ADENOSINE TRIPHOSPHATASE LOCALIZATION IN AMPHIBIAN EPIDERMIS

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ABSTRACT

The localization of ATPase[1] activity has been studied by light and electron microscopy in the epidermis of *Rana pipiens*, *Rana catesbiana*, and *Bufo marinus*. The reaction was carried out on skin (glutaraldehyde-fixed or fresh) sectioned with or without freezing. Best results were obtained with nonfrozen sections of fixed tissue. The incubation mixture was either a Wachstein-Meisel medium, or a modification which approximates assay systems used in biochemical studies of transport ATPases. The reaction product was found localized in contact with the outer leaflet of all cell membranes facing the labyrinth of intercellular spaces of the epidermis. It was absent from: (*a*) membrane areas involved in cell junctions (desmosomes, *zonulae* and *maculae occludentes*); (*b*) cell membranes facing the external medium (i.e., those on the distal aspect of the ultimate cell layer in *s. corneum*); (*c*) cell membranes facing the dermis (those on the proximal aspect of cells in *s. germinativum*). In the presence of ($Na^+ + K^+$) the localization did not change, but the reaction was not appreciably activated. A similar though less intense reaction was obtained with ITP, but not with ADP, AMP, and GP as substrates. The results are discussed in relation to available data on transport ATPases in general, and on the morphology and physiology of amphibian skin in particular. Assuming that the ATPase studied is related to transport ATPase, the findings suggest a series of modifications to the frog skin model proposed by Koefoed-Johnsen and Ussing. The salient feature of this modified model is the localization of the Na^+ pump along all cell membranes facing the intercellular spaces of the epidermis.

The inward transport of Na^+ across the frog skin is an active process (1, 2) inhibited by cardiac glycosides (3, 4) and dependent on energy provided by oxidative metabolism (5, 6), presumably oxidative phosphorylation (7). The transport mechanism, usually referred to as "the Na^+ pump," has been tentatively localized by Koefoed-Johnsen and Ussing (8) on an "inward facing membrane" which they assume to be the cell membrane on the basal aspect of the *stratum germinativum* (*s. basale*) of the epidermis.

A membrane-bound ATPase[1] has been implicated in the active transport of ($Na^+ + K^+$) in a number of cells and organs, e.g., crab nerves (9, 10), mammalian erythrocytes (11, 12), kidney (13–17), brain (14, 18–21), muscle (22, 23), and many others (24–32). The implication is based on the finding that cation transport and membrane ATPase (sometimes referred to as "transport ATPase") have a number of features in common

[1] The abbreviations used in this paper are: ATPase, adenosine triphosphatase; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate, or 5′-adenylic acid; GP, Na β glycerophosphate; ITP, inosine triphosphate; ($Na^+ + K^+$), Na^+ and K^+ present and acting (or being transported) concomitantly; *s.*, *stratum*; Tris, tris(hydroxymethyl)aminomethane.

which have been discussed in detail by Skou (9, 10), Post et al. (11), and Dunham and Glynn (12). Prominent among these features are activation by K^+ in the presence of Mg^{2+} and Na^+, and inhibition by certain cardiac glycosides. A similar "transport ATPase" has been found in frog skin (14, 24, 33) where, as in several other cells and organs investigated, the amount of enzymatic activity can be correlated with the magnitude of cation fluxes (25).

Qualitative cytochemical techniques for the localization of a Mg^{2+}-activated ATPase in tissue sections at the subcellular level are now available (34, 35) and have already been applied to a number of organs specialized in active cation transport (34–39). We have used similar techniques to study the distribution of Mg^{2+}-activated ATPase in the epidermis of frog and toad skin.[2] For reasons given in the Discussion, we assume that this enzyme is involved in cation transport, although its exact relationship to "transport ATPase" is still debated (cf. 24, 41 and especially 42, 43). Our results indicate that ATPase activity is associated with all cell membranes facing the intercellular spaces of the epidermis. Hence, they do not support the original Koefoed-Johnsen-Ussing model (8). They yield, however, a more satisfactory picture of the functional organization of the epidermis, when integrated with our morphological findings (44) and with biochemical and physiological data recently obtained by Ussing (7), Koefoed-Johnsen (45), Hansen and Zerahn (46), Curran and Cerijido (47), and Chowdhury and Snell (48).

MATERIALS AND METHODS

Materials

The tissue studied was the abdominal skin of frogs (*Rana pipiens* and *Rana catesbiana*), and toads (*Bufo marinus*).

ATP, ITP, ADP, AMP, and GP were obtained as sodium salts from Sigma Chemical Company, St. Louis.

Preparation of Fixed Specimens

FIXATION: Skin strips (1 x 5 cm) were fixed at 4° in either 1.5% glutaraldehyde[3] in 0.067 M cacodylate buffer (49) for 2 to 4 hr, or Baker's formol-calcium (50) for 16 to 24 hr. After glutaraldehyde fixation, the strips were washed and stored at 4° in 0.1 M cacodylate buffer containing 7% sucrose; after formol-calcium, washing and storage were carried out in unbuffered 7% sucrose. Storage up to several weeks did not reduce ATPase activity.

SECTIONING: For enzyme tests, sections of either 10 μ or 50 μ were cut with a tissue sectioner (51, 52)[4] which avoids specimen freezing. For comparison, sections of similar thickness were cut with a Leitz freezing microtome.

INCUBATION: The sections were incubated for 10 to 90 min at 25° or 37°, in one of the following media:

(*a*) A Wachstein-Meisel medium (53) (0.83 mM ATP, 10 mM $MgSO_4$, 80 mM Tris-maleate buffer, pH 7.2) in which the Pb $(NO_3)_2$ concentration was reduced from 3.6 to 2.4 mM. This medium contains Na^+ at a concentration of ~82 mM, since it is prepared with the disodium salt of ATP, and NaOH is used to adjust the pH.

(*b*) A similar medium in which the cation content was adjusted to approach that used for biochemical assays by Skou (9, 10) and Post et al. (11) (5 mM ATP, 5 mM $MgSO_4$, 100 mM NaCl, 30 mM KCl, 2.4 mM $Pb(NO_3)_2$ and 80 mM Tris-maleate buffer, pH 7.2).

(*c*) Medium (*b*) to which 0.1 mM ouabain was added.

Control sections were incubated in (*a*) without Mg^{2+}, in (*a*) or (*b*) without ATP, and in (*a*) with ATP replaced by equimolar concentrations of ITP, ADP, AMP, or GP.

SUBSEQUENT PROCESSING: For light microscopy, the 10-μ sections were treated with 1% $(NH_4)_2S$ (to convert the reaction product, lead phosphate, into black lead sulfide), and mounted in glycerogel.

For electron microscopy, the 50-μ sections were rinsed in acetate-Veronal buffer (pH 7.4) containing 7% sucrose, fixed for 45 min in 1% OsO_4 in either the same buffer or 0.1 M phosphate buffer[5] (pH 7.6), dehydrated in graded ethanols, and embedded in Araldite. The reaction product is opaque enough to be visualized directly in the electron microscope, in specimens processed as described. Since contrast in cell membranes remains low in these preparations, some of the 50-μ sections were treated with 0.5% uranyl acetate in acetate-Veronal buffer for 1 to 2

[2] These results have been published in preliminary form in reference 40.

[3] The glutaraldehyde stock solution, obtained from Union Carbide Corp., New York, was purified by distillation before use, as described in reference 55.

[4] Initially a McIlwain Mechanical Tissue Chopper, in which the lower setting for section thickness is 50 μ, was used to cut nonfrozen sections; more recently, however, a modified instrument capable of cutting sections down to 10 μ was employed (52).

[5] Similar results were obtained with the two buffers, indicating that rinsing effectively washed away Pb^{2+} from the tissue.

hr prior to dehydration (44). This procedure also removed many of the nonspecific lead phosphate precipitates developed during incubation. If extended beyond 1 hr, it reduced the deposits of ATPase reaction product.

Thin sections (500 to 700 A) were cut with diamond knives on a Porter-Blum MT-2 microtome, mounted on carbon-coated grids, stained with uranyl acetate alone or doubly stained with uranyl and lead as previously described (44, 54), and examined in a Siemens Elmiskop I, operated with a double condenser and a 50-μ aperture in the objective. Micrographs were taken at original magnifications varying from 2,300 to 30,000.

Sections 1 to 2 μ thick were routinely cut from Araldite-embedded blocks, affixed to a glass slide, treated with 1% $(NH_4)_2S$, stained with crystal violet (cf. 55), and examined in the light microscope. Such preparations proved useful in monitoring the results of the cytochemical tests and in demonstrating the general distribution of reaction product in the whole skin.

Preparation of Fresh Specimens

Sections ~50 μ thick, cut with the tissue sectioner[4] from strips of fresh, unfixed frog and toad skin, were incubated immediately at 25°, for 15 to 60 min, in the media already described supplemented with 6% sucrose. Controls were run in the same media without ATP. After incubation, the sections were rinsed in 0.1 M Tris-maleate buffer, fixed in 1% OsO_4 in 0.1 M phosphate buffer for 90 min, and subsequently processed like aldehyde-fixed specimens.

OBSERVATIONS

Most of the work was carried out on frog skin (*Rana pipiens*). The best results, in terms of structural preservation and precise localization, were obtained with glutaraldehyde-fixed tissue, sectioned without freezing before incubation. Hence, the description that follows is based primarily on findings made on this type of material. ATPase localization was similar in formol-calcium fixed specimens, but structural preservation was less satisfactory. Observations on differently prepared specimens are only briefly mentioned.

Light Microscopy

Frog

FIXED SKIN, NONFROZEN SECTIONS: In 10-μ sections, black deposits of reaction product (lead sulfide) outlined the cells of the epidermis (keratinocytes), giving the appearance of an irregularly polygonal network of dark cement lines in a mosaic of light tiles (Fig. 1). The deposits were most abundant in the *s. spinosum* and *s. granulosum*, more sparse along the lateral aspects of the *s. germinativum*, and absent or only occasionally present on the outer and inner fronts of the entire epidermis. Dendritic cells (cf. 56) in the *s. germinativum* and *s. spinosum* generally gave a more intense reaction than keratinocytes.[6] The results were the same irrespective of the fixation or incubation procedure used. The presence of $(Na^+ + K^+)$, as in medium (*b*), did not modify the localization of the reaction product and did not increase the intensity of the reaction. Addition of ouabain, as in medium (*c*), did not noticeably depress the reaction.

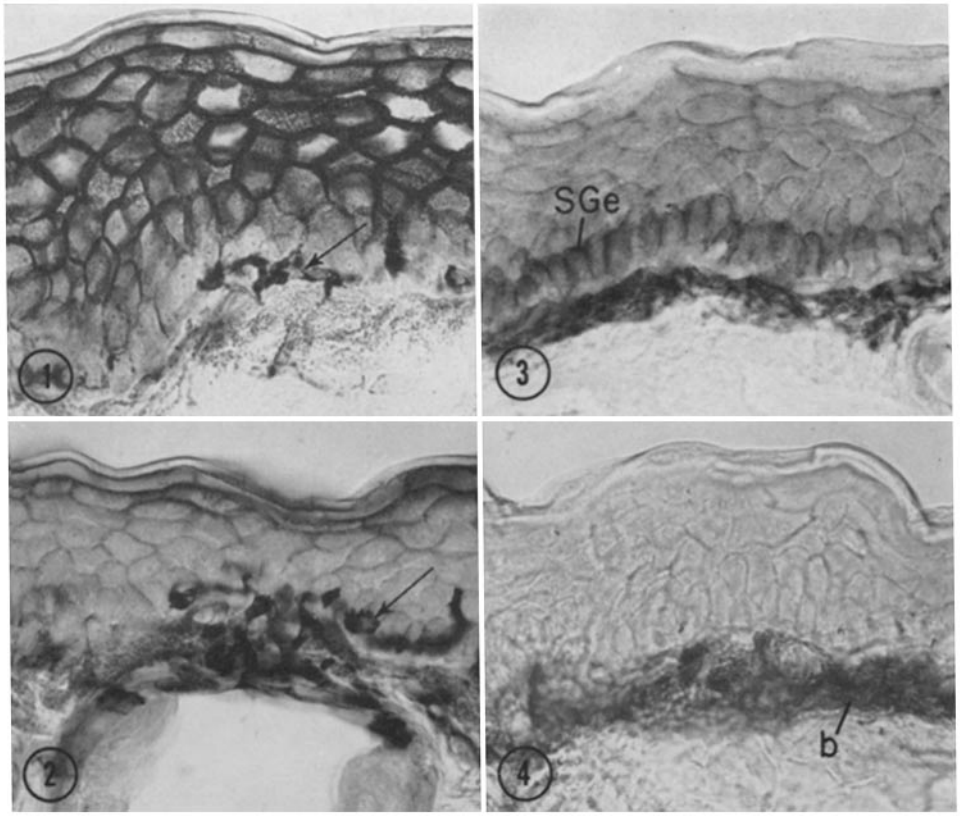
In all cases, the deposits of reaction product were restricted mainly to the outer epidermal strata after 30-min incubation at 37°; by 60 min, they were evident in all strata, and by 90 min appeared to outline completely the whole network of epidermal intercellular spaces. The restricted localization of the ATPase reaction product to cell surfaces and intercellular spaces appeared with particular clarity in 1- to 2-μ thick sections of specimens postfixed in OsO_4 and embedded in Araldite (Fig. 5).[7]

With ITP as substrate, the reaction was less intense, but the distribution of the reaction product was similar to that given by ATP. With ADP as substrate, reaction product appeared occasionally along the *s. corneum—s. granulosum* boundary after a 30-min incubation. After 90 min, such deposits were more frequent, a faint and variable reaction developed along all intercellular spaces, and an intense reaction was regularly detected along the dendritic cells of the deeper layers of the epidermis (Fig. 2). AMP and GP gave similar results only as far as the *s. corneum—s. granulosum* boundary was concerned. In addition, with AMP and occasionally with GP (Fig. 3), reaction product outlined a discontinuous series of cells in the *s. germinativum* after long incubation periods (90 min).

In the absence of substrate, no lead deposits were formed anywhere in the epidermis (Fig. 4).

[6] Such elements are known to give an intense reaction for ATPase in human epidermis (57–59).

[7] Such preparations also showed that ~50 μ is the maximum thickness at which sections give a uniform reaction in depth. When thicker than 50 μ, they react only at their periphery, irrespective of their preincubation history (fixed-nonfrozen, fixed-frozen, or fresh).



General Abbreviations

SC: *stratum corneum*
SG: *stratum granulosum*
SS: *stratum spinosum*
SGe: *stratum germinativum*
B: basement membrane
Co: collagen fibrils
D: dermis
De: dendritic cell
Is: intercellular space
Zo: *zonula occludens* (occluding zonule)
cm: cell membrane
d: desmosome
cd: composite desmosome
md: modified desmosome

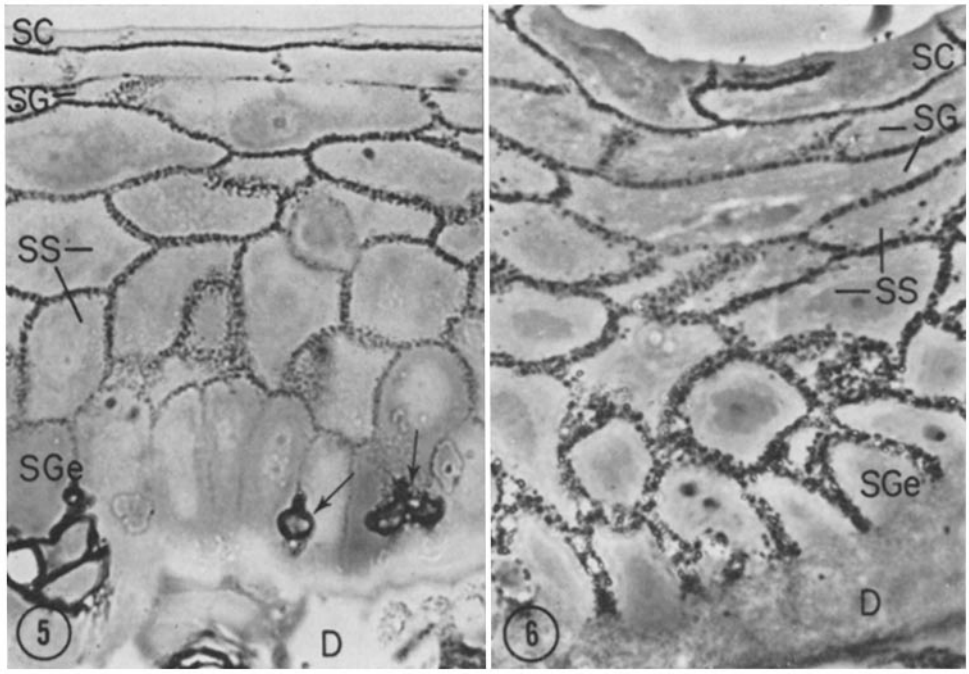
FIGURES 1 to 4 Photomicrographs of sections of frog skin incubated in Wachstein-Meisel media with different substrates. For the specimen in Fig. 1, the substrate was ATP. Deposits of ATPase reaction product (lead sulfide) outline the cells in black throughout the epidermis, presumably by filling the intercellular spaces. The deposits are most abundant in the middle layers (*s. spinosum* and *s. granulosum*) and more sparse in the basal layer, except for dendritic elements (arrow) which give an intense reaction.

In Fig. 2, with ADP as substrate, a moderate reaction is seen around the cornified cells and along the *s. corneum-s. granulosum* boundary; an intense reaction is present along dendritic cells (arrow) of the deeper layers. Cell boundaries in the rest of the epidermis show only a faint reaction.

In Fig. 3, incubated with GP, only the cells in the *s. germinativum* (*SGe*) are heavily outlined by reaction product. Most of the other intercellular boundaries are unreactive or show only a faint reaction. There is, however, a diffuse cytoplasmic staining of all epidermal cells (compare with Fig. 4).

In Fig. 4, a control preparation incubated without substrate, no lead deposits are seen anywhere in the epidermis. The broad black band (*b*) present in the dermis is formed by pigment cells and presumably by naturally occurring deposits of calcium phosphate.

Tissues fixed in glutaraldehyde; nonfrozen sections incubated for 1 to 1½ hr, treated with $(NH_4)_2S$, and mounted in glycerogel. × 375.



FIGURES 5 and 6 Photomicrographs showing the general distribution of ATPase reaction product in the frog (Fig. 5) and toad (Fig. 6) epidermis. In both cases, deposits of lead sulfide are restricted to cell boundaries, presumably intercellular spaces; none are present along the outer membrane of the cells of the cornified layers (*SC*) or along the basal membrane of the *s. germinativum* cells (*SGe*) which face the dermis (*D*). In the frog, the reaction is most intense in the middle layers (*SG* and *SS*) and faint or indiscernible along the lateral surfaces of the basal cells (*SGe*). In the deeper epidermal strata, only dendritic elements (arrows) are heavily outlined by reaction product. In the toad, the reaction is generally "displaced" toward the deeper epidermal strata: it is light in the upper layers (*SC* and *SG*) and heavy in the *s. spinosum* (*SS*) and along the distal and lateral surfaces of the basal cells (*SGe*).

Tissues fixed in glutaraldehyde, incubated in ATPase medium for 60 min, postfixed in OsO_4, and embedded in Araldite. 1-μ sections treated with $(NH_4)_2S$ and stained with crystal violet. Fig. 5 is a bright-field, and Fig. 6 a phase contrast photomicrograph. × 900.

Dense deposits occurred regularly, however, in the dermis, namely, in the layer of extracellular material between the *s. spongiosum* and the *s. compactum* (see Fig. 1 in reference 44) where they might reveal the presence of natural deposits of calcium (60), probably calcium phosphates (61). Less extensive deposits appeared in two other locations in the dermis: (1) in a broad subepithelial band in the *s. spongiosum*, and (2) in a band between the *s. compactum* and the *tela subcutanea*. The extent of these deposits is difficult to assess since at their level the dermis contains large populations of pigment cells.

In the absence of Mg^{2+}, only small amounts of reaction product were seen in the epidermis after 90-min incubation with ATP as substrate.

FIXED SKIN, FROZEN SECTIONS: In 10-μ frozen sections, the reaction was more diffuse and apparently slower than in nonfrozen specimens. Localized deposits of reaction product became evident only after 60-min incubation at 37°, and occurred not only along the intercellular spaces, but also within the cytoplasm at the periphery of the cells.

TOAD

In the epidermis of the toad skin, the ATPase reaction had the same general distribution as in the frog. It was, however, more intense: the intercellular spaces were already heavily outlined by reaction product after 30 min at 37°. In addition, the reaction was "displaced" towards the deeper

strata of the epidermis: it was light in the *s. corneum* and *s. granulosum* and heavy in the *s. spinosum* and along the distal and lateral aspects of the cells in *s. germinativum* (Fig. 6). As in the frog, little or no reaction product was found along the distal and proximal fronts of the epidermis.

Electron Microscopy

Frog

FIXED SKIN, NONFROZEN SECTIONS: *Morphology.* The organization of epidermal cells was in general satisfactorily preserved and showed few changes that could be ascribed to the incubation procedure. Blebs and small myelin figures, produced during glutaraldehyde fixation, were occasionally encountered along the cell surfaces, but otherwise the cell membranes appeared well preserved and clearly showed their typical stratified structure after staining in block with uranyl (44). All junctional elements characteristic of frog skin (44) remained unaffected by the incubation procedure.

Relationship of ATPase reaction product. The reaction product (lead phosphate) appeared in the form of relatively large (diam. = 200 to 700 A) globular deposits formed by aggregated fine particles (Figs. 7, 11 to 14). The size and frequency of these globules increased with incubation time. The majority of the deposits was found in the intercellular spaces in close contact with the cell membranes (Figs. 7 to 14). In uranyl-treated specimens, it was clear that they "grew" on the outer leaflet of the plasma membrane without disturbing the other layers of the structure (Figs. 13 and 14). This relationship was particularly evident in specimens incubated for short periods (10 to 20 min at 37°), in which the deposits also appeared more or less evenly spaced on the surface of the cell membrane. With longer incubation (30 to 60 min at 37°), the deposits increased in size, became irregular in shape, and, as a result, their relationship to the cell membrane was obscured. Many deposits appeared "free" in the intercellular spaces, presumably as a result of oblique sectioning (Fig. 10).

Little or no reaction product was found in the junctional elements of the epidermis, irrespective of type, i.e., desmosomes, or *zonulae* or *maculae occludentes* (Figs. 7, 8, and 10 to 12).

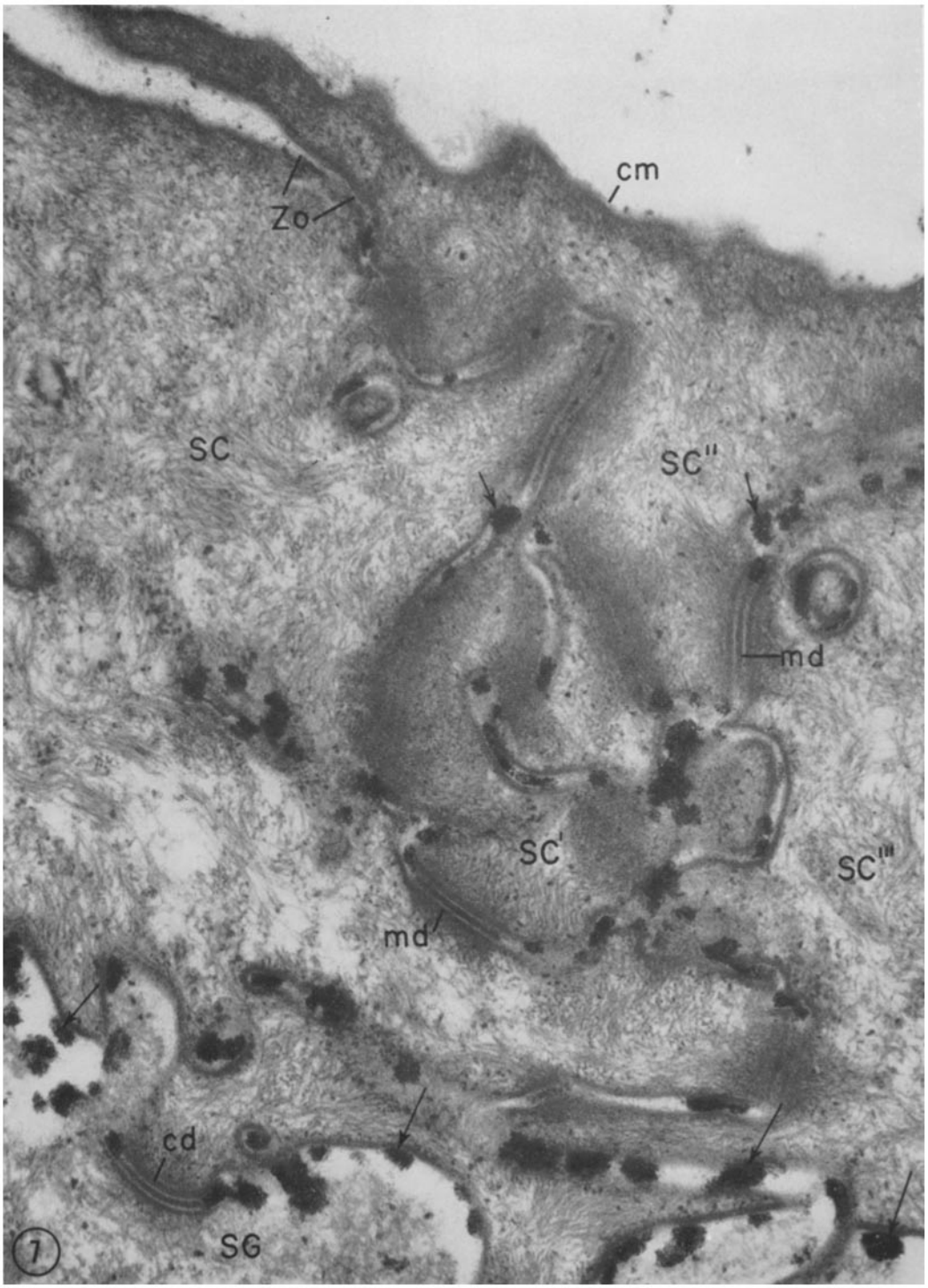
Distribution of ATPase reaction product within the epidermis. Electron microscope observations confirmed in detail and established, without reservations imposed by limited resolution, the distribution pattern found by light microscopy.

Up to 30-min incubation, there were few or no deposits on the cell membranes that form the distal front of the epidermis, i.e., on those parts of the plasma membrane of the cells in the outermost cornified layer that are in direct contact with the external medium (Fig. 7). Deposits appeared on the lateral aspects of the cells in this layer immediately proximal to the first row of occluding zonules (44) (Fig. 7) and occurred with increased frequency along the cell membranes facing the intercellular spaces of the *s. corneum* (Fig. 11), *s. granulosum*, and *s. spinosum* (Fig. 8). The deposits were more sparse on the lateral aspects of the cells in the *s. germinativum*, and were absent or only rarely encountered on the proximal aspects of the latter, i.e., on that part of the membrane of the *s. germinativum* cells that faces the dermis and is covered by the basement membrane (Figs. 9 to 10).

Much heavier deposits were regularly found on the cell membrane of the dendritic elements of the epidermal cell population (Fig. 9).

FIGURE 7 ATPase preparation from the outer front of the frog epidermis, showing junction lines among four profiles of cornified cells (*SC*, *SC'*, *SC''*, and *SC'''*). Globular deposits of reaction product (lead phosphate) are found in the intercellular spaces along the lateral (short arrows) and basal (long arrows) surfaces of the cornified cells, in close association with the cell membrane. Note that little or no reaction product is present along the outer membranes (*cm*) of the cornified cells or along the cell junctions, namely, the occluding zonule (*Zo*) and the modified (*md*) and composite (*cd*) desmosomes characteristically found along the lateral and proximal aspects, respectively, of cornified cells.

Specimen fixed in glutaraldehyde in cacodylate buffer (pH 7.4). Nonfrozen section incubated 20 min with ATP, postfixed in 1% OsO_4 in phosphate buffer (pH 7.6), and embedded in Araldite. Section doubly stained with uranyl and lead. × 56,000.


Zo
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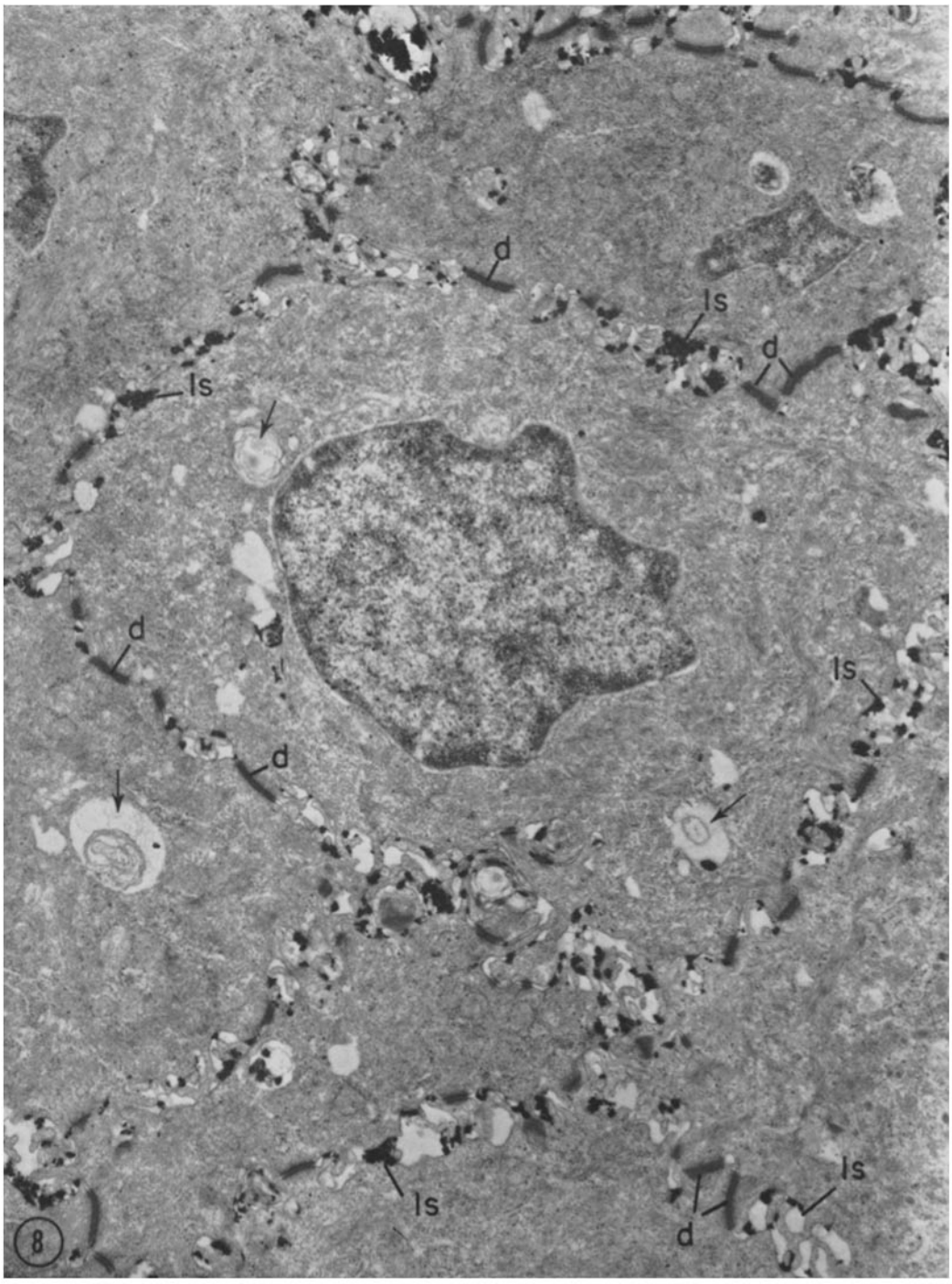

FIGURE 8 Group of cells in the *s. spinosum* of a frog skin preparation incubated with ATP. Dense deposits of lead phosphate are present throughout the intercellular spaces (*Is*) except along the desmosomes (*d*) which are free of reaction product. A fine, presumably nonspecific precipitate appears in the nuclei. Vacuoles, some of which contain myelin figures, can be seen in some cells (arrows); they probably represent artifacts produced during fixation or incubation. Specimen fixed in glutaraldehyde in cacodylate buffer (pH 7.4). Nonfrozen section incubated 1½ hr with ATP; postfixed in 1% OsO_4 in acetate-Veronal buffer; stained in block in uranyl acetate; and embedded in Araldite. Section doubly stained with uranyl and lead. × 12,000.

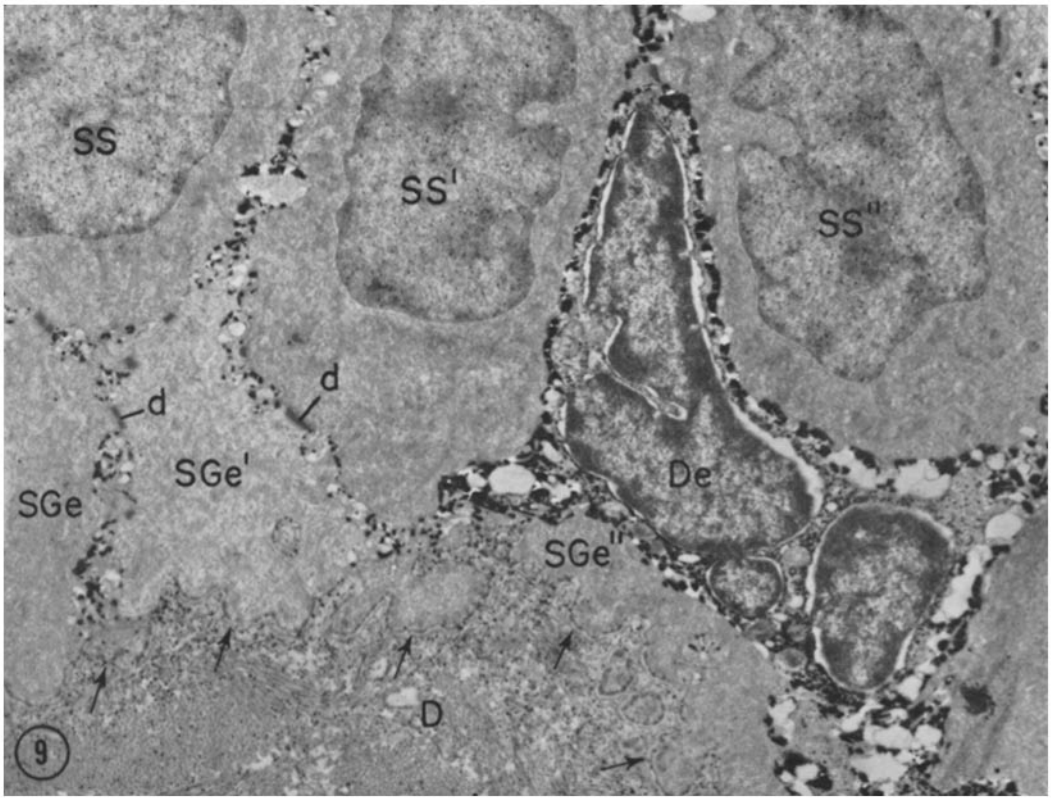

FIGURE 9 Base of the epidermis in an ATPase preparation of frog skin. The field includes the dermo-epidermal junction (arrows) and a dendritic cell (*De*) insinuated between the regular cells of the *s. germinativum* (*SGe''*) and *s. spinosum* (*SS'*, *SS''*). Dendritic elements are distinguished by the angular shape of their profiles and by the absence of desmosomal attachments to adjoining cells. Their cytoplasm is poorly preserved by the procedure used here. Globular deposits of reaction product are present along all cell membranes facing the system of intercellular spaces. Those found around the dendritic cell are larger and more extensive than those around the remaining epidermal cells. Note that no deposits are seen along the basal cell membrane of the *s. germinativum* cells where they face the basement membrane. A fine precipitate is present throughout the dermis (*D*).

Specimen preparation as for Fig. 8. × 8,000.

Reaction patterns with other substrates. With ADP as substrate and long incubation periods, deposits of reaction product were frequently found along the cell membranes outlining the intercellular spaces in the *s. corneum* and between the latter and the *s. granulosum*. As previously shown (44), those spaces represent partially or completely isolated subcompartments of the labyrinth of intercellular spaces. Much smaller and sparser deposits occurred along the cell membranes facing the rest of the labyrinth. The reaction was intense only along the cell membrane of dendritic cells (Fig. 15). Limited observations made on specimens incubated with AMP and GP showed a similar accumulation of reaction product in the distal subcompartments of the extracellular spaces and occasional small deposits along other cell membranes.

FIXED TISSUE–FROZEN SECTIONS AND FRESH TISSUE: The preincubation history of the specimen affected the distribution of the reaction product in ways already suggested by the light microscope observations.

In specimens fixed and frozen before incubation, deposits of reaction product were found in the intercellular spaces but, in addition, appeared ir-

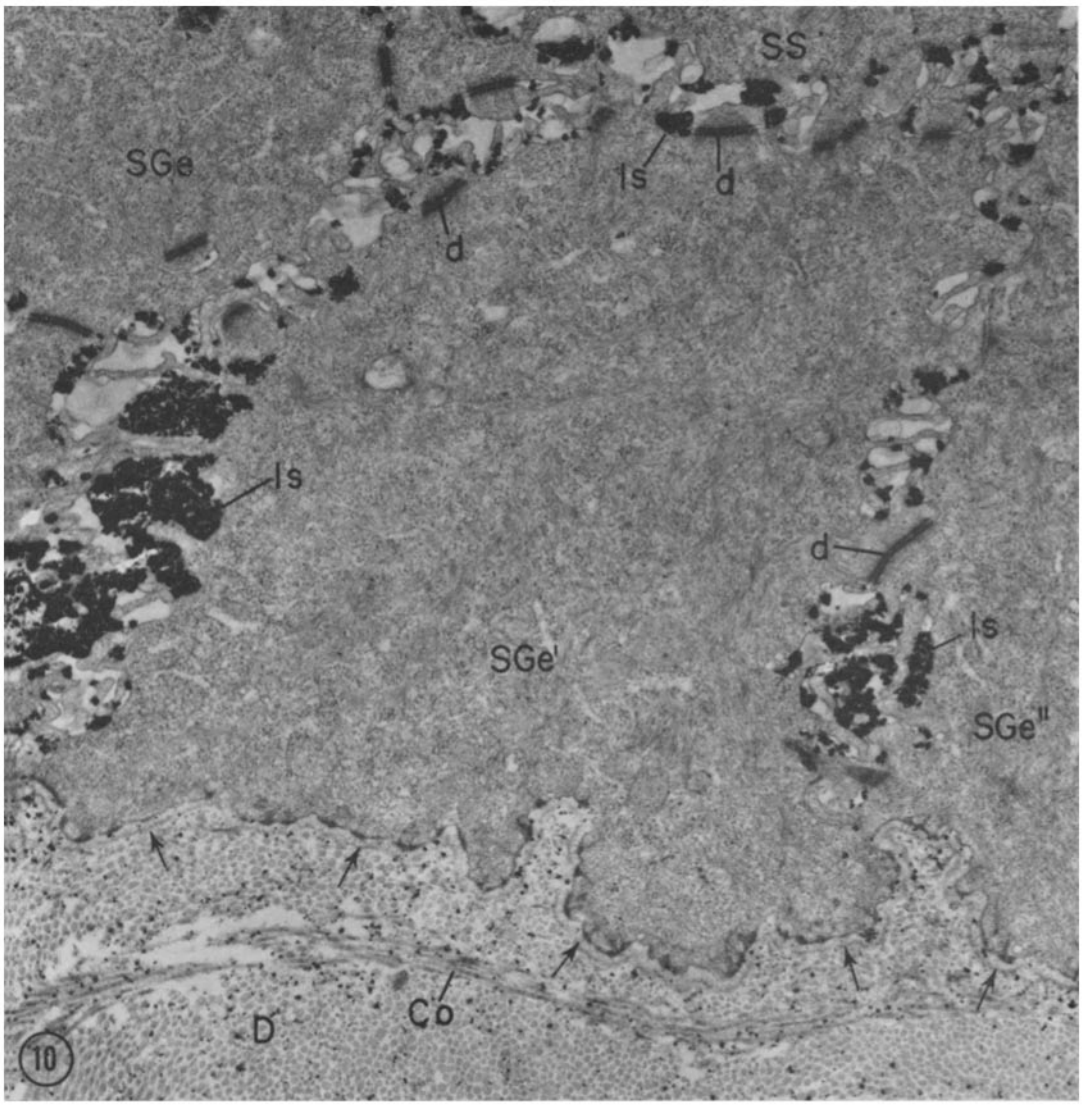

FIGURE 10 Portions of several *s. germinativum* cells from the inner front of the frog skin, in a preparation similar to that shown in Fig. 9. As in the previous figure, lead phosphate deposits are present in the intercellular spaces along their distal surfaces which face the *s. spinosum* (*SS*) and along their lateral surfaces where *SGe'* interdigitates with the adjoining cells (*SGe* and *SGe''*). No deposits are present, however, along their basal surface which is covered by basement membrane (arrows) and faces the dermis (*D*). All desmosomes (*d*) are also free of deposits. The heavy deposition of lead phosphate on the left side of *SGe'* probably indicates the vicinity of a dendritic cell. As in the previous figure, fine deposits are scattered throughout the dermis.

Specimen preparation as for Fig. 8. × 14,000.

regularly scattered in the peripheral cytoplasm without preferential association with any subcellular component. These peripheral deposits varied in extent from specimen to specimen, were smaller than the intercellular deposits, and decreased in size and frequency with distance from the cell membrane. Hence, they appeared to be artifacts produced by the diffusion of either the

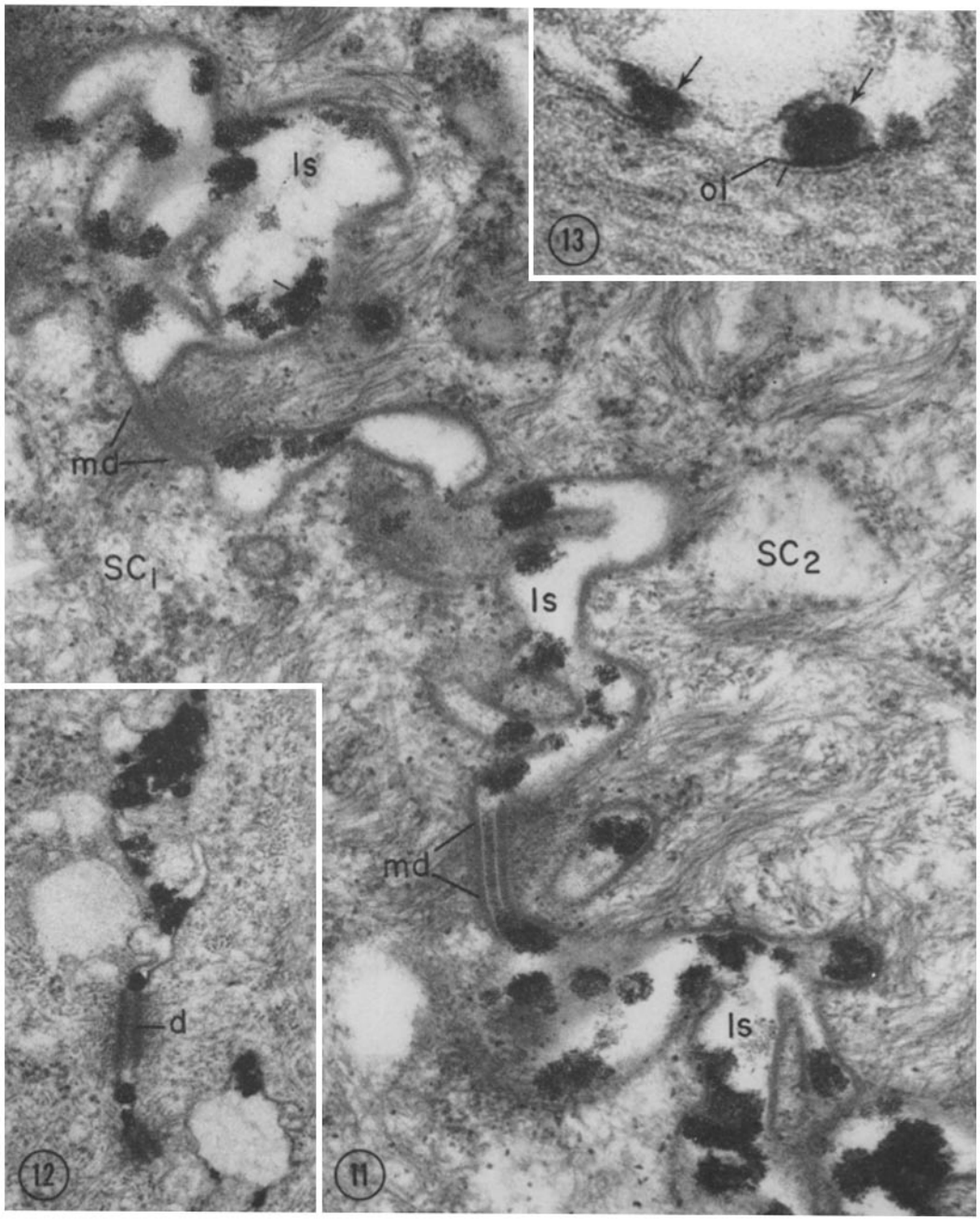

FIGURE 11 ATPase reaction product in the intercellular space (*Is*) between two cells (SC_1 and SC_2) of the *s. corneum* in frog epidermis. The lead phosphate deposits occur in the form of globular aggregates irregularly distributed along the cell surface. Each aggregate is composed of smaller (~50 A) particles. No such deposits are seen in the intercellular space along the modified desmosomes (*md*).

Specimen preparation as for Fig. 7. × 72,000.

FIGURE 12 Enlargement of a small field in Fig. 8 to show absence of ATPase reaction along desmosomes (*d*) in the *s. spinosum*. × 42,000.

FIGURE 13 Small portion of the cell membrane from an ATPase preparation. Globules of reaction product (arrows) are located in close association with the outer membrane leaflet (*ol*) which appears thicker and denser in the vicinity of the deposits.

Specimen preparation is the same as for Fig. 8. × 225,000.

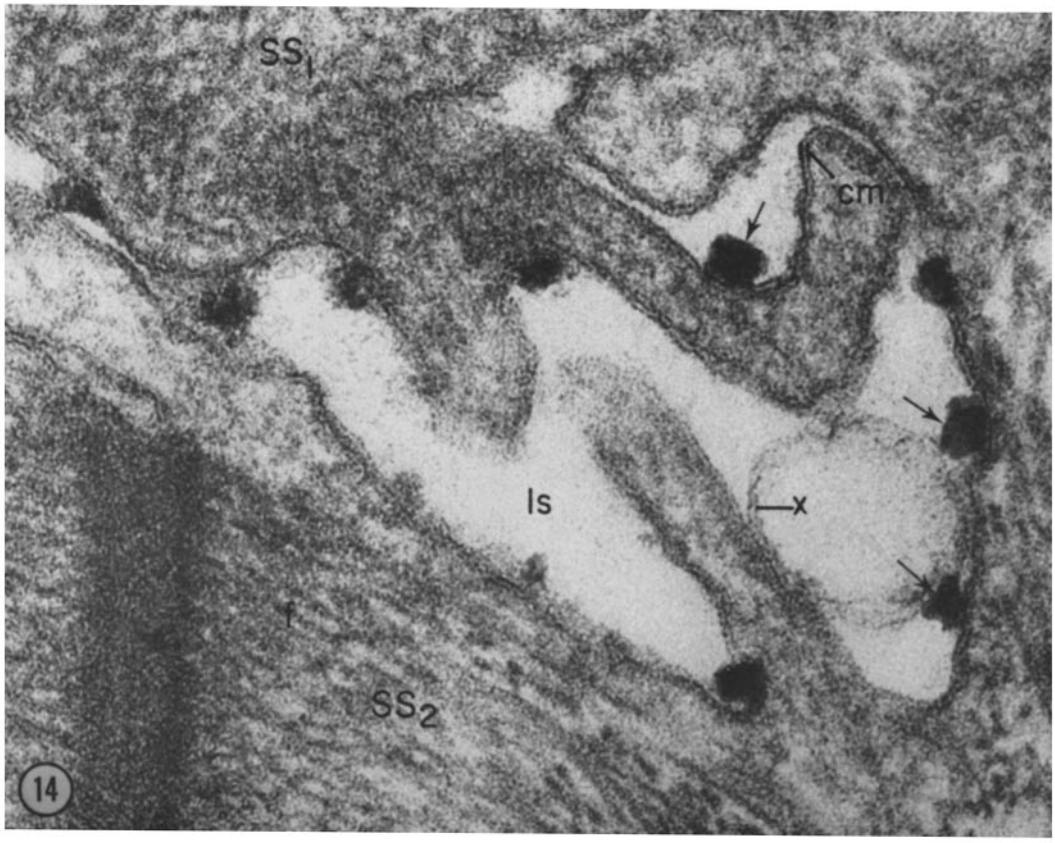

FIGURE 14 Frog epidermis reacted for ATPase. Small field in the *s. spinosum* showing at high magnification an intercellular space (*Is*) and parts of two adjacent cells (SS_1 and SS_2). In this preparation the trilaminar structure of the cell membrane (*cm*) is clearly visible in a number of places. Globular deposits of lead phosphate occur along the outer surface of the cell membrane at more or less regularly spaced intervals. In several places (arrows) where the membranes are cut normally, the deposits are located in close association with the dense outer leaflet of the membrane. In the lower left corner, numerous cytoplasmic filaments (*f*) insert on an obliquely cut desmosome. A bleb or small myelin figure, presumably produced during fixation, is marked *x*.

Specimen preparation as for Fig. 8. × 180,000.

enzyme or the reaction product as a result of the freezing and thawing inherent in the frozen section procedure.

In specimens incubated without prior fixation or freezing, cell structure was less satisfactorily preserved: many nuclei were pyknotic and most mitochondria were swollen even though sucrose had been added to the media. As a rule, deposits of reaction product were found only on cells that had been cut or damaged during sectioning. Otherwise, their appearance and distribution throughout the epidermis were similar to that already described in fixed skin. It is noteworthy that in fresh as well as in fixed epidermis the reaction product was regularly found deposited on the outer leaflet of the cell membranes. Most intracellular deposits were located in mitochondria.

TOAD

Relations of reaction product deposits with cell membranes were the same as described in frog epidermis. The general distribution of the ATPase reaction was also similar to that found in the frog, except for the shift already mentioned in reaction

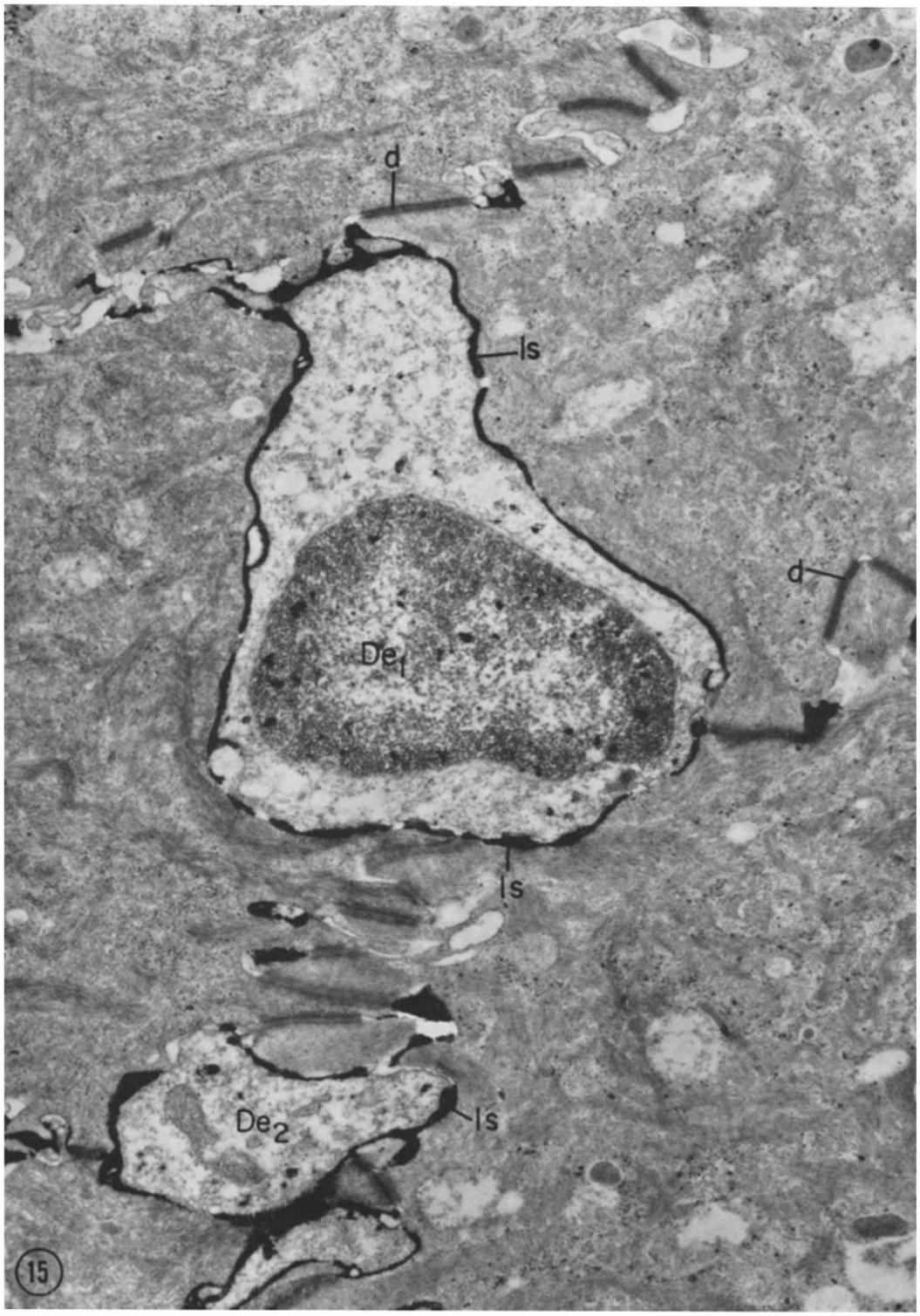

FIGURE 15 Group of cells from the *s. spinosum* of a frog skin preparation incubated with ADP. Portions of the same or different dendritic cells (De_1 and De_2) are present in the field. Deposits of reaction product fill entirely the intercellular space (*Is*) around dendritic elements, but appear sparsely and discontinuously scattered along the remaining intercellular spaces.

Specimen preparation as for Fig. 8, except that incubation was carried out for 1½ hours with ADP. × 18,000.

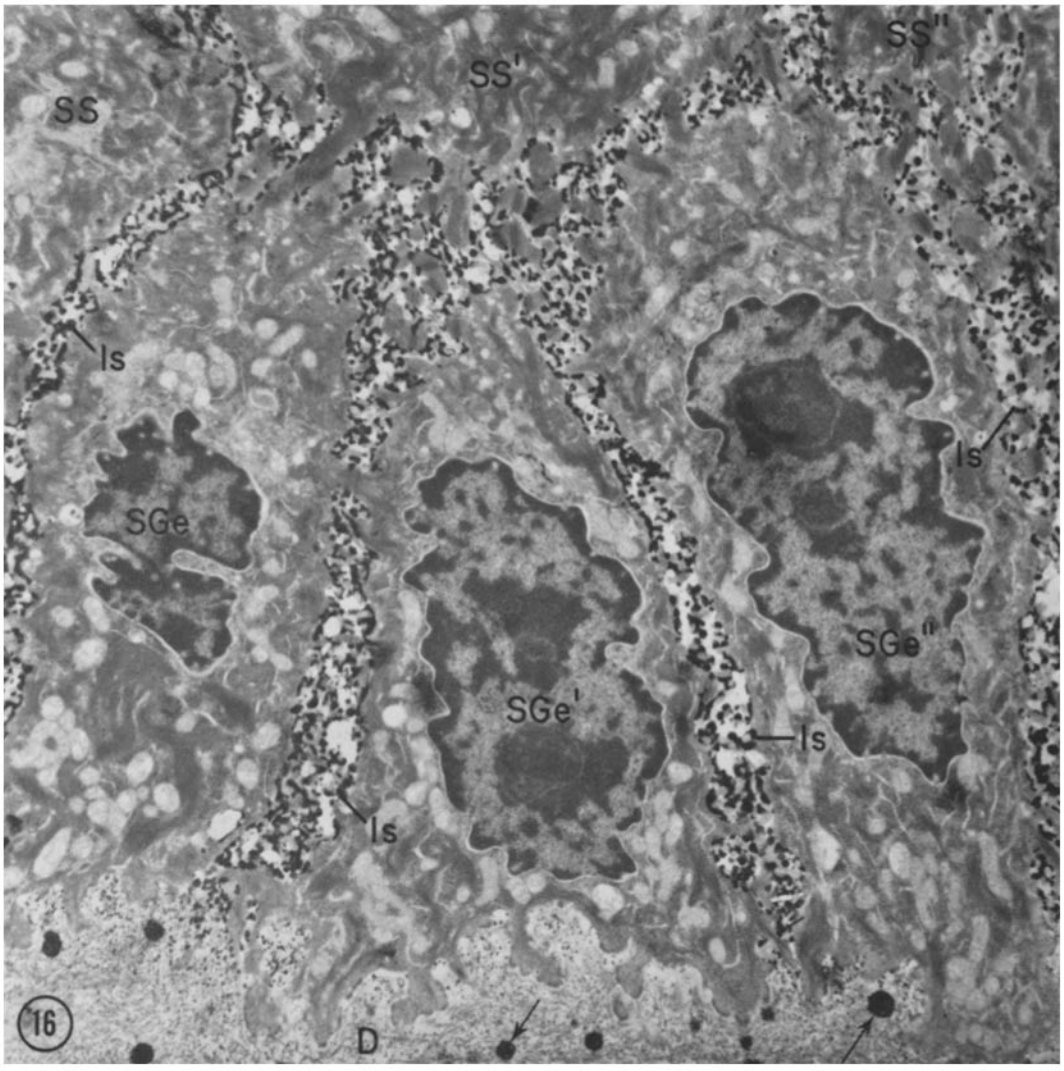

FIGURE 16 Dermo-epidermal junction in an ATPase preparation of toad skin, similar to Fig. 10 (frog). Three large cells of the *s. germinativum* (*SGe*, *SGe'*, *SGe''*) are shown. The intercellular spaces along their lateral surfaces (*Is*) and distal surfaces facing cells of the *s. spinosum* (*SS*, *SS'*, *SS''*) are filled with reaction product, but the cell membranes along their basal surfaces are not reactive. While the distribution of reaction product is the same as in the frog, the intensity of the reaction in the basal layer is greater (see also Figs. 5 and 6). Fine dense deposits occur throughout the dermis (*D*). The larger deposits (arrows) in the dermis are present in unincubated preparations (see Fig. 2 of reference 44) and are believed to be due to naturally occurring deposits of calcium phosphate.

Specimen preparation as for Fig. 7. × 7500.

intensity toward the *s. spinosum* and *s. germinativum* (Fig. 16).

DISCUSSION

Our results demonstrate the existence of a Mg^{2+}-activated ATPase in the epidermis of frog and toad skin. The enzyme is present in all cell membranes that face the labyrinth of epidermal intercellular spaces, with the exception of membrane areas involved in cell junctions. In all locations, the reaction product is found in close association with the outer leaflet of the cell membrane. The activity is

missing from the membranes that face the external medium and the interstitia of the dermis: namely, the outer membrane of the cells in the ultimate cornified layer and the basal membrane of the cells in the *s. germinativum* (*s. basale*). These membranes constitute the outer and inner front of the epidermis, respectively.

Admittedly, our findings are subject to a number of important limitations: (*a*) a large percentage of ATPase activity is lost during fixation (60 to 70%, according to preliminary determinations); (*b*) further inhibition by Pb^{2+}, used as a phosphate-trapping agent, (cf. 24) should be considered; and (*c*) an essentially qualitative technique has been used to study a problem in which quantitation is important for the reasons discussed below.

Membrane ATPase is activated by Mg^{2+} and further stimulated by Na^+ and K^+ acting together (9–12). Only the $(Na^+ + K^+)$–dependent activity is glycoside-sensitive, and only this activity, sometimes referred to as "transport ATPase," (16) shares common features with active cation transport (11, 16). The ratio $(Mg^{2+} + Na^+ + K^+)$–activated enzyme/Mg^{2+}–activated enzyme varies with the tissue (14, 24, 25, 42, 43) and the preparation procedure (12, 14, 15) and, in the case of the frog skin, it is known to be not greater than 2 (14, 24, 25). Hence the chances of detecting activation by $(Na^+ + K^+)$ and inhibition by ouabain through a qualitative histochemical procedure are rather limited owing to the large number of variables involved, the most important of which is section thickness. We could ascertain that the distribution pattern did not change in the presence of $(Na^+ + K^+)$, or $(Na^+ + K^+)$ and ouabain, but the concomitant variations in the intensity of the reaction were too small and not reproducible or consistent enough for further conclusion. Accordingly, our results do not bear directly on the localization of $(Na^+ + K^+)$–dependent, glycoside-sensitive ATPase.

A substantial body of evidence indicates, however, that the ratio $(Mg^{2+} + Na^+ + K^+)/Mg^{2+}$–activated enzyme can be changed experimentally by a variety of procedures such as aging (12, 19), sonication (12), dialysis (15), preincubation (62), or treatment with deoxycholate (14, 63), urea, and *N*-ethylmaleimide (42, 43, 64). A certain amount of total ATPase is lost through these procedures, but the $(Mg^{2+} + Na^+ + K^+)$–dependent activity increases concomitantly with a roughly proportional loss in Mg^{2+}-dependent activity. For this reason, these two activities have been considered different but convertible forms of the same enzyme (63–65). Moreover, the existence of a relatively high initial level of Mg^{2+}–activated ATPase which shows limited response to $(Na^+ + K^+)$ has been ascribed to the presence of endogenous, enzyme-bound $(Na^+ + K^+)$ in the corresponding tissues (15, 63). Although the relationship between the two forms of activity is not fully understood (cf. 42, 43, 64) and different interpretations have been advanced over the years (14, 66, 67), recent results definitely favor the one enzyme hypothesis (64).

Assuming that we are dealing with two forms of a single transport ATPase, our results can be taken to indicate that active transport of $(Na^+ + K^+)$ is located in all cell membranes that face the labyrinth of intercellular spaces in the frog and toad epidermis. The approach used allows direct localization in situ at sufficiently high resolution and indicates clearly that the enzyme is associated with the cell membrane, as it is in erythrocytes (11, 12, 68) and nerves (69, 70). In this respect, it has an advantage over cell fractionation procedures in which the derivation of various vesicles, "microsomes," and "submicroscopic particles" remains open to question (cf. 12, 10). An ATPase or nucleoside phosphatase activity restricted to, or predominantly associated with, cell membranes has already been described in a number of electron microscope studies of epithelia engaged in active transport, e.g., in mammalian kidney tubules (34, 37, 38), gall bladder (71), ciliary epithelium (72, 73), corneal endothelium and epithelium (74), and in toad bladder (39).

To the extent we tested it, the specificity of the reaction in the frog epidermis is not strictly limited to ATP in sectioned tissue; slightly lower activity can be demonstrated with ITP and considerably lower activity with ADP. In other organs (e.g., kidney, toad bladder), a cytochemical reaction of even broader specificity, extending to all nucleoside triphosphates and some diphosphates, has been observed, the reaction being considerably less intense with the latter (34, 39). It should be pointed out that all these cases involve tissue sections in which enzymic nucleotide interconversions are possible. In some isolated membrane preparations specificity is more restricted (11) and in certain erythrocyte membranes it appears to be limited to ATP (68).

Although many aspects of the membrane-bound

ATPase have been investigated extensively, the sequence of chemical reactions involved in transport remains unknown, and the same pertains to the mechanism(s) by which the energy released upon ATP-splitting is used to move cations across the membranes. Many findings suggest that the chain of reactions involves a phosphorylated "intermediate" (27, 75–82), identified as a phosphoprotein (25, 75, 78–80, 83), which could be either the enzyme itself (79) or another component ("carrier") of the system (80). Recently Skou (42) has reviewed the evidence on this problem and concluded that the "intermediate" represents an alternative pathway, possibly an artifact, which appears only in the absence of K^+. Presumably, under normal conditions with Na^+ and K^+ present at the appropriate sites, ATP is directly split into ADP and inorganic phosphate. If a phosphorylated intermediate were involved, our reaction product would reveal its location, or that of the enzyme that hydrolyzes it, rather than the location of an ATPase. In any case, it should be understood that at present biochemical assays as well as cytochemical reactions deal with a complex system ("the pump"), as a whole, rather than with the specific enzymes of which it is comprised.

According to Hoffman (68), the Mg^{2+}-activated ATPase splits extracellular substrate, whereas the (Mg^{2+} + Na^+ + K^+)-activated enzyme acts on intracellular ATP. These findings could provide an explanation for the fact that our reaction product was regularly found on the outer leaflet of the cell membrane. In an alternative explanation, ATP has access to the inner and outer surface of the cell membrane in fixed tissue but, upon its splitting, phosphate is released into the intercellular spaces, an interpretation which is supported by the results we obtained with fresh skin. This unexpected and a priori wasteful move may represent a peculiarity of the amphibian skin, which apparently has large reserves of phosphate in the form of Ca phosphate deposits in the dermis (60, 61).

The distribution of ATPase (and, by implication, of Na^+ pumps) we found has a number of potentially interesting features: it utilizes a large membrane surface and the pumping capacity of the entire cell population of the epidermis; it takes advantage of the special geometry of the intercellular spaces which, being closed towards the external medium and open towards the dermis, could restrict the diffusion of "captured" Na^+ towards the interstitial fluid of the organism.

The absence of ATPase activity on the outer front of the epidermis is in agreement with physiological data which indicate that Na^+ efflux is a passive process of limited magnitude. The lack of activity at the level of junctional elements could reflect either lack of enzyme or limited access of substrate. The absence of ATPase activity on the inner (dermal) front of the epidermis is difficult to explain, unless we assume that the entire membrane surface involved is needed for the free movement of K^+.

These results, taken together with our previous findings on the morphology of the epidermis, suggest that a number of modifications should be introduced into the model originally proposed by Koefoed-Johnsen and Ussing (8) to explain the functional features of the frog skin. The model postulated the existence of an "outward facing membrane," freely permeable to Na^+ but impermeable to K^+, localized on the outer aspect of the *s. germinativum* (Fig. 17). Our results suggest that this membrane should be moved outward since at the postulated level there is no structurally continuous barrier (44). The most likely candidate for the function ascribed to the "outward facing membrane" is the ATPase-free membrane on the outer front of the epidermis, or another continuous barrier immediately proximal to it, but not deeper than the outer front of the *s. granulosum*. A comparable modification has been recently considered by Ussing and Windhager (84, 85) and by Ussing (7).

The model also postulated an "inward facing membrane" characterized by free permeability to K^+ and low permeability to Na^+ and by active Na^+ transport, probably coupled with K^+. This membrane was assumed to be located on the inner aspect of the *s. germinativum*. In the light of our findings, the functions of the "inward facing membrane" seem to be dissociated, the (Na^+ + K^+) pumps being located in the cell membranes facing the intercellular spaces, and the ATPase-free membrane on the inner front of the epidermis being a likely candidate for the structure freely permeable to K^+ (Fig. 18).

To work, this modified model requires free or almost free movement of Na^+ and K^+ from cell to cell throughout the epidermis (44). A similar postulate has been recently considered by Ussing and Windhager (84) and substantiated by the

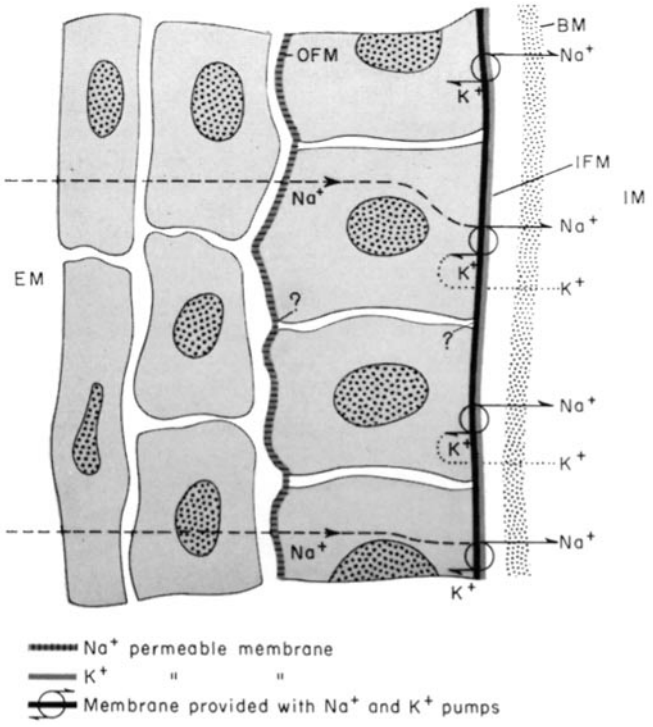

FIG. 17

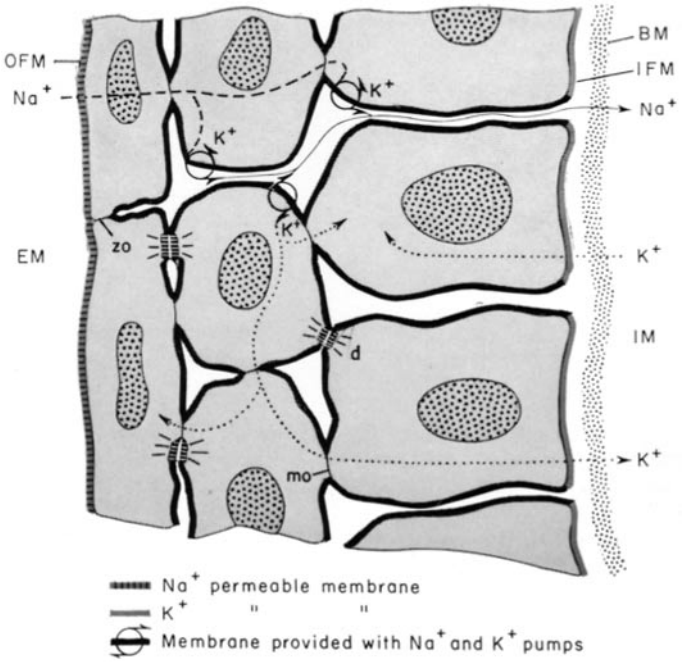

FIG. 18

FIGURES 17 and 18 Fig. 17 shows the Koefoed-Johnsen-Ussing frog skin model, redrawn to place it within the structural framework of the amphibian epidermis.

Fig. 18 depicts the modified model of amphibian epidermis suggested by the present findings.

In both figures, the 3 cell layers represent schematically, from left to right, the *s. corneum*, *s. spinosum*, and *s. germinativum*. The intercellular space is shown in white and the intracellular space in gray. The nuclei appear in stippled gray.

EM, external medium; *OFM*, outward facing membrane; *IFM*, inward facing membrane; *BM*, basement membrane; *IM*, internal medium (interstitia of the dermis); *zo*, zonula occludens; *mo*, macula occludens; *d*, desmosome. The question marks at the entry and exit of the *s. germinativum* in Fig. 17 indicate that the Koefoed-Johnsen-Ussing model does not specify the condition (open or closed) of these spaces.

results of Koefoed-Johnsen (45) who showed that ^{42}K introduced on the inner side of the skin diffuses throughout the entire thickness of the epidermis. Hansen and Zerahn (46) also found that external Li^+, which behaves like Na^+, diffuses rapidly inward and accumulates evenly throughout the middle and deep layers of the epidermis; only in the *s. corneum* the concentration remains low. Ussing and Windhager (84, 85) assume that the sites of free cell-to-cell diffusion are the desmosomes. We favor the *zonulae* and *maculae occludentes*, recently described in the epidermis (44), because of their basic structural similarity to the nexus (86, 87) and electrotonic synapses (88, 89) which are known to be sites of low resistance cell-to-cell coupling (cf. 86).

Taking into consideration more general problems, our findings show how an organism could use the ($Na^+ + K^+$) pumps located in the plasma membrane of each individual cell to move Na^+ across an epithelium, in this case from the external medium to the interstitial fluid. The operation is achieved by a redistribution of mechanisms involved in free diffusion and active transport along the cell membranes, and by transforming the intercellular spaces of the epithelium into a trap drained only towards the subepithelial spaces. As a result of the redistribution mentioned, the plasma membrane of the epidermal cells, especially of the cells in the *s. corneum* and *s. germinativum*, becomes a functional mosaic, with the proviso that the mosaic in question is rather coarse, a "tessera" being as large as a whole side of the cell. Moreover, our findings show that this mosaic is not set for the duration of the entire life of the cell: it changes with cell differentiation to give, at the end of the process, a pattern of ATPase distribution opposite to that inherited in the *s. germinativum*.

This research was supported by research grants AM-09090 and HE-05648 and research career program award I-K3-GM-25, 109, from the United States Public Health Service.

We gratefully acknowledge the technical assistance of Mrs. Karin Taylor, Mrs. Suzanne Leiss, Mrs. Jean Sarris, Miss Irene Rudolph, and Miss Cassandra Lista.

Received for publication 6 January 1966.

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