Movement of lipolytic products to mitochondria in brown adipose tissue of young rats: an electron microscope study

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Abstract Lipolysis occurred and lamellar structures with a periodicity of 40 Å developed in glutaraldehyde-fixed brown adipose tissue of suckling rats when the tissue was incubated at 25°C. The lamellar structures were found in capillaries, associated with chylomicrons, in intracellular channels of capillary endothelium, in extracellular space, and in channels near lipid droplets in adipocytes in tissue of fed rats injected intravenously with chylomicrons. They were also found in channels near mitochondria and inside mitochondria in adipocytes in incubated-fixed tissue of rats exposed to 4°C for 2 hr or unsuckled overnight. In addition, aqueous spaces developed adjacent to lipid droplets in incubated tissue of cold-exposed and unsuckled rats. Development of lamellar structures under conditions causing lipolysis and accumulation of fatty acids in fixed tissue indicated the lamellae were composed primarily of fatty acids. We conclude that fatty acids formed by lipolysis of chylomicrons in tissue from fed rats accumulated in a continuum of the outer leaflets of cell membranes extending from capillary lumen to lipid droplets of adipocytes, and fatty acids formed by lipolysis of intracellular lipid in tissue from cold-exposed or unsuckled rats accumulated mostly in a continuum extending from lipid droplets to the interior of mitochondria. When fatty acids overcrowded the continuum in fixed tissue, they formed lamellar extensions of the continuum at different sites along its course through the tissue.—**Blanchette-Mackie, E. J., and R. O. Scow.** Movement of lipolytic products to mitochondria in brown adipose tissue of young rats: an electron microscope study. *J. Lipid Res.* 1983. **24:** 229–244.

Supplementary key words lamellar structures • chylomicrons • fatty acids

Brown adipose tissue is an important site of heat production in young rats (1, 2). The tissue consists of well vascularized lobules of adipocytes (2), and heat is produced in mitochondria by oxidation of long chain fatty acids (2–4) derived mostly from triacylglycerol in circulating chylomicrons and very low density lipoproteins (VLDL) (5, 6). Uptake of fatty acids from chylomicrons and VLDL requires hydrolysis of the triacylglycerol to fatty acids by lipoprotein lipase acting at the luminal surface of capillary endothelium (7, 8). Lipoprotein lipase activity is increased in this tissue by fasting (9) and by exposure to cold (10–12). Some of the fatty acids taken up are esterified to triacylglycerol and stored in lipid droplets in brown adipocytes before being oxidized. Movement of fatty acids from the droplets to mitochondria requires hydrolysis of triacylglycerol to fatty acids by tissue (hormone-sensitive) lipase (13), which is also increased by fasting (14) and exposure to cold (14).

The mechanism for transport of long chain fatty acids from capillary to lipid droplets and mitochondria in brown fat cells is not known. We have developed a model for transport of fatty acids from chylomicrons to parenchymal cells in extrahepatic tissues by lateral movement in an interfacial continuum composed of external leaflets of plasma and intracellular membranes of endothelial and parenchymal cells (8, 15–17). The model proposes that fatty acids enter the continuum in capillaries, at the site of lipolysis, and leave the continuum in endoplasmic reticulum of adipocytes where they are reesterified to triacylglycerol and accumulate as lipid droplets (lenses) between leaflets of the reticulum. Recent studies in white adipose tissue of young rats provide histochemical evidence for transport of fatty acids from capillary to white adipocytes by lateral movement in an interfacial continuum of membranes (18). Fatty acids produced by lipolysis of chylomicrons in fixed tissue accumulated in the continuum and formed lamellar extensions at various sites along the continuum, thereby marking its course through the tissue. Similar findings in hearts of young rats support the concept of a continuum between capillary and parenchymal cells (myocytes) and provide evidence for extending the continuum to mitochondria (19).

This report describes morphological studies of movement of lipolytic products in fixed brown adipose tissue. The findings constitute new evidence for transport of fatty acids from intracellular lipid droplets to mito-

chondria in adipocytes, as well as transport of fatty acids from capillary lumen to lipid droplets in parenchymal cells (adipocytes), by lateral movement in an interfacial continuum of cell membranes.

MATERIALS AND METHODS

Animals

Female CD rats pregnant for 15 days were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA) and allowed free access to water and Purina NIH Open Formula Rat and Mouse Ration #5018 (Ralston Purina Co., St. Louis, MO). Two days after delivery, the size of each litter was adjusted to eight pups.

Preparation of chylomicrons

Chylomicrons were isolated from thoracic duct lymph (20) collected for 6 hr from adult rats tube-fed, after an overnight fast, 0.5 ml of corn oil. Chylomicrons containing triacylglycerol labeled with [^{3}H]oleic acid were collected from rats tube-fed corn oil containing tri[9,10-^{3}H]oleoylglycerol (545 mCi/mmol, TRA 191, Batch 45, Amersham Corp., Arlington, IL). The chyle was centrifuged in a swinging head rotor SW 50.1 at 24,000 rpm for 60 min at 3°C with a Spinco Model L-2-65B ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA). The compact floating layer of chylomicrons formed during centrifugation was collected and suspended, as described elsewhere (20), in 4% albumin in Tyrode's solution at a triacylglycerol concentration of 80–120 mM. The albumin-Tyrode's solution contained 40 mg of bovine serum albumin powder (Fraction V, Lot J-40409, Armour Pharmaceutical Co., Kankakee, IL) per ml of glucose-free Tyrode's solution.

Morphological analyses

Interscapular brown fat tissue was immediately removed from decapitated rats and cut into small pieces. The tissue was fixed with 3% glutaraldehyde and 2% tannic acid (Mallinckrodt, Inc., St. Louis, MO) in 0.2 M sodium cacodylate solution, pH 7.4, for 30 min at 4°C. Tissue was rinsed (5 min) with cold cacodylate solution to remove excess glutaraldehyde. Some pieces were immediately post-fixed with 2% OsO_4 in 0.2 M sodium cacodylate solution, pH 7.4, for 2 hr at 4°C. Other pieces of tissue were incubated with 1% tannic acid in 0.2 M sodium cacodylate solution, pH 7.4, for 15–120 min at 25°C before being post-fixed with 2% OsO_4.

The tissues were then dehydrated rapidly with acetone (21) and embedded in Epon 812. Some sections were stained with $Pb(OH)_2$ and all were examined in a Phillips EM-300 electron microscope.

Morphometry

The effect of incubation at 25°C on development of lamellae in fixed tissue was studied in interscapular brown fat tissue taken from 8-day-old rats. The tissue, weighing about 3 mg, was diced into small pieces with a razor blade and fixed with a mixture of 3% glutaraldehyde and 2% tannic acid in 0.2 M sodium cacodylate solution (pH 7.4) at 4°C for 30 min. The tissue pieces were rinsed twice in 1% tannic acid in 0.2 M sodium cacodylate solution (pH 7.4) at 25°C. Three blocks of tissue from each animal were selected at random for immediate post-fixation with 2% OsO_4 in 0.2 M sodium cacodylate solution at 4°C for 2 hr (0 min incubation), and another three blocks were incubated in tannic acid-cacodylate solution for 60 min at 25°C, and then post-fixed with OsO_4. All specimens were dehydrated, embedded, and sectioned in the same manner as above. Twelve thin sections, 1 mm × 0.5 mm, were cut from each block; the sections were cut serially in sets of four with an interval of 10 μm between each set, and each set was placed as a ribbon on a hexagonal grid. One micrograph was taken at random from each section (18, 22) at 4,000× magnification and printed at a final magnification of 10,000×. The printed micrographs measured 18 × 24 cm.

The point counting method was used to estimate the volume fraction of lamellae and cells (22). The points counted were intersections of lines of square lattices. The lattices, measuring 18 × 22 cm, were drawn on transparent sheets with spaces of 3.0, 1.0, or 0.5 cm between lines. The coarser lattices were used for measuring cell volumes, while the fine lattice (0.5 cm), containing 1536 intersections, was used for measuring volume of lamellae. The number of points falling on a specific structure relative to the total number of points falling on the tissue was determined for each micrograph. In addition, the location of lamellae was recorded: in capillary lumen and endothelium, in extracellular space, or in adipocytes associated with either lipid droplets or mitochondria. Lamellae present in invaginations of cell surfaces were placed in the category "extracellular space". Lamellae in lipid droplets or extending from lipid droplets were placed in the category "associated with lipid droplets". Lamellae in mitochondria or in contact with the outer mitochondrial membrane were placed in the category "associated with mitochondria". The volume fraction of structures was measured by the relative number of points falling on each structure. Ten micrographs, selected at random, were used to determine the volume fraction of lamellae and tissue components in each tissue block, and three

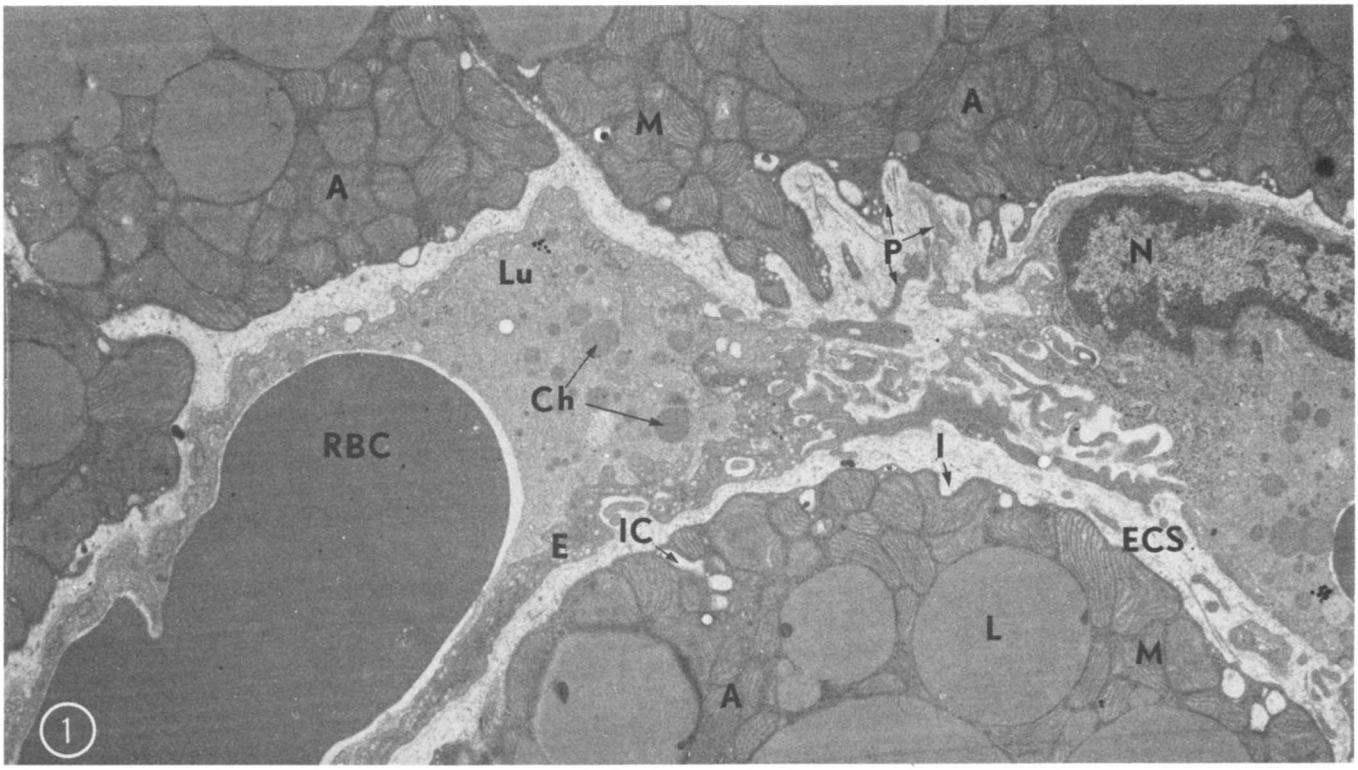

Fig. 1. A section of brown adipose tissue from a fed 8-day-old rat injected with chylomicrons showing a portion of capillaries and adipocytes. The capillary lumen (Lu) contains numerous chylomicrons (Ch). Mitochondria (M) and lipid droplets (L) occupy most of the interior of adipocytes (A). Adipocytes have numerous invaginations (I) of the surface and intracellular channels (IC) near the surface. Cytoplasmic processes (P) of both endothelial cells (E) and adipocytes extend across extracellular space (ECS) toward each other. N, nucleus; RBC, erythrocyte. Incubated at 25°C: 0 min. ×8,500.

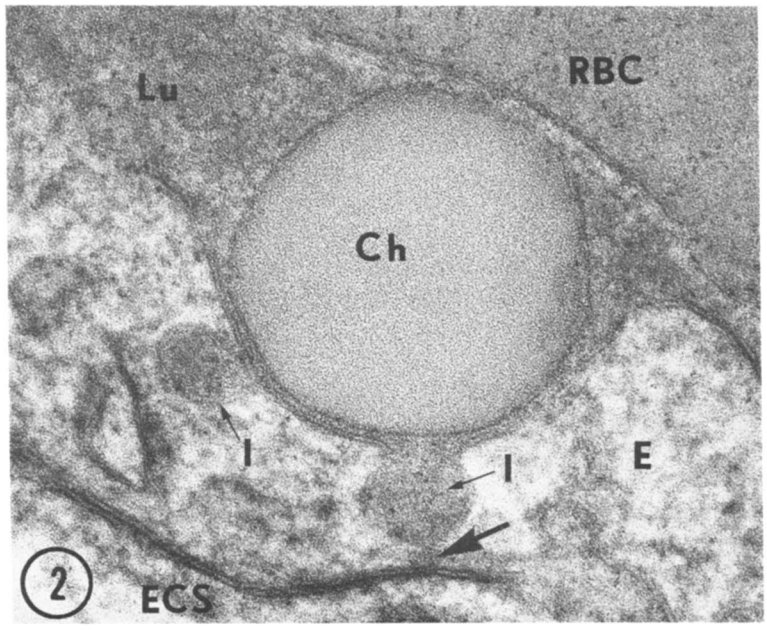

Fig. 2. A section of brown adipose tissue from a fed 8-day-old rat injected with chylomicrons showing a portion of capillary. Electron opaque material fills the capillary lumen (Lu) and invaginations (I) of the luminal surface of the endothelial cell. Note at large arrow that one of the invaginations is in contact with the basal surface of the endothelial cell (E). Ch, chylomicron; ECS, extracellular space; RBC, erythrocyte. Incubated at 25°C: 15 min. ×148,500.

blocks were examined at each time interval for each rat. The values given in Tables 1 and 2 for rats injected with chylomicrons or exposed to cold are the mean ± standard error of measurements made on tissue blocks from three animals (N = 3), whereas the values given for fed and unsuckled rats are the mean ± standard error of measurements made on three blocks of tissue (N = 3) from one animal.

Biochemical analyses

Lipoprotein lipase activity was assayed in clear aqueous extracts of brown adipose tissue with rat chylomicron triacylglycerol as substrate (23). The chylomicrons contained triacylglycerol labeled with [^{3}H]oleic acid. Production of fatty acid was linear with both time and amount of sample assayed. One milliunit of activity represents production of 1 nmol of fatty acid/min.

Hydrolysis of chylomicron triacylglycerol in glutaraldehyde-fixed brown adipose tissue was measured in tissue taken from 13-day-old rats injected with chylomicrons containing triacylglycerol labeled with [^{3}H]oleic acid. The rats were anesthetized with sodium pentobarbital, 4 mg/100 g body wt, given intraperitoneally 10–12 min before they were injected with chylomicrons. A suspension of chylomicrons containing about 20 μmol of triacylglycerol in 0.15 to 0.2 ml was injected into the left saphenous vein over a period of 15–25 sec, and 1.5 min later the rat was decapitated and three pieces of brown fat tissue were taken for study. One piece was put immediately into hexane-isopropanol (24) at 4°C (unfixed specimen), and the rest were cut into smaller pieces, fixed in 5 ml of 3% glutaraldehyde in 0.2 M sodium cacodylate solution (pH 7.4) for 30 min at 4°C, and rinsed twice with 5 ml of cold 0.2 M sodium

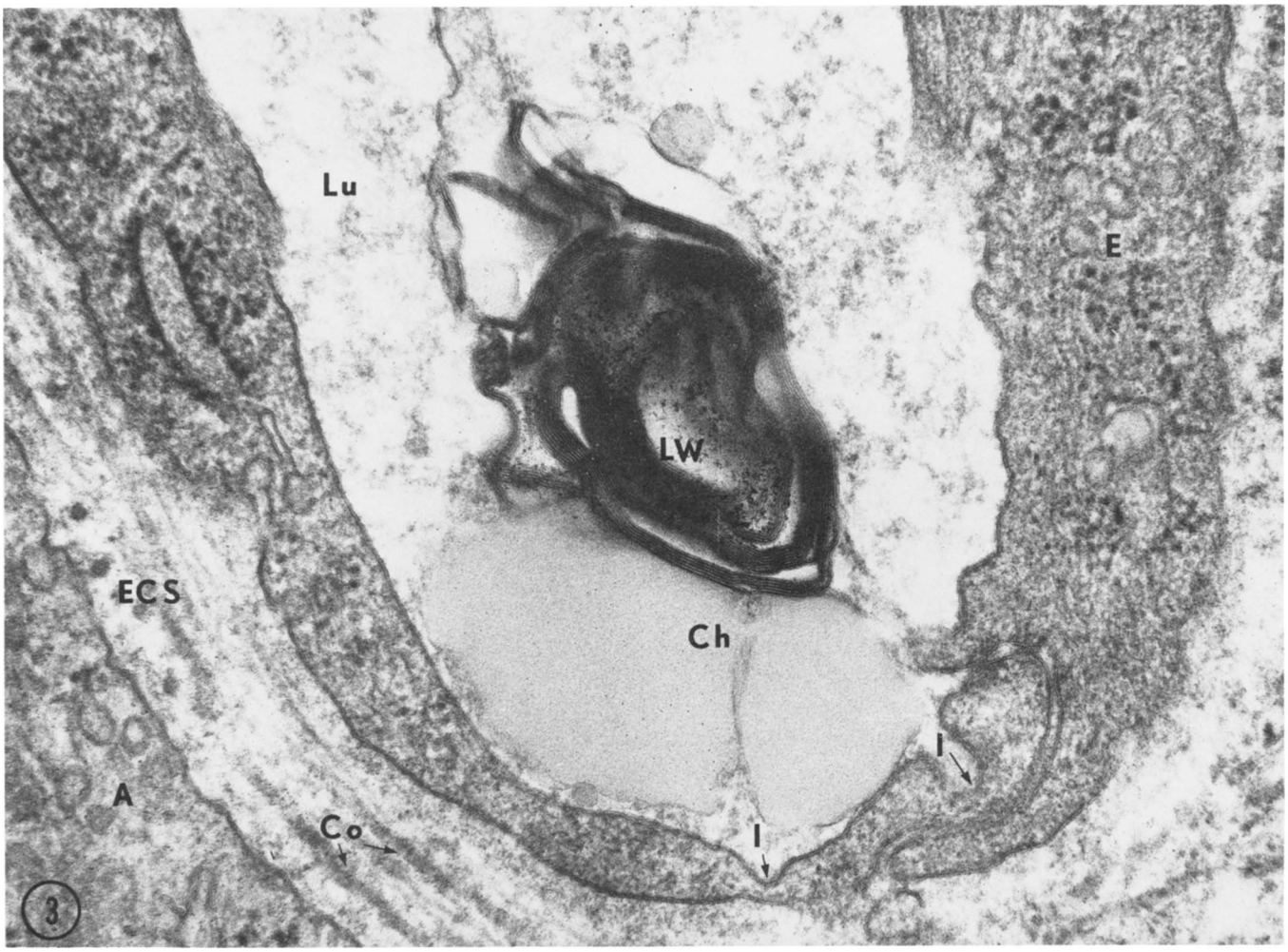

Fig. 3. A section of brown adipose tissue from a fed 8-day-old rat injected with chylomicrons showing a portion of capillary and adipocyte. Lamellar whorls (LW) are associated with chylomicrons (Ch) in the capillary lumen (Lu). A, adipocyte; Co, collagen; E, endothelium; ECS, extracellular space; I, invagination. Incubated at 25°C: 15 min. ×77,500.

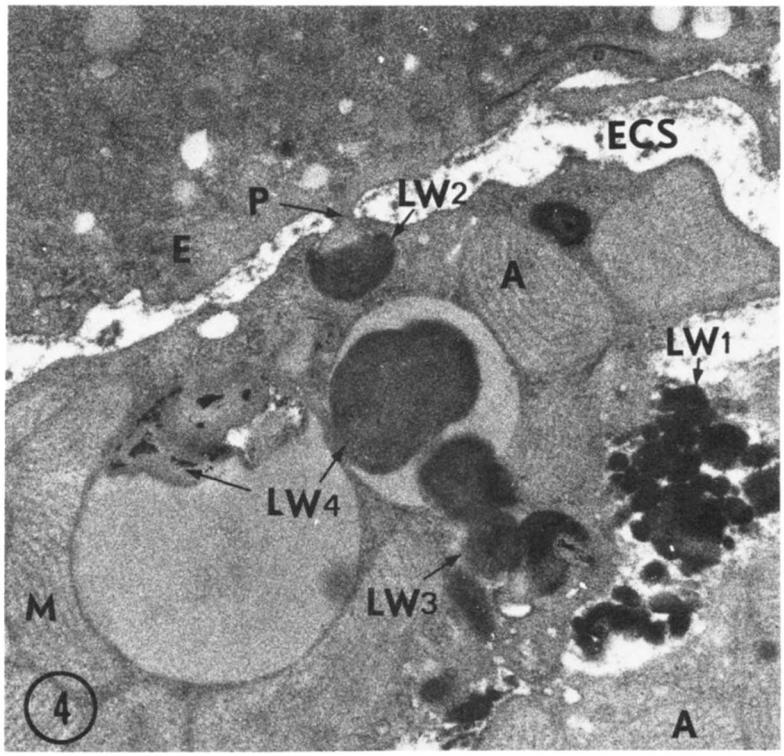

Fig. 4. A section of brown adipose tissue from a fed 8-day-old rat injected with chylomicrons showing a capillary and adipocytes. A basal projection (P) of capillary endothelium (E) extends into an invagination of an adipocyte (A). Lamellar structures are present in extracellular space (LW_1), near the area of intercellular contact (LW_2), in invaginations of adipocytes (LW_3), and associated with lipid droplets in adipocytes (LW_4). The periodicity of the lamellar whorls is not apparent at this low magnification. ECS, extracellular space; M, mitochondria. Incubated at 25°C: 60 min. ×22,000.

cacodylate solution (pH 7.4). Half of the fixed pieces were put immediately into cold hexane-isopropanol (fixed specimen) whereas the others were incubated in 1% tannic acid–0.2 M sodium cacodylate solution (pH 7.4) for 30 min at 25°C before being put into cold hexane-isopropanol (incubated-fixed specimens). Neutral lipids and fatty acids in the specimens were extracted into hexane (24) and separated into triacylglycerol and fatty acid fractions by thin-layer chromatography (25). Lipids were dissolved in Beckman GP Scintillation Solvent for measurement of ^3H content with a Beckman LS-8000 β Spectrometer. Acylglycerol content of chylomicrons was measured by the method of Rapport and Alonzo (26).

RESULTS

The adipocytes in interscapular brown adipose tissue of 8-day-old rats contained numerous mitochondria and lipid droplets of various sizes and had irregular surfaces, formed by large surface invaginations and cytoplasmic processes (**Fig. 1**). The cytoplasmic processes extended outward to capillary endothelial cells, and sometimes curved back to the cell to form circular areas resembling vacuoles in section (Fig. 1). The adipocytes also had intracellular channels.

Capillary endothelial cells in brown adipose tissue had cytoplasmic processes that extended from the basal surface toward processes of adipocytes (Fig. 1). Basement membrane, which surrounded both capillaries and adipocytes, was absent at points of contact between capillary cells and adipocytes. Capillary endothelial cells had invaginations of luminal and basal surfaces (**Figs. 2, 3 and 5**) which sometimes appeared to span the width of the endothelium (Fig. 2). Chylomicrons in the capillary lumen were in contact with the endothelium (Figs. 2 and 3), often at sites of invaginations of the luminal plasma membrane (Fig. 2).

Lamellar structures were found in capillaries, extracellular space, and adipocytes in brown adipose tissue of young rats, especially when the tissue was incubated at 25°C after being fixed with glutaraldehyde (Figs. 3–12). The lamellar structures often had a regular pattern

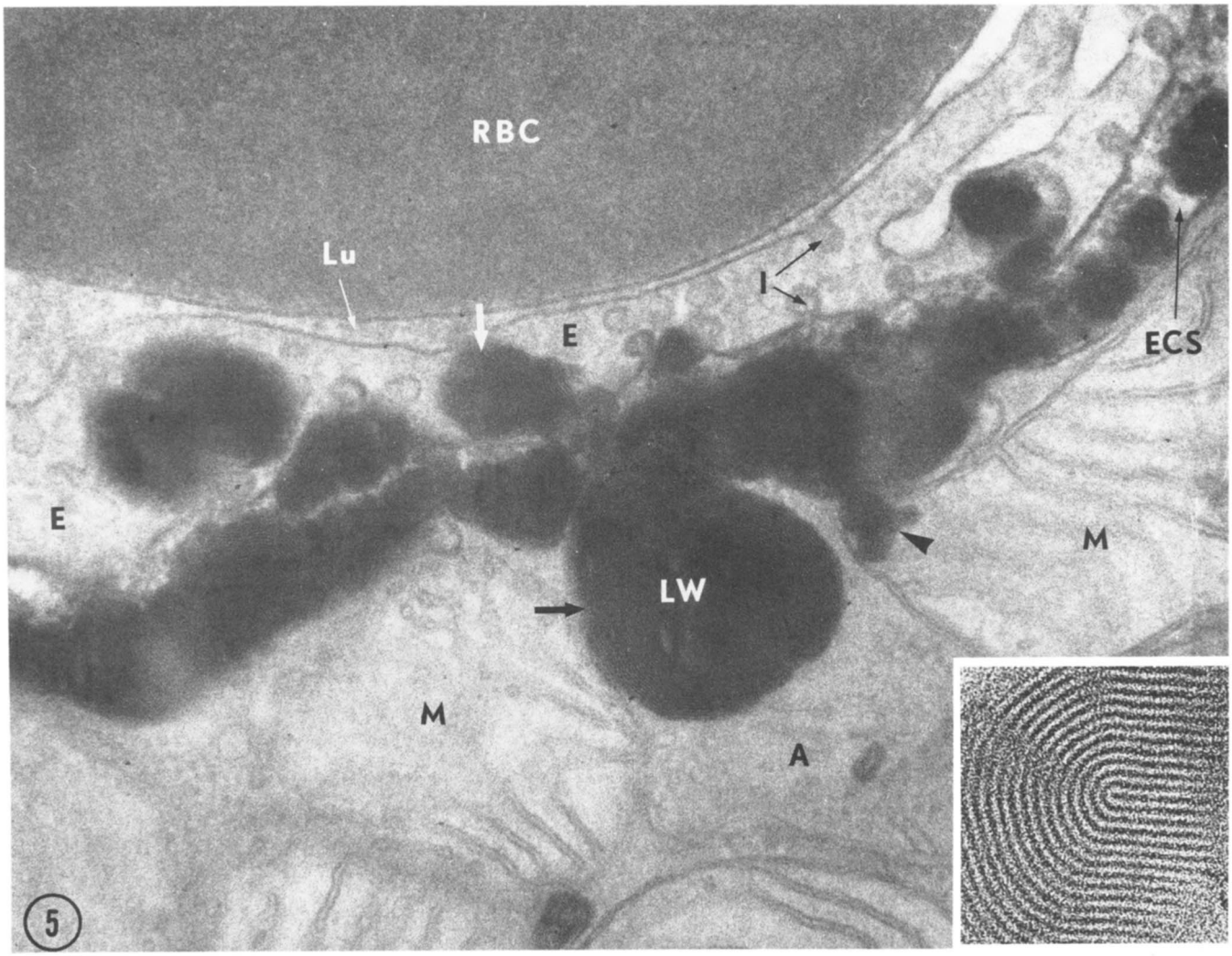

Fig. 5. A section of brown adipose tissue from a fed 8-day-old rat showing a capillary and adipocyte. Lamellar whorls (LW) extend from the luminal surface of the endothelial cell (E) (white arrow), across the endothelial cell and extracellular space (ECS), into a surface invagination of an adipocyte (A) (black arrow). A small amount of lamellae is associated at arrowhead with a mitochondrion (M). The inset shows an intracellular lamellar whorl at higher magnification to demonstrate its 40 Å repeating pattern. I, invagination; Lu, capillary lumen; RBC, erythrocyte. Incubated at 25°C: 60 min. ×88,000; Inset, ×400,000.

of alternating dark and light lines with a periodicity of 40 Å in tissue (Fig. 6). Large lamellar whorls within adipocytes sometimes had areas of irregular banding patterns and staining, and contained aqueous spaces (Fig. 6). Lamellae were found in the lumen of capillaries (Fig. 3), sometimes associated with chylomicrons (Fig. 3), in channels inside capillary endothelial cells (Fig. 5), in extracellular space (Figs. 1 and 4–6), at sites of contact between endothelium and adipocytes (**Fig. 4**), and in adipocytes associated with lipid droplets (Figs. 1 and 6–10) and mitochondria (Figs. 9–12). Some of the lamellae in adipocytes could be localized to the lumen of intracellular channels (**Figs. 6–8**), and some of the channels containing lamellae were open to extracellular space (Fig. 7). Lamellae were sometimes adjacent to aqueous spaces near lipid droplets (Figs. 8 and 10). The lamellae associated with mitochondria were found in channels near mitochondria and in contact with the outer mitochondrial membrane (Figs. 8–10); between the outer and inner mitochondrial membranes (**Fig. 11**); and between membranes of the cristae (**Fig. 12**). Lamellae were also found between lipid droplets and mitochondria (**Figs. 9 and 10**).

Effect of incubation and metabolic state of tissue donor

The effect of incubation at 25°C and the metabolic state of the tissue donor on the amount and distribution

of lamellar structures in glutaraldehyde-fixed brown adipose tissue was determined with morphometric analyses of micrographs of tissue from fed rats, rats injected with chylomicrons, rats exposed to 4°C for 2 hr, and rats unsuckled overnight (**Table 1** and **Table 2**). The tissue specimens were fixed in glutaraldehyde-tannic acid solution at 4°C for 30 min, rinsed in tannic acid-cacodylate solution, and incubated in the latter solution at 25°C for 0 or 60 min, and post-fixed with OsO_4 at 4°C. Table 1 gives the relative volume of capillaries, extracellular space, and certain intracellular components of adipocytes in the tissue specimens used for this study. The values for each tissue compartment were similar in all groups indicating that the sampling procedure used was satisfactory.

The volume of lamellae was very small at the start

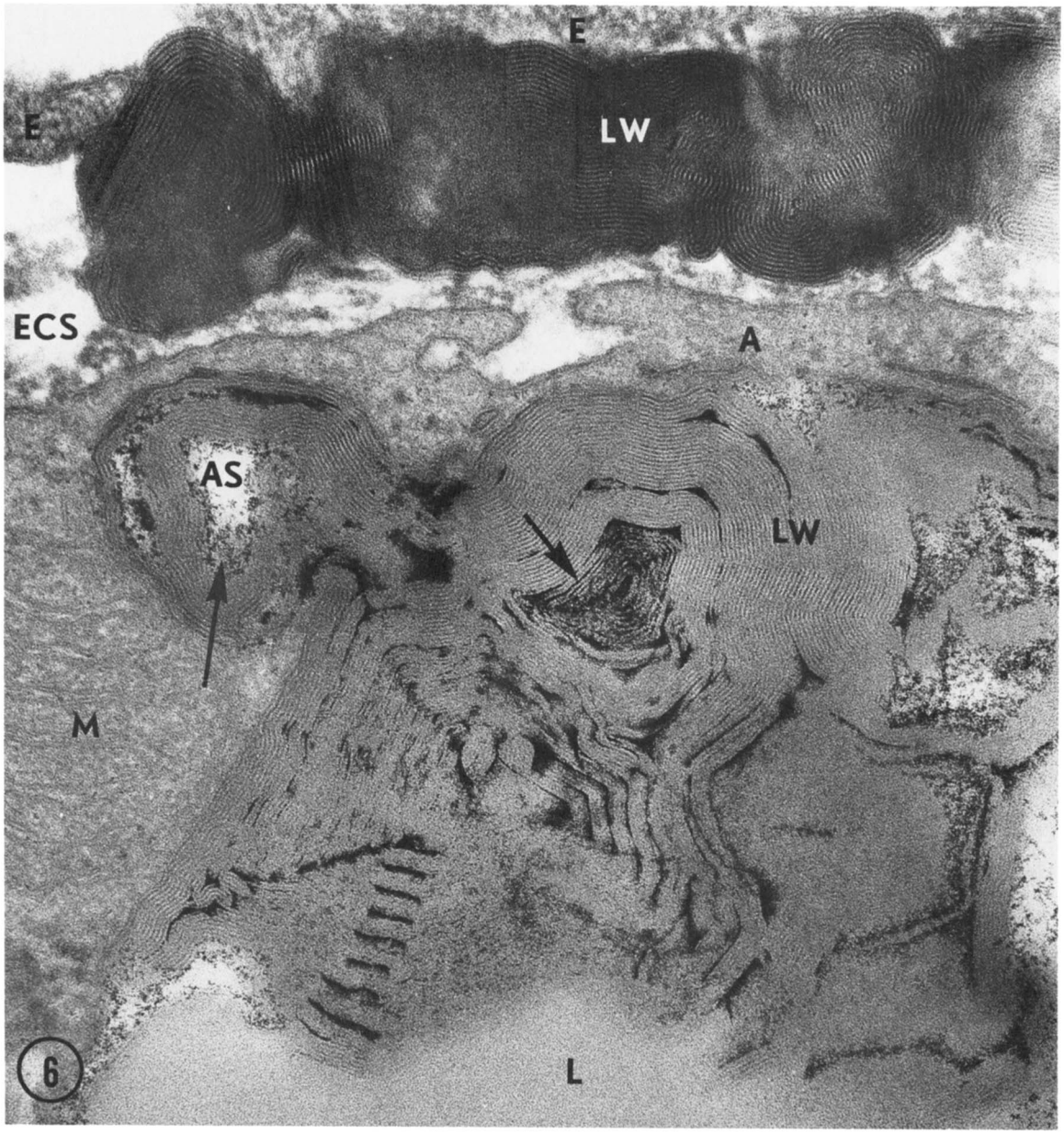

Fig. 6. A section of brown adipose tissue from a fed 8-day-old rat injected with chylomicrons showing a portion of an adipocyte, extracellular space, and capillary endothelium. Note the large lamellar whorl (LW) in the extracellular space (ECS) and another near a lipid droplet (L) in the adipocyte (A). The lamellar whorl near the lipid droplet has patchy electron-opaque areas (arrows), probably due to tannic acid. The lamellar whorl in extracellular space has regularly aligned lamellae and a uniform staining pattern. AS, aqueous space; E, capillary endothelium; I, invagination; M, mitochondrion. Incubated at 25°C: 60 min. ×140,000.

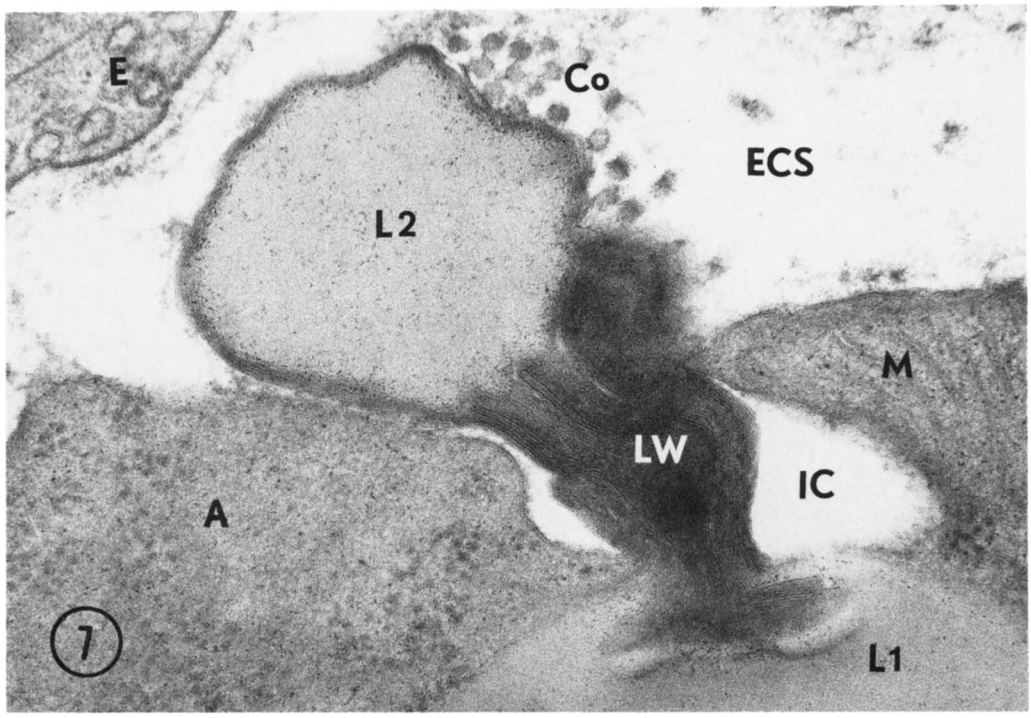

Fig. 7. A section of brown adipose tissue from a fed 8-day-old rat injected with chylomicrons showing a portion of an adipocyte and capillary endothelium. Note the lamellar whorl (LW) in a surface-connected channel of an adipocyte (A), located between an intracellular lipid droplet (L_1) and an extruded portion of the droplet (L_2). The lamellar whorl is continuous with both the intracellular lipid droplet and the extruded droplet. Note lamellae inside the intracellular droplet and at the surface of the extruded droplet. A, adipocyte; Co, collagen; E, capillary endothelium; ECS, extracellular space; IC, intracellular channel; M, mitochondria. Incubated at 25°C: 15 min. ×82,000.

of incubation (0 min) and increased markedly during incubation in all groups (Table 2). Distribution of lamellae in incubated tissue was dependent on the metabolic state of the tissue donor. In tissues from uninjected fed rats and fed rats injected with chylomicrons, lamellae developed most in extracellular space ($P < 0.025$), less in capillaries ($P < 0.025$) and in adipocytes near lipid droplets ($P < 0.025$), and rarely in or near mitochondria. In tissues from cold-stressed and unsuckled rats, lamellae developed most in and near mitochondria of adipocytes ($P < 0.05$), less near lipid droplets and in extracellular space, and the least in capillaries. Aqueous spaces developed near intracellular lipid droplets (Figs. 8 and 10) in tissue from cold-exposed and unsuckled animals ($P < 0.025$) (Table 2).

Lipoprotein lipase activity was present in brown adipose tissue of 9–14-day-old suckling rats (**Table 3**), as reported by others (27, 28). The activity in the tissue was increased 150% when the rats were not suckled for 18 hr. The findings presented in **Table 4** clearly indicate that lipoprotein lipase was active in glutaraldehyde-fixed brown adipose tissue of young rats. Triacylglycerol of injected chylomicrons was hydrolyzed to fatty acids during fixation as well as during incubation of fixed tissue at 25°C. The findings also show that lipoprotein lipase activity was >150% higher in tissue from rats unsuckled overnight than fed rats, confirming the data reported in Table 3.

DISCUSSION

These studies show that lamellar structures developed in brown adipose tissue of young rats when tissue fixed with glutaraldehyde was incubated at 25°C. The lamellar structures had a regular pattern of alternating dark and light bands with a periodicity of 40 Å, the same as observed in sections of sodium oleate fixed with osmium at pH 7.4 (29). They were found in capillaries, extracellular space, and adipocytes. Distribution of lamellae in the tissue was affected by the metabolic state of the tissue donor (Table 2). They were located primarily in extracellular space and near lipid droplets of adipocytes in tissue from control and chylomicron-injected fed rats, whereas they were located mostly near or in mitochondria in tissue from rats exposed to 4°C for 2 hr or unsuckled overnight. Biochemical studies showed that lipoprotein lipase was active in glutaral-

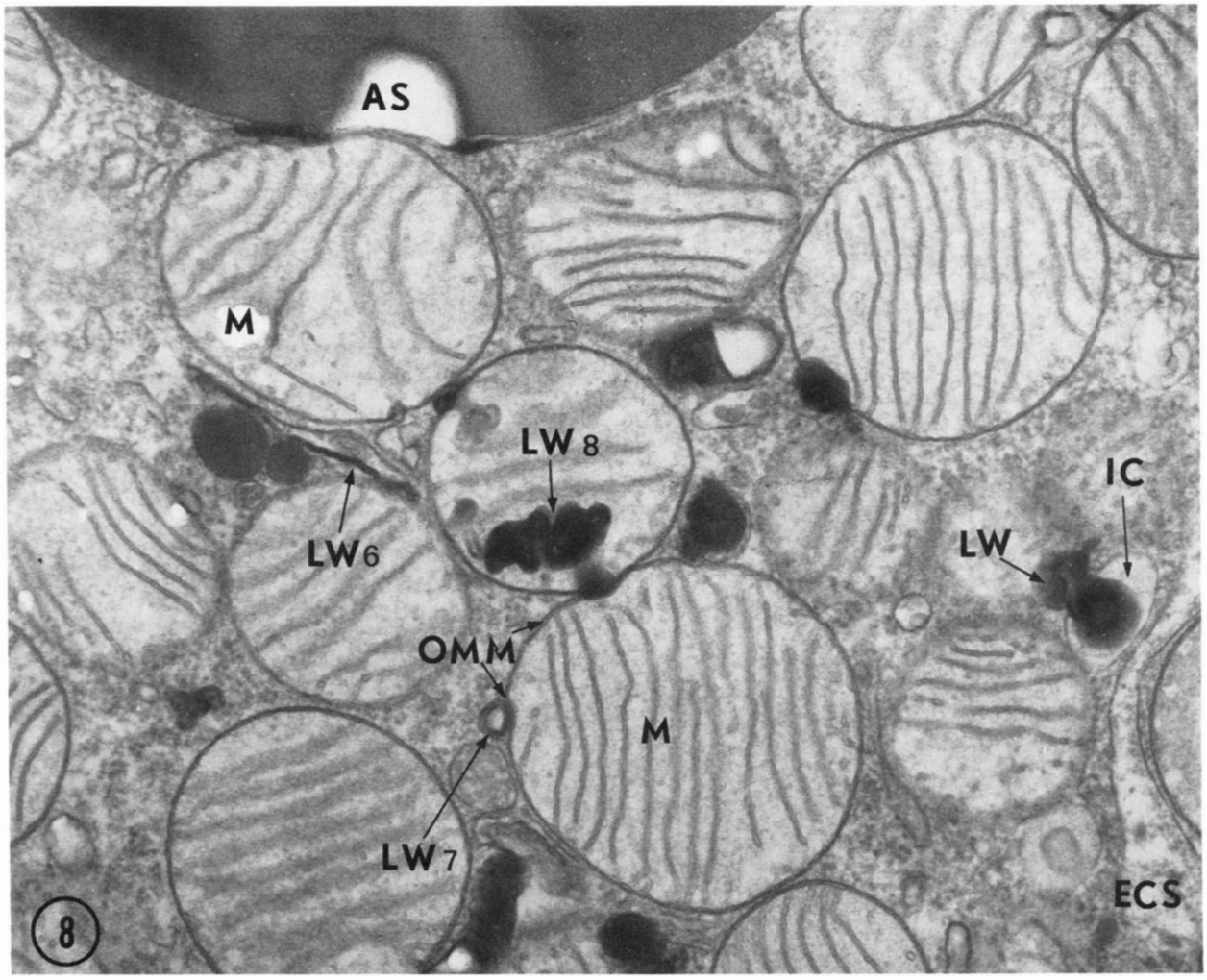

Fig. 8. A section of brown adipose tissue from a fed 8-day-old rat exposed to cold showing a portion of an adipocyte. Lamellar whorls are present in intracellular channels (LW_6), near the outer mitochondrial membrane (LW_7), and inside mitochondria (LW_8). ECS, extracellular space; IC, intracellular channel; OMM, outer mitochondrial membrane; M, mitochondria. Incubated at 25°C: 60 min. ×45,000.

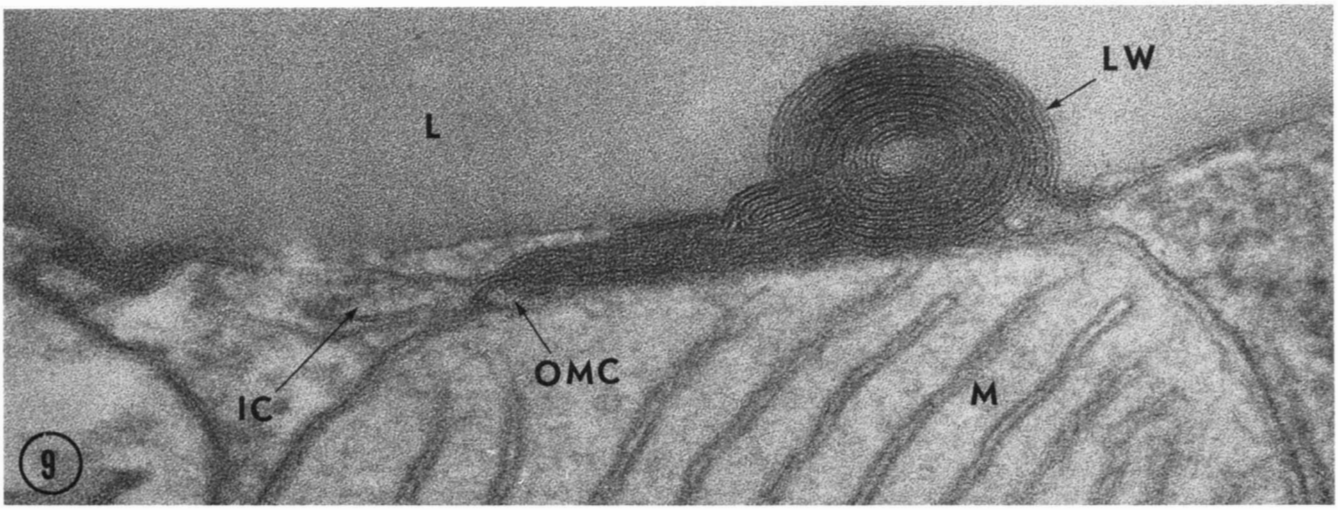

Fig. 9. A section of brown adipose tissue from a fed 8-day-old rat exposed to cold showing a portion of an adipocyte. A lamellar whorl (LW) extends from the surface of a lipid droplet (L) into an intracellular channel (IC) and the outer chamber (OMC) of a mitochondrion (M). Incubated at 25°C: 60 min. ×147,000.

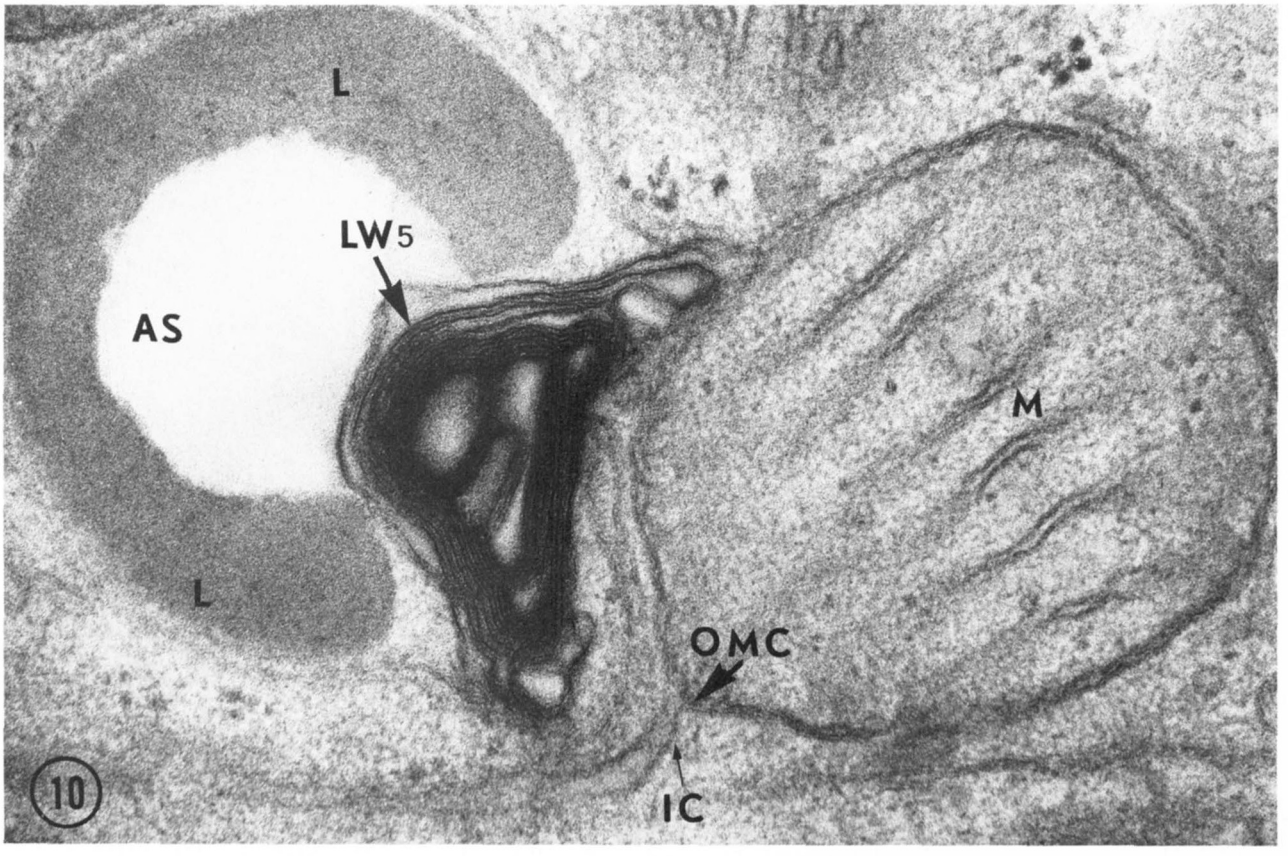

Fig. 10. A section of brown adipose tissue from a fasted 8-day-old rat showing a portion of an adipocyte. Lamellar whorls (LW_5) in the aqueous space (AS) extend from the lipid droplet (L) to the mitochondrion (M). An intracellular channel (IC) appears continuous with the other mitochondrial chamber (OMC) at arrow. Incubated at 25°C: 60 min. ×156,000.

dehyde-fixed brown adipose tissue (Table 4), and that the fatty acids produced remained in the tissue, confirming our earlier findings in white adipose tissue (18, 21) and heart (19). Development of aqueous spaces near intracellular lipid droplets (Table 2), an indication of lipolytic activity (18, 29–31), suggests that tissue (hormone-sensitive) lipase (13) was active in fixed tissue from cold-exposed and unsuckled animals.

The development of lamellar structures under conditions causing lipolysis and accumulation of fatty acids in fixed tissue, the effect of the metabolic state of the tissue donor on distribution of lamellae in the tissue (Table 2), and the similarity in periodicity between lamellar structures in tissue and in sections of sodium oleate suggest that the lamellar structures found in incubated-fixed brown adipose tissue are primarily fatty acids formed by lipolysis in fixed tissue.

Additional evidence that lipolysis is involved in development of lamellar structures in incubated fixed tissue was obtained in white adipose tissue from young rats (18). Lamellar structures were present in capillary lumen, associated with chylomicrons, at the start of incubation in adipose tissue from chylomicron-injected fed rats, whereas they appeared in capillary lumen in tissue from uninjected fasted rats only after 30 min of incubation. In contrast, lamellar structures near lipid droplets in adipocytes developed earlier and were much larger in tissue from fasted rats than in tissue from fed rats. These distributions of lamellae correspond to the direction of flow of fatty acids in white adipose tissue in vivo, from capillaries to adipocytes in the fed state and from adipocytes to capillaries in the fasted state. It was also observed that glucose and insulin, which enhance reesterification and lower fatty acid content in adipose tissue (32), prevented development of lamellar structures in white adipose tissue from fed rats incubated at 25°C before being fixed with glutaraldehyde (18). Lamellar structures also developed in incubated fixed heart of rat under conditions causing lipolysis and accumulation of fatty acids in that tissue (19).

Three mechanisms have been proposed for transport of fatty acids in tissues: transport by molecular diffusion through aqueous phase and across cell membranes (33), transport through cells by fatty acid-binding protein

(34), and transport by lateral movement in cell membranes (8). Limitations of the first two mechanisms, especially in fixed tissues, have been discussed in detail in earlier publications (16, 18). Transport of fatty acids by lateral movement in membranes is feasible because cell membranes are composed of lipid bilayers (35), amphipathic lipids can move rapidly in the plane of membranes (36), and long chain fatty acids are amphipathic (37, 38).

Our concept of transport of fatty acids from blood to tissue by lateral movement in cell membranes presupposes an interfacial continuum between capillary lumen and the interior of parenchymal cells (8, 15, 17). Studies in adipose and other tissues have shown transcellular channels in endothelial cells, some formed by interconnected vesicles, that connect capillary lumen with extracellular (extravascular) space (Fig. 2) (8, 18, 39). Since the external (luminal) leaflet of the membrane lining such channels connects the external leaflet of the luminal plasma membrane with that of the basal plasma membrane, it provides an interfacial continuum across capillary endothelium.

Areas of apparent contact between endothelial and parenchymal cells have been observed in white adipose tissue (18, 40) and heart (19), as well as in brown adipose tissue (Fig. 4). Recent findings in white adipose tissue suggest that the external leaflets of plasma membrane of adjoining cells are continuous in areas of contact between endothelial and parenchymal cells (40).

Studies in white adipose tissue of young rats, using tannic acid as an extracellular marker, showed channels in adipocytes that extended from extracellular space to the surface of lipid droplets in cells (40). Similar studies in brown adipose tissue of young rats, using both tannic acid and lanthanum as extracellular markers, showed marker substance present in intracellular channels surrounding lipid droplets in brown adipocytes (41). This finding indicates that the lipid droplets protrude into the lumen of endoplasmic reticulum in brown adipocytes (41). The small distance found between the lumen

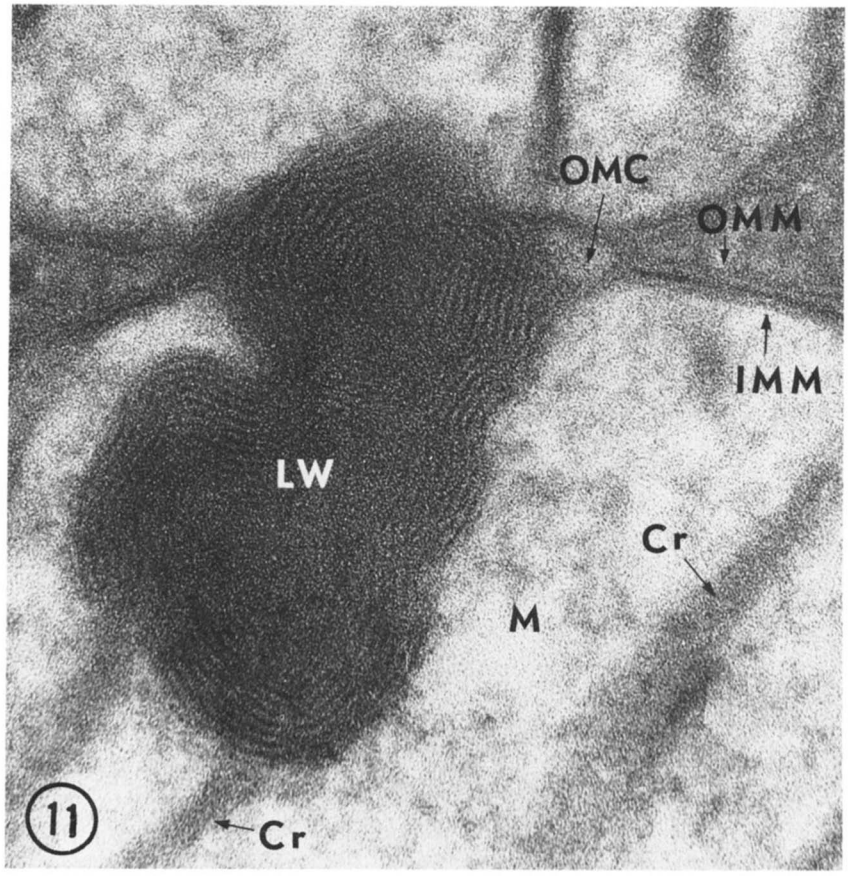

Fig. 11. A section of brown adipose tissue from a fasted 8-day-old rat exposed to cold showing a portion of an adipocyte. A lamellar whorl (LW) is located within the outer mitochondrial chamber (OMC) and the intracristal space. Cr, crista; IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane. Incubated at 25°C: 60 min. ×240,000.

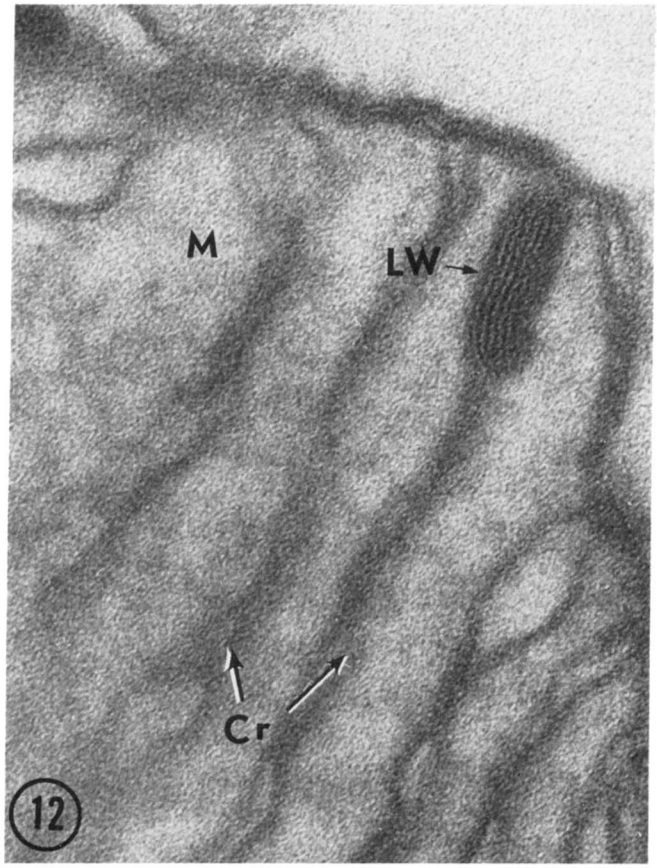

Fig. 12. A section of brown adipose tissue from a fed 8-day-old rat exposed to cold showing a portion of an adipocyte. Lamellae (LW) are present in the intracristal space of a mitochondrion (M). Cr, crista. Incubated at 25°C: 60 min. ×195,000.

of the channel and the lipid droplet suggests that a monolayered structure, perhaps a leaflet of the membrane lining the channel, separated the lipid droplet from the lumen. This finding supports the concept that newly synthesized triacylglycerol accumulates between the luminal and cytoplasmic leaflets of endoplasmic reticulum (8, 16, 17, 42). Thus, the luminal leaflet of the membrane lining these channels provides an interfacial continuum between the external leaflet of the plasma membrane of adipocytes and the surface of lipid droplets in such cells. Although membrane continuity between endoplasmic reticulum and the external membrane of mitochondria has not been observed in brown adipocytes, it has been demonstrated in several other tissues, rat liver (43, 44) and diaphragm muscle (45), canine heart (46), and mouse oocytes (47). The above constitute the morphological evidence at present for an interfacial continuum of membranes extending from the luminal surface of capillaries to endoplasmic reticulum and mitochondria of parenchymal cells.

Our studies with incubated chylomicrons (29) and lipid monolayers (38) showed that lipolytic products formed by hydrolysis of long chain triacylglycerol locate in the interface between lipid/gas and aqueous medium at pH 7.4, and spread towards zones of decreased surface pressure, resulting from either removal of lipolytic products from the interface or extension of the interface. According to our model for fatty acid transport in tissue, lipolytic products would be removed from the interfacial continuum in vivo by either esterification, oxidation, or binding to albumin in plasma (17). When

TABLE 1. Volume of capillaries, extracellular space, and components of adipocytes in brown adipose tissue of 8-day-old rats

Group	Tissue Incubated at 25°C	No. of Rats	Volume of				
			Capillaries	Extracellular Space	Adipocytes		
					Lipid Droplets	Mitochondria	Other
	min				*% of tissue volume*		
Fed	0	1	11 ± 3[a]	8 ± 2	48 ± 2	30 ± 2	3 ± 1
	60	1	10 ± 2	6 ± 2	52 ± 5	30 ± 5	2 ± 1
Fed + chylomicrons IV[b]	0	3	15 ± 1[c]	6 ± 1	35 ± 4	40 ± 3	3 ± 1
	60	3	14 ± 1	7 ± 1	35 ± 1	40 ± 2	5 ± 1
Exposed to 4°C for 2 hr[d]	0	3	11 ± 1[c]	6 ± 1	50 ± 1	33 ± 1	1 ± 1
	60	3	11 ± 0.5	6 ± 0.2	40 ± 3	39 ± 1	5 ± 2
Unsuckled for 18 hr[e]	0	1	10 ± 0.4[a]	5 ± 0.4	42 ± 1	37 ± 1	5 ± 2
	60	1	11 ± 0.4	8 ± 0.4	45 ± 3	32 ± 2	5 ± 2

[a] Values given for this group are mean ± standard error of measurements made in three blocks of tissue from one rat (N = 3). The measurements were made using lattices with 1 cm between lines.

[b] Tissue was taken 1.5 min after intravenous injection of chylomicrons.

[c] Values given for this group are mean ± standard error of measurements made in tissues of three rats (N = 3). The measurements were made using lattices with 3 cm between lines.

[d] Rats were kept at 4°C without suckling for 2 hr.

[e] Unsuckled rats were kept at 20°C.

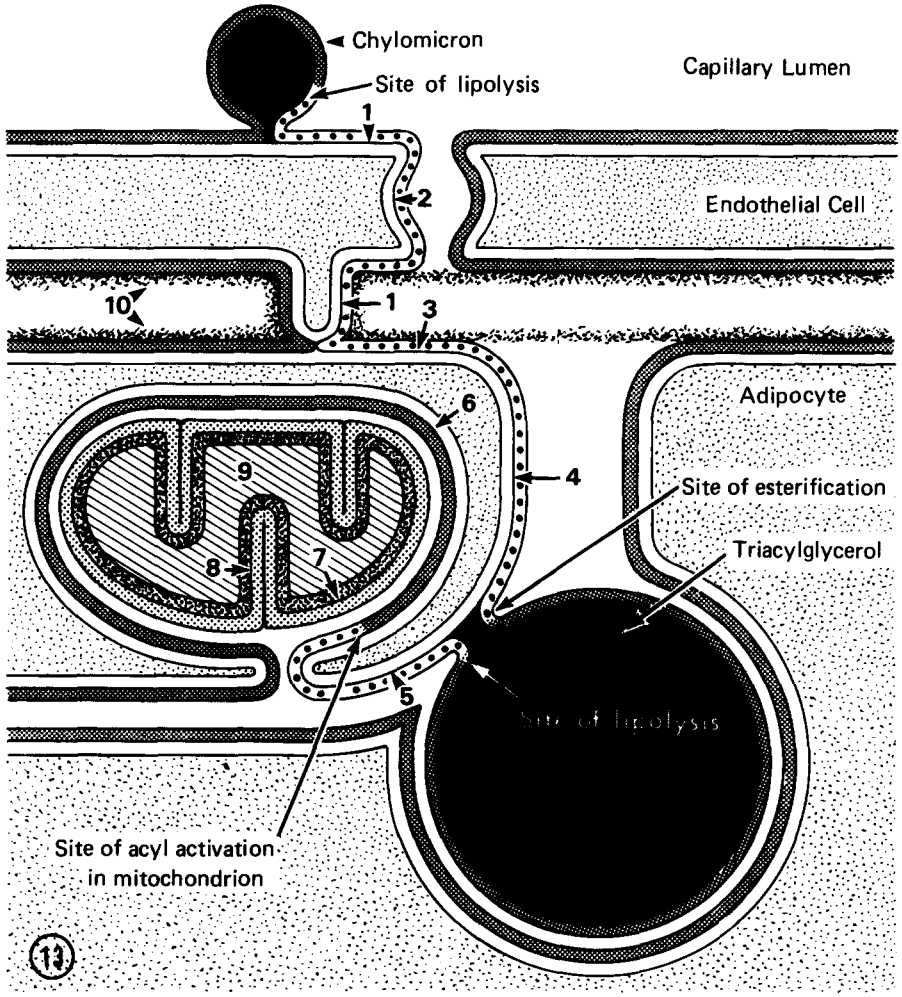

Fig. 13. Route proposed for transport of fatty acids from capillary lumen to adipocytes in brown adipose tissue by lateral movement in an interfacial continuum of cell membranes. The interfacial continuum, represented by the broad stippled line, is composed of the external leaflets of the plasma membrane of endothelium (*1*), the membrane of transcellular channels in endothelium (*2*), the plasma membrane of adipocytes (*3*), and the membrane of surface-connected channels (*4*), the membrane of endoplasmic reticulum (*5*), and the outer mitochondrial membrane (*6*) in adipocytes. The external leaflet of the plasma membrane of the endothelial cell (*1*) and that of the adipocyte (*3*) are continuous at the site of contact between the cells (33). The interfacial continuum would include the chylomicron surface film in fed animals when the surface film is fused temporarily with the external leaflet of the endothelial plasma membrane (*1*) (16). Fatty acids (black dots) formed by action of lipoprotein lipase on chylomicron triacylglycerol, as in fed animals, would enter the continuum in capillary lumen, move in the continuum to endoplasmic reticulum in the adipocyte, and leave the continuum when they are reesterified to triacylglycerol and accumulate as lipid droplets (lenses) between leaflets of the reticulum. Fatty acids formed by action of tissue (hormone-sensitive) lipase on intracellular triacylglycerol, as in cold-stressed animals, would reenter the continuum at the site of lipolysis in endoplasmic reticulum, move in the continuum to mitochondria, and leave the continuum when fatty acids are activated in the outer mitochondrial membrane (49) for transfer to the inner mitochondrial membrane (*7*) (50, 51). Fatty acids produced by intracapillary lipolysis could bypass the pedicel of the lipid droplet and leave the continuum in mitochondria. Other components of mitochondria labeled in the drawing are crista (*8*) and inner mitochondrial matrix (*9*). Note that the cellular projection of the endothelial cell penetrates the basement membrane (*10*) to make contact with the other cell. Cytoplasmic leaflets of membranes are represented by broad white lines. (Adapted from references 17, 19, and 41).

these removal processes are reduced in glutaraldehyde-fixed tissues, fatty acids accumulating in a crowded interface could be expected to form a bilayered extension of the interface at collapse pressure and thereby create space for themselves in the interface (48). Our recent findings in white adipose tissue of young rats demonstrated that long bilayered structures, which appeared continuous with the external leaflets of plasma membrane, extended out from the surface of adipocytes in glutaraldehyde-fixed tissue, and formed tightly wound lamellar whorls in tissue treated with tannic acid (18). As noted above, lamellar structures also developed in

TABLE 2. Development of lamellae in glutaraldehyde-fixed brown adipose tissue of 8-day-old rats

Group	Tissue Incubated at 25°C	No. of Rats	Volume of Lamellae in				Volume of Aqueous Spaces Associated with Lipid Droplets
			Capillaries	Extracellular Space	Adipocytes associated with Lipid Droplets	Adipocytes associated with Mitochondria	
	min		*×10⁴/tissue volume*				*×10⁴/tissue volume*
Fed	0	1	1 ± 1[a]	2 ± 1	4 ± 3	1 ± 1	0
	60	1	14 ± 3[b]	96 ± 14[b]	73 ± 4[b]	0	0
Fed + Chylomicrons IV[c]	0	3	10 ± 4[d]	6 ± 3	8 ± 5	0	0
	60	3	19 ± 7	161 ± 91[e]	53 ± 25	0	3 ± 1
Exposed to 4°C for 2 hr[f]	0	3	1 ± 1[d]	1 ± 1	1 ± 1	10 ± 1[g]	4 ± 1[g]
	60	3	9 ± 1[b]	31 ± 23	14 ± 3[b]	63 ± 16[b,g]	34 ± 3[b,g]
Unsuckled for 18 hr[h]	0	1	1 ± 1[a]	0	7 ± 3	16 ± 4	3 ± 1
	60	1	24 ± 11	34 ± 24	58 ± 5[b]	120 ± 19[b]	23 ± 1[b]

[a] Values given for this group are mean ± standard error of measurements made in three blocks of tissue from one rat (N = 3).
[b] Value significantly different from that at 0 min ($P < 0.025$).
[c] Tissue was taken 1.5 min after intravenous injection of chylomicrons.
[d] Values given for this group are mean ± standard error of measurements made in tissues of three rats (N = 3).
[e] Value significantly different from that at 0 min by paired comparison of geometric means ($P < 0.05$).
[f] Rats were kept at 4°C without suckling for 2 hr.
[g] Value significantly different from that for the chylomicron-injected group at the corresponding time ($P < 0.025$).
[h] Unsuckled rats were kept at 20°C.

intracellular channels in capillary endothelium and adipocytes, at sites of intercellular contact, and in capillary lumen. Thus, the lamellar structures that developed during incubation in fixed white adipose tissue were considered to be fatty acids that overcrowded the interfacial continuum and formed spiralling lamellar extensions of the continuum along its course through the tissue (18).

We conclude that the lamellar structures found in brown adipose tissue also represent long chain fatty acids formed by lipolysis in glutaraldehyde-fixed tissue. Distribution of lamellae suggests that the fatty acids accumulated and moved in an interfacial continuum extending from the capillary lumen to lipid droplets and mitochondria in adipocytes. We suggest that the continuum in brown adipose tissue is composed of the outer (luminal) leaflets of plasma membranes of endothelium, the membrane of transcellular channels in endothelium, the plasma membrane of adipocytes, and the membrane of channels in adipocytes that are continuous with endoplasmic reticulum and external mitochondrial membranes (**Fig. 13**).

We propose that in vivo fatty acids would also move in an interfacial continuum of outer leaflets of membrane extending from capillary lumen to the interior of adipocytes in brown adipose tissue. In fed animals, lipolytic products formed by action of lipoprotein lipase on chylomicrons would enter the continuum in capillary lumen, move in the continuum to endoplasmic reticulum, and leave when they are reesterified and accumulate as triacylglycerol droplets (lenses) between leaflets of the reticulum (Fig. 13). In animals exposed to cold or unsuckled, lipolytic products would be formed by action of tissue (hormone-sensitive) lipase on triacylglycerol in adipocytes, reenter the continuum at the site of lipolysis, move in the continuum to mitochondria, and leave the continuum in the outer mitochondrial membrane when they are activated for transfer to the inner mitochondrial membrane (49), the first step in transfer of fatty acids from the outer membrane to the inner matrix for oxidation (50, 51).

TABLE 3. Effect of nonsuckling on lipoprotein lipase activity of brown adipose tissue in young rats

Age	Lipoprotein Lipase Activity	
	Fed	Unsuckled 18 hr[a]
	munit/mg wet wt	
8–9 days	9.2 ± 1.0	24.1 ± 3.5
14 days	6.9 ± 1.3	16.9 ± 1.7

Lipoprotein lipase activity was assayed in clear homogenates of interscapular brown fat with rat chylomicron triacylglycerol as substrate. 1 munit of activity = 1 nmol of fatty acid produced per min. Values are means ± standard error of measurements made in tissues from nine animals per group. Each group consisted of approximately equal numbers of males and females.
[a] Unsuckled rats were kept at 20°C.

TABLE 4. Effect of nonsuckling on hydrolysis of chylomicron tri[^{3}H]acylglycerol in glutaraldehyde-fixed brown adipose tissue of 13-day-old rats

Group[a]	[^{3}H]Acyl Lipid Recovered as Fatty Acid in Tissue[b]				
	Not Fixed	Fixed	Fixed and Incubated 30 min	Increase During[c]	
				Fixation	Incubation
			% of Total		
Fed (4)	1.8 ± 0.2	8.1 ± 1.0	12.0 ± 1.3	6.3 ± 0.8	3.9 ± 0.9
Unsuckled 18 hr (3)[d]	13.2 ± 4.8	29.1 ± 6.9	44.3 ± 6.2	15.9 ± 2.7	15.2 ± 0.8

[a] Number of rats per group is given in parentheses.
[b] Tissue was taken 1.5 min after injection of chylomicrons, fixed 30 min in glutaraldehyde solution at 4°C, and incubated 30 min in tannic acid-sodium cacodylate solution, pH 7.4, at 25°C.
[c] Values are means ± standard error.
[b] Unsuckled rats were kept at 20°C.

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