubations were diluted to 4.0 ml with 0.35 M-sucrose-TKM buffer. The suspensions (2.0 ml) were layered over discontinuous sucrose gradients in Beckman 16.0 ml cellulose nitrate tubes containing 2.0 ml of each of the following sucrose solutions, all in TKM buffer, pH 7.6 at 20°C (from top to bottom): smooth microsomes: 1.0 M; 1.3 M; 1.5 M; 1.7 M; 1.9 M; 2.1 M; 2.3 M; rough microsomes: 1.5 M; 1.7 M; 1.9 M; 2.0 M; 2.1 M; 2.2 M; 2.3 M. The tubes were centrifuged at 94 000g in the SW 27 rotor of a Beckman L2-65B ultracentrifuge for 3 h, after which the interface bands were aspirated from the top with a Pasteur pipette. The fractions were stored at -20°C before analysis for glucose 6-phosphatase activity (usually the next day), protein and RNA content. It should be noted that a single cycle of freezing and thawing destroys 30% of microsomal glucose 6-phosphatase activity, so that the specific activity of stored fractions is always lower than that of unfrozen material.

Chemical assays

Protein was assayed by the method of Lowry *et al.* (1951), after precipitation with trichloroacetic acid, by using α -chymotrypsin as standard; RNA by the method of Fleck & Munro (1962) and P_i by the method of Chen *et al.* (1958).

Enzyme assays

Glucose 6-phosphatase activity was assayed by measuring the release of P_i from glucose 6-phosphate in an incubation mixture of final volume 1.0 ml containing 20 μ mol of glucose 6-phosphate, 50 μ mol of sodium cacodylate buffer, pH 6.6 at 30°C, and 0.05–0.10 mg of microsomal protein (the amount of P_i released increases linearly with the amount of protein added within this range). Incubations were carried out for 20 min at 30°C and results are expressed as μ mol of P_i released/20 min per mg of protein.

5'-Nucleotidase was assayed in a similar fashion but by using 5 μ mol of 5'-AMP in 50 mM-Tris-HCl buffer, pH 7.4 at 37°C. Incubations were carried out at 37°C for 20 min.

Electron microscopy

Samples (approx. 0.5 ml) of the fractions were diluted to 6.0 ml with 0.05 M-sodium cacodylate buffer, pH 7.4, and the suspension made 3% with respect to glutaraldehyde. The tubes containing the suspension [MSE polypropylene ultracentrifuge tubes (8.0 ml)] were centrifuged for 1 h at 105 000g in the 8 $\times$ 10 ml rotor of an MSE Superspeed 50 ultracentrifuge. The pellets were rinsed with 0.25 M-sucrose–0.05 M-sodium cacodylate buffer, pH 7.4, and then post-fixed with 1% osmium tetroxide in the same buffer. Fixative was removed by aspiration and after several rinses with 0.03 M-veronal buffer, pH 6.0, the pellets were block stained with 0.5% uranyl acetate in the same buffer. After dehydration through graded alcohols and clearing with propylene oxide, the pellets were infiltrated with a mixture of equal volumes of propylene oxide and Araldite for 30 min. The pellets were then transferred to Araldite and left overnight. After trimming and orientation the blocks were embedded in an Araldite mixture (Glauert *et al.*, 1956). Ultrathin sections (grey to silver) were cut with an LKB Ultratome mark 1 by using glass knives, deposited on copper grids and doubly stained with aq. 1% uranyl acetate and alkaline lead citrate (Reynolds, 1963). In some cases lead citrate staining was omitted. Sections were examined in a Philips EM 300 electron microscope operating at 80 kV with an anti-contamination device fitted.

Materials

The following were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. and were of AnalaR grade wherever possible; sucrose, magnesium acetate, potassium acetate, $\text{Pb}(\text{NO}_3)_2$, EDTA, trichloroacetic acid and propylene oxide. Trizma and AMP were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A., and sodium cacodylate, glutaraldehyde, osmium tetroxide, uranyl acetate and lead citrate from Taab Laboratories Ltd., Reading, Berks., U.K. Glucose 6-phosphate (disodium salt) was supplied by Boehringer Corp. (London) Ltd., Ealing, London, U.K., and α -chymotrypsin (bovine; grade 1A) by Miles-Seravac, Maidenhead, Berks., U.K.

Results and Discussion

The exploitation of the Gomori cytochemical technique for biochemical use depends on the trapping of phosphate, released by enzymic activity, as lead phosphate, thus increasing the density of the vesicles

Fig. 1. Schematic representation of the biochemical adaptation of the Gomori cytochemical technique

PO_4^{3-} ions released from glucose 6-phosphate by the action of glucose 6-phosphatase are trapped within microsomal vesicles as insoluble lead phosphate. After centrifugation and resuspension, the vesicles are fractionated on discontinuous sucrose density gradients to give bands of material according to the amount of lead phosphate in the vesicles.

Table 1. Effect of incubation with and without lead and glucose 6-phosphate on specific activity of microsomal glucose 6-phosphatase

Microsomes were incubated for 20 min at 30°C in the presence of 0.16 mM- $\text{Pb}(\text{NO}_3)_2$ (lead/protein ratio 0.4 μmol of Pb/mg of protein), 30 mM-glucose 6-phosphate or both, and then reisolated by centrifugation. Glucose 6-phosphatase activity was then assayed; corrections for trapped phosphate were made for incubations with both Pb^{2+} and glucose 6-phosphate by measuring the P_i present in the sample and subtracting it from that present in the enzyme assay. Uncorrected values are given in parentheses. Specific activity is expressed as μmol of P_i released/20 min per mg of protein. In this experiment the specific activities were assayed on material that had not been previously frozen. Thus the values are somewhat higher than those for unfractionated smooth and rough microsomes given in Tables 2 and 3.

Microsomes	Treatment of microsomes ...	Specific activity of glucose 6-phosphatase			
		None	Incubated	Incubated + Pb^{2+}	Incubated + glucose 6-phosphate
Smooth		4.55	4.73	4.60	4.50
Rough		7.10	5.80	7.40	7.05
					Incubated + Pb^{2+} + glucose 6-phosphate
					5.05 (5.30)
					7.85 (8.20)

in question. Both Leskes *et al.* (1971b) and Widnell (1972), in their studies on microsomal glucose 6-phosphatase and 5'-nucleotidase respectively, took care to ensure that Pb^{2+} ions were present in the incubation medium at a higher concentration than the P_i released from the substrate. If a quantitative heterogeneity of enzyme distribution were to exist in a subcellular fraction, it could be demonstrated if Pb^{2+} ions were limiting in the incubation so that the more active vesicles would, in a given period of incubation, tend to form more lead phosphate than the less active ones. The products of the incubation could then be analysed by fractionation on a sucrose density gradient as shown in Fig. 1.

In such a study it is important to know the effect of lead itself on enzyme activity. Glucose 6-phosphatase assays in the presence of Pb^{2+} ions (0.0–2.0 mM) in the incubation medium, showed that no

inhibition occurred up to 2.0 mM- $\text{Pb}(\text{NO}_3)_2$ with 0.2 mg of microsomal protein in the incubation mixture. This upper limit represents a lead/protein ratio of 10.0 μmol of Pb/mg of protein, which is much higher than that used in the incubation before sub-fractionation, which is discussed below.

Rosenthal *et al.* (1966) showed that lead causes a non-enzymic hydrolysis of ATP. However, incubation of glucose 6-phosphate and $\text{Pb}(\text{NO}_3)_2$ under the incubation conditions used in the present work does not lead to release of phosphate. These results indicate that lead itself does not affect the glucose 6-phosphatase incubation, and that it may be possible to incubate microsomes in a lead-glucose 6-phosphate medium, reisolate the microsomes and measure their glucose 6-phosphatase activity. Table 1 shows the results of such an experiment. Microsomes were incubated for 20 min and the incubation mixture was

Fig. 2. Diagram depicting the subfractionation obtained following incubation at various lead/microsomal protein ratios

Smooth or rough microsomes were incubated, with $\text{Pb}(\text{NO}_3)_2$ and glucose 6-phosphate, centrifuged and subfractionated on discontinuous sucrose density gradients as described in the Materials and Methods section. Abbreviations are: Nil, microsomes not subjected to incubation, pelleting and resuspension; 0.6(-G6P), microsomes incubated with 0.25 mM- $\text{Pb}(\text{NO}_3)_2$ but no glucose 6-phosphate; 0.1, 0.6 and 1.0, microsomes incubated with 0.04 mM- $\text{Pb}(\text{NO}_3)_2$, 0.25 mM- $\text{Pb}(\text{NO}_3)_2$ and 0.40 mM- $\text{Pb}(\text{NO}_3)_2$ respectively. The positions of the sucrose interfaces are indicated at the far left.

then centrifuged and resuspended as described in the Materials and Methods section. The glucose 6-phosphatase activity of the resultant microsomes is in all cases essentially the same as that of unincubated microsomes. In particular, incubation with both lead and glucose 6-phosphate appears to only slightly affect the activity after correction for phosphate trapped within the microsomal vesicles during incubation. These results indicate that, after incubation with lead and subfractionation on sucrose gradients, it is possible to re-examine the glucose 6-phosphatase activities of the isolated fractions. Leskes *et al.* (1971b) were unable to do this, as their incubation medium contained a higher concentration of lead (2.0 mM), which causes aggregation of rough microsomes thus necessitating dialysis with consequent inactivation of the enzyme.

Preliminary experiments showed that it was necessary to use washed microsomes to avoid contamination by endogenous substrates. The preparation

procedure described above was developed to obtain, relatively quickly, rough and smooth microsomes free of endogenous substrate and homogeneous with regard to banding on the sucrose gradients used. The fractions do not represent all the endoplasmic reticulum of the liver, but only some 40–50% of that normally obtained by a single centrifugation, which is in turn only 30–40% of the total endoplasmic reticulum present in the liver (J. A. Lewis & J. R. Tata, unpublished work). Results reported here are those obtained only with fractions corresponding to the smooth and 'heavy-rough' microsomes described by Tata & Williams-Ashman (1967).

Fig. 2 depicts the effects of different concentrations of $\text{Pb}(\text{NO}_3)_2$ in the incubation medium. It was found that the ratio of lead/microsomal protein was critical for optimum resolution when microsomes incubated with lead and glucose 6-phosphate were subsequently subfractionated on sucrose gradients, and with the two gradient types used in this work a value of

0.6 μmol of Pb/mg of protein gave the best results. Resolution is very much limited by this ratio, as can be seen from tubes in which the lead/protein ratios are 0.1, 0.6, and 1.0 μmol of Pb/mg of protein respectively. Microsomes incubated in the presence of lead but not of glucose 6-phosphate sediment at the same position as unincubated microsomes. Widnell (1972), who used much higher concentrations of $\text{Pb}(\text{NO}_3)_2$ (1.0 mM), found that microsomes incubated with lead alone sedimented slightly further into his gradients than unincubated microsomes, presumably owing to lead binding to the microsomes.

A photograph of a typical band pattern, obtained at the optimum lead/protein ratio of 0.6 μmol of lead/mg of protein, is shown in Plate 1. The tubes were stored overnight in ice before photography so that some diffusion occurred, but as a routine six sharp bands can be obtained for both smooth and rough microsomes. Possibly such a result might be obtained if an aggregate of lead, glucose 6-phosphate and microsomes were to form. It would be expected that such a complex would be dissociated by EDTA, and hence a control experiment was carried out in which the incubated microsomes were resuspended in either 0.35 M-sucrose–50 mM-Tris–acetate buffer (pH 7.6) or 0.35 M-sucrose–50 mM-Tris–acetate (pH 7.6)–1.0 mM-EDTA. EDTA did not significantly alter the banding pattern, thus eliminating the possibility of the formation of artifactual aggregates of lead, glucose 6-phosphate and microsomes.

Plates 2–4 are electron micrographs representative of the fractions shown in Plate 1. The single band from the rough-microsome control tube is represented in Plate 2(a), and it can be seen that the microsomal

membranes are well preserved in spite of having been incubated with $\text{Pb}(\text{NO}_3)_2$ at 30°C for 20 min and subjected to extensive handling and centrifugation. The membranes are well covered with ribosomes, which is in agreement with the fairly high RNA/protein ratio of about 0.30 which is usual for this preparation. Occasional smooth vesicles, i.e. vesicles not visibly associated with ribosomes, are observed, as also, though less frequently, are mitochondria and elements of the Golgi apparatus. There is no evidence of lead binding to any of the vesicles or ribosomes, as shown by the absence of electron-dense particles.

Electron micrographs of the material present in band R-1 of Plate 1 are shown in Plates 2(b) and 2(c). Smooth vesicles are probably more frequent, possibly because many of them represent components that do not contain glucose 6-phosphatase, such as plasma membrane, lysosomes and Golgi elements. Some of the vesicles contain para-crystalline electron-dense deposits (shown by arrows) which we believe to be lead phosphate. That they are not artifacts of the staining procedures (alkaline lead citrate occasionally results in deposits of electron-dense lead carbonate if exposed to atmospheric CO_2) was checked by omitting lead citrate staining. In such cases (results not shown) the electron-dense deposits were still observed with the same frequency, suggesting that they were not lead carbonate. Further, the electron-dense deposits are virtually always found within vesicles and not in surrounding parts of the section, showing that they are specific to the microsomal material. At higher magnification (Plate 2c) the trilaminar nature of the membranes is clearly visible and within this structure grains of electron-dense

EXPLANATION OF PLATE 1

Banding pattern on subfractionation of smooth and rough microsomes after incubation with $\text{Pb}(\text{NO}_3)_2$ and glucose 6-phosphate

Smooth and rough microsomes were incubated with lead (–G6P) and with lead and glucose 6-phosphate (+G6P) for 20 min at 30°C, pelleted and then subfractionated on discontinuous sucrose density gradients as described in the Materials and Methods section. The lead/protein ratio was 0.6 μmol of Pb/mg of protein.

EXPLANATION OF PLATE 2

Electron micrographs of rough-microsomal subfractions R-1 and R-2 obtained after incubation with $\text{Pb}(\text{NO}_3)_2$ and glucose 6-phosphate

(a) Rough microsomes incubated with lead (lead/protein ratio 0.6 μmol of Pb/mg of protein), but without glucose 6-phosphate, and subfractionated as described in the text. Large numbers of ribosomes attached to membranous vesicles are visible. Magnification $\times 29\,200$. (b)–(d) Rough microsomes incubated with lead (lead/protein ratio 0.6 μmol of Pb/mg of protein) and glucose 6-phosphate and subfractionated as described in the text. (b) Fraction R-1 of Plate 1. Note Golgi apparatus (G) and plasma membrane (P1). Some vesicles contain para-crystalline electron-dense deposits of lead phosphate. Magnification $\times 28\,000$. (c) Fraction R-1 of Plate 1. Note black grains within trilaminar membrane (arrows). Magnification $\times 118\,000$. (d) Fraction R-2 of Plate 1. Note increased number of vesicles containing para-crystalline electron-dense deposits. Magnification $\times 37\,600$.

material are seen; these are sometimes quite extensive (shown by arrows).

The fractions recovered from band R-2 (Plate 1) are represented in Plates 2(d) and 3(a). In this fraction little other than rough vesicles is observed; no recognizable Golgi or plasma-membrane elements are found, although a few lysosome-like structures were observed. The number of para-crystalline electron-dense deposits is probably higher in this fraction than in band R-1.

The material of band R-5 (Plate 1) is represented in Plate 3(b), and again little other than rough vesicles is observed. Although the para-crystalline electron-lense deposits are again abundant, a different form of deposition is found (shown by arrows). This is more granular than the para-crystalline material and it is often difficult to ascertain whether it is within the vesicles. Close examination, however, suggests that these deposits are always within vesicles and that these vesicles are usually small. These deposits closely resemble those described by Leskeš *et al.* (1971b). It is possible that with the relatively low $\text{Pb}(\text{NO}_3)_2$ concentration used in our incubation medium, large centres of deposition of lead phosphate occur only where local concentrations of substrates are high, e.g. in the apices of vesicles of high glucose 6-phosphatase activity. Thus where the section cuts the highly curved part of such a vesicle these intense granular deposits might be seen. The appearance of material from band R-6 (Plates 3c and 3d) is similar to that of material

from band R-5 except that the granular deposits are more intense and more frequent. More smooth vesicles are found in this fraction, which appears to contradict the increasing RNA/protein ratio shown in Table 3 (but see below). The vesicles are often deformed in contrast to the rounded structures seen in the material from bands R-1–R-5 and this is presumed to be due to the high osmolality (2.3 M-sucrose) to which these vesicles have been subjected. At high magnification (Plate 3d) many of the granular deposits can be seen to be within the membrane (see encircling arrows).

Smooth-microsomal subfractions (Plate 4) show much the same features as those described for the rough-microsomal subfractions. Few ribosomes are observed in any of the fractions. Band S-1 contains few deposits, but those present are of the para-crystalline type. At higher magnification (Plate 4b) electron-dense grains are often seen in the membrane structure (cf. band R-1), and similar observations have been made in all the subfractions from both smooth and rough microsomes. In band S-4 the granular deposits (cf. band R-5) are again found, and the para-crystalline deposits are more intense than in band S-1. The inset of Plate 4(d) shows very clearly the grains of dense material within the trilaminar structure of the membrane. It is absent from parts of the membrane, scattered in others and intense in others (shown by arrows).

In summary, this series of electron micrographs

EXPLANATION OF PLATE 3

Electron micrographs of rough-microsomal subfractions R-2, R-5 and R-6 after incubation with $\text{Pb}(\text{NO}_3)_2$ and glucose 6-phosphate

(a) Fraction R-2 of Plate 1. As in Plate 2(d), many vesicles have para-crystalline electron-dense deposits. Nearly all structures are membranous vesicles with ribosomes attached. Magnification $\times 37600$. (b) Fraction R-5 of Plate 1. In addition to para-crystalline electron-dense deposits many vesicles contain granular dense deposits (arrows). Magnification $\times 50000$. (c) Fraction R-6 of Plate 1. Granular dense deposits are widely distributed. Many vesicles devoid of ribosomes can be seen. Magnification $\times 54000$. (d) Higher magnification of fraction R-6. The granular dense deposits are seen in detail and in some cases the surrounding membrane and ribosomes can be picked out (arrows around deposits). Magnification $\times 132000$.

EXPLANATION OF PLATE 4

Electron micrographs of smooth-microsomal subfractions S-1, S-4 and S-5 after incubation with $\text{Pb}(\text{NO}_3)_2$ and glucose 6-phosphate

(a) Fraction S-1 of Plate 1. Vesicles with contents of various electron density are seen, but no ribosome-studded vesicles can be found. A few vesicles contain electron-dense specks. Magnification $\times 27000$. (b) High magnification of fraction S-1. The trilaminar nature of the membrane is clear and in places black grains can be seen (arrows). Magnification $\times 65000$. (c) Fraction S-4 of Plate 1. Many vesicles containing dense specks and others with a more granular deposit of lead phosphate are seen. Magnification $\times 71000$. The inset shows a vesicle containing a para-crystalline electron-dense deposit. Magnification $\times 200000$. (d) Fraction S-5 of Plate 1. Vesicles appear much as in the preceding fraction (Plate 4c). Magnification $\times 80000$. The inset shows a vesicle with black grains scattered within its membrane. Magnification $\times 142000$.

suggests that, with the exception of bands R-1 and S-1, the vesicles contained in the various subfractions are predominantly derived from the endoplasmic reticulum, which is particularly clearly seen in the rough microsomal subfractions. The increasing amounts of lead phosphate deposit in vesicles that sediment further into the gradient is in agreement with the principle that subfractionation occurs according to glucose 6-phosphatase activity and corroborates the results obtained by subsequently assaying the subfractions for glucose 6-phosphatase activity. It should, however, be borne in mind that the absence of visible deposits in many vesicles does not mean that these vesicles are devoid of either deposit or glucose 6-phosphatase activity, since deposits may have been missed in the particular plane in which the vesicles are sectioned. Leskes *et al.* (1971b), using a much higher $\text{Pb}(\text{NO}_3)_2$ concentration in the incubation medium, also found vesicles that contained no deposit.

The specific activities of glucose 6-phosphatase in the bands obtained by the technique described in this paper are given in Tables 2 and 3. For smooth microsomes (Table 2), where the mean of four separate experiments is given, the specific activity clearly increases with the distance that the membrane vesicles have sedimented, thus showing that the resolution of smooth microsomes into several bands is indeed a consequence of the unequal distribution of glucose 6-phosphatase in the microsomal vesicles. It is also noteworthy that the heterogeneity, with respect to this enzyme at least, is maintained after exposure to $\text{Pb}(\text{NO}_3)_2$ and subsequent centrifugation

through the sucrose gradient. This lack of harmful effects caused by the low concentrations of $\text{Pb}(\text{NO}_3)_2$ used in our technique may extend to other properties of microsomes allowing this technique to be used in a more general sense, such as the assay of rough-microsomal subfractions for cell-free amino acid-incorporating activity.

Results of the assay of rough-microsomal subfractions were variable, and the specific activities of glucose 6-phosphatase did not always follow a simple linear increase with sedimentation. Results from two typical experiments are shown in Table 3 and specific activities based on both protein and RNA are given. In some experiments the pattern obtained in Expt. 1 was obtained, where a steady rise in specific activity (in terms of protein) is seen. In others a less clear pattern of the type seen in Expt. 2 was found. In both cases the RNA/protein ratio of the bands decreases in the upper fractions but increases towards the bottom of the tubes. If the glucose 6-phosphatase activities are expressed per mg of RNA, the values for the two experiments agree fairly closely, thus suggesting that the banding of rough-microsomal subfractions was not due to some random process. Electron micrographs of the fractions (see Plate 3c) showed that many smooth vesicles were present in the bottom fractions, and we suggest that the incubation and centrifugation to which the vesicles are subjected causes some stripping of ribosomes. To explain differences in specific activities and the effects of detergents and NH_3 on smooth and rough microsomes, Stetten & Ghosh (1971) have suggested that glucose 6-phosphatase is more accessible to substrate

Table 2. *Specific activities of glucose 6-phosphatase and 5'-nucleotidase, and relative protein distribution in smooth-microsomal subfractions obtained after incubation with $\text{Pb}(\text{NO}_3)_2$ and glucose 6-phosphate*

Smooth microsomes were incubated with $\text{Pb}(\text{NO}_3)_2$ and glucose 6-phosphate, centrifuged and subfractionated as described in the Materials and Methods section with a lead/protein ratio of $0.6 \mu\text{mol}$ of Pb/mg of protein. The resulting bands, numbered as in Plate 1, were assayed for glucose 6-phosphatase, 5'-nucleotidase and protein. Relative protein content is expressed as % of total protein recovered from discontinuous sucrose density gradients. This was always 50–60% of that added to the incubation mixture. The mean $\pm$ s.d. of three separate experiments is given. Specific activity is expressed as μmol of P_i released/20 min per mg of protein. Values for glucose 6-phosphate are means $\pm$ s.d. of four separate experiments. Values for 5'-nucleotidase are from a typical experiment.

Microsomal subfraction	Relative protein content	Specific activity	
		Glucose 6-phosphatase	5'-Nucleotidase
S-1	12.0 ± 2.2	1.79 ± 0.81	6.49
S-2	23.2 ± 9.4	2.96 ± 0.96	5.57
S-3	41.6 ± 5.3	3.68 ± 0.63	4.46
S-4	17.7 ± 5.3	5.31 ± 2.01	3.28
S-5	4.1 ± 2.8	5.66 ± 2.14	4.35
S-6	2.3 ± 0.3	5.85 ± 0.40	5.83
Smooth microsomes (unfractionated)	—	3.27 ± 0.13	3.53

Table 3. *Specific activities of glucose 6-phosphatase in rough-microsomal subfractions obtained after incubation with Pb(NO₃)₂ and glucose 6-phosphate*

Rough microsomes were incubated with Pb(NO₃)₂ and glucose 6-phosphate, centrifuged and subfractionated as described in the Materials and Methods section with a lead/protein ratio of 0.6 μ mol of Pb/mg of protein. The resulting bands, numbered as in Plate 1, were assayed for glucose 6-phosphatase activity, protein and RNA. Results are expressed as μ mol of P_i released/20 min per mg of protein or per mg of RNA.

Microsomal subfraction	Specific activity of glucose 6-phosphate					
	(μ mol of P _i /20 min per mg of protein)		(μ mol of P _i /20 min per mg of RNA)		RNA/protein ratio	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
R-1	2.70	1.00	—	—	—	—
R-2	5.50	6.35	9.91	9.69	0.555	0.655
R-3	6.85	6.43	16.51	16.08	0.415	0.400
R-4	7.50	6.95	28.20	20.32	0.266	0.342
R-5	8.30	5.70	19.08	21.11	0.435	0.270
R-6	9.70	6.07	16.17	16.32	0.600	0.372
Rough microsomes (unfractionated)	4.50	5.80	15.00	18.07	0.300	0.321

in the rough- than in the smooth-microsomal vesicles and that this difference may be due to the presence of ribosomes. In this connection Rabin and co-workers have shown that the presence or the absence of ribosomes affects the activity of the disulphide-interchange enzyme of microsomal membranes (Rabin *et al.*, 1971). Our results may be consistent with such an effect, but with an intrinsic heterogeneity of enzyme distribution within the membrane superimposed on this effect of ribosomes.

Table 2 also shows the specific activity of 5'-nucleotidase assayed in the smooth-microsomal fractions. This enzyme, although heavily concentrated in the plasma membrane (Benedetti & Emmelot, 1968), is also believed to be present in the endoplasmic reticulum. The plasma membrane has been shown cytochemically to be unreactive for glucose 6-phosphatase (Tice & Barrnett, 1962; Ericsson, 1966) and biochemically to have very low glucose 6-phosphatase activity (Emmelot *et al.*, 1964), and hence the fragments derived from plasma membranes and present in the microsomal fraction should remain at the 1.3/1.0M-sucrose interface after incubation. This probably accounts for the high 5'-nucleotidase activity in fraction 1. However, the continuing presence of the enzyme at high specific activity even in the fractions taken from the bottom of the gradients supports the findings of Widnell (1972) that 5'-nucleotidase is also present in fragments of the endoplasmic reticulum.

In conclusion, two salient features arise from the work described in the present paper. First, it can be seen that smooth- and rough-microsomal fractions are heterogeneous with respect to the distribution of

glucose 6-phosphatase activity. To some extent this is a consequence of the multicomponent nature of the microsomal fraction itself, i.e. the presence of endoplasmic reticulum, plasma membrane, Golgi vacuoles and lysosomes. However, our method of subfractionation should exclude this from consideration for all the bands except band 1, since elements other than those derived from the endoplasmic reticulum should be specifically excluded from bands 2-6; this is supported by the electron micrographs. Thus the banding pattern of fractions 2-6 represents a true heterogeneous distribution of glucose 6-phosphatase in the endoplasmic reticulum. We cannot tell, however, whether this reflects the situation within a single cell or within the liver lobule. In this respect Chiquoine (1953) and Novikoff (1959) have reported that glucose 6-phosphatase staining at the light-microscope level is stronger in peripheral cells than in centrolobular cells. Whatever the case may be, the presence of heterogeneity in isolated fractions should perhaps be borne in mind when using glucose 6-phosphatase as a marker enzyme for microsomal preparations (see Glaumann & Dallner, 1970). More importantly perhaps, the findings raise the question of the significance *in vivo* of our observation of vesicles with different glucose 6-phosphatase specific activities. If the heterogeneity reflects different cell types this may be linked to the supply of glucose from the portal blood. If, however, heterogeneity exists within an individual cell, many interesting questions may be posed; e.g.: does this heterogeneity correspond to a specialization of different portions of the endoplasmic reticulum? Could the presence of

vesicles with different enzyme specific activities reflect the turnover of specific membrane proteins?

The other main feature arising from this work is that the technique described here may be particularly useful in exploiting situations where the activity of glucose 6-phosphatase, or other membrane-bound phosphatases, is altered so that one might investigate newly formed membrane and compare it with pre-existing membrane, e.g. in the neonatal rat liver (see Leskes *et al.*, 1971*a,b*), phenobarbital-induced proliferation of the hepatic endoplasmic reticulum (Orrenius *et al.*, 1965; Jones & Fawcett, 1966), and the induction of glucose 6-phosphatase during metamorphosis of tadpoles (Frieden & Mathews, 1958). Such systems in which a constitutive membrane component is involved may give further insight into the mechanisms of membrane biosynthesis and induction of membrane-bound enzymes. The method that we have described may also be regarded as a general one, which may be adapted to separate cellular structures, such as endoplasmic-reticulum fragments, from contaminating components which are devoid of the particular enzyme used, e.g. in this case Golgi elements and plasma membrane.

J. A. L. thanks the Medical Research Council for a Scholarship.

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