The Induction by Serum of Lipid Storage in Cells of the Cornea Grown in Culture

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DESPITE ITS RELATIVELY SIMPLE STRUCTURE, the cornea is a highly specialized tissue with several unique properties. It possesses a characteristic curvature, is crystal clear and transparent to light, and is one of the few avascular tissues. The excised cornea is markedly hydrophilic, yet normally remains in a deturgescent state in the intact organism.[1] The abundant collagenous fibers of the cornea differ from those of most other tissues in having an extremely regular diameter.[2] Moreover, corneal collagen can be differentiated from cutaneous collagen with respect to its amino acid composition and its behavior during thermal denaturation.[3] The cornea possesses a characteristic glycosaminoglycan content. Chondroitin, chondroitin-4 sulfate (chondroitin sulfate-A), and keratan sulfate (keratosulfate) occur in the cornea under normal circumstances. Of these, chondroitin has never been clearly demonstrated in any tissue other than the cornea; moreover, although keratan sulfate, the major glycosaminoglycan component of the cornea, exists in cartilage and bone, the question of structural identity with the material found in the cornea is not yet completely answered.[4–8] In addition to the above reflections of the corneal cellular elements, the corneal cells manifest other remarkable qualities. For example, the corneal epithelium appears to be capable of the biosynthesis of collagen in at least some species,[9,10] and while the corneal stromal cells have the attributes of fibroblasts they also have decided phagocytic capabilities.[11] The corneal cells also possess the propensity to synthesize and store lipid.[12–19]

Some of the above-mentioned biological attributes may be ascribed to specific properties resulting from corneal differentiation, others may be accounted for by the precise environment in which the cells of the cornea reside. Included among the normal milieu of the corneal cells is the unique chemical composition of the extracellular fluid. This com-

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position differs from that in other tissues and potentially is derived from several sources including serum, tears, aqueous humor, and the secretions of the cellular constituents. Several corneal diseases probably result from either an intrinsic metabolic disturbance of the corneal cells, or from a derangement of the environment that the corneal cells inhabit. The present study attempts to establish whether abnormalities will occur in the corneal cellular elements if they are exposed to an extracellular environment which differs from the natural one. The induction of lipid by both homologous and heterologous sera in the rabbit cornea grown in culture is described, and evidence as to the intracellular site of lipid synthesis within the corneal cells is also presented.

Material and Methods

Adult albino rabbits were killed by intravenous injection of sodium pentobarbital into the marginal ear vein. The corneas were immediately removed under aseptic conditions and placed in Hank's balanced salt solution. Using a Zeiss operating microscope, the epithelium and endothelium were stripped from the corneal stroma as described by Stocker *et al.*[20] Each layer was transferred to a separate petri dish and rinsed 5 times in the medium within which it was to be cultured. The tissue was then sectioned into 1–2 mm fragments and transferred with a Pasteur pipette into Falcon 30-ml plastic culture bottles. The various cellular elements of the cornea were cultured in McCoy's medium with 30% calf serum or a modified Eagle's medium[21] containing variable quantities of normal rabbit or calf serum (0–30%). The media contained penicillin and streptomycin in concentrations of 100 units and 100 μg/ml, respectively. All cultures were incubated in a 5% CO_2 air-humidified tissue culture chamber at 37°C. The media were replenished when growth became evident and whenever indicated by an alteration in the pH of the medium (usually 2–3 times/week). The living cells were regularly observed in the culture flasks with phase-contrast microscopy, sometimes with time-lapse cinephotomicrography. Cultures were maintained without subculture for variable periods (up to 42 days) and then fixed for electron microscopy or trypsinized and subcultured.

Cells were prepared for electron microscopy by replacing the culture medium with cold veronal-buffered 2% osmium tetroxide (pH 7.4) for 1 hr or cold sodium cacodylate-buffered glutaraldehyde for variable periods. In some instances lipid was extracted from the cultures by exposing the cells to increasing strengths of ethanol prior to fixation. Following glutaraldehyde fixation, the cells were sometimes rinsed with 5% sucrose or cacodylate buffer (pH 7.4) and postfixed in 2% osmium tetroxide for 1 hr. All were dehydrated through graded alcohols, exposed to a mixture of Epon containing DMP-30 (2,4,6-dimethylaminomethyl-phenol) and absolute alcohol (1:1) for 1½–3 hr, and then passed through an Epon DMP-30 mixture for 30 min. The cells were then incubated in an Epon DMP-30 mixture at 45°C for 16–18 hr and at 60°C for 24 hr. After the Epon had hardened, the Falcon plastic tissue bottle was removed from it. The embedded cells were fixed with epoxy resin onto a block of Epon. Thin sections of the monolayer of embedded cells were cut with a Sorvall Porter-Blum microtome, mounted on 300 mesh copper grids, and stained for 10 min with uranyl acetate or lead citrate.[22] Sections were cut perpendicular or horizontal to the long axis of the cells. Electron microscopic examination was made with an RCA EMU-3G electron microscope at 50 kv.

After trypsinization with 0.25% trypsin the cells were subcultured, and then delivered with culture medium to cover slips (45 × 50 mm, No. 2 thickness) to which a glass ring (55 mm diameter, 7 mm height) was attached with a nontoxic, heat-stable, silicone lubricant (Dow-Corning). To prevent evaporation of the medium a glass lid was placed over the ring. This unit was set in a petri dish humidified with moistened gauze and returned to the incubator. The medium was replenished whenever required. At variable periods (5–14 days) the monolayer of cells was examined in the living state by phase-contrast, polarization, and fluorescent microscopy; and after various cytochemical or staining procedures, including hematoxylin and eosin, sudan dyes, osmium tetroxide, periodic acid-Schiff, Schultz's histochemical variant of the Liebermann-Burchandt reaction, and techniques for acid phosphatase (Burstone's and Gomori's).[23] The cells were examined with and without prior alcohol or acetone extraction and after incubation at 37°C for 2 hr in 0.1 M phosphate buffer (pH 7.4) containing pancreatic lipase (Worthington) in a concentration of 1 mg/ml.

Results

The cells of the rabbit cornea grew readily in the various media employed. Growth from the explant first became evident after 5–10 days and a good outgrowth of cells was usually obtained within 4–8 weeks. In subcultures abundant growth was usually present in 4–7 days.

After growth in media containing homologous or heterologous nonhyperlipemic serum the epithelium, endothelium, and the fibroblasts of the cornea all progressively accumulated osmiophilic and sudanophilic intracytoplasmic lipid droplets. These were scattered haphazardly throughout the cytoplasm though they often tended to be in the vicinity of the nucleus, sometimes displacing the latter eccentrically (Fig 1–4). This phenomenon was ostensible in some cells in less than 7 days and was strikingly apparent in most after 14 days. Under phase-contrast microscopy the lipid appeared as refractile bodies (Fig 1). The lipid was neither birefringent nor autofluorescent and gave a negative Schultz reaction. The lipid droplets were digested with lipase and could be extracted readily with organic solvents, such as ethanol and acetone (Fig 2). The lipid was not consistently associated with acid phosphatase activity. The incidence of affected cells, as well as the number and size of the droplets within involved cells, progressively increased with the duration of exposure to the serum-containing medium. In older cultures the individual droplets often reached 17 μ in diameter. The lipid accumulation was also directly proportional to the quantity of serum in the medium.

When it was viewed with the electron microscope, the appearance of the intracytoplasmic lipid varied with the fixation used, being markedly electron-dense after osmium tetroxide fixation, slightly electron-dense with fixation in glutaraldehyde followed by osmium te-

troxide, and almost electron-lucent after glutaraldehyde fixation or when alcohol extraction preceded osmium tetroxide fixation. Individual osmiophilic particles had a maximum diameter of about 570 mμ and aggregates of these corresponded to the much larger droplets visualized by light microscopy.

The individual osmiophilic droplets were usually distorted and irregular in outline, and rarely retained a spherical form following fixation and dehydration. The lipid was usually scattered randomly throughout the cytoplasmic matrix and was not consistently related to organelles like the rough or smooth endoplasmic reticulum, mitochondria, the Golgi apparatus, or lysosomes. In some cells the lipid aggregated adjacent to the nucleus (Fig 5–7). The lipid was clearly not ingested in the form seen within cells. Extracellular osmiophilic material was not noted in any of the preparations and there was no evidence of pinocytosis or phagocytosis of the lipid. In the least severely involved cells when the initial stage of the phenomenon could be observed, osmiophilic particles appeared in the cytoplasmic ground substance, suggesting that this might be a site of synthesis (Fig 8). Though older cultures often contained lysosomes and lysosomal-like particles with dense osmiophilic material within them, most cytoplasmic accumulates were not related to such structures (Fig 9).

Even when cells contained lipid deposits they were actively motile as evidenced by time-lapse cinephotomicrography. Following trypsinization and subculturing of lipid-laden cells, the younger daughter cells lacked overt intracytoplasmic lipid until the subcultures had grown in a serum-containing medium for about 1–2 weeks (depending on the concentration of the serum).

When cells were grown in media lacking serum or lipid, growth was not as prolific as when serum was present, but intracytoplasmic lipid storage was not observed.

Discussion

The storage of lipid within cells grown in media containing normal, nonhyperlipemic serum is not unique to the cornea. We have observed it in cutaneous fibroblasts and several investigators have described the phenomenon in some, but not all, cells from several species.[24–47] The fact that the stored material is sudanophilic, osmiophilic, soluble in lipid solvents, and hydrolyzed by lipase implies that it consists largely of triglycerides—a conclusion compatible with chemical analyses of the same phenomenon in other cells.[34,37]

Several possibilities for the origin of the fat in the present experi-

mental model need to be considered: (1) ingestion by phagocytosis, (2) metabolic product synthesized by cell, (3) degenerative product of cytoplasmic organelles, and (4) inhibition in degradation of normal lipid.

That the cellular fat is derived in some way from constituents of the external environment is supported by several observations: (1) fat accumulation fails to occur when serum or other lipid sources are omitted from the medium; [32] (2) the quantity of lipid accumulation in cultured cells is directly correlated with the concentration of serum in the medium;[29] (3) the addition of long-chain fatty acids, such as oleic acid, to tissue culture media enhances the accumulation in the cells; [16,30,39,45] (4) the fatty acid constituents of the lipid of cells grown in media containing serum reflects in large measure that of the serum; [25,28] (5) toxic injuries to cells do not result in fat accumulation if the cultures are maintained in lipid-free media.[30]

Knowledge about the metabolic pathway involved in the lipid storage of cultures grown in media containing serum is rudimentary. Several investigators have attempted to isolate lipogenic compounds from serum.[27,37,39,43-48] The lipogenic activity of serum varies with the species, being notably high in rabbit serum, but low in horse serum.[37] The lipid-inducing factor resists exhaustive dialysis and appears to be a macromolecule with most activity in the albumin fraction of the serum.[35,37,46] Though serum contains several lipids, there is evidence that the intracellular lipid accumulates are more related to free fatty acids than to any of the other lipids. It is well established that fatty acids readily ionize in aqueous solutions and that ionized fatty acids in serum combine almost immediately with the albumin. These albumin-bound fatty acids may serve as the cells' main source of fatty acids for both triglyceride and phospholipid synthesis.[37] In cell culture long-chain fatty acids are readily assimilated, esterified, and stored as triglycerides by cells in the absence of serum.[39] Besides possessing compounds which induce lipid storage, serum also contains substances that counteract its lipogenic activity.[43,45] These may include serum proteins, such as albumin. Indeed, albumin has been noted to counteract the lipogenic effect of oleates.[16]

It is clear from the present study that fatty acids need not be added to the serum for lipid storage to occur in the corneal cell cultures. Some years ago, Cogan *et al* [14-17] investigated the same phenomenon by incubating explants of corneal tissue from several species in media containing homologous and heterologous sera. Under the conditions of their experimental model they found that both serum and additional fatty acids were required for intracellular lipid storage to occur. Based

upon these studies Cogan and Kuwabara [16] questioned whether fatty acids alone were sufficient to account for increased lipid accumulation, or whether some additional cofactors from the serum were also required. They suggested that oleic acid, protein, calcium, and magnesium might be involved in the sequence of events leading to the biosynthesis of the lipid.

Several observations fail to support the possibility that the fat accumulation resulted from cell injury, and that the cytoplasmic organelles underwent degeneration with subsequent transformation into lipid droplets. The lipid-laden cells were viable, actively motile, and capable of replication. Indeed, on subculture they produced progeny that were indistinguishable from the non-lipid-laden mother cells. More important, sequential observations with the electron microscope revealed no evidence of cellular degeneration prior to the onset of lipid accumulation. In this regard, it is of interest that Virchow's [49] concept of lipophanerosis has thus far not been substantiated in any tissue. In situations where it was postulated, the fat accumulates cannot be accounted for by an alteration in the visibility of the lipids; the lesions not only contain more lipid than normal but lipid with different properties.[50,51] The chance of lipid storage resulting from an inhibition in the degradation of lipid seems unlikely in view of the lack of lipid deposits in cells grown in the absence of serum. Intracellular lipid storage in the present model, as in experimental studies on the incorporation of lipid by cells from such varied tissues as the intestinal mucosa, adipose tissue, and vascular endothelium, clearly does not result significantly from the ingestion of lipid by pinocytosis or phagocytosis.[48,52–55]

When the aforementioned observations and published evidence concerning lipid storage in cells grown in culture are considered in the light of current knowledge about lipid absorption, metabolism, and transport, a possible explanation for the intracellular lipid storage in the present system comes to mind. The findings are consistent with the hypothesis that fatty acids which are normally bound to serum albumin enter the cell possibly by simple diffusion, and that these become esterfied into triglycerides within the cytoplasmic ground substance. This postulated site of fatty acid esterification differs from that suggested for the intestine where fatty acids and monoglycerides appear to be taken up and incorporated into triglycerides within the smooth endoplasmic reticulum.[56] Although the cornea can synthesize sterols and fatty acids from acetate and glucose,[12] it is unlikely that such precursors constitute an important role in the present system, as lipid accumulation

was not observed when cells were incubated in media containing such compounds.

Despite the fact that the lipid storage in corneal cells grown in media containing serum does not seem to result from cellular degeneration, one might still pose the question whether it represents a pathologic state. In some conditions the intracellular storage of lipid is clearly a manifestation of a disease process. This is exemplified by conditions like Niemann-Pick's disease, Tay-Sach's disease, generalized gangliosidosis, Gaucher's disease, Krabbe's disease, Fabry's disease, metachromatic leukodystrophy, Farber's disease, Refsum's disease, atherosclerosis, chloroquine toxicity, and hypercholesterolemia.[57-62] However, it is clear that lipid storage by itself does not signify cellular injury. Under physiologic circumstances lipid droplets accumulate in such diverse cells as white and brown fat cells, adrenal cortical cells, lactating mammary epithelium, lutein cells, and the absorptive cells of the alimentary tract. Because of the evidence offered above, it would appear that in the present model the intracellular lipid is a metabolic product of the cell, and that the fat accumulation reflects the external milieu from which the cells derive their nutrition. Although the occurrence of lipid within the corneal cells does not preclude the possibility that they have a detrimental effect on the cell, our observations, as well as those of others [38, 40, 46] argue against the contention that lipid storage in cell culture reflects significant cell injury.

That the cellular elements of the cornea can manifest abnormalities when exposed to normal constituents of serum raises the question whether an analogous situation will arise in the intact organism if the relevant constituents of serum reach the cornea in sufficient quantities. This may have bearing on the rare lipid corneal disorders which do not appear to be associated with hyperlipemia, hypercholesterolemia, or with an underlying corneal disease. The deposition of lipid within the cornea is a well-established phenomenon. It may follow an underlying corneal lesion or occur in the absence of a known predisposing local condition. Sometimes the accumulation of lipid is associated with a disorder in the serum lipids. However, the association between lipid-containing corneal lesions and an elevated serum lipid is not a constant one. Either may occur in the absence of the other.[63]

Summary

The induction by serum of lipid storage in monolayer cultures of the cells of the rabbit cornea is described. Intracellular lipid accumulates occurred in the epithelium, endothelium, and corneal stromal cells

following growth in media containing nonhyperlipemic homologous and heterologous serum. The extent of the phenomenon was directly related to the quantity of serum in the medium, and to the duration of growth within it. The lipid was sudanophilic, osmiophilic, soluble in organic solvents, and digested by pancreatic lipase. On the basis of these attributes, the lipid is presumed to consist largely of triglycerides, a conclusion compatible with chemical analyses of the same phenomenon in other cells. Sequential observations in the electron microscope failed to provide evidence of lipid ingestion by pinocytosis or phagocytosis. The lipid storage was not preceded by overt degenerative alterations in the cellular organelles and did not appear to reflect significant cell injury, but rather an altered homeostatic state resulting from the chemical composition of the environment from which the cells derived their nutrition.

When these observations were viewed in light of current knowledge concerning the phenomenon in corneal and other cells, it was postulated that triglycerides are synthesized within the cytoplasmic matrix from fatty acids derived from the serum in the medium. That the cellular elements of the cornea do manifest the propensity to accumulate lipid when exposed to normal serum, raises the question whether an analogous situation will arise in the intact organism in the event of an altered homeostatic state.

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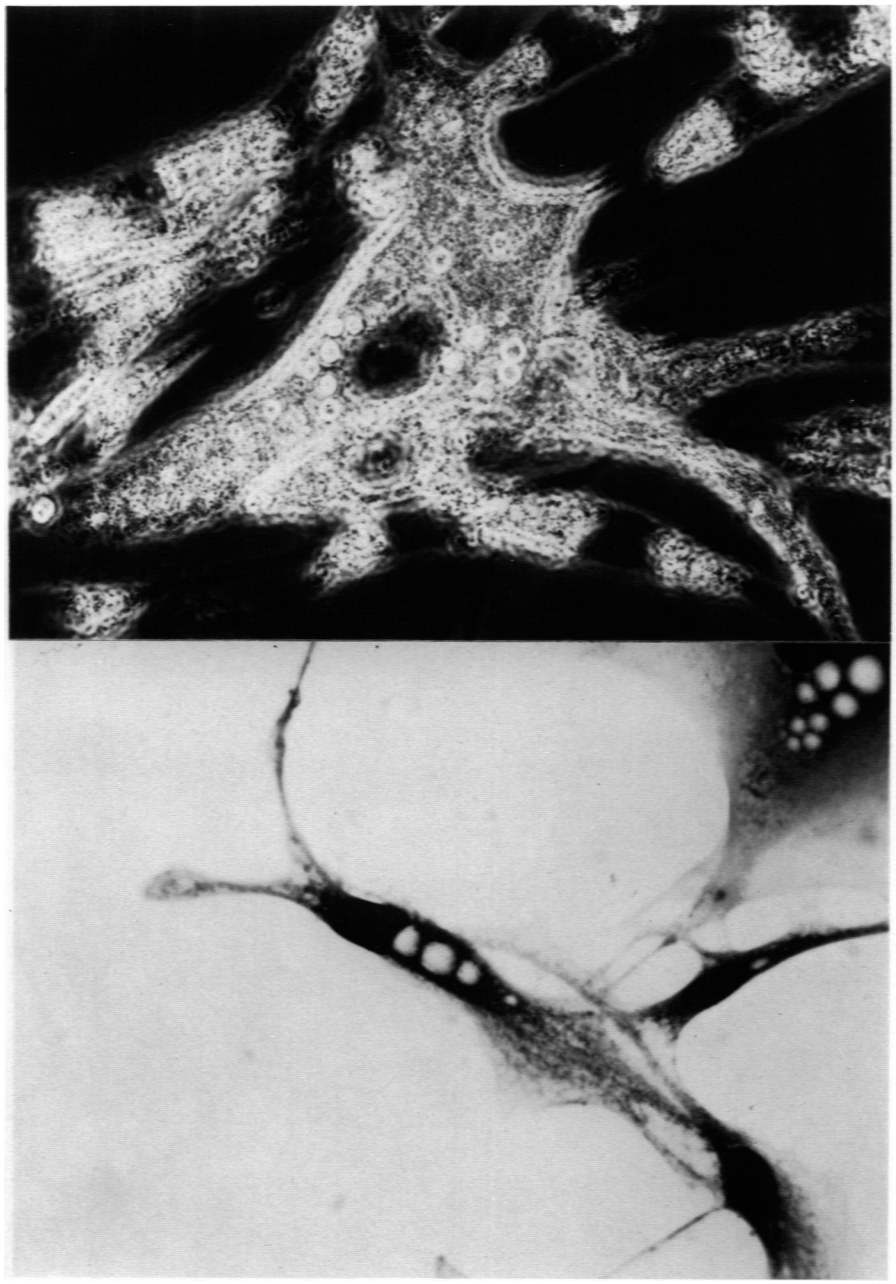
Legends for Figures

Fig 1. With phase-contrast microscopy numerous spherical refractile bodies can be identified within cytoplasm of this unstrained corneal fibroblast grown in modified Eagles' medium containing 30% rabbit serum. × 500.

Fig 2. Cells grown in media containing homologous or heterologous serum contain intracytoplasmic vacuoles after exposure to organic solvents. H & E, × 520.



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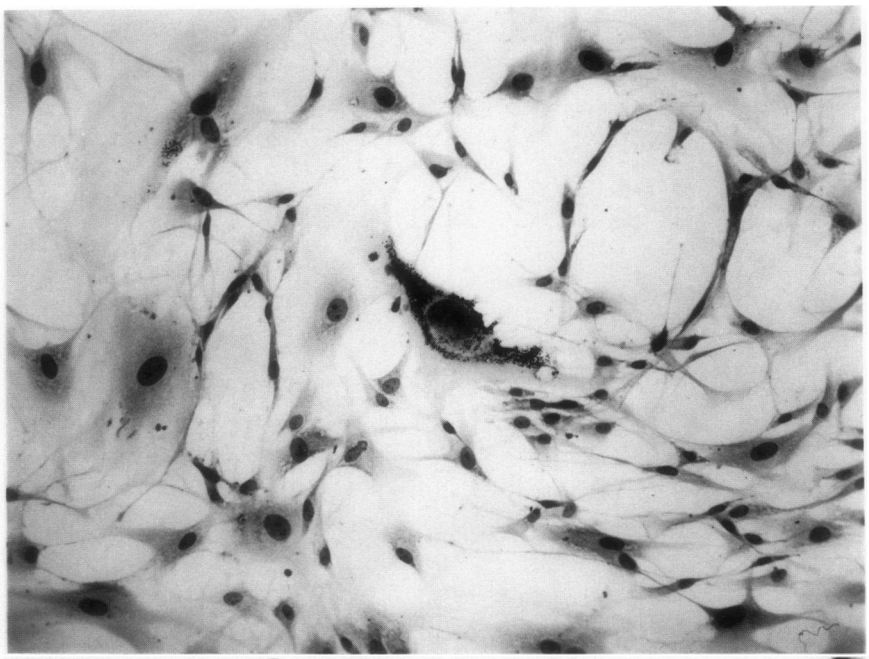

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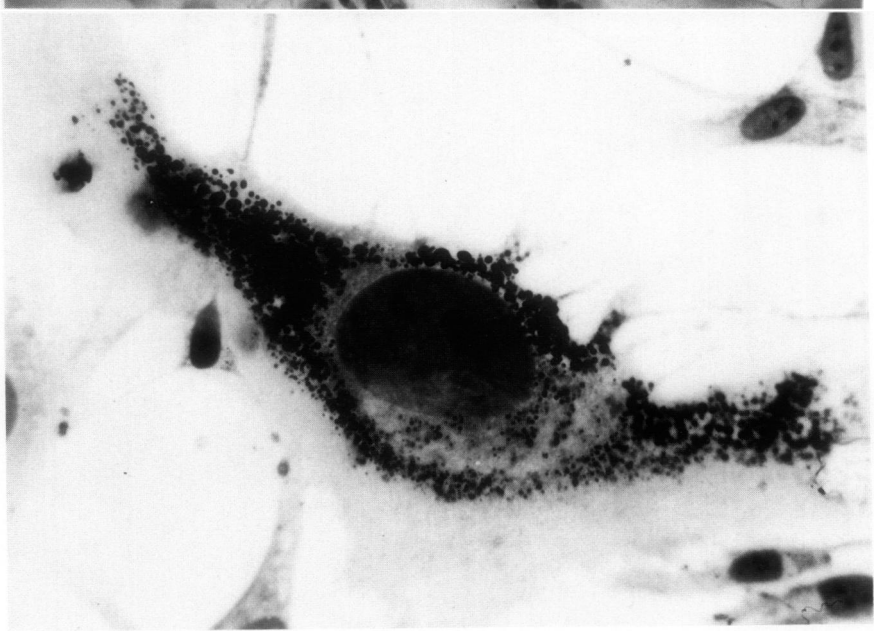


Fig 3. After less than 7 days growth in media containing normal nonhyperlipemic serum, sudanophilic particles are evident in some cells. Though these cells were exposed to a similar environment, the individual cellular elements did not manifest the same metabolic attributes, as evidenced by the lack of obvious stainable lipid. Such sudanophilic accumulates were absent in cells exposed to lipase or lipid solvents prior to staining. × 130.

Fig 4. Higher magnification of lipid-laden corneal fibroblast shown in center of Fig 3. × 520.

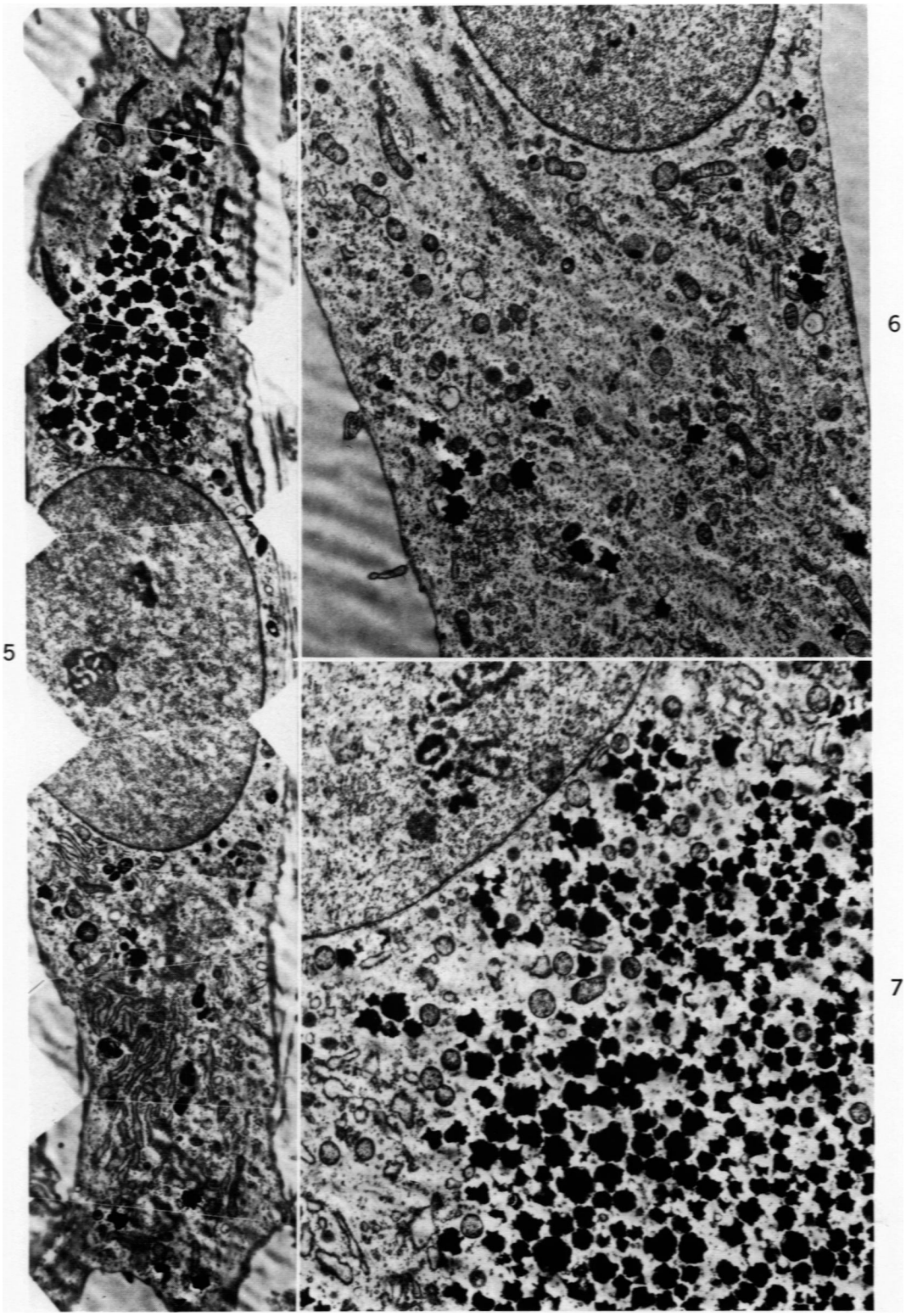
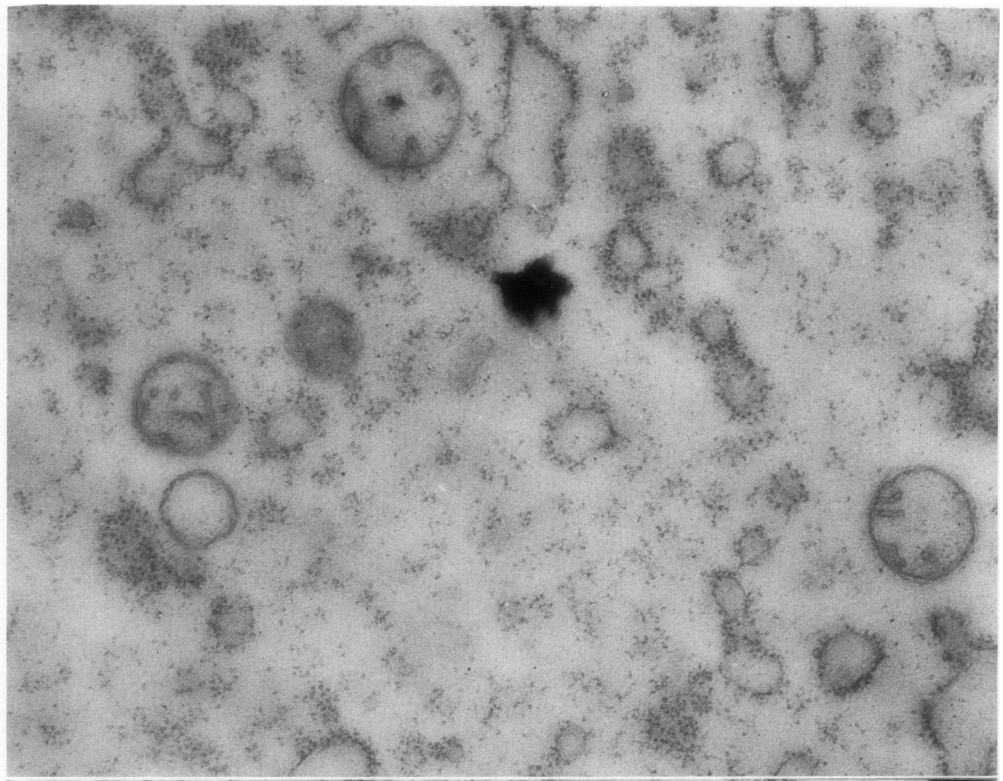


Fig 5. In this keratocyte, most of the lipid is located adjacent to nucleus. × 7000.

Fig 6. Abundant electron dense particles scattered throughout cytoplasm between various organelles of this corneal fibroblast grown in serum-containing medium. × 8000.

Fig 7. Lipid particles evident between organelles of this corneal fibroblast. × 18,000.

8



9

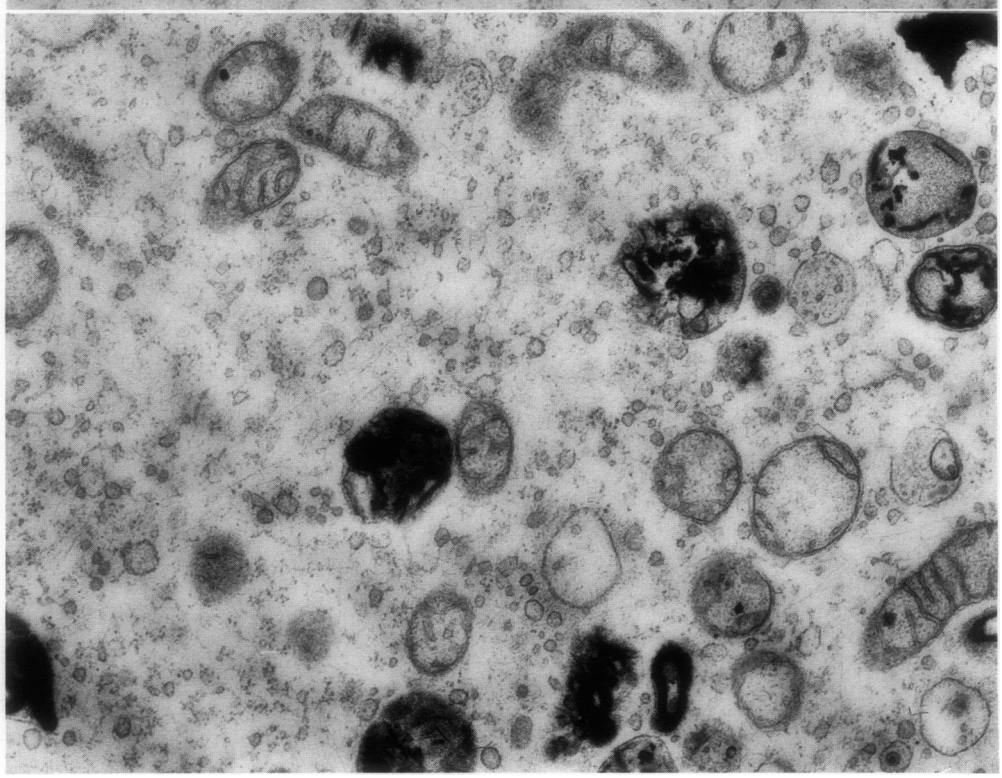


Fig 8. In least severely involved cells when initial stage of phenomenon could be observed, osmiophilic material appeared in cytoplasmic ground substance as shown here, suggesting that this might be a site of synthesis. × 31,300.

Fig 9. Lysosomes and lysosomal-like particles containing dense osmiophilic material are evident in this corneal fibroblast. × 31,300.