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Salt Transport Across Isolated Frog Skin

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Salt transport across isolated frog skin

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[Plates 26 to 27]

We have measured on isolated epithelia of *Rana* skin the amount of tissue sodium that equilibrates with the sodium present in the solution in contact with the outside surface. Only about 12% of the sodium in the tissue equilibrated with outside sodium. Antidiuretic hormone (0.1 u/ml) and ouabain (10^{-4} mol l^{-1}) had no effect on the amount of cell sodium that equilibrated with outside sodium.

We have also studied with the electron microscope the localization of the permeability barriers of frog skin epithelium using as tracers ruthenium red and colloidal and ionic lanthanum. Our observations indicate that there are two barriers to diffusion in frog skin epithelium. The first is at the s. corneum, the second at the s. granulosum. Of these, the first is the least selective.

In other experiments the effects of acetazolamide and amiloride on active transport of both sodium and chloride were determined. Acetazolamide (10^{-4} mol l^{-1}) blocked chloride transport without affecting sodium transport. Amiloride (10^{-4} mol l^{-1}) blocked sodium transport and did not modify chloride transport.

These results and others available in the literature are used to raise some defined questions on the relationship between structure and function and the coupling of ion fluxes in frog skin.

In this paper I will describe experiments initiated with the aim of clarifying two problems of the salt transport across the frog skin: (1) What are the boundaries and the identity of the transepithelial transport compartment? (2) How are the sodium and chloride fluxes coupled? Although so far we have only obtained partial answers, the consideration of our results taken together with other findings in the literature allow us to restate these questions in more explicitly defined terms.

Sodium in the epithelium

To improve the estimations of the Na content in the epithelium and to study its behaviour during variations in the rate of transepithelial transport, we have devised a simple preparation in which the epithelium is separated from the corium. The method is based on the finding that hydrostatic pressure (about 150 cmH_2O; 15 kPa) applied from inside the skin results in the formation of blisters (Reid 1890; Ussing 1965). The back of these blisters is constituted by the corium while the outer wall of the blister is mainly formed by epithelial cells. In skins of *Rana* species the isolated epithelia obtained by using these high hydrostatic pressures had extremely low levels of potential difference and short-circuit current. However, when the skins were treated with collagenase the application of a pressure of 30 cmH_2O (3 kPa) resulted in the formation of blisters. The epithelia obtained from these blisters have satisfactory anatomical and physiological properties (Erlij & Aceves 1969; Aceves & Erlij 1971). Isolated epithelia obtained from the skins of *Bufo bufo* by using high hydrostatic pressures (140 cmH_2O; 14 kPa) alone retain satisfactory physiological properties (Rawlins, Mateu, Fragachan & Whittembury 1970).

The general organization and fine structure of epithelia obtained with the combined use of collagenase and hydrostatic pressure are similar to those of the whole skin (figure 1, plate 26).

So far we have found (Erlij & Aceves, 1969; Aceves & Erlij, 1971) that in the isolated epithelia: (1) the values of short-circuit current and potential difference are similar to those observed in whole skins; (2) short-circuit current and sodium flux are equivalent; (3) the potential difference is a function of the sodium concentration in the outside solution and the potassium concentration in the inside solution; (4) the sodium net flux, the short-circuit current and the potential difference are enhanced by antidiuretic hormone and abolished by ouabain.

In the isolated epithelium the average intracellular sodium concentration was 24 mmol/l and the potassium concentration was 133 mmol/l (Aceves & Erlij 1971). These values were calculated by subtracting the volume of water in the extracellular space, determined as the inulin space, from the total tissue water. It was also assumed for those calculations that the composition of the extracellular space thus determined was identical with the Ringer's solution bathing the skin and that Na and K are uniformly distributed in the cell water.

Our figures are lower than the values presented in other publications in which the Na concentration in skin epithelium was calculated to be around 70 mmol/l (Cereijido, Reisin & Rotunno 1968). Probably the discrepancy arises because in these studies the contributions of the extracellular solution was not considered. Figures obtained from recent work made in slices of epithelium and in which the contribution of the extracellular spaces was used for the calculation are in agreement with our findings (Zerahn 1969).

The experiments summarized in table 1 were undertaken to determine what fraction of the Na analytically measured in the epithelium participated in transepithelial transport. When the

TABLE 1. AMOUNT OF INTRACELLULAR SODIUM THAT EQUILIBRATES WITH SODIUM IN EITHER THE OUTSIDE OR THE INSIDE SOLUTIONS

	^{22}Na	$[Na_i]$/mmol/l	total Na
control	inside	11.1 ± 1.34	
ouabain		55.9 ± 7.6	
control	outside	3.0 ± 1.4	24.7
ouabain		3.1 ± 1	
control	outside	1.75 ± 0.9	
ADH		1.57 ± 0.8	

radioisotope was added to the external solution, only 3 mmol of the total 24 mmol in the epithelium equilibrated with the outside solution. An unexpected finding was that the amount of epithelial Na equilibrating with Na in the outside solution was not altered by either antidiuretic hormone or by ouabain, although these substances produced their full effects in these preparations. A similar effec of ouabain on the amount of epithelial sodium equilibrating with the Na in the external solution has been described recently by Dörge & Nagel (1970) in whole skins.

The small fraction of epithelial Na equilibrating with sodium in the external solution could be explained if the movement of Na across the inside border of the epithelium were about seven times larger than the flux across the outer membranes. Radioactive sodium would be then continually diluted by sodium arising in the inside solution. However the Na flux across the inside membrane is much lower than the Na flux across the outside membrane (for references see Aceves & Erlij 1971).

The finding that the amount of sodium originating in the outside solution was not altered by ouabain nor antidiuretic hormone has no clear-cut explanation. A simple explanation would be to postulate that ouabain blocks both the active movement of sodium from the outside solution

into the epithelium, and the active extrusion of Na from the cells into the inside solutions. There is some evidence favouring the possibility of active transport of cations from the outside solution into the skin. Biber (1970) found that under certain circumstances this movement could be blocked by ouabain. The amount of Li accumulated in the epithelium from the outside solution is above the level predicted from passive distribution (Hansen & Zerahn 1964). However, the information is still insufficient to settle the point.

Table 1 also shows that about 11 mmol/l of cell sodium equilibrated with Na in the inside solution and that this amount was markedly increased by ouabain. This action of the glycosides is in line with its effects on other cells.

The identity of the transport compartment

I believe that there are two main clues on which an attempt to build an image of the transport compartment can be based: (1) only a small fraction of the sodium contained in the epithelium participates in epithelial transport (Aceves & Erlij 1971; Cereijido & Rotunno 1967; Zerahn 1969); (2) during the increase in rate of transepithelial sodium transport caused by short circuiting the skin, there is an increase in cell volume restricted almost exclusively to the outer layer of the s. granulosum.

These two findings could be combined to propose that the sodium transported across the epithelium moves only through the cells in the outer layer of the stratum granulosum, i.e. the first reactive cell layer of Voute & Ussing (1968). An implication of this explanation is that very little sodium moves through the cell junctions linking the cytoplasm of cells in different layers. Very little Na would move inwards through the cell junctions if the cells in the outer layer of the s. granulosum possess a very efficient active transport system. The possibility that an extremely active pump exists at such a point is supported by some recent observations of Voute & Ussing (1970). They found that the extracellular spaces of the epithelium are dilated during active sodium transport. This finding can be explained along the lines of the model proposed by Diamond & Bossert (1967) by postulating that a large secretion of sodium occurs at the distal end of the extracellular channels.

A radically different model has been proposed by Cereijido & Rotunno (1967). They postulate that transepithelial transport of sodium occurs along the surfaces of the epithelial cells instead of through the cells. They base this conclusion on the limited equilibration of the sodium in the epithelium with the sodium in the outside solution as well as in two other observations made in slices of epithelium cut parallel to the surface of the skin with a freezing microtome. First, determinations of the Na–K activated ouabain sensitive ATPase showed that all the enzyme present in the epithelium is necessary to maintain Na transport if it is assumed that for every ATP molecule hydrolysed three sodium ions are transported (Rotunno, Pouchan & Cereijido 1966). Secondly, when radioactive sodium is placed in the outside solution, the fraction of tissue sodium that equilibrates with the isotope is equal in slices obtained in all levels of the epithelium (Cereijido & Rotunno 1967). These experiments cannot be interpreted without some reservations. It is doubtful whether during the homogenization procedure there is a full preservation of ATPase activity. Furthermore, in the interpretation of the isotope equilibration experiments it is assumed that there is a correspondence between the slices cut on the microtome and the anatomical layers of the skin. If we consider that deviations of a few micrometres between boundaries of the anatomical layers and the slicing plane will considerably homogenize the composition of

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the slices, it is very unlikely that the composition of a particular anatomical layer will be drastically reflected in any of the slices. Furthermore, as discussed above, it is probable that the solution filling the extracellular channels of the epithelium will have a high proportion of isotopes originating in the outside solution since the salt absorption by the skin is accompanied by little water movement. This probable high content of isotope within the extracellular channels will further homogenize the specific radioactivities of the slices. Apart from these reservations, the extracellular transport model does not explain the changes in cell volume associated with modifications in the rate of sodium transport. Neither does it explain the finding that the resistance between a micro-electrode placed within the cells near the outer border of the skin and the outside solution is greatly increased when sodium is eliminated from the outside solutions (Rawlins *et al.* 1970).

It is clear from these considerations that the evidence available at the moment is more conveniently explained by assuming that the transepithelial sodium transport occurs through a single layer of cells.

The organization of the outer barrier

Since Farquhar & Palade (1965) found that the cells in the epithelium are sealed together by tight junctions at two levels, the s. corneum and the s. granulosum, it became important to decide the relative role of each of the two barriers thus formed (Bracho, Erlij & Martinez-Palomo 1971; Martinez-Palomo, Erlij & Bracho 1971).

The first approach was to use ruthenium red and colloidal lanthanum to determine whether both sets of junctions are true tight junctions. These markers have been recently introduced to distinguish whether cell junctions are either 'true' tight junctions or 'gap' junctions. In 'true' tight junctions the intercellular space is obliterated and no extracellular markers are detected within these specialized junctions: 'gap' junctions are permeable to the extracellular tracers (Revel & Karnovsky 1967; Brightman & Reese 1969; Goodenough & Revel 1970; Martinez-Palomo 1970).

In specimens treated with ruthenium red (figures 1 and 3, plates 26 and 27) an electron dense material was found along the surface coat of the outer border of the cells of the s. corneum and also along the border of the cell membranes throughout the s. germinativum, s. spinosum and s. granulosum. Ruthenium red was not found in the space between the cells of the s. corneum and s. granulosum nor in the cytoplasm of any of the epithelial cells. The barriers that limit the movement of ruthenium red into the space formed between the s. corneum and s. granulosum appear to be the tight junctions joining the cells in these layers. 'Colloidal' lanthanum produced identical images of penetration through the internal side to those obtained with ruthenium red. When applied from the outside, 'colloidal' lanthanum did not penetrate beyond the surface coat at the outside border of the s. corneum. However, in a few instances (figure 2, plate 27) the electron dense reaction of 'colloidal' lanthanum was observed within the cytoplasm of the cornified cells and the space between the 's. corneum' and 's. granulosum'. It appears that lanthanum can cross the membrane of the cornified cells in these preparations since no tracer was found inside the tight junctions joining the cornified cells.

These observations indicating the presence of a space at the external border of the epithelium, limited between the outer anatomical border of the skin and the outer border of the cells in the s. granulosum were made in skins treated with glutaraldehyde. It was then necessary to obtain

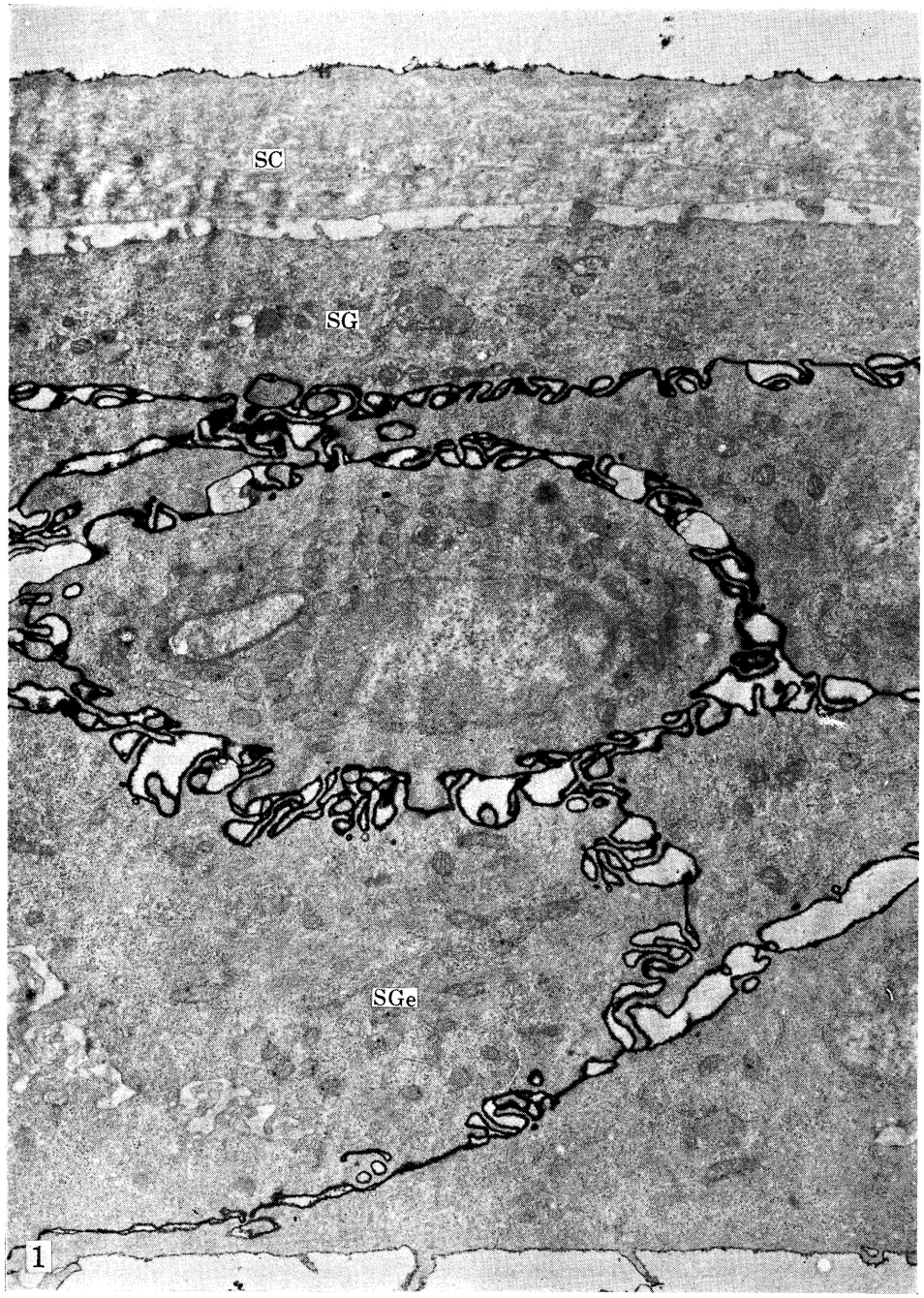

FIGURE 1. Isolated epithelium of the frog skin. Notice the adequate preservation of epithelial cells. No basement membrane is seen under the stratum germinativum cells (SGe). Ruthenium red has penetrated into the intercellular spaces forming a dense deposit on the cell membranes; however, no dense deposit is seen in the intercellular space separating the stratum corneum (SC) from the stratum granulosum (SG). Glutaraldehyde, osmium tetroxide, ruthenium red, Epon, lead citrate. (Magn. × 9000.)

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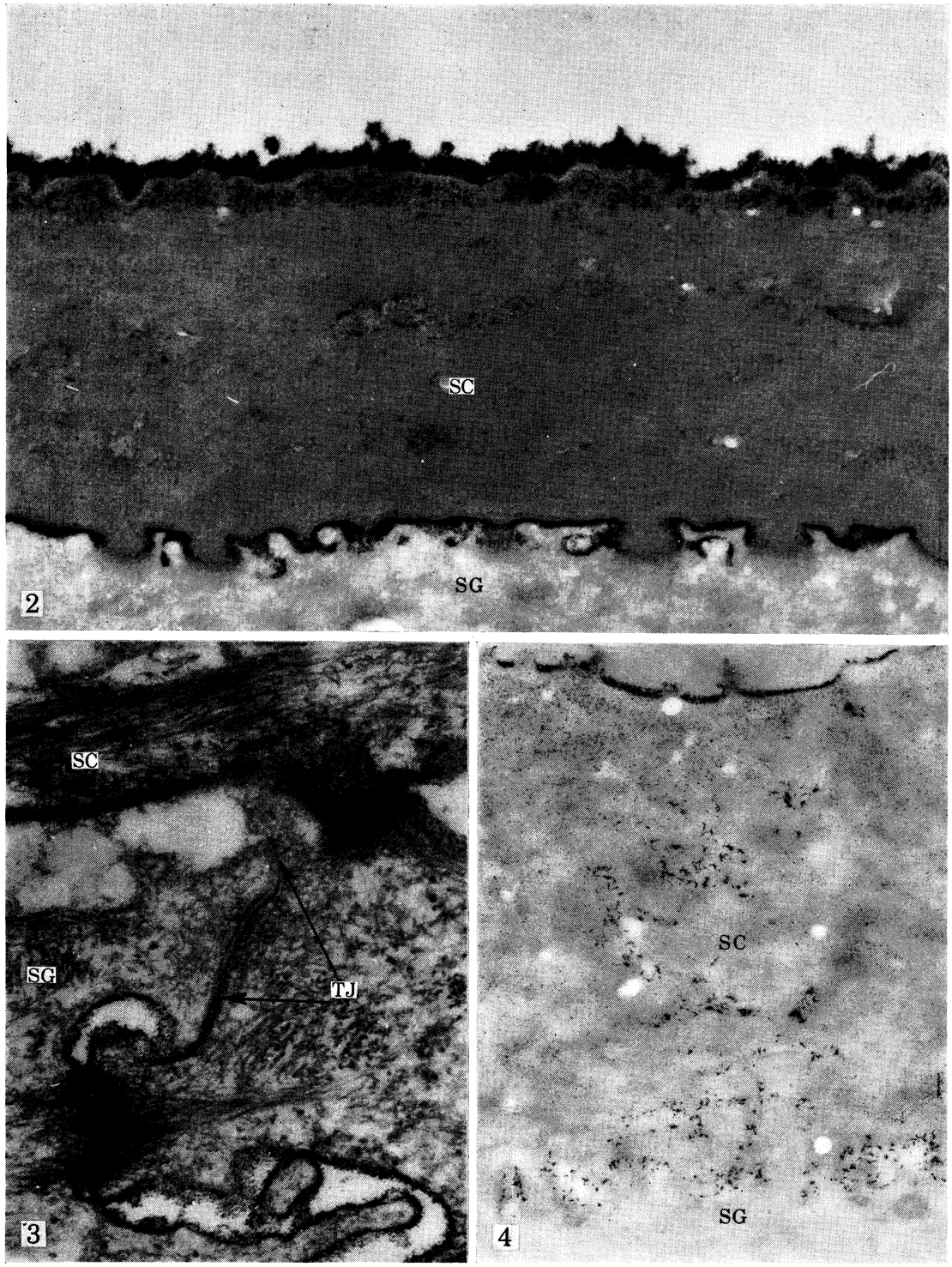

FIGURE 2. Frog skin treated with colloidal lanthanum. A dense precipitate is seen at the surface coat of the stratum corneum (SC) in the cytoplasm of the cornified cells and in the intercellular space separating the s. corneum from the stratum granulosum (SG). Glutaraldehyde, osmium tetroxide and colloidal lanthanum, Epon, lead citrate. (Magn. × 18000).

FIGURE 3. Tight junction (TJ) between two cells of the s. granulosum (SG) Ruthenium red is prevented from entering into the first intercellular space by the fusion of cell membranes at the junction. Similar results are obtained when colloidal lanthanum or lanthanum chloride are applied from the inside. Glutaraldehyde, osmium tetroxide and ruthenium red. Epon, lead citrate. (Magn. × 40000.)

FIGURE 4. Frog skin treated from the outside with lanthanum chloride. A finely granular or microcrystalline precipitate is seen at the surface coat of the s. corneum (SC) and in the first intercellular space. No precipitates are seen in the cytoplasm of the s. granulosum cells. Lanthanum chloride, glutaraldehyde, Epon. Unstained section. (Magn. × 21000.)

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evidence for the presence of such a space in unfixed skins. With this aim, we tested the effects of lanthanum on skins mounted on Ussing-type chambers, bathed with Ringers solution on both sides. We found that lanthanum (0.1 to 1 mmol/l) had no effects on the short circuit current nor on the potential difference from the inside, while when added to the external solution it produced an increase in short-circuit current and potential difference. Measurements of fluxes with isotopes of sodium and chloride showed that the increases resulted exclusively from an increase in the inward net flux of sodium. We also measured the fluxes of lanthanum into and across the skin using the radioactive isotope ^{140}La. Lanthanum moved rapidly into the skin. After an initial phase of rapid uptake, lasting about 5 min, the amount of lanthanum within the skin reached a steady level. However, no lanthanum moved across the skin even after 5 h of exposing the skin to Ringer's containing 1 mmol/l lanthanum. When the skins exposed to La^{3+} on the outside surface were immersed in the glutaraldehyde solutions almost no lanthanum was lost into the fixative.

The skins that had been exposed to Ringer's solution containing lanthanum and then fixed in glutaraldehyde were examined with the electron microscope without staining (figure 4, plate 27). A dense finely granular or microcrystalline precipitate was observed at the surface coat and randomly distributed within the cytoplasm of the cells of the s. corneum and also at the intercellular space between the s. corneum and s. granulosum. The dense precipitates were never observed within the cells of the s. granulosum or at deeper levels when the specimens were treated from the outside with lanthanum. These findings were constant in all examined specimens. Skins treated only with Ringer's solution and processed similarly for electron microscopy did not show any precipitate.

These findings show that on the external border of the skin there are two barriers of different properties. The first barrier formed by the cells in the s. corneum and their tight junctions is less selective at least as far as lanthanum is concerned. Furthermore, the results raise two questions: (1) What role does the external compartment play in the recently measured (Biber & Curran 1970; Rotunno, Villalonga, Fernandez & Cereijido 1970) large movements of Na from the outside solution into the skin? (2) Which of the two barriers correspond to the outside barrier of the transport compartment?

The first question cannot be answered until more is known about the nature of the Na movement from the outside solution into the skin. With respect to the second question, the natural tendency would be to identify the outside barrier of the transport compartment with the outside border of the s. granulosum since this is a tighter barrier. However, some caution is necessary before accepting this suggestion. Lindemann & Thorns (1967) measured the resistance between a microelectrode and the external solution and localized the tip of the micropipettes by positioning it with a precision step motor and also by direct observation with a high power microscope. They concluded that in most of the skins the main resistive barrier was at the outermost surface of the epithelium. They were careful, however, to point out that perhaps the cornified cells were missing in their preparation due to the possibility that the skins could have been in a particular stage of their monthly regeneration cycle. It is then possible that during a certain part of the cycle—perhaps immediately after shedding—the outer anatomical border of the skin and the outside barrier of the transport compartment coincide.

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The Cl^- movements

A question that so far has not received a satisfactory answer is whether there is a true difference between the mechanism of Cl^- absorption through the skin in whole frogs and the Cl^- absorption in isolated skins.

The evidence for an independent absorption of Cl^- through the skin of whole animals is irrefutable. Krogh (1937) found that intact frogs (*R. esculenta*) absorbed Cl^- and Br^- through the skin from dilute solutions. He also showed that Cl^- was absorbed when no cation uptake was observed and presented results that suggested an exchange of Cl^- for bicarbonate. Some time

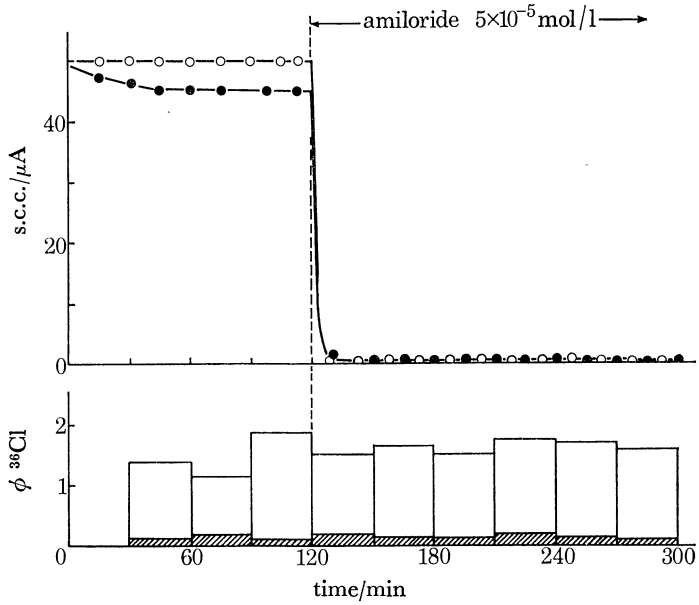

Figure 5. The effects of amiloride on the short-circuit current and the chloride fluxes of a pair of skins obtained from the same frog. The empty bars and circles show the results from the skin used to measure Cl influx. The filled circles and hatched bars correspond to the skins used to measure Cl outflux. The upper part of the figure illustrates the behaviour of the short-circuit current; the lower part the ^{36}Cl fluxes. Both are expressed in μA per cm^2 of skin.

later, Jorgensen, Levi & Zerahn (1954) extended Krogh's observations to other species (*Bufo bufo*, *R. arvalis*, *R. esculenta* and *R. temporaria*). More recently Garcia-Romeu and his co-workers (Salibian, Pezzani-Hernandez & Garcia-Romeu, 1968; Garcia-Romeu, Salibian & Pezzani-Hernandez, 1969) have carried out observations in *Leptodactylus ocellatus* and *Callyctocephalella gayi* showing that under several conditions Cl^- can be absorbed without any concomitant movements of Na^+. In addition, they found a ratio of 2:3 between the net Cl^- absorption and the $^-HCO_3$ excreted.

Attempts to show active Cl^- transport in isolated skins have been less uniformly successful. The ratio of unidirectional chloride fluxes in isolated frog skins (*R. temporaria* and *R. esculenta*) bathed with 0.1 Ringer's outside and normal Ringer's inside conformed with the predictions of the flux ratio equation for passive fluxes (Koefoed-Johnsen, Levi & Ussing 1952). Chloride also moves passively in the isolated short-circuited skin bathed by Ringer's solution on both surfaces (Koefoed-Johnsen, Ussing & Zerahn 1952). On the other hand, the isolated skin of *Leptodactylus ocellatus* transports chloride actively (Zadunaisky, Candia & Chiarandini 1963). More recently,

Martin & Curran (1966) have shown that an active transport of chloride can be detected in isolated skins of *Rana* species if they are immersed in solutions containing only 2 mmol/l Cl^-.

In experiments carried out in my laboratory (in collaboration with Mr I. Galar) we have obtained further evidence for the existence of an independent active chloride transport across the isolated skins of *Rana*. As in the experiments of Martin & Curran (1966) we have used a Ringer's solution that contains only 2 mmol/l of chloride in which most of the anion was sulphate. Figure 5 compares the effects of amiloride on the short-circuit current and the chloride influx and outflux of two pieces of skin obtained from the same frog. Two features are of interest in this experiment; (1) in agreement with Martin & Curran (1966) the chloride influx exceeded by severalfold the outflux; (2) amiloride abolished the short-circuit current without altering the chloride fluxes. Figure 6 shows the converse observation; in this case chloride influx was blocked by acetazolamide without perceptible alteration in the short-circuit current. In spite of the presence of a net chloride transport in these skins, the short-circuit current is essentially equivalent to net sodium flux because the chloride movement is very small when compared with the sodium movement.

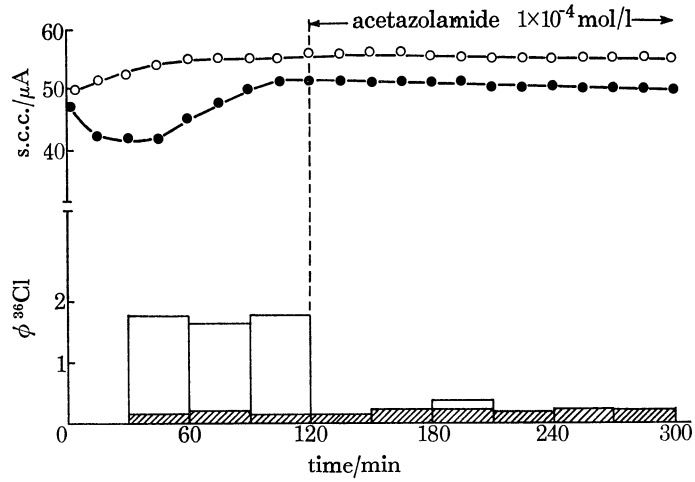

FIGURE 6. The effects of acetazolamide (1×10^{-4} mol/l) on the short-circuit current and chloride fluxes of a pair of skins obtained from the same frog. Symbols and scales as in figure 5.

The difference in chloride absorption observed between isolated skin and whole animal experiments may depend on the conditions in which the frogs were kept before the observations. Krogh (1937) explicitly stated that 'a frog taken directly from a pond or kept in tap water for any length of time will not absorb Cl from a dilute solution'. All the whole animal experiments described at the beginning of this section were carried out in animals preadapted to solutions of low ionic content while the isolated skins used in the experiments of Martin & Curran (1966) and in our work came from frogs not preadapted to solutions with low ionic content.

We have measured Cl movements in skins of animals kept in distilled water for 2 weeks before making flux determinations. In the isolated skins of some of these animals we found net inward chloride fluxes as high as 1.3 μmol cm^{-2} h^{-1}. However, the response to distilled water adaptation was not uniform: some skins had Cl fluxes similar to those observed in skins of animals kept in tap water. In spite of the variability of these results, it can be tentatively suggested that a major contribution to the discrepancy between whole animal and isolated skin experiments may arise from the difference in treatment to which the animals were subjected before the flux determinations.

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Two other problems have to be considered. The first is whether the action of acetazolamide on chloride transport is mediated through the inhibition of carbonic anhydrase. So far the determinations of the enzyme in the frog skin have given negative results (Maren 1967). It may be, however, a case similar to the turtle urinary bladder. Originally no carbonic anhydrase had been found in this tissue but when acetazolamide sensitive chloride and bicarbonate transports were found (Gonzalez 1969), the enzyme was looked for with greater care and its presence detected (Scott, Shamoo & Brodsky 1970).

The second problem concerns the coupling of sodium and chloride fluxes across frog skin, specially since under certain circumstances either Cl^- or Na^+ might be transported independently. This problem is evident even in the open circuited isolated skin where in several instances the net absorptions of Na and Cl do not have the one to one relationship expected if chloride were passively following the transported sodium (Ussing 1949; Huf, Parrish & Weatherford 1951). An exchange of H^+ for Na^+ is the most likely mechanism to account for a Na transport not coupled with chloride movements (Ussing 1949; Garcia-Romeu *et al.* 1969). The same question arises in the case of independent chloride movements during which the most likely candidate for exchange is bicarbonate. It is not yet clear whether these couplings result from an obligatory exchange mediated through a carrier or whether the exchanged ion moves as a result of the potential difference created by electrogenic transport.

Conclusions

Figure 7 summarizes the problems on salt transport discussed in this paper. I have purposefully avoided supporting, as far as possible, a broad term model. The state of our knowledge of

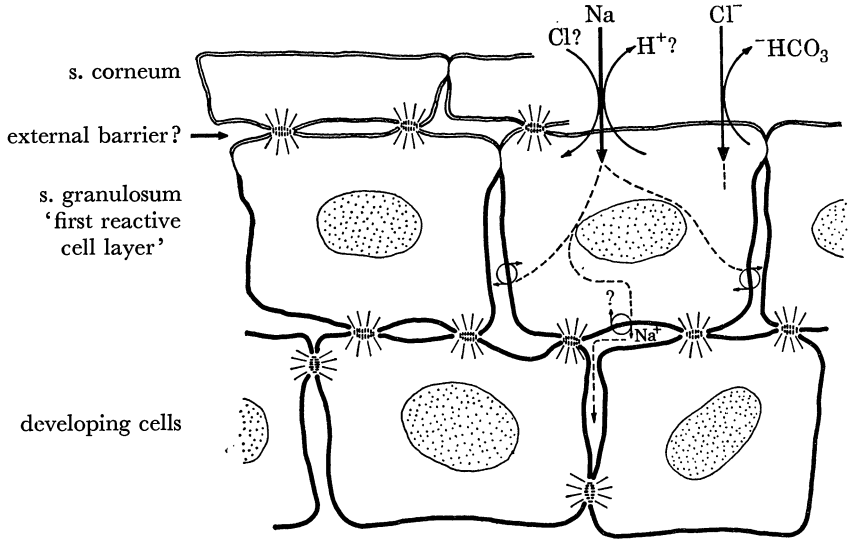

Figure 7. The problem of active salt transport discussed in this communication and their proposed connexion with the given cell components of the epithelium are schematically illustrated. The problems considered are: (*a*) If the active transport of Na is carried out mainly by the 'first reactive cell layer', what is the role of the more proximal cell layers? Are they mainly involved in the process of cell proliferation? (*b*) Which of the two groups of barriers at the outer edge of the epithelium corresponds to the outer border of the transport compartment? (*c*) Also localized at the outer border are two independent mechanisms for the absorption of Cl^- and Na^+ respectively. We know very little about their nature and the coupling with other ionic species necessary to maintain electroneutrality.

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salt transport across the frog skin is such that it is more valuable to concentrate our interest in considering specific questions and their possible alternative explanations.

I am specially indebted to Dr A. Martinez-Palomo who prepared the electron micrographs used in figures 1 to 4. I wish to express my gratitude to Drs J. Aceves, H. Bracho and I. Galar. The original work described here is the result of our collective efforts. These investigations were supported by a grant from the Life Insurance Research Fund.

References (Erlij)

Aceves, J. & Erlij, D. 1971 *J. Physiol.* **212**, 195.
Biber, T. U. L. 1970 *Fedn. Proc. Fedn Am. Socs exp. Biol.* **29**, 724.
Biber, T. U. L. & Curran, P. F. 1970 *J. gen. Physiol.* **56**, 83.
Bracho, H., Erlij, D. & Martinez-Palomo, A. 1971 *J. Physiol.* **213**, 50P.
Brightman, M. W. & Reese, T. S. 1969 *J. Cell Biol.* **40**, 648.
Cereijido, M., Reisin, I. & Rotunno, C. A. 1968 *J. Physiol.* **196**, 237.
Cereijido, M. & Rotunno, C. A. 1967 *J. Physiol.* **190**, 481.
Diamond, J. M. & Bossert, W. H. 1967 *J. gen. Physiol.* **50**, 2061.
Dörge, A. & Nagel, W. 1970 *Pflügers Arch. ges. Physiol.* **319**, R 89.
Erlij, D. & Aceves, J. 1969 *Biophys. J.* **9**, A163.
Farquhar, M. G. & Palade, G. M. 1965 *J. Cell Biol.* **26**, 263.
Garcia Romeu, F., Salibian, A. & Pezzani-Hernandex, S. 1969 *J. gen. Physiol.* **53**, 816.
Gonzalez, C. F. 1969 *Biochim. biophys. Acta* **193**, 146.
Goodenough, D. A. & Revel, J. P. 1970 *J. Cell Biol.* **45**, 272.
Hansen, H. H. & Zerahn, K. 1964 *Acta Physiol. scand.* **60**, 189.
Huf, E. G., Parrish, J. & Weatherford, C. 1951 *Am. J. Physiol.* **164**, 137.
Jorgensen, C. B., Levi, H. & Zerahn, K. 1954 *Acta physiol. scand.* **30**, 178.
Koefoed-Johnsen, V., Levi, H. & Ussing, H. H. 1952, *Acta physiol. scand.* **25**, 150.
Koefoed-Johnsen, V., Ussing, H. H. & Zerahn, K. 1952 *Acta physiol. scand.* **27**, 38.
Krogh, A. 1937 *Skand. Arch. Physiol.* **76**, 60.
Lindemann, B. & Thorns, U. 1967 *Science, N.Y.* **158**, 1473.
Maren, T. H. 1967 *Physiol. Rev.* **47**, 595.
Martin, D. W. & Curran, P. F. 1966 *J. cell. comp. Physiol.* **67**, 367.
Martinez-Palomo, A. 1970 *Lab. Invest.* **22**, 605.
Martinez-Palomo, A., Erlij, D. & Bracho, H. 1971 *J. Cell Biol.* (in the Press).
Rawlins, F., Mateu, L., Fragachan, F. & Whittembury, G. 1970 *Pflügers Arch. ges. Physiol.* **316**, 64.
Reid, W. 1890 *J. Physiol.* **11**, 312.
Revel, J. P. & Karnovsky, M. J. 1967 *J. Cell Biol.* **33**, C7.
Rotunno, C. A., Pouchan, M. I. & Cereijido, M. 1966 *Nature, Lond.* **210**, 597.
Rotunno, C. A., Vilallonga, F. A., Fernandez-M. & Cereijido, M. 1970 *J. gen. Physiol.* **55**, 716.
Salibian, A., Pezzani-Hernandez, S. & Garcia-Romeu, F. 1968 *Comp. Biochem. Physiol.* **25**, 311.
Scott, W. N., Shamoo, Y. E. & Brodsky, W. A. 1970 *Biochem. biophys. Acta* **219**, 248.
Ussing, H. H. 1949 *Acta physiol. scand.* **17**, 1.
Ussing, H. H. 1965 *Harvey Lect.* **59**. 1.
Voute, C. L. & Ussing, H. H. 1968 *J. Cell Biol.* **36**, 625.
Voute, C. L. & Ussing, H. H. 1970 *Expl Cell Res.* **62**, 375.
Zadunaisky, J. A., Candia, O. A. & Chiarandini, J. 1963 *J. gen. Physiol.* **47**, 393.
Zerahn, K. 1969 *Acta physiol. scand.* **77**, 272.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

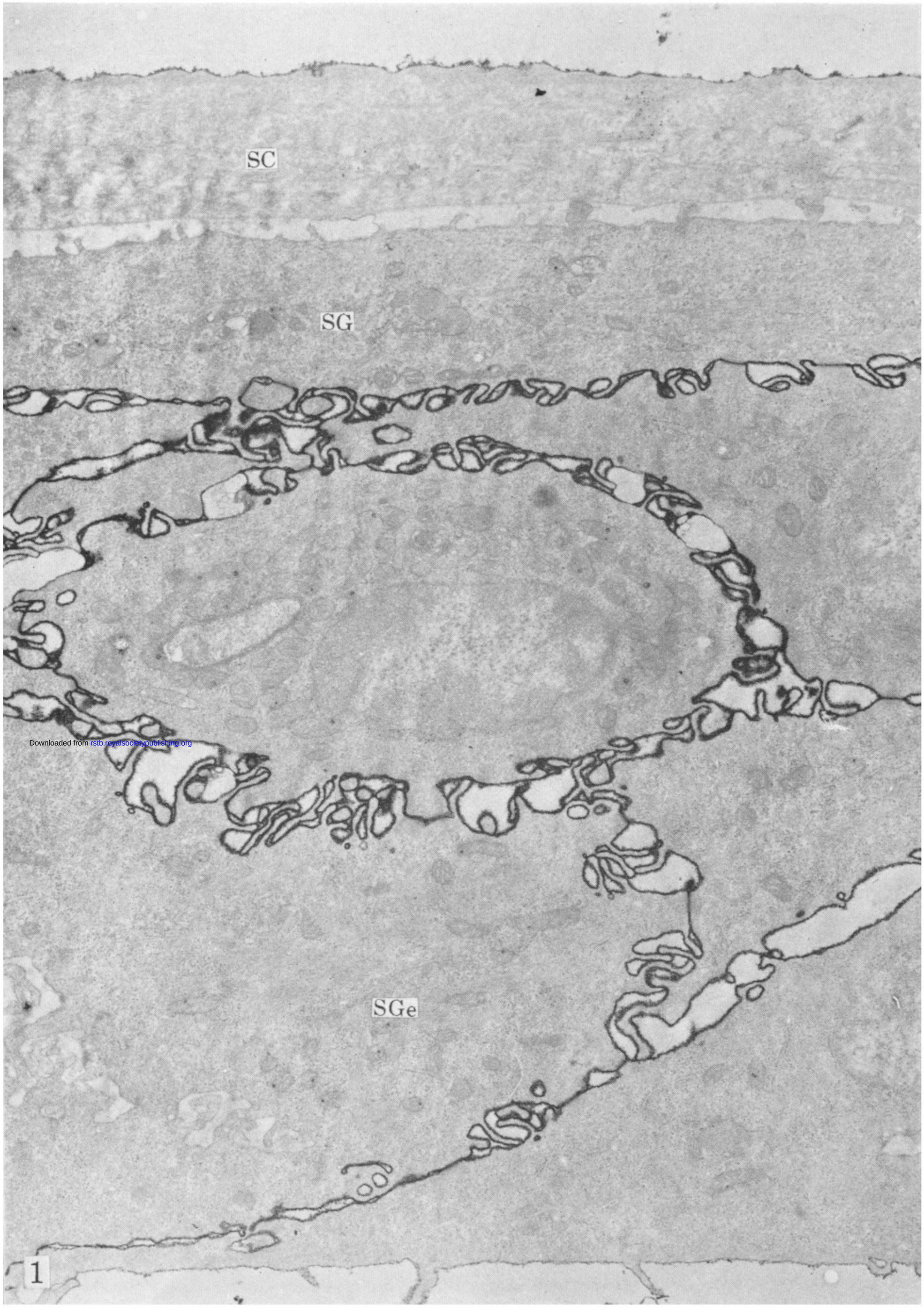
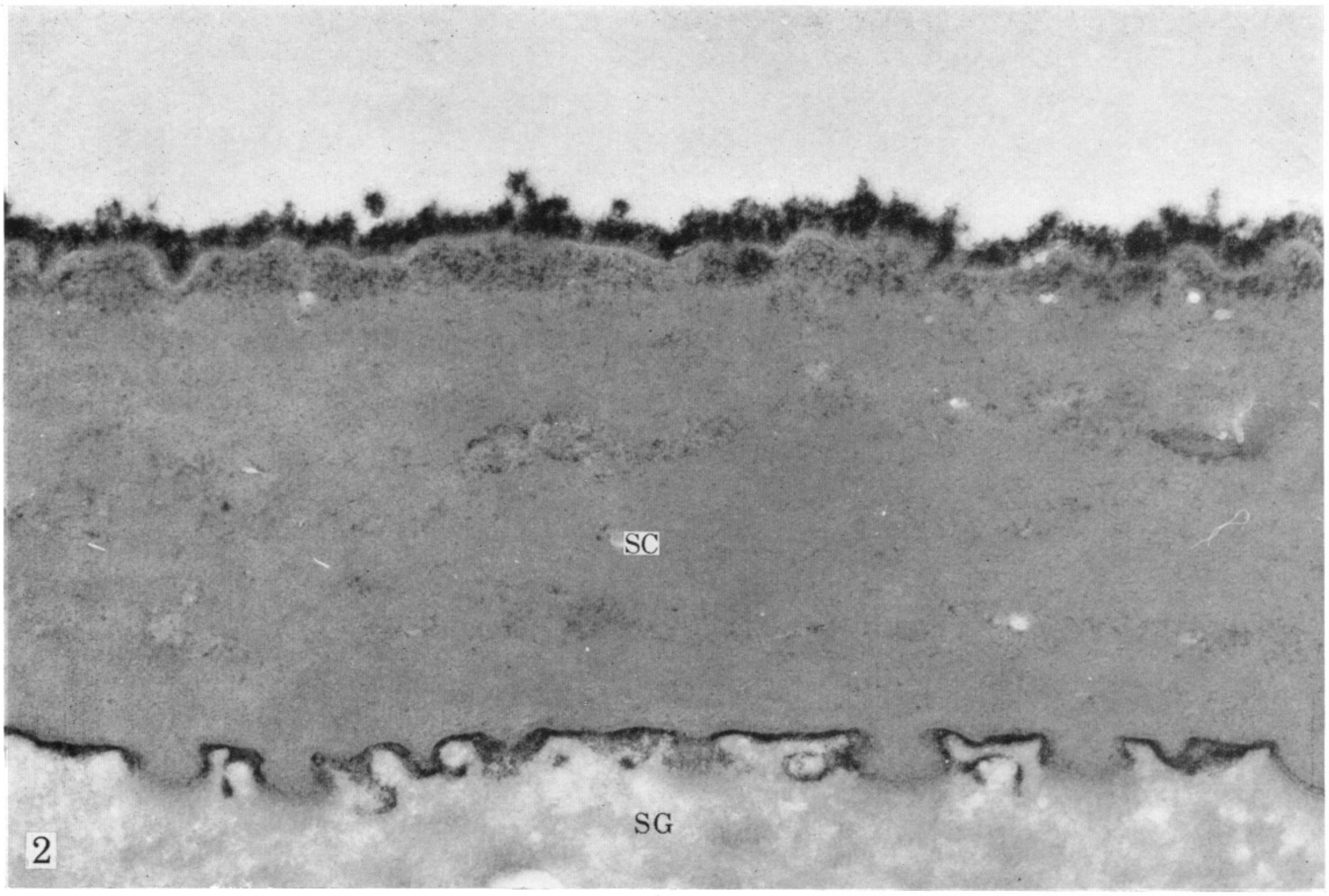
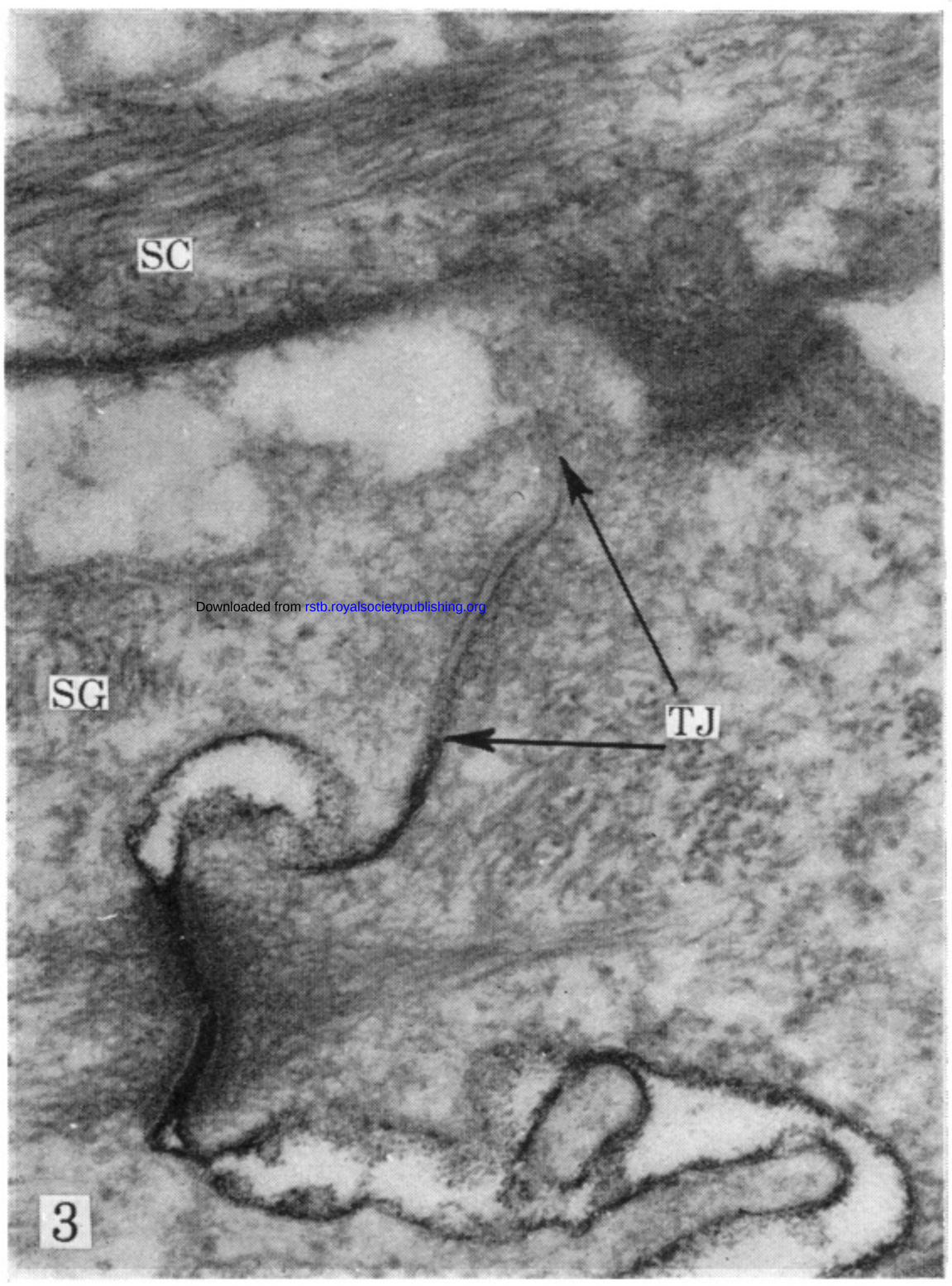


FIGURE 1. Isolated epithelium of the frog skin. Notice the adequate preservation of epithelial cells. No basement membrane is seen under the stratum germinativum cells (SGe). Ruthenium red has penetrated into the intercellular spaces forming a dense deposit on the cell membranes; however, no dense deposit is seen in the intercellular space separating the stratum corneum (SC) from the stratum granulosum (SG). Glutaraldehyde, osmium tetroxide, ruthenium red, Epon, lead citrate. (Magn. $\times 9000$.)





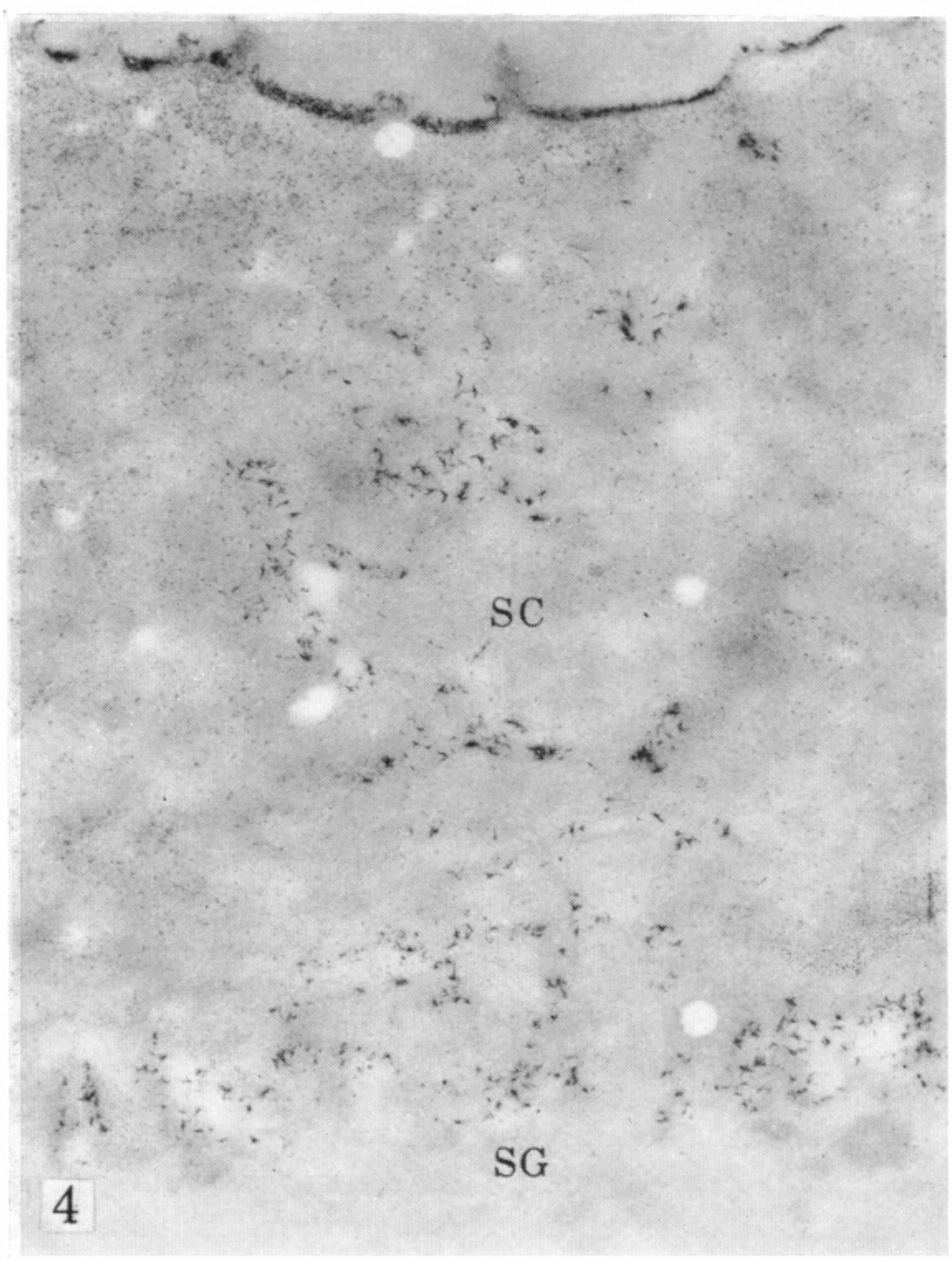


FIGURE 2. Frog skin treated with colloidal lanthanum. A dense precipitate is seen at the surface coat of the stratum corneum (SC) in the cytoplasm of the cornified cells and in the intercellular space separating the s. corneum from the stratum granulosum (SG). Glutaraldehyde, osmium tetroxide and colloidal lanthanum, Epon, lead citrate. (Magn. $\times 18000$).

FIGURE 3. Tight junction (TJ) between two cells of the s. granulosum (SG) Ruthenium red is prevented from entering into the first intercellular space by the fusion of cell membranes at the junction. Similar results are obtained when colloidal lanthanum or lanthanum chloride are applied from the inside. Glutaraldehyde, osmium tetroxide and ruthenium red. Epon, lead citrate. (Magn. $\times 40000$.)

FIGURE 4. Frog skin treated from the outside with lanthanum chloride. A finely granular or microcrystalline precipitate is seen at the surface coat of the s. corneum (SC) and in the first intercellular space. No precipitates are seen in the cytoplasm of the s. granulosum cells. Lanthanum chloride, glutaraldehyde, Epon. Unstained section. (Magn. $\times 21000$.)