Enzymic Sulfation of Corneal Mucopolysaccharides by Beef Cornea Epithelial Extract*

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On an anatomical basis, cornea may be divided into three major parts: the fibrous stroma, the overlying epithelium, and endothelium. Epithelial and endothelial cells contribute approximately 20% of the total wet weight of beef cornea, and their integrity is essential for stromal maintenance (1). Of the three major parts of cornea, epithelial cells have the highest oxygen consumption that can be compared with that of liver (1). In agreement with the high oxygen consumption, the quantitation of glycolytic enzymes in cornea has shown higher activities in the epithelium than in the stroma (2, 3). A recent study has shown that all parts of beef cornea contain phenol sulfotransferase, which is also more active in the epithelium than in the stroma (4).

Gregory and Lipmann (5) suggested that phenol sulfotransferase might be coupled with other sulfotransferases so that it would act as a feeder system which could be assayed spectrophotometrically. When phenol and steroid sulfotransferases were tested as a coupled enzyme system, no transfer of sulfate to a steroid acceptor was detected because of an inhibitory effect of 3' phosphoadenosine 5' phosphate. Nevertheless, this mechanism cannot be eliminated as a feasible assay procedure. Its successful use would facilitate the characterization of sulfotransferases.

The present communication demonstrates the presence of phenol and mucopolysaccharide sulfotransferase activities in beef cornea epithelial extract. Furthermore, it reopens the possibility of using phenol sulfotransferase as a feeder system for the assay and characterization of mucopolysaccharide sulfotransferase.

EXPERIMENTAL PROCEDURE

Materials and Methods

Preparation of Beef Cornea Epithelial Extract—Epithelial cells were scraped from fresh beef corneas and homogenized in 4 volumes (weight per volume) of 0.005 M Tris-HCl, pH 7.4, and centrifuged for 1 hour at 34,800 × g. The supernatant fluid was dialyzed for 1 hour against 0.005 M phosphate buffer, pH 6.8-0.5 mM EDTA. At the end of the dialysis period, the extract was analyzed for protein concentration (6) and stored in small aliquots at −20°C. Some preparations were stored without dialysis. All operations were conducted in the cold (0-4°C), unless otherwise stated.

Isolation of Beef Cornea Mucopolysaccharides—Previously described methods were employed to isolate and purify corneal mucopolysaccharides (7). No attempt was made to separate the various types of mucopolysaccharides present in beef cornea, and therefore the isolated mixture was used as the sulfate acceptor in the present study.*

Chemicals—p-Nitrophenyl sulfate and p-nitrophenol were obtained from Sigma Chemical Company. PAP was present as a 2% contaminant in ADP lot 602 from Pabst Laboratories (5). p-Nitrophenyl sulfate-S35 was synthesized enzymically from p-nitrophenol and inorganic sulfate-S35 (obtained from Oak Ridge National Laboratories as carrier-free H2SO4-S35); beef corneal extract served as the source of phenol sulfotransferase and the sulfate-activating system (8). p-Nitrophenyl sulfate-S35 was separated from incubation mixtures by paper electrophoresis and chromatography. The product was eluted from the paper with water, and the radioactivity of the solution of p-nitrophenol sulfate S35 so obtained was determined by spotting an aliquot on Whatman No. 3 MM paper and measuring the radioactivity on the paper. No correction was made for absorption of radioactivity by the paper. The concentration of p-nitrophenol sulfate was determined indirectly by the spectrophotometric measurement of p-nitrophenol at 400 nm after hydrolysis of an aliquot of the p-nitrophenol sulfate-S35 in 1 N HCl for 10 minutes at 100°C. As measured by this procedure, the product contained 29 × 10⁶ c.p.m. per μmole of p-nitrophenyl sulfate.

Sulfotransferase Assay—The following reaction sequence is presented as the basis of the assay procedure used in this study:

Phenol Sulfotransferase—

p-Nitrophenyl sulfate + PAPS → p-nitrophenol + PAP + PAPS

Mucopolysaccharide Sulfotransferase—

PAPS + corneal mucopolysaccharides → "sulfated" corneal mucopolysaccharides + PAP

The transfer of sulfate is followed by the appearance of p-nitrophenol from p-nitrophenyl sulfate. The absence of PAP and corneal mucopolysaccharides is rate-limiting in the above reactions. Some characteristics of the sulfotransferases in beef cornea epithelial extract were studied, and the assay was based on the quantitative measurement of p-nitrophenol. At the end

* The preparation of corneal mucopolysaccharides contained: heparonic acid as glucuronic acid, 16.2%; hexosamine as glucosamine HCl, 27.7%.

† The preparation used were: EDTA, ethylenediaminetetra-acetate; PAP, 3' phosphoadenosine 5' phosphate; and PAPS, 3' phosphoadenosine 5'-phosphosulfate.
of a given incubation reaction which varied with the experimental design, strong alkali was added and the optical density recorded at 400 nm. The amount of p-nitrophenol formed from p-nitrophenyl sulfate during the enzymic transfer of sulfate was calculated from p-nitrophenol standards carried throughout all experimental procedures.

**Paper Electrophoresis and Chromatography**—Incubation reactions designed to demonstrate the enzymic transfer of sulfate-S\(^{35}\) were applied to Whatman No. 3MM paper sheets in either spots or bands. Descending chromatography was conducted in ethanol-ammonium acetate solvent, pH 7.4, at room temperature (9). Horizontal paper electrophoresis was conducted in 0.05 M citrate buffer, pH 4.5, at a gradient of approximately 12 volts per cm for 2 to 3 hours in the cold.

**Localization and Measurement of Radioactivity**—The presence of radioactivity was detected by the following procedures. The localization of radioactive substances on paper after electrophoresis and chromatography, as described above, was accomplished by direct contact with Kodak Blue Brand x-ray film for several hours. After the radioactive substances were located, an area of the paper containing the radioactivity was either counted directly or eluted with water, and an aliquot of the radioactive solution was counted. A sufficient number of counts was accumulated with a thin window gas flow detector (Nuclear-Chicago) so that the counting error was less than 3%; all experimental values were corrected for background counts.

**RESULTS**

**Acid Stability of Sulfate-S\(^{35}\)-containing Substances with Electrophoretic Mobility of Chondroitin Sulfate**—Extracts of epithelium, stroma, and endothelium from beef and rabbit corneas were found to synthesize a sulfate-S\(^{35}\)-containing substance with an electrophoretic mobility similar to that of authentic chondroitin sulfate. In this respect, extracts from the corneas of both species were similar; the results obtained with beef cornea epithelial extract are shown in Fig. 1. Approximately 50% of the S\(^{35}\) was hydrolyzed in 5 to 10 minutes in 0.1 N HCl at 38\(^{\circ}\) (Table I). In the case of beef cornea epithelial extract, approximately 44 To of the sulfate-S\(^{35}\)-containing substance remains charcoal-adsorbable after acid treatment. These data indicate that beef cornea epithelial extracts can synthesize a mixture of sulfate-S\(^{35}\)-containing compounds which demonstrate similar electrophoretic mobilities.
Table II
Transfer of sulfate-S35 from p-nitrophenyl sulfate-S35 to corneal mucopolysaccharides by beef corneal epithelial extract

<table>
<thead>
<tr>
<th>Incubation*</th>
<th>Radioactivity</th>
<th>S35O4- Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c.p.m.</td>
<td>μmole</td>
</tr>
<tr>
<td>1. Complete system, 3 hours at 38°</td>
<td>542</td>
<td>6.9</td>
</tr>
<tr>
<td>2. Complete system, 3 hours at 0-4°</td>
<td>66</td>
<td>0.8</td>
</tr>
<tr>
<td>3. Corneal mucopolysaccharides omitted, 3 hours at 38°</td>
<td>24</td>
<td>0.3</td>
</tr>
<tr>
<td>4. Boiled extract 3 hours at 38°</td>
<td>21</td>
<td>0.3</td>
</tr>
</tbody>
</table>

* Incubation mixtures contained: 30 μmoles of phosphate buffer, pH 7.8; 1.29 mg of corneal mucopolysaccharides; 497 μg of extract protein; and 0.1 mmole of p-nitrophenyl sulfate-S35, in a total volume of 251 μl. At the end of the incubation period, reaction mixtures were boiled for 3 minutes and chromatographed on paper as described in text.
† Radioactivity on a 2-cm diameter circle at the origin of the chromatogram was detected; recovery of S35O4- at the origin was based on activity of the p-nitrophenyl sulfate-S35 solution measured under conditions described in text.
‡ Less than 2%.

![Fig. 2. Effect of beef epithelial extract protein concentration on sulfate transfer. X—X, complete system; O—O, corneal mucopolysaccharides omitted; •—•, PAP omitted. (a) Incubation mixtures contained: 19.4 μmoles of phosphate buffer, pH 6.9; 54.8 mmoles of p-nitrophenyl sulfate; 0.2 μmole of PAP; 39 μg of corneal mucopolysaccharides; varying amounts of beef cornea epithelial extract protein. After incubation for 2 hours at 38°, 6 μl of 5 N NaOH were added and the optical density at 400 nm recorded. (b) NaF was added to all incubations to a concentration of 2.1 mM. (c) Beef corn epithelial extract, 1 ml, was dialyzed against 3 liters of 0.005 M phosphate buffer, pH 6.7-0.5 mM EDTA for 1 hour and assayed as in Fig. 2a. The abbreviation used in the figures is: NP, nitrophenol.](http://www.jbc.org/)

Effect of Incubation Time—The production of p-nitrophenol depends on corneal mucopolysaccharides. The addition of PAP at the lower concentration of extract protein produced some inhibition in the appearance of p-nitrophenol (Fig. 2a) increased activity in the absence of added PAP demonstrates that an adequate amount of PAP is present in the extract for sulfotransferase activity to manifest itself. At the highest concentration of extract protein, the activity was the same with or without added PAP. NaF caused an inhibition of appearance of p-nitrophenol at the highest extract protein concentration in the system containing exogenous PAP and an inhibition in the system not receiving added PAP at the lower protein level (Fig. 2b). Dialysis of epithelial extract in the presence of EDTA results in the removal of some PAP and divalent cation activators, e.g. Mg++ (Fig. 2c). A short period of dialysis is necessary because of an inactivation of phenol sulfotransferase with dialysis periods greater than 2 hours. After dialysis, a greater PAP and corneal mucopolysaccharide dependence was found, although there remains some increased activity at the higher extract protein concentration in the absence of added PAP. It was not always possible to remove endogenous PAP during short periods of dialysis.

Effect of PAP and Corneal Mucopolysaccharide Concentration—The endogenous concentration of PAP is at saturation levels for phenol sulfotransferase. As more PAP is added to the incubation, an inhibitory level is reached, until at approximately 5 μM exogenous PAP, only phenol sulfotransferase is functional, and the dependence on corneal mucopolysaccharides is no longer evident (Fig. 3a).

The addition of increasing amounts of corneal mucopolysaccharides to incubations showed that in the PAP-dependent transfer of sulfate, corneal mucopolysaccharides appear to have an optimal concentration in excess of 4 μg per μl of incubation volume (Fig. 3b).

![Fig. 3. Symbols are as in Fig. 2. (a) Effect of PAP concentration on sulfate transfer. Incubation mixtures contained: 19.4 μmoles of phosphate buffer, pH 6.9; 54.8 mmoles of p-nitrophenyl sulfate; 226 μg of corneal mucopolysaccharides; 192.5 μg of beef cornea epithelial extract protein; varying concentrations of PAP. After incubation for 2 hours at 38°, 6 μl of 5 N NaOH were added and the optical density at 400 nm recorded. (b) Effect of corneal mucopolysaccharides concentration on sulfate transfer. Incubation mixtures contained: 19.5 μmoles of phosphate buffer, pH 6.9; 55 mmoles of p-nitrophenyl sulfate; 0.2 mmole of PAP; 192 μg of beef cornea epithelial extract protein; varying concentrations of corneal mucopolysaccharides (CM). After incubation for 6 hours at 38°, 6 μl of 5 N NaOH were added and the optical density at 400 nm recorded.](http://www.jbc.org/)
polysaccharides proceeds at a rapid rate for the first hour and then at a lower rate for the remaining time (Fig. 4a). In the presence of exogenous PAP, a delay of 1 hour was followed by a rate almost equal to that of the system without added PAP. In the presence of fluoride, the addition of PAP produced slight additional effect until after the first hour, at which time exogenous PAP appeared to have a slight stimulatory effect (Fig. 4b).

**pH Activity Curves**—At pH values greater than 6.0, there was no detectable hydrolysis of p-nitrophenyl sulfate because of arylsulfatase activity (Fig. 5). At lower pH values, arylsulfatase activity is noted. In beef cornea epithelial extract, phenol sulfotransferase has its pH optimum between 7.0 and 7.2. Higher activity for the system dependent on corneal mucopolysaccharides was observed between 7.6 and 8.0, although most of the data reported in the present study were obtained at pH 6.9. At lower pH values, PAP dependency disappears, and the appearance of p-nitrophenol seems to be dependent on corneal mucopolysaccharides and possibly related to arylsulfatase activity.

**Discussion**

When the earlier observations were made (4), no obvious physiological function could be suggested for phenol sulfotransferase in beef cornea as could be in liver where it may serve to detoxify phenolic compounds (10). The existence of PAP, and of phenol and mucopolysaccharide sulfotransferases in beef cornea epithelium is strongly suggestive of their physiological role in the synthesis of sulfated mucopolysaccharides. Previous studies of inorganic sulfate-S\textsuperscript{35} incorporation have shown that rabbit corneal stroma is almost completely dependent upon an intact epithelium for incorporation of inorganic sulfate-S\textsuperscript{35}, but beef stroma is not (4). Additional species differences were found during a preliminary study of the sulfate-activating system (8) in extracts of parts of beef and rabbit corneas. The sulfated material synthesized by beef epithelial extract was probably a mixture of PAPS and sulfated mucopolysaccharides or some other sulfated polymer. These observations could also mean differences in the sulfation mechanism or in the distribution and in the concentration of necessary enzymic components in parts of corneas of these two species.

The present communication offers no specific information as regards the mechanism of synthesis in vivo of corneal mucopolysaccharides or at which site or stage of polymerization sulfate may be added or exchanged. The measurement of p-nitrophenol and the recovery of sulfate-S\textsuperscript{35} from p-nitrophenyl sulfate-S\textsuperscript{35} in a substance with a chromatographic R\textsubscript{P} similar to that of corneal mucopolysaccharides are not the proof of an enzymic transfer of sulfate. Nevertheless, corneal mucopolysaccharides are necessary for the appearance of p-nitrophenol from p-nitrophenyl sulfate. These facts strengthen the interpretation of the data to suggest an enzymic transfer of sulfate from p-nitrophenyl sulfate to corneal mucopolysaccharides via phenol and mucopolysaccharide sulfotransferases.

Measurement of mucopolysaccharide sulfotransferase is complicated by the presence of relatively large amounts of PAP in epithelial extracts. It is estimated that beef cornea epithelium contains at least 68 \textmu m moles of PAP per g of wet weight of tissue or almost 4 times as much as that reported for liver (5). PAP concentration of 2 \textmu M has been reported as the saturation level for phenol sulfotransferase, whereas concentrations above 20 \textmu M are inhibitory (11). Mucopolysaccharide sulfotransferase is inhibited by a much lower level of PAP than is phenol sulfotransferase (see Fig. 3b). It was estimated that incubation mixtures in the present study contained approximately 2 \textmu M endogenous PAP. Inasmuch as phenol sulfotransferase will function with PAP concentrations as low as 0.2 \textmu M (11), PAP dependency is not always as evident as is the dependency on corneal mucopolysaccharides.

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3 B. Wortman, unpublished observations.
neal mucopolysaccharides for the appearance of p-nitrophenol from p-nitrophenyl sulfate.

Suzuki and Strominger (12) have shown that chondroitin from beef cornea and chondroitin sulfate C produce the highest reaction rates when used as sulfate acceptors for mucopolysaccharide sulfotransferase in the hen oviduct. Chondroitin sulfate A demonstrated approximately one-half the maximal reaction velocity of the other two mucopolysaccharides, and keratosulfate was inactive in that system. The various mucopolysaccharides present in beef corneal stroma were not tested individually as sulfate acceptors in the corneal system used in the present study. Until the individual corneal mucopolysaccharides can be investigated separately, it is assumed that the preparation used in the present study contained chondroitin, chondroitin sulfate A, and keratosulfate in the proportions of 1:1:2 (7). If keratosulfate also does not accept sulfate in the corneal system, then less than 50% of the dry weight of corneal mucopolysaccharides would function as a sulfate acceptor. Chondroitin and chondroitin sulfate A functioned maximally in concentrations of approximately 1.5 and 1 µg per µl of incubation volume, respectively, in the hen oviduct system. It is interesting to note that the preparation of corneal mucopolysaccharides approaches a maximal reaction velocity when used as an acceptor in concentrations in excess of 4 µg per µl of incubation volume. If chondroitin were the only active sulfate acceptor in the corneal system, then 4 µg of corneal mucopolysaccharides per µl of incubation volume would represent approximately 1 µg of active acceptor compound per µl of incubation volume.

Beef cornea is known to contain arylsulfatases existing in multiple forms which can be inhibited by KH₂PO₄ and by NaF (13). In the present study, phosphate buffer was used routinely because of its inhibitory action on sulfatases. An interesting phenomenon can be observed at lower pH values which approach the optimal range of arylsulfatase. At pH values of less than 6.7, an increased content of corneal mucopolysaccharides in the reaction mixtures stimulated the appearance of p-nitrophenol from p-nitrophenyl sulfate and the dependency on PAP was no longer evident (see Fig. 5). Current searches for a function in vivo for arylsulfatases are in progress in other laboratories (14). Except for the transfer of sulfate to water, arylsulfatases have not been found capable of transferring inorganic sulfate from p-nitrophenyl sulfate to acceptor compounds, such as polysaccharides (15). Such possible functions for arylsulfatases in beef cornea merit further consideration.

SUMMARY

1. Phenol and mucopolysaccharide sulfotransferase activities have been demonstrated in beef cornea epithelial extract. These enzymes have been coupled to serve as an assay procedure for the study of the latter enzyme.
2. Beef cornea epithelial extract was used to synthesize a sulfate-S³⁵-containing substance with the same electrophoretic and chromatographic mobility as authentic chondroitin sulfate.
3. The dependency on corneal mucopolysaccharides for the appearance of p-nitrophenol from p-nitrophenyl sulfate is evident from the data. Under certain experimental conditions, a dependency on 3'-phosphoadenosine 5'-phosphate can be shown. 3'-Phosphoadenosine 5'-phosphate is inhibitory to mucopolysaccharide sulfotransferase at approximately 5 µM. Beef cornea epithelium contains a relatively large amount of 3'-phosphoadenosine 5'-phosphate which can be partially removed by dialysis.
4. Phenol and mucopolysaccharide sulfotransferase demonstrated pH optima at approximately 7.0 and 7.8, respectively. NaF and KH₂PO₄ were used as inhibitors of nucleotidase and sulfatase.
5. Beef cornea epithelial extracts also contain sulfate-activating enzymes which were not separated from sulfotransferases in this study.

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REFERENCES

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