Subcellular localization of the sulphation reaction of heparan sulphate synthesis and transport of the proteoglycan to the cell surface in rat liver

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We report on the incorporation of radiolabelled sulphate into proteoglycan in the '*in situ*'-perfused rat liver. After 5 min virtually all of the [³⁵S]sulphate was incorporated into heparan sulphate; no partially sulphated precursors were detected. Pulse-chase experiments, followed by centrifugation in gradients of sucrose and metrizamide, showed that, at 5 min, the heparan sulphate was associated predominantly with the Golgi membranes. Over the next 20 min, intact proteoglycan appeared at the plasma membrane. At intermediate times the heparan sulphate was detected simultaneously in two distinct populations of membrane vesicles. Whether the heparan sulphate in these two populations has two different destinies (e.g. plasma membrane or secretion) is not yet clear. Subfractionation of the Golgi membranes showed that the *N*-sulphotransferase co-purified with the heparan [³⁵S]sulphate and was separable from the galactosyltransferase of glycoprotein synthesis, confirming that the Golgi membrane system is functionally segregated. Subfractionation also permitted an almost 100-fold purification of the *N*-sulphotransferase over the homogenate: this will provide an excellent starting material for isolation and further characterization of the enzyme.

INTRODUCTION

Proteoglycans can interact, sometimes in a highly specific manner (determined in part by their polyanionic character), with a variety of other molecules, including proteins of the extracellular matrix, growth factors, enzymes (and their inhibitors) and bivalent cations. They are therefore involved in the cohesion of cells within tissues; they can mediate cell regulatory functions of the external domain and they are involved in the control of embryogenesis and cell development (for references, see Gallagher *et al.* [1]).

Changes in the degree and specificity of the sulphation process, which appears to be tightly regulated, have been correlated with the differentiation of monocytes into macrophage-like cells [2,3], the proliferation of arterial smooth-muscle cells [4], the transformation of tissue-culture cells by virus [5–7] and the selection of highly tumorigenic variants from cloned cells [8,9].

The heparan sulphate class of proteoglycans, within which we include heparin, a specialized product of mast cells, is synthesized by most cells and associated primarily with plasma membranes and basement membranes (see Gallagher *et al.* [1]), as well as being secreted and found in minor amounts in other subcellular sites. The characteristic disaccharide repeat of heparan sulphate (glucuronic acid and *N*-acetylglucosamine) is modified by a series of post-polymerization reactions which appear to occur in a sequential, but concerted, manner: *N*-deacetylation, the first step, occurs in conjunction with *N*-sulphation of the amino group thus liberated [10–13]. The introduction of *N*-sulphated residues generates the recognition sites for the subsequent reactions: epimerization of the glucuronic acid residue to iduronic acid [14,15] and selective *O*-sulphation [11,16].

Golgi membranes are the site of the terminal glycosylation [17–19] and sulphation of a number of macromolecules, including proteoglycans [20,21]. Relatively little is known about the proteoglycan sulphotransferases; most of the early work has been concentrated on broad acceptor specificities demonstrated by crude microsomal preparations or partially purified solubilized enzymes, usually containing multiple activities consistent with the existence of multienzyme complexes [22]. It is clear, however, both for heparan sulphate [13] and chondroitin sulphate [23], that the products generated *in vitro* do not correspond with the sulphation patterns seen in the corresponding glycosaminoglycan produced by intact cells. These results indicate that further progress will require the study of intact Golgi systems.

We have chosen the perfused rat liver to study the sulphation and synthesis of heparan sulphate and the transport of this molecule from its site of synthesis to the hepatocyte surface. We have (a) investigated the localization and organization of the sulphation process in the Golgi, particularly in its relation to other known functions such as galactosylation of glycoproteins and (b) attempted to identify vesicles containing newly synthesized heparan sulphate before their incorporation into the plasma membrane or secretion.

MATERIALS AND METHODS

³⁵S-labelling

Under sodium pentobarbitone anaesthesia, the superior vena cava and superior mesenteric veins of an adult male Wistar rat (250–300 g) were cannulated and the vena cava, aorta and splenic veins ligated. To prevent

clotting of blood within the liver and within the afferent and efferent blood vessels, 1 ml of sulphate-free Hanks solution containing 2 units of heparin/ml was injected from syringes attached to the cannulas. The liver was then exsanguinated and the vascular space cleared of sulphate by pumping 200 ml of sulphate-free Hanks solution [maintained at 37 °C and equilibrated with O₂/CO₂ (19:1)] via the superior mesenteric vein. Hanks solution contained, in 1 litre: 0.186 g of CaCl₂·2H₂O, 0.4 g of KCl, 0.06 g of KH₂PO₄, 0.2 g of MgCl₂·6H₂O, 8 g of NaCl, 0.35 g of NaHCO₃, 0.048 g of Na₂HPO₄ and 1 g of glucose. A closed perfusion system was then established in which the efferent flow from the superior vena cava was recirculated to the liver through the superior mesenteric vein via a 5 ml reservoir containing sulphate-free Hanks solution also kept at 37 °C and gassed with O₂/CO₂ (19:1). The total volume of the perfusion system (excluding the vasculature of the liver, but including the reservoir) was about 12 ml, and the perfusion rate was 20 ml·min⁻¹.

At zero time, 1 mCi of carrier-free ³⁵SO₄²⁻ (Amersham International) was added to the reservoir. After the labelling period, the efferent from the superior vena cava was diverted to a waste trap and the liver perfused with Hanks solution containing 1 mM-Na₂SO₄ for a 'chase' period as required. Finally the liver was flushed out with 100 ml of ice-cold 0.25 M-sucrose/1 mM-sulphate/5 mM-Tris/HCl, pH 8.0, and then homogenized in 30 ml of this medium by using five strokes of the pestle of a Potter-Elvehjem homogenizer (0.09 mm clearance and 500 rev·min⁻¹). It should be noted that, for both the pulse-chase experiments and also for experiments comparing labelling *in vivo* with sulphotransferase activity measurements *in vitro*, separate animals were required for each condition.

Fractionation in a B14 zonal rotor

The homogenate was adjusted to 1.84 M-sucrose and a total volume of 140 ml. An MSE B14 zonal rotor was then loaded sequentially, at about 25 ml·min⁻¹, via the wall with the following solutions: 100 ml of 0.25 M-sucrose, a 300 ml linear 0.8–1.8 M-sucrose gradient, 140 ml of homogenate and 2.25 M-sucrose/1 mM-MgCl₂ to fill the rotor (about 110 ml). All the solutions contained 5 mM-Tris/HCl, pH 8.0. After centrifugation at 40 000 rev·min⁻¹ for 2.5 h, the rotor was unloaded from the centre by pumping 2.35 M-sucrose to the wall. The effluent from the rotor was collected in 52 fractions; the first and last were 50 ml in volume, the intervening 50 fractions (2–51) were 10 ml.

Isolation of crude Golgi membrane fractions

The homogenate was centrifuged at 1000 *g*_{av} for 5 min and the supernatant re-centrifuged at 10 000 *g*_{av} for 20 min; the pellet was resuspended by gentle homogenization in 10 ml of 1.55 M-sucrose/5 mM-Tris/HCl, pH 8.0, and the Golgi membranes were isolated by flotation through a discontinuous sucrose gradient as described by Fleischer & Fleischer [24], using 4 ml of sample, 2.5 ml of 1.33 M-, 2 ml of 1.20 M-, 2 ml of 1.10 M-, 1 ml of 0.77 M- and 1 ml of 0.25 M-sucrose; all solutions contained 5 mM-Tris/HCl, pH 8.0. After centrifugation at 100 000 *g*_{av} for 1 h, the banded material in the gradient was harvested by dilution with 2 vol. of 5 mM-Tris/HCl, pH 8.0, and centrifugation at 100 000 *g*_{av} for 30 min.

Subfractionation of membranes in metrizamide gradients

Sedimented membranous material was resuspended in 3–4 ml of metrizamide (Analytical grade; Nycomed, Birmingham, U.K.) of the appropriate concentration and rehomogenized by using 10 strokes of the pestle of a Potter-Elvehjem homogenizer (500 rev·min⁻¹). The sample was then overlaid with a discontinuous gradient so that the total volume was 12.0–12.5 ml. All centrifugations were carried out at 100 000 *g*_{av}, and all solutions contained 5 mM-Tris/HCl, pH 8.0. The precise conditions of the gradient and centrifugation time, which varied with the type of membranous material, are given in the legends to Figs. 5 and 6 (below).

Analysis

Enzymes. In all enzyme assays the concentration of sucrose or metrizamide was never more than 5%. Galactosyltransferase (EC 2.4.1.22) was assayed by using *N*-acetylglucosamine [25] as an acceptor; acid phosphatase (EC 3.1.3.2) was measured with *p*-nitrophenyl phosphate as described by Engstrom [26], and 5'-nucleotidase (EC 3.1.3.5) was assayed radiometrically [27]. Succinate dehydrogenase (EC 1.3.99.1) was determined by using either cytochrome *c* [28] or 2-*p*-iodophenyl-3-*p*-nitrophenyltetrazolium chloride [29] as electron acceptors.

Protein. Gradient samples (either sucrose or metrizamide) were assayed directly by spotting (up to 8 µl, containing 0.5–8 µg of protein) on to Whatman 3 MM paper (using a 1 cm square grid) and staining in 0.04% Coomassie Blue R250 as described by Winterbourne [30]. Membrane samples in 0.25 M-sucrose were assayed either by the same method or that of Lowry *et al.* [31]. Bovine serum albumin was used as the standard.

Quantification and identification of ³⁵S-labelled macromolecules. Aliquots (50 µl) to be assayed for ³⁵S-labelled macromolecules were mixed with 10 µl of heparin (10 mg/ml; Sigma Grade II) as carrier and precipitated by incubating for 1 h at 37 °C with 1 ml of 50 mM-Na₂SO₄/1% (w/v) cetylpyridinium chloride. Precipitates were collected by filtering through Whatman GF/C filters, washed with 50 mM-Na₂SO₄/0.02% (w/v) cetylpyridinium chloride and the dried filters were counted in 1 ml of 1% PPO (2,5-diphenyloxazole)/0.05% POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene] in toluene. Exclusion gel chromatography was performed in 1 cm × 48 cm columns of Sepharose CL4B or CL6B (Pharmacia) equilibrated and eluted at 5 ml/h with 1 M-LiCl/0.1% SDS/10 mM-sodium Mops, pH 7.0. Samples were boiled for 5 min in 2% (w/v) SDS before application, and fractions (1 ml) were counted in 5 ml of NE260 (Nuclear Enterprises). Analysis of glycosaminoglycans by papain digestion, DEAE-cellulose chromatography and HNO₂ digestion were as described previously [8].

Sulphotransferase assay. Sulphotransferase acceptors were prepared by chemical desulphation of heparin grade I (Sigma; 170 units/mg) as described previously [32]. *N*-Sulphotransferases were assayed with de-*N*-sulphated heparin and *O*-sulphotransferases with chemically re-*N*-sulphated extensively desulphated heparin. Radiolabelled PAPS was produced as described by Balasubramanian *et al.* [33], with minor modifications,

Fig. 1. Fractionation of rat liver homogenate by flotation through sucrose gradient in a B14 zonal rotor: enzyme activities

The fractionation was carried out as described in the Materials and methods section. (a) Galactosyltransferase; (b) acid phosphatase; (c) succinate dehydrogenase; (d) 5'-nucleotidase. In (c), ● represents gradient density profile (% w/w, sucrose).

from a cytosolic fraction of rat liver. The product had a radiochemical purity of 98% as judged by electrophoresis.

Sulphotransferase activity was determined by modifications of the assay described by Hart [34]. Each assay mixture contained 1–40 μg of protein, 50 μg of acceptor and 0.5 μCi of [³⁵S]PAPS (100 μM) in 50 μl of 20 mM-KF/10 mM-MgCl₂/0.5% Nonidet P40/10 mM-phosphate/citrate buffer, pH 7. Reactions were initiated by the addition of PAPS as the last component in the mixture, incubated at 37 °C for 30 min and stopped by boiling for 3 min. The products were isolated by paper chromatography [35], measuring radioactivity at the

origin in 1 ml of 1% (w/v) PPO/0.05% (w/v) POPOP in toluene at 56% counting efficiency.

RESULTS

Time-dependent incorporation of ³⁵S into vesicle-associated heparan sulphate

After subcellular fractionation of a liver homogenate by flotation through a sucrose gradient in a zonal rotor, the distribution of the four enzymes galactosyltransferase, 5'-nucleotidase, acid phosphatase and succinate dehydrogenase in the zonal gradient was as shown in

Fig. 2. Fractionation of ^{35}S -labelled rat liver homogenate by flotation through sucrose gradient in a B14 zonal rotor: distribution of ^{35}S -labelled macromolecules

The fractionation was carried out as described in the Materials and methods section. (a) 5 min pulse with $^{35}\text{SO}_4^{2-}$; (b) 5 min pulse with $^{35}\text{SO}_4^{2-}$ + 5 min chase with unlabelled sulphate; (c) 5 min pulse + 10 min chase; (d) 5 min pulse + 20 min chase. In (c) ● is the gradient density profile (% w/w, sucrose).

Fig. 1. Golgi membranes (galactosyltransferase) in the lightest part of the gradient (fractions 7–18) are clearly resolved from other membranes. Although the 5'-nucleotidase (plasma membrane) is broadly spread through the middle of the gradient and overlaps both the lysosomes (acid phosphatase) and mitochondria (succinate dehydrogenase), its peak activity is quite clearly resolved from the other three enzymes.

After a 5 min pulse (Fig. 2a) with $^{35}\text{SO}_4^{2-}$, the bulk of macromolecular ^{35}S radioactivity (band I) is associated

with the Golgi, with a minor peak which co-purifies with the lysosomes and mitochondria (band III). The latter becomes a major ^{35}S -containing region after a 5 min chase with unlabelled sulphate (Fig. 2b); under these conditions band I remains highly labelled, and a new shoulder of ^{35}S -labelling becomes apparent in the region of the zonal gradient which exhibits maximum 5'-nucleotidase activity (band II) and may represent the plasma membrane or an internal pool of membrane vesicles destined to fuse with the plasma membrane.

Fig. 3. Exclusion gel chromatography of material labelled by a 5 min perfusion

Samples were analysed by Sepharose CL6B as described in the Materials and methods section. ●, Undegraded Golgi-enriched fraction obtained by sucrose-gradient fractionation; △, glycosaminoglycan obtained from ion-exchange chromatography; ○, glycosaminoglycan as above, but after degradation with HNO_2 .

After a 10 min chase with unlabelled SO_4^{2-} (Fig. 2c) the labelled material of band II now predominates over that of band III, but much of the label still resides in band I. Only after a 20 min chase period (Fig. 2d) does this diminish somewhat (note the different ordinate scales), while band II is now the major site of ^{35}S -labelling and labelling in the denser part of the gradient has been reduced considerably.

Analysis of Golgi-enriched material from a liver perfused for 5 min with $^{35}\text{SO}_4^{2-}$, with no chase period, resulted in a single well-defined peak on a Sepharose CL6B column with a K_{av} of 0.22, characteristic of the heparan sulphate proteoglycan from rat liver (Fig. 3). Papain digestion of pulse-labelled homogenates followed by ion-exchange chromatography showed that 86% of the radioactivity was eluted at the same, or a higher, salt concentration when compared with an internal standard of heparan sulphate from 210 C cells [8] (results not shown). This material eluted with a K_{av} of 0.42 on Sepharose CL6B (Fig. 3) and was quantitatively converted into material of low molecular mass (K_{av} 0.96; Fig. 3) by HNO_2 , confirming the original material as heparan sulphate proteoglycan. High-voltage paper electrophoresis at pH 1.7 of the HNO_2 -degraded material demonstrated that the *N*- to *O*-sulphate ratio was 1.2. Despite the short pulse duration, no evidence for immature, partially sulphated, precursors was found. The remaining 14% of radioactivity from the papain-digested homogenate was eluted as a discrete peak on ion-exchange chromatography at a low salt concentration. This material had a K_{av} of 0.56 on Sepharose CL6B, which was unaltered by HNO_2 treatment, indicating that it was not related to heparan sulphate.

There is no evidence that the macromolecule containing the ^{35}S marker is different in the three regions of

the zonal gradient. The radioactivity from membranes in the three regions ran identically on Sepharose CL 6B columns in the presence of SDS. Moreover, autoradiographs of SDS/polyacrylamide gels showed that, in all fractions which contained ^{35}S -labelled macromolecules (results not shown), these did not enter the gel. These results are consistent with the presence of high-molecular-mass, highly glycosylated, material; there was no evidence for the existence of any labelled breakdown products in any fraction.

Rapid labelling of band I (Fig. 2) certainly indicates that this is Golgi, but the apparent inability to 'chase' effectively the ^{35}S -label out of this material in the zonal gradient is puzzling. The persistence of label in the Golgi region was not due to failure to wash out the $^{35}\text{SO}_4^{2-}$ from the perfusate. After a 10 min wash-out of the labelled liver with ice-cold 0.25 M-sucrose, the concentration of labelled sulphate passing through the liver was less than 1% of that during the pulse phase. Moreover, the transfer of macromolecular ^{35}S to the other two compartments (bands II and III) is very clearly time-dependent; in particular, the almost complete disappearance of labelling from band III between 10 and 20 min suggests that poor lavage is not the explanation. However, it is noticeable that there is a pronounced broadening of band I with increasing chase times and some intimation of the presence of two components in the pulse-with-chase experiments but not in the pulse-only experiment. These features may reflect the presence of a low-density compartment which co-migrates with the Golgi in the zonal gradient, but which becomes labelled at a later time.

The weight of liver and the concentration of $^{35}\text{SO}_4^{2-}$ in the perfusate used in the four experiments described in Fig. 2 was approximately the same; it is worth noting, therefore, that the total ^{35}S -labelled macromolecules (total area under each plot) in Figs. 2(b) and 2(c) was approximately twice that in Figs. 2(a) and 2(d). These discrepancies were due neither to incomplete perfusion of the livers nor to leakage of perfusate from the system. The increase in total heparan sulphate between Figs. 2(a) and 2(b) is likely to be due to the continued incorporation of the $^{35}\text{SO}_4^{2-}$ during the 5 min chase, of label taken up during the 5 min pulse. In the pulse-only experiment, this would have been curtailed by the rapid decrease in temperature caused by the incoming ice-cold iso-osmotic sucrose. The subsequent fall in total recovered macromolecular ^{35}S at 20 min is probably due to secretion of the heparan sulphate from the liver into the perfusate; it seems unlikely that the label is degraded in lysosomes during the perfusion, since the macromolecule which is present is intact heparan sulphate proteoglycan as determined by exclusion gel chromatography. Moreover the acid phosphatase (Fig. 1b) is broadly distributed in the denser regions of the zonal gradient, whereas the ^{35}S -labelling of band III (Fig. 2b) is rather sharp.

To identify rigorously the membranes in Bands I, II and III which contain ^{35}S -labelled macromolecules, we used techniques specifically designed to isolate the membranes contained within these bands, followed by fine resolution of membrane components in gradients of metrizamide.

Golgi membranes

Low-density Golgi-containing membranes were prepared by flotation of a mitochondrial pellet through a

Fig. 4. Fractionation of ^{35}S -labelled rat liver mitochondrial pellet by flotation through a sucrose gradient (swing-out rotor): enzyme and ^{35}S -labelled-macromolecule distributions

The fractionation was carried out as described in the Materials and methods section. (a) ^{35}S -labelled macromolecules after 5 min pulse of $^{35}\text{SO}_4^{2-}$; (b) ^{35}S -labelled macromolecules after 2.5 h perfusion with $^{35}\text{SO}_4^{2-}$; (c) galactosyltransferase; (d) 5'-nucleotidase; (e) acid phosphatase; (f) succinate dehydrogenase.

discontinuous sucrose gradient. This discontinuous sucrose gradient, designed to resolve the Golgi membranes, also resolves 5'-nucleotidase and acid-phosphatase-rich fractions (see Fig. 4) from the mitochondrial pellet in a manner analogous to that of the fractionation of the whole homogenate in the continuous zonal sucrose gradient. Figs. 4(a) and 4(b) also compare the distribution of ^{35}S -label in these membranes after a 5 min pulse with $^{35}\text{SO}_4^{2-}$ with that after a continuous perfusion with the radiolabelled sulphate for 2.5 h. Between these times there is a clear shift of ^{35}S -labelled macromolecules from the Golgi to the major 5'-nucleotidase-rich band (band 4). This confirms results of the pulse-chase experiments

Table 1. Solubilization of *N*-sulphotransferase from Golgi membranes

Golgi membranes were homogenized in 0.25 M-sucrose/5 mM-Tris/HCl, pH 7.4, and centrifuged to yield Supernatant A. The pellet was homogenized in 5 mM-Tris/HCl, pH 7.4, and centrifuged to yield Supernatant B. The pellet was homogenized in 0.5% Nonidet P40 and centrifuged to yield Supernatant C. All centrifugations were at 100 000 g_{av} for 1 h.

Supernatant	<i>N</i> -Sulphotransferase activity (% of total)
A	11
B	13
C	77

and the work of other groups (see [1]), namely that the plasma membrane is the major ultimate site of cell-associated heparan sulphate.

In a separate series of experiments, unlabelled membranous material from sham-perfused rat livers was collected for measurement of sulphotransferase activity. It is necessary to perform separate experiments for the measurement of heparan sulphate synthesis and sulphotransferase activity, since both rely on the assay of ^{35}S -labelled macromolecules. In preliminary experiments it was shown that all the *N*-sulphotransferase was membrane-associated: no activity was detected in the cytosol fraction. Moreover, when the Golgi fractions were washed sequentially in iso-osmotic sucrose, hypo-osmotic Tris/HCl buffer and the non-ionic detergent Nonidet P40 (Table 1), only the latter effectively solubilized the sulphotransferase activity, indicating that the enzyme is, at least predominantly, membrane-bound.

About 70% of the galactosyltransferase and heparan [^{35}S]sulphate were recovered in the top two fractions of the discontinuous sucrose gradient (Fig. 4). A similar percentage of the sulphotransferase activity was also recovered here (results not shown). Subsequently, these two bands of material were harvested, combined and refractionated in metrizamide gradients (Fig. 5). The clear dichotomy of the sulphotransferase (Fig. 5a) and the glycoprotein galactosyltransferase (Fig. 5b) indicates that a specific region of the Golgi is responsible for sulphation in the synthesis of proteoglycan molecules. Only the *N*-sulphotransferase activity is shown; the *O*-sulphotransferase, although showing an identical profile, had an activity less than 10% of that of the *N*-sulphotransferase activity.

Not surprisingly, after a 5 min pulse of $^{35}\text{SO}_4^{2-}$ the major band of ^{35}S -labelled macromolecules (Fig. 5c) coincided with the sulphotransferase, but there was a small, but distinct, labelling of a low-density fraction which coincided with neither enzyme. After a 5 min pulse + 5 min chase experiment this low-density fraction then contained most of the ^{35}S -labelled macromolecules (Fig. 5e), yet neither the visually observed membrane banding patterns in the sucrose and metrizamide gradients nor the galactosyltransferase distribution (Fig. 5d) were changed. A similar ^{35}S distribution in metriz-

Fig. 5. Metrizamide gradient fractionation of Golgi fractions: comparison of 5 min pulse with $^{35}\text{SO}_4^{2-}$ and 5 min pulse + 5 min chase

The two lightest bands from the sucrose gradient (see Fig. 4) were combined, harvested by centrifugation and resuspended in 30% (w/v) metrizamide, and 2.5 ml of this was overlaid with 2.5 ml each of 25, 20, 15 and 10% metrizamide. All solutions contained 5 mM-Tris/HCl, pH 8.0. After centrifugation at 100000 g for 16 h, the gradients were unloaded low-density-end-first in 8×1.5 ml fractions. (a) Sham perfused liver; (b) and (c) 5 min pulse with $^{35}\text{SO}_4^{2-}$; (d) and (e) 5 min pulse + 5 min chase with unlabelled sulphate. Histograms show specific activity of N -sulphotransferase (a), galactosyltransferase (b and d), ^{35}S -labelled macromolecules (c and e).

amide was observed for the 5 min pulse + 20-min chase experiment. It appears, therefore, that after the ^{35}S label appears in the Golgi membranes between 0 and 5 min, it is transferred to a second compartment (at about 10 min), whose density is much lower than that of either the Golgi or the plasma membrane (see the next subsection) and was only separated from the Golgi

Fig. 6. Isolation of lysosomes from a light mitochondrial pellet by metrizamide gradient centrifugation

After a 5 min pulse with $^{35}\text{SO}_4^{2-}$ + 5 min chase with unlabelled sulphate, a light mitochondrial fraction was prepared by centrifugation of the liver homogenate post-nuclear supernatant at 25000 $g_{av.}$ for 10 min. The pellet was resuspended in 35% (w/v) metrizamide and 2×3 ml overlaid with 2 ml each of 30, 20, 24 and 3 ml of 19% metrizamide (all solutions contained 5 mM-Tris/HCl, pH 7.6). After centrifugation at 100000 g for 2 h, 10×1 ml fractions were collected (low-density-first) together with a pellet. Histograms show the specific activity of ^{35}S -labelled macromolecules (a); acid phosphatase (b); 5'-nucleotidase (c); succinate dehydrogenase (d).

fraction isolated on sucrose gradients by a subsequent high-resolution metrizamide gradient.

Lysosomes and plasma membrane

To investigate the source of the ^{35}S -labelling detected in band III of the sucrose zonal gradient in the 5 min pulse + 5 min chase experiment, a lysosome-rich fraction was further resolved in a metrizamide gradient by the method of Wattiaux & Wattiaux-De Coninck [36]. The distributions of ^{35}S -labelled macromolecules, acid phosphatase, 5'-nucleotidase and succinate dehydrogenase were compared (Fig. 6). Clearly the heparan sulphate (Fig. 6a) is located in a low-density membrane compartment distinct from the lysosomes (Fig. 6b), the mitochondria (Fig. 6d) or the 5'-nucleotidase activity (Fig. 6c).

From the same homogenate used to prepare the lysosome-rich fraction, a plasma-membrane fraction, enriched in 5'-nucleotidase (see Fig. 4), was also subfractionated in a 10–30% metrizamide gradient run at 100000 $g_{av.}$ for 16 h. Both the 5'-nucleotidase and ^{35}S -

labelled macromolecules banded similarly to each other, at densities greater than $1.08 \text{ gm} \cdot \text{ml}^{-1}$, in contrast with the two low-density compartments, which banded at less than $1.05 \text{ gm} \cdot \text{ml}^{-1}$ and which were essentially devoid of 5'-nucleotidase activity. All the evidence indicates that the 5'-nucleotidase-rich material shown in Figs. 1 and 4 contains only plasma membrane.

DISCUSSION

Our pulse-chase experiments have shown a rapid (5 min) incorporation of sulphate into macromolecules in Golgi membranes, confirming the observations of other workers [20,37]. The appearance of heparan [^{35}S]sulphate at the plasma membrane within 10–15 min of the start of the labelling period is in broad agreement with data on the time course of secretion of proteoglycans [20,37–39].

Although it is not known whether or not functional specialization occurs between individual Golgi complexes [40], it is now well established that the Golgi apparatus is polarized into *cis* and *trans* faces [41], where sequential processing, of glycoproteins at least, are both temporally and spatially segregated. A number of workers [11,12] have speculated on the influence of the subcellular organization of the biosynthetic apparatus on the structure of the final product. In an immunohistochemical study of chondrocytes, elaboration of chondroitin sulphate chains was co-localized to the medial-*trans* region of the Golgi, together with ricin-binding sites, which are presumed to be sites rich in galactosyltransferase activity [18]. Although this result might suggest that both glycoprotein and proteoglycan processing can occur in the same Golgi sacs, it should be noted that chondroitin sulphate proteoglycan bears a significant number of galactose-containing side chains, including complex-type glycoprotein units, and these proteoglycans are the major secretory products of chondrocytes. By contrast, our results indicate that, in liver, the major sites of glycoprotein galactosyltransferase and heparan sulphotransferase may be partially separated physically. Although further work is required to determine whether this segregation is within or between Golgi systems, Mitchell & Hardingham [42] noted that monensin totally inhibited secretion of glycoproteins but only partially inhibited that of chondroitin sulphate proteoglycan.

As to the route by which synthesized proteoglycan is transferred to the surface relatively little is known beyond the time course for the process of secretion (see above). In chondrocytes vesicle shuttles involved in chondroitin sulphate synthesis and secretion, between the endoplasmic reticulum and Golgi and between the Golgi and the plasma membrane respectively, have been identified [39]. Our own data have demonstrated two low-density compartments that are apparently involved in the translocation of heparan sulphate to the surface: in metrizamide gradients they have a very similar density, so the reason for their distinctly different rates of flotation in the zonal sucrose gradient must be due to their sizes. Band III in the zonal gradient (Fig. 2b) clearly demonstrates the characteristic transient labelling pattern expected of a vesicle responsible for transporting heparan sulphate from the Golgi to the cell surface for incorporation into the plasma membrane or for secretion. The lack of any involvement of the lysosomes themselves, suggested by the resolution of the ^{35}S -labelled macro-

molecules from these organelles in metrizamide gradients and the lack of any evidence for degradation, supports the finding that no degradation of proteoglycan in chondrocytes occurs before secretion, nor do lysosomal inhibitors have any effect on this process [43].

The relevance of the second low-density compartment (i.e. that which co-migrates with the Golgi in the zonal gradient: most evident in Fig. 2d) is not clear. Although rapidly labelled in the same way as the 'lysosomal' vesicles, it does not show the same rapid clearance; rather the heparan sulphate seems to exit rather slowly from this compartment. It is noteworthy there may be two forms of proteoglycan: that which contains a hydrophobic amino acid tail and is anchored to the membrane and that which does not and is secreted [1]. Moreover, the heparan sulphate in lysates of neuroblastoma cells has a different *O*-sulphation pattern to that of the secreted molecule [44]. In further studies it will be of interest to determine whether the heparan sulphate proteoglycans in the two post-Golgi low-density compartments have different fates or are different in their fine structure.

There are few reports on the purification and characterization of heparan sulphotransferase [45,46]. We find no evidence of soluble *N*-sulphotransferase in rat liver, the enzyme behaving as a intercalated membrane protein which is readily solubilized by a mild non-ionic detergent. Reports of soluble enzymes are doubtful, as either centrifugation conditions used were unlikely to sediment even quite large membrane vesicles [34] or enzymes were isolated from tissues exposed to harsh conditions [47].

For both heparan sulphate [13] and chondroitin sulphate [23] products of sulphation *in vitro* indicate that such reactions are not subjected to the controls that exist in intact cells. We have established conditions which allow the isolation of highly purified Golgi vesicles. The extent of purification by subcellular fractionation alone (about 100-fold) compares very favourably with that obtained by others after extensive biochemical fractionation from whole tissue or from crude membranes [46–48]. These Golgi preparations should permit the isolation of the individual sulphotransferases for biochemical analysis and production of antibodies in addition to permitting studies of the role that organization within the Golgi apparatus plays in the regulation of proteoglycan maturation.

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