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Quantitative tear lysozyme assay in units of activity per microlitre

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Fleming (1922) discovered lysozyme, and in 1932 he assayed the tear level using a dilution technique (Fleming, 1932). He showed that the concentration of lysozyme in tears was up to a thousandfold greater than in serum.

Accurate lysozyme assay has been possible only since the establishment of hen egg lysozyme (HEL) and human lysozyme (HL) standards. Their activity can be accurately measured. HEL standards have recently been used for lysozyme assay in urine, peritoneal fluid, and serum and HL standards have been used for assay in cerebrospinal fluid (Parry, Chandian, and Shahani, 1965; Harrison, Lunt, Scott, and Blainey, 1968; Wardle, 1973; Cacatian, Newman, Josephson, and Tsang, 1974).

Tear fluid is most easily collected by filter paper disc absorption. Results of the total quantity of lysozyme on the discs, have been expressed in diameters of zones of lysis of *Micrococcus lysodeikticus*. Various authors have reported different normal diameters (Bonavida and Sapse, 1968; Bijsterveld, 1969; el Gammel and Salah, 1971; Pietsch, Pearlman, and Durham, 1973; Bijsterveld 1974). Results have also been expressed in μg/μl of uncalibrated HL (Pietsch and others, 1973), of HEL (Covey, Perillie, and Finch, 1971), and calibrated HEL (Osserman and Lawlor, 1966; Friedland, Anderson, and Forster, 1972). Direct comparison of these results is unreliable, because HEL and HL vary in their activity per μg, as shown by the different levels quoted (Harrison and others, 1968; Bonavida and Sapse, 1968; Friedland and others, 1972; Bijsterveld, 1974). In addition, batches of lysozyme standards are prepared by different commercial laboratories; further confusion arises because HL has eight to 12 times the activity of HEL per μg (Osserman and Lawlor, 1966).

In this study, calibrated HEL standards were used in each assay alongside the test discs. The activity of this HEL standard was measured in 'units of activity' per μg, so that direct comparison with other results, in units, could be made. A 'unit of activity' is defined below.

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Method

Dried, autoclaved Whatman No. 1 filter paper discs 6 mm in diameter, were placed inside bijoux bottles which were weighed and put into clear plastic envelopes. The disc was placed in the lower fornix of the conjunctiva with a forceps, allowed to absorb tear fluid, and then replaced in the bottle. Care was taken to avoid collecting the mucus thread in the lower fornix. The bijou bottle, with the wet disc, was reweighed so that the volume of tear fluid could be calculated. Other discs were wetted with standard HEL at a concentration of 1, 3, 5, and 10 μg/μl and the volume absorbed was again estimated by weighing. A control disc of HEL was similarly treated.

Twenty colonies of *M. lysodeikticus*, from a purity plate, were innoculated into 20 ml nutrient broth (Oxoid), and shaken for 2 min. This was flooded on to a 20 cm² plate, containing 250 ml Direct Sensitivity Test Agar (DST-1·2 per cent Oxoid), and dried (Bijsterveld 1974). The discs for the tests, standards, and controls were placed on the surface of the plate, which was incubated at a temperature of 37°C for 18 h. Zones of lysis of the *M. lysodeikticus* occurred around the discs. The diameters of these zones were measured with precision calipers.

The HEL standard was calibrated in a spectrophotometer by measuring the rate of lysis of a fresh substrate of *M. lysodeikticus* grown for 48 h on Meyer and Hahnel medium (Meyer and Hahnel, 1946; Shugar, 1952). The *M. lysodeikticus* was suspended in M/10 phosphate buffer at pH 7·0. The absorption of the suspension in a 3 ml spectrophotometer cuvette was adjusted to between 0·5 and 0·6 with a wavelength of 450 nm at a temperature of 25°C. Samples of 10, 20, 30, and 40 μl of the 1, 3, 5, and 10 μg/μl HEL standards, each diluted to 0·3 μg/μl, were added to the cuvette so that the concentration of the HEL was known in μg/ml buffer substrate. The decrease in absorption during the first minute was measured.

One 'unit of activity' was defined as that amount of enzyme required to cause a decrease in absorption of the *M. lysodeikticus* substrate, in M/10 phosphate buffer at pH 7·0 and at a temperature 25°C, of 0·001 in the first minute of the reaction, at a wavelength of 450 nm.

The HEL standards had to be kept deep frozen (−20°C) as they would have lost some of their activity

at temperatures above 4°C. The standards used for the plate assay were checked monthly on the spectrophotometer, against the calibration curve, to assess whether they had lost any activity.

Results

A straight line graph was obtained between the logarithms of the total quantities of the HEL in μg on the discs and the squares of the diameters of the zones of lysis of *M. lysodeikticus* on the plate, in the range equivalent to that in human tears (Fig. 1); a non-lysozyme antibacterial factor has been described in human tears (Friedland and others, 1972). It has been neither identified nor assayed, and it has not been taken into account in this method.

A straight line graph was obtained with the HEL calibration on the spectrophotometer (Fig. 2) (Parry and others, 1965). A decrease in absorption during the first minute of 0·030 was obtained by 1 μg HEL/ml buffer-substrate, under stated conditions. This represented, by our definition, 30 'units of activity'/μg HEL (30 000 units/mg).

It was necessary for the standard HEL, which was used in the test, to be calibrated in the laboratory. Lysozyme chloride ex egg white* was stated to have an activity of approximately 15 000 units/mg. On repeated testing of freshly prepared solutions, however, an activity of 30 000 units/mg was recorded.

The lysozyme concentration in units of activity/μl tear fluid, of the normal eyes of 54 volunteers (aged 18 to 86 years) was assayed (Fig. 3). The

*Supplied by Koch-Light Laboratories

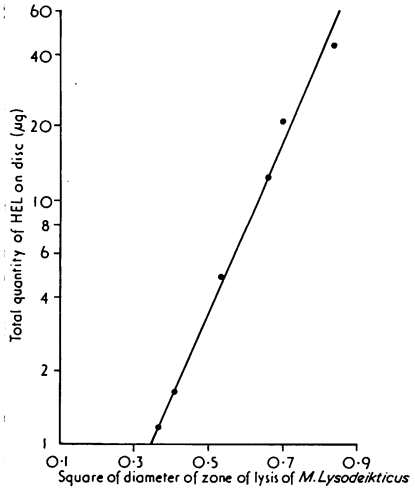

FIG. 1 *Calibration graph of HEL on plate of* M. lysodeikticus

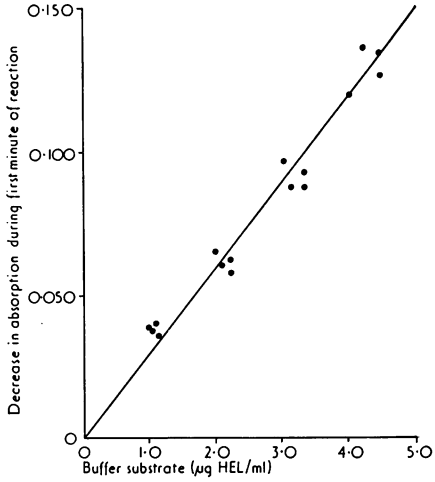

FIG. 2 *Calibration graph of HEL on spectrophotometer*

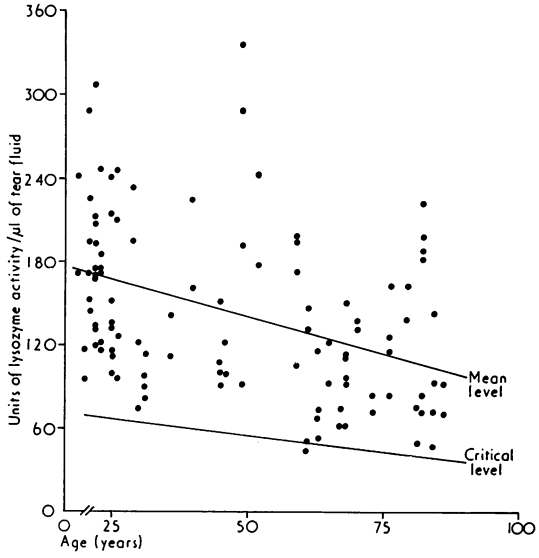

FIG. 3 *Lysozyme concentration by age in* 54 *normal subjects*

mean level of this distribution decreased by one unit of activity/μl tear fluid for each year. A limit of 70 units of activity/μl tear fluid at 20 years of age had decreased to 40 units/μl tear fluid by 85 years, and this was considered to represent the lowest normal concentration, although one in 108 eyes lay below it; it is called the critical limit.

The variability of the volume of tear fluid absorbed by the Whatman filter paper discs, which were considered 'wet' on inspection, is shown in Table 1. In all age groups, the volume collected on a 'wet' disc could vary by threefold, and between

Table I *Volume of tear fluid absorbed on to filter paper discs from 54 normal volunteers*

Age-group (years)	No. per group	Range of volume (μl)	Mean (μl)	Standard deviation
20–39	24	2·0 to 8·5	5·1	1·5
40–59	9	2·3 to 6·5	4·5	1·2
60–89	21	2·2 to 6·5	4·3	1·2

the ages of 20 and 40 years, by fourfold. The mean volume collected varied from 5·1 μl for 20 to 39 years of age to 4·3 μl for 60 to 89 years.

Out of 54 normal volunteers, four were slow in their response to produce enough tear fluid to wet the disc, and 11 were quick, producing copious quantities of tears, see Fig. 4. The other volunteers were classified as 'medium tear producers'. It was demonstrated that tear fluid produced slowly could have a high or low normal lysozyme concentration, as was also the case for tear fluid produced quickly and copiously.

There was no relationship between the lysozyme concentration, measured in units of activity/μl tear fluid, and the total quantity of tear fluid absorbed by the disc (correlation coefficients: −0·1 for left eye, −0·1 for right eye) (Fig. 5). This meant that volunteers producing smaller quantities of tear fluid (2 to 3 μl absorbed by the disc) might have a high or low normal lysozyme concentration. This was also the case for volunteers producing larger quantities of tear fluid (6 to 7 μl absorbed by the disc).

A significant association between the lysozyme concentration in the left and right eyes of the volunteers was calculated (correlation coefficient: 0·7).

The details of six patients with a clinical diagnosis of keratoconjunctivitis sicca, and three control patients with severe autoimmune disease, but with normal eyes, are summarized in Table II. Five out of the six patients with keratoconjunctivitis sicca had tear lysozyme concentrations well below the critical limit for the lowest normal concentration for their age; one, with quiescent systemic lupus erythematosus and keratoconjunctivitis sicca, had a lysozyme concentration around the limit. The three control patients had lysozyme concentrations well above the critical limit.

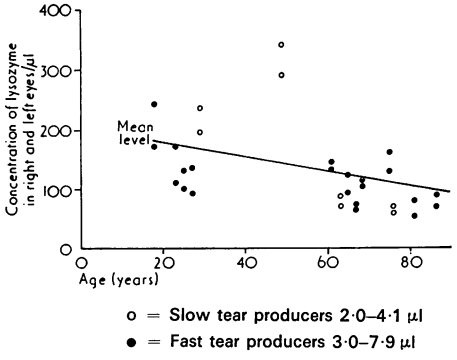

FIG. 4 *Comparison of lysozyme concentrations in slow and fast tear producers*

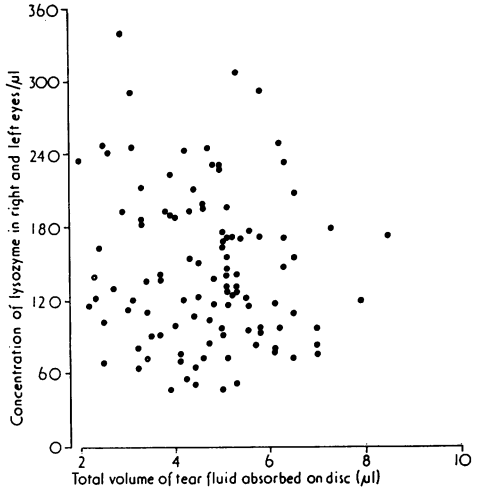

FIG. 5 *Total volume of tear fluid absorbed by disc compared with lysozyme concentration*

Discussion

Bonavida and Sapse (1968) stated that the variables affecting the diameter of the zone of lysis of the *M. lysodeikticus* include incubation time, temperature, concentration of micrococcus, and thickness of agar. Bijsterveld (1974) investigated different media for growing the *M. lysodeikticus* in order to find a medium on which to standardize the test. Pietsch and others (1973) stated that the reporting of results, compared with a lysozyme standard was not necessary because the zone of lysis obtained with a standard might be erroneous because of impurities.

In our experience the growth of the *M. lysodeikticus* on DST agar (1·2 per cent Oxoid) is never exactly the same, and direct comparison between zones of lysis is unreliable. It is only by direct comparison with a reliable standard on each plate that the biological variable (that is the growth of the *M. lysodeikticus*) can be compensated. If the lysozyme standard is calibrated on the spectrophotometer into units of activity, compensation can be made for impurities and different strengths

Table II *Clinical details of six patients and three controls*

	Age of patient (years)	*Diagnosis*	*Volume of tear fluid*		*Lysozyme concentration (units/μl)*	*Critical limit* (units/μl)*
			Eye	*(μl)*		
Sicca syndrome	23	Hypergammaglobulinaemia with sicca	Right	2·5	39	69
			Left	2·4	39	
	33	Quiescent systemic lupus erythematosus and sicca	Right	4·0	60	65
			Left	2·5	71	
	52	Sicca	Right	6·2	15	56
			Left	4·8	15	
	60	Sicca	Right	5·6	24	52
			Left	4·2	30	
	66	Sicca	Right	2·9	< 9	49
			Left	2·4	< 9	
	82	Sicca	Right	3·6	19	41
			Left	1·8	44	
Controls with other autoimmune diseases	54	Severe rheumatoid arthritis Normal tear film and eyes	Right	2·9	87	55
			Left	2·4	72	
	55	Raynaud's disease Scleroderma Skin biopsy positive for systemic lupus erythematosus Normal tear film and eyes	Right	2·8	84	54
			Left	4·0	120	
	67	Sjögren's syndrome (dry mouth and rheumatoid arthritis) Normal tear film and eyes	Right	2·7	64	48
			Left	2·2	78	

*Lowest normal level for age

It is essential to weigh the filter paper discs to estimate the quantity of tear fluid absorbed. Otherwise, the diameter of the zone of lysis of the *M. lysodeikticus* will be increased by 40 per cent for each doubling of the quantity of tear fluid on the disc.

Erickson (1955) and McEwen and Kimura (1955) reported the absence of lysozyme in the sicca syndrome, and Minton (1965) reported its absence in one case of systemic lupus erythematosus, with some reduction in other cases.

We have shown in this study that the tear lysozyme concentration is likely to be abnormal, if it lies below a critical limit, which varies with age.

Bijsterveld (1973) found that the probability of misclassification of a patient with keratoconjunctivitis sicca by Schirmer's test was 15 per cent, by rose bengal test 5 per cent, and by his lysozyme method 1 per cent. Pinschmidt (1970) showed that Schirmer's test was inconsistent. We suggest that the tear lysozyme concentration, in units of activity/μl tear fluid, provides the ophthalmologist with a good guide to lacrimal gland function.

Summary

An accurate method of assaying the tear lysozyme concentration in units of activity/μl has been developed. Normal physiological levels which vary with age have been determined.

It has been demonstrated that measurement of the volume of tear fluid collected and assay with a calibrated standard are essential for the accurate determination of the precise concentration of lysozyme in the tear fluid.

Tear lysozyme concentration can be used as an index of lacrimal gland function, and in the diagnosis of the sicca syndrome and associated conditions.

We believe that this method of tear lysozyme assay has important applications in drug toxicity states in humans (for example, practolol toxicity) and also in animal drug toxicity studies.

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