ENDOTHELIAL PERMEABILITY

II. The Passage of Particles Through the Lymphatic Endothelium of Normal and Injured Ears*

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Theoretically there are 4 possible paths by which particles can pass through an endothelial barrier. They can go between the cells, or they can traverse them. If they traverse the cells they may do so through pores, *via* cellular organelles, or by direct penetration of the cytoplasm. Each of these possible routes has been suggested by some workers.

Some light microscopists considered that material passes through the " intercellular cement ", which they held to occupy the intercellular junctions (Chambers and Zweifach, 1940 and 1947 ; Zweifach, 1959). The electron microscope has revealed that the junctions are 50 times narrower than was thought and that the cement, in the sense used by these workers, does not exist (Palade, 1953 ; Buck, 1958 ; Florey, Poole and Meek, 1959). In addition, the adhesion plates (attachment belts), which connect many of the cells are sometimes considered to render the junctions impermeable (Bennett, Luft and Hampton, 1959). Injected particles are rarely seen in the junctions between the endothelial cells of normal blood vessels (Buck, 1958 ; Policard and Collet, 1958 ; Wissig, 1958 ; Palade, personal communication and 1960 ; Jennings, Marchesi and Florey, 1962). However they are often seen in the junctions of traumatized venules (Alksne, 1959 ; Majno and Palade, 1961 ; Marchesi, 1962). In tissues where there is much movement—intestinal villi, the diaphragm and active skeletal muscle in general—the lymphatics have frequent open junctions in which many injected particles may be found (Palay and Karlin, 1959*b* ; French, Florey and Morris, 1960 ; Casley-Smith and Florey, 1961 ; Casley-Smith, 1961, 1962*a*, 1964).

Pappenheimer (1953) and his school considered that material passed through pores of about 9 mμ diameter. These have not been seen with the electron microscope, although they would be well within its resolving power (Bennett *et al.*, 1959). Fenestrae of about 50 mμ diameter do occur, but only in some specialized tissues and in severely hypoxic vessels (Luft and Hechter, 1957 ; Bennett *et al.*, 1959). Injected particles are seen in some of these openings (Farquhar, Wissig and Palade, 1961).

Particles have been shown to cross endothelium in the many small vesicles ($\sim$50 mμ.) which occur in the cells (Palade, 1960 ; Jennings *et al.*, 1962 ; Casley-Smith, 1964). This had already been suggested by many workers including Palade (1953), Bennett (1956), Moore and Ruska (1957), Wissig (1958) and Bennett *et al.* (1959).

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Some particles, *e.g.* ferritin and thorium dioxide, may be able to pass directly into the cytoplasmic matrix ; other particles cannot do this (Hampton, 1958 ; Wissig, 1958 ; Bessis and Breton-Gorius, 1959 ; Jennings *et al.*, 1962 ; Casley-Smith, 1964). It is possible that this is a further method by which certain particles can traverse the cells. (However, Bruns (1963) and Wissig (personal communication) have suggested that the " free " particles are artefacts of osmium fixation.)

It can be seen, therefore, that particles can pass through endothelium in all of the four theoretically possible ways. The relative importance of the different paths depends on a number of factors. One of these is the site of the endothelium, *e.g.* particles pass from the peritoneal cavity into the diaphragmatic lymphatics almost entirely via open endothelial junctions ; open junctions are of much less importance for some of these particles when they pass out of the lymphatics into the surrounding connective tissue (Casley-Smith, 1964). Again, fenestrae are only found in the blood capillaries of some specialized tissues. The nature of the particle is also of great importance in determining the relative amount of it which passes via each of the different paths (Casley-Smith, 1964).

In addition to the site of the endothelium and the nature of the test particles, it is well known that many physiological and pathological factors profoundly affect endothelial permeability (Landis, 1934 ; Yoffey and Courtice, 1956 ; Spector, 1958 ; Rusznyák, Földi and Szabó, 1960). Apart from the open junctions found in the endothelium of traumatized venules, little is known of the morphological endothelial changes produced by these conditions, or of the alterations they induce in the relative amounts of the different substances traversing the various paths. Therefore, in the present work, the effects of some of these conditions have been studied by observing carbon, ferritin and thorium dioxide passing through normal and traumatized endothelium.

The endothelium used was that of the small lymphatics in the external ears of mice and guinea-pigs. The particles were chosen because of the great differences in behaviour which they showed when passing through the endothelium of diaphragmatic lymphatics (Casley-Smith, 1964). In these, some of the ferritin passed through open junctions, but the majority traversed the cells in small ($\sim$50 mμ.) vesicles or by direct penetration of the cytoplasm. Nearly all of the carbon which penetrated the endothelium did so via open junctions. While much carbon entered the small vesicles, these coalesced to form large (0·1–1 μ.) ones. Material in these large vesicles stayed in the cells for at least 3 months ; very little escaped from them. Some ferritin also occurred in large vesicles. This amount was, relative to the amount in small vesicles, much less than the amount of carbon. The behaviour of thorium dioxide was intermediate between that of ferritin and that of carbon.

The permeability of ear lymphatics has been studied by injecting coloured substances into the vessels (Hudack and McMaster, 1932 ; McMaster and Hudack, 1932, 1934 ; Pullinger and Florey, 1935 ; McMaster and Parsons, 1949 ; Miles and Miles, 1958). These workers found that the permeability of lymphatics normally approximates that of blood capillaries (Landis, 1934 ; Grotte, 1956 ; Yoffey and Courtice, 1956 ; Rusznyák *et al.*, 1960). Carbon particles, if they escape at all, do so in small discrete ecchymoses ; dyes escape all along the vessels. The relative rates of passage of the dyes are determined by their size, adsorption onto proteins, and the local fluid flow. However, very mild injuries, such as mild

heat, weak chemical irritants and poisons, or light strokes (even the animal scratching itself) can all cause a most marked and rapid increase in the permeability of the vessels. This change lasts for some hours. For this reason lymphatics have been studied in ears suffering from mild heat oedema, light stroking, raised intra-lymphatic pressure and cyanide poisoning.

The particles have been observed both entering and leaving the lymphatics. There is no good evidence that the direction of passage itself affects the permeability of endothelium, and much evidence to show that it does not do so (Yoffey and Courtice, 1956; Rusznyák *et al.*, 1960).

MATERIALS AND METHODS

Animals.—The mice and guinea-pigs were of albino strains. They weighed about 20 and 90 g., respectively. The anaesthetics were nembutal and urethane, supplemented with open ether.

Injectants.—Ferritin was obtained from horse spleens by the method of Farrant (1954). It was made up as a 5 per cent solution (w/v) in Tyrode's medium. Colloidal carbon (" Pelikan " Indian Ink, Günther Wagner, Hanover) was centrifuged and the supernatant diluted 1 : 3 with Tyrode's medium to give a 5 per cent solution. Thorium dioxide (" Thorotrast ", Testager and Co., London) was diluted 1 : 4 with Tyrode's medium to give a 5 per cent solution. These substances were often injected together. Potassium cyanide was added (0·01 M) in some of the experiments. If observations were to be made with the light microscope and carbon was not included in the injectant, Pontamine sky blue (5 per cent was added to it.

Micro-injection.—Pullinger and Florey's (1935) modification of Hudack and McMaster's (1932) technique was used. A micro-needle was pushed into the ears while they were placed over an illuminated rod. A small amount of the fluid was injected. If the needle was inside a lymphatic, the network of connecting vessels rapidly filled. If the needle was not inside a lymphatic, none became visible and a small blob of injectant was formed. If desired, the needle could then be moved for another attempt at finding a lymphatic. Thus it was possible to inject material either into the lymphatics, or into the connective tissues. However it was not possible to be certain that there was no spillage of injectant. At times some seemed to leave the vessels through the tear where the needle had entered them. Similarly, if the fluid had initially been injected into the connective tissues, it occasionally appeared to enter some lymphatics where they had been torn by the needle. The passage of the dye or carbon into, or out of, the lymphatics was easily observed with a dissecting microscope.

Method of producing oedema.—The animal was anaesthetized and one ear was immersed in water, at 54°, for 4 min. This caused moderate oedema and redness. These were still visible after 6 hr., but subsided after 12–18 hr. The micro-injections were made 1 hr. after the induction of oedema.

Method of traumatising the lymphatics by light strokes.—A fine wire was lightly drawn across the ear to produce a slight " white line " as described by McMaster and Hudack (1932). Material was injected distal to the line and the injectant was seen escaping from the lymphatics where they intersected the line.

Method of raising the intra-lymphatic pressure.—The lymphatics were injected and then occluded with a light rubber-band ligature.

The preparation and observation of the specimens.—After injury the animals were kept anaesthetized. (If there was no injury they were kept anaesthetized from the time of the micro-injection.) Specimens were obtained after intervals of from 1 min.–18 hr. after the micro-injection. If the injection had been made into the connective tissue, a portion of the ear near the centre of the blob was selected. If the injection had been made into a lymphatic, the tissue was studied about 3–5 mm. from the injection site.

Small pieces of the ear were excised under the dissecting microscope. They were rapidly transferred to Caulfield's (1957) osmium tetroxide fixative. This was allowed to act for 2 hr. at 4°. The blocks were then quickly dehydrated and embedded in Methacrylate and Araldite (Glauert, 1961). Some of the blocks were stained by adding phosphotungstic acid to the dehydrating alcohols. Sections were cut with a Huxley ultramicrotome (Cambridge

Instrument Co., London). Some sections were examined unstained to eliminate the possibility of small staining artefacts being confused with the injected particles. Other sections were stained with Lead acetate or with Uranyl acetate (Glauert, 1961). The sections were examined unsupported, or on Formvar or carbon membranes, in a Philips 100B electron microscope at 40 and 60 Kv.

RESULTS

No significant differences are observed between the lymphatics of mice and guinea-pigs.

Morphology of the Lymphatics

Normal Lymphatics.—The morphology of the lymphatics, as revealed by the electron microscope, has been described by Weiss (1955), Palay and Karlin (1959*a*), French *et al.* (1960), Casley-Smith and Florey (1961), and Fraley and Weiss (1961). In particular Casley-Smith and Florey studied the lymphatics in the ears of mice and guinea-pigs.

The diameters of the lymphatic capillaries are much larger than those of blood capillaries (Figs. 1, 17). The endothelium is very similar to that of the blood capillaries, but is usually rather thicker and possesses a less dense cytoplasmic matrix (Figs. 1, 17). The basement membranes of the lymphatics are much less evident than those of blood capillaries (Figs. 2–6) ; there may even be none visible. The endothelial intercellular junctions of normal ear lymphatics are usually closed and lack adhesion plates (Figs. 1, 3, 4, 6). A few junctions are seen open over part or all of their length. These are rare—about one in every 10–20 cross-sections of the vessels. Apart from these differences, the lymphatic endothelium resembles that of blood vessels in possessing all the normal cellular organelles. In particular there are many small vesicles ($\sim$50 mμ.), a little endoplasmic reticulum partly covered with RNA-containing particles, a Golgi complex, and the cytoplasmic matrix (Figs. 2–6).

Heat injury.—The most impressive alteration is the presence of many open and partly open endothelial junctions (Figs. 7–10). There are 1 or 2 of these in nearly every cross-section of a lymphatic, *cf.* 1 per 10–20 cross-sections in normal ears. The cells are sometimes very widely separated, as if they had been pulled apart (Fig. 9).

Some of the cells appear quite normal (Fig. 8) ; others are very swollen with pale, almost empty cytoplasms (Figs. 10–15). These probably contain fewer small vesicles than do normal cells. The plasma membranes of the injured cells sometimes have large gaps and may be absent altogether over much of the cell (Figs. 13–15). There are often large, irregular, nearly empty vacuoles in the cells (Figs. 7, 10–13). Some of these have RNA-containing granules on their outer surfaces (Figs. 10, 12). It would seem therefore that these represent dilated elements of the " rough " endoplasmic reticulum. The " smooth " elements of this organelle presumably give rise to the vacuoles which do not possess granules. The vacuoles sometimes contain a little plasma protein, or a few of the injected particles. They are quite distinct from the large vesicles which are densely packed with particles (*vide-infra*). At times dilated and distorted mitochondria are seen (Fig. 13).

The basement membranes are sometimes visible (Figs. 7, 9–11, 13, 14). However, it is not possible to be sure if they are altered as they are normally so variable.

There is often a gap between the cell and the connective tissue (Figs. 10, 12, 14).

Light Stroking.—There are many open junctions (Fig. 16). One or two are seen in each lymphatic cross-section. At times it appears that the cells may have been torn. Other than this they appear normal.

Raised intra-lymphatic pressure.—Many open junctions are seen (1–2 per lymphatic cross-section). The cells are often widely separated, as if they had been pulled apart by the dilation of the vessel. This dilation is readily observed with the dissecting microscope. The cells themselves appear normal.

Cyanide poisoning.—Here also there are many open and partly open junctions (Figs. 17–19). Again one or two are seen in each lymphatic cross-section (Fig. 17).

The cells often appear abnormal. There are frequent localized pale blebs on their luminal surfaces (Figs. 17, 19). Many mitochondria are dilated and distorted (Fig. 18). Often there are large vacuoles, some with external RNA-containing granules indicating their endoplasmic reticular origin. There is frequently a gap separating the cells from the connective tissue.

The sites in the endothelium where the particles are seen

These observations are summarized in the Table.

TABLE.—*The Amounts of the Particles in the Various Endothelial Sites under Different Conditions*

		Junctions	Small vesicles	Large vesicles	Free
Normal	C	−	+	+++	−
	T	+	++	+++	+
	F	+	+++	++	++
Heat Oedema	C	+++	+	+++	++
	T	+++	+	+++	+++
	F	+++	++	++	+++
Light Touch	C	+++	+	+++	−
	T	+++	++	+++	+
	F	+++	+++	++	++
Raised Intra-lymphatic Pressure	C	+++	+	+++	−
	T	+++	++	+++	+
	F	+++	+++	++	++
Cyanide poisoning	C	+++	+	+++	−
	T	+++	++	+++	++
	F	+++	+++	++	+++

These results are based on visual estimation of the amounts of material in the various sites. The symbols C, T, and F stand for carbon, Thorium dioxide and ferritin, respectively.

When the junctions are open, many particles are seen in them (Figs. 7–9). Closed junctions occasionally contain a few particles of ferritin or thorium dioxide (Figs. 3, 4). Usually they are empty (Fig. 6). Carbon is never seen in closed junctions.

All 3 types of particles are often seen entering the cells *via* the normal, small (~50 mμ.) caveolae intracellulares (Figs. 2, 3, 11, 20) and, less frequently, *via* large (0·1–1 μ.) ones. The particles in these larger indentations sometimes seem to have been enfolded by a cellular projection (Fig. 5).

EXPLANATION OF PLATES

FIG. 1.—Normal mouse ear, 1 min. after the injection of thorium dioxide into the lymphatics. Some of the particles are visible in the lumen (L) of a lymphatic. The endothelium (E) of the lymphatic capillary is thicker, but less electron-opaque, than that of a blood capillary (BC). The lymphatic capillary is quite a small one, yet it is much larger than the blood capillary. There are about seven intercellular junctions (J) in the lymphatic endothelium. All of them are closed. The connective tissue (CT) contains many collagen fibres (COL) embedded in the ground substance. (Methacrylate; stained with Phosphotungstic acid. ×4000.)

FIG. 2.—Normal guinea-pig ear, 15 min. after the injection of ferritin into the lymphatics. There are many molecules in the lumen (L), and considerable numbers in the endothelium (E) and in the connective tissue (CT). Ferritin occurs in the endothelium in small vesicles (SV), small caveolae intracellulares (CI) and a large vesicle (LV). Some molecules are lying free in the cytoplasm (small arrows). The direction of travel of the microtome knife is shown by the large arrow. (Methacrylate; unstained. ×80,000.)

FIG. 3.—Normal mouse ear, 20 min. after the injection of ferritin into the lymphatics. There are many molecules in the lumen (L), the connective tissue (CT) and in the endothelium (E). The long, closed junction (JJJ) contains few particles. In the cells ferritin is seen in small caveolae (CI), small vesicles (SV), free in the cytoplasm (small arrows) and, occasionally, very close to the plasma membranes. (The direction of the microtome knife is shown by the large arrow.) A basement membrane is visible (BM). It seems to offer little hindrance to the passage of the molecules. (Methacrylate; stained with Lead. ×60,000.)

FIG. 4.—Normal mouse ear, 2 hr. after the injection of ferritin into the lymphatics. Molecules are visible in the lumen, connective tissue, small vesicles, large vesicles, free in the cytoplasm and perhaps penetrating the plasma membrane (small arrows). Some of these last particles were only reached by the knife after it had traversed almost empty cytoplasm, hence they are unlikely to be particles which have been carried from elsewhere. (The direction of the knife is shown by the large arrow.) A closed junction (JJ) contains some molecules. (Methacrylate; unstained. ×60,000.)

FIG. 5.—Normal mouse ear, 2 hr. after the injection of ferritin into the lymphatics. Particles can be seen in two large vesicles (LV) and in some small ones (SV). Some are enfolded by a projection of the endothelium. (Methacrylate; unstained. ×80,000.)

FIG. 6.—Normal mouse ear, 1 min. after the injection of ferritin into the lymphatics. There are many molecules of ferritin free in the cytoplasm (small arrows). A closed junction (J) is present. (The direction of the microtome knife is shown by the large arrow.) (Methacrylate; unstained. ×60,000.)

FIG. 7.—Guinea-pig ear, injured by heat, 5 min. after the injection of ferritin into the lymphatics. There is a complex, partly open junction (JJJ). There is a little ferritin in the cells, but more in the open junctions. A basement membrane (BM) is visible. In one area it seems to have slightly impeded the passage of the ferritin, but many particles have entered the connective tissue. One cell has two large, nearly empty vacuoles. These are probably dilated elements of the endoplasmic reticulum. (Methacrylate; unstained. ×44,000.)

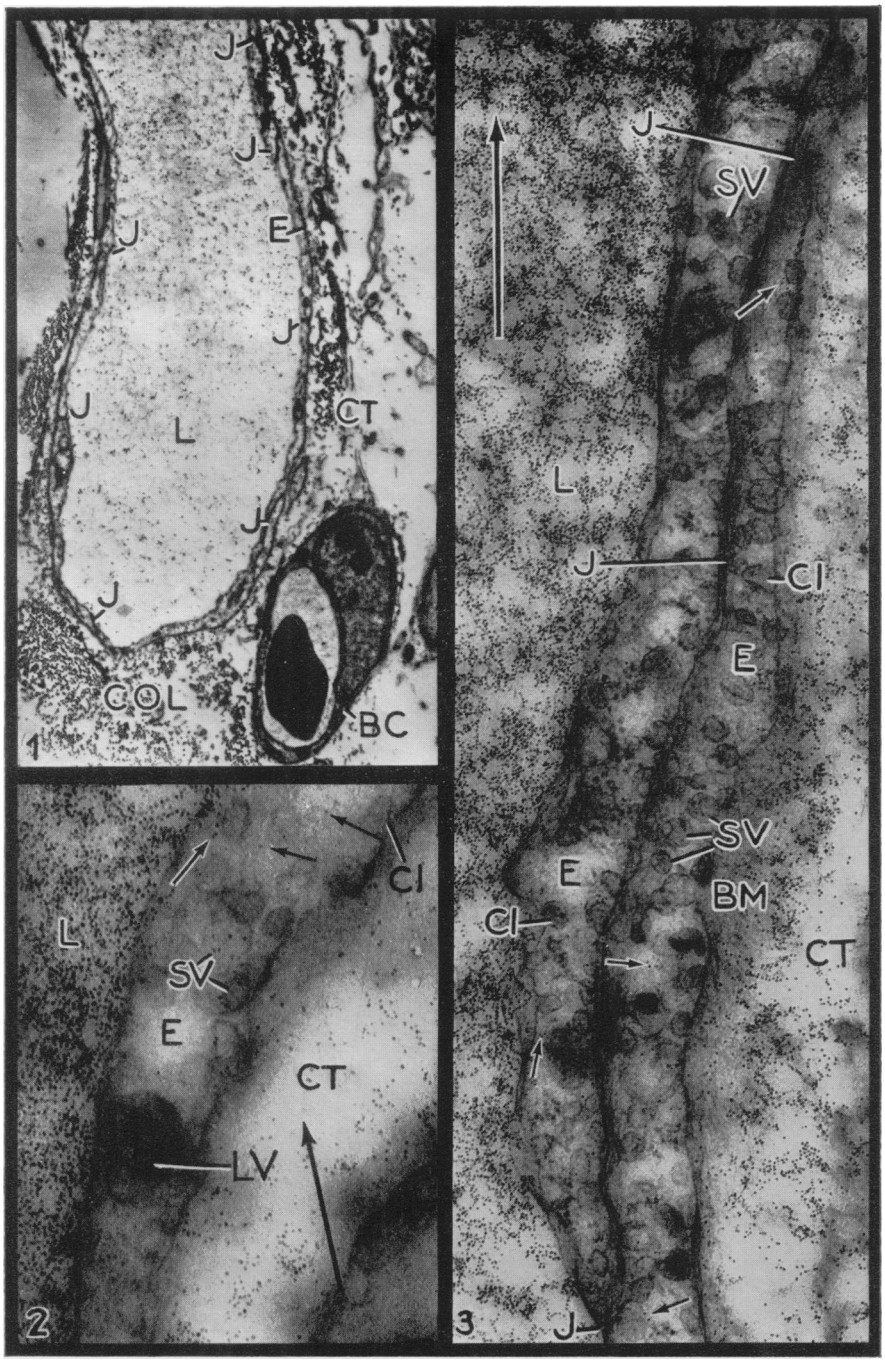
FIG. 8.—Mouse ear, injured by heat, 30 min. after the injection of carbon into the lymphatics. Many particles are leaving the vessel via an open junction (JJ). Others occupy a large vesicle (LV). (Araldite; stained with Uranyl acetate. ×71,000.)

FIG. 9.—Mouse ear, injured by heat, 5 min. after the injection of ferritin into the connective tissues. There is an open junction (JJJ) between two widely separated endothelial cells (E). A basement membrane (BM) does not at all impede the passage of large numbers of ferritin molecules into the lumen. (Araldite, stained with Phosphotungstic acid. ×28,000.)

FIG. 10.—Mouse ear, injured by heat, 1 min. after the injection of carbon into the connective tissues. No particles have yet reached this area. The cells are very swollen, with pale cytoplasmic matrices and few small vesicles. There is a large, pale, nearly empty vacuole (arrow) which has many RNA-containing particles attached to its exterior. These demonstrate its endoplasmic reticular origin. A junction (J) is open over part of its length. Around much of the vessel a basement membrane (BM) can be seen. In some areas (X) there is a gap between the cells and the basement membrane. (Methacrylate; unstained. ×20,000.)

FIG. 11.—Guinea-pig ear, injured by heat, 15 min. after the injection of ferritin into the connective tissues. Some molecules are visible in the cell in small vesicles and caveolae. Free particles are also seen. There is a large, irregular, nearly empty vacuole. (Araldite, stained with Phosphotungstic acid. ×60,000.)

FIG. 12.—Guinea-pig ear, injured by heat, 1 hr. after the injection of ferritin into the connective tissues. There is a large gap (X) between the endothelium and the connective tissue. In the endothelium there is a large, nearly empty vacuole, whose exterior is studded with many RNA-containing granules. Ferritin is seen in the connective tissue, in the gap external to the cell, free in the cytoplasm (small arrow), and in the lumen. (Araldite; stained with Phosphotungstic acid. ×35,000.)



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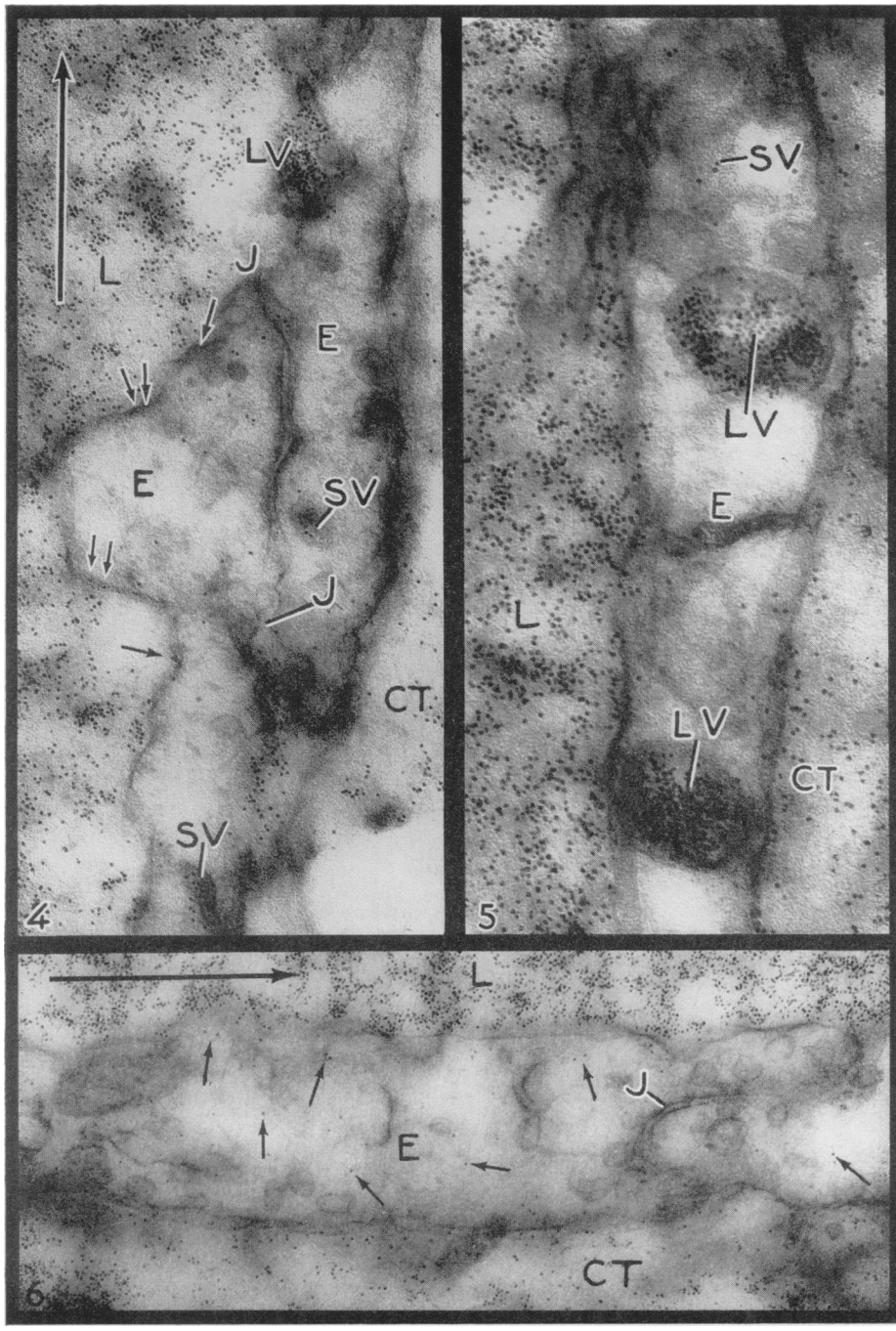

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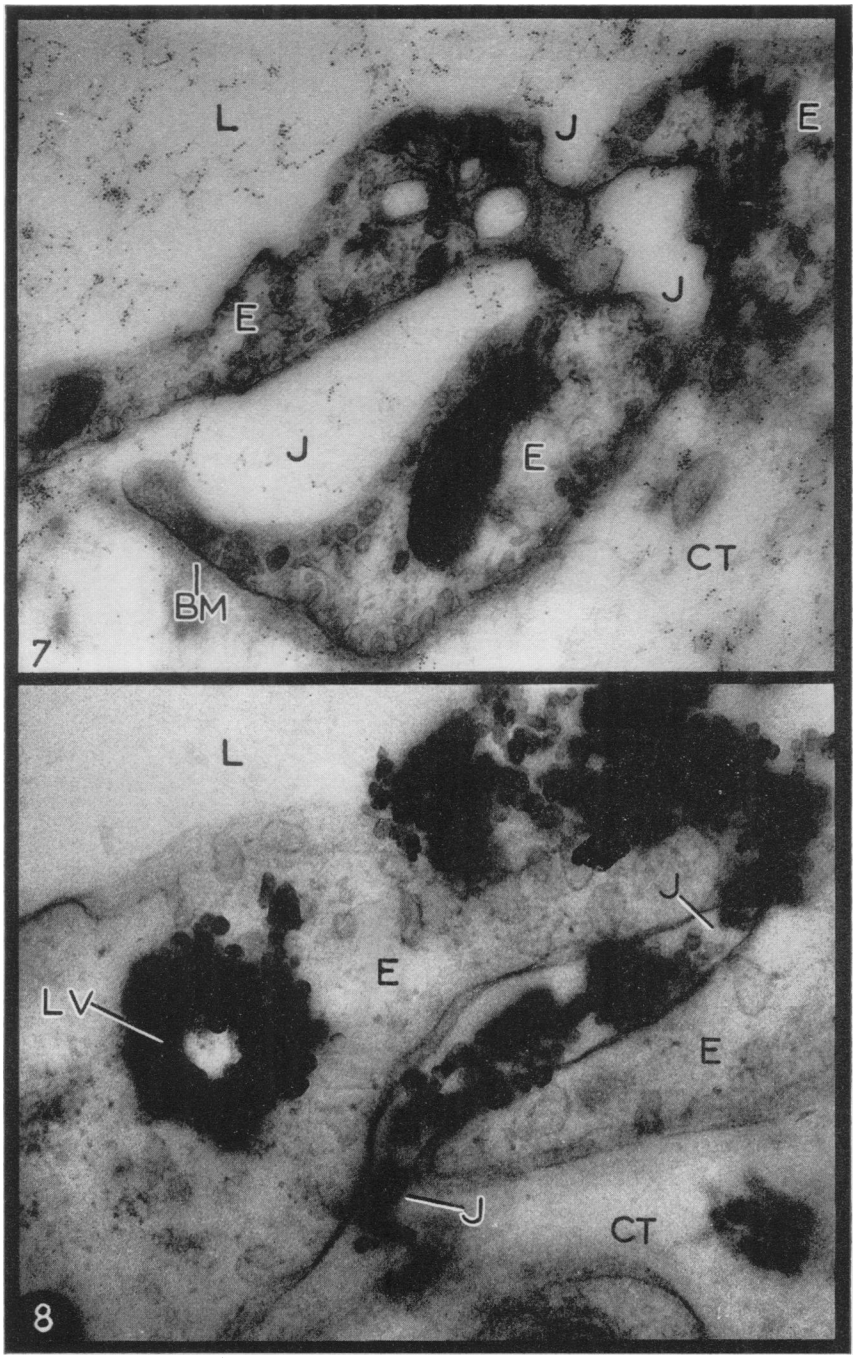

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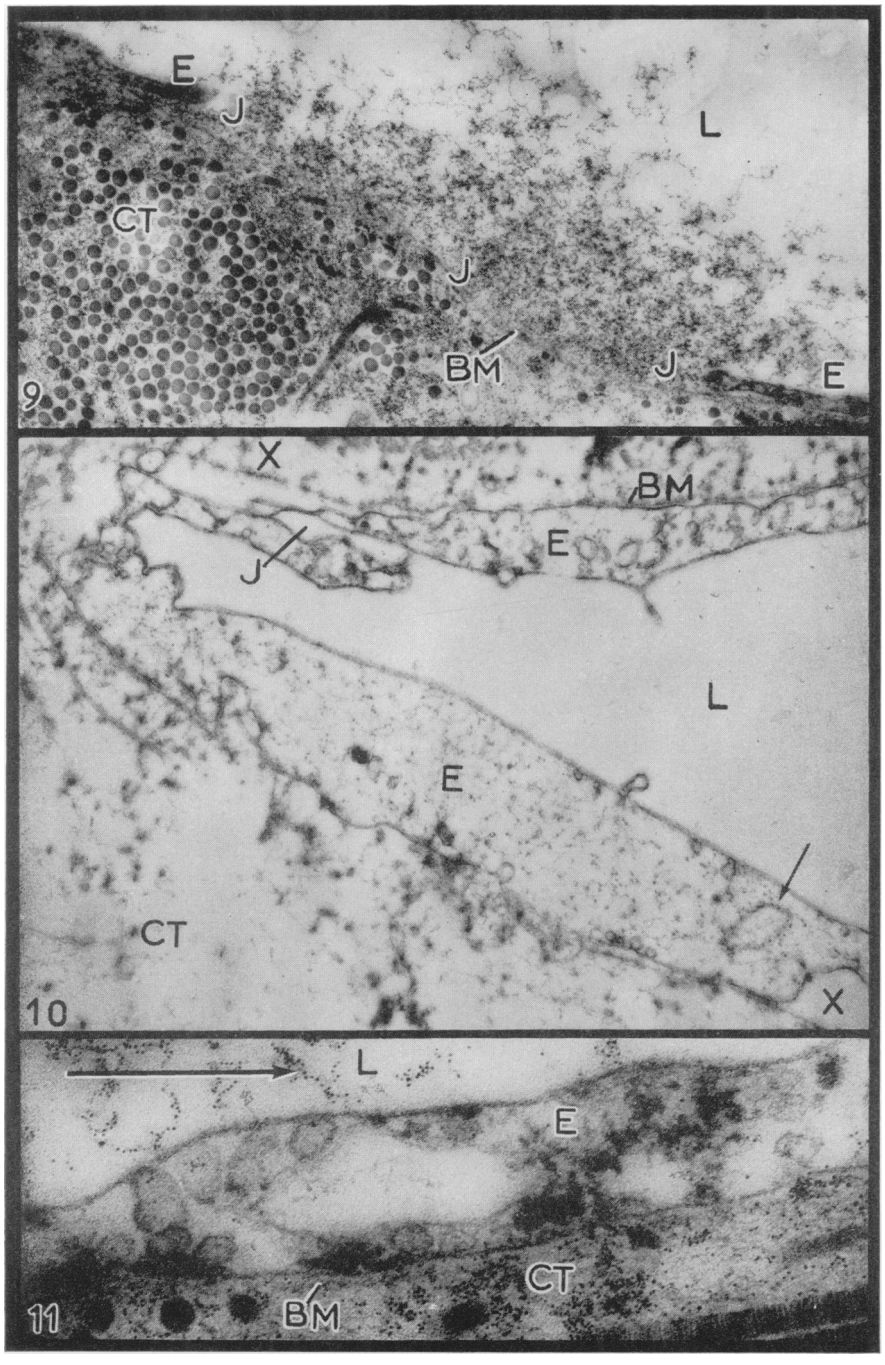

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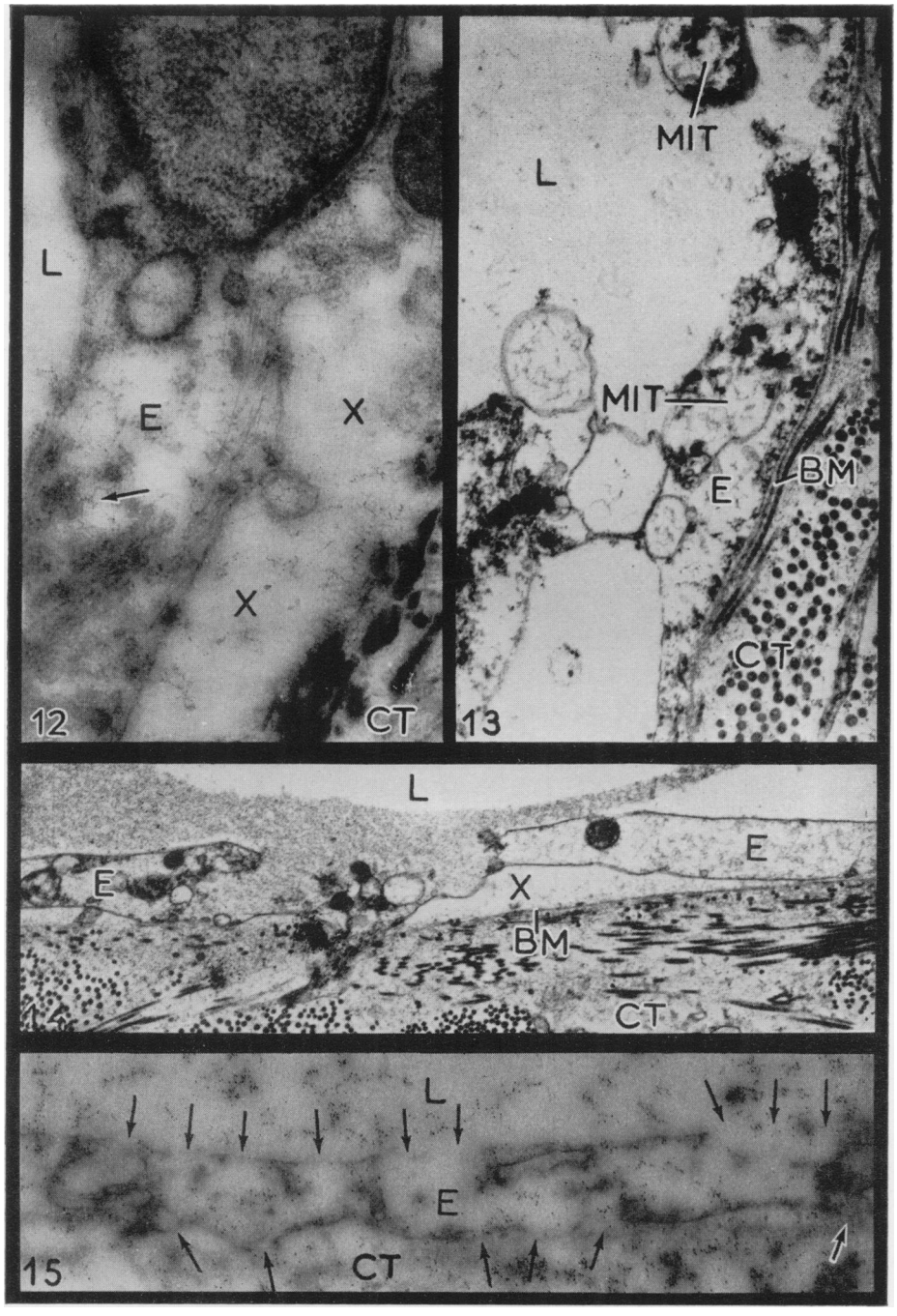
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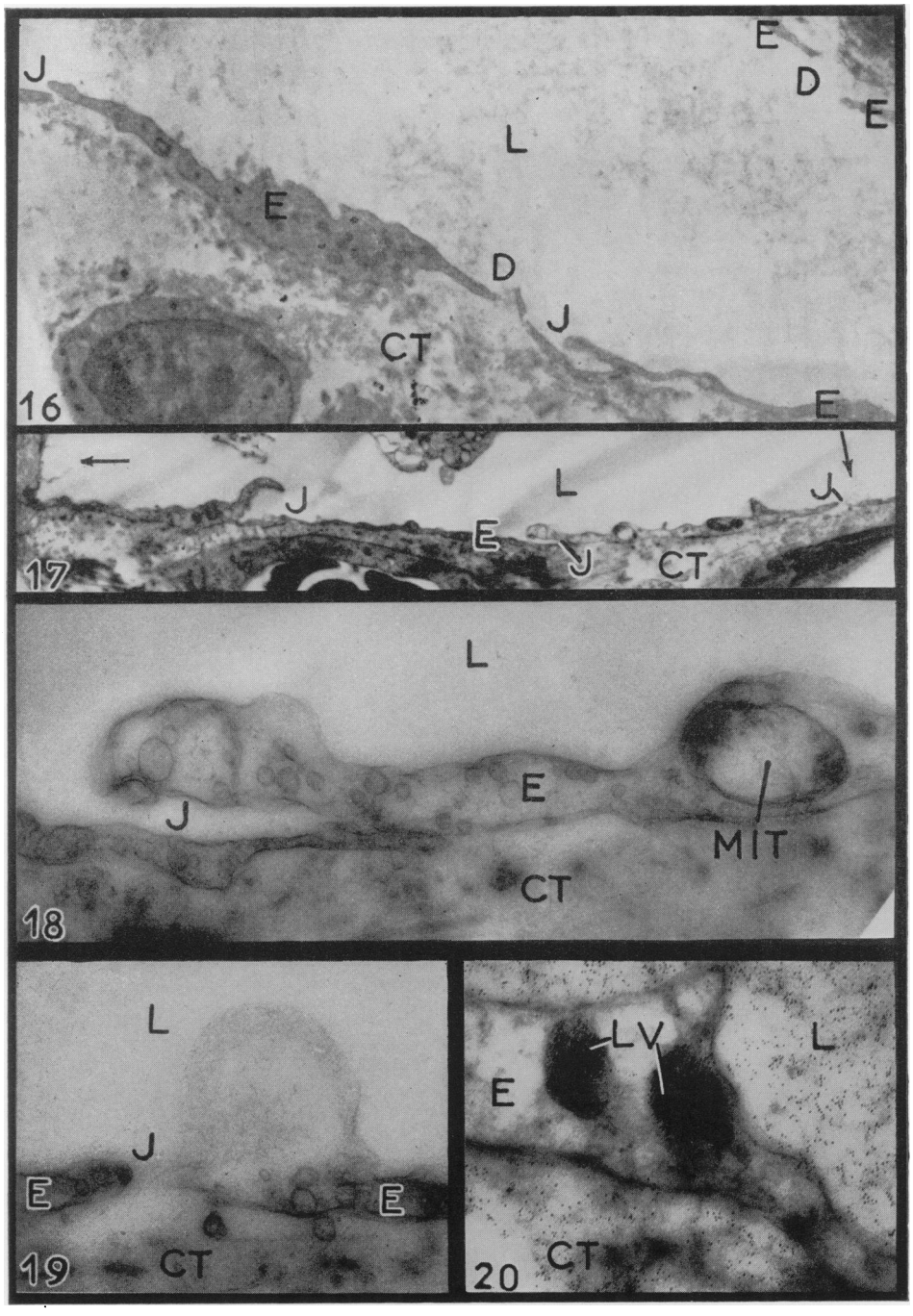

Casley-Smith.

Inside the cells the particles are seen in small (~50 mμ.) vesicles (Figs. 2–6, 11). They are also seen densely packed in large (0·1–1 μ.) ones (Figs. 2, 5, 8, 20). These seem to form by the coalescense of many small vesicles which are often found attached to them. (Others may be formed from the large caveolae). There are many more ferritin particles in small vesicles than there are in large and many more carbon particles in large vesicles than small.

Particles are often seen in small caveolae on the surface of the cell opposite to that on which they were injected (Figs. 2, 3, 11). *i.e.* If the particles are injected into the lymphatics, they are seen on the connective tissue (" anti-luminal ") surface of the cell and vice versa. This would imply that the particles have traversed the cells. Ferritin and thorium dioxide are seen like this much more commonly than carbon. (Occasionally the large vesicles are connected by small (~50 mμ.) openings with the plasma membrane). There are, however, always many particles on both sides of the cells. (This is presumably caused by spillage at the injection site and passage through the endothelium.) In these circumstances it is not possible to be certain in which direction any individual group of particles is passing. Thus one cannot here be sure that some particles, apparently leaving the cells' anti-luminal surfaces, are not really entering them there (Casley-Smith, 1964).

Many ferritin and fewer thorium dioxide particles are seen lying in the cytoplasmic matrices of normal cells, free of any encircling membrane (Figs. 2–4, 6).

EXPLANATION OF PLATES—*cont.*

FIG. 13.—Guinea-pig ear, injured by heat, 1 min. after the injection of ferritin into the connective tissues. The cell is grossly swollen and damaged. No plasma membrane is visible on its luminal surface and large portions of the cytoplasm appear to be passing into the lumen. There are some large, irregular, nearly empty vacuoles and two bodies with some vestiges of cristae suggesting that they were mitochondria (MIT). The basement membrane (BM) is well developed and preserved. (Araldite ; stained with Phosphotungstic acid. ×20,000.)

FIG. 14.—Mouse ear, injured by heat, 1 min. after the injection of carbon into the connective tissue. There is a large gap in the luminal plasma membrane. Much material, probably plasma protein, has entered the cell. There is a gap (X) between the cell and the basement membrane (BM). The cell is swollen, with a pale cytoplasm and few small vesicles. (Araldite ; stained with Phosphotungstic acid. ×16.000.)

FIG. 15.—Mouse ear, injured by heat, 5 min. after the injection of ferritin into the lymphatics. There are many gaps (arrows) in both the luminal and the anti-luminal plasma membranes. Many ferritin molecules are passing through these. The cell is swollen ; its cytoplasm is pale, with few small vesicles and much free ferritin. (Methacrylate ; unstained. ×40,000.)

FIG. 16.—Mouse ear, lightly stroked, 15 min. after the injection of carbon into the connective tissues. There is one open junction and one partly open one (J). Two other endothelial discontinuities (D) are present. It is possible that these latter are tears through the cells rather than open junctions. (Araldite ; stained with Uranyl acetate. ×4,300.)

FIG. 17.—Mouse ear, poisoned with cyanide, 5 min. after the injection of ferritin into the connective tissues. There are two partly open junctions (J) and one open one. Two localized, pale blebs are seen on the luminal surface of the cells (arrows). (Araldite ; unstained. ×5,100.)

FIG. 18.—An enlargement of Fig. 17. A partly open junction (J) is seen. The endothelium contains a mitochondrion (MIT) which is swollen and has distorted cristae. It is most unlikely that this is merely a polymerization artefact, for the tissue is embedded in Araldite. (×43,000.)

FIG. 19.—An enlargement of Fig. 17. An open junction (J) is visible. There is a localized, pale, cytoplasmic bleb on one of the cells. (×34,000.)

FIG. 20.—Guinea-pig ear, poisoned with cyanide, 2 hr. after the injection of ferritin into the lymphatics. There are two large vesicles, densely packed with particles. Ferritin is also seen in small vesicles, caveolae and free in the cytoplasm. (Methacrylate ; unstained. ×26,000.)

Using data on the extent to which ferritin is carried by the microtome knife (Casley-Smith, 1962*b*), it is possible to show that at least some of these " free " particles are not artefacts from this cause. Thus in Figs. 3, 4, and 6 it is highly unlikely ($P<0{\cdot}001$) that all the free particles have reached their present positions through the agency of the microtome knife. How these enter the cytoplasm is uncertain. Presumably they must pass through one of the cellular membranes—either vesicular or cytoplasmic. Particles are frequently seen very close to these membranes (Figs. 2–6). (It is hard to be certain that the appearance of particles actually passing through the membrane is not due to their minute movement by the knife.) In the ears injured by heat there is little problem. There are many free particles in the cells (Figs. 12, 15). Their mode of entry is evident for there are often large gaps in the plasma membranes. Plasma proteins, which increase in concentration under these conditions, are also seen entering the cells in large amounts (Fig. 14). Free carbon particles are seen in these heat-injured cells but are not seen in normal ones.

It is certain that the free particles leave the cells, for after some hours none are visible. It is as difficult to establish their method of exit as it is to prove how they enter. Again it is probable that they pass directly through the plasma membranes which they often approach very closely.

Alterations with the passage of time

Within a few minutes of their injection, particles are seen in open junctions and in the cellular sites (Figs. 2, 3, 6, 7, 9, 11, 15). As time passes the large vesicles become more numerous, more densely packed and of greater diameters (Figs. 4, 5, 8, 20).

The ferritin particles in the connective tissues, the lumens, the small vesicles and the cytoplasm all disappear after an interval. In normal ears this is 12–18 hr. In oedematous ones it is 6–12 hr. This occurs irrespective of whether the ferritin was injected into the connective tissues or into the lumens. Thorium dioxide behaves like ferritin, but rather more slowly. Carbon particles are still slower to disappear.

The large vesicles increase in size and density of packing as time passes. Relatively more carbon than ferritin accumulates in these bodies. Hence, most of the intra-cellular ferritin disappears from the cells ; most of the intracellular carbon remains. Like the carbon, much of the thorium dioxide stays in large vesicles, but a relatively greater amount is seen in the rest of the cell and eventually disappears.

The relative amounts of the different particles seen in the various sites in normal and injured endothelia

The data are presented in detail in the Table. They may be summarized as follows :—

In normal vessels ferritin is much more frequently seen in small vesicles and free in the cytoplasm than in large vesicles. With thorium dioxide the reverse applies. Carbon is even less common in small vesicles and never seen free in the cytoplasm. The frequent closed junctions occasionally contain a little ferritin or thorium dioxide ; the rare open ones contain all 3 types of particles.

The experimental procedures—heat, light stroking, raised intra-lymphatic pressure, and cyanide poisoning—all cause many intercellular junctions to open (Figs. 7–10, 16–19). These are 20–40 times more common than in normal ears. All the types of particles are frequently found in them. The experimental procedures cause little change, however, in the amounts of the different particles seen in the intra-cellular sites. There are two exceptions. The gaps in the plasma membranes of the cells injured by heat allow many more particles, including carbon, to enter the cytoplasm (Figs. 14, 15). The swelling of the cells is probably associated with the entry of water and the " dilution " of the cytoplasmic matrix. Hence the cells can contain more particles and these can move freely in the less viscous medium. (More free ferritin and thorium dioxide are also seen in the cells injured by cyanide). The second exception is that there are probably fewer small vesicles in cells injured by heat (Figs. 10, 14, 15). It is possible, but unlikely, that this appearance is just caused by the swelling of the cells. It is of interest that none of the experimental conditions seem to alter the occurrence of particles in large vesicles (Figs. 8, 20).

DISCUSSION

The endothelial paths

Junctions.—When the intercellular junctions are open much material passes through them. This has also been described in the lymphatics of jejunal villi (Palay and Karlin, 1959*b* ; Casley-Smith, 1964), of the diaphragm (French *et al.*, 1960 ; Casley-Smith and Florey, 1961 ; Casley-Smith, 1964) and in cardiac and active skeletal muscle (Casley-Smith, 1961). It is also seen in venules which have been injured with histamine or serotonin (Alksne, 1959 ; Majno and Palade, 1961 ; Majno, Palade and Schoefl, 1961), or by light touch (Marchesi, 1962). It is evident that many light microscopists saw the phenomenon without realizing exactly what was happening (reviewed by Landis, 1934 ; Majno *et al.*, 1961). In particular, some workers using the light microscope saw gaps, through which particles passed, in the lymphatic endothelium of living ears (Henry, 1933 ; Clark, 1936).

Vesicles.—Many workers have suggested that particles can traverse endothelium in small (~50 mμ.) vesicles (Palade, 1953 ; Bennett, 1956 ; Moore and Ruska, 1957 ; Wissig, 1958 ; Bennett *et al.*, 1959 ; Brandt and Pappas, 1960). This has been demonstrated by Palade (1960), Jennings *et al.* (1962), and Casley-Smith (1964). The present results also support this. As with the lymphatics of the diaphragm (Casley-Smith, 1964), it appears that much more ferritin than carbon actually crosses the cells via the vesicles. However vesicles seem to play very little part in the increased permeability of the injured lymphatics. Indeed in the case of heat-injury they seem even fewer than normal. Similarly, the vesicles contribute little to the increased permeability of injured venules (Alksne, 1959 ; Majno and Palade, 1961 ; Marchesi, 1962) and Florey (1961) considered that they did not increase in number after stimulation with mustard oil.

The occurrence of particles in large (0·1–1 μ.) densely-packed vesicles is identical with that which happens in the lymphatics of the diaphragm (Casley-Smith, 1964). There it was seen that the various types of particles tended to induce the formation of this type of large vesicle to the same extent as they tended to form aggregations in the lumen of the lymphatic. It seems that the large vesicles are

formed by the coalescence of many small vesicles and the coherence of their contents. This coalescence of small vesicles to form large ones has also been recorded by Buck (1958), Farquhar and Palade (1960, 1962), French *et al.* (1960), Miller (1960) and Majno and Palade (1961)

A number of investigators (Gordon and King, 1960) have shown that particles of ~50 mμ. diameter or less can enter the cells, presumably via small vesicles, without the need of cellular energy. The present results show that ferritin, thorium dioxide and carbon can be seen in small vesicles after poisoning the cells with 0·01M KCN. The formation of large vesicles, densely packed with particles, is also not affected by this. The large vesicles formed by the coalescence of many small ones remain in the cells for at least 3 months (Casley-Smith, 1964). (It may be that a few of the densely-packed large vesicles are also formed by the ingestion of all the particles, *en masse*, *via* large indentations in the cell. This would be similar to the ingestion and retention of material by macrophages (Essner, 1960 ; Karrer, 1960).)

The ingestion of large (~1 μ.) particles does require cellular energy and is prevented by cooling the cells to 4°, a number of metabolic poisons, or prolonged severe anoxia (Gordon and King, 1960). The passage of chylomicra (~1 μ.) through lymphatic endothelium is very rapid and presumably needs cellular energy (Casley-Smith, 1962*a* ; 1964). These large particles occur in large vesicles which are formed en masse and which rapidly transfer their contents across the cell. It is evident that the two types of large vesicle differ markedly in their properties, notably in the cellular energy required for their formation and in the fate of their contents.

The small vesicles are too small to be resolved with the light microscope ; the large ones are certainly visible. The type which remains for long periods in the cells evidently correspond to the " phagocytic vesicles " of light microscopists.

Particles free in the cytoplasm.—Ferritin and thorium dioxide have been seen lying in the cytoplasmic matrix, free of any encircling membrane (Hampton, 1958 ; Wissig, 1958 ; Bessis and Breton-Gorius, 1959 ; Jennings *et al.*, 1962 ; Casley-Smith, 1964). Some workers (Alksne, 1959) consider that this may be due to the particles being carried by the microtome knife. While this artefact does exist it is possible to allow for it (Casley-Smith, 1962*b*). This has been done for the present results, and for the particles in the endothelium of lymphatics in the diaphragm (Casley-Smith, 1964). It could be argued that the apparently free particles lie in vesicles which are only partly included in the section and whose oblique membranes therefore escape detection. This has been excluded by taking stereo-electron micrographs (Casley-Smith, 1961). It is therefore certain that at least some of the particles which are observed free in the cytoplasm are actually in this situation at the time of embedding. However Bruns (1963) and Wissig (personal communication) have observed that, while free particles are seen following osmium fixation, none are observed after fixation with gluteraldehyde. They suggest that the free particles are artefacts caused by the action of osmium, perhaps by the production of small holes in the plasma membranes. This point requires confirmation, but even if it is established there still remain the very great differences, in the numbers and types of free particles, seen between normal and heat-injured endothelia. One possibility would be that heat-injury alters the plasma membranes so that they are more vulnerable to the effects of osmium. However, the sizes and numbers of the free particles are so large and the gaps in

the heat-injured plasma membranes so extensive, that it would seem much more probable that these observations are representative of the *in vivo* situation.

It is evident that the particles which can traverse the cytoplasm cross the cells in greater quantities than would be possible if they were confined solely to vesicular passage. It is also evident that conditions which allow more particles to pass into and through the cytoplasm than normal (*e.g.* heat injury) increase the permeability of the endothelium to the particles. This applies especially for carbon which normally does not enter the cytoplasm at all.

Fenestrae.—These are not seen in normal lymphatic endothelia. The claim of Ashworth, Stembridge and Sanders (1960) to have observed them in the endothelium of jejunal lacteals, has not been substantiated by other workers (Palay and Karlin, 1959*a*; Casley-Smith, 1962*a*) Fenestrae are seen in normal endothelium of blood capillaries in some specialized tissues (Bennett *et al.*, 1959) and in severely hypoxic endothelium (Luft and Hechter, 1957). They allow the passage of much material through renal glomerular endothelium (Farquhar *et al.*, 1961), thus increasing the permeability of this endothelium. However not all fenestrae seem to be patent (Florey, 1961).

Endoplasmic reticulum.—The endoplasmic reticulum is often very dilated in endothelium injured by heat or cyanide. It appears as large (0·1–1 μ.) irregular, nearly empty vacuoles, which often have some RNA-containing granules attached to their exteriors. Only a few of the injected particles are seen in these vacuoles. These are very similar to one type of " vesicle " found by Alksne (1959) in the endothelium injured by histamine. They were not observed by Majno and Palade (1961), but the injuries caused by these workers seem milder than those of Alksne and the present experiments.

The relative importance of the paths at different sites and under various conditions

Normal lymphatics.—The ferritin particles which cross the endothelial barrier of normal ear lymphatics must do so via the cells. (When one considers the amounts which are in the cells it is evident that there are far too few open junctions for these normally to be a significant factor in the passage of the molecules across this endothelial barrier). This is made especially evident by the rapidity with which the ear tissues are cleared of ferritin, presumably via the lymphatics, and the rapidity with which large numbers of these particles can traverse the endothelial cells of the lymphatics of the diaphragm (Casley-Smith, 1964). Very little carbon is able to cross the endothelial cells of ear or diaphragmatic lymphatics. Similarly light microscopists have previously shown that normal ear lymphatics are " impermeable " to carbon (Hudack and McMaster, 1932; Pullinger and Florey, 1935). Thorium dioxide occupies an intermediate position between these two extremes.

Very few particles, of any type, pass through the intercellular junctions of normal ear lymphatics. The lymphatics in other relatively motionless regions (*e.g.* penile skin—Fraley and Weiss, 1961) also possess very few open junctions and probably have similar permeability characteristics.

Lymphatics with artificially increased permeability.—These all have one thing in common. They all have many open junctions, through which much material is seen to pass. Inevitably one concludes that it is the open junctions which give the injured vessels their increased permeability. It is true that in some conditions

other paths are also of increased importance. Thus to some extent in the poisoned lymphatics, and more obviously in those in ears injured by heat, many more particles are seen free in the cytoplasm. Again it is possible that some of the cells are torn in the vessels subjected to light touch. However the most obvious abnormal feature of all of these vessels (and the only abnormality under most conditions) is that there are many open junctions.

Normally very permeable lymphatics.—The permeability of lymphatics, in relatively motionless regions of the body, to dyes, proteins and carbon is approximately the same as that of capillaries and venules (Hudack and McMaster, 1932 ; McMaster and Hudack, 1932, 1934 ; Pullinger and Florey, 1935 ; Grotte, 1956 ; Yoffey and Courtice, 1956 ; Rusznyák *et al.*, 1960). Lymphatics in regions where there is much movement are much more permeable (Drinker, 1942 ; Yoffey and Courtice, 1956 ; Rusznyák *et al.*, 1960). In all of these regions which have been examined with the electron microscope, many open junctions have been found and many particles have been seen pouring through them, *e.g.* jejunal lacteals (Palay and Karlin, 1959*a*, 1959*b* ; Casley-Smith, 1962*a*) ; diaphragmatic lacunes (French *et al.*, 1960 ; Casley-Smith and Florey, 1961 ; Casley-Smith, 1964) ; cardiac and stimulated skeletal muscle (Casley-Smith, 1961). In many of these instances it is probable that some particles also pass through the cells, but in at least one situation, *viz.* the passage of material from the peritoneal cavity to the diaphragmatic lacunes, almost all of them pass through open junctions.

Endothelium of blood vessels.—Normally very little material passes through the endothelial junctions of small blood vessels (Buck, 1958 ; Policard and Collet, 1958 ; Wissig, 1958 ; Palade, personal communication and 1960 ; Jennings *et al.*, 1962). They remain closed even if there is much tissue movement. However, injured venules have many open junctions through which much material passes (Alksne, 1959 ; Majno and Palade, 1961 ; Marchesi, 1962). It is well known that these injured vessels have a greatly increased permeability.

Conclusion :

Particles traverse the endothelial barrier of normal small blood vessels and normal lymphatics, in quiescent regions, almost entirely through the cells. The great increases in the permeability of these endothelia after various injuries is predominantly caused by the opening of many intercellular junctions. In addition, severe trauma may so damage the cells that more material also passes through their cytoplasm.

Why do the junctions open?

It is well known (Yoffey and Courtice, 1956 ; Rusznyák *et al.*, 1960) that lymphatics are dilated in oedematous regions. Pullinger and Florey (1935) showed that this was due to the fluid separating the connective tissue fibres and causing those " attached " to the lymphatics to pull the vessels open. It is evident that this tension could separate the cells. This would explain the open junctions seen in the ears injured by heat and cyanide. In these conditions some, or much, oedema is seen. Indeed many of the cells are seen to be separated by large distances as if they have been pulled apart as the vessel was dilated. The same applies to the vessels which were subjected to raised intra-lymphatic pressure. (Only here the cells were being pushed apart rather than pulled.)

Most of the lymphatic junctions do not possess adhesion plates (Fraley and Weiss, 1961 ; Casley-Smith and Florey, 1961). The poor development, or absence, of basement membranes also give the lymphatic endothelial cells more freedom of movement. These may account for the ease with which tissue movements separate these endothelial cells, while those of blood vessels remain in contact.

In addition to these " remote " effects on the vessels, it is also probable that there are " direct " effects on the cells. It is of interest that histamine and serotonin seem able to open the junctions of venules but not of arterioles or " true " capillaries (Majno and Palade, 1961). At present it is not possible to speculate about possible mechanisms by which the various present experimental procedures could have affected the relatively unknown forces holding the cells together.

The importance of the type of particle

The open junctions of the injured vessels allow much carbon to cross the endothelial barrier; little traverses the normal vessel walls. The open junctions also allow more ferritin to pass, but a considerable amount normally goes through the cells. Hence the relative increase in permeability is much greater in the case of carbon. This is very relevant to the findings of the light microscopists. They found that normal endothelium is " impermeable " to carbon, but that dyes, even if absorbed to proteins, pass fairly easily through the endothelia of both blood vessels and lymphatics (Landis, 1934 ; Grotte, 1956 ; Yoffey and Courtice, 1956 ; Rusznyák *et al.*, 1960). It was observed that injury increases the permeability of the vessels to the dyes which behave much like ferritin and escape through the cells. Trauma also allows much carbon to leave the vessels in discrete ecchymoses, as the particles pass through the open junctions.

There are other examples of the nature of the particle determining the amount which passes via a certain route under various conditions. Thus no carbon enters the cytoplasm unless large gaps are torn in the plasma membrane. Similarly in the endothelium of normal diaphragmatic lymphatics the nature of the particle profoundly affects the amount of it able to pass *via* each of the paths (Casley-Smith, 1964).

Factors determining the permeability of endothelium

It will be seen, from the present results and those of other workers mentioned in this discussion, that the amount of a substance which passes through a portion of endothelium is dependent on a number of factors. The total amount is the sum of the amounts which pass through each of the four possible paths. The amount of the substance which passes through an individual path depends on :—

1. The nature of the the substance. (*e.g.* Carbon in small vesicles tends to coalesce into large ones and remain in the endothelium ; ferritin has much less tendency to form large vesicles and readily passes through the cells.)

2. The nature of the path and its environment. (*e.g.* The gaps in the plasma membranes and lessened viscosity of the cytoplasm of cells injured by heat allow more and larger particles to pass through them.)

3*a*. The site and type of endothelium. (*e.g.* Junctions of lymphatic endothelium open much more readily than do those of the endothelium of blood vessels.)

3*b*. The conditions affecting the endothelium. (*e.g.* Lymphatics in quiescent areas have few open junctions ; those subjected to much movement have many.)

SUMMARY

Ferritin, carbon and thorium dioxide have been studied as they passed through the lymphatic endothelium in the normal and injured ears of mice and guinea-pigs. The injuries were mild heat, light strokes, raised intra-lymphatic pressure and cyanide poisoning.

The normal endothelium rarely possessed open intercellular junctions. All the injured endothelia had many of them. It was considered that they were the predominant reason for the increased permeability shown by the injured vessels. Large numbers of all types of particles were seen passing through the open junctions.

Many ferritin, some thorium dioxide and a few carbon particles were seen in small ($\sim$ 50 mμ.) vesicles. The amounts of these did not increase after the various injuries. Much of the carbon and thorium dioxide, and some of the ferritin, occurred in large (0·1–1 μ.), densely packed, vesicles. These seemed to be formed by the coalescence of many small ones. Their formation was not affected by the above injuries. The material in the large vesicles stayed in the cells; particles contained in small vesicles rapidly left the cells.

Much ferritin and some thorium dioxide were seen lying free in the cytoplasm. This was not a knife-carry artifact. These amounts were greatly increased after the cells were injured by heat which caused many large gaps in the plasma membranes. Even carbon particles entered these pale and swollen cells. Carbon was never seen lying free in the cytoplasm of normal cells.

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