ARTIFACTUAL LOCALIZATION OF FERRITIN IN THE CILIARY EPITHELIUM *IN VITRO*

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ABSTRACT

The accumulation of ferritin by the ciliary epithelium of the adult albino rabbit has been studied by electron microscopy. The experiments have been carried out under *in vitro* conditions, such that any uptake observed should be the result of passive diffusion of the tracer particles rather than the product of active metabolic processes. The cells were fixed in osmium tetroxide and embedded in Araldite. Ferritin was found localized in three areas: in rows of apparent vesicles, free in the cytoplasmic matrix, and in the basement membrane. Some of the conclusions reached are as follows. The appearance of tracer in rows of vesicles is not in itself an adequate demonstration of pinocytosis. The permeability of the plasma membrane is drastically increased by osmium tetroxide fixation, so that tracer particles are free to diffuse across the membrane and wander through the cytoplasm. These results indicate the serious danger of being misled by artifacts when colloidal particles are used as tracers.

INTRODUCTION

Colloidal particles have been used as tracers in a number of electron microscope studies of permeability and cellular uptake, and in general the technique has yielded valuable results. Nevertheless, insufficient attention seems to have been paid to the possibility that some of the observed localization of colloidal tracers might be artifactual.

The present paper describes certain aspects of the *in vitro* uptake of ferritin by the ciliary epithelium of the rabbit eye. Two types of experiment have been carried out. In one, the cells were bathed in ferritin after they had been removed from the eye and left in ice cold, non-oxygenated buffer for 15 minutes. In the other, the cells were bathed in the tracer after they had been fixed in osmium tetroxide. Both experiments were designed so that any uptake observed would be due to passive diffusion of the ferritin rather than to active metabolic processes.

Under these artificial conditions, the tracer particles are found localized in two places of especial interest, namely, in long rows of apparent vesicles and scattered about within the cytoplasmic matrix. Reasons will be given supporting the conclusion that the accumulation of tracer in these two places is artifactual.

Since there is no reason to believe that the artifacts observed here are peculiar to the ciliary epithelium, this paper should serve to point out certain general hazards in the use of colloidal particles as tracers.

METHODS

Eyes were enucleated from adult albino rabbits and dissected in ice cold 0.15 M phosphate buffer (pH 7.4) (5). The lens was enucleated, and the lens capsule was carefully dissected away from the ciliary processes by directly cutting the zonular fibers. The

tissue was bathed in the buffer for a total of approximately 15 minutes before further procedures were carried out.

Horse spleen ferritin (Nutritional Biochemicals Corp., Cleveland, Ohio) was employed as a tracer. The initial concentration of ferritin from this source was approximately 60 mg/ml in distilled water. Both routinely crystallized and "cadmium free" preparations were used.

In one set of experiments, the ciliary epithelium, after being bathed in the buffer for 15 minutes, was transferred to an ice cold ferritin solution. This solution was made by mixing stock ferritin directly with phosphate buffer, and contained approximately 30 mg ferritin per ml in 0.15 M buffer (5) at pH 7.4. The tissue remained in the ferritin solution for 10 to 15 minutes before being briefly washed in buffer and fixed in cold OsO_4.

In the other set of experiments, the tissue, after being in buffer for about 15 minutes, was fixed directly in cold OsO_4. After a brief wash in distilled water or 0.15 M sucrose, the tissue was soaked for 10 to 15 minutes in ice cold ferritin solutions. The ferritin in this case was either used directly from the bottle, or made up into a 30 mg/ml solution in water or 0.15 M sucrose. The pH of these solutions was 6.6–7.2. (Phosphate buffer was used as a vehicle for ferritin in some of these experiments but was found to have a detrimental effect on plasma membrane preservation.) After being soaked in ferritin, the tissue was briefly washed and then dehydrated.

One per cent OsO_4 was used as the fixative. Veronal-acetate buffer with balanced salts added (17) and 0.15 M phosphate buffer (5), both at pH 7.4, were used interchangeably as vehicles for the fixative, without demonstrable differences. Fixation times were varied from 6 to 120 minutes. Dehydration was done in cold ethanol or acetone, and was carried out both rapidly (to absolute in 5 minutes) and slowly (to absolute in 1 hour) without obvious differences. The material was embedded in Araldite 6005 (10), sectioned with diamond knives, stained with lead citrate (9), and examined in an RCA EMU 3-F microscope.

RESULTS

The observations reported here were made at or near the free surface of the non-pigmented layer of the ciliary epithelium. Although the author has described these cells before (12, 13), it seems wise to review here several pertinent aspects of their morphology.

The free surface of the epithelium is covered by a basement membrane about 300 A thick, herein referred to as the internal limiting membrane. The apparent paradox of having a "free" cell surface covered by a basement membrane deserves some comment. This surface is free in the sense that, aside from the internal limiting membrane, it is immediately bounded by no other structure and is in direct contact with the posterior chamber of the eye. The internal limiting membrane reaches its position as a result of the invagination of the optic vesicle during embryogenesis.

Many "infoldings" of the plasma membrane occur at the surface of the non-pigmented layer. Serial sections of these infoldings show that they are actually interdigitations from adjacent cells (12). The space between the paired plasma membranes of an interdigitation is intercellular space and is in direct continuity with the free surface of the epithelium.

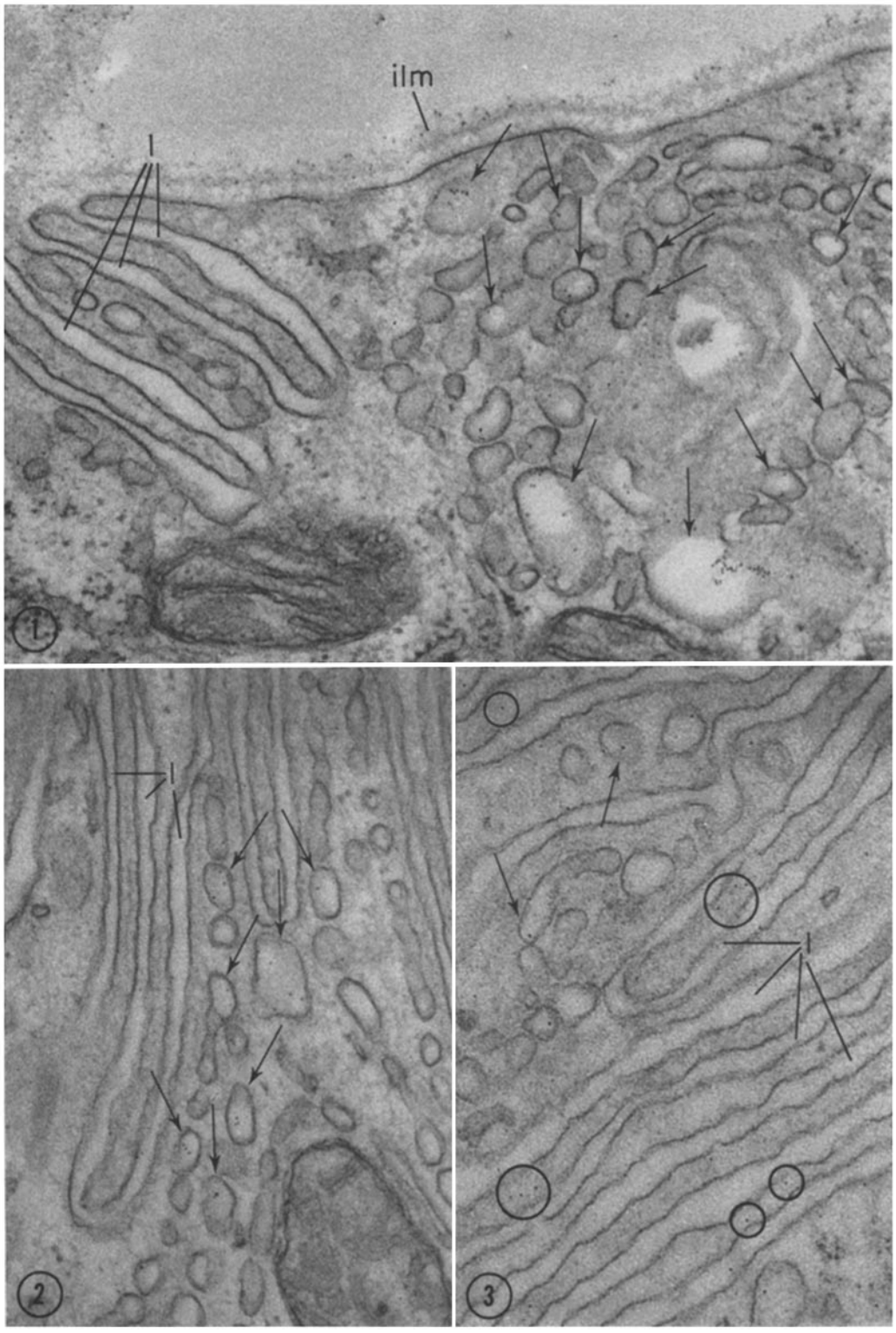
In OsO_4-fixed material, many rows of vesicles and areas of interconnected tubules are found. The tubules are arranged in the form of sheets, and the apparent rows of vesicles are actually sheets of tubules cut in cross-section (12). In glutaraldehyde-fixed material these sheets of tubules and apparent rows of vesicles are not seen.

FIGURES 1 TO 3 Micrographs of ciliary epithelium treated with ferritin *before fixation*. All three areas shown are at or near the free surface of the non-pigmented cell layer. Ferritin-containing "vesicles" and tubules are indicated by arrows. Ferritin found free in the cytoplasmic matrix is indicated by circles (Fig. 3). The intercellular space between cytoplasmic interdigitations is indicated by *I*. × 60,000.

Fig. 1 demonstrates ferritin in "vesicles" and tubules and also enmeshed in the basement membrane (internal limiting membrane, *ilm*). Few tracer particles are found within the interdigitations and the cytoplasmic matrix.

Fig. 2 also demonstrates the tracer within apparent rows of vesicles, with little localized elsewhere.

Fig. 3 shows ferritin predominantly within the cytoplasmic matrix, although not in large concentrations.


ilm
1
2
3

The author has reviewed this discrepancy and has concluded that these structures are artifacts of OsO_4 fixation (13). Evidently, OsO_4 does not completely fix the paired plasma membranes of the interdigitations, and, as a result, some of the interdigitations partially break down and reorganize during tissue preparation. The membranes fuse across the intercellular space. As a result, normally separate cells are joined together into an artificial syncytium. More important for the purposes of this paper, the normally continuous intercellular space is pinched off and segregated within the newly formed tubules.

A total of 13 eyes—6 treated with ferritin before fixation, and 7 treated with ferritin after fixation—were examined. Within each group of eyes, the results were consistent from eye to eye.

Localization of Ferritin

TUBULES AND VESICLES: When the cells are bathed in ferritin *before* fixation, tracer particles are frequently found in tubules and apparent rows of vesicles near the cell surface (see Figs. 1 to 3). The appearance of ferritin in rows of vesicles closely resembles what one would expect to see if the tracer were taken up by pinocytosis. On the other hand, very little ferritin is found within the intact intercellular spaces between the interdigitations. When the cells are bathed in ferritin after fixation, the tracer is not found in the tubules and vesicles (see Fig. 6).

CYTOPLASMIC MATRIX: In those experiments in which ferritin is used *before* fixation, the frequency with which tracer particles are found in the cytoplasmic matrix is rather variable. Sometimes very little ferritin is present in the matrix, as in Figs. 1 and 2; but at other times a quite definite, although widely scattered, accumulation is found there, as in Fig. 3. In all instances, however, the quantity of ferritin present surpasses that found previously by the author in normal cells untreated with ferritin (12).

When ferritin is used *after* fixation, the cytoplasmic matrix consistently accumulates quite marked quantities of the tracer, as in Figs. 4 to 6. It should be noted that the plasma membrane appears intact, with no evident holes through which the ferritin might have gained access to the cytoplasm.

INTERNAL LIMITING MEMBRANE: For the sake of completeness, it should also be noted that the tracer is found caught up in the internal limiting membrane, whether the cells are bathed in ferritin before or after fixation (see Figs. 1, 4, and 5). The particles are most commonly observed on the outer surface of this membrane. Evidently, the membrane acts as a very leaky barrier in which some of the large ferritin particles are trapped, but through which many more are able to diffuse without great difficulty.

DISCUSSION

Two of the foregoing observations seem to be of sufficient importance to warrant further discussion.

Tubules and Vesicles

The presence of tracer in rows of vesicles is, of course, the pattern one would expect to see if this material were being taken up by pinocytosis. Yet this "uptake" occurs under experimental conditions in which active transport across the ciliary epithelium is known to be completely inhibited (1, 2).

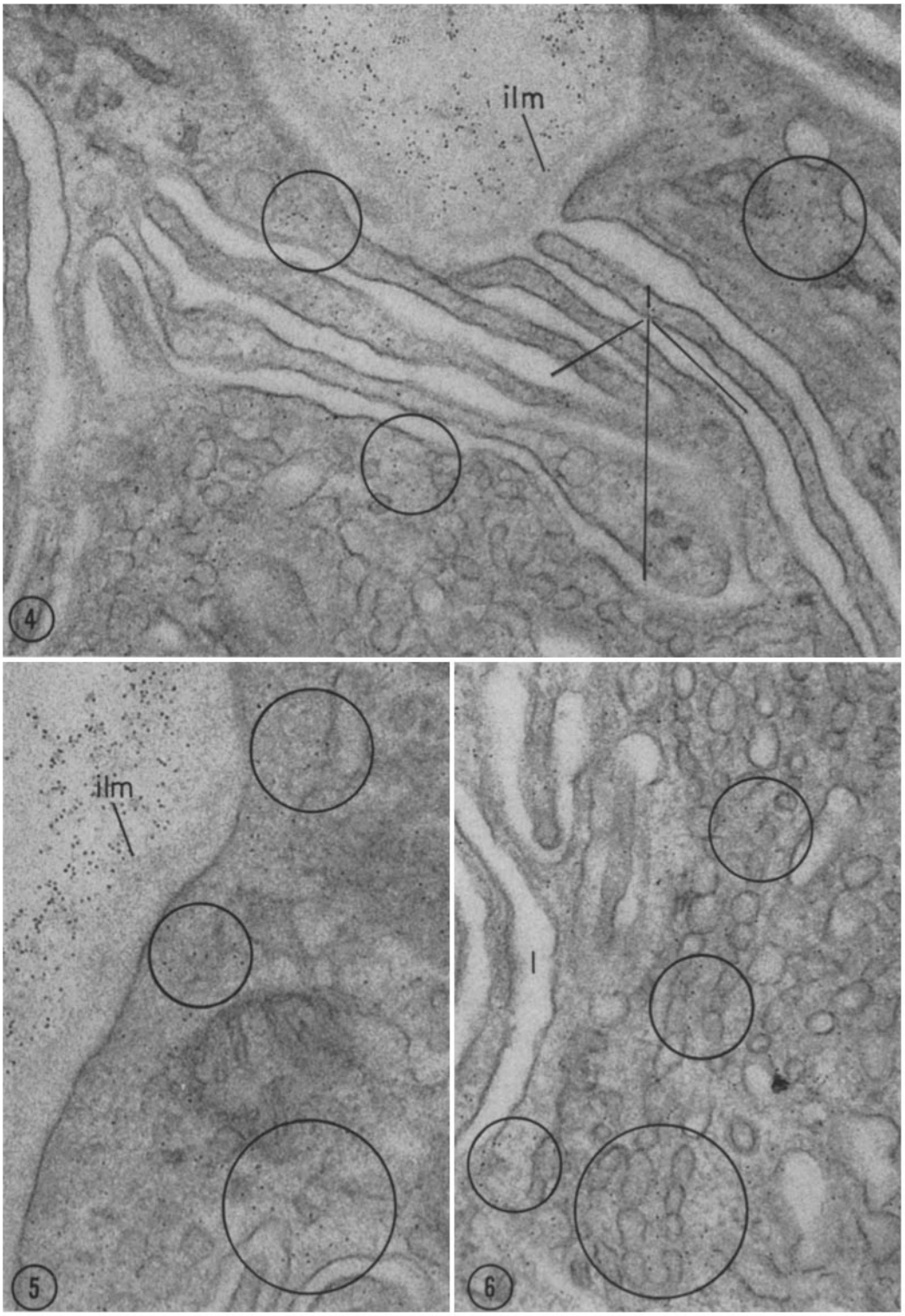
An adequate basis for understanding the mechanism of this ferritin uptake is provided by the view, discussed earlier in this paper, that the rows of apparent vesicles are artifacts of OsO_4 fixation. The following sequence probably occurs. When the

FIGURES 4 TO 6 Ciliary epithelium treated with ferritin *after fixation*. Areas shown are at or near the free surface of the epithelium. Portions of the ground cytoplasm containing substantial concentrations of ferritin are encircled. The intercellular space between cytoplasmic interdigitations is indicated by *I*.

Fig. 4 shows an abundance of ferritin that is free within the cytoplasmic matrix. It is also found enmeshed in the basement membrane (internal limiting membrane, *ilm*). Note that there are no obvious breaks in the plasma membrane. × 60,000.

Fig. 5 also demonstrates ferritin in the cytoplasm and basement membrane, with no evident discontinuities in the plasma membrane. × 71,000.

Fig. 6 shows ferritin particles scattered in the cytoplasmic matrix, with none localized within the vesicular profiles. × 60,000.


ilm
4
ilm
5
6

cells are bathed in ferritin before fixation, the tracer diffuses through the internal limiting membrane and then between the cells into the interdigitations. As fixation begins, the tracer particles begin to diffuse back out; but, before they can do so, the membranes around them begin to break down and reorganize into tubules. As a result, some of the ferritin is trapped inside the tubules. The rest continues to diffuse out. Thus, there results the pattern observed here: ferritin in tubules and rows of apparent vesicles, and no ferritin in the intact intercellular spaces between the interdigitating cells.

Pappas and co-workers (7, 8), injecting Thorotrast particles directly into the living eye, have also observed that the ciliary epithelium incorporates tracer into rows of apparent vesicles; and they have considered this to be direct evidence for active pinocytotic transport away from the free surface of the epithelium. Since OsO_4 promotes the breakdown of the interdigitations regardless of whether the eye is fixed *in vivo* at 37°C (12) or *in vitro* at 0°C, it seems likely that those authors were merely observing the phenomenon described here.

The tendency of OsO_4 fixation to cause the fragmentation of continuous membranes into vesicle-like structures is now coming to be recognized in a number of systems (4, 11). The appearance of colloidal particle tracers in rows of vesicles, therefore, can no longer be regarded as a demonstration of pinocytosis, unless it is also shown that these vesicles are not artifacts.

Cytoplasmic Matrix

Relatively large quantities of tracer accumulate within the cytoplasmic matrix when the cells are bathed in ferritin *after* fixation. Evidently, OsO_4 fixation so grossly alters the permeability of the plasma membrane that it becomes quite permeable to the ferritin.

This finding indicates the probable explanation for the small but definite accumulation of tracer particles found free in the cytoplasm of cells bathed in ferritin *before* fixation. The following sequence seems likely. Before fixation the plasma membrane excludes the tracer from the cytoplasm. When fixation begins, most of the tracer is either washed away or bound down *in situ* by the fixation process. Some of the tracer, however, is not completely immobilized. This "wandering" tracer can then pass through the OsO_4-fixed membranes and into the cytoplasmic matrix, where it is subsequently held in place.

Alternative explanations for the accumulation of tracer in the cytoplasm of cells bathed in ferritin *before* fixation seem unlikely. The possibility that the living membrane has 100-A pores is contrary to current physiological concepts of membrane permeability. And the possibility that the phosphate buffer damages the cells finds no warrant in the physiological literature.

The manner in which the ferritin particles cross the *fixed* plasma membranes is uncertain. One possibility is the existence of pores about 100 A in diameter within the fixed membranes. Such pores are not evident in our material, but this does not rule out the possibility of their presence. Since the sections are some 600 to 800 A thick, any pores, if present, would tend to be masked by the density of the membranes in which they were located. Another possibility, of course, is the existence of frank disruptions in the fixed membranes, but such breaks, if present at all, are extremely rare in this material.

An unpublished observation of the author (14) may be useful as an indication of how the plasma membrane is altered by fixation. When the cells are bathed in Thorotrast before fixation, the Thorotrast particles are distributed in a pattern very similar to that obtained with ferritin, *i.e.*, tracer in rows of apparent vesicles and in the cytoplasmic matrix. The Thorotrast particles, however, exhibit considerable variability in size; some of them have apparent diameters considerably greater than 100 A. The point to be made is that, although many of the smaller Thorotrast particles are found in the cytoplasmic matrix, few of the larger particles are found there. This would seem to indicate that the OsO_4-fixed plasma membrane still retains some selectivity and filters out particles above a certain diameter. This would be compatible with the presence of holes about 100 to 200 A in diameter.

Whatever the exact explanation may be, the fact is that colloidal particles can traverse OsO_4-fixed membranes. Furthermore, some of the particles seem to be able to move about during fixation and thus to cross membranes and reach areas which are closed to them in living tissues. This possibility indicates a need for great caution on the part of investigators who would use colloidal particles as indicators of cellular permeability.

These results probably explain certain seeming discrepancies in the literature (compare, *e.g.*, 3, 6, 15, 16), according to which tracers have been found free in the cytoplasm of certain cell types in some instances but not in others.

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