DIFFERENCES IN MEMBRANE CONFIGURATION BETWEEN OSMIUM TETROXIDE-FIXED AND GLUTARALDEHYDE-FIXED CILIARY EPITHELIUM

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INTRODUCTION

Early electron microscope studies of the ciliary epithelium of the rabbit eye reported large numbers of vesicles at the free surface of the non-pigmented cell layer (4, 7). Characteristically, these vesicles were arranged in long rows, and it was suggested that they were engaged in pinocytosis.

More recently, the present author reported a different interpretation of these rows of vesicles (12). This interpretation, like those of previous workers, was based on observations of OsO_4-fixed material. It is summarized in Fig. 1 A. The apparent rows of vesicles are seen to be actually sheets of tubules cut in cross-section. Because these tubules are extensively interconnected, they were identified as an unusual "sheeted" form of smooth-surfaced endoplasmic reticulum. The original paper (12) should be consulted for details of this interpretation.

Another prominent feature of the free surface of the ciliary epithelium is extensive interdigitations or "infoldings" of the plasma membrane. The basic geometry of these structures is illustrated in Fig. 1 *B*, and, again, the original paper (12) should be consulted for details.

A comparison of Figs. 1 *A* and *B* reveals that the over-all configurations of both the sheets of tubules and the interdigitations are essentially identical, *i.e.*, both structures are in the form of two parallel sheets joined together into a U-loop. It is also noteworthy that the tubules, as seen in cross-section, have a variable length but a fairly constant width which is approximately the same as the width of the intercellular space separating the paired plasma membranes of the interdigitations. Also of significance is the fact that both types of structure are limited to the same part of the cell, and usually are closely associated with one another.

This similarity between the two structures raises

an interesting possibility, namely, that the sheets of tubules could be artifacts, formed by the partial breakdown of the paired plasma membranes of the interdigitations and their subsequent reorganization into tubules during OsO_4 fixation. Such a mechanism would involve fusion of paired plasma membranes across the intercellular space which normally separates them.

To investigate this possibility, an alternate means of fixation was tried, namely glutaraldehyde followed by OsO_4 (11). This fixation was originally selected because it was reputed (2) to give results superior to OsO_4 in preventing the breakdown of continuous membrane structures.

METHODS

Freshly excised pieces of ciliary body from the eyes of adult albino rabbits were fixed in glutaraldehyde (11). Various concentrations of fixative and buffer were employed, all of which gave identical results so far as the main purpose of this paper is concerned. Three per cent glutaraldehyde in 0.05 M phosphate buffer at room temperature was found most satisfactory. Specimens were subsequently washed in 0.15 M phosphate buffer for up to several hours. They were then post-fixed in 1 per cent OsO_4 in 0.15 M phosphate buffer. For purposes of comparison, other specimens were directly fixed in OsO_4 buffered with 0.15 M phosphate. The material was then dehydrated in ethanol and embedded in either Epon 812 (6) or Araldite 6005 (9). Thin sections were stained with lead citrate (8) and examined in a RCA EMU-3F microscope.

OBSERVATIONS

After glutaraldehyde fixation, the fine structure of the ciliary epithelium of the rabbit eye appears well preserved. In fact, the general quality of preservation seems as good as that achieved with OsO_4 alone. Fig. 3 shows typical glutaraldehyde-fixed material, and is to be compared with Fig. 2 which represents a similar area of OsO_4-fixed tissue.

There is one major difference between the structures demonstrated with the two fixatives. The tubules and vesicles of the "sheeted form of smooth-surfaced endoplasmic reticulum" are completely absent from Fig. 3 and from all the glutaraldehyde-fixed material the author has examined. In approximately one hundred micrographs of ma-

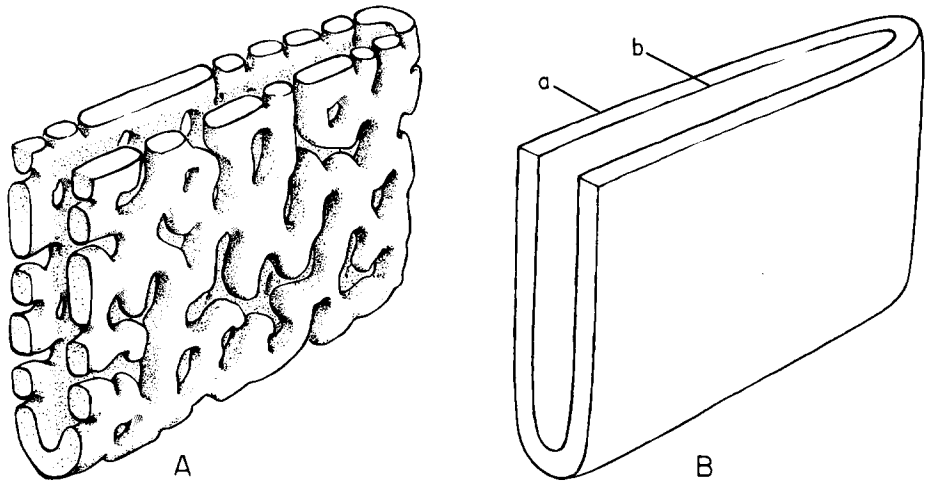


FIGURE 1 *A* Three-dimensional representation of the author's original interpretation of the apparent rows of vesicles found in OsO_4-fixed ciliary epithelium. It shows how sheets of interconnected tubules when cut in cross-section give rise to the appearance of rows of vesicles. It also shows that these sheets of tubules are commonly found in pairs joined together to form U-loops. (Drawing is modified from illustration in author's original paper (12).)

FIGURE 1 *B* Three-dimensional, cut-away representation of the form of an interdigitation in the ciliary epithelium. The plasma membrane (*a*) of one cell is invaginated into a long fold. Within this fold lies a narrow cytoplasmic projection from an adjacent cell; this projection is invested with its own plasma membrane (*b*). The two membranes are separated by a uniform intercellular space. Compare the over-all configuration with that shown in Fig. 1 *A*, which is drawn to the same scale.

terial from six eyes, not even a single row of apparent vesicles has been found. This stands in marked contrast to Fig. 2 and to OsO_4-fixed ciliary epithelium generally, in which the author has found rows of apparent vesicles to be very common. It is quite clear that this difference between the two fixatives has a very high level of statistical significance.

Although the sheet-like arrays of tubules are absent, all other components of the cell seem to be well preserved by glutaraldehyde. Neither the cell itself nor any of its components (with the possible exception of the mitochondria) show obvious signs of shrinkage or swelling or of fragmentation. Although not shown in Fig. 3, other smooth-surfaced membranous elements are well preserved, *i.e.*, loosely arranged, non-sheeted endoplasmic reticulum, and Golgi elements. Except in the Golgi area, the only vesicles seen are few in number and widely scattered. "Microtubules" in modest numbers course through the cytoplasm, a feature not well preserved by OsO_4. In brief, there are no obvious indications that the glutaraldehyde fixation is at all inadequate.

DISCUSSION

The one outstanding difference between OsO_4-fixed and glutaraldehyde-fixed ciliary epithelium is that the apparent rows of vesicles (sheets of tubules) which are so abundant in the former are absent in the latter. Clearly we are dealing with some sort of fixation artifact. The question is, which fixative has best preserved the true structure of the cell?

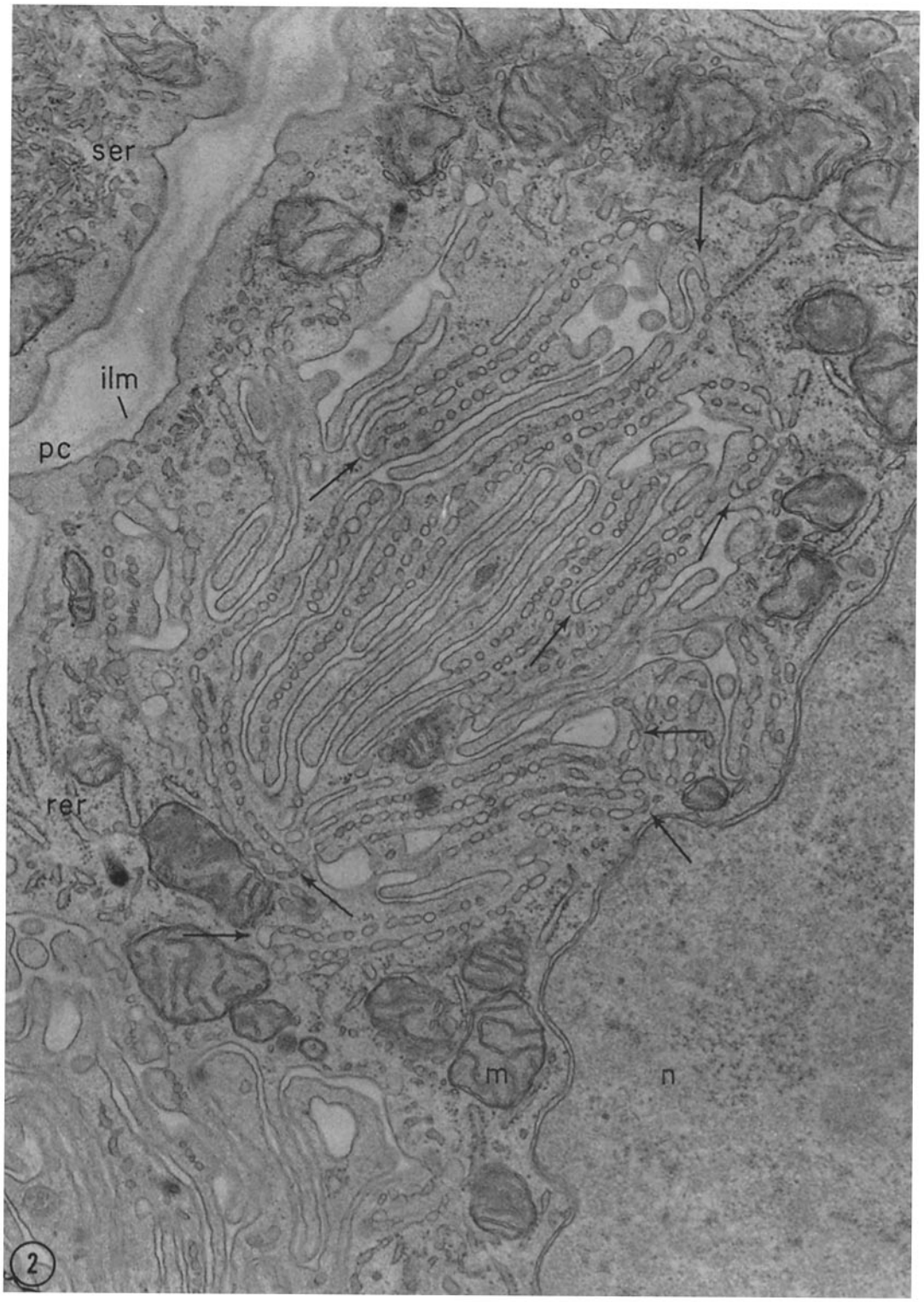
The data at hand fit well the hypothesis that the sheets of tubules are artifacts of OsO_4 fixation. According to this hypothesis, osmium tetroxide causes the membranes of the interdigitations to become physically unstable. In this unstable state, some of the membranes, which are normally separated by the intercellular space, come together and fuse at intervals along their length. Thus, continuous plasma membrane sheets are reorganized into sheets of tubules. This hypothesis explains particularly well the fact that the configuration of the sheets of tubules is identical to the configuration of the interdigitations.

The principal alternative to this seems much less likely, namely that glutaraldehyde fixation has selectively destroyed the sheets of tubules at the same time it has produced good preservation of every other cell component.

Another possibility is that the "sheeted reticulum" is a step in the biosynthesis of new interdigitations and that glutaraldehyde simply promotes the transformation of the tubules into continuous membranes. There is nothing in the data at hand to either prove or disprove this possibility, nor is it easy to imagine an experiment which might conclusively establish it. However, the author has recently studied the uptake of colloidal particles by the ciliary epithelium *in vitro* (13); the results of these experiments are much more readily explained by the OsO_4 artifact hypothesis than by any other. Furthermore, there is no reason to suppose that a mature cell would need to employ such an elaborate mechanism to make new plasma membrane.

The case for the OsO_4 artifact hypothesis is greatly strengthened by other data. There is substantial evidence from other cell systems both that OsO_4 can cause the partial breakdown of continuous membrane structures and that glutaraldehyde can prevent such breakdown. For example, the

FIGURE 2 Micrograph of OsO_4-fixed rabbit ciliary epithelium. The area shown is at the free surface of a cell of the non-pigmented layer. Complex plasma membrane foldings occupy most of the central portion of the picture; these represent interdigitations from one or more adjacent cells. Intimately associated with these interdigitations are many long rows of vesicular profiles. The configuration of the rows is very similar to that of the interdigitations, and arrows indicate several places where two rows are joined together to form U-loops in the manner of the interdigitations. These vesicles are interpreted as fixation artifacts. Other structural features are similar to those of glutaraldehyde-fixed cells. Smooth-surfaced endoplasmic reticulum (*ser*) is shown in the upper part of the micrograph. This smooth ER is not to be confused with the "sheeted form of endoplasmic reticulum" described in the text, and, although it is not demonstrated in Fig. 3. it is found with equal frequency in glutaraldehyde- and OsO_4-fixed material. Also shown are: *pc*, posterior-chamber of the eye; *ilm*, basement membrane (internal limiting membrane); *rer*, rough surfaced endoplasmic reticulum; *m*, mitochondrion; *n*, nucleus. × 24,000.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smooth-surfaced endoplasmic reticulum of testicular interstitial cells fragments into isolated vesicles readily with OsO_4 fixation (1) but very slightly with glutaraldehyde (2). A similar situation seems to exist in the case of the gastric parietal cell (5). Rosenbluth (10) found that what appeared as a continuous invagination of the plasma membrane in permanganate-fixed cells appeared as a long row of vesicles in OsO_4-fixed material. Particularly helpful is a recent study by Franzini-Armstrong and Porter (3) on the transverse tubular (or T) system in muscle. In OsO_4-fixed preparations the T system closely resembles the sheets of tubules of the ciliary epithelium. After glutaraldehyde fixation, however, the T system has the form of a continuous plasma membrane invagination. It seems fairly certain, on both morphological and physiological grounds, that the glutaraldehyde picture of the T system is the truer one.

Also, it is worth noting that preliminary observations by the author (14) indicate that the "sheeted endoplasmic reticulum" is not found in permanganate-fixed ciliary epithelium.

In short, all the currently available evidence seems to support the view that the sheets of tubules found in the ciliary epithelium are artifacts of OsO_4 fixation. Several implications of this conclusion will now be considered.

Because some interdigitations break down while others remain intact within the same cell (as in Fig. 2), the factors responsible for the breakdown of one set of membranes but not the next must be very delicately balanced. Therefore, variations of fixation and embedding coupled with alterations of experimental conditions might, in some cases, alter the extent of membrane breakdown. This may explain why some workers (4, 7) have seen changes in the number of apparent vesicles under certain experimental conditions whereas others (12) have not.

Since most, and possibly all the membrane folds found at the free surface of the ciliary epithelium are probably true interdigitations between adjacent cells (12), the breakdown of the interdigitations by OsO_4 produces an artificial syncytium; *i.e.*, the sheets of tubules demarcate the borders between the cytoplasms of normally separate cells, and the cells communicate directly through the gaps in the tubular sheet.

If the observations of this paper are correctly interpreted, then the rows of vesicles and sheets of tubules previously reported in the ciliary epithelium are without counterpart in the living cell. Therefore, the elucidation of the structural basis of the water and electrolyte-secreting function of these cells is now simplified, if only because there is one less structure to be taken into account.

This should also serve as a warning that rows of vesicles and tubular systems found in other cell types might also be, in some cases, artifacts of OsO_4 fixation.

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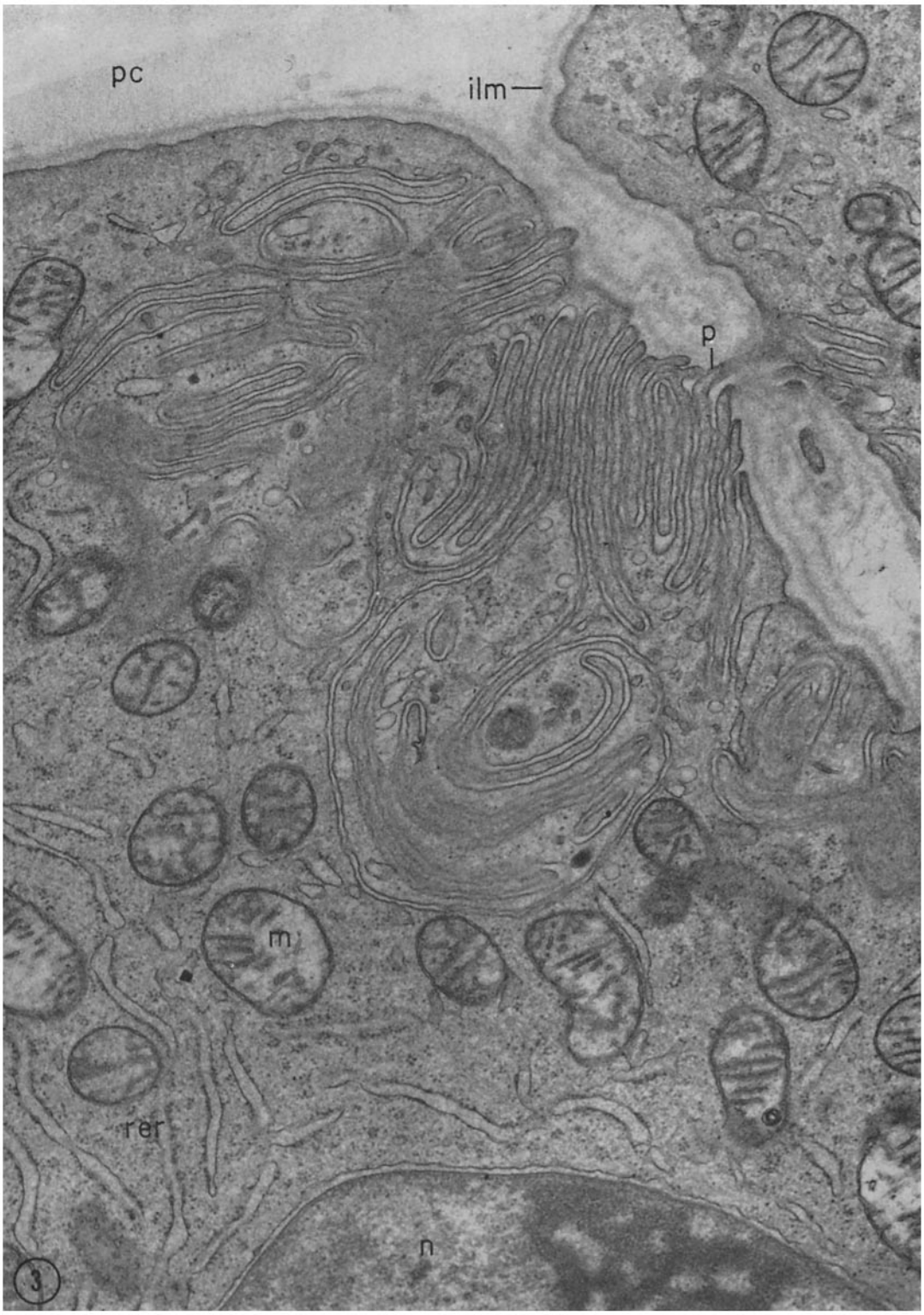
Note Added in Proof: In a recent publication (L'ultrastructure des tissus oculaires, *Bull Soc. Belge Opht.*, 1964, **136,** 1), L. Missotten mentions that rows of vesicles are absent from permanganate-fixed and forma-

FIGURE 3 Micrograph of glutaraldehyde-fixed ciliary epithelium. The area shown is similar to that shown in Fig. 2. The central part of the micrograph shows complex membrane foldings which represent interdigitations from adjacent cells. One (labeled *p*) is clearly a projection from the neighboring cell shown in the upper right corner. The membranes are blurred where their orientation is oblique to the plane of section. Particular attention is called to the absence of rows of vesicles. A relatively small number of solitary vesicles remains; some of these appear as isolated blebs or evaginations of the interdigitating membranes. This contrasts strikingly with the many rows of vesicles in Fig. 2. Other features shown are: *pc*, posterior chamber of the eye; *ilm*, basement membrane (internal limiting membrane); *rer*, rough-surfaced endoplasmic reticulum; *m*, mitochondrion; *n*, nucleus with nucleolus. × 24,000.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lin-fixed ciliary epithelium. Unfortunately, his paper contains no micrographs which demontrate this point, nor does it discuss whether these alternate fixatives produce otherwise high quality preservation. He concludes that the vesicles are artifacts of OsO_4 fixation.

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