Rabbit tear proteins.

I. Detection and quantitation of lysozyme in nonstimulated tears

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Rabbit tear lysozyme was detected in low concentration in rabbit tears collected without any stimulation. Quantitation of rabbit tear lysozyme was found possible through the use of sensitive techniques that measure its specific enzymic activity on the bacterial cell wall of Micrococcus lysodeikticus. Rabbit tear lysozyme has an electrophoretic mobility identical to that of hen egg-white lysozyme on cellulose and acrylamide gel. The experimental use of rabbits for tear lysozyme studies is discussed.

In human eye pathology, much attention has been paid to lysozyme in tears and its variation in pathological conditions, allergic manifestations, and autoimmune diseases. Recently, smog eye irritation has been correlated with a low tear lysozyme level and workers around fumes also have a reduced lysozyme concentration.

In order to be able to study the effect of environmental conditions on tear lysozyme, a suitable experimental animal is a necessity. A favored subject in experimental eye investigation has been the rabbit, but heretofore it has been reported that lysozyme was absent from rabbit tears.

This study seeks to determine whether rabbit tears contain lysozyme, and consequently whether the rabbit can be used to study the environmental effect of different experimental pathological conditions on the tear lysozyme. In this report, lysozyme will be identified: (1) by its lytic activity on Micrococcus lysodeikticus, (2) by its electrophoretic mobility as compared with hen egg-white lysozyme, and (3) by the fact that lysozyme can be efficiently adsorbed by bentonite.

Materials and methods

Experimental white albino rabbits of different ages and sexes were chosen randomly to determine the lysozyme content of their tears. Standard Schirmer strips or microcapillary tubes were used for tear collection. When microcapillary tubes were employed for tear collection, care was taken to collect only the tears that were already present in the external canthus of the eyes under the lids. The amount usually collected from each eye was 5 to 10 µl. When the Schirmer method was used, only the first few millimeters of the paper were allowed to moisten with tears. Either 2.5 µl tears or a 5 mm² Schirmer strip was enough for assaying lysozyme.

Protein determination. Protein concentration was determined by the Lowry method and bo...
Table I. Quantitative determination of lysozyme in rabbit tears

<table>
<thead>
<tr>
<th>Rabbit No.</th>
<th>Protein concentration (mg./ml. tears)</th>
<th>Schirmer plate</th>
<th>Smolelis and Hartsell</th>
<th>Cellulose acetate electrophoresis</th>
<th>After adsorption with bentonite</th>
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The radii of the zones of lysis obtained surrounding the putative lysozyme bands were measured. The amounts of rabbit tear lysozyme present in the tear samples tested were extrapolated from a reference standard curve prepared with various concentrations of pure hen egg-white lysozyme (HEL).

D. Acrylamide gel electrophoresis. Acrylamide gel electrophoresis was performed according to the method described by Reisfeld and colleagues, for the separation of basic proteins, with a voltage of 320 V, 5 Ma. per tube, for 45 minutes. Staining was done with 1 per cent Amido Schwartz for 1 hour and destaining was done electrophoretically with 7 per cent acetic acid for several hours.

E. Bentonite adsorption. Lysozyme has been found to be removable from serum by adsorption onto bentonite particles, because of its basic ionic characteristic. The rabbit tear samples were mixed with a small quantity of dry bentonite and then centrifuged. The supernatant was tested for the presence of lysozyme by the Schirmer lysoplate method.

Results

Lysozyme Quantitation in Rabbit Tears. The concentrations of lysozyme in non-stimulated rabbit tears are summarized in Table I. Eight rabbits were tested which were chosen randomly.

A. The Schirmer lysoplate method. In tears collected on Schirmer paper strips, the lysozyme concentration ranged between 60 and 170 µg per milliliter of tears with an average of 102 µg per milliliter. A control normal rabbit serum has a lysozyme concentration of 30 µg per milliliter of serum (Table I).
Equalizing the protein concentrations in tears and serum, we find that the lysozyme concentration in rabbit tears is about 14 times greater than in serum. The protein content of rabbit tears is greater than that of human tears; in the latter, the protein content is 7.2 mg. per milliliter of tears, whereas in rabbits it averages 12.6 mg. per milliliter. Human tear lysozyme (HTL) comprises 20 to 40 per cent of the total tear protein, whereas in rabbit tears, lysozyme comprises only 1 per cent.

B. The Smolelis and Hartsell method. The results obtained with this method agree very closely with those obtained with the use of Schirmer lysoplate method (Table I).

C. Cellulose acetate electrophoresis method. Rabbit tears upon electrophoresis on cellulose polyacetate strips give 6 to 7 bands. In only 1 out of 8 cases were we able to visualize a stained protein band that corresponded in position to a control hen egg lysozyme (HEL) band. This rabbit had the highest lysozyme concentration in its tears (170 µg per milliliter, rabbit No. 2).

Following the electrophoresis of tears on cellulose acetate strips, the strips were cut longitudinally into two halves. One half was layered on a Micrococcus plate and left to incubate 24 hours; the other half of the strip was stained. The lysozyme concentrations of the tear samples were extrapolated from the standard curve shown in Fig. 1 (Table I). The results with this assay show that: (1) the lysozyme content of each tear sample appears to be decreased relative to the Schirmer lysoplate method; and (2) the cathodal position of the lytic zone obtained from the tear samples corresponded to the electropho-
retic position of HEL, despite the absence of an observable, stained band on the strip.

D. Acrylamide gel electrophoresis. The normal pattern of rabbit tear proteins on acrylamide gel electrophoresis will be described elsewhere. Under conditions used for migration of basic proteins, rabbit serum gave many bands, whereas rabbit tears gave 8 bands, the most cathodal of which corresponded in migration to a sample of purified HEL (Fig. 2). When rabbit tears and HEL were mixed before layering the sample on the acrylamide tube, this most cathodal band was the only one which appeared more dense, since HEL staining was coincident with this band in rabbit tears.

E. Bentonite adsorption. Since bentonite has been found to adsorb lysozyme completely,\textsuperscript{12} treatment of the rabbit tear sample with bentonite should result in the absence of any lysozyme activity. This was found to be the case, as is shown in Table I, where the assay used was the Schirmer lysoplate.

Discussion

The results obtained from these experiments indicate the presence of a protein component in rabbit tears which (1) has an enzymatic activity on Micrococcus lysodeikticus cell walls, (2) migrates electrophoretically toward the cathode to a position identical with that of hen egg lysozyme (HEL), and (3) is removable upon bentonite adsorption. These properties are also shared by all lysozymes so far studied\textsuperscript{13}; thus the protein component presumably is a lysozyme.

The presence of lysozyme in rabbit tears was sought by several investigators, none of whom were able to demonstrate it in nonstimulated rabbit tears. Goldsworthy and Florey\textsuperscript{1} showed the absence of lysozyme in rabbit tears using a method based on the reduction in turbidity of a suspension of Micrococcus lysodeikticus as a measure of the amount of lysozyme. Erickson and colleagues\textsuperscript{2} used a filter paper electrophoresis technique and noticed the absence of lysozyme in rabbit tears; their
results were confirmed by McDonald, Leopold, and Sery.\textsuperscript{14} Kimura and colleagues\textsuperscript{15} reported the presence of lysozyme in tears when the rabbit eyes had been infected or irritated. The lysozyme concentration in rabbit tears is very low compared to that of humans.\textsuperscript{16, 17} However, its concentration is greater than in the circulation. Its isolation and purification from rabbit tears in sufficient quantity for characterization is not feasible; however, Jollès\textsuperscript{18} has isolated and purified lysozyme from rabbit spleen. Its over-all amino acid composition is quite different from HEL\textsuperscript{19} and human lysozymes.\textsuperscript{16, 14} Since it showed a similar electrophoretic mobility as HEL, we can assume that rabbit tear lysozyme has a net positive charge similar to that of HEL.

In the quantitation of rabbit tear lysozyme, it has been estimated that its specific enzymic activity is similar to HEL. In fact this was demonstrated with rabbit spleen lysozyme,\textsuperscript{18} which we assume has a similar enzymatic activity as rabbit tear lysozyme (RTL).

We have used methods based upon enzymatic activity to demonstrate the presence of lysozyme in rabbit tears. Even though our assay systems were basically identical with those of other investigators, they do differ in their sensitivity. We have used a method of measuring lysozyme in a final concentration of less than 10 µg per milliliter of solution.\textsuperscript{19} We have also shown that lysozyme detection solely by electrophoretic and staining techniques is not sensitive enough for detecting low levels of lysozyme. There exists a certain threshold below which paper electrophoresis techniques are not capable of detecting the relatively low level of lysozyme in rabbit tears. From our staining experiments on cellulose acetate strips after electrophoresis, we have shown that in 7 out of 8 cases it was impossible to detect the protein unless its enzymatic activity was utilized to localize its position on the strip.

The presence of lysozyme in rabbit tears permits the use of this valuable experimental animal in studies of the tear proteins. For example, it has recently been shown that lysozyme levels in human subjects with smog eye irritation are markedly decreased.\textsuperscript{7} Experiments under defined atmospheric conditions in rabbits may lead to an understanding of the causative agent(s) responsible for the alteration in the tear lysozyme level and possibly smog eye irritation.

We wish to thank Mr. Solomon Bonavida for his technical assistance.

REFERENCES

13. Jollès, P., Charlemagne, D., Petit, F. F.,


