PASSAGE OF LIPID ACROSS VASCULAR ENDOTHELIUM IN NEWBORN RATS

An Electron Microscopic Study

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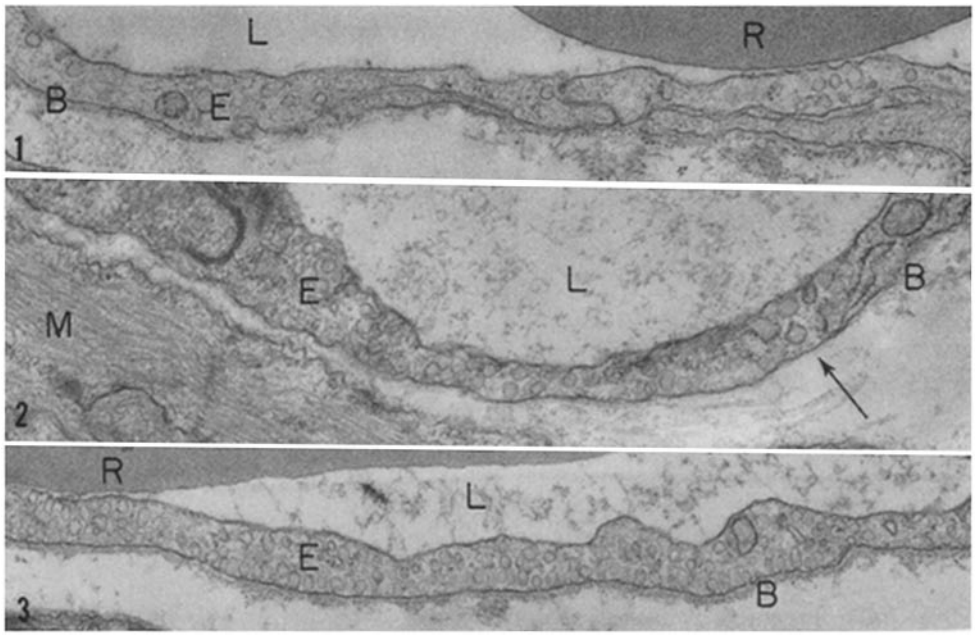
ABSTRACT

An electron microscopic study of the fine blood vessels in the skin and muscle of 25 newborn rats (sucklings, and therefore subject to physiologic lipemia) has shown that blood-borne lipid particles may leave the lumen of these vessels by two pathways, intercellular and intracellular. (*a*) An intercellular pathway: Some capillaries, venous capillaries and venules contain intramural, extracellular deposits of lipid which is presumably hematogenous. In some animals these deposits are quite numerous; available evidence suggests that they are a consequence of intercellular gaps, too small or too transient to be observed except in rare instances. Plasma apparently escapes through these gaps and filters across the basement membrane, while lipid particles are retained, usually in sufficient number to fill the small defect; some lipid particles are then taken up by endothelial cells and pericytes, while a few escape and are incorporated into free phagocytes. These focal defects, though few in number, may explain the apparent incapacity of blood vessels of newborn rats to leak any further after a local injection of histamine. Discontinuities in the endothelium were found also in the renal glomerulus, sometimes accompanied by extensive interstitial accumulations of lipid particles. Similar intercellular gaps are known to exist in other types of immature endothelia. (*b*) An intracellular pathway: This is best demonstrated in the capillaries, venous capillaries and venules which supply the developing subcutaneous adipose tissue. Here the lipid particles adhere in large numbers to the endothelial surface; the morphologic evidence suggests that they are also taken up into the endothelium through phagocytosis by "flaps," or into pockets or crevices. The lipid is apparently metabolized in the vascular wall; some is found in the multivesicular bodies. There was no evidence of active transport by vesicles or vacuoles. Neither pathway was demonstrable in the adult.

The passage of materials across the endothelial barrier has been studied, at the level of the electron microscope, mainly with the aid of tracer particles injected intravenously. With the exception of ferritin, these tracers are colloidal suspensions of substances foreign to the body. The chylomicrons, which are normally carried by the blood stream, could be considered physiologic tracer particles; they are recognizable by electron microscopy, and when sufficiently numerous they can be used for "labeling" leaking vessels almost as effectively as foreign materials (1). The present study was undertaken for two purposes. In the first place, the physiologic lipemia of the suckling rat offered an opportunity to study the behavior of the endothelium towards blood-borne lipid particles. Furthermore, it appeared useful to study the capillaries of the newborn, since relatively little is known about immature endothelia. A detailed survey of the literature on the fine



Key to Abbreviations for Figures

B, basement membrane
E, endothelial cell
F, endothelial flap
L, lumen
M, striated muscle fibre
MB, multivesicular body
N, nucleus
O, osmiophilic material
P, pericyte
R, red blood cell
S, extravascular space

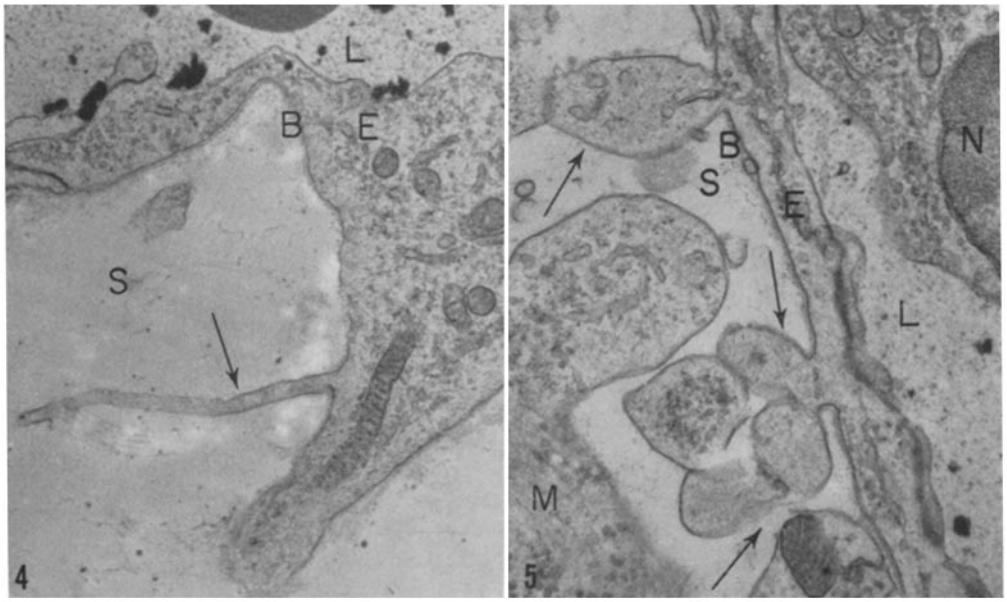
FIGURES 1 to 3 Comparison of pericapillary basement membranes in a newborn rat (Figs. 1 and 2) and in a young adult rat (360 gm) (Fig. 3). Fig. 1 (skin) illustrates the thin, somewhat irregular basement membrane typical of many capillaries in the newborn. In Fig. 2 the basement membrane is more poorly developed; the arrow points to an area in which it is virtually absent. Fig. 3 shows that the adult basement membrane is thicker, denser, and more even in width. (Figs. 2 and 3, striated muscle. Uranyl acetate, lead citrate.) × 33,000.

structure of the endothelium, both mature and immature, is available elsewhere (2).

MATERIALS AND METHODS

Pregnant female rats of the Sprague-Dawley and Wistar strains were procured. Preliminary experiments indicated that competition among sucklings in large litters could lead to very different levels of milk consumption and thus of lipemia. Therefore, shortly after delivery, the size of each litter was reduced to 4 to 6 sucklings. Despite these precautions, the degree of lipemia remained quite variable, a factor which made this study very time-consuming. In an attempt to circumvent this difficulty, intravenous injections of homologous chyle were administered; the resulting lipemia was short-lived and did not compare in intensity with the spontaneous lipemia (when present). The sucklings were sacrificed when they were 1 to 2 days old and weighed 6 to 8 gm. Tissues examined from 19 normal sucklings included skin, subcutaneous adipose tissue and striated muscle from the lower abdominal quadrants, heart, lung, kidney, liver, and adrenal gland. Six additional animals were examined 3 to 70 minutes after intravenous injection of homologous chyle.

Thirteen adult male Holtzman rats weighing 250 to 300 gm were made lipemic by 2 cc of melted butter



FIGURES 4 and 5 Cellular processes (arrows) arising from the outer surface of the endothelium in small vessels of the newborn rat. Where the surface of these processes is cut at right angles, the lack of basement membrane is obvious. Note chylomicrons in the lumen (*L*). Fig. 4: skin. Fig. 5: muscle. Lead hydroxide, × 16000.

or corn oil fed by stomach tube after a fasting period of 24 hours. The tissues sampled from these rats (and 4 normal controls) were the same as for the newborn animals.

All the tissues mentioned above were also examined by light microscopy, on frozen sections of formol-fixed samples stained with oil red O and Fettrot, as well as on whole mounts of tissue cleared in glycerin and stained with Sudan Black and Fettrot. Some of the thick Epon sections were also stained for fat (3).

Tissues for electron microscopy were removed from the living animals under ether anesthesia, fixed for 2½ to 3½ hours in Palade's fixative (4) with 2 per cent OsO_4, dehydrated in ascending concentrations of acetone, and embedded in Epon. Some blocks were stained with $KMnO_4$ following dehydration (5). Sections were cut on an LKB ultratome with diamond knives, stained with Karnovsky's method A (6), or with lead citrate (7) preceded by saturated uranyl acetate, and examined with an RCA 3D or a Philips 200 electron microscope. Thicker sections from the same blocks, stained with 0.1 or 1 per cent toluidine blue, were used for orientation. To obtain the greatest number of small blood vessels it was found practical to embed the skin with the epithelium towards the cutting surface; this was then trimmed away to the depth of the tips of the papillae. Perpendicular sections through the whole thickness of the skin were also studied.

Chyle was obtained by cannulation of the main lacteal of an adult rat made lipemic as described above. Aliquots (0.005 or 0.02 cc) were immediately injected intravenously, into newborn animals which were sacrificed after 3, 20, 30, and 70 minutes.

RESULTS

Electron Microscopy

In the description which follows, the osmiophilic bodies suspended in the plasma of the lipemic animals will be referred to as lipid particles or chylomicrons. Their size is comparable to that given for chylomicrons isolated from rat lymph: 1000 to 3000 A (maximum 35,000 A) (8) and 1000 A to 1 μ (9). Lipoproteins isolated from rat chyle measure 100 to 1,000 A in diameter (9). The lipid nature of the larger, extravascular deposits or droplets was confirmed by light microscopy, as indicated.

NEWBORN ANIMALS

SKIN AND MUSCLE: The ultrastructure of the small blood vessels in these two tissues differs

from that of the adult vessels in two respects: (*a*) the basement membrane is not yet uniformly developed; it is, on the average, thinner and less dense than in the adult (Figs. 1 to 3); and (*b*) short cell processes occasionally arise from the outer surface of the endothelium and penetrate through the basement membrane (Figs. 4 and 5). Though not so frequent, these processes were clearly reminiscent of those previously described in regenerating capillaries (10), and may be similarly interpreted as buds of new vascular channels.

In both skin and muscle, there was a rather clear-cut relationship between the type of vessel and its behavior with regard to blood-borne lipid. In the arterioles and larger veins, there was very little evidence that lipid particles could traverse the endothelial barrier; the only pertinent finding was the presence of occasional osmiophilic droplets, usually membrane-bounded, within an endothelial cell. Many more observations could be made on the finest vessels (presumably capillaries) and on vessels of similar structure but greater diameter, probably venous capillaries and venules (diameter 5 to 20 μ). Here the findings fell into two groups: (*a*) evidence of massive penetration of chylomicrons into the vascular wall; and (*b*) evidence of isolated penetration of chylomicrons into endothelial cells.

The former finding is illustrated in Figs. 6 to 9. The lipid deposits are mainly extracellular, either between endothelial cells and pericytes (Figs. 8 and 9) or piled up against the basement membrane (Figs. 6 to 9). This barrier is eventually overcome by some of the particles which then appear free in the extravascular space (Figs. 6, 7, and 9). Abundant deposits of lipid particles within the vascular wall are usually accompanied by the finding of electron-opaque (and presumably lipid) inclusions in the pericytes (Fig. 6); and lipid particles disseminated in the extravascular space are usually found in conjunction with osmiophilic bodies within free cells (macrophages and possibly fibroblasts).

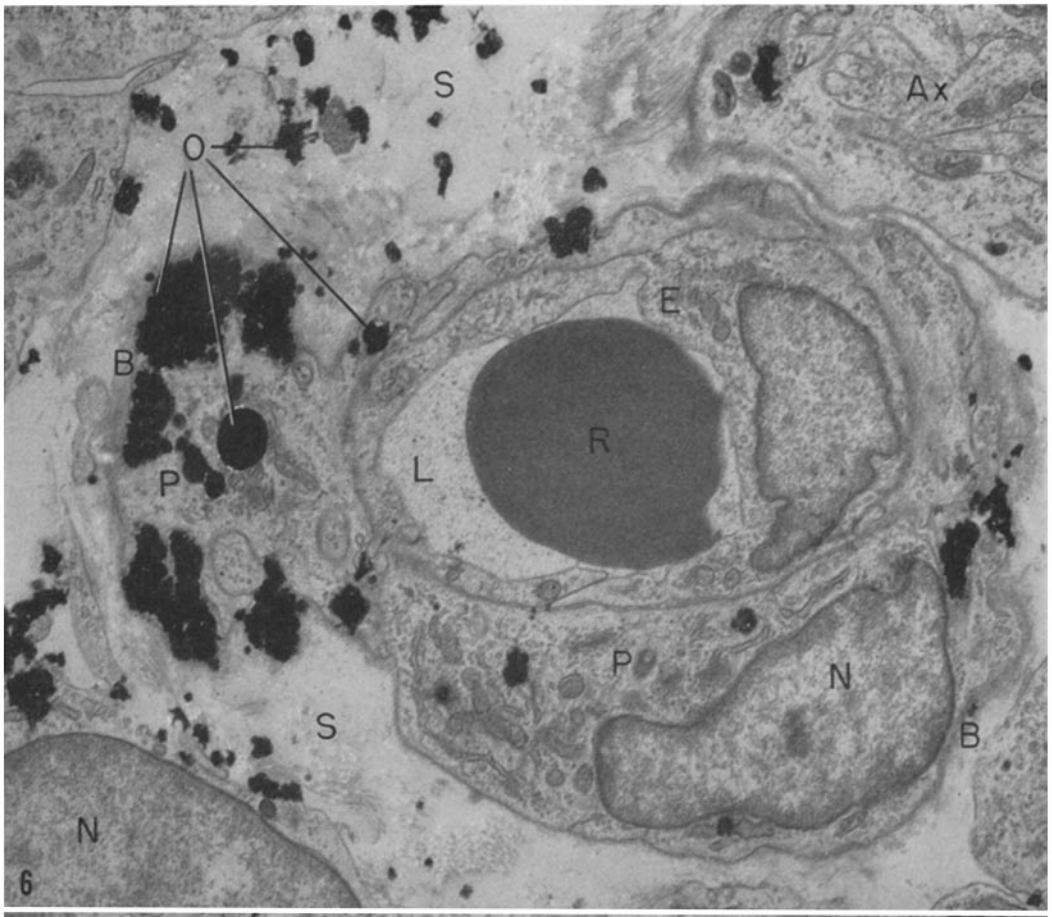
The larger deposits of chylomicrons within the vascular wall (Fig. 8) are very reminiscent of the intramural carbon deposits in vessels induced to leak by histamine and labeled with carbon black (11). In the latter case, the deposits arise by the formation of gaps between endothelial cells, followed by escape of plasma which filters across the basement membrane. An intensive search led to the finding of a few similar gaps in the vessels of the newborn animals (Fig. 10). Some of these gaps were sealed off by one or more platelets. Erythrocytes, or parts of erythrocytes, were also found trapped within the vascular wall; occasional ones were free in the extravascular spaces, and it was not infrequent to encounter macrophages containing (together with other materials, sometimes quite abundant) recognizable parts of blood cells.

The other finding, relatively frequent in the endothelium of these vessels, is the presence of single intracellular osmiophilic bodies comparable in size to chylomicrons (Fig. 7). These bodies are not surrounded by a distinct membrane; the latter may be actually absent, but the same image could also be interpreted as a membrane-bounded vacuole with its contour obscured by the very dense content. Chylomicrons are not infrequently seen in contact with the endothelial surface (Figs. 7 and 9), sometimes in what appears to be a pocket. Multivesicular bodies in the endothelium often contain one or more osmiophilic droplets, especially in the subcutaneous adipose tissue.

SUBCUTANEOUS ADIPOSE TISSUE: This tissue is poorly developed in the newborn rat and its structure differs considerably from that of the

FIGURE 6 Small cutaneous vessel (newborn rat). Osmiophilic material (*O*) within a pericyte (*P*) and apposed against the basement membrane (*B*). Similar particles have crossed the basement membrane and are scattered in the extravascular space (*S*). Upper right: axons (*Ax*) within a Schwann cell. Lead hydroxide, × 10,000.

FIGURE 7 Small cutaneous vessel (newborn rat). Note osmiophilic particles in the lumen (*L*), in the endothelial cells (*E*), in pericytes (*P*), and in the extravascular space (*S*). Larger accumulations of osmiophilic material (*O*) against the basement membrane (*B*). Red blood cell (*R*) contains mitochondria. Upper right: Axons (*Ax*) surrounded by Schwann cell (*N*, nucleus of Schwann cell). Lead hydroxide, × 10,000.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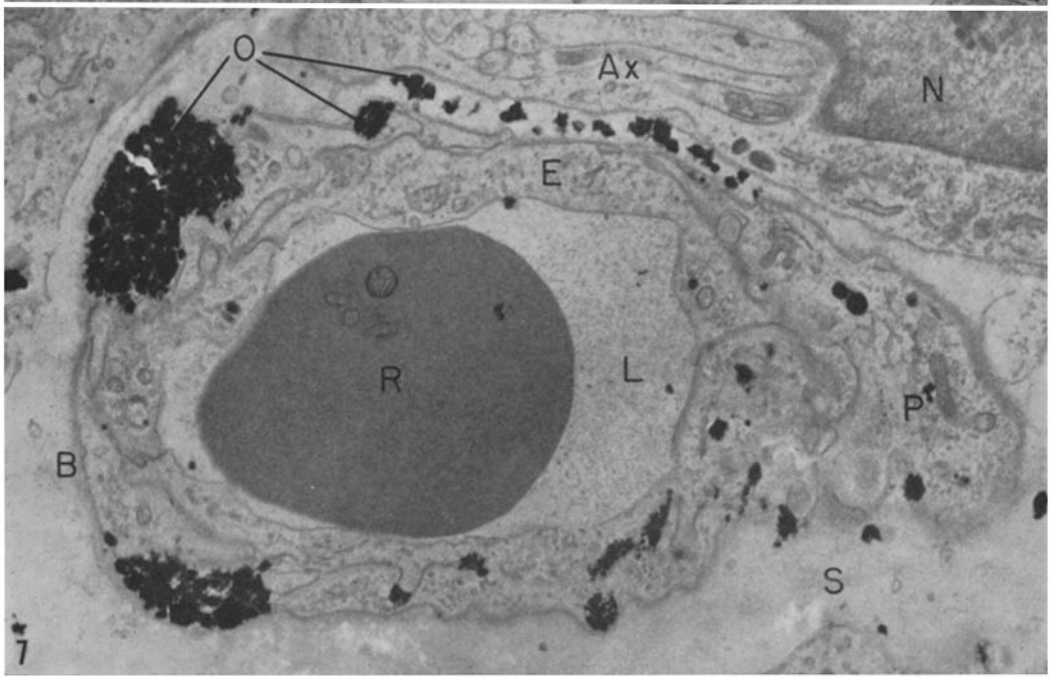
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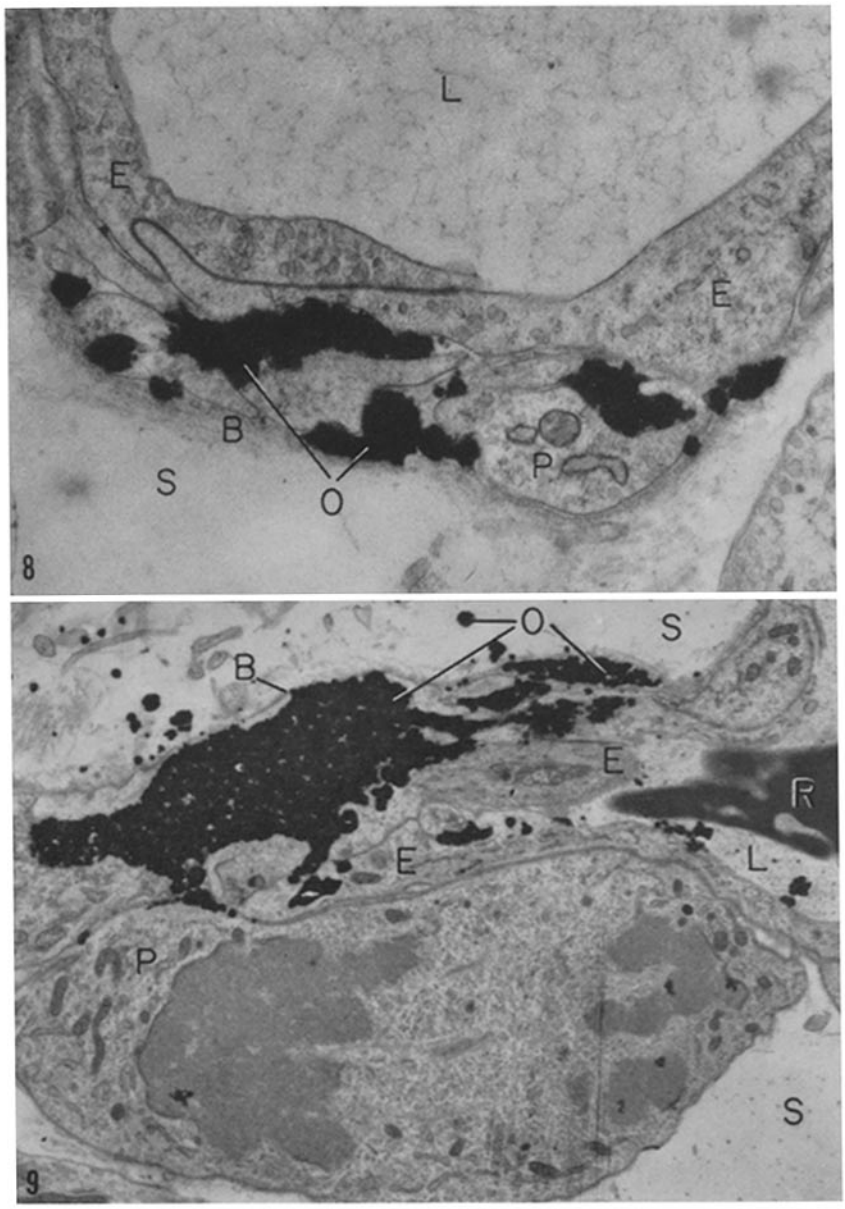


FIGURE 8 Detail of small cutaneous vessel (newborn rat). The presence of osmiophilic material (*O*) between endothelial cells (*E*) and pericytes (*P*), and against the basement membrane (*B*), indicates that this vessel is—or has been recently—leaking. Lead hydroxide, × 20,000.

FIGURE 9 Small cutaneous vessel sectioned obliquely (newborn rat). Large accumulations of lipid (*O*) against the basement membrane (*B*). In the lumen (*L*), visible on the right, are portions of a red blood cell (*R*) and some chylomicrons. Pericyte (*P*) in mitosis. Upper left: lipid particles which have penetrated the basement membrane lie free in the extravascular space (*S*). Lead hydroxide, × 6000.

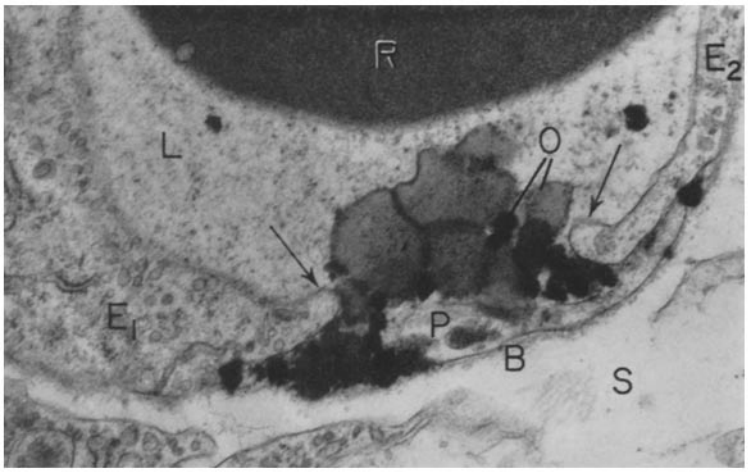


FIGURE 10 Detail of small vessel in striated muscle (newborn rat). Note gap (arrows) between two endothelial cells (E_1 and E_2). If the cell labeled P (pericyte?) is interpreted as endothelial, then there are two gaps (between E_1 and P, and between P and E_2). Lead hydroxide, × 20,000.

adult. The immature fat cells, interspersed with fibroblasts and mast cells, are widely spaced and lack a basement membrane wrapping (Fig. 11); they are about half-filled with lipid bodies of various dimensions, and contain many mitochondria and vesicles (especially towards the periphery). Numerous capillaries supply the fat cells and frequently come into close contact with them; chylomicrons adhere in great numbers to the luminal surface of the endothelium (Fig. 11). At higher magnifications (Figs. 12 to 19) these areas present many features suggestive of lipid phagocytosis by the endothelium. Some chylomicrons appear trapped by endothelial flaps (Fig. 18), which are often wrapped around them (Figs. 14 to 16); others lie in deep recesses in the luminal surface of the endothelium (Figs. 12, 13, 16, and 17). Within the endothelial cells, osmiophilic droplets are frequent (Figs. 13, 16, and 18); some appear to be membrane-bounded (Fig. 13). One or more osmiophilic droplets are seen in most of the multivesicular bodies (Figs. 15, 16, and 19) which are quite numerous. By contrast, no droplets are found in the extravascular spaces, between the capillaries and the fat cells.

KIDNEY CORTEX: GLOMERULAR CAPILLARIES: In some normal and chyle-injected newborn rats extensive lipid deposits are seen in the central region of many glomeruli (Figs. 20 and 21). The basement membrane does not have the retaining effect it has in the systemic vessels; it seems to be freely penetrable, and the chylomicrons spread along it (Fig. 20); often they form masses which indent the "third cells" (12) (Fig. 21). Here again, as in muscle and skin, it is much easier to show that lipid has crossed the endothelial barrier, rather than to find the pathway which led it there from the lumen. Fig. 22 illustrates one of the rare instances in which the passage was found. In some areas the basement membrane looks edematous and contains disseminated lipid particles. Fig. 23 is interpreted as showing three successive stages of lipid phagocytosis. Occasionally, and certainly less frequently than in the glomeruli, chylomicrons are also scattered along the basement membrane of *peritubular* capillaries; however, the endothelial fenestrae, which are much less numerous in the newborn than in the adult (especially in the glomeruli), never appeared to be involved in the passage of lipid.

OTHER TISSUES: In the heart vessels there was no evidence of lipid migration across the endothelium. The latter contained an occasional vacuole filled with osmiophilic material, much as did all endothelia. In the lung the macrophages contained great numbers of large osmiophilic inclusions.[1] Chylomicrons were often numerous in

[1] Since completion of this work, we have noticed that comparable lipid droplets are present also in pulmonary septal cells of the rat fetus at term ob-

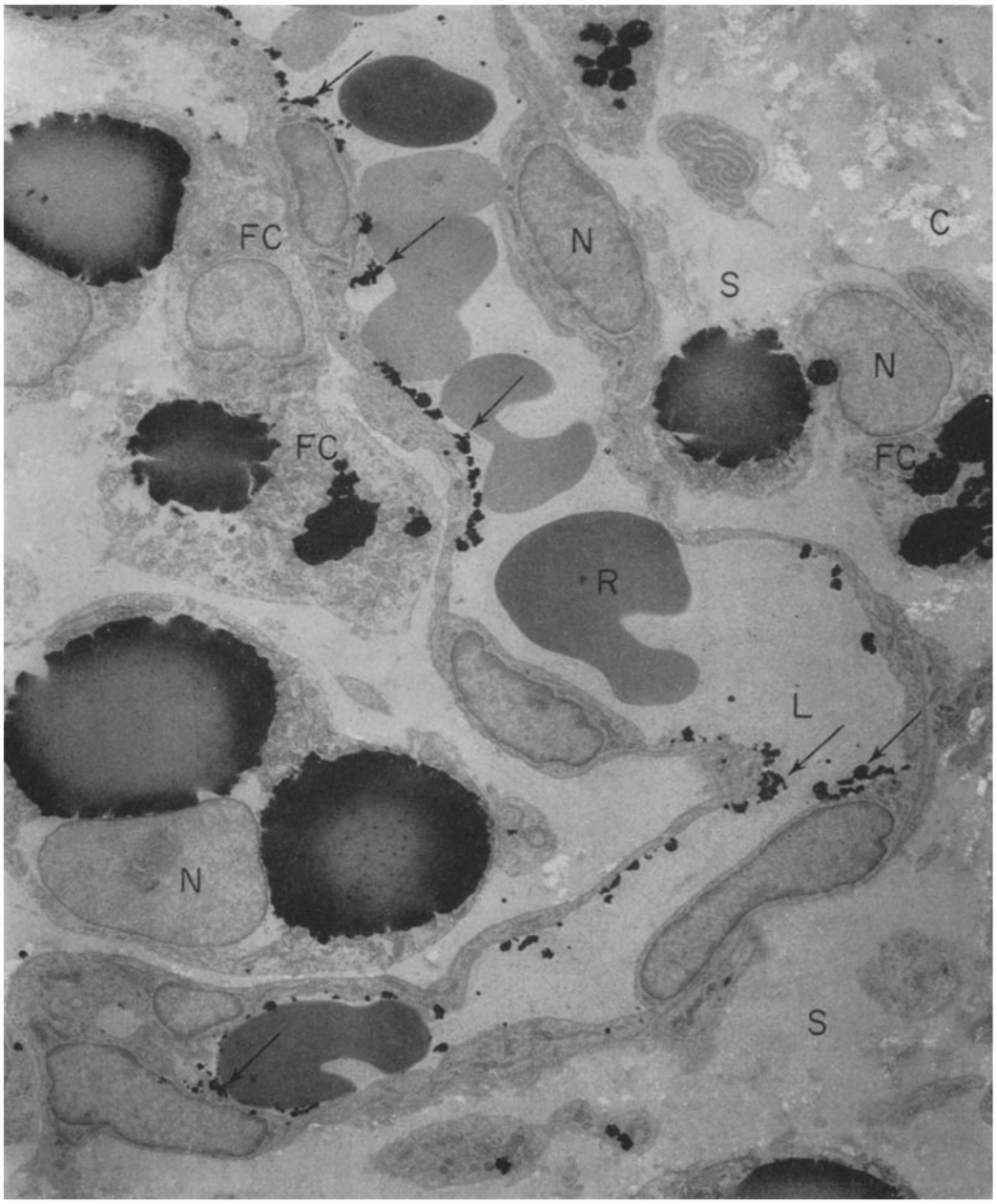


FIGURE 11 Capillary, sectioned longitudinally, in subcutaneous adipose tissue (newborn rat). Arrows point to chylomicrons which are adhering to the endothelium. Note immature fat cells (*FC*) with lipid droplets of various sizes. The extravascular space (*S*) contains bundles of collagen fibers (*C*). Lead hydroxide, × 3300.

the lumen of the capillaries, but there were no signs that they might cross the endothelium. In the liver many chylomicrons entered the space of Disse through the sinusoidal openings, and hence appeared to be taken up by the parenchymatous cells which showed inclusions of comparable size and density, as well as more centrally located, large vacuoles with an osmiophilic content. The Kupffer cells were never loaded with fat. Similar observations have been reported on livers of adult animals (13, 14). In the innermost zone of the adrenal cortex the capillary endothelium was occasionally interrupted by wide gaps through which processes of endocrine cells sometimes projected. A number of endothelial cells contained osmiophilic bodies some small, others much larger than usual and resembling in size and density the lipid inclusions of the endocrine cells. Smaller lipid droplets, similar to chylomicrons, were present in the pericapillary space, either free or surrounded by processes of endocrine cells.

Adult Animals

LIPEMIC RATS: In some instances the degree of lipemia was comparable to the physiologic lipemia of the newborn; yet even in these animals there was no evidence of lipid passing across the endothelium, elsewhere than in the liver.

Light Microscopy

Fat stains were used in order to assess the nature of the largest osmiophilic deposits or vacuoles observed by electron microscopy. In this manner it was ascertained that fat droplets were present in numerous cells of the subcutaneous tissue, in or against the wall of small blood vessels, and in the interstitial cells of the lung. Detailed observations on intramural lipid of blood vessels could not be made, because intraluminal fat was usually present in sufficient amounts to cloud the picture.

DISCUSSION

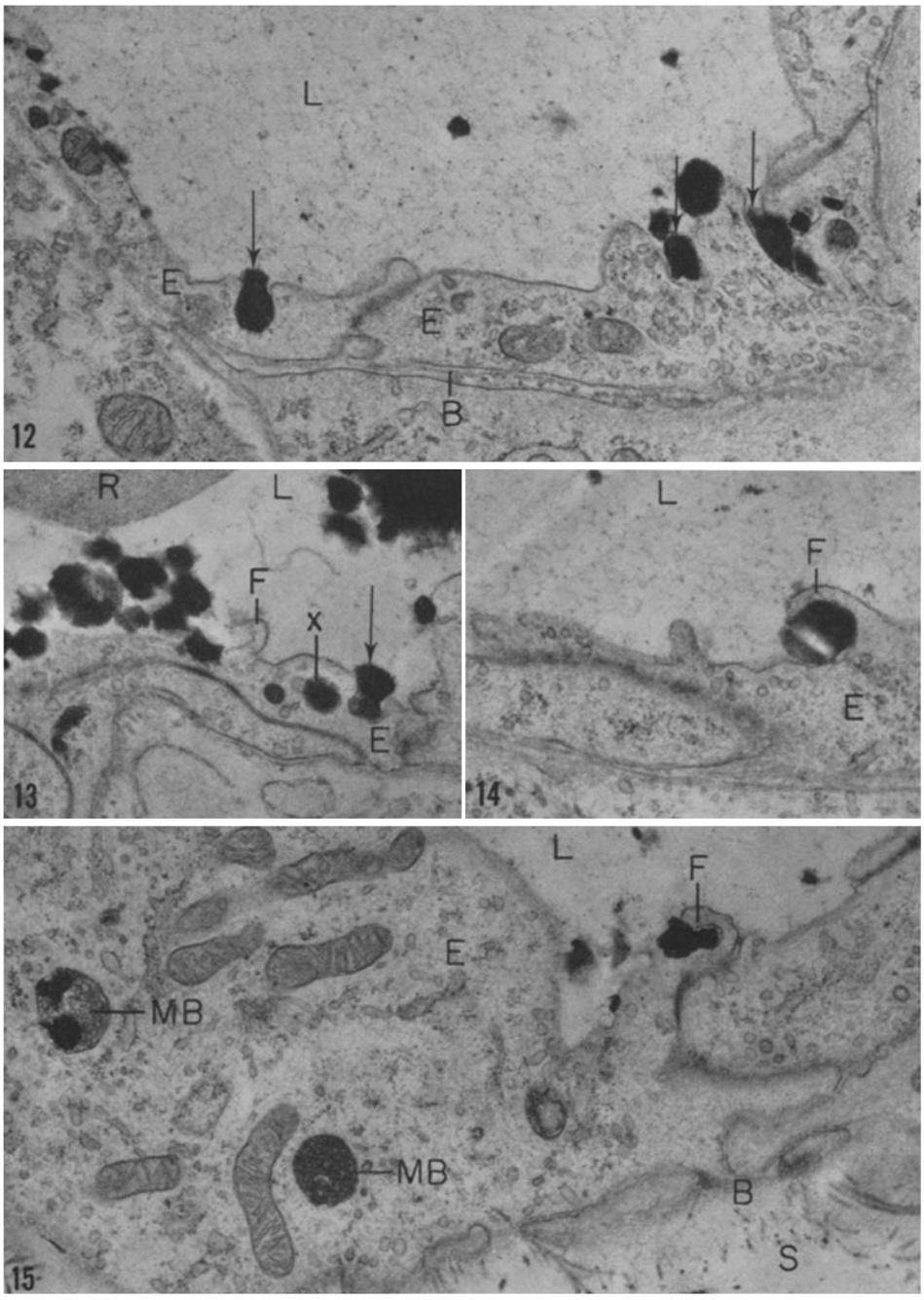
Our electron microscopic observations suggest that the pattern of chylomicron clearance from the blood is different in newborn and in adult rats: lipid particles can be seen crossing the endothelial barrier in newborn rats, but not in lipemic adults. Two pathways can be identified: intracellular and intercellular. Of these, the intercellular is by far the easiest to demonstrate, especially in the skin and muscle, but also in the renal glomerulus. It seems quite clear that the chylomicrons penetrate into the vascular wall through endothelial gaps (Figs. 10 and 22) and then accumulate at this site because of a filtering action of the basement membrane. The presence of a few particles with all the appearances of chylomicrons in the perivascular space suggests that the basement membrane is not a wholly impermeable filter with regard to these bodies. The barrier may be overcome in areas where it is particularly thin or absent; but it is also likely that small openings eventually form under the pressure of growing aggregates, and that single particles may be squeezed out. It has been shown that carbon particles about 300 A in diameter can cross the adult basement membrane, especially at certain weak spots where it splits to surround a pericyte (15). Intramural deposits of lipid are much more commonly found than actual endothelial gaps; this may mean that the gaps are very small, and possibly transient. It is noteworthy that protein does not seem to accumulate against the basement membrane together with particles, whereas, in the adult, prolonged leakage from endothelial gaps leads to the formation of protein "pools" within the wall (16). This may reflect a greater permeability of the basement membrane, since it is thinner than in the adult (Figs. 1 to 3) (2), and perhaps also a transient nature of the openings.

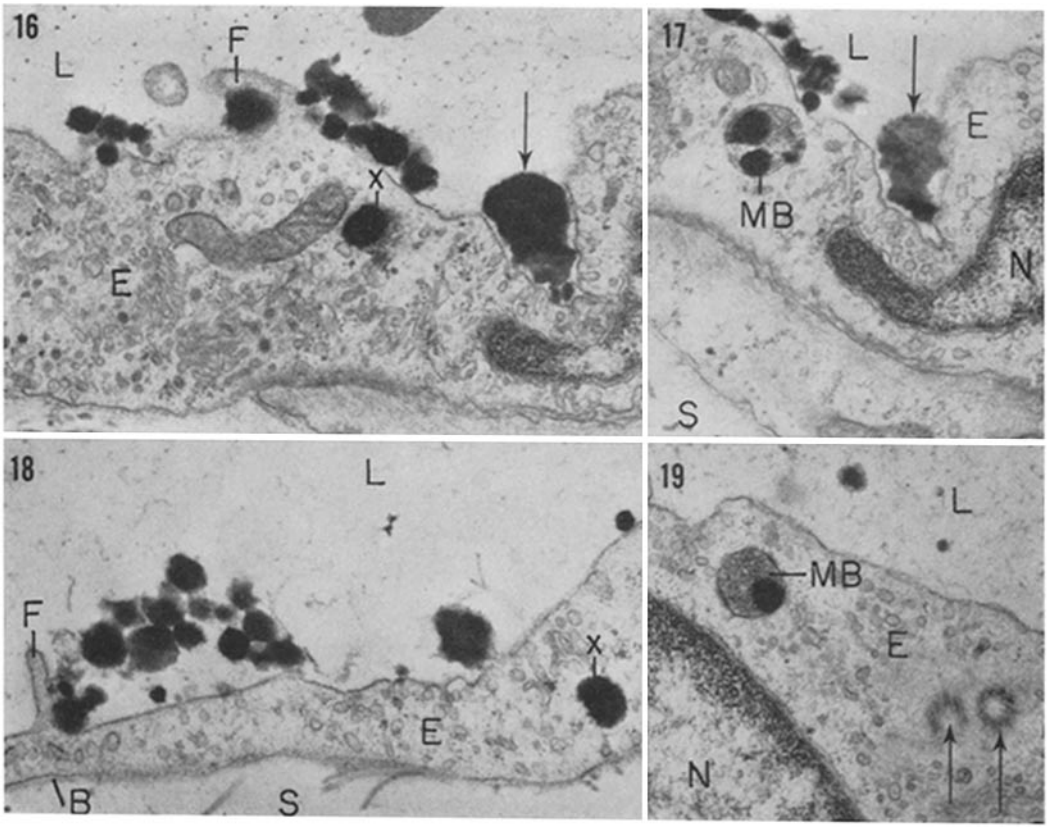
The intracellular pathway is more difficult to prove, and it may be qualitatively different in that it probably leads to intracellular breakdown of the lipid, rather than to the transfer of whole chylomicrons across the endothelium. However, numerous images were highly suggestive of chylomicron phagocytosis by the endothelium (Figs. 12 to 19, and 23); some of these implied that the particles may be trapped by endothelial flaps, a mechanism stressed by Fawcett (17). The further fate of the phagocytized particles cannot be definitely established electron-microscopically, but as there is no suggestion that single lipid droplets can be extruded towards the basement membrane, it may be assumed that the lipid is metabolized within the endothelial cell. The multivesicular bodies may well be involved in this breakdown process, since they often contain osmiophilic bodies resembling chylomicrons. In

tained by caesarean section; thus these droplets seem to be unrelated to the physiologic lipemia of the suckling.

fact, these bodies, quite numerous in the endothelia of the newborn, are often diffusely osmiophilic.

Among all the endothelia examined, the most active in phagocytizing chylomicrons is that of the capillaries in the subcutaneous adipose tissue; another striking characteristic of these same vessels is that chylomicrons seem to adhere to the endothelium in greater numbers. Adhesion, of course, is a necessary preliminary to phagocytosis. A similar "selective sticking" was observed by Wassermann





FIGURES 12 to 19 A series of images taken from capillaries of adipose tissue which may be interpreted as corresponding to various phases of lipid uptake by the endothelium. Note adhesion of chylomicrons to the endothelium, an occurrence typical for these vessels. Arrows point to osmiophilic particles lodged in deep pockets of the endothelial surface; similar particles are present inside the endothelial cells (*x*) as well as in multivesicular bodies (*MB*). A lipid droplet which appears to be free in the endothelium in Fig. 16 (*x*) corresponds to a multivesicular body in a serial section (Fig. 17). Figs. 13 to 16, and 18 show endothelial "flaps" (*F*) variously related to chylomicrons which they may be trapping and engulfing. Uranyl acetate, lead citrate, × 25,000.

in the blood vessels of adipose tissue in mice 1 to 2 days old, starved and refed (18). Because the chylomicrons tended to form clusters, Wassermann suggested that they might be responsible for the stickiness. Other findings from this same study tally with ours (particularly the presence of chylomicrons embedded in pockets in the endothelial surface) but we did not notice the dark, amorphous material against the endothelial surface which Wassermann interpreted as disintegrated chylomicrons and which sometimes appeared to coincide with the presence of numerous vesicles in the cytoplasm. We had no evidence that the endothelial vesicles take part in lipid transport.

The relative importance of the two pathways, intra- and intercellular, in removing chylomicrons from the blood stream cannot be established on the basis of our findings. It can be safely assumed, however, that both pathways decrease in importance with age, because in adult rats, despite comparable degrees of lipemia, we found no evidence of lipid transfer across normal endothelium. This is in agreement with the observations by Williamson (19), who also failed to identify lipid in the endothelium in his electron microscopic study of lipid mobilization and impletion in young adult rats. He did see, however, chylomicrons adhere to the endothelium of adipose

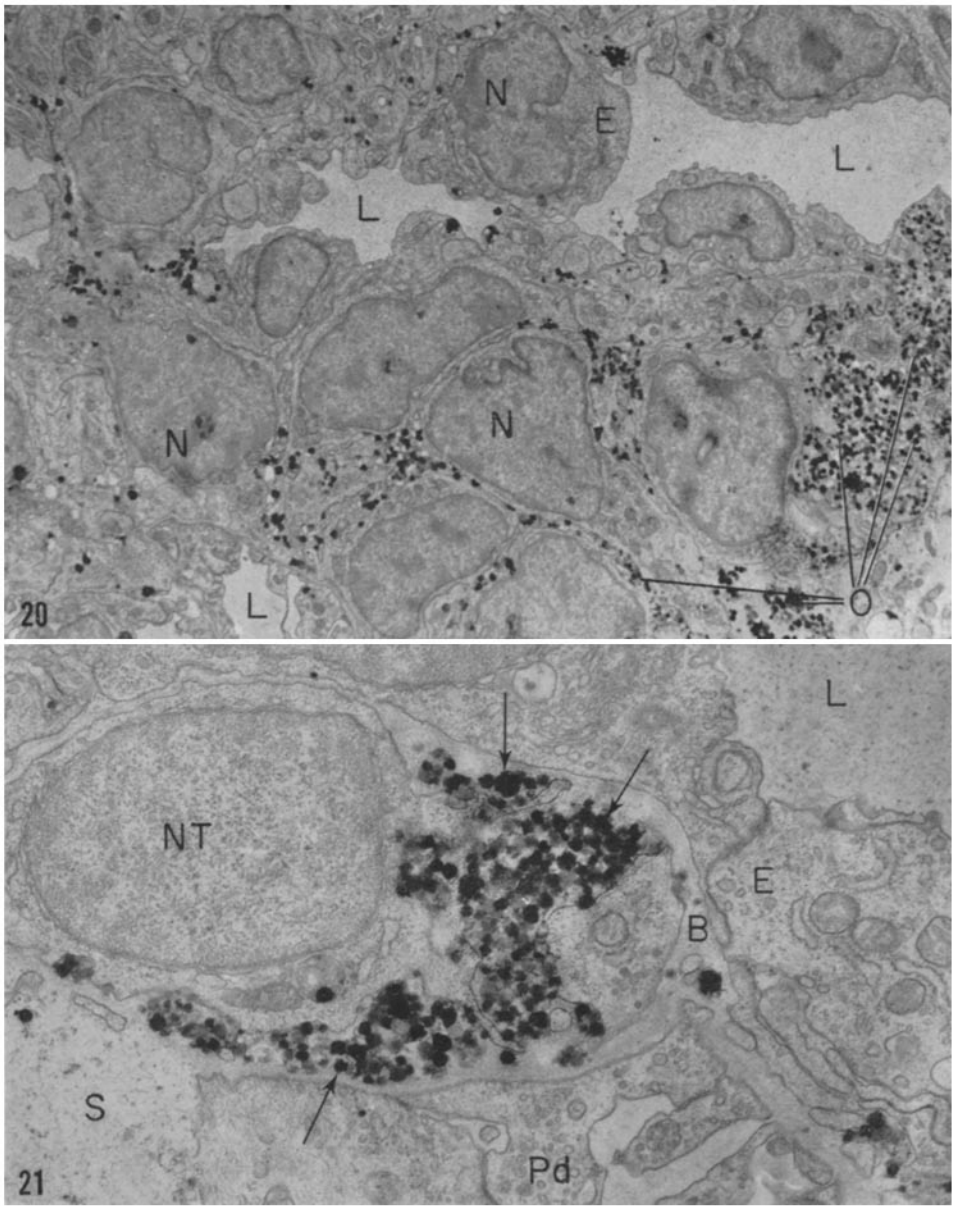


FIGURE 20 Central region of a glomerulus (newborn rat, 70 minutes after intravenous injection of chyle). Extravascular chylomicron accumulations (*O*). Uranyl acetate, lead citrate, × 5000.

FIGURE 21 Detail from central region of a glomerulus (newborn rat, 20 minutes after intravenous injection of chyle). Arrows point to accumulations of extracellular lipid particles. *NT*, nucleus of a "third cell." Extravascular space at *S* looks edematous. *Pd*, podocyte. Uranyl acetate, lead citrate, × 20,000.

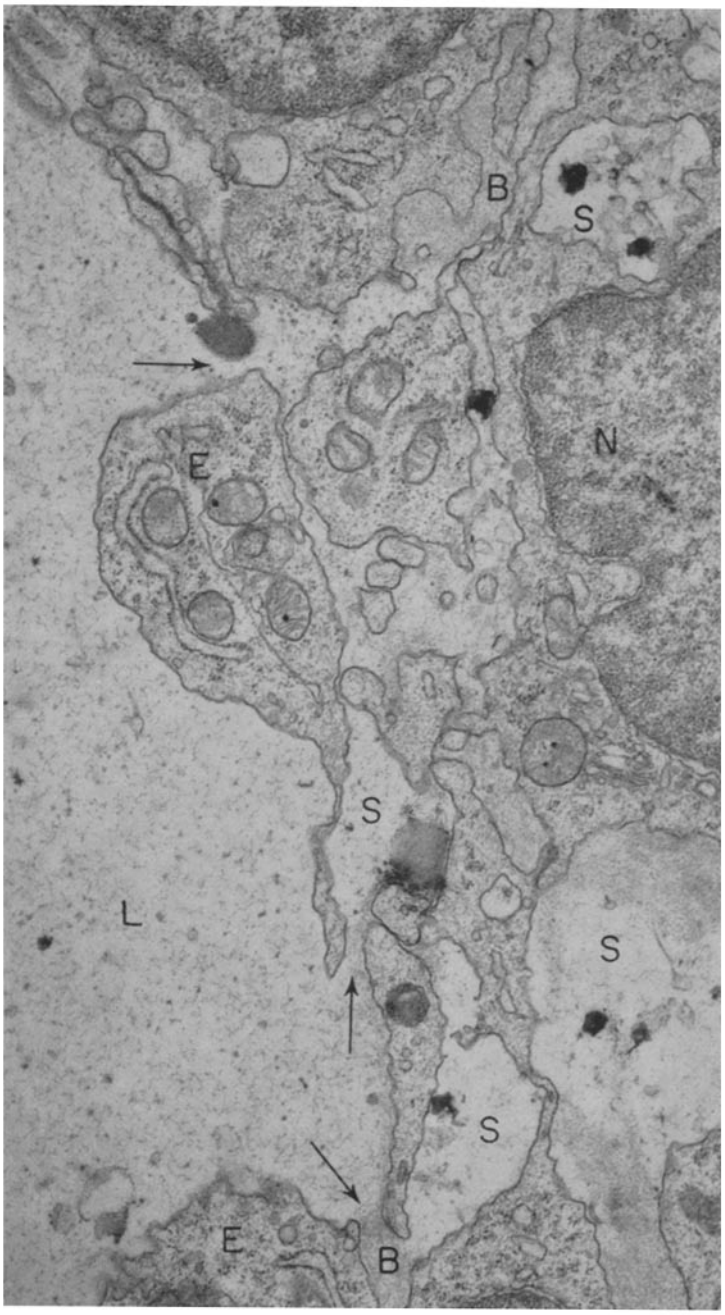


FIGURE 22 Detail of "leaking" glomerular capillary (newborn rat, 20 minutes after intravenous injection of chyle). Arrows point to discontinuities in endothelial lining. Notice exposed basement membrane between the two bottom arrows. Several lipid particles lie free beyond the endothlial barrier. Uranyl acetate, lead citrate, × 25,000.

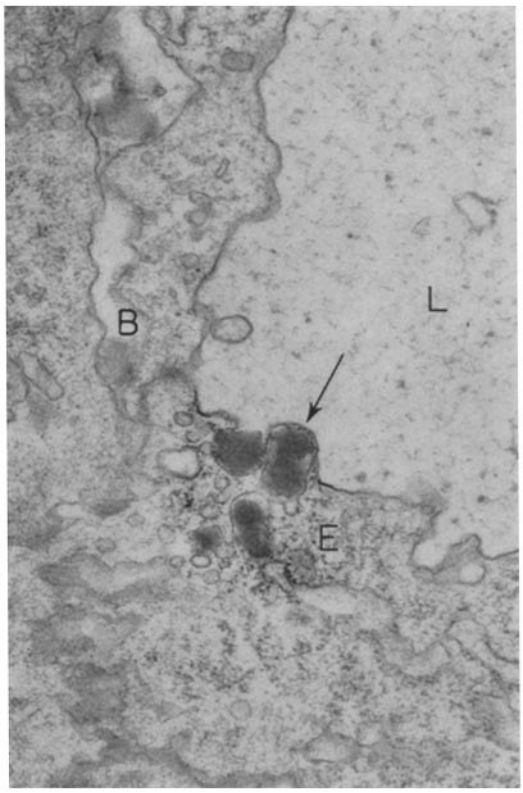


FIGURE 23 Detail of glomerular endothelium (newborn rat, 20 minutes after intravenous injection of chyle). Arrow points to area of endothelial cell (*E*), which shows a chylomicron invaginating the cell membrane, as well as two lipid-filled vacuoles. Uranyl acetate, lead citrate, × 25,000.

tissue capillaries; in our material this occurred only in the newborn. As a possible mechanism of lipid transport across the endothelium, Williamson proposes that chylomicrons in contact with the endothelial surface are hydrolyzed by lipoprotein lipase; free fatty acids would then induce vesicle formation by the plasma membrane, and be carried across the cell by these vesicles.

Although we did not find any evidence that *normal* adult endothelium can be traversed by chylomicrons, an extensive survey of adult tissue led to the finding of a few very obvious endothelial injuries of microscopic dimensions, such as small platelet thrombi overlying a disrupted endothelial cell; in one case the basement membrane was exposed and chylomicrons were present in the gap. Physiologic studies have shown that a few chylomicrons do escape from the systemic capillaries, since they can be found in small numbers in the lymph flowing from the limbs of adult lipemic animals (20). Presumably these chylomicrons escape through endothelial gaps, which are either reversible or so few in number as to be extremely rare to encounter by electron microscopy. Courtice and Garlick (21) suggest that the major route of transfer may be between the endothelial cells, even though "no permanent fenestrae or pores" have been seen. It is conceivable that this may not be, strictly speaking, a physiologic phenomenon but the expression (in part at least) of inevitable microscopic injuries of the endothelium, such as those we observed.

Apart from the problem of lipid transport across the endothelial barrier, our findings on the blood vessels of the newborn rat correlate with other observations on immature endothelia (2). Typical are, for example, the cell processes penetrating across the basement membrane (10). Intercellular gaps have been described in the kidney of the human fetus (22) and in regenerating vessels of the adult rat (10, 23). (None were found in the cerebral capillaries of the rabbit fetus, reference 24.) In the newborn, because of its physiological lipemia, the gaps are filled with lipid particles; a probable counterpart of this phenomenon in the adult is the accumulation of sudanophilic material in the granulation tissue of lipemic animals (25). The high water content of immature tissues (fetal, newborn, and adult regenerating) may be partially dependent upon incompleteness of the endothelial barrier.

In appearance and in diameter, the endothelial gaps in the newborn rat are very similar to those induced by histamine and other mediators in the endothelium of the venules in the adult (11). On the other hand, exogenous histamine does not induce any noticeable edema in the tissues of the newborn rat (1). It may be that the physiological leakage at this age is already so abundant that further leakage is partially or completely prevented.

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