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SECRETION AND ELECTROGENESIS OF THE SUPPORTING CELL IN THE OLFACTORY EPITHELIUM

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SUMMARY

1. Electrophysiological study disclosed that chloroform and some other odours generate long-lasting positive potentials in the olfactory epithelium of the bullfrog, while electron microscopical study showed that they elicit vigorous protrusion of the distal cytoplasmic portion of the supporting cell containing the secretory granules.

2. The secretory process of the supporting cell is as follows: The first detectable indication is the protrusion of the apical portion of the supporting cell (Pls. 3 and 4); the protruded part detaches from its maternal supporting cell (Pl. 7*B*), floats as a droplet in the mucus (Pl. 5), and finally the secretory granules inside the droplet disintegrate into the mucus (Pls. 6 and 7*A*).

3. The secretion was not elicited by the odours which elicit the negative potentials.

4. In Cl^--free Ringer solution, neither the positive potential nor the protrusion and secretion occurred.

5. When 1–2 mM-Ba^{2+} in Ringer solution was dripped on the epithelium, both the positive potential and the protrusion and secretion resulted. Subsequent application of chloroform vapour only advanced further disintegration of the secretory granules, but it elicited neither a new protrusion of the granules nor the positive potential.

6. In the olfactory epithelium in which the olfactory cells had degenerated but the supporting cells survived, both the positive potential and the protrusion and secretion occurred, but the negative potential did not.

7. It is concluded that Cl^- entry which mainly generates the long-lasting positive potential triggers the secretion of the supporting cell.

INTRODUCTION

Since Hosoya & Yoshida (1937) and Yoshida (1950) studied the electrical responses generated by odorous stimulation in the olfactory epithelium of the dog, and also since Ottoson (1956) studied them extensively in the frog and named them 'Electro-olfactogram or EOG', five types of responses have been found (Takagi, 1969). They are (1) 'negative-on-', (2) 'negative-off-' (Takagi & Shibuya, 1959, 1960*a*, *b*, *c*), (3) 'positive-on-' (Takagi, Shibuya, Higashino & Arai, 1960), (4) 'positive-off-' potentials (Shibuya, 1960) and (5) 'positive afterpotential' (MacLeod, 1959) or long-lasting positive potential (Takagi, Wyse & Yajima, 1966). Recently Ottoson (1971) defined the EOG as the 'monophasic negative potential' evoked by odours in the sensory region of the nasal mucosa. However, in the retina the term 'ERG' is used to define the complicated potentials with the positive and negative components all of which are elicited in the retina by light illumination. Since there is a probability that the potential elicited in the olfactory epithelium by odours contains not only the negative components but also the positive components as will be proven in this paper, and since in most cases it is impossible to discriminate strictly the negative and the positive components, the authors propose that the word 'EOG' should be used in a wide sense, namely for all kinds of potentials which are evoked by odours in the olfactory epithelium. Thus, the word 'EOG' is used in a wide sense in this paper.

In order to clarify the origins of these EOGs, Takagi & Yajima (1964, 1965) sectioned the olfactory nerve and studied both the degeneration of the olfactory epithelium and the change of the EOGs. They found that the olfactory cells degenerated and disappeared in proportion to the decrease in amplitude and disappearance of the negative EOGs, but that the supporting and basal cells survived together with the long-lasting positive potential. Consequently, the origin of the negative EOGs was attributed to the olfactory cell, but the origin of the long-lasting positive potentials remained unsolved.

The present experiment was undertaken to clarify the generative mechanisms of the long-lasting positive potential. Changes in the olfactory epithelium studied by means of an electron-microscope after sectioning the olfactory nerve will be explained in detail. Also, the changes in the normal epithelium and the degenerating epithelium immediately and 1–3 min after the application of several kinds of odours will be outlined below. Since Cl^--free Ringer solution and 1–2 mM-Ba^{2+} in Ringer solution are known to depress the positive potential (Takagi, Wyse, Kitamura & Ito, 1968), histological and electrical changes caused by these solutions will also be presented here. The origin of the positive potential will be discussed

toward the end of this article. A preliminary report was published elsewhere (Takagi & Okano, 1971).

METHODS

Material

One hundred and forty-four bullfrogs were used. Among them, sixteen bullfrogs were used to study the normal olfactory epithelia, and the rest were used to study the degenerating and degenerated olfactory epithelia 19, 39 and 112 days after sectioning the olfactory nerves unilaterally and bilaterally. Under local anaesthesia, the olfactory eminentia were exposed by removing the overlying skin and bone of the nasal cavity after the bullfrogs were immobilized by D-tubocurarine.

Odorous stimulation

Amyl acetate has been well known to be one of the odours which elicits the greatest negative EOG. Sixteen out of one hundred and twenty two odorous substances were found to elicit the positive EOGs (Takagi, Aoki, Iino & Yajima, 1969). Among them, chloroform produces the most remarkable long-lasting positive potential. Consequently, these two odours were mainly used as representatives of the negative and positive EOGs generating odours. In a few cases, however, *n*-butyl alcohol and t-butyl alcohol were used to elicit the negative and positive EOGs. The original solutions of amyl acetate and chloroform were diluted to 0·1 M and 0·5 M by adding odourless mineral oil (Nujol, plough, Inc. Memphis Tenn., U.S.A.). 1 c.c. of these and original solutions were stored in 30 c.c. syringes respectively. The vapours in these syringes were used as stimulants. The vapours were described as 0·1 M, 0·5 M and original vapours.

Recording

Non-polarizable Zn-$ZnSO_4$ Ringer-gelatin electrodes were used as recording electrodes. This sort of electrode has already been recommended as best by Kimura (1961) and Mozell (1962), since with this electrode non-biological artifactual responses were either non-existent or so small that they could not be measured. An exploring electrode was placed on the exposed olfactory eminentia and an indifferent one was put on the Ringer-soaked cotton-wool placed around the head. EOGs were recorded with an ink-writing oscillograph through a DC amplifier.

Preparations for the electron-microscope

Since this study was commenced in 1966, several different types of fixatives have been recommended and in fact some of them have been used. This might be confusing to the readers. It is, therefore, to be emphasized that the old recipes of fixatives have also remarkable property of preserving the fine structures of the olfactory epithelia. The authors believe that the use of different fixatives does not hinder the essential evaluation of the secretory phenomena.

The fixatives used were 2·5, 4 and 6·5 per cent of glutaraldehyde adjusted to pH 7·2–7·4 with phosphate buffer solution (Sabatini, Bensch & Barnett, 1963), 1 per cent osmium tetroxide adjusted to pH 7·2 to 7·4 with veronal acetate (Caulfield, 1957), and 2 per cent osmium tetroxide solution buffered with phosphate solution (Millonig, 1962). The olfactory epithelia were prefixed with one of the osmium solutions for 2 hr. Then, it was post-fixed with one of the glutaraldehyde solutions for 1 hr. In some cases, the epithelia were prefixed with a glutaraldehyde solution for 1 hr and post-fixed with an osmic solution for 2 hr. The concentrations of these solutions are stated in the explanation of each Figure. Then, the epithelia were

dehydrated by immersing them in aceton or ethanol and finally they were imbedded with Epon 812 (Luft, 1961). Ultrathin tissue preparations were made by means of Porter-Blum microtome or LKB ultratome with glass knives. After they were double-stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963), they were examined by means of electron-microscopes of the Akashi TRS-80W DC type, and Hitachi HS-6.

For the light microscope, the serial tissue preparations with thickness of about 0·8 μm were made and stained with 0·5 per cent toluidine blue solution. This was performed in order to ascertain whether or not the protruded part detaches itself from the supporting cell and is thus the initial phenomenon of the secretory processes.

Experimental procedures

By means of syringes, each of the above mentioned odours was applied from 1 to several seconds. Only one odour was applied to each bullfrog. Immediately after the beginning of the odour application, one of the cooled fixatives was poured onto the olfactory epithelium. The starting time of pouring was chosen so that the epithelium could be fixed at the peak of the EOG elicited. Some normal olfactory epithelia were fixed 1–3 min after the application of chloroform vapour in order to examine the changes of the epithelium in later stages. Then, the olfactory eminentia were excised from the surrounding tissue and were immersed into the fresh fixative for 2 hr. These procedures were repeated in the normal and degenerated olfactory epithelia by applying the four kinds of odour vapours. As controls, the normal and degenerated olfactory epithelia were fixed, as well. Thus, eleven groups of olfactory epithelia were prepared for electron-microscopic investigation.

RESULTS

I. *The normal non-stimulated olfactory epithelium*

In the present experiment, the free surface and adjacent parts of the olfactory epithelium were the prime object of our study, although the entire epithelium was examined.

The supporting cell. The characteristics of this cell are bushy microvilli, a large quantity of cytoplasmic organelles and rich secretory granules. Mitochondria, smooth and rough surfaced endoplasmic reticular membranes, Golgi apparatus and minute granules are also found in the cytoplasm.

The secretory granules are found in the upper part of the cytoplasm, in particular immediately under the free surface of the cell. There were often pit-like structures in the free surface which suggested a vestige of liberated granules (arrow 2 in Pl. 1*A*). In general, the supporting cell resembles the shape of the goblet cell in the epithelium of the small intestine (Neutra & Leblond, 1969).

The olfactory cell. In Pl. 1*A*, the olfactory vesicles, the cilia therefrom and a part of the so-called olfactory dendrite are shown, but the oval nuclei, perikarya and axon of the olfactory cell are not. The border area between the olfactory vesicle and the olfactory dendrite is narrower in

width and looks as though it is anchored by the invagination of the adjacent supporting cells and the terminal bars.

The olfactory vesicle (Pl. 1*A*) is round and has a diameter of 3–4 μm, and contains a small number of mitochondria and basal bodies of the olfactory cilia. It also has a few minute corpuscles and a few masses of the corpuscles. These corpuscles resemble in shape the synaptic vesicles described by Gray (1964) and Gray & Guillery (1966) and also resemble the small granules in the multivesicular body found in the olfactory vesicle of the dog (Okano, Weber & Frommes, 1967). The olfactory vesicles seem to be buried by the numerous bushy microvilli of the adjacent supporting cells. The long cilia extend from all parts of the olfactory vesicle. They run obliquely almost in a straight line for a short distance and then bend to run in parallel with the surface of the olfactory epithelium. These cilia compose a thick mat in the upper part of the deep mucus layer.

The mucus layer. In the non-stimulated epithelia, this layer is 10–15 μm in depth, and can be divided into the following four parts.

(1) A part where the olfactory vesicles and microvilli are found.

The depth of the mucus which imbeds the olfactory vesicle and microvilli is about 3 μm.

(2) A part where the cilia extend straight.

This part is about 5 μm in depth. Here, the cilia are many in number but much less than in the next part. This part corresponds to what Reese (1965) refers to as the 'proximal segment'.

(3) A part where the cilia run in parallel with the mucus surface.

This part is about 3 μm in depth. Many cilia run horizontally, piling up, and forming a seemingly rigid mat just underneath the mucus surface. This part corresponds with the 'distal segment' of Reese (1965).

(4) A part of the mucus proper.

This part, about 1 μm in depth, forms the surface of the mucus and has no special structure, except a structure mentioned below.

These findings coincide very well with those which were obtained in the frog, *Rana esculenta* and *R. pipiens* by Bloom (1954), Reese (1965) and Yamamoto, Tonosaki & Kurosawa (1965). Besides, the authors discovered an entirely new structure. It was a crypt-like structure in the surface of the mucus (arrow 1 in Pl. 1*A*). This crypt could be followed into the depth of the mucus in the serial sections. Although a continuity between the crypt and the secretory duct of the Bowman's gland has been suspected, there was not found any such evidence for this. Nor was any relationship found between it and the other cells.

II. *The degenerated non-stimulated olfactory epithelium*

This may be the first electron-microscopic work on the degenerating and degenerated olfactory epithelia due to the section of the olfactory nerve (Pl. 1*B*).

The supporting cell. Remarkable morphological changes were found in the apical cytoplasm of this cell. The part of the cell between the nucleus and the apical surface became thicker and columnar, and is full of the secretory granules in this case. In some other cases, however, the secretory granules either decreased in number or they disappeared entirely. No special changes in the microvilli were evident.

The olfactory cell. In most preparations, this cell was not found, but only rarely there were found olfactory cells devoid of complete cilia and cell organelles.

The mucus layer. The thickness of the mucus layer was found to be very thin about 3–4 μm, much thinner than the normal one, and the above mentioned four structural divisions of the normal mucus layer were not evident. This phenomenon may occur due to the loss of the olfactory cilia.

III. *The normal olfactory epithelium stimulated with amyl acetate vapours*

In general, electronmicrographs of this epithelium (Pl. 2*A*) closely resembled those in the normal non-stimulated epithelium (Pl. 1*A*). There were never found any active protrusions of the supporting cells as were found in Pls. 3 and 4.

EOG. It has been made clear that amyl acetate and most other odours elicit the negative EOGs in the normal olfactory epithelium (Text-fig. 1) (Takagi *et al.* 1969).

IV. *The degenerated olfactory epithelium stimulated with amyl acetate vapours (Pl. 2B)*

The mucus layer was again very thin (3–4 μm) and there was practically no secretory activity in the supporting cell (Pl. 2*B*). Takagi & Yajima (1964, 1965) proved that the negative EOG can not be elicited in this epithelium (Text-fig. 1, see also Fig. 2 in Takagi *et al.* 1969).

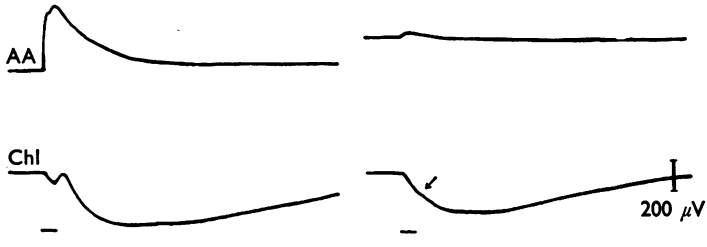
The findings in III and IV indicate that amyl acetate vapours produce neither noticeable secretions in the supporting cell nor the positive potential in the olfactory epithelia.

V. *The normal olfactory epithelium stimulated with chloroform vapour ((Pls. 3A, B and 4A).*

The most remarkable findings were obtained in this epithelium.

The supporting cell. Protrusion en masse of the apical part was found in each cell, showing a powerful secretory activity. In parallel with the increase

in concentration of the chloroform vapours, the grades of protrusion were generally increased (Pl. 3*A*, *B*). The most vigorous protrusions were found after the application of the original vapour (Pl. 4*A*). These findings also coincide with the increase in amplitude of the long-lasting positive potential (Pl. 1*A*: Takagi *et al.* 1969). In the sections made in parallel with the longitudinal axis of the supporting cell, the protrusions had shapes of Indian clubs, contained many secretory granules and were about 11 μm in height.



Text-fig. 1. Negative and positive EOGs. The negative and positive EOGs elicited in the normal olfactory epithelium are shown on the left and the ones elicited in the degenerated epithelium are shown on the right. It is clear that the negative EOGs are not elicited at all or very small artifactual potentials are produced in the degenerated epithelium. The negative off-EOG elicited by chloroform (Chl) vapour also disappears in the degenerated epithelium and only a vestige is seen as indicated by an arrow (bottom records). AA, amyl acetate. The horizontal bars at the bottom indicate 4 sec.

The mucus layer. The well organized mucus layer which was observed in the normal epithelium was not found, but the thickness of this layer increased considerably. Serial sections were made from the olfactory epithelia fixed 1 min after the chloroform application in various concentrations (0·1 M, 0·5 M, and original), and continuity was examined between the protruded parts and their maternal cell bodies by means of a light microscope. It was found that the heights of the protrusions were always less than 15 μm, as long as the continuity was found, and that the protruded parts which were situated higher than 20 μm never had continued connexions with their maternal cells, but floated freely in the mucus (Pl. 5). However, even the protruded parts which were found situated 5 μm high, frequently showed discontinuity with the supporting cells (Pl. 7*A*). Hereafter the protruded parts without connections are called secretory droplets. Thus, the finding of the droplets can be interpreted as indicating that the protrusion is the initial process of the secretion. The second process of the secretion is shown in Pl. 7*B*. It is seen that the protruded parts are going to detach from their maternal supporting cells. Many stages of this sort of detachment were found.

The secretory droplets were examined by means of an electron-microscope. The olfactory epithelia were also fixed 2, and 3 min after the application of chloroform vapours in three concentrations. In the mucus layer, many stages of disintegrations and dissolutions were found. In Pl. 6, for instance, secretory granules G show that the outer limiting membranes of the protruded parts disappeared at first and the clear secretory granules are floating in the mucus. In contrast, the secretory granules G′ look like ghosts and the outer limiting membrane can still be traced in some cases (Pl. 6). Pl. 7*A* shows that the membrane which had surrounded a droplet began to disintegrate partly as indicated by arrows and that the secretory granules inside the droplet eventually dissolved into the mucus. In the epithelia fixed 2 or 3 min after the application, protrusions were still found in some preparations, but in others partial or full recovery to the normal appearance was found.

Under the epithelial surface, it was found that the bodies of the supporting cells thinned down, and there were many clear intercellular spaces which had not been apparent previously (Pl. 7*B*). These pictures show that the protrusions are not produced by the swelling of the surrounding cells.

The olfactory cell. Because of the protrusions of the supporting cells, the olfactory vesicles were seen as if they were hidden in the bottom of the valleys. The olfactory cilia extended straight upwards from the bottom.

EOG. It has been shown that in the normal olfactory epithelium a long-lasting positive potential with or without a negative off-potential appears in response to chloroform vapours in three concentrations (Text-fig. 1; Takagi *et al.* 1966, 1969).

VI. *The degenerated olfactory epithelium stimulated with chloroform vapour* (*Pl.* 4*B*).

The supporting cell. Striking protrusion of the apical part of this cell was found. They were about 12 μm in height. In this respect, the protrusions in the degenerated epithelium resembled the ones in the normal epithelium. The only difference was the shapes of the protrusions. In contrast to the shapes of the Indian clubs in the normal tissue, the protrusions in the degenerated ones sometimes had thin, irregularly elongated, and columnar shapes as seen in Pl. 4*B*. The number of the secretory granules was often found to be smaller in degenerated epithelia.

EOG. It was shown before that chloroform elicits a long-lasting positive potential in the degenerated olfactory epithelium (Text-fig. 1; Takagi *et al.* 1969). Since this positive potential was devoid of the negative off-potential, it was generally difficult to discriminate the positive on- and after-potential. In this case, we simply termed it a long-lasting positive potential. How-

ever, there was sometimes a step which indicated a junction between the positive on- and after-potentials (Text-fig. 1).

The findings in V and VI show that chloroform vapours produce both vigorous secretion in the supporting cell and the long-lasting positive potential in the olfactory epithelium.

VII. *The effect of Cl^--free Ringer solution in the normal olfactory epithelium*

Takagi *et al.* (1966) showed that the positive EOG in response to chloroform vapour is not seen, when the olfactory epithelium is immersed in a modified Ringer solution in which Cl^- is replaced by SO_4^{2-} or propionate$^-$.

The electron-microscope showed that chloroform vapour produces practically no secretion of the supporting cell in the olfactory epithelium which has been treated in Cl^--free Ringer solutions (Pl. 8*A*, *B*). On the contrary, even a slight indentation of the surface was evident in the supporting cell, as seen in Pl. 8*B*. This finding indicates that in the case when no positive potential is seen, no secretion occurs in the supporting cell.

VIII. *The effect of Ba^{2+} upon the olfactory epithelium*

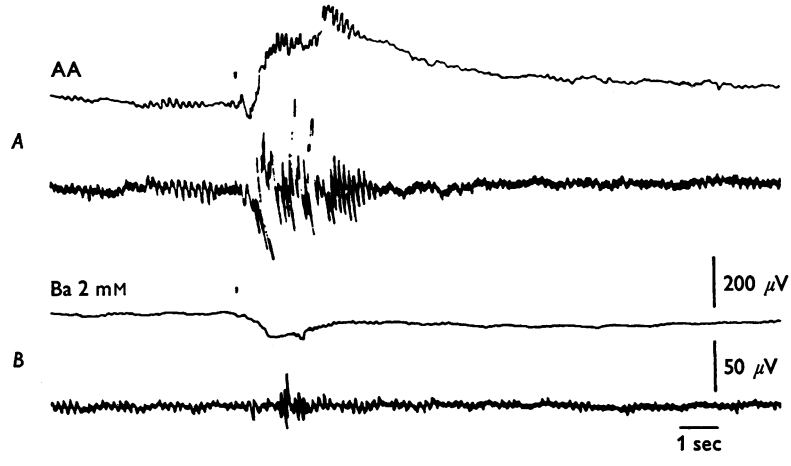
When chloroform vapour is applied to the olfactory epithelium which has been immersed in a Ringer solution containing 1–2 mM-Ba^{2+}, the positive potential is much reduced in size. It is known that Ba^{2+} has a depressing action upon the positive EOGs and that such action is due to the selective blocking of the Cl^- influx in the positive EOG producing membrane (Takagi *et al.* 1968). However, both the histological and potential changes caused by this Ba^{2+} solution *per se* have never been studied.

The electron-microscope disclosed that in response to the Ba^{2+} solution, the supporting cells protrude their apical parts containing secretory granules (Pl. 9*A*). The protrusions have the shape of Indian clubs and closely resemble those produced by chloroform vapour. However, an important difference from the findings in the normal olfactory epithelia is that the surface of the (Indian club-like) protrusions were not smooth, but bumpy due to the cytoplasmic projections of many secretory granules. Often disintegration and dissolution into the amorphous substances of a part of secretory granules were seen in the protruded cytoplasm (Pl. 9*A*). Since normal Ringer solution does not produce such a phenomenon, the above findings can be attributed to the effect of Ba^{2+}.

Since the solution flowed over the olfactory epithelia, to record the EOG the recording electrode was placed on the olfactory nerve as close to the epithelia as possible. This permitted observation of both the electronically conducted EOG from the olfactory cells and the extrinsic EOG originated from the supporting cells. Ba^{2+} solution elicited a positive potential (Text-

fig. 2). However, it was found to be relatively small when compared with the negative potential elicited by amyl acetate solution. The generative mechanism of the small positive potential will be explained in the Discussion (3).

Next, chloroform vapour was applied onto the olfactory epithelium which had been pre-treated with the Ba^{2+} solution. Many secretory granules inside the Indian club-like protrusions which had been elicited by Ba^{2+}, disrupted and almost disappeared (Pl. 9*B*). When compared with Pl. 9*A*,



Text-fig. 2. EOGs elicited by amyl acetate and Ba^{2+}. In *A*, it is found that a large negative EOG was elicited by a drop of 0·5 M amyl acetate solution (top record) and that it was accompanied by remarkably induced waves in the olfactory bulb (bottom record). In *B*, it is observed that a positive EOG was produced by a drop of Ringer solution containing 2 mM-Ba^{2+}, accompanied by small induced waves in the olfactory bulb. The small induced waves are supposed to be due to the stimulative effect of Ba^{2+} upon the olfactory cells. Small dots above each record indicate dripping of the solutions. Further explanation are found in the text.

it is seen that application of chloroform vapour promotes disintegration of the granules already secreted, but does not effect protrusion of new secretory granules. In conforming to the work of Takagi and his associates (1968), application of chloroform vapour to Ba^{2+} pre-treated olfactory epithelium no longer elicited the positive potential.

From these studies, it is clear that a long-lasting positive potential is also elicited by the application of Ba^{2+}, and it is always accompanied by the release of the secretory granules, but that the application of chloroform vapour after Ba^{2+} produces neither further secretion nor a positive potential.

DISCUSSION

(1) *Changes in the supporting cell after sectioning the olfactory nerve*

The retrograde degeneration of the olfactory cell after the sectioning of the olfactory nerve or the removal of the olfactory bulb has been studied by many workers (Nagahara, 1940; Clark & Le Gros, 1957; Sen Gupta, 1967; Takagi & Yajima, 1964, 1965; Andres, 1965, 1966; cf. Takagi, 1971 for further reference). However, no one has described the various changes in the supporting cell after nerve sectioning.

In the present electron-microscopical study, marked changes were evident in the supporting cell (cf. Result II). They seem very significant, because the supporting cell belongs to the ependymal cell and hence is supposed to be a kind of glial cell. A close relationship between the sensory neurone and the glial cell has been studied in the retina by Svaetichin, Negishi, Fatehchand, Drujan & Selvin De Testa (1965). The changes found in the cytoplasm of the supporting cell after the degeneration of the olfactory cell indicate a close relationship between the olfactory cell and the supporting cell.

(2) *Secretory function of the supporting cell*

In his pioneering observations of the olfactory epithelium with electron-microscopy, Bloom (1954) mentioned granules released from the supporting cells into the surface mucous layer. Since then, secretion of granules from the supporting cells in many animals had been presumed by several investigators (Bronshtein & Ivanov, 1965; Reese, 1965; Frisch, 1967; Graziadei, 1971). However, severe criticism has been centred on their presumption. Their criticism maintains that simple observation of the protrusion of a part of the supporting cell does not mean secretion, and also that it should be proved by the technique of serial sections as to whether or not the protruded parts are detached from their maternal supporting cells and eventually lead to disintegration and dissolution of the secretory granules. In response to such criticism, a considerable part of the present research was directed to clarify the secretory process of the supporting cell.

In the normal non-stimulated olfactory epithelium, there were found small pit-like structures which suggested the occasional release of secretory granules from the surface membrane of the supporting cells (arrow 2 in Pl. 1 *A*). Consequently, it is supposed that even in the non-stimulated state the olfactory epithelium at times releases the secretory granules in small quantities into the mucus layer in accordance with microapocrine secretion (Kurosumi, 1961). In fact, such a secretory process is suggested by the arrow 3 in Pl. 1. On the other hand, the apical portion of the supporting

cell, full of secretory granules, is projected en masse when chloroform vapour is applied (Pls. 3 and 4*A*). This corresponds to macroapocrine secretion (Kurosumi, 1961). These phenomena remind us of the neuromuscular junction, where acetylcholine is constantly and randomly released in small quantities in the resting state, but it is released in large quantities at one time when an impulse reaches the junction.

The Indian club-like projections are surrounded by the original cell membranes in the beginning. It seems so even when the projected parts are freed from their maternal cells. Later, in olfactory epithelia fixed 1–3 min after application of the chloroform, dissolution of the cell membrane was often observed, freeing the secretory granules into the mucus where they disintegrated. In other cases, the granules seemed to disintegrate into amorphous substances which were later freed into the mucus. These detached secretory processes were also found in droplets situated more than 20 μm away that had no continued connexion with the supporting cells. Thus, the secretion of the supporting cell was fully proved with the aid of the electron-microscope.

(3) *Electrogenesis and secretion of the supporting cell*

The normal olfactory epithelia which were fixed instantly while they were eliciting the positive potentials in response to chloroform and t-butyl alcohol, showed vigorous secretory activities of the supporting cells. On the contrary, the normal olfactory epithelia which were fixed instantly while they were eliciting the negative EOGs in response to amyl acetate and *n*-butyl alcohol did not show any active secretion of the supporting cells. Next, the degenerated olfactory epithelia which were fixed instantly while they were eliciting the long-lasting positive potential in response to chloroform and t-butyl alcohol revealed remarkable secretory activities of the supporting cells. On the contrary, the degenerated olfactory epithelia which were fixed instantly while they were being stimulated with amyl acetate vapour, showed neither the EOGs nor noticeable secretory activities. These results indicate a close parallel relationship between the generation of the long-lasting positive potential and the secretory function of the supporting cell.

The olfactory epithelium which had been immersed in Cl^--free Ringer solution did not show any noticeable secretory activity of the supporting cell. It was proved before that practically no positive potential appears in this olfactory epithelium in response to chloroform vapour (Takagi *et al.* 1966). In contrast, in response to a Ringer solution containing 1–2 mM-Ba^{2+}, the olfactory epithelium showed both striking secretory activity of the supporting cell and a positive potential. Since it has been well known that Ba^{2+} is toxic to the excitable membrane, these phenomena can be

attributed to a direct action of Ba^{2+}. The relatively small amplitude of this positive potential (Text-fig. 2) can be explained by the loss of internal K^+ ions only (and possibly of other ions), because in this case the secretion is initiated by Ba^{2+} and this ion was already shown to selectively block the entry of the Cl^-, but not the exit of the K^+ in the positive EOG (Takagi *et al.* 1968). Consequently, the influx of the Cl^- ion in this case has nothing to do with the occurrence of both the positive potential and the secretory activity. Recording of the extrinsic EOG may also be one of the causes of the small amplitude.

The olfactory epithelium which has been pretreated with Ba^{2+}, did not show any positive potential in response to chloroform vapour. This can be explained by the fact that chloroform vapour did not elicit any new secretions from the supporting cells, but only disintegration and dissolution of the granules already liberated by Ba^{2+} (Pl. 9*B*). From all these results a close connexion between the secretion of the supporting cell and the generation of the long-lasting positive potential is evident.

(4) *Ionic mechanism of secretion in the supporting cell*

In 1966, Takagi *et al.* proved that the long-lasting positive potential depends primarily upon the influx of the Cl^- ion through the olfactory receptive membrane and that some contribution of the K^+ ion (and possibly of other ions) to the positive potential may be present. When these ionic mechanisms are considered with the above-mentioned close relationship, it is concluded that the long-lasting positive potential (or after-potential) elicited by the application of chloroform and other vapours is generated by the entry of the Cl^- ion through the surface membrane of the 'supporting cell', and that this entry of the Cl^- ion becomes a trigger for the secretory activity of the supporting cell, and thus secretory granules are excreted together with the internal K^+ ions.

In case of the sublingual gland, Lundberg (1957*a*, *b*) concluded that the secretory potential resulting from stimulation of the chorda nerve is mainly or solely due to an active transport of the Cl^- ion through the outer cell membrane into the gland cell. It is interesting that the positive potential of the supporting cell resembles the above chorda electrogram in that the transport of the Cl^- ion into the cell elicits the potential. In the positive EOG, however, the entry of the Cl^- ion was thought to be passive on the basis of pore size hypothesis (Takagi *et al.* 1966). From the results on the sublingual gland, it is conceivable that the entry of the chloride ion is partly or wholly due to the active transport mechanism initiated by the adsorption of the chloroform or other odorous molecules on the membrane of the supporting cell. In the present stage of our research, however, it remains a subject for future investigation.

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EXPLANATION OF PLATES

PLATE 1

Non-stimulated normal and degenerated olfactory epithelia.

A, normal olfactory epithelium: olfactory cells (OC) have olfactory vesicles (OV) and long olfactory cilia (CI). They are evenly interposed among the supporting cells (SUP) which are enriched with numerous microvilli (VI) and secretory granules (SG). There is seen a pit-like structure that suggests a vestige of liberated secretory granules (arrow 2). Crypt-like structure of unknown nature is seen on the surface of the mucus layer (arrow 1). Arrow 3, see Discussion (2). 2 per cent osmic acid fixation only. Uranyl acetate and lead citrate staining. × 6000.

B, degenerated olfactory epithelium: olfactory cells are not found, but the supporting cells (SUP) which are enriched with numerous secretory granules (SG) and provided with typical bushy microvilli (VI) remain. The surface of the epithelium is covered with a much shallower mucus layer than that of the normal epithelium in *A*. 2 per cent osmic acid and 2·5% glutaraldehyde fixation. Uranyl acetate and lead citrate staining. × 6000.

PLATE 2

Normal and degenerated olfactory epithelia stimulated with amyl acetate vapour.

A, normal olfactory epithelium: there is no special discovery in the olfactory vesicles (OV) with numerous long cilia (CI), and in the mucus layer. The supporting cells (SUP) also look normal. VI, microvilli. 2 per cent osmic acid and 2·5 per cent glutaraldehyde fixation. Uranyl acetate and lead citrate staining. × 6000.

B, degenerated olfactory epithelium without an olfactory cell. The supporting cells (SUP) are full of the secretory granules (SG), and are provided with typical microvilli (VI). The surface of the epithelium is covered with shallow mucus. 2 per cent osmic acid and 2·5 per cent glutaraldehyde fixation. Uranyl acetate and lead citrate staining. × 6000.

PLATE 3

Normal olfactory epithelia stimulated with diluted chloroform vapours.

A, stimulation with 0·1 M chloroform vapour. Protrusions in light grades of the secretory granules are observed. 2 per cent osmic acid and 2·5 per cent glutaraldehyde fixation. Uranyl acetate and lead citrate staining. × 6000.

B, stimulation with 0·5 M chloroform vapour. Protrusions in heavier grade of the secretory granules can be seen. 2 per cent osmic acid and 2·5 per cent glutaraldehyde fixation. × 6000. Abbreviations as before.

PLATE 4

Normal and degenerated olfactory epithelia stimulated with original chloroform vapour.

A, normal olfactory epithelium: notice the typical Indian club-like protrusions of the supporting cells (SUP). Microvilli (VI) are also seen among them. A cytoplasmic fragment with a few secretory granules (arrow) is found in the mucus layer. CI, olfactory cilia. SG, secretory granules. OV, olfactory vesicles. 6·5 per cent glutaraldehyde and 1 per cent osmic acid fixation. Uranyl acetate and lead citrate staining. × 6000.

B, degenerated olfactory epithelium: degenerated olfactory epithelium stimulated with chloroform vapour. The protrusions of the supporting cell (SUP) are columnar or irregularly elongated. Membranous debris (arrows) are scattered among the olfactory cilia (CI). OV, degenerating olfactory vesicles. SG, secretory granules. VI, microvilli. 6·5 per cent glutaraldehyde and 1 per cent osmic acid fixation. Uranyl acetate and lead citrate staining. × 6000.

PLATE 5

Light micrographs of serial sections of the normal olfactory epithelium which was stimulated with 0·5 M chloroform vapour.

The pictures *A* to *H* are sections of nos. 13, 14, 15, 17, 21, 22, 23 and 25 respectively from a series cut perpendicular to the surface of the epithelium. Micrographs of nos. 16, 18, 19, 20 and 24 are not shown here due to the limitation of the space. These pictures and Pl. 6 were shown here to prove that some of the droplets and secretory granules are really free from their maternal supporting cells.

The secretory droplets shown by SD are the clusters of numerous secretory granules and cellular organelles enveloped in the bags of the plasma membranes as shown in Pls. 6 and 7*A*. The thick arrows indicate that a droplet appears in *A*, becomes largest in *C* and disappears in *E*. The thin arrows show that another droplet appears in *B*, becomes largest in *D* and disappears in *H*. Thus, these two droplets are floating entirely freely and have no continued connexion with the supporting cells as seen in the bottom.

Staining with 0·5 per cent toluidine blue solution. A vertical bar shows 10 μm. × 1500.

PLATE 6

Normal olfactory epithelium stimulated with 0·5 M chloroform vapour and fixed 1 min after the stimulation. Various stages of the disintegration of the secretory droplets (SD 1, 2, 3 and 4) and granules (G and G′) are shown. Secretory droplet 1 is almost liquified into the surrounding mucus and no granule is seen except the ghosts (G′) of the secretory granules. It appears that the secretory droplet 4 is becoming detached from the supporting cell. This is supposed to be the second process of secretion. The droplet 4 also indicates that a part of the membrane disrupted and disintegration and dissolution of this droplet has already started. Further explanation in the text. Cl, olfactory cilia. G, secretory granules. G′, ghosts of the secretory granules. OC, olfactory cells. OV, olfactory vesicle. SD, secretory droplets. SUP, supporting cells. VI, microvilli. 2 per cent osmic acid and 2·5 per cent glutaraldehyde fixation. Uranyl acetate and lead citrate staining. × 6000.

PLATE 7

Normal olfactory epithelium fixed 1 min after stimulation. *A*, disintegration of the secretory granules. Many of the secretory granules are disrupting. The membrane detached from the maternal supporting cell still enclose these granules except the parts indicated by arrows. In the indicated parts, the contents of the granules are flowing into the surrounding mucus. Stimulation with original chloroform vapour. CI, olfactory cilia. OV, olfactory vesicles. 2 per cent osmic acid and 2·5 per cent glutaraldehyde fixation. Uranyl acetate and lead citrate staining. × 12,000.

B, normal olfactory epithelium stimulated with 0·5 M chloroform vapour.

Notice the remarkably wide intercellular space, as indicated by the arrows, while protrusions are in progress. The two protrusions on the right are going to part from their maternal supporting cells. This is supposed to be the second process of the secretion. OC, olfactory cell. SUP, the supporting cell. 2 per cent osmic acid and 2·5 per cent glutaraldehyde fixation. Uranyl acetate and lead citrate staining. × 6000.

PLATE 8

Normal olfactory epithelium pre-treated with Cl^--free Ringer solution and stimulated with chloroform vapour.

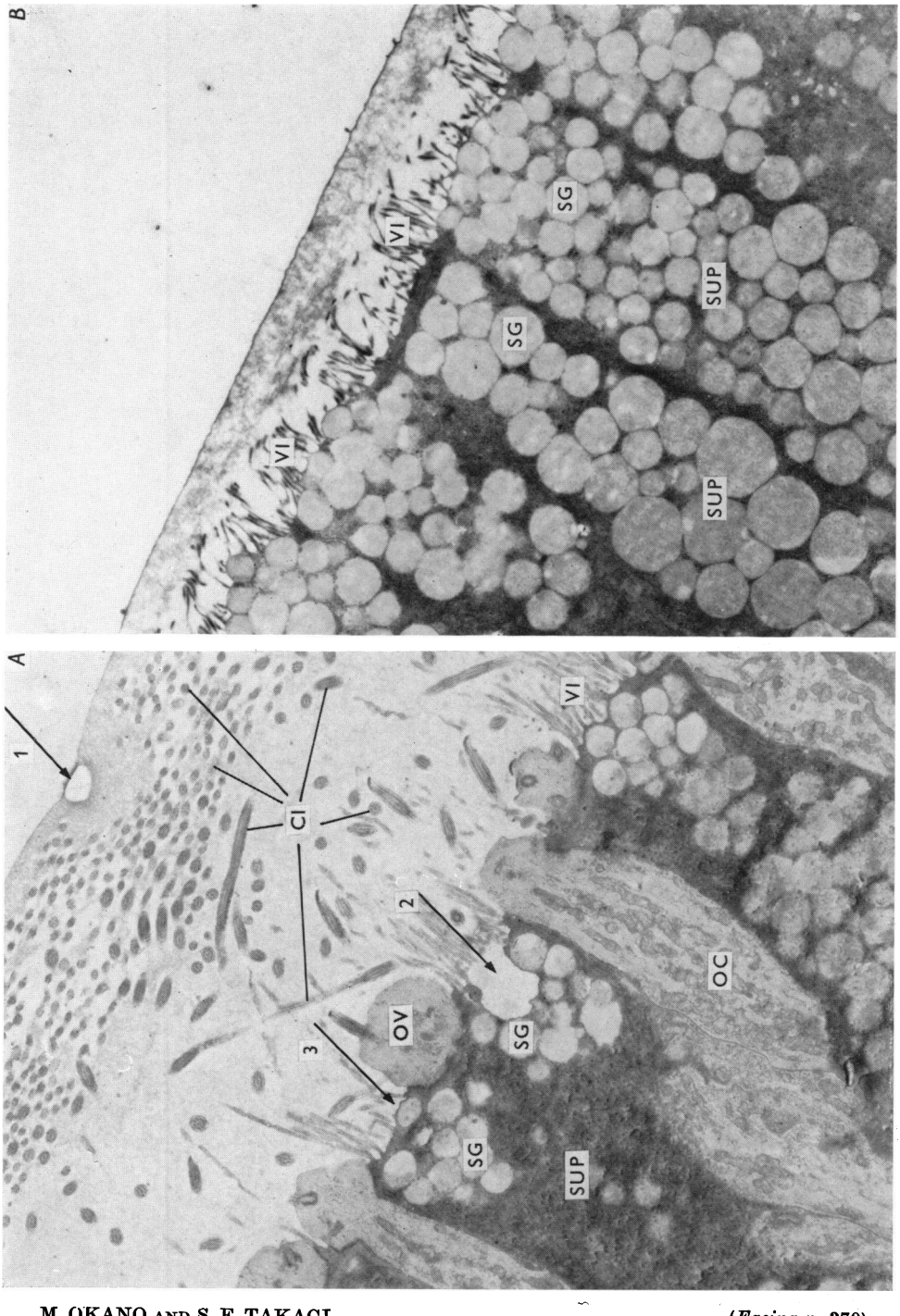
A and *B* are the same epithelium. In *A*, magnification is × 6000 and in *B*, × 12,000. It is noteworthy that the free surfaces of the supporting cells are just flat or even recessed. This sort of dent can not be found in the untreated epithelia and looks very unnatural. Besides, in spite of stimulation with original chloroform vapour no protrusion of the supporting cell is observed. OV, olfactory vesicles. SUP, supporting cells. 2 per cent osmic acid and 2·5 per cent glutaraldehyde fixation. Uranyl acetate and lead citrate staining.

PLATE 9

Normal olfactory epithelia affected by Ba^{2+}.

A, dripping of Ba^{2+} elicit remarkable Indian club-like protrusions from the supporting cells (SUP). Note the bumpy contours of these cytoplasms in contrast with the smooth ones in case of chloroform stimulation (Pl. 4*A*). In the apical part of the protrusion, the secretory granules are disintegrating. CI, olfactory cilia. OC, olfactory cells. 2 per cent osmic acid and 4 per cent glutaraldehyde fixation. Uranyl acetate and lead citrate staining. × 6000.

B, normal olfactory epithelium pre-treated with Ba^{2+} and later stimulated with original chloroform vapour. When compared with *A*, it is found that chloroform vapour facilitated the disintegration of the secretory granules in the protruded cytoplasms. In the apical and middle part of the protrusions the granules completely disappeared while in the basal part they still remain. CI, olfactory cilia. OV, olfactory vesicles. SUP, supporting cells. 2 per cent osmic acid and 4 per cent glutaraldehyde fixation. Uranyl acetate and lead citrate staining. × 6000.



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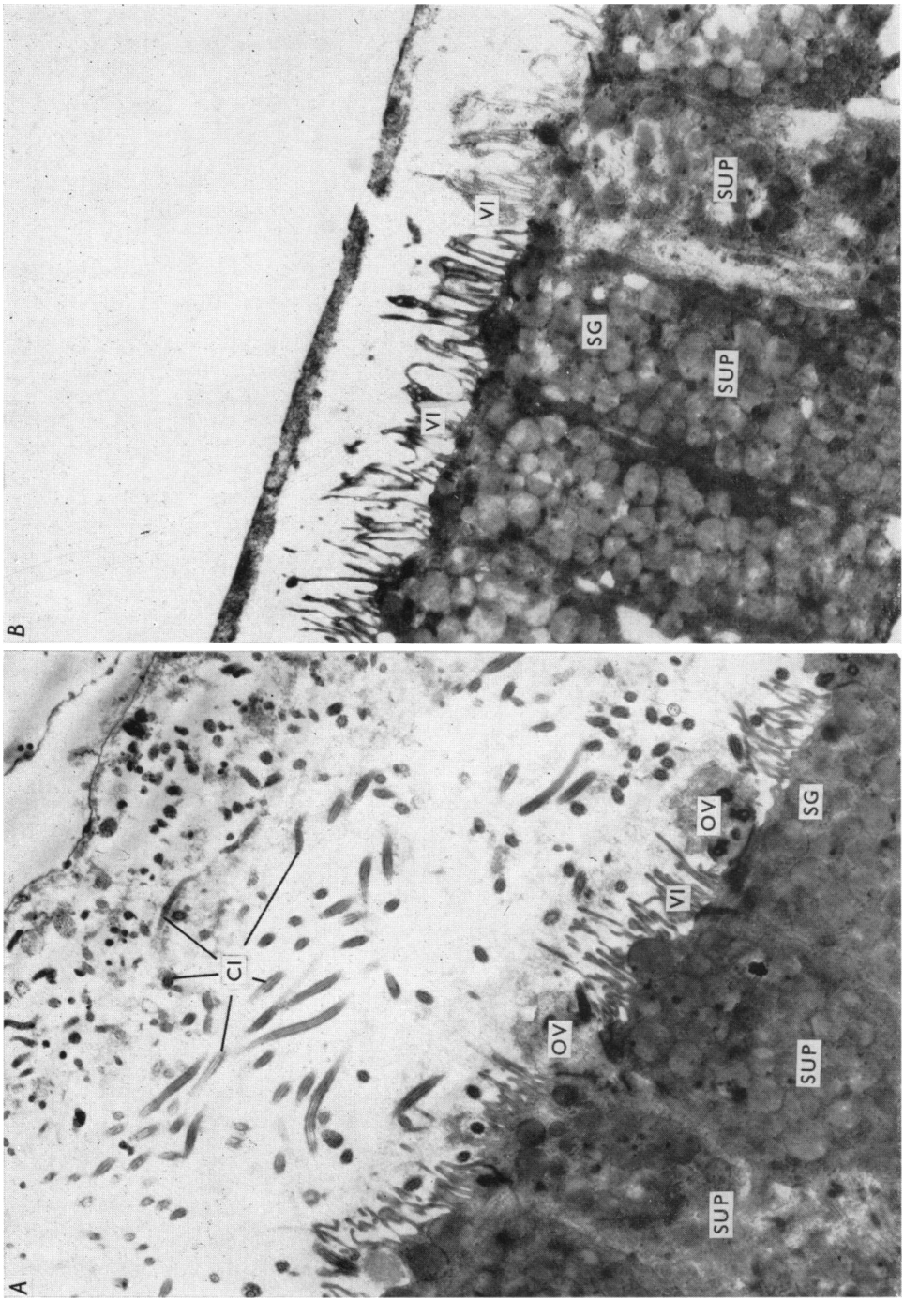

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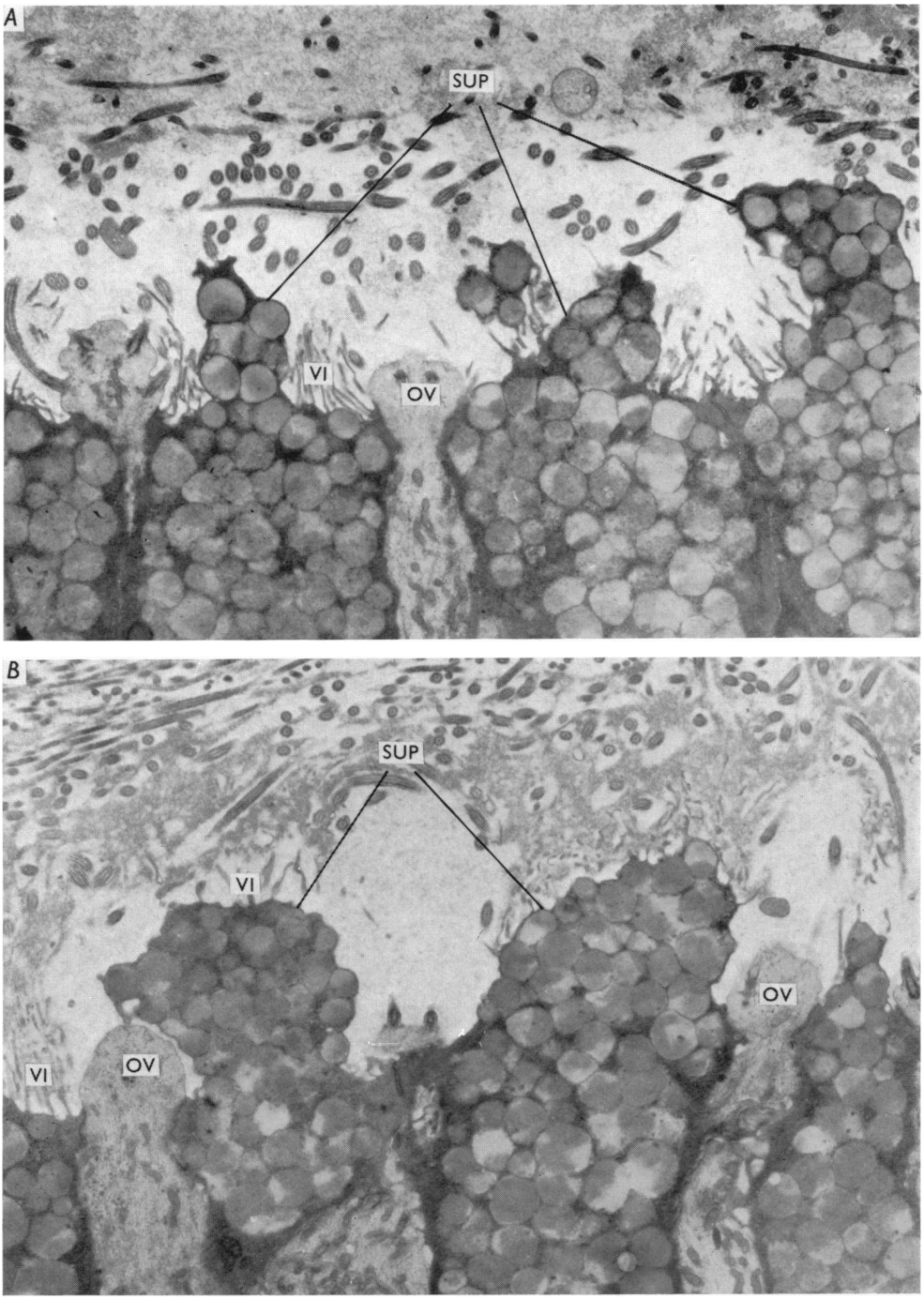
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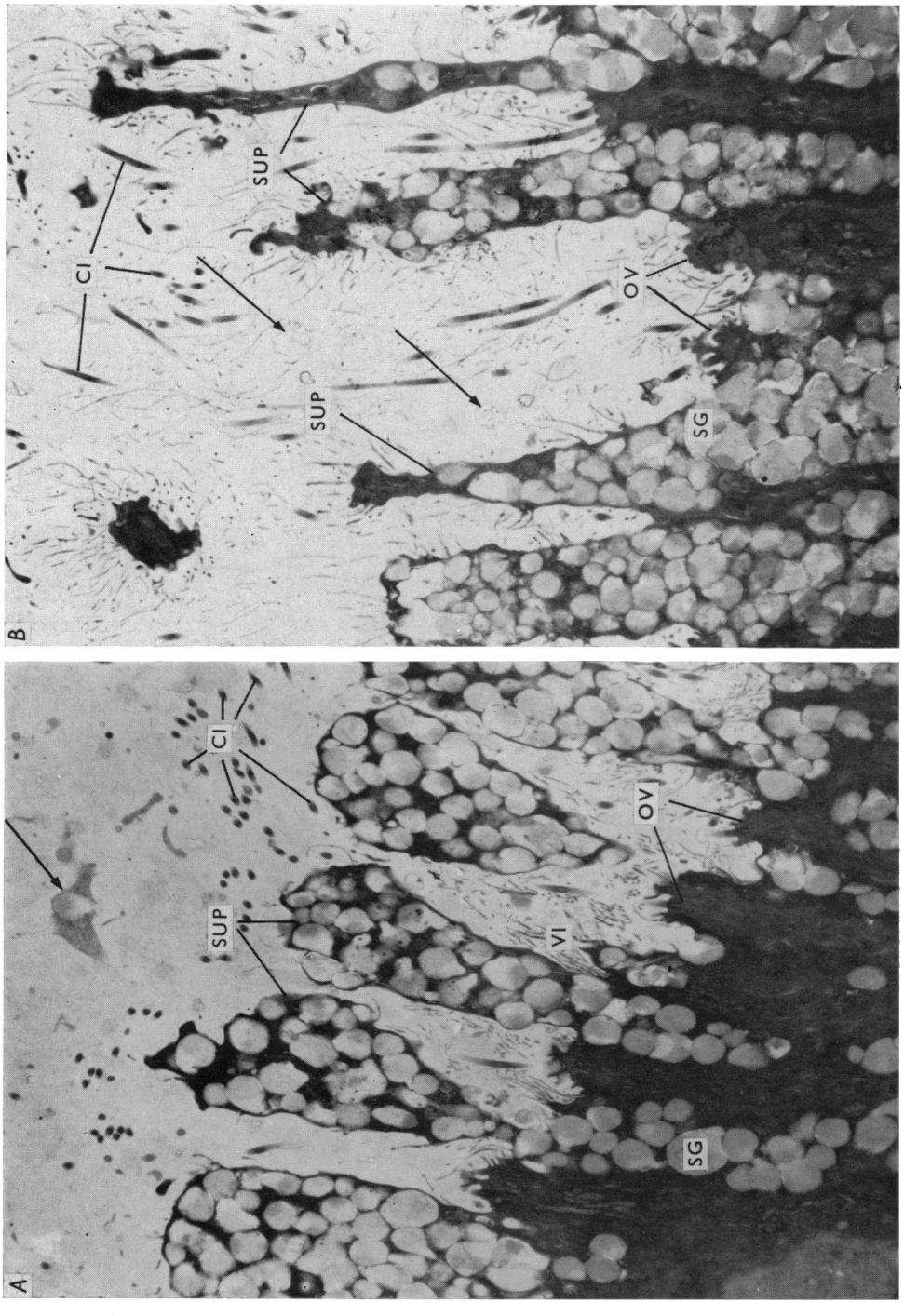
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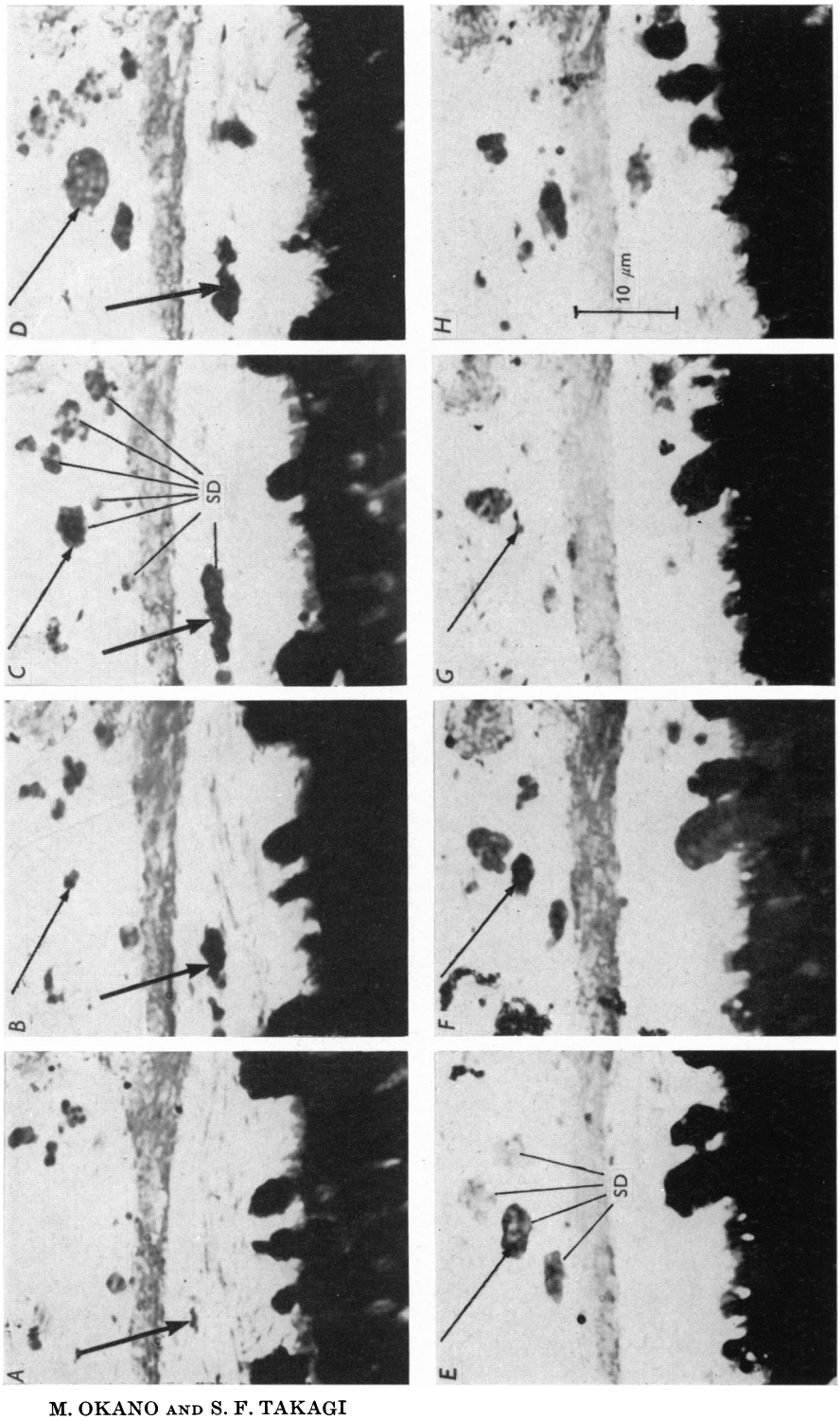
A
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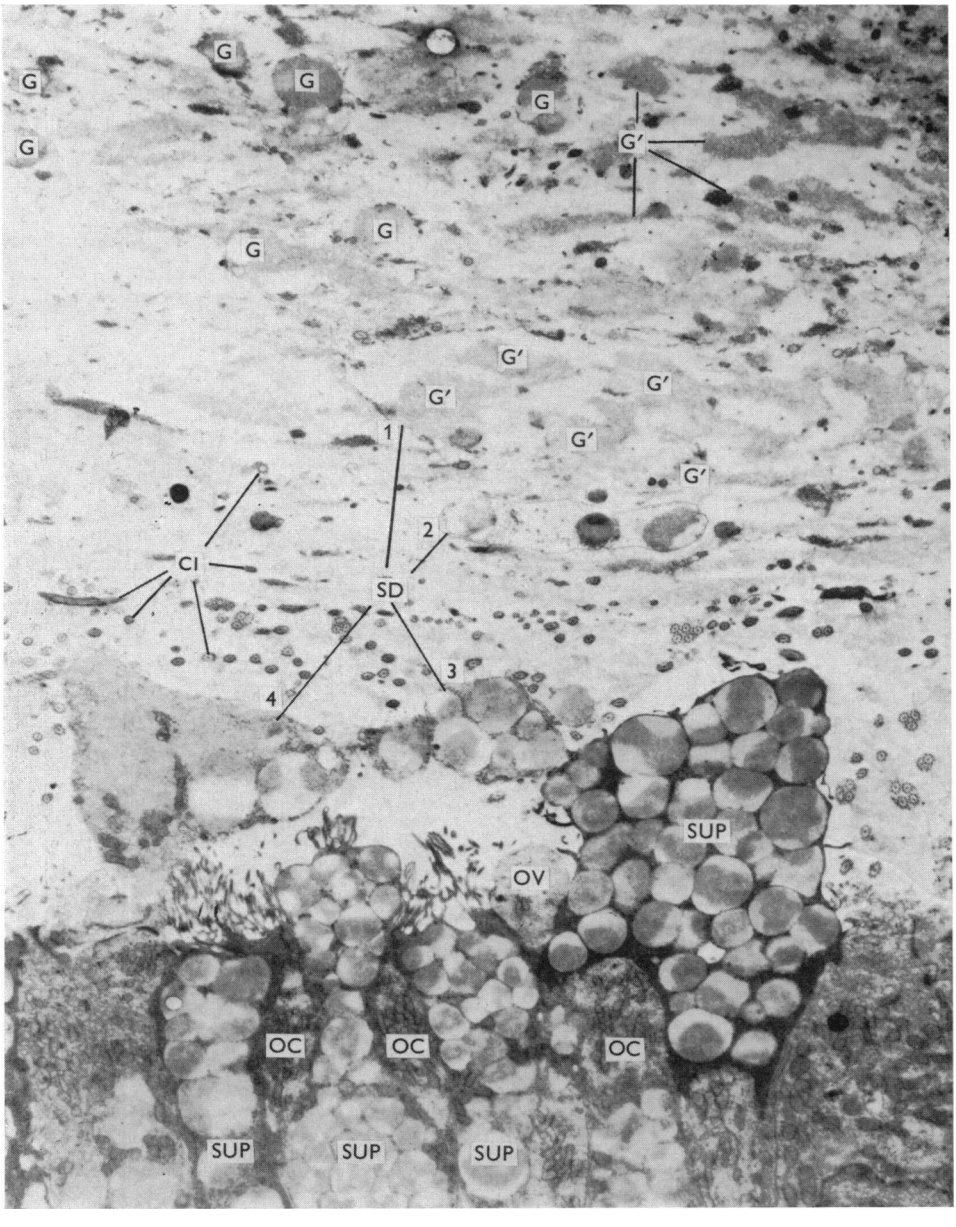
10 μm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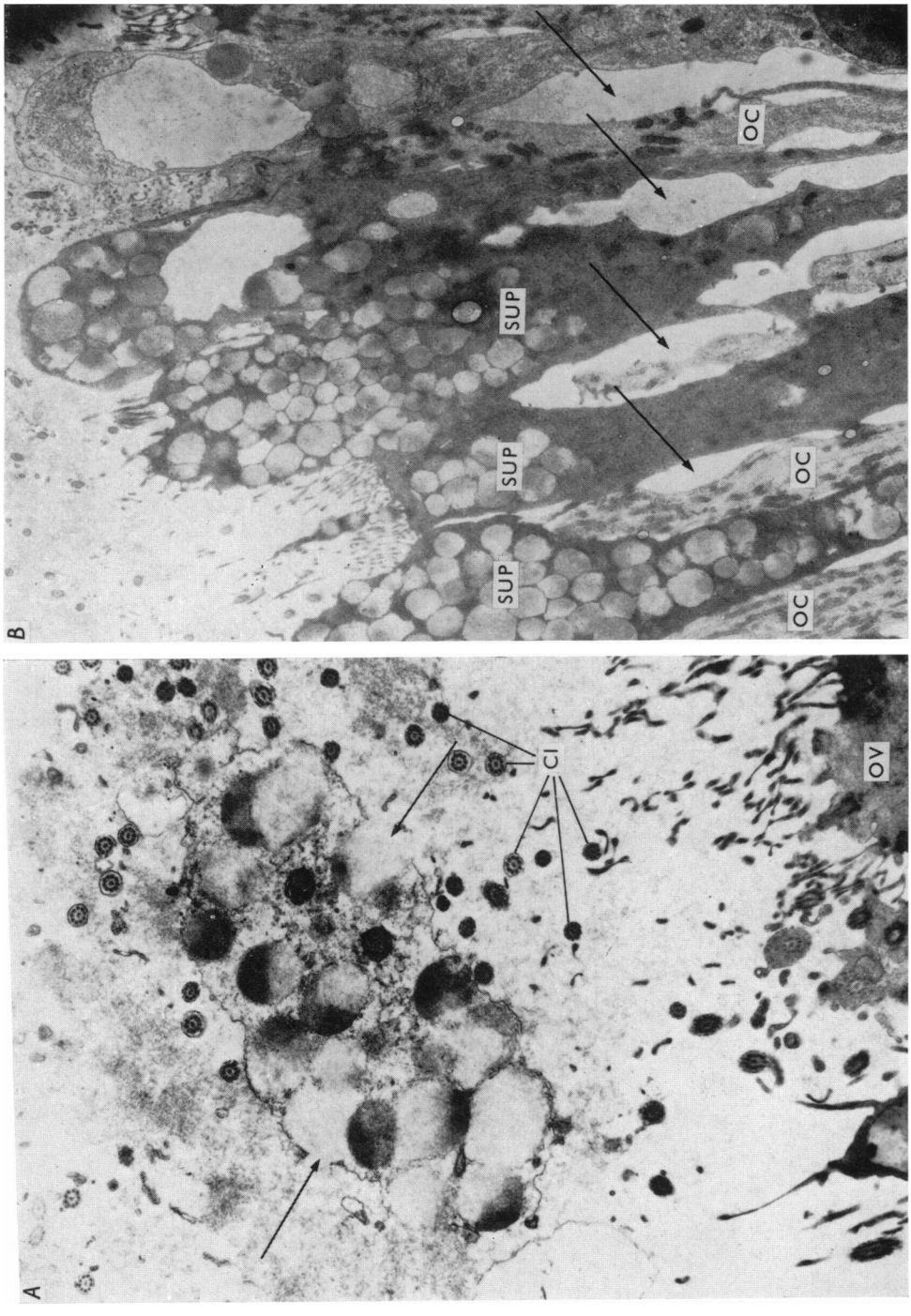
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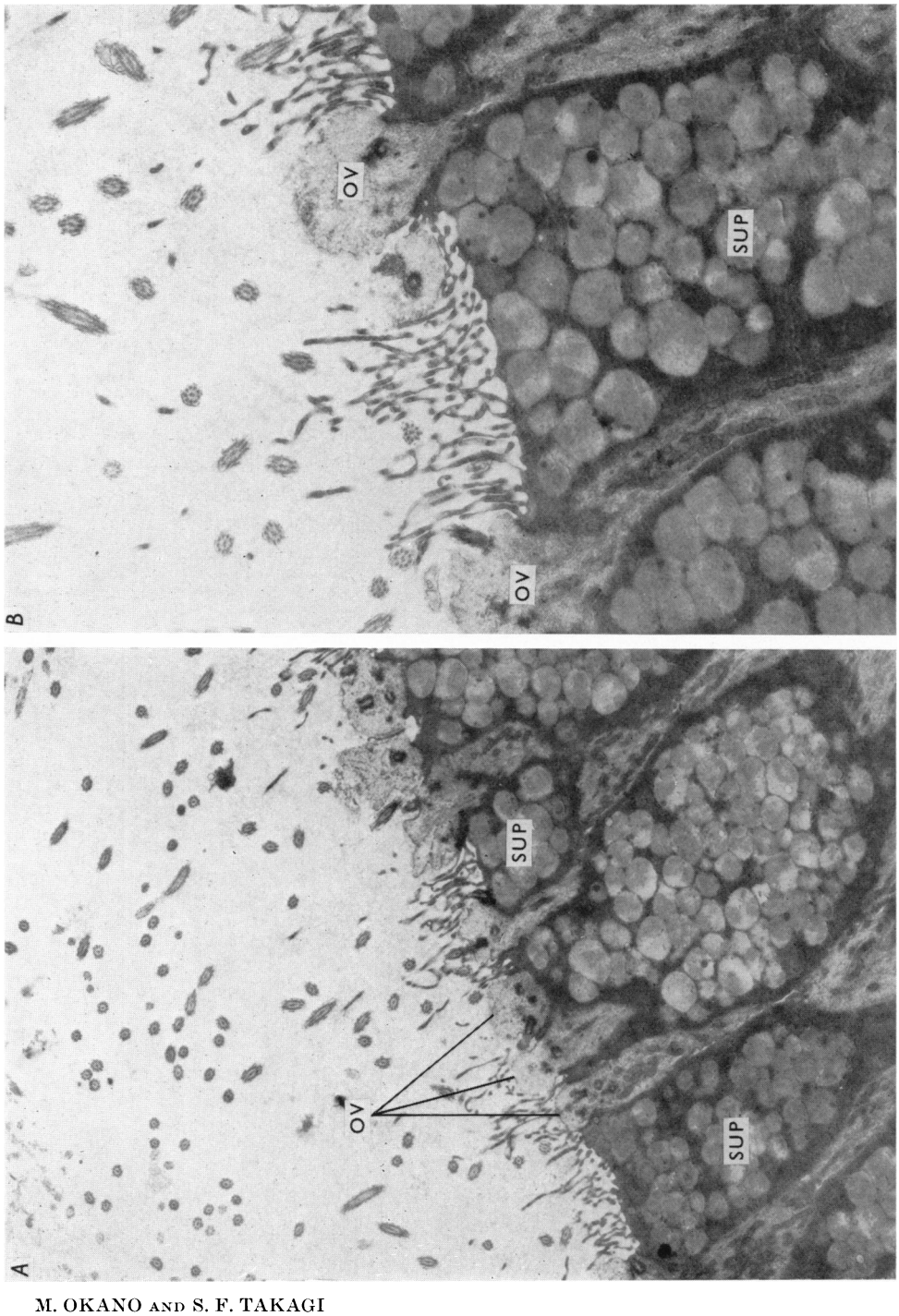

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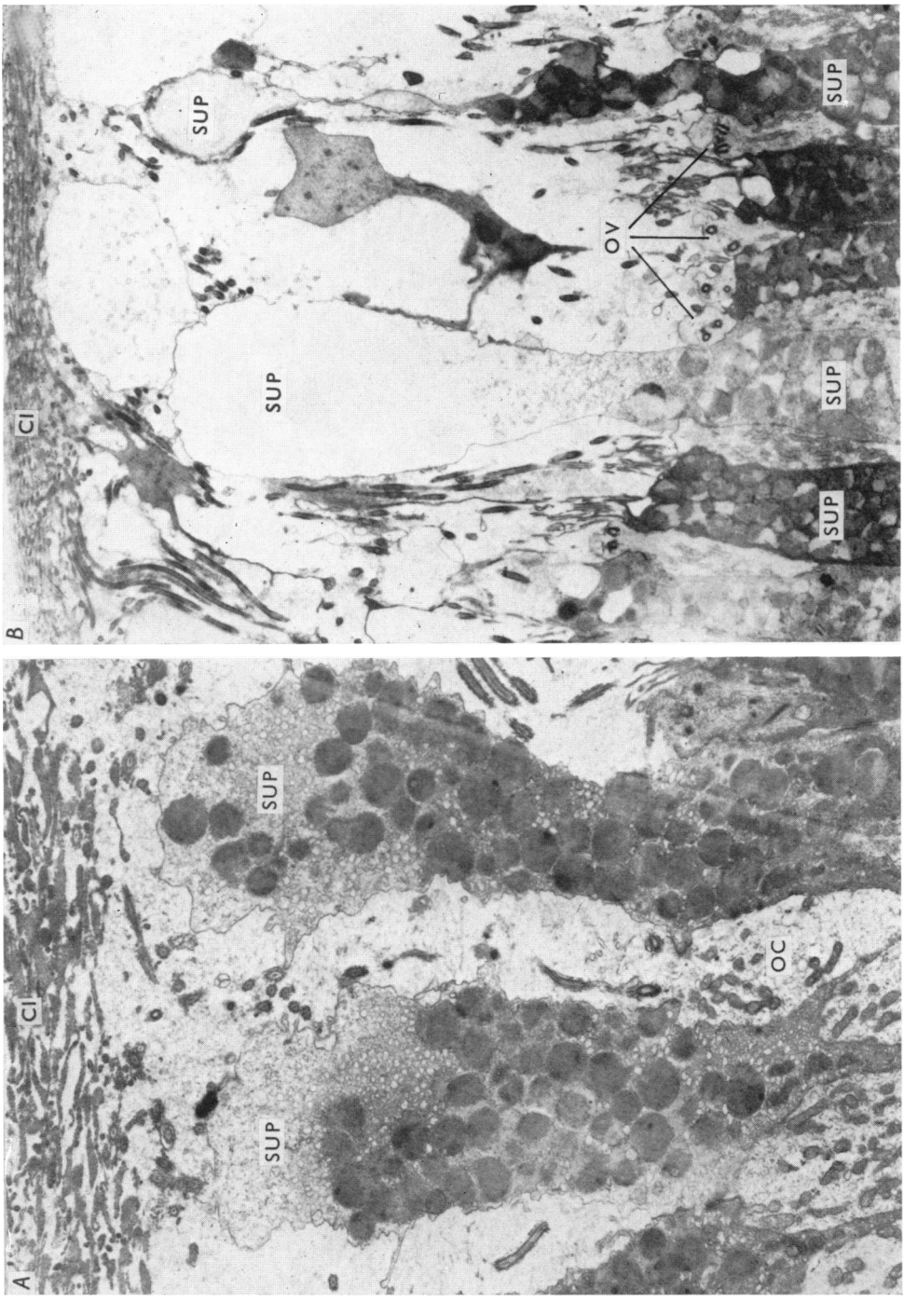

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