A New Small Molecular Tracer for Permeability Studies with the Electron Microscope

The paths taken by various substances in passing across endothelial and other barriers have been fairly extensively studied[1-12]. It has been shown that most small lipid-insoluble molecules pass through intercellular junctions, even in the presence of zonulae occludentes, and also through fenestrae. However, large molecules are confined to a much slower path via the smooth small vesicles, again unless fenestrae are present. This accounts for the great difference in the permeabilities of these two classes of molecules through endothelium. The permeability to large molecules is greatly increased by fenestrae, or by open junctions in lymphatics or injured blood vessels. Small protein molecules, e.g. horseradish peroxidase (mol. wt. ~38,000), are unable to pass through some 'closed' junctions, such as those in the brain, but can pass through others. However, all junctions are readily permeable to ions. While factors other than size (e.g. charge) may be important in determining whether a substance can cross a particular barrier, it was felt that a tracer molecule smaller than peroxidase, but larger than ions, would be very useful in permeability studies.

Such a tracer should be non-toxic and have a high mass-density[13], or react with some substance so as to form a product of high mass-density. Ferrous gluconate (mol. wt. 482) was found to fulfill these requirements. It is precipitated by the phosphate ions in MILLONIG's[14] fixative, and can therefore be fixed at the same time as the tissues.

The experiments were performed on the 'sleepy lizard', *Trachydosaurus rugosus* because these animals are easily handled under operative conditions and also little work of this nature has been done on reptiles. The animals were heated to 35 °C, spinalized, and attached to a respirator. Approximately 5 ml of ferrous gluconate (1% in Ringers solution) was then injected into the lumen of the ventricle over a period of 1 min. A 7% solution stopped the heart within a few seconds, whereas the 1% solution allowed it to continue beating. After 5 or 10 min, portions of the heart and aorta were excized and fixed for 2 h in a 4% solution of glutaraldehyde in MILLONIG's buffer. (The buffer contained glucose as a 9% solution.) The tissues were post-fixed in 2% Osmium tetroxide in buffer, dehydrated in acetone and embedded in araldite. Some sections were stained with Lead citrate, but most were examined unstained to avoid confusing tracer with deposits of stain. The tissues from control animals were fixed and embedded in the same manner.

In other experiments the abdomen was opened and the urinary bladder was completely drained. 10 ml of a 7% solution of ferrous gluconate was then instilled into the bladder. After 30 min a piece of the bladder was removed and treated in the same way as the tissues from the heart.

The blood vessels of the heart and bladder, and the intima of the aorta were very similar to those of mammals. The endothelial junctions were closed with zonulae occludentes, although these may have had brief interruptions as shown by KARNOVSKY[1]. The lymphatics in the heart and bladder had open junctions, similar to those in mammals. The controls showed no deposits of tracer but were morphologically identical with the experimental animals. The latter had deposits (20–50 nm) in the vascular lumens, junctions, vesicles and connective tissue (Figures 1 and 2). Some very small deposits (2–5 nm) were attached to the luminal plasma membranes, the membranes of some vesicles and in the zonulae occludentes (Figure 2). There were often continuous deposits through the zonulae; frequently reaching from the lumen to the connective tissues. Occasionally the deposits in

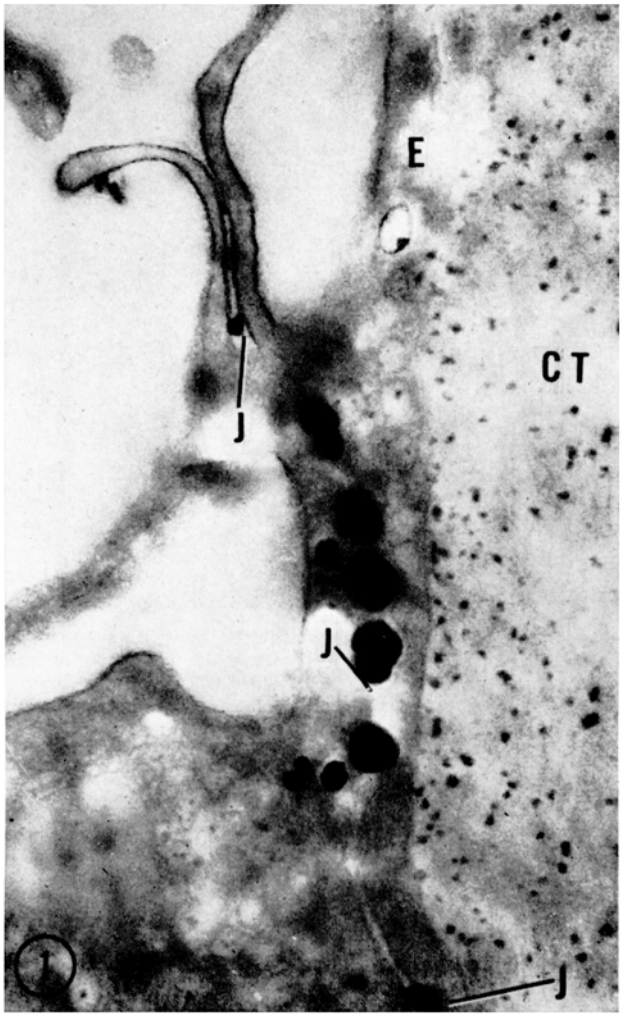

Fig. 1. Aorta. 2 endothelial cells (E) are shown, separated by a junction (JJJ) in which precipitates of tracer can be seen. Other particles appear in the lumen, in the connective tissue (CT) and in some endothelial vesicles. The cellular membranes have poor contrast because the section was unstained to avoid confusing deposits of stain with deposits of tracer. It is evident, then, that some portions of the plasma membranes have tracer adhering to them – notably on the luminal side of the cells and in front of the junction. ×70,000.

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the junctions had aggregated, and presumably distorted the adjacent plasma membranes, to form quite large deposits (Figure 1); usually they were quite small and discrete (Figures 1 and 2). In the heart the tracer had passed through the connective tissues and into the lymphatics. No tracer was found in unexpected, and possibly artefactual places, such as the cytoplasmic matrix, the nucleus, etc.

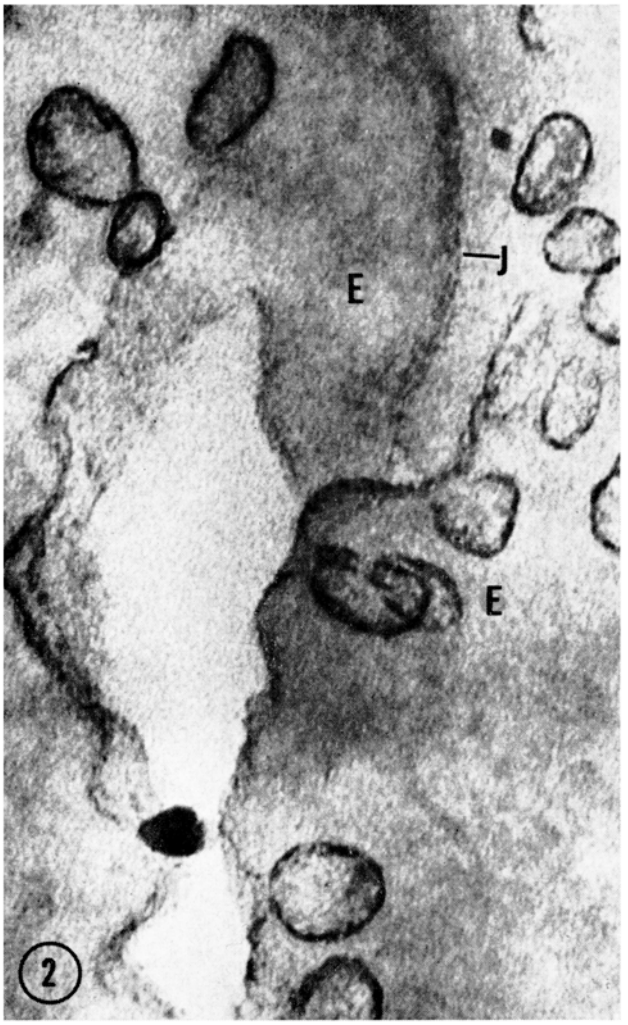

Fig. 2. Heart capillary. Small particles of tracer can be seen in a zonula occludens in a junction (J) and on the membranes of small vesicles in the cells. Unstained. ×170,000.

The bladder showed a similar localization of tracer to that of the blood vessels, i.e. in continuous lines down the junctions from the lumen, through the zonulae occludentes, to the connective tissue, and in the small vesicles. It had also passed into the vascular endothelial barriers, but any in the lumens had been washed away. These findings for the bladder should be regarded with some caution, because a 7% solution, as used in the bladder, was toxic to the heart.

These results show that Ferrous gluconate, with a mol. wt. of ~500, is a useful tracer. It passes through the endothelial barriers, and that of the epithelium of the bladder, via the junctions and the vesicles. Since, however, the vesicles only offer very slow transport relative to the junctions[1-3,6,8,12], we conclude that the junctional pathway is almost certainly the most important in the present instance. (Obviously the vesicles which open at the lumen will pick up some tracer, just as they pick up some of all the molecules in the plasma.) A priori, it would seem that any molecule smaller than 2000–5000 mol. wt. should pass mostly through the junctions[6], which almost certainly represent the pores of PAPPENHEIMER[15]. The zonulae occludentes usually do not stop flow completely, but supply the restraints necessary for molecular sieving[1,7,8,10].

Résumé. Le gluconate de fer a la propriété de suivre les molécules d'un poids moléculaire de ~500. Il traverse avec elles l'endothélium des vaisseaux sanguins et l'épithélium de la vessie en passant par les joints ou, pour une faible part, entre les vésicles.

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[16] We are most grateful for the skilful assistance of Mr. B. R. DIXON and the partial support of the Australian Research Grants Committee.

A Method for Studying the Ultrastructure of Intercellular Contacts in Leukocyte Cultures

To study the ultrastructure of the contacts between cells grown in tissue culture as well as the attachment of these cells to the substratum, it is often desirable to fix and embed the cells in situ in order to preserve the spatial relationship of these contacts. Previous investigators[1,2] have attempted to embed in epoxy resins cells cultured directly on glass coverslips, but, in contrast to results with the less satisfactory methacrylate embedding material[3-8], were unable to separate the coverslips from the epoxy-embedded cells unless the coverslips had been coated prior to culture with extraneous materials. We have briefly reported[9] a method to remove blocks of Epon-embedded leukocytes from uncoated glass coverslips, thereby making it unnecessary to complicate standard culturing procedures by exposing the cells to such extraneous coating substances. FLAXMAN et al.[10] also have recently reported a method by means of which a thin layer of Araldite, another epoxy resin, could be removed from an uncoated glass coverslip containing fibroblasts. In this paper we describe in detail our technique and its application to provide the first electron microscopic study of the unaltered, intercellular contacts between macrophages and lymphocytes grown on glass coverslips in human, peripheral blood leukocyte cultures stimulated to undergo blastogenesis.

Materials and methods. An individual's heparinized, peripheral, venous blood was centrifuged at 300*g* for 5 min. The resulting leukocyte-rich, supernatant plasma