Electron Microscopy: Sodium Localization in Normal and Ouabain-Treated Transporting Cells

Abstract. *When rabbit corneas are fixed in an osmic acid fixative to which potassium pyroantimonate has been added, an electron-opaque precipitate of sodium pyroantimonate is localized along the cellular side of the lateral plasma membrane of the endothelial cells. This heavy precipitate represents areas of high Na^+ concentration and may represent the site of a saturated, high-affinity Na^+ carrier. Significantly heavier precipitate occurs at the same site when ouabain is injected into the aqueous humor prior to fixation of the cornea.*

After removal of approximately 0.2 ml of aqueous humor from each eye of rabbits anesthetized with Diabutal, approximately 0.2 ml of artificial aqueous humor (*1*) was injected into the anterior chamber of one eye and 0.2 ml of ouabain ($3 \times 10^{-4}M$) (*2*) was injected into the anterior chamber of the other eye. At regular intervals before and after these injections, corneal thickness was measured with an optical pachometer (*3*). The thickness of the control corneas did not change after injection, whereas the thickness of the ouabain-treated corneas increased 20 to 30 percent within approximately 40 minutes. Corneas were fixed *in situ* with 1 percent OsO_4 + 2 percent $KSb(OH)_6$ in an acetate buffer (*p*H 7.6) by the method used by Komnick to demonstrate sodium in the avian salt gland (*4*). Both control and ouabain-treated corneas show localization of an electron-opaque precipitate of what is probably $NaSb(OH)_6$ along the cellular side of the lateral plasma membrane of the endothelial cells (Figs. 1 and 2). In the control corneas (Fig. 1), as in untreated corneas, this localization is spotty; in the treated corneas (Fig. 2) the heavy precipitate almost covers the entire inner surface of the lateral membrane. In addition, in treated corneas there is a moderate quantity of precipitate along the basal and apical membranes of the endothelial cells. In the controls these sites show no precipitate.

The increased precipitate following the ouabain treatment is significant in relation to previous findings on transport in this tissue (*5*), on the effects of ouabain on transporting systems (*6*, *7*), and on current models of hypothetical carriers in Na^+ transport (*7*, *8*). The lead phosphate end product of the adenosine triphosphatase reaction localizes along the extracellular side of the lateral plasma membrane of corneal endothelial cells (*9*). Biochemical studies (*10*) have shown that ouabain is a potent inhibitor of so-called transport enzymes (Na^+- and K^+-activated adenosine triphosphatases). The inhibitory effect of ouabain on transport in the cornea has been shown by studies on corneal swelling, Na^+ flux, and pinocytosis (*6*). The effects of ouabain in this system are reversible at concentrations below $10^{-4}M$ (*6*). In the experiments reported here dilution of the ouabain in the anterior chamber and leakage of ouabain from the eye through the injection path (which in all cases was rapid) reduce the effective ouabain concentration by a factor of at least 10.

According to models of Na^+ transport (*7*, *8*), a carrier would necessarily have a high affinity for Na^+ on the cellular side of the membrane and would be converted to a low Na^+-affinity state at the extracellular

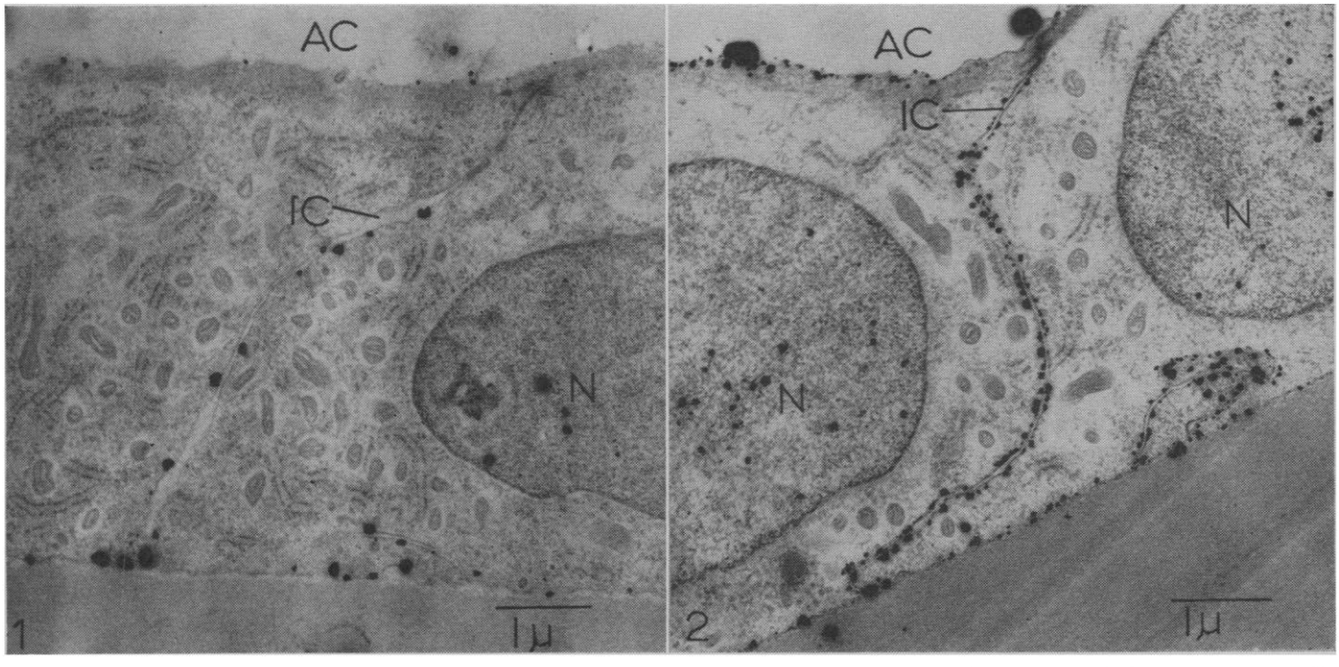

Fig. 1 (left). Electron micrograph of two adjacent endothelial cells of a control cornea fixed in 1 percent OsO_4 + 2 percent $KSb(OH)_6$. The distribution of $NaSb(OH)_6$ is spotty and follows the cellular side of the lateral plasma membrane. *N*, nucleus; *AC*, anterior chamber; *IC*, intercellular space. Fig. 2 (right). Electron micrograph of two adjacent endothelial cells of the right cornea of the same rabbit whose control cornea is shown in Fig. 1. Ouabain (0.2 ml; $3 \times 10^{-4}M$) was injected into the anterior chamber of this eye and the cornea was fixed in 1 percent OsO_4 + 2 percent $KSb(OH)_6$. This cornea had swelled approximately 30 percent between the time of injection and the time of fixation. There is a heavy precipitate along the entire length of the cellular side of the lateral plasma membrane. In addition, there is some precipitate at the apical and basal surfaces of these cells. The increased precipitate after ouabain treatment may represent saturation of Na^+ carrier in the membrane after inhibition of transport. *N*, nucleus; *AC*, anterior chamber; *IC*, intercellular space.

side of the membrane. Some evidence suggests that adenosine triphosphate (ATP) may be associated with the carrier in its high Na^+-affinity state and that conversion of the carrier to a low Na^+-affinity state is the result of the hydrolysis of the ATP by adenosine triphosphatase (*7, 8*).

The precise localization of the lead phosphate end-product of the adenosine triphosphatase reaction (*9*) and $NaSb(OH)_6$ (Figs. 1 and 2) on opposite sides of the lateral plasma membrane of corneal endothelial cells supports this theory. The increase of $NaSb(OH)_6$ precipitate after exposure of the cornea to ouabain may be the indirect result of adenosine triphosphatase inhibition. If this inhibition stops the removal of Na^+ from the carrier, all of the carrier may, therefore, remain in its high Na^+-affinity state and continue to concentrate Na^+ on the cellular side of the membrane until the carrier is saturated. The precipitate which occurs at the basal and apical membranes after ouabain treatment may indicate sites which are capable of transport but which are not active under normal conditions.

GORDON I. KAYE
JEANNE D. COLE
Division of Surgical Pathology, College of Physicians and Surgeons, Columbia University, New York 10032
ANTHONY DONN
Institute of Ophthalmology, Presbyterian Hospital, New York

References and Notes

1. B. Schwartz, B. Danes, P. J. Leinfelder, *Am. J. Ophthalmol.* **38**, 182 (1954).
2. Ouabain (G-strophanthin) USP for intravenous injection in phosphate-buffered saline solution.
3. D. M. Maurice and A. A. Giardini, *Brit. J. Ophthalmol.* **35**, 169 (1951).
4. H. Komnick and U. Komnick, *Z. Zellforsch.* **60**, 163 (1963).
5. G. I. Kaye and G. D. Pappas, *J. Cell Biol.* **12**, 457 (1962); ———, A. Donn, N. Mallett, *ibid.*, p. 481; G. I. Kaye, *ibid.* **15**, 241 (1962); A. Donn, D. M. Maurice, N. Mills, *Arch. Ophthalmol.* **62**, 741, 748 (1959).
6. B. Lambert and A. Donn, *Arch. Ophthalmol.* **72**, 525 (1964); G. I. Kaye and A. Donn, *Invest. Ophthalmol.* **4**, 844 (1965).
7. J. C. Skou, *Progr. Biophys. Mol. Biol.* **14**, 133 (1964); H. J. Schatzmann, *Helv. Physiol. Acta* **11**, 346 (1953).
8. W. Wilbrandt and T. Rosenberg, *Pharmacol. Rev.* **13**, 109 (1961); W. Wilbrandt, *Intern. Rev. Cytol.* **13**, 203 (1962).
9. G. I. Kaye and L. Tice, *Anat. Record* **148**, 298 (1964); *Invest. Ophthalmol.*, in press.
10. S. L. Bonting, L. L. Caravaggio, N. M. Hawkins *Arch. Biochem. Biophys.* **98**, 413 (1962).
11. Supported in part by PHS grants GM-11343 and NB-02343 and grant P-362 of the American Cancer Society. G.I.K. is recipient of a career scientist award of the Health Research Council of the City of New York (contract I-320).

2 September 1965

Sulfur: Incorporation into the Transfer Fraction of Soluble Ribonucleic Acid

Abstract. *When S^{35}-labeled soluble RNA from* Escherichia coli *K 38 is subjected to gel filtration, four fractions of RNA are obtained by elution. Only one RNA fraction, the transfer RNA, contains sulfur, presumably as thionucleotides. Treatment with ribonuclease suggests that the incorporated sulfur is an integral part of the polynucleotide chain; digestion with alkali yields a mixture of products containing sulfur, the major one being eluted in a position similar to uridine diphosphate upon Dowex-1 chromatography. Analysis by countercurrent distribution of S^{35}-labeled transfer RNA from* E. coli *B reveals that the incorporated sulfur is found in many RNA's that accept amino acids, but the possibility remains that not all acceptor RNA's contain sulfur.*

The presence of sulfur-containing nucleotides in the sRNA (*1*) of *Escherichia coli* has recently been established (*2, 3*). However, the sRNA of several strains of *E, coli,* prepared by phenol extraction and elution from DEAE-cellulose with molar NaCl, is a composite of RNA's of various sizes, only one of which has the biologically important ability to accept amino acids (*4, 5*). In view of this, experiments were undertaken to determine the distribution of radiosulfur in the fractions of differing sizes in sRNA from *E. coli* K 38 (*6*).

Figure 1 shows the elution patterns after gel filtration on Sephadex G-100 equilibrated with 1.0*M* NaCl (column, 0.9 by 150 cm, *7*) of H^3-uracil-labeled sRNA and S^{35}-labeled sRNA (*8*). The sRNA's were obtained by phenol extraction (*9*) of *E. coli* K 38 grown to the late logarithmic phase in Difco leucine-assay medium (10 g/liter) containing 0.1*M* phosphate buffer (*p*H 7.0) supplemented with either H^3-uracil (5 μc/ml, 8 μg/ml) or S^{35}-sulfate (25 μc/ml, total sulfate 0.4m*M*). The H^3-uracil labels elution region 1, which is a blend of messenger and ribosomal RNA (*4*); region 2, which contains an RNA of unknown identity (*4*); region 3, which is an RNA of major nucleotide composition more similar to tRNA than to either messenger or ribosomal RNA, but lacking minor nucleotides (*4*); and region 4 which is tRNA (*4*). Such a distribution of H^3-uracil indicates biosynthesis of each of these fractions. The S^{35} label, on the other hand, appears at elution region 4 (the tRNA) and is also present to the extent of 30 percent of the total S^{35} radioactivity in elution region 5, the low-molecular-weight area of the elution profile.

That sulfur is an integral part of the RNA emerging in elution region 4 is illustrated in Fig. 2, which shows the gel filtration pattern of S^{35}-labeled sRNA digested with ribonuclease. Digestions were carried out by addition of 50 μg of ribonuclease A per milliliter (Worthington, protease free) to the S^{35}-labeled sRNA solution (2 mg/ml) in 1.0*M* NaCl and subsequent incubation of the mixture at 35°C for 27 hours. All of the label (*8*) shifts with the

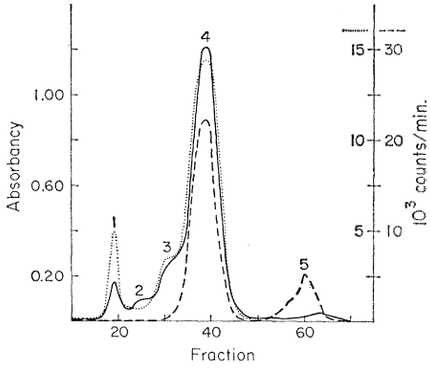

Fig. 1. Elution profiles for S^{35}-labeled sRNA and H^3-uracil–labeled sRNA after gel filtration on Sephadex G-100. Solid line, absorbancy at 260 mμ; dotted line, radioactivity of H^3, counts per minute; broken lines, radioactivity of S^{35} counts per minute. The presenece of H^3-uracil in region 5 was not determined.

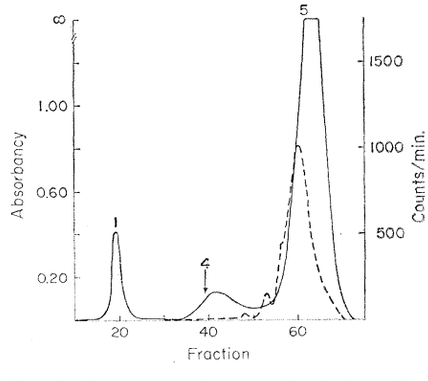

Fig. 2. Elution profile (on Sephadex) for ribonuclease-digested S^{35}-labeled sRNA. Peak 1 (void volume), in this instance, is the absorbancy of blue-dextran dye which is added to the digestion mixture prior to gel filtration. Solid line, absorbancy at 260 mμ; broken line, radioactivity, counts per minute.