Sodium-potassium dependent inorganic
\( ^{35}S \)-sulfate incorporation into cornea

Bernard Wortman and Raymond K. Locke

Cornea requires the presence of sodium and potassium ions in the medium for in vitro incorporation of inorganic \(^{35}S\)-sulfate. The effects of other cations and chemical inhibitors of Na\(^+\)-K\(^+\) activated ATPase are also demonstrated. The possible role of Na\(^+\)-K\(^+\) activated ATPase, of sulfate transport, and of protein-polysaccharide interactions is discussed with relation to corneal physiology.

Key words: corneal hydration, corneal swelling, active biological transport, sulfate ions, sodium, potassium, mucopolysaccharides, ouabain, chlorpromazine, pharmacodynamics.

Transcorneal potentials have been reported by several laboratories and have been related to an active transfer of sodium ions across the cornea. Both epithelial and endothelial cells have been implicated as sites of a metabolic pump. The active transport of ions across the cornea is involved in the maintenance of hydration, thickness, and transparency. The effect of these ions may be indirect and may involve the presence of sulfated-proteinpolsaccharides which behave as polyanions. When the corneal stroma are denuded of epithelial and endothelial cells, their in vitro incorporation of inorganic sulfate and of amino acids is less than normal; therefore, the stroma show some dependency upon these cells. It is unlikely, however, that the epithelial and endothelial cells synthesize the stromal macromolecules. It is more likely that these cells exert an indirect control over the stromal cells, which are the primary sites of biosynthesis of the macromolecules found in the extracellular spaces. This communication demonstrates one possible control mechanism which may act in the cornea. The in vitro incorporation of inorganic \( ^{35}S \)-sulfate into the cornea appears to be cation dependent and is probably related to Na\(^+\)-K\(^+\) activated ATPase and transport phenomena.

Materials and methods

Incubation medium. A balanced salt solution was used for the basic incubation medium (see Table I for composition). Glucose was present in a concentration of approximately 5.6 mM and served as the only possible exogenous energy source. Potassium or sodium salts of the various components of the incubation medium were substituted according to the experimental design; additional sodium or potassium was added as the chloride (Fig. 1). Tris-(hydroxymethyl)aminomethane base or ammonium chloride was used to maintain osmolarity when NaCl and KCl were omitted from the medium. The inorganic \( ^{35}S \)-sulfate was added as carrier-free \( ^{35}S \)-sulfuric acid (10 mc. \( ^{35}S \)-sulfate per milliliter).

Na\(^+\), K\(^+\), and water contents. Guinea pig corneas were analyzed for water, Na\(^+\), and K\(^+\) contents before and after incubation. The corneas were taken to dryness at 105° C. and the residue was used for Na\(^+\) and K\(^+\) analyses. The corneal...
Fig. 1. Effect of varying the concentration of NaCl or KCl on inorganic 35S-sulfate uptake. NaCl was held at 116.2 mM, and KCl concentration was varied over the range shown (x—x); KCl was held at 5.4 mM, and NaCl concentration was varied over the range shown (O—O). Approximately 3.5 μc 35S-sulfate per milliliter of incubation medium was added to each flask. Incubation medium contained: 116.2 mM NaCl; 5.4 mM KCl; 0.8 mM MgSO4; 1.0 mM NaH2PO4; 5.6 mM glucose; 1.8 mM CaCl2; 26.2 mM NaHCO3; pH 7.2 to 7.6. Potassium or sodium salts of the various components were substituted according to the design of the experiment. Each experimental point is based on two incubation flasks which contained two corneas each.

Tissue preparation and radioactivity detection. Pairs of fresh guinea pig corneas were incubated in 10 ml. of media at 37°C. At the end of the incubation period corneas were frozen to stop metabolic sulfate incorporation, washed three times with water, and incubated for 3 hours at 37°C in 0.1M Na2SO4 to remove the unincorporated sulfate ions from the tissue. The corneas were then dissolved in NCS (Nuclear-Chicago Corp.) reagent, and the radioactivity detected by scintillation counting. The reported values are corrected for background counts.

Results

The in vitro incorporation of inorganic 35S-sulfate into cornea is linear during the first 3 hours of incubation under the conditions of this study. Therefore, all experiments are based on 3 hours of incubation at 37°C and 4°C. The latter temperature served as the control to indicate the washout of free inorganic sulfate at the end of the incubation (Table I).

Na+, K+, and water contents. In the absence of Na+ or K+ from the incubation medium, less than 2 per cent and 21 per cent of endogenous corneal Na+ and K+, respectively, is retained after 3 hours (see Table I). This is accompanied by an inhibition of sulfate incorporation into protein-polysaccharides. Visual examination revealed that corneal swelling had also occurred. Previous studies have documented the inverse relationship between corneal hydration and Na+ transport. The higher Na+ content of cornea found after incubation suggests that more Na+ binding sites have been made available as a result of these experimental conditions. This would result in a change in the ionic content of the cornea or an alteration of active trans...
Table I. Water, Na\(^+\) and K\(^+\) content of whole guinea pig cornea

<table>
<thead>
<tr>
<th>Condition</th>
<th>Water (%)</th>
<th>Na(^+) (meq./Kg. H(_2)O)</th>
<th>K(^+) (meq./Kg. H(_2)O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preincubation*</td>
<td>78.8</td>
<td>115.0</td>
<td>37.1</td>
</tr>
<tr>
<td>Incubated(f)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na(^+), K(^+)</td>
<td>82.1</td>
<td>204.0</td>
<td>34.0</td>
</tr>
<tr>
<td>Na(^+), minus K(^+)§</td>
<td>83.2</td>
<td>225.0</td>
<td>7.1</td>
</tr>
<tr>
<td>K(^+), minus Na(^+)|</td>
<td>85.3</td>
<td>2.6</td>
<td>45.0</td>
</tr>
</tbody>
</table>

*Values are the average of 2 to 4 corneas per determination; corneas were frozen immediately upon removal and stored for analysis.
\(f\) Incubation for 3 hours at 37° C. in 10 ml. of the appropriate medium.
§ Medium contained 143.5 mM. Na\(^+\) and no K\(^+\).
\| Medium contained 32.6 mM. K\(^+\) and no Na\(^+\).

Table II. Effect of cation substitution on inorganic \(^{35}\)S-sulfate incorporation into cornea*

<table>
<thead>
<tr>
<th>Substituted cation</th>
<th>(^{35})S (c.p.m./mg. wet tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (4° C.)</td>
<td>7</td>
</tr>
<tr>
<td>None (37° C.)</td>
<td>144</td>
</tr>
<tr>
<td>Minus Na(^+)(f)</td>
<td>48</td>
</tr>
<tr>
<td>Minus K(^+)(f)</td>
<td>13</td>
</tr>
</tbody>
</table>

*Guinea pig corneas were incubated for 3 hours in 10 ml. of the basic medium at 37° C. (see Table I) containing inorganic \(^{35}\)S-sulfate, 3.3 \(\mu\)c per milliliter. Na\(^+\) and K\(^+\) salts were substituted according to the experimental design.
\(f\) The concentration of K\(^+\) was held at 5.4 mM and the cations listed were substituted for Na\(^+\) at a concentration of approximately 116 mM.

Effect of Na\(^+\), K\(^+\), and other cations. KCl concentration was kept at 5.4 mM and NaCl concentration was varied over the range of 0 to 225 mM. In the absence of NaCl, the incorporation of inorganic \(^{35}\)S-sulfate was insignificant. The stimulatory effect of NaCl was observed over a broad range of concentrations with a maximum stimulation between 75 and 125 mM concentration. The corollary experiment was to keep the concentration of NaCl at 116.2 mM and vary the concentration of KCl over the range of 0 to 125 mM. The optimal concentration of KCl is in a much smaller range than that of NaCl and peaks at approximately 15 mM. KCl. Half maximal activities were at approximately 4 and 40 mM. KCl and NaCl, respectively (Fig. 1).

When tris-(hydroxymethyl)aminomethane, lithium, rubidium, thallium, and ammonium chlorides were substituted for NaCl in the presence of 5.4 mM. KCl, tris\(^+\) and Li\(^+\) had a slight stimulatory effect. However, only RbCl substituted for KCl in the presence of 116.2 mM. NaCl (Table II).

Effect of ouabain and chlorpromazine. These two drugs were tested over the range shown in Fig. 2; at concentrations between 10\(^{-6}\)M and 10\(^{-5}\)M they caused 50 per cent inhibition of inorganic \(^{35}\)S-sulfate incorporation. The calculated p\(_{50}\) values of ouabain and chlorpromazine were approximately 5.7 and 5.0, respectively (see Fig. 2), which is in the range of the p\(_{50}\) values reported for these drugs with respect to partially purified Na\(^+\)-K\(^+\) activated ATPase.\(^9\)

The effect of the Na\(^+\)/K\(^+\) ratio on inorganic \(^{35}\)S-sulfate incorporation. At each of the three concentrations of NaCl tested, there is a single concentration of K\(^+\) necessary for optimal incorporation of inorganic \(^{35}\)S-sulfate. When expressed as a Na\(^+\)/K\(^+\) ratio, the maximum incorporation of inorganic \(^{35}\)S-sulfate occurs at a 7.7 ratio (Fig. 3). This value is in accord with the ratio required for maximal activity of Na\(^+\)-K\(^+\) activated ATPase in rabbit intestinal mucosa.\(^10\)

Discussion

These experiments were designed to show that control mechanisms are involved in the in situ incorporation of inorganic sulfate into corneal proteinpolysaccharides.
The biosynthesis of the macromolecules takes place at intracellular locations in fibroblasts, such as the Golgi apparatus. Among the first levels of control would normally be the cell membrane through which the essential precursors must pass to reach the intracellular sites where biosynthesis may then take place.

Bonting, Simon, and Hawkins have demonstrated that a high specific activity of Na⁺-K⁺ activated ATPase is found in the membranes of corneal epithelial cells. The activity is more than ten times greater in epithelium than in stroma when expressed on a wet tissue weight basis (see Table III). The epithelium contributes approximately 10 per cent of the total wet weight of the cornea and contains at least twice as many cells as the stroma. On the basis of such information, it can be estimated that stromal cells have an enzyme activity within the range of epithelial cells, but neither is as active as endothelial cells. This type of calculation does not consider the volume of cellular membranes in the two structures. Na⁺-K⁺ activated ATPase is localized in cell membranes in which it may exert its regulatory effects. Calculations to illustrate that stromal cells are metabolically active cells have been presented in numerous reports. The stromal cell is probably as active as the epithelial cell when evaluated on an individual basis.

At least two metabolic systems compete for the available corneal ATP and are pertinent to this discussion. The Na⁺-K⁺ activated ATPase uses ATP in a transfer function at a particular rate according to one set of kinetics. The sulfate-activation system uses ATP according to the kinetics of another set of reactions. The sulfate ion must be put into an "active" form before it is incorporated into macromolecules. Activation requires 2 moles of ATP per
Table III. Corneal Na\(^+\)-K\(^+\) activated ATPase

<table>
<thead>
<tr>
<th></th>
<th>Wet weight (mg)(^a)</th>
<th>No. of cells (×10(^6))(^b)</th>
<th>mmoles/mg wet tissue/hour(^c)</th>
<th>Total activity in mmoles/tissue</th>
<th>mmoles/10(^6) cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelium</td>
<td>70-90</td>
<td>400-1,000</td>
<td>13.3</td>
<td>931-1,195</td>
<td>2.3-1.2</td>
</tr>
<tr>
<td>Stroma</td>
<td>400-600</td>
<td>200-500</td>
<td>1.0</td>
<td>400-600</td>
<td>2.0-1.2</td>
</tr>
<tr>
<td>Endothelium</td>
<td>7-8</td>
<td>10-20</td>
<td>6.9</td>
<td>48-55</td>
<td>4.8-2.8</td>
</tr>
</tbody>
</table>

\(^a\)Estimated wet weight and number cells in ox cornea.\(^9\)
\(^b\)Specific activity reported for cat cornea.\(^9\)

mole of sulfate ion in order to produce one mole of 3'-phosphoadenosine 5'-phosphosulfate (active-sulfate).\(^9\) The inhibition of "transfer-ATPase" by drugs which duplicate the effect of Na\(^+\)-K\(^+\) deficiency should increase the amount of ATP available for other reactions. However, if ATP is present in quantities in excess of that required by both "transport ATPase" and "sulfate-activation," the inhibition of one system is not necessarily observed as the enhancement of the other. An excess of ATP may well exist in cornea, since an inhibition of "transfer-ATPase" by ouabain and chlorpromazine also leads to a decrease in sulfate incorporation rather than to an increase as might be expected. The synthesis of both "active-sulfate" and sulfated-proteinpolysaccharides can be demonstrated in cell-free systems and is independent of a Na\(^+\)-K\(^+\) requirement.\(^9\) One can only conclude from the present observations that in situ membrane phenomena are involved in the control of sulfate incorporation into proteinpolysaccharides of corneal cells.

It is possible to envision a membrane-controlled active transport of inorganic sulfate ions into stromal cells which could be a tremendous physiological advantage, enabling the cells to concentrate these ions from a dilute environment during periods of demand.\(^9\) Studies with Chlorella\(^16\),\(^17\) verify that sulfate ions can enter this organism against a concentration gradient and be retained as a nonexchangeable ion. These investigators have proposed the existence of an intracellular "effector" which controls sulfate ion absorption. The "effector" may appear in the presence of sulfate ions and disappear in the absence of sulfate ions. An earlier report\(^17\) demonstrated that corneal epithelial cells have the capacity to transfer sulfate ions from "active-sulfate" to an unidentified intermediate under a specific set of experimental conditions. A re-evaluation of such data raises the possibility that the cornea may also synthesize an "effector" substance which may be involved in the penetration of sulfate ions into corneal cells. Because of the large stromal extracellular space, studies with pure cell cultures would be necessary in order to determine whether inorganic \(^35\)S-sulfate can be concentrated intracellularly as a free ion prior to its incorporation into a larger molecule.

KCl and NaCl concentrations directly affect the incorporation of inorganic \(^35\)S-sulfate into cornea (Fig. 1). The higher incorporation in the absence of KCl in the medium may be explained on the basis of the information in Table I. Na\(^+\) can be almost completely washed out of the cornea; however, a residual amount of K\(^+\) does not wash out and is sufficient to effect inorganic \(^35\)S-sulfate incorporation. In addition, the cornea will concentrate Na\(^+\) at a level which is greater than that of its preincubation level, but a greater concentration of K\(^+\) than that of the incubation medium is observed only after incubation of cornea in the medium deficient in Na\(^+\) (Table I).

When the incorporation of inorganic \(^35\)S-sulfate is regarded as a function of Na\(^+\)/K\(^+\) ratio, the optimal activity occurs at 7.7 (Fig. 3). A similar Na\(^+\)/K\(^+\) ratio for maximal activation of rabbit intestinal mucosa ATPase has been reported by Richardson.\(^10\) K\(^+\) has significant stimulatory and inhibitory effects at lower concentrations than does Na\(^+\). The inhibitory effects of K\(^+\) are more pronounced than its stimulatory effects, as evidenced by the askew curve (Fig. 3). It
is conceivable that the observed control mechanism of sulfate incorporation into proteinpolysaccharides of the cornea is due to a cationic influence on the activity of corneal Na⁺-K⁺ activated ATPase coupled to an active transport of inorganic sulfate.

The techniques used in this study have produced data which closely parallel those obtained with Na⁺-K⁺ ATPase coupled with transport systems. The effects of a Na⁺-K⁺ activated ATPase inhibitor such as ouabain, and of an inhibitor of electron movement such as chlorpromazine, combined with a clear Na⁺-K⁺ dependency, support the concept that the movement of inorganic sulfate may be directly related to an active transport mechanism.⁹¹⁰ The inhibition of sulfate incorporation by these compounds may be explained as a direct interference with the mechanism of sulfate ion transport, as the result of cationic and drug interference with intracellular metabolic processes, or as the interference with regulation of energy production and biosynthetic mechanisms.¹⁵ However, the exact mechanisms remain unclear and invite further investigation of corneal control mechanisms as related to corneal physiology and their interrelationships with proteinpolysaccharides.⁷²⁰

The authors express their appreciation for the technical assistance of Jean Johnson and Bert E. Fry, Jr.

REFERENCES


