markedly modified; no effect was seen at pressure below 40 mm. Hg, very small reduction occurred above that level which did not increase with higher IOP levels. This suggests that the mechanism of IOP effect on flow rate under this condition was greatly modified or virtually eliminated. Of interest is the finding that when ocular pressure level is raised above systolic arterial pressure in the intact preparation, the reduction in monitored flow rate is slightly greater than that obtained when the SPCA are ligated just outside the globe. This may reflect the inadequacy of the ligating technique.

It becomes of crucial importance to implicate nutrient flow in a more specific fashion. Here, the results of tissue $P_0_2$ studies are of great interest. This measurement reflects nutrient flow and is not sensitive to flow in adjacent large vessels. The results demonstrate that tissue $P_0_2$, 1 mm. deep into optic nerve substance 4 mm. behind the globe is dependent upon ocular pressure level. Of interest is the finding that as ocular pressure is elevated, there is initially an increase in $P_0_2$ followed by a decline. This increase may represent the expected increase in flow rate to extraocular branches of SPCA or those derived from the CRA and supply this region as the resistance of the intraocular portion of their vascular bed is increased with elevation of ocular pressure. However, at higher IOP levels, there is a marked reduction in tissue $P_0_2$ at this site which remains remarkable even after correcting for the simultaneous reduction in tissue temperature. The reduction in $P_0_2$ occurs at levels of IOP that are similar to those associated with rapid reduction in flow rate as monitored by the heated thermocouple technique. Thus, we cannot escape including nutrient flow as one of the components reflected in the heat conductivity measurement concluding that it is dependent upon ocular pressure level.

So far, the results indicated that the mechanism of IOP effect is intraocular, is sensitive to systemic arterial pressure and sensitive to SPCA ligation; the most likely target is the recurrent branches of the SPCA. It would appear, therefore, that flow rate at the monitored site and at least 1 mm. deep into optic nerve substance must receive a major contribution from these branches. The effect of ocular pressure on flow rate in these arterial branches will be small at lower levels of IOP until a level high enough is reached to overcome the intravascular pressure and, thereafter, produces great reduction in flow rate. This model will also explain the interaction between IOP level and systemic arterial pressure level described previously.1

From the clinical standpoint, it would seem that with moderate elevation of ocular pressure level there is slight increase in nutrient flow to the distal 6 mm. of the optic nerve. Whereas with marked elevation of IOP, there is great reduction in nutrient flow which might result in primary lesions of the optic nerve at this site due to significant vascular insufficiency and tissue anoxia. Anatomic studies and those of experimental glaucoma are in progress to elucidate this point and their results will be reported in future publications.

From the Department of Ophthalmology, The George Washington University Medical Center, 2150 Pennsylvania Ave., N.W., Washington, D. C. 20037. This investigation was supported in part by National Eye Institute Grant No. EY00692. Submitted for publication Jan. 17, 1975. Reprint requests: Dr. Armaly.

REFERENCE


A spectrophotometric micromethod for lysozyme determination in tears is described, which enables the estimation of very low concentrations of lysozyme in individual tear samples, and which
can be performed rapidly. The method is based on collecting the tears by a microcapillary tube and diluting them in a special manner which increases the volume of the tear sample, thus making possible analysis of other tear constituents in addition to lysozyme. Lysozyme activity in tears is determined by reduction of optical density (OD) of Micrococcus luteus suspension. The sensitivity of the method was determined on the basis of repetitive readings. The level of lysozyme in tears of 60 healthy people was determined by this method and found to be 6.1 mg. per milliliter hen egg lysozyme (HEL) with standard deviation of 1.57 mg. per milliliter HEL or 1.5 mg. per milliliter human tear lysozyme (HTL) with standard deviation of 0.39 HTL. Lysozyme level in tears of both eyes of each individual is equal, and any difference observed between the two eyes is due to the variability of the method.

Lysozyme is a mucolytic enzyme, widely distributed in nature, and may be found in tissues, vegetable, egg white, saliva, and white blood cells. It is a normal constituent of human tears, amounting to 30 per cent of the tear protein. In 1922, Fleming was the first to notice that the lytic activity of tears is due to the presence of lysozyme therein. The lytic action of the enzyme, which is expressed in the hydrolysis of the polymer-N-acetyl glucosamine N-acetyl muramic acid in the cell wall of bacteria, was used in several studies for quantitative determination of lysozyme. The lysoplate method described by Bonavida and Sapse is the most commonly used for routine determination of lysozyme in tears. By use of this method, a study on normal subjects demonstrated that lysozyme concentration in human tears is not affected by diurnal variation. Increasing age slightly affects lysozyme level in tears.

In this study, another routine method has been developed for determining lysozyme concentration in tears, which is (1) applicable to small quantities of tears; (2) sensitive to low levels of the enzyme; and (3) performed rapidly. This method was used to screen the lysozyme level in normal human tears.

**Material and methods.**

**Preparation of a standard curve of lysozyme activity.** Shugar’s method for determining lysozyme activity was modified by us and adapted to estimate the lysozyme level in human tears. This method is based on measuring the decrease in optical density (OD) of a suspension of Micrococcus luteus caused by lysozyme. The decrease in OD was determined by means of a Gilford spectrophotometer at a wave length of 450 mλ. Immediately before reading the activity of the enzyme, a fresh batch of substrate was prepared containing a mixture of 0.71 mg. of lyophilized freeze-dried *M. luteus* (Miles Laboratories) in 3 ml. phosphate buffer, pH 6.24, 0.15 M, per each lysozyme reading. Three milliliters of the substrate were placed in a cell within the spectrophotometer and the OD usually ranging from 1.0 to 1.2 was read at room temperature. A volume of 25 mλ of the tested solution was then quickly added to this cell and the contents rapidly mixed with pipette by mouth. Optical density was read again after two minutes, this time interval having been found to be optimal. A standard curve of lysozyme activity was established by measuring a succession of concentrations prepared by weighing standard hen’s egg lysozyme salt-free (Worthington Biochemical). The activity of each concentration of the standard enzyme was read twice. The standard curve was obtained from the mean value of two readings of each concentration.

**Quantitative determination of lysozyme in a tear sample.** Lysozyme activity in a tear sample was measured in the same way as described above for the standard enzyme solutions. The concentration of lysozyme in the tear sample was determined by reading off the standard curve, the standard enzyme concentration which caused the same reduction in OD. The lysozyme concentration in a tear sample is thus expressed in milligrams of hen egg lysozyme per milliliter (HEL milligrams per milliliter). A conversion to human tear lysozyme (HTL) units can be done by dividing the values obtained in HEL units into the conversion factor 4 found by Jolles and Jolles and Bonavida and Sapse.

**Collection of tears.** An attempt was made to collect the tears by inserting a capillary tube in the lateral side of the upper fornix, near to the lacrimal gland. However, this method caused discomfort to the donor and was time-consuming. It was, therefore, decided to use reflexory method by trigeminal irritation. A swab dipped in alcohol solution (25 Gm. camphor in 100 c.c. alcohol 95 per cent) was placed into the nose, thus causing a considerable flood of tears. A comparison of tears collected by the two methods showed no significant differences in lysozyme level. The provoked tears were collected from the lower fornix of the eye near the puncta lacrimalia by a Vitrex-Micro-Hematocrit Capillary tube, nonheparinized, having a capacity of 75 mλ (CHR Bardam, Denmark). The capillary tubes were then fixed in a miniseal plate of Medidenta Company, and refrigerated at 4° C.

**Dilution of tear sample.** The tear sample was later removed from the capillary tubes by a vacuum dropper to a parafilm paper, thus preventing dispersion of the tear drop to a minimum loss of material. In order to find a convenient dilution of tears for enzyme determination, a
Fig. 1. Rate of lysis of *M. lysodeikticus* (measured by decrease in optical density after 2 minutes reaction), by standard hen’s egg lysozyme concentrations in M/15 phosphate buffer, pH 6.24. (A) general activity curve; (B) linear range of activity.

volume of 2 \( \lambda \) was removed from the tear drop on the parafilm paper to a test-tube containing 0.1 ml. phosphate buffer, pH 6.24, 0.15 M, and a volume of 10 \( \lambda \) to a test tube containing the same volume of buffer. Thus, dilutions of 1:50 and 1:10 were obtained, respectively. Determination of lysozyme concentration was performed on the diluted and undiluted tears.

**Sensitivity of the assay.** In order to estimate the sensitivity of the assay, tears were collected from both eyes of five volunteers, once a day, for two consecutive days, 20 samples in all. Lysozyme determination was repeated three times on the 1:10 dilution of each tear sample, yielding 20 sets of three repetitive readings. The sensitivity of the assay was measured by means of the pooled standard deviation of repetitive readings (Spr) of the 20 sets.

**Selection of normal population for tear lysozyme screening.** The tears were collected from both eyes of healthy people, comprising 60 men and women of different age groups, ranging from 16 to 70 years of age. The people tested had been examined clinically and were found to be completely free of any eye disease. In addition, swabs from both eyes of each person were taken and inoculated on various media for the detection of infectious agents. Sabouraud dextrose agar medium was used for the isolation of fungi; blood agar and thioglycolate media were chosen for bacterial examinations. Swabs taken for virologic examinations were shaken in Mm with 5 per cent fetal bovine serum and then inoculated on rabbit kidney primary tissue culture and on Vero cell line. No growth on Sabouraud dextrose agar appeared and no cytopathic effect was detected in tissue cultures. Bacteriologic examinations showed that of the eyes tested, 70.1 per cent coagulase-negative gram-positive cocci were isolated. These cocci were considered as part of the normal flora of the eye.

**Results.**

**Standard curve of lysozyme activity.** In order to determine the linear range of enzyme activity, a succession of standard HEL concentrations, ranging from 0.05 mg. per milliliter to 14 mg. per milliliter were prepared. The decrease in OD of the substrate solution versus (vs) concentrations of the standard enzyme, is described in
Fig. 1. A. From the curve obtained, it is evident that the linear range applies to at least a concentration of 2 mg. per milliliter. Accordingly, a series of many gradually increasing, closely spaced concentrations of the standard enzyme, ranging from 0.05 mg. per milliliter to 2 mg. per milliliter, were prepared in phosphate buffer, and their activity determined and described in Fig. 1, B. The linear curve obtained served as a standard curve for determination of lysozyme level in the diluted tears. A new calibration curve was prepared whenever a fresh stock of bacteria or a newly prepared buffer was put into use.

Sensitivity of the assay. As described above, the sensitivity of the method was measured by means of the pooled standard deviation of repetitive readings (Spr) of the 20 sets of three repetitive readings. The Spr was 0.90 mg. per milliliter HEL or 0.25 HTL. In one reading, the 95 per cent confidence interval was ± 1.8 mg. per milliliter HEL (0.45 HTL) of the observed value; in three readings, it was ± 1.1 mg. per milliliter HEL (0.27 HTL) of their mean value. Obviously, as the number of repetitive readings increases, a more precise estimate of the real level of lysozyme is obtained.

Dilution of tear samples. The most convenient dilution for lysozyme determination was found by testing three dilutions of each of 10 tear samples obtained from both eyes of five healthy people. The results are summarized in Table I. The results show that by diluting the tear samples, the enzyme activity increases. We chose the dilution of 1:10 as being the most convenient for routine work. All our examinations were made on 1:10 diluted tears.

Tear lysozyme in healthy subjects. Comparison of tear lysozyme level in both eyes of each subject: the pooled standard deviation, measuring the within pair variability, was calculated for the 55 pairs of eyes of the normal subjects on whom data on both eyes were available. This pooled standard deviation was found to be 1.1 mg. per milliliter HEL. It did not differ significantly (p (F) > 0.10) from the Spr indicating that lysozyme concentration does not differ in tears of both eyes of the individual. The distribution of lysozyme level in the 60 normal subjects studied is described in Fig. 2. The distribution seems to be somewhat skewed to the right. Since the lysozyme level can be assumed to be equal in each pair of eyes, the mean value for both eyes

*The pooled standard deviation Spr represents the mean of the individual standard deviations of the sets of repetitive readings. The standard deviation of each set indicates the extent of variation of the repetitive readings around their own mean. The more sensitive method the smaller the variability between repetitive readings, and the smaller the value of the Spr.
was used for each individual. The mean value of tear lysozyme for the subjects was 6.1 mg. per milliliter HEL with standard deviation of 1.57 mg. per milliliter HEL or 1.5 mg. per milliliter HTL with standard deviation of 0.39 mg. per milliliter HTL and a range of 3.8 to 7.7 mg. per milliliter HEL or 0.95 to 1.9 mg. per milliliter HTL.

**Discussion.** The relationship between tear lysozyme level and pathologic eye conditions have been discussed in a number of reports. These findings impelled us to look for a rapid micro-method to determine lysozyme level in tears, which could be used easily and routinely for diagnostic purposes. At present, the most commonly used method for tear lysozyme determination is the lysoplate one. This method is based on collecting a tear sample on a strip of blotting paper and incubating the strip on agar plate seeded with *M. lysodeikticus*. Thus, the result is obtained only after 24 hours. The lysoplate method uses human tear lysozyme (HTL) as a standard for lysozyme determination in human tears. Since HTL is not available commercially, routine work becomes difficult. In addition, the tear sample taken on the strip cannot be used for any other test. This is a general problem with tears, since the volume of a tear sample obtained is very small. Consequently, in previous reports, the analysis of tear constituents, such as antibodies, complement, etc., were made on a pool of tears from the eyes of several persons. This precluded the use of such analysis in pathologic conditions, which requires individual samples. These problems are overcome by the advantages of the spectrophotometric method, as follows: (1) tears are drawn in a capillary tube and diluted in a special procedure which enables diversified analysis of the content of individual tear samples; (2) a diluted tear sample can be refrigerated and stored for further analysis; and (3) only a short time is required for the determination of lysozyme, since all stages can be performed in a few minutes. Bonavida and Sapse found in their normal series that the mean level of the enzyme in tears, when measured against standard HTL, was 1.7 mg. per milliliter, while in our series, where the standard was HEL, the mean level was 6.1 mg. per milliliter. Jolles and Jolles and Bonavida and Sapse found that the ratio between the specific activity of HEL is 3 to 4. This ratio makes possible

<table>
<thead>
<tr>
<th>Concentration of lysozyme in tear samples</th>
<th>1:10 Diluted tears</th>
<th>1:50 Diluted tears</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undiluted tears</td>
<td>2.8</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>1.76</td>
<td>1.76</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>2.7</td>
<td>2.14</td>
</tr>
<tr>
<td></td>
<td>2.4</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>1.8</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. Distribution of tear lysozyme level (mean value of lysozyme level in both eyes of each individual) in 60 healthy individuals (in milligrams per milliliter).
the conversion of our results (as expressed in HEL) to HTL units. By dividing the mean value of lysozyme (6.1 mg per milliliter HEL) into the conversion factor, a value of 1.5 to 2.0 mg per milliliter HTL was arrived at. This value correlates with the results obtained by the lysozyme method of Bonavida and Sapse. Our experiments indicate that when the tears are further diluted, the activity of the enzyme increases. This might be explained by the presence of inhibitors in tears whose activities are reduced by dilution. Another possibility might be that a dimer form of the enzyme with no activity was present to an appreciable extent initially and was changed to the active monomer form upon dilution.15, 14

The collection and dilution procedure used here does not affect the reading appreciably. This conclusion is supported by the fact that tear lysozyme readings from both eyes, varied to the same extent as repetitive readings of the same diluted sample. In other words, the variability of the method stems mainly from the assay procedure itself.

The spectrophotometric method can be used to describe the distribution of tear lysozyme level in a population as well as to compare normal subjects to a group of patients with a certain disease. For this purpose, one reading per person is sufficient. However, when the aim is to establish the individual tear lysozyme level for diagnostic purposes, one reading may not be sufficient, depending on the sensitivity of the method used.

The method was used to determine the normal level of tear lysozyme in both eyes of 60 healthy subjects. The group was analyzed as a whole, since previous reports had indicated that tear lysozyme level was not affected by sex and only very slightly by age. One of our findings is that lysozyme level is the same in both eyes. Accordingly, it is possible to use the mean lysozyme level of both eyes to obtain a better estimate of the level in each healthy person.

From the Department of Human Microbiology, Sackler Medical School, Tel Aviv, Department of Ophthalmology and Department of Clinical Epidemiology, The Chaim Sheba Medical Center, Tel-Hashomer, Israel. Submitted for publication May 7, 1974. Reprint requests: Dr. E. Eylan, Dept. of Human Microbiology, Tel Aviv University Medical School, Tel Aviv, Israel.

*Established Investigator of The Chief Scientist’s Bureau, Ministry of Health.

Key words: lysozyme, human tears, M. lysodeikticus.

REFERENCES


A surgical approach to full-thickness eye wall biopsy is proposed as a method that may be applied to poorly understood diseases of the retina and choroid. An eye basket is sutured to the eye for stabilization, and two trephines mark an area to which diathermy is applied until penetration is achieved. The specimen is removed with fine