MIGRATION OF LYMPHOCYTES ACROSS SPECIALIZED VASCULAR ENDOTHELIUM

V. PRODUCTION OF A SULPHATED MACROMOLECULE BY HIGH ENDOTHELIAL CELLS IN LYMPH NODES*

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SUMMARY

High endothelial cells lining the post capillary venules in the paracortical areas of rat lymph nodes were found by autoradiography to incorporate [35S]sulphate, whether it was injected into the footpad to reach the draining popliteal lymph node or added to short-term cultures of cervical lymph node slices. The early localization of [35S]sulphate was confined to the Golgi apparatus, but before it disappeared from the cell radioactivity was associated with cytoplasmic vesicles. Sulphated material in macromolecular form was extracted from lymph nodes that had been labelled in vivo and was also found in the supernatant of lymph node cultures. The labelled material was not proteoglycan in nature. High endothelial cells apparently secrete a sulphated macromolecule but its relationship to the only known function of high-walled endothelium – the selective extraction of lymphocytes from the blood – remains to be clarified.

INTRODUCTION

The vascular endothelium lining the post-capillary venules of the lymph node paracortex was originally distinguished on morphological grounds (Thomé, 1898; Schulze, 1925). Compared to the thin, flat endothelial cells lining small venules elsewhere, these specialized endothelial cells are increased in height as measured from the lumen of the vessel to the basement membrane, so that they have been described as high, plump or cuboidal. As reviewed by Andrews, Ford & Stoddart (1980), these high endothelial cells differ quantitatively from flat venular endothelium in many histochemical and ultrastructural characteristics. Gowans and his colleagues (Gowans & Knight, 1964; Marchesi & Gowans, 1964) discovered that the great majority of recirculating lymphocytes that enter lymph nodes (LN) from the blood do so by crossing the walls of high endothelial venules (HEV), and recently the dynamic interaction between lymphocytes and HEV has been the subject of detailed ultrastructural study (Schoefl, 1972; Van Ewijk, Brons & Rozing, 1975; Anderson & Anderson, 1976). However, the relationship between the only known function of HEV – the selective extraction of lymphocytes from the blood – and their structural peculiarities remains totally obscure. It is particularly curious that an equally efficient mechanism

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for the selective extraction of lymphocytes from the blood is found in the marginal zone of the spleen, which lacks HEV.

Early after the injection of sodium $[^{35}S]$sulphate into the footpad of rats a heavy autoradiographic localization of $^{35}$S in the HEV of the popliteal LN was consistently observed, as has been reported in review papers (Andrews et al. 1980; Ford, Smith & Andrews, 1978). This signified that $^{35}$S had been incorporated into molecules that are rendered insoluble by fixation. The original reason for injecting $[^{35}S]$sulphate was the suggestion that certain staining characteristics of HEV supported the presence of proteoglycan (Smith & Henon, 1959), although the validity of this conclusion has been seriously questioned on the basis of more detailed histochemical study (Ropke, Jørgensen & Claesson, 1972). The aims of the present paper are: first, to report studies on the time-course and precise site of $[^{38}S]$sulphate incorporation both in vivo and in vitro; and second, to describe the initial characterization of the sulphated molecule. This led to the conclusion that $[^{38}S]$sulphate is incorporated into a macromolecule that is not a proteoglycan. Experiments intended to elucidate the relationship between the synthesis of this sulphated macromolecule, the structure of HEV and lymphocyte traffic across HEV will be reported in later papers.

MATERIALS AND METHODS

Animals

The rats used in this study were adult males or females of the highly inbred AO or PVG strains. Occasionally (AO x PVG) F$_1$ hybrids were used.

Reagents

Biochemicals were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K.; all other chemicals were of analytical grade where possible and were purchased from British Drug Houses, Poole, Dorset, U.K., unless otherwise stated. Methacrylate resin (JB-4) was obtained from Polysciences Inc., Warrington, Pennsylvania, U.S.A., whilst Epon 812 resin and glutaraldehyde were supplied by TAAB Laboratories Equipment, Reading, Berks., U.K. Dulbecco's balanced salt solution was purchased from Oxoid Ltd, Basingstoke, Hants., U.K. and sheep erythrocytes supplied by Tissue Culture Services Ltd, Slough, Berks., U.K. Carrier-free sodium $[^{35}S]$sulphate (SJ$^S$-2P) was obtained from The Radiochemical Centre, Amersham, Bucks, U.K.

In vivo labelling of the high endothelial venule (HEV)

The popliteal LN was studied because it receives a well-defined drainage from the hind footpad, which ensured that material injected into the footpads had rapid access to this LN (Drayson, Smith & Ford, 1981).

Both hind footpads were injected with 0.1 ml of 10% (v/v) washed sheep erythrocyte suspension to increase the size of the popliteal LN (Drayson et al. 1981) and the volume of HEV (Anderson, Anderson & Wylie, 1975). Five days later at the height of the proliferative response 1.0 $\mu$Ci $g^{-1}$ body weight of sodium $[^{38}S]$sulphate was injected into each footpad. The rats were killed by cervical dislocation at intervals from 15 min to 24 h after injection. Both popliteal LN were removed, cleaned of adherent fat and washed in Dulbecco’s balanced salt solution A + B (DAB) before fixation by immersion in 3.0% (v/v) glutaraldehyde in 0.1 M-sodium cacodylate buffer (pH 7.4) at 4 °C for 2–3 h.
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Embedding for autoradiography

Tissues to be examined by light microscopy were dehydrated through a graded ethanol series and embedded in methacrylate resin, according to the manufacturers, instructions (Polysciences Data Sheet, 123, May 1976). Sections were cut at 1.5 µm and prepared for autoradiography according to the method of Kopriwa & Leblond (1962) using Ilford G-5 emulsion. The sections were developed after 14 days using Amidol developer (Rogers, 1973) and stained with toluidine blue.

After fixation, specimens for electron microscopy were thoroughly washed in 0.1 M sodium cacodylate (pH 7.4) containing 3.0 mM calcium chloride. Samples were post-fixed in 0.1% (w/v) osmium tetroxide in 0.1 M sodium cacodylate (pH 7.4) for 90 min at 4 °C. All specimens were dehydrated in graded ethanol and embedded in Epon 812 resin before sectioning. Thin sections (100-150 nm) were cut on a Reichert OM U3 ultramicrotome and prepared for electron microscopic autoradiography by a loop technique (Caro & van Tubergen, 1962) using Ilford L-4 emulsion. After appropriate exposure, the preparations were developed with D-19 developer (Eastman Kodak Co.), double stained with uranyl acetate and lead citrate before examination in a Philips EM301 microscope.

Extraction of sulphated material from lymph nodes labelled in vivo

The popliteal LN of six rats were antigenically stimulated and [35S]sulphate was administered as described above. At 2.5 h after the injection of the [35S]sulphate-labelled LN were excised and stripped of their adherent fat. The cleaned LN were then partially incised and washed before being gently crushed between two flat plastic surfaces to remove as many lymphocytes as possible from the nodes before solubilization.

The LN residue was washed twice in 0.15 M NaCl, 50 mM Tris·HCl (pH 7.4) (Tris/saline) and transferred to a Dounce homogenizer at 0 °C. The labelled material was extracted from the LN fragments by gentle homogenization in Tris/saline containing 5 0 % (v/v) Tween 40, using a loose-fitting pestle. After centrifugation at 300 g (av.) for 15 min to remove nuclei and large insoluble debris, the supernatant was decanted and brought to 2% (w/v) with sodium deoxycholate. Solubilization of the membrane vesicles was assayed by homogenization with a tight-fitting pestle. Insoluble sub-cellular components remaining after 30 min at 0 °C were removed from the supernatant by centrifugation at 100 000g (av.) for 1 h.

The resulting particle-free supernatant was passed down a column of Sephadex G-25 (1.6 cm x 25 cm) equilibrated with Tris/saline containing 5% (v/v) Tween 40 and 2% (w/v) sodium deoxycholate, at 18 °C, to separate the high molecular weight components from free [35S]sulphate or intermediates of low molecular weight.

The material excluded from Sephadex G-25 was applied to a column of DEAE-cellulose (1.6 cm x 10 cm) equilibrated with Tris/saline at 4 °C. The sample was applied directly to the column at a rate of 1.0 ml min⁻¹ and washed through with Tris/saline until the u.v. absorbance (at 205 nm) of the eluate returned to zero. The column was eluted with a linear salt gradient ranging from 0.15 M to 1.0 M NaCl in 50 mM Tris (pH 7.4). The fractions in the region of the elution profile (Fig. 4) containing the two peaks of radioactivity were collected, pooled and dialysed against excess Tris/saline overnight at 4 °C before application to a column of epichlorhydrin, triethanolamine (ECTEOLA)-cellulose (1.6 cm x 5.0 cm). Binding and elution was carried out as for DEAE-cellulose but using a reduced flow rate of 0.2 ml min⁻¹.

Extraction of sulphated material secreted by LN slices maintained in vitro

Superficial and deep cervical LN were excised from six rats and cleaned as before. The cleaned LN were cut into slices approximately 1.0 mm thick with sharp scissors, thoroughly washed and equilibrated with DAB containing 7 mM-glucose. The slices were finally resuspended in 20 ml of DAB containing 7 mM-glucose and 100 µCi carrier-free [35S]sulphate. Following incubation at 37 °C for 1 h the medium was discarded and the slices were thoroughly washed in DAB containing 7 mM-glucose. The labelled slices were then incubated for a further 2 h at 37 °C in 10 ml of DAB containing 7 mM-glucose. This medium was carefully decanted from the tissue slices and centrifuged at 300 g (av.) for 15 min to remove free lympho-
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cytes. The resultant cell-free supernatant was fractionated on Sephadex G-25 equilibrated with Tris/saline and on DEAE-cellulose columns, as described above. As first experiments showed that no further purification was obtained by fractionation on (ECTEOLA)-cellulose this step was subsequently omitted (Fig. 4).

Equilibrium density-gradient centrifugation

The purified radioactive material was fractionated under both dissociative and associative conditions. Experiments performed under dissociative conditions (Hascall & Sajdera, 1969) used aqueous mixtures (12 ml) containing the radioactive sample, with bovine serum albumin (2 mg) as protein/glycoprotein carrier and bovine tendon proteoglycan (2 mg) as proteoglycan carrier. Guanidinium chloride was added to a final concentration of 4 M and sufficient caesium chloride to give an initial density of approximately 1.4 g ml\(^{-1}\). The samples were centrifuged for 72 h at 20 °C in an MSE superspeed 75 ultracentrifuge (6 x 14 ml swing-out rotor; 100,000 g (av.)). After centrifugation, the gradient was divided into 12 fractions, the densities of which were determined gravimetrically. The fractions were dialysed against running tap water overnight at room temperature and analysed for protein (Lowry, Roseborough, Farr & Randall, 1951) and hexuronic acid (Bitter & Muir, 1962). Radioactivity was measured by liquid scintillation spectroscopy (Ford & Hunt, 1973).

Experiments under associative conditions were carried out as described above except that guanidinium chloride was omitted and the starting density was approximately 1.3 g ml\(^{-1}\).

Discontinuous polyacrylamide gel electrophoresis

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis was performed under non-reducing conditions in the presence of 0.1% SDS as described by Neville (1971). The gels were prepared in glass tubes of internal dimensions 0.6 cm x 7.4 cm, and consisted of a separating gel (6.0 cm) containing 7.5% (w/v) polyacrylamide and a stacking gel (1.0 cm) containing 3% (w/v) polyacrylamide.

Solubilization of the samples was carried out after the method of Laemmli (1970) in the absence of β-mercaptoethanol. After clearing the mixture by centrifugation (40,000 g (av.) for 30 min) 50 µl and 100 µl samples were loaded onto the gels and electrophoresed at room temperature at 2.0 mA per gel.

Gels were fixed and stained for protein with Coomassie Brilliant blue R (Fairbanks, Steck & Wallach, 1971).

RESULTS

Light and electron microscopic autoradiography

Injection of \([^{35}S]\)sulphate into the hind footpads of rats resulted in a marked localization of radioactivity to the majority of high endothelial (HE) cells, as determined by autoradiography of 1.5 µm sections (Fig. 1). The maximum diameter of a high endothelial cell is 20–30 µm and consequently if radioactivity were localized to a

Fig. 1. A. Low-power light autoradiograph of the paracortical region of a labelled rat LN, 15 min after injection of \([^{35}S]\)sulphate into the footpad. Three HE venules are visible (arrows), two cut in cross-section and the third cut obliquely through a bifurcation in the vessel. Note the irregular distribution of autoradiographic grains across the vessel walls. B. High-power light autoradiograph across HE venule of rat LN paracortex, showing grains associated with HE cells. Again the uneven concentration of label is clear. Lymphocytes are visible within the lumen of the vessel (l) and in the basement membrane (bm). The basement membrane itself is marked bm and the outline indicated by arrowheads.
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small part of the cell, as is suggested below, it appears probable that all HE cells are labelled, but in some cells the section missed the site of localization. In contrast, injection of [\(^{3}\)H]leucine, [\(^{3}\)H]galactose and [\(^{3}\)H]fucose failed to produce such a well-defined localization of radioactivity to the HEV at any stage.

The time-course study of LN removed between 15 min and 24 h after footpad injection indicated that the heaviest labelling was found at 15 min. The intensity of radioactivity had begun to wane by 1 h after administration and continued to diminish, so that at 24 h there was little evidence of a specific localization anywhere in the LN except for certain mast cells (Ford et al. 1978).

![Fig. 2. Electron micrograph autoradiograph of high endothelial cells in LN removed 15 min after injection of [\(^{38}\)S]sulphate into the footpad. Three HE cells (he) are visible in the micrograph, one occupying the majority of the field (he*). The lumen of the vessel is present at the top right (lu) and the basement membrane (bm) is visible across the bottom of the micrograph. A lymphocyte (ly) can be seen in transit between the endothelial cells whilst a second (ly*) is seen held up by the basement membrane. At this time radioactivity is exclusively confined to a small vesicular region of the he* cell (in the middle of the field), which contains the Golgi apparatus.](image)

Apart from the HEV, the mast cells in the vicinity of the sub-capsular sinus were the only other type of cell within the lymph node to show appreciable labelling with [\(^{38}\)S]sulphate, although the time-course of this labelling was clearly different from that of the HEV. No significant labelling of mast cells was noted until approximately 3 h after injection and no other vascular endothelium, including that of the spleen,
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liver, lung and kidney was labelled at any time. However, mucus-secreting areas of the larynx and gut were found to be heavily labelled within 1 h of footpad injection.

Electron microscopic (EM) autoradiography revealed that by 30 min after footpad injection the radioisotope was localized to the Golgi apparatus and associated smooth-walled vesicles (Fig. 2). Much of the label had been lost from the Golgi apparatus after 1 h, without any substantial increase in radioactivity associated with the smooth-walled vesicles. This trend continued until virtually all the label had left the Golgi apparatus by 2 h after injection. Vesicles throughout the HE cell cytoplasm were sparsely labelled and in some instances the nuclear membrane was seen to be lightly labelled (Andrews et al. 1980). At no time was any concentration of label seen near the plasma membrane and no evidence was obtained to show that [35S]sulphate-labelled material was incorporated into the plasma membrane. Since the Golgi apparatus and most of the labelled vesicles were usually found on the luminal side of the nucleus (Ford et al. 1978), it is conceivable that this material was secreted directly into the lumina of the venules and so was swept away.

![Graph](image)

Fig. 3. Rate of incorporation of [35S]sulphate into macromolecules synthesized by the HE cells within rat LN. Material isolated by detergent extraction of labelled LN was passed down a Sephadex G-25 column (O—O). Mean recoveries of high molecular weight (high Mr) radioactivity from 8 rats; (●—●) mean recoveries of low molecular weight (low Mr) sulphate from the same experiments.

**Extraction of [35S]sulphate-labelled material from lymph nodes labelled in vivo**

An attempt was made to isolate the [35S]sulphate-labelled molecule from the HEV by the methods described. Before purification of the molecule was attempted it was necessary to determine the *in vivo* incubation time when differential incorporation of radioactivity into the HEV was maximal with respect to other cell types within the LN.

Fig. 3 shows the distribution of high and low molecular weight radioactive material in labelled popliteal LN removed at various times after footpad injection of [35S]sulphate. Measurement of the high molecular weight sulphated material (excluded from Sephadex G-25) showed a steady rise in macromolecular [35S]sulphate recovered over the initial 2-h period, which reached a peak approximately 2.5 h after injection before gradually tailing off. The radioactivity associated with the low molecular weight
material (included by Sephadex G-25) was found to be maximal at the earliest time intervals observed, after which it declined rapidly at a rate similar to that seen by autoradiography. The early autoradiographic localization appears to be a reflection of the total radioactivity in both the high and low molecular weight labelled components. The nature of the low molecular weight labelled material is unknown. Paper electrophoresis of the low molecular weight fraction revealed the presence of a substantial amount of a low molecular weight, radiolabelled component other than free sulphate. Whether this represented nucleotide sulphate (Goldberg, 1961; George, Singh & Bacharvat, 1970) or some other low molecular weight carrier or molecule (Hardingham & Muir, 1972) was not determined.

The time between injection and removal of the LN that produced the highest proportion of high molecular weight, $^{35}$S-labelled material was 2.5 h. By this time the extractable high molecular weight labelled fraction was at a maximum, whilst no other cell type within the LN was significantly labelled, as judged by autoradiography. It was therefore concluded that the bulk of $^{35}$S-sulphate-labelled material extracted at this time was of HE origin and that most of the HE cells within the lymph nodes contributed to this pool of labelled material.

**Purification of $[^{35}]S$-labelled material from LN incubated in vivo and in vitro**

Partial purification of this labelled material was carried out by the methods described. Table 1 shows the recovery of radioactivity from two typical experiments at selected stages of the purification procedures. Relative specific activities were not calculated, since the protein content of the fraction could not be determined because of the high content of detergent in the early stages of the isolation procedure in vivo,

<table>
<thead>
<tr>
<th>Source</th>
<th>Total activity (d.p.m.) recovered after incubation in vivo</th>
<th>Total activity (d.p.m.) recovered after incubation in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>$1.58 \times 10^{10}$</td>
<td>$1.96 \times 10^{10}$</td>
</tr>
<tr>
<td>Lymph node</td>
<td>$1.54 \times 10^{7}$</td>
<td>$1.44 \times 10^{6}$</td>
</tr>
<tr>
<td>Particle-free supernatant</td>
<td>ND</td>
<td>$3.93 \times 10^{4}$</td>
</tr>
<tr>
<td>Cell-free medium</td>
<td>$2.15 \times 10^{7}$</td>
<td>ND</td>
</tr>
<tr>
<td>G-25 excluded</td>
<td>$2.06 \times 10^{8}$</td>
<td>$3.42 \times 10^{4}$</td>
</tr>
<tr>
<td>G-25 included</td>
<td>$1.71 \times 10^{7}$</td>
<td>$6.9 \times 10^{4}$</td>
</tr>
<tr>
<td>DEAE retarded</td>
<td>$1.85 \times 10^{8}$</td>
<td>$2.54 \times 10^{4}$</td>
</tr>
<tr>
<td>DEAE unretarded</td>
<td>$1.3 \times 10^{8}$</td>
<td>$7.95 \times 10^{3}$</td>
</tr>
<tr>
<td>ECTEOLA retarded</td>
<td>ND</td>
<td>$2.5 \times 10^{4}$</td>
</tr>
<tr>
<td>ECTEOLA unretarded</td>
<td>ND</td>
<td>$1.0 \times 10^{4}$</td>
</tr>
</tbody>
</table>

Initial activity refers to that administered to the animal or added to the incubation medium. Particle-free supernatant refers to the activity in the 100000 g (av.) supernatant, whereas, cell-free medium refers to the activity remaining in the analogous supernatant after incubation in vitro.

ND, not determined.
In vitro and in vivo

Suphadex G-25

Fraction no.

Fig. 4. Protein and radioactivity elution profiles from the various columns used in the isolation procedures. Both the material isolated from in vitro and in vivo labelled LN were purified under the same conditions. The profiles on the left of the figure represent the extraction from in vitro labelled LN and those on the right show the profiles from in vivo labelled LN on comparable columns. (-) u.v. profile (205 nm); (-.-) radioactivity profile; (x) point of application to column, wash, and gradient application.
the low absorption at 205 nm of the purified molecule and its uncertain chemical nature.

Clearly a higher recovery of macromolecular [35S]sulphate was obtained using the in vitro system. Ultraviolet light absorption at 205 nm (see Fig. 4) indicated that this difference in radioactivity was not entirely due to a higher specific radioactivity of the molecule but also to a substantially higher total amount of u.v.-absorbing material recovered.

The major loss of [35S]sulphate in vivo appeared to be to other sulphate-utilizing sites of the body. Mucus-secreting areas of the gut and larynx were found, by autoradiography, to be extremely heavily labelled by 1 h after footpad injection. No such loss occurred during incubation in vitro. Consequently, a substantial quantity of free [35S]sulphate was carried over into the chase medium both by absorption to the tissue and from within the HE cells themselves. These cells, like other endothelium, are capable of transporting material by diacytosis (Simionescu, 1980). This involves the cells randomly ingesting material from the luminal surface by micropinocytosis, transporting it through the cytoplasm within the vesicle and releasing it at the other side. Consequently, maintaining the lymph node slices in a relatively high concentration of [35S]sulphate in serum-free medium resulted in the continual release of [35S]sulphate into the chase medium (Andrews et al. 1980). Such a process explains the presence of the substantial peak of low molecular weight [35S]sulphate recovered from the Sephadex G-25 column after incubation in vitro.

Preliminary characterization of the sulphated macromolecule

The nature of the 35S-sulphated macromolecules released into maintenance medium and isolated from labelled lymph nodes was investigated by caesium chloride equilibrium density-gradient centrifugation. Under dissociative conditions selected to disrupt the strong binding between protein and proteoglycan (Fig. 5A), more than 90% of the radioactivity was located with the protein carrier in the fractions with a density of less than 1.35 g ml⁻¹. These results showed that the sulphate had not been incorporated into proteoglycan. In experiments designed to determine whether the sulphate had been incorporated into protein or lipid, the labelled material was analysed under associative conditions. Fig. 5B demonstrates that the sulphate-labelled sample sedimented as two peaks of approximately equal activity, one with a buoyant density less than 1.20 g ml⁻¹, the other with a buoyant density close to that of the protein carrier, bovine serum albumin. This suggested that the [35S]sulphate was being incorporated into a protein as well as a low-density material, possibly lipid.

The compositions of the purified samples were investigated further by polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS) (Fig. 6). The results showed that the sample obtained from LN culture medium contained only one major protein-staining band while the equivalent detergent-extracted sample appeared more heterogeneous. In neither gel was there a clear relation between the profiles of radioactivity and protein. However, there was a small peak of radioactivity associated with the major protein band from the sample of LN culture medium, but this was very small compared with the broad peak running ahead of the dye front. This suggests
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Fig. 5. Equilibrium CsCl density-gradient centrifugation of purified [35S]sulphate-labelled macromolecules in: A, the presence of 4 M-guanidinium chloride, and B the absence of guanidinium chloride. When analysed under associative conditions the sample had a starting density of 1.3 g ml⁻¹ and included serum protein and tendon proteoglycans as carrier macromolecules. In the presence of guanidinium chloride the starting density was 1.4 g ml⁻¹. After centrifugation the fractions were assayed for radioactivity (x—x); protein (□—□); hexuronic acid (○—○) and density (——). Under dissociative conditions no protein was detected in fractions 5-10 inclusive.

that the label was incorporated into lipid, since these molecules are reported to run ahead of the dye front under the conditions described (Lenard, 1970; Carraway & Kobyła, 1970; Glossman & Neville, 1971).

A significant amount of protein was found to be unable to enter the running gels, particularly in the detergent-extracted sample. Reduction of the samples with β-mercaptoethanol resulted in the migration of all Coomassie brilliant blue staining material into the gel but did not change the distribution of radiolabel.

DISCUSSION

The rapid incorporation of [35S]sulphate into the high endothelial cells of LN has been detected by autoradiography after administering [35S]sulphate either in vivo or in vitro. The early localization of 35S in the HE cell is confined to the Golgi apparatus,
Fig. 6. SDS–polyacrylamide gel electrophoresis of the $[^{35}S]$sulphate-labelled material isolated from: A, LN culture medium, or B in vivo labelled LN. The top half of the figure shows a densitometric scan of the gels after staining with Coomassie brilliant blue R, whilst the lower half shows the profile of radioactivity from each gel. The dye front is marked with an arrow (D/F) and the positions of molecular weight marker proteins are also indicated.
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suggesting that to be the site of incorporation. The sulphated material is believed to be secreted by the cell rather than broken down internally because: (1) electron microscope autoradiographs prepared from LN removed serially after injection indicated that $^{35}$S-labelled material persisted in cytoplasmic vesicles after it had been lost from the Golgi apparatus (this is compatible with the conclusion of Cläesson, Jørgensen & Röpke (1971) that the prominent Golgi apparatus and associated vesicles of HE cells indicates active secretion); and (2) high molecular weight $^{38}$S-labelled material was consistently released from LN slices into the culture medium.

No evidence was found of any localization in the vicinity of the cell membrane and it was not certain whether the secretion of the material occurred all around the periphery of the cell or predominantly in one direction. Although the Golgi apparatus was most frequently situated on the luminal side of the nucleus, suggesting that secretion occurs into the blood stream, the possibility remains open that the sulphated material may be secreted through the basement membrane into the extravascular space of the LN.

The possible significance of the large Golgi apparatus of the HE cells has been stressed previously by several groups (Röpke et al. 1972; Van Deurs & Röpke, 1975; Wenk, Orlic, Reith & Rhodin, 1974; Kittas & Henry, 1979). The present work suggest an approach to the elucidation of the secretory products of these cells. It is natural to assume that this secretory activity is somehow related to selective lymphocyte migration from the blood and several possibilities for the precise role of the secretion have been suggested (Andrews et al. 1980). We have started on three approaches towards clarifying the glandular activity of HE cells as follows: (1) functional studies on the influence of partially purified $^{38}$S-labelled material on lymphocyte traffic (preliminary observations have already been reported; Andrews et al. 1980). (2) Comparative measurement of rates of incorporation of $^{36}$S-sulphate and the secretion of the product under a number of conditions including a deficit or a surplus of recirculating lymphocytes. This may determine whether the secretory activity of HE cells facilitates lymphocyte traffic or is a consequence of lymphocyte traffic. (3) Biochemical characterization of the sulphated material. The preliminary findings included in this paper indicate that Röpke et al. (1972) were justified in their scepticism of the evidence that HE cells contain an acidic proteoglycan.

Whether the $^{35}$S-labelled material was obtained from the LN homogenate after administering $^{35}$S-sulphate in vivo, or from the culture medium of LN slices, the behaviour of the radioactive material during gel permeation chromatography clearly showed that the $^{38}$S-sulphate was in macromolecular form. Isopycnic density-gradient centrifugation in cesium chloride under dissociative conditions, showed that at least 90% of the applied radioactivity was not associated with proteoglycan. This was further supported by the failure to detect hexuronic acid in the purified fractions (Andrews et al. 1980) and the behaviour of the radiolabelled molecules on ECTEOLA-cellulose (Ringertz & Reichard, 1959; Anseth & Laurent, 1961; Anseth, Antonopoulos, Bjella & Fransson, 1970). Under the conditions described it was found that proteins, glycoproteins and proteoglycans had different elution characteristics. Bovine serum albumin was found to be unretracted by the column. Ovalbumin and
chondroitin sulphate were both retarded. However, whereas ovalbumin eluted within the range of the NaCl gradient used, chondroitin sulphate required a substantially greater Cl− concentration for elution.

Moreover, distribution of the label following caesium chloride centrifugation under associative conditions suggested that the HE cells incorporated [35S]sulphate into molecules containing both lipid and protein.

The elution characteristics of the labelled molecule from DEAE-cellulose were found to vary depending on the source of material. At least two distinct radioactive peaks were obtained from detergent-solubilized LN whereas only one broad peak was obtained from material isolated from LN maintenance medium (Fig. 4c). Such a difference can be accounted for if each labelled peak from the detergent-extracted sample represented the sulphated molecule at different stages of its synthesis and secretion. As the smaller peak in Fig. 4d is eluted at a similar salt concentration (approx. 0.25–0.3 M-NaCl) to the radioactive peak in Fig. 4c this material is likely to represent the complete, secreted molecule. Thus, the major radioactive peak in Fig. 4d may therefore consist of material isolated from within the HE cell at an earlier stage of synthesis.

In the next paper of the series the complexity of the 35S-labeled material will be confirmed and biochemical characterization will be carried further by enzymic hydrolysis and gel permeation chromatography of the labelled molecule. Application of these techniques points to the conclusion that the HE cell secretes a sulphated glycolipid in close and possibly significant association with one or more glycoproteins.

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REFERENCES


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