THE ACTION OF EXCESS OF VITAMIN A ALCOHOL ON THE FINE STRUCTURE OF RAT DERMAL FIBROBLASTS

MARY R. DANIEL, J. T. DINGLE, AUDREY M. GLAUERT, and J. A. LUCY

From Strangeways Research Laboratory, Cambridge, England

ABSTRACT

Rat dermal fibroblasts were grown as monolayers, and changes in the fine structure of the cells that occurred during 12 hr incubation in a medium containing protein and excess of retinol (vitamin A alcohol) were studied by electron microscopy.

There is little change during the first 6 hr, although some of the nuclei have highly convoluted membranes. During the subsequent 3 hr, there is some disorganization of the mitochondrial cristae; the cisternae of the rough-surfaced endoplasmic reticulum diminish in number; and the amount of smooth membranous material and free ribosomes increases. There is a rapid decline in the respiratory activity of the cells after 6 hr exposure to the vitamin. It is concluded that the primary action of excess of retinol is to cause alterations in the membranes of the cells and that these alterations affect the functions of the mitochondria and endoplasmic reticulum.

INTRODUCTION

Lipoprotein membranes have been found to be concerned in a number of the actions of excess of vitamin A (for a review article, see Dingle and Lucy, 1965). It appears that, as a result of its amphipathic structure, retinol (vitamin A alcohol) is highly surface active (Bangham, Dingle, and Lucy, 1964) and that some of the effects of excess retinol spring initially from the ability of the compound to interact with the plasma and intracellular membranes of cells. At 37°C, added retinol rapidly expands and then destroys the plasma membrane of the rabbit erythrocyte (Glauert, Daniel, Lucy, and Dingle, 1963), but closely related derivatives of retinol that are inactive physiologically are not similarly membrane-active (Dingle and Lucy, 1962). This relationship between chemical structure and function indicates that membranes might also be concerned in the action of vitamin A under physiological conditions. Studies of the destructive action of retinol on lipoprotein membranes may thus lead indirectly to an improved understanding of the normal biochemical functions of vitamin A.

As previously described in preliminary reports, the addition of retinol to fibroblasts in buffered saline at 37°C was observed to distend the plasma membranes which then quickly disintegrated (Dingle, Glauert, Daniel, and Lucy, 1962; Lucy, 1964). The nuclear membranes also showed signs of damage in the treated cells, while the mitochondria became grossly swollen and their internal structure badly distorted. These observations demonstrated that excess of retinol disorganizes the structure of several different membranes but, as the cells took up the vitamin very rapidly, the drastic nature of the changes produced often made it impossible to follow the progressive alterations in the structure of the membranes. In this paper, the action of retinol on the fine structure of rat fibroblasts

grown in a nutritive medium containing protein is described. By means of this technique, it has been possible to investigate the effects of excess of retinol on the membranes of cells that are not rapidly degenerating in response to the hypervitaminosis as they do in the absence of protein. The results of the present experiments may help to elucidate the morphological changes produced by excess of vitamin A in more complex systems such as organ cultures of bone and skin (Fell and Mellanby, 1952, 1953).

MATERIALS AND METHODS

Culture Method

The cells were obtained by trypsinization of the dorsal skin of fetal rats near term, by the technique described by Daniel, Dingle, and Lucy (1961), and were grown as monolayers under a medium consisting of 80% Eagle's medium, 10% unheated bovine serum, and 10% fowl embryo extract. A cover slip coated with collagen by the method of Heyner (1963) was placed in each culture bottle before the cells were pipetted into it. The medium was changed after 4 and 7 days of incubation.

After the change of medium on the 7th day, when a dense layer of fibroblasts with a few myoblasts and muscle straps, had formed, retinol (dissolved in ethanol) was added to some of the cultures to give a final concentration of 33 μg of vitamin A per milliliter of medium. The final dilution of the solvent was 1:1000, and an equivalent volume of ethanol was added to the control cultures. Cover slips were removed for examination after further periods of incubation of 1, 3, 6, 9, and 16 hr.

Measurement Of Respiration

The cells attached to the floor of the culture bottle were removed by trypsinization, washed, and suspended in Krebs-Ringer phosphate, with 1.8% glucose and 10% neutralized serum (Friend and Hastings, 1940); respiration was measured by the method described by Daniel, Dingle, Webb, and Heath (1963); and oxygen uptake was expressed as QO_2 (μl $O_2/10^6$ viable cells per hour).

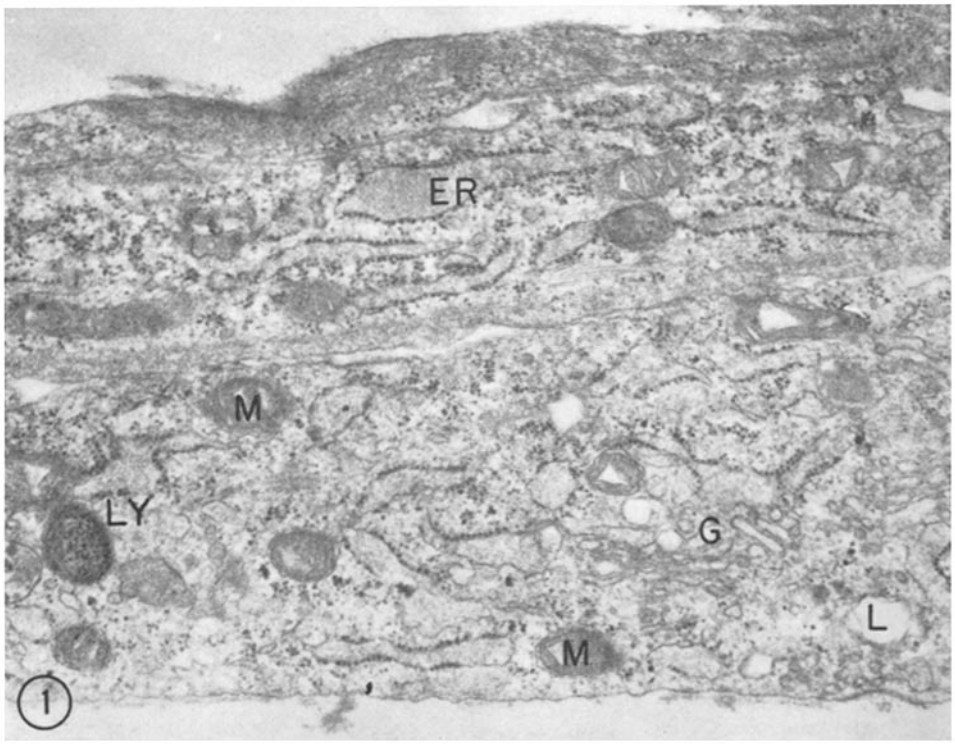

FIGURE 1 Control culture. Incubation for 12 hr. The culture is 3 cells thick. The cytoplasm contains elongated profiles of the endoplasmic reticulum (*ER*), an extensive Golgi zone (*G*), mitochondria (*M*), lysosomal granules (*LY*) and low-density droplets (*L*). Lead tartrate stain. × 30,000.

Light Microscopy

Cultures were examined before fixation with a Cooke, Troughton, and Simms phase-contrast microscope.

Electron Microscopy

The monolayers of fibroblasts were fixed in situ by immersing the cover slips in the fixative contained in a Columbia staining dish. After a brief rinse in Tyrode's solution, the monolayers were fixed overnight in 3% glutaraldehyde in phosphate buffer (Sabatini, Bensch, and Barrnett, 1963) at pH 7.4 at 4°C, washed in two changes of Tyrode's solution and then postfixed in Zetterqvist's buffered osmium tetroxide at pH 7.4 for 1 hr at 4°C. After fixation, the preparations were dehydrated in ethanol. The coverslips were brought to room temperature in 70% ethanol in water, and at this stage the monolayers were scraped off the cover slips with a razor blade and placed in small bottles. The monolayers rolled up loosely on removal from the glass and were then handled in the same way as pieces of tissue; the collagen layer peeled off with the cells. The dehydration was then completed and the cells were embedded in Araldite.

Thin sections, which were cut with glass knives on a Huxley microtome, were stained with uranyl acetate, lead tartrate (Millonig, 1961), or lead citrate (Reynolds, 1963). Some sections were double stained with uranyl acetate and lead citrate.

Micrographs were taken on a Siemens Elmiskop I operating at 60 kv with a 50 μ objective aperture.

RESULTS

Fine Structure of Untreated Cells

The cells grow as a thin layer on the collagen surface and are flat, with long cytoplasmic processes. In places the processes of adjacent cells overlap and the culture is several layers thick (Fig. 1).

The fine structure of the untreated cells in control cultures does not change during the 12 hr incubation and they have the typical fine structure of mammalian fibroblasts that are active in collagen synthesis (see reviews by Chapman, 1962; Porter, 1964). The nuclei have fairly smooth outlines (Fig. 2, *N*) and prominent nucleoli.

The mitochondria are elongated and possesnumerous transverse cristae, and the matrix mas

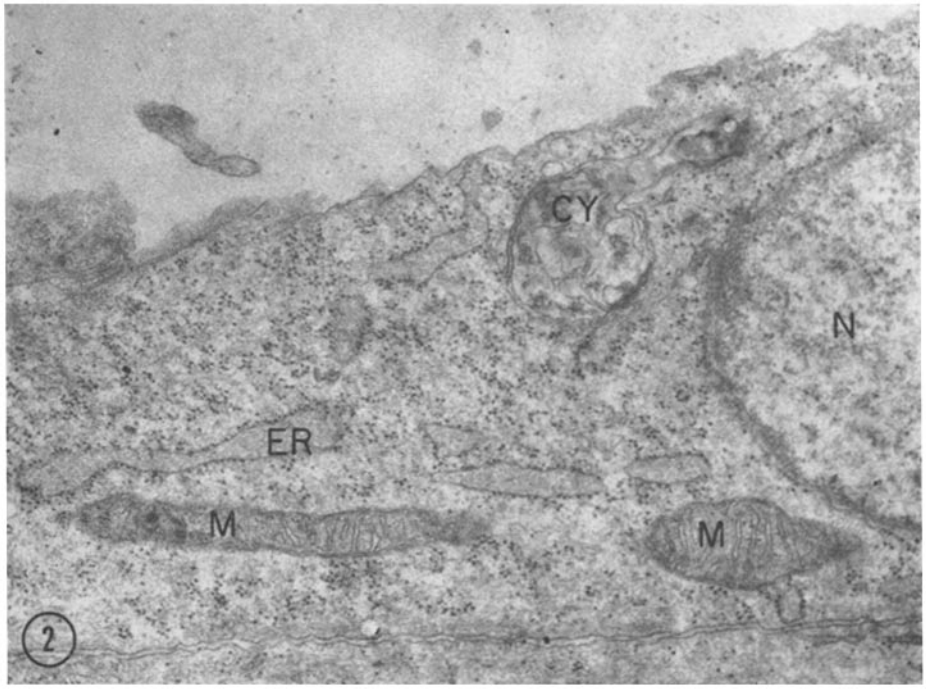

FIGURE 2 Mitochondria (*M*), profiles of the endoplasmic reticulum (*ER*) and a cytolysome (*CY*) in the cytoplasm of a cell in a control culture after 12 hr incubation. Part of the nucleus (*N*) is also visible. Lead tartrate stain. × 30,000.

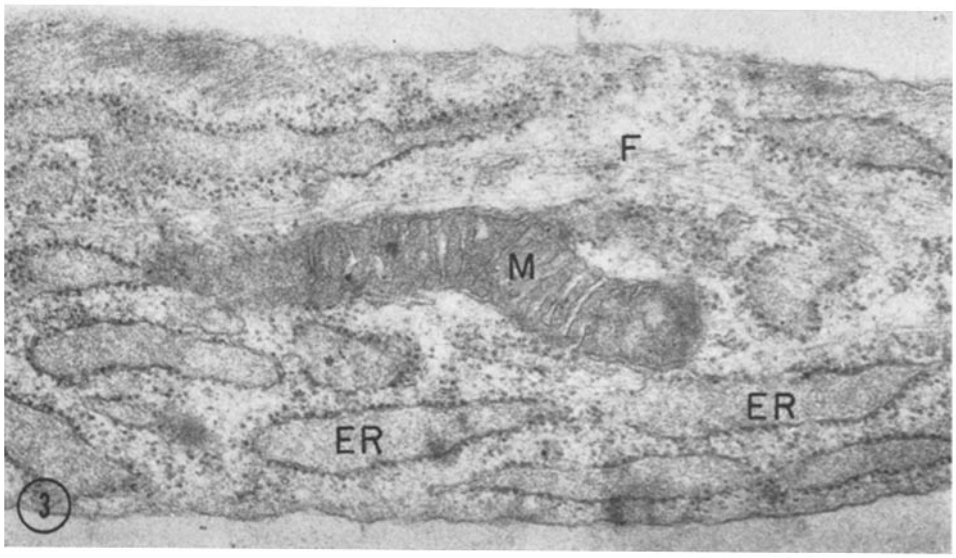

FIGURE 3 Control culture. Incubation for 12 hr. A cytoplasmic process contains a mitochondrion (*M*), cisternae of the endoplasmic reticulum with dense contents (*ER*), and fine fibrils (*F*). Lead tartrate stain. × 40,000.

terial has the high opacity that is usually observed after fixation in glutaraldehyde (Figs. 2 and 3, *M*).

The cisternae of the rough-surfaced endoplasmic reticulum are distended and contain fine granular or flocculent material of moderate density (Figs. 2 and 3, *ER*). The majority of the ribosomes are associated with the membranes of the reticulum, but some are apparently free in the cytoplasm and are arranged either singly or in groups and chains (Fig. 2).

Dense granules, similar to lysosomes (Fig. 1, *LY*), and membrane-bounded regions of local cytolysis (cytolysomes) (Novikoff, 1960; Novikoff and Essner, 1962) (Fig. 2, *CY*) are present. Large, clear areas in the cytoplasm (Fig. 1, *L*) are probably the sites of extracted lipid granules or mucopolysaccharide secretion droplets. There is usually an extensive Golgi zone containing smooth lamellae and vesicles (Fig. 1, *G*).

Densely packed, thin fibrils, typical of fibroblasts, are visible just beneath the plasma membrane and parallel to it; similar fibrils (Fig. 3, *F*) and microtubules (Fig. 4, arrows) are also observed throughout the cytoplasm of elongated cell processes.

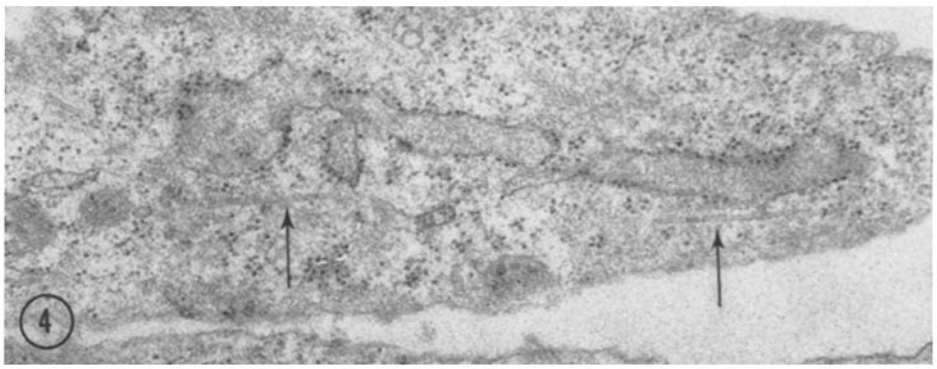

FIGURE 4 Microtubules (arrows) in the cytoplasm of a control cell. Lead tartrate stain. × 40,000.

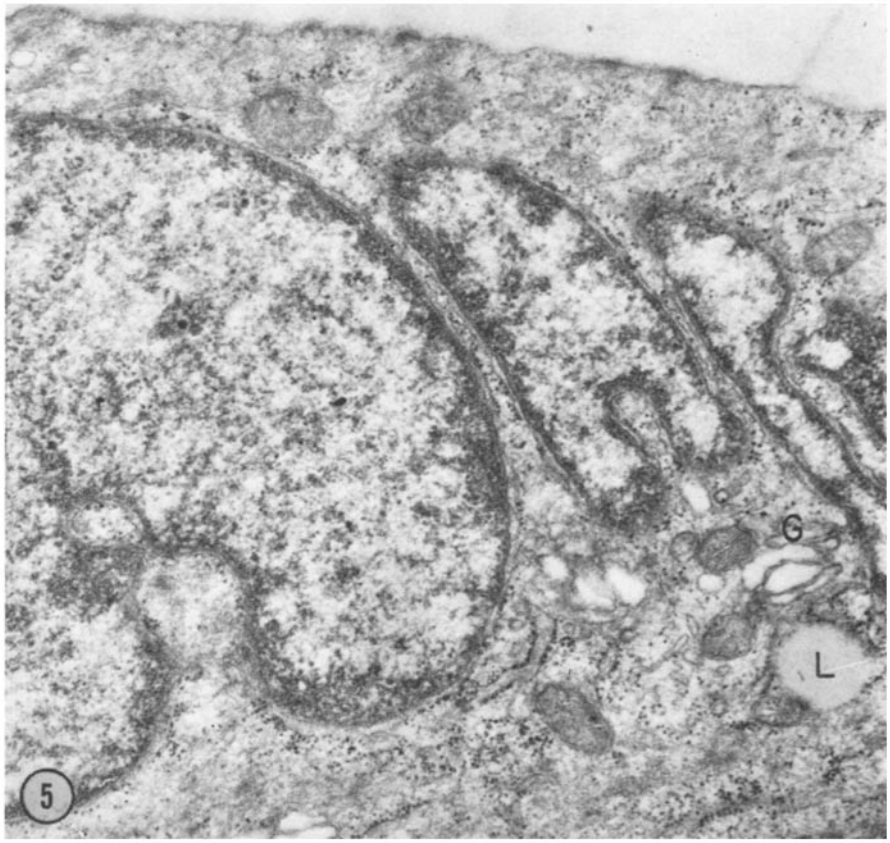

FIGURE 5 Incubation with retinol for 1 hr. There are deep invaginations in the nuclear membrane. Vesicles in the Golgi zone (*G*) are swollen and a low-density droplet (*L*) is present. Uranyl acetate and lead citrate stains. × 30,000.

Alterations in Fine Structure in the Presence of Retinol

Phase-contrast microscopy of living cells shows that the whole upper surface swells upwards under the influence of excess of retinol. At a later stage, deep infoldings of the nuclear membrane are visible, the vesicles in the cytoplasm become swollen, and refractile granules appear.

There is considerable variation in the rate at which the fibroblasts respond to the vitamin; this may reflect differences in the metabolic state of the cells when the vitamin is added.

The nuclei of most cells are little changed in fine structure during the first 3 hr growth in the presence of retinol, and typical mitoses are observed. A few nuclei have highly convoluted nuclear membranes with deep infoldings (Fig. 5); after 6 hr, this appearance is often seen.

The mitochondria are apparently unchanged for several hours (Fig. 5). After 12 hr, some of the cristae become disorganized and indistinct (Fig. 6, *M*); the mitochondria also appear to be slightly swollen but the degree of swelling is much less than that previously observed in the absence of protein (Lucy, 1964).

The cisternae of the rough-surfaced endoplasmic reticulum remain distended and filled with fine granular material for some hours, but after 9 hr they seem to disintegrate since they are no longer distinguishable in many cells. The remaining cisternae are flattened and appear to have lost much of their contents (Fig. 6, *ER*). This disappearance of the granular endoplasmic reticulum is accompanied by a marked rise in the number of free ribosomes (Fig. 7), some of which are organized in apparently helical aggregates

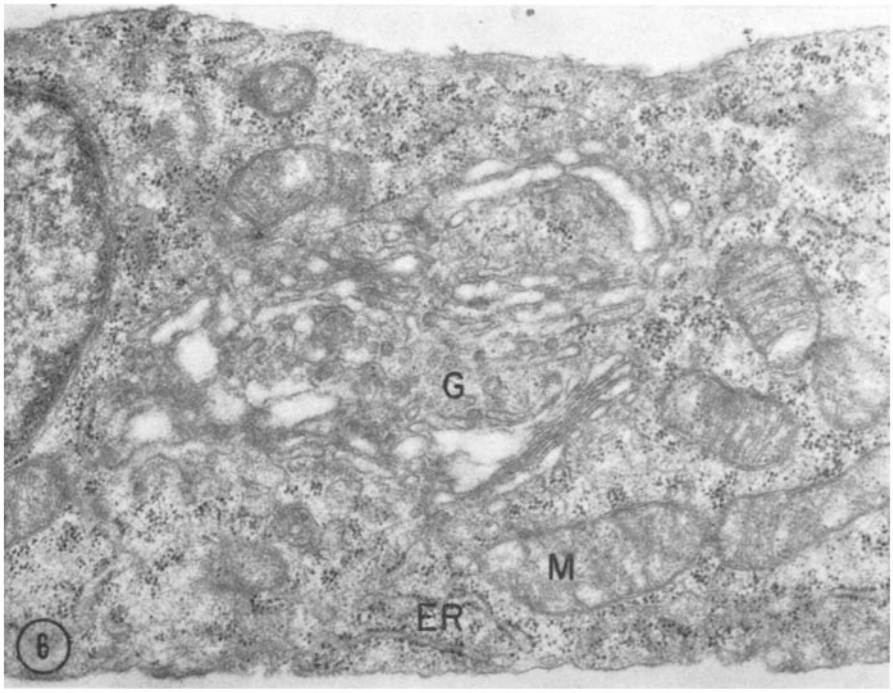

FIGURE 6 Incubation with retinol for 12 hr. The vesicles in the Golgi zone (*G*) and the mitochondria (*M*) appear to be slightly swollen. The cisternae of the endoplasmic reticulum (*ER*) have collapsed. Uranyl acetate and lead citrate stains. × 30,000.

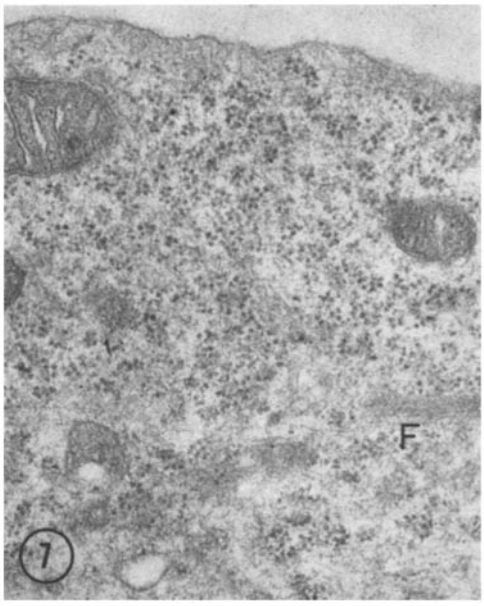

FIGURE 7 Many free ribosomes and fine fibrils (*F*) are present after 9 hr incubation with retinol. Lead tartrate stain. × 40,000.

(Fig. 8, arrow). The amount of agranular or smooth membranous material in the cytoplasm also increases; this may arise either from the Golgi zone, which is often very extensive (Figs. 6 and 9, *G*), or from the granular reticulum after loss of ribosomes. The Golgi vesicles appear slightly swollen after 1 hr growth in the presence of the vitamin (Fig. 5, *G*) and remain so during the extension of the zone (Figs. 6 and 9, *G*).

Dense "lysosomal" granules are visible throughout the 12 hr of culture with retinol (Fig. 9, *LY*); the number of cytolysomes (Fig. 8, *CY*) appears to increase considerably. There is little change in the low-density granules (Fig. 8, *L*) or in the fine fibrils (Fig. 7, *F*) and microtubules.

Small invaginations of the plasma membrane, with very dense inner borders (Fig. 10, arrow), are apparently more frequent in vitamin A–treated fibroblasts than in the controls. The plasma membrane appears to remain intact throughout the 12 hr of culture, and no breaks are visible in the membrane in the sections.

Myelinlike structures composed of concentric layers of membranes are seen in the treated cells

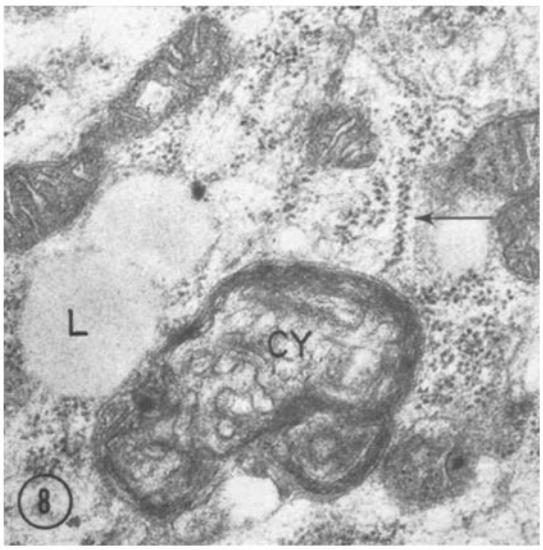

FIGURE 8 After 9 hr incubation with retinol the cytoplasm contains a cytolysome (*CY*) and an apparently helical aggregate of ribosomes (arrow). *L*, low-density granules. Lead tartrate stain. × 40,000.

(Fig. 11, *MY*) and may correspond to the refractile bodies seen in the phase-contrast microscope. Similar granules also have been observed in electron micrographs of fibroblasts incubated in buffered saline with retinol (Lucy, 1964). It is possible, however, that these myelin forms are not produced as a direct result of the action of the vitamin, since similar structures have been observed in degenerating cells by many workers, including Karrer (1960), Vickerman (1962), and Yasuzumi et al. (1960). They have also been seen in cells that have ingested bacteria, and under many other conditions. The nature of the bodies in retinol-treated fibroblasts requires further study.

Cell Respiration

There is little change in the QO_2 of the cells for the first 6 hr exposure to retinol. Untreated cells had a QO_2 of 2.1 and after 3 hr treatment, the QO_2 was 2.0. Subsequently, between 6 and 9 hr, respiratory activity rapidly declines (QO_2 1.7 at 6 hr, QO_2 zero at 9 and 12 hr). This depression in

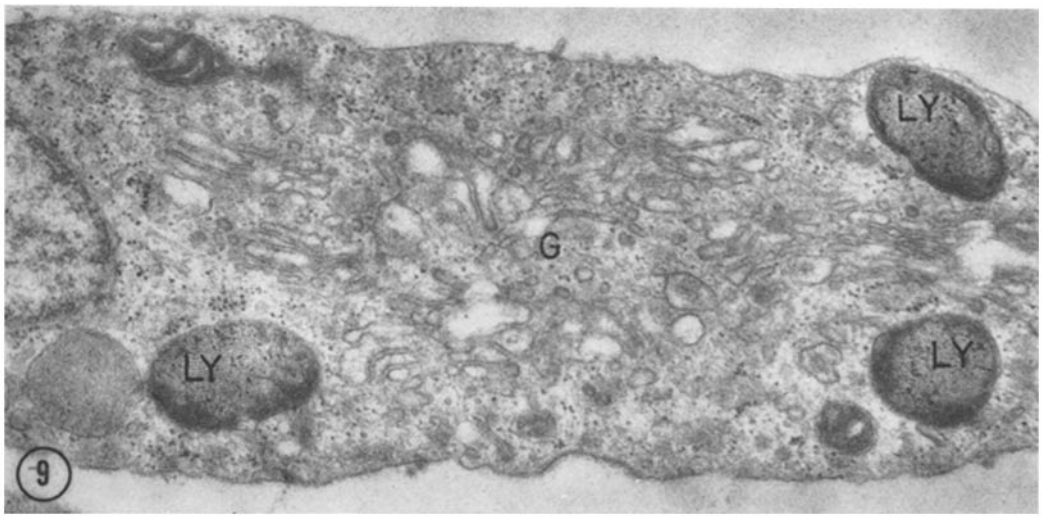

FIGURE 9 An extensive Golgi zone (*G*) and lysosomal granules (*LY*) in the cytoplasm of a cell after 12 hr incubation with retinol. Uranyl acetate and lead citrate stains. × 30,000.

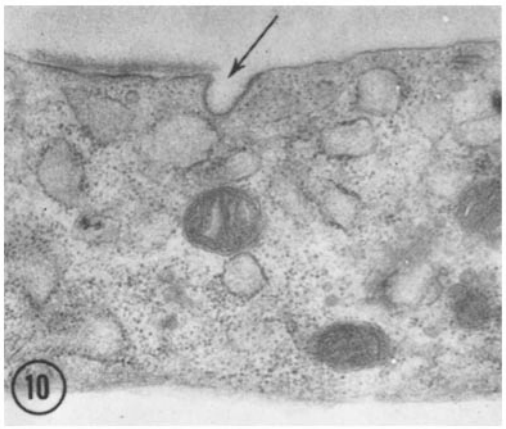

FIGURE 10 Incubation with retinol for 6 hr. A small invagination of the plasma membrane (arrow) has a dense inner border. Lead tartrate stain. × 30,000.

respiration may be correlated with the morphological changes produced in the mitochondria after 6 hr treatment with the vitamin. Untreated cells show no change in respiration during 12 hr incubation in this medium (Daniel et al., 1963).

DISCUSSION

Fibroblasts growing in a culture medium containing protein react much more slowly to the presence of retinol than similar cells that are isolated and then incubated with the vitamin in buffered saline. It has been pointed out previously that the ability of excess retinol attached to serum proteins to affect tissues in vitro probably depends on digestion of the carrier protein by the tissue and the simultaneous release of the vitamin (Lucy, 1964). In the present experiments, normal mitoses were observed during the first 3 hr exposure to the vitamin, and the plasma membranes of growing cells were intact after 12 hr, whereas in the absence of protein the membranes of isolated cells disintegrate after 10 min incubation with 20 μg/ml of retinol. Similarly, the mitochondria in the cultured fibroblasts only begin to show signs of damage after approximately 9 hr treatment, while in buffered saline the initial changes appear in a few minutes and swelling is complete in 10 min (Dingle et al., 1962; Lucy, 1964). Mitochondria isolated from rat liver and kidney begin to swell almost immediately on treatment with low concentrations of retinol in sucrose (Lucy, Luscombe, and Dingle, 1963), while mitochondria in organ cultures of embryonic chick skin (Jackson and Fell, 1963) and mouse bone rudiments (Glauert and Fell, unpublished observations, see Fell, 1964) often take some days to show a similar degree of disorganization.

The disorganization of the internal structure of

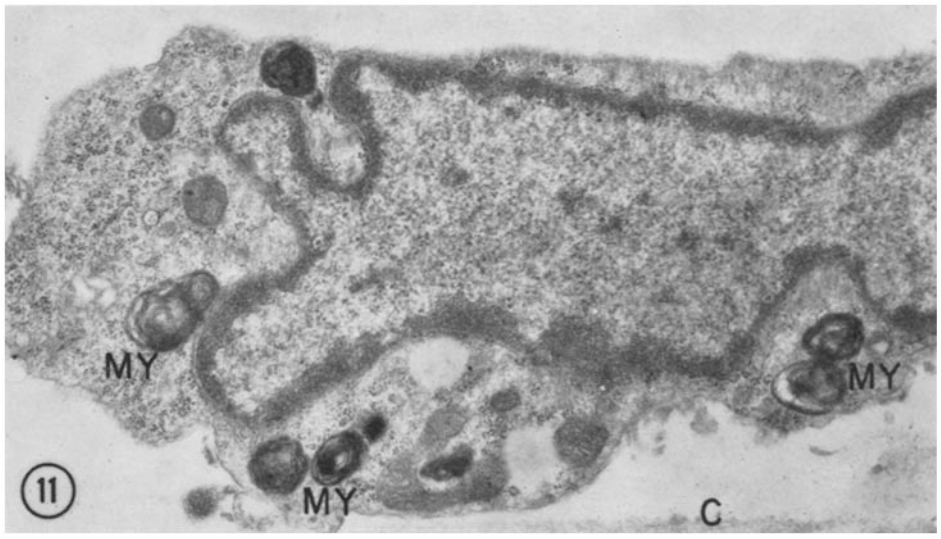

FIGURE 11 Incubation with retinol for 6 hr. The nuclear membrane of the cell is highly convoluted and myelinlike bodies (*MY*) are present in the cytoplasm. The collagen layer is visible at (*C*). Lead tartrate stain. × 20,000.

the mitochondria is probably the cause of the reduction in respiration noted in these experiments, since the same duration of exposure was required to produce both effects. It is probable that there is a similar relationship between the swelling of isolated mitochondria exposed to retinol (Lucy, Luscombe, and Dingle, 1963) and the inhibition of pyruvate metabolism in isolated mitochondria noted by DeLuca and Dingle (unpublished work). These observations may offer an explanation of the depression of respiration in chick limb bone rudiments treated in culture with retinol (Dingle, Lucy, and Fell, 1961).

Many lysosomal granules can be seen, under the light microscope, in preparations of cells stained by the Gomori technique for acid phosphatase; the number and size of these acid phosphatase–positive particles are similar in control and retinol-treated cultures (Dingle and Daniel, unpublished observations). However, the electron micrographs indicated that the relative proportions of the different types of lysosomes may have been altered by hypervitaminosis A. In the cultures exposed to retinol, the number of cytolysomes appeared to be much greater than in the controls. It has been suggested that cytolysomes may be less stable than the storage granules and residual bodies (Dingle and Lucy, 1965); the formation of cytolysomes may therefore be associated with the extracellular digestion that occurs in embryonic chick cartilage treated with excess of retinol in vitro (Dingle et al., 1961).

The cisternae of the rough-surfaced endoplasmic reticulum in fibroblasts suspended in buffered saline are quickly affected by retinol and in a few minutes swell into spherical forms. Throughout this swelling the ribosomes remain attached to the membranes, and there is no apparent loss of the enclosed granular material (Dingle et al., 1962; Lucy, 1964). In contrast, the cisternae of the rough endoplasmic reticulum in fibroblasts growing in culture medium containing protein respond more slowly to the presence of retinol and remain unaltered for about 9 hr. Eventually, the cisternae collapse and seem to lose their ribosomes; swollen cisternae are seen only among cell debris. This collapse of the cisternae also occurs in osteoblasts in organ cultures of mouse bone rudiments grown for 6 days in the presence of excess of retinol (Glauert and Fell, unpublished observations, see Fell, 1964) and presumably reflects a decrease in the capabilities of the cells to synthesize extracellular materials.

The apparent loss of ribosomes from the cisternae in the cultured fibroblasts suggests that the vitamin may affect the binding between the lipoprotein membranes and the ribonucleoprotein particles. The ribosomes themselves do not appear to be altered, as they do not diminish in number and are still able to aggregate into the helical polyribosomal structures that are a feature of many differentiating cells (Behnke, 1963; Waddington and Perry, 1963). Although it is known that attachment of ribosomes to the endoplasmic reticulum is not essential for protein synthesis, it is of interest that the major protein-synthesizing activity of the liver cell is associated with the ribosomes attached to the endoplasmic reticulum (Campbell, Cooper, and Hicks, 1964). It is conceivable, therefore, that degranulation of the endoplasmic reticulum under the influence of excess of vitamin A might be responsible for the suppression of keratinization found by Fell and Mellanby (1953) in cultures of embryonic chick skin grown in medium containing excess of the vitamin. Such a mechanism would be consistent with the more recent observations of Jackson and Fell (1963) that few profiles of the endoplasmic reticulum remain in the basal cells of chick epidermis after 6 to 7 days' cultivation with excess of vitamin A.

The small invaginations of the plasma membrane observed in retinol-treated fibroblasts growing in vitro are somewhat similar to the invaginations of the surface membrane that have been associated with pinocytosis in other cells (Novikoff, Essner, and Quintana, 1964). Both the invaginations observed in the present experiments, and those seen previously in erythrocytes treated with vitamin A (Glauert et al., 1963), are also reminiscent of the observations of Paul and Pearson (1960), and of Barrnett and Ball (1960) on the ultrastructural changes induced by insulin. The addition of this hormone to mouse fibroblasts and rat adipose cells in vitro encourages pinocytosis. The similarity between the actions of vitamin A and of insulin on the ultrastructure of certain cell membranes is interesting in view of the possibility that both compounds may exert physiological effects through interactions with the membranous components of cells.

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