Immunoelectrophoretic Identification of Lysozyme in Saliva (35519)

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The presence of lysozyme in saliva is well known (1–3), but its location in immunoelectropherograms of saliva is not. Here we describe its identification by immunoprecipitin cross-reaction with tear lysozyme. This, purified by agarose electrophoresis, was itself identified by lysis of *Micrococcus lysodeikticus* directly over its acid-disassociated immunoelectrophoresis precipitin arc. Although saliva lysozyme cross reacted with tear lysozyme, it was significantly less cathodal in immunoelectropherograms, probably because of an anodal carrier.

Materials and Methods. Tears. Human tears were collected by Pasteur pipette from two females who lachrymated spontaneously. The turbid fluid was frozen immediately and stored at −19°.

Saliva. Whole unfiltered saliva was expectorated and used immediately. To retain maximum lysozyme, the saliva was not filtered or dialyzed.[1]

Tear lysozyme. This was purified by zone electrophoresis. Tears were electrophoresed on a microscope slide in 2.5 ml of 1.5% agarose. A center trough as origin was used instead of a round hole, and, following fractionation, a 3-mm area of the agarose was cut from the center of the area containing lysozyme as determined immunoelectrophoretically (Fig. 1). Enzymatic identification of this arc is described below. Lysozyme was eluted by freezing and thawing the gel and pressing it through a fine-mesh, stainless steel screen. Lysozyme was assayed by the Arnheim and Wilson method (6), using 5-ml aliquots of a suspension of 0.25 mg of dried *M. lysodeikticus* (Worthington)/ml of buffer (0.066 *M* sodium phosphate and 0.05 *M* NaCl; pH 6.2) and 0.1-ml samples of the electrophoresed enzyme. One unit of lysozyme activity caused a 1% change of transmittance/min at 540 mμ in a Bausch and Lomb Spectronic-20 spectrophotometer. By this criterion, our eluate contained 11.40 units/ml.

Antiserum to tear lysozyme. This antiserum was obtained from rabbits immunized with electrophoresis-purified tear lysozyme. They received 1.0 ml of w/o emulsion consisting of 2 parts of the agarose–lysozyme harvest, Virtis-homogenized before emulsification, and 1 part of 1:4 Myverol:*n*-hexadecane mixture (7) injected subcutaneously on days 0 and 7. Bleeding 6 weeks later was preceded by three successive daily boosters of the electrophoretically purified tear lysozyme. Boosting injections contained 0.114, 1.14, and 10.2 units, respectively. After another 11 days each rabbit received 0.5 ml of tears and then was bled 5 days later. The high concentration of lysozyme in tears used for the final boosting provided an anamnestic response to the lysozyme; concomitant primary responses to other tear antigens were insignificant, so that bleeding was done before precipitins to tear antigens other than lysozyme appeared.

Antiserum to tears. Rabbits were injected subcutaneously with 0.9 ml of water-in-oil emulsion consisting of 2 parts of antigen solution (37 and 30% on days 0 and 7, respectively) in physiologic phosphate buffer and one part of 1:4 Myverol:*n*-hexadecane mixture. One month later they received three successive daily subcutaneous boosters of 0.01, 0.05, and 0.9 ml, respectively, of undi-

[1] Josephson and Weiner (4) have found leakage of lysozyme across a semipermeable membrane, and our observation (5) indicates that removal of insoluble mucin known to complex with lysozyme results in loss of enzymatic activity in the filtrate.

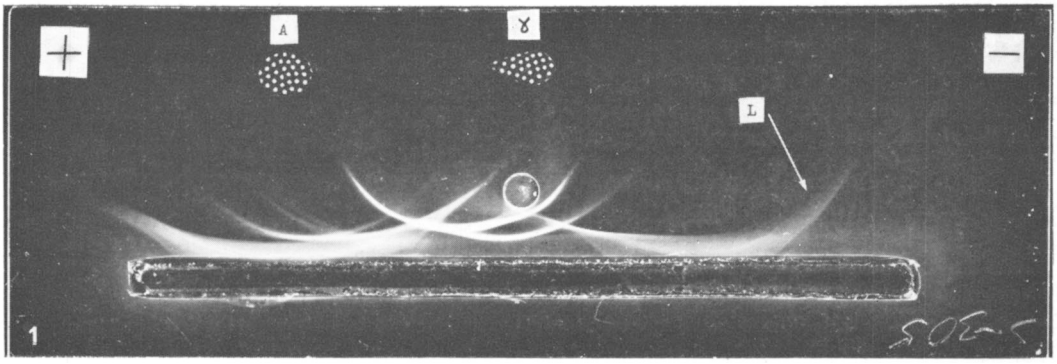


FIG. 1. Tear immunoelectropherogram as developed by As/tears comparing the location of lysozyme (L) with the theoretical locations of gamma globulin and albumin as indicated diagrammatically.

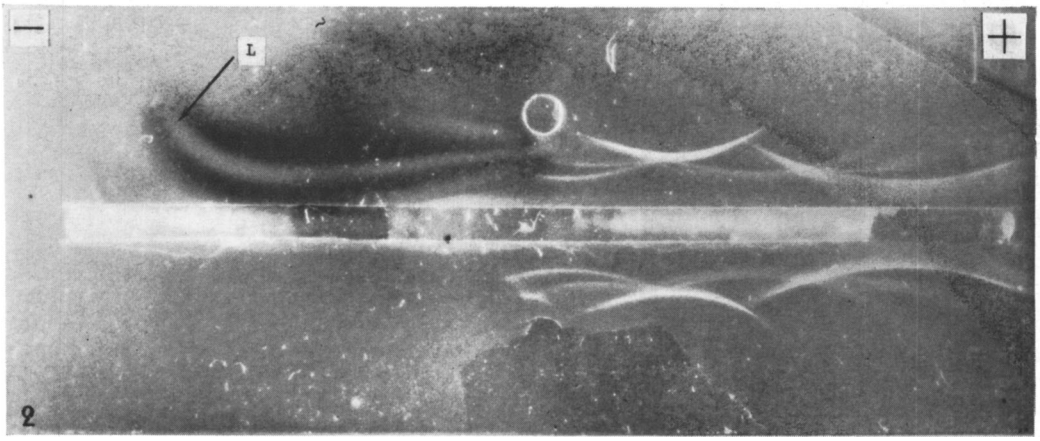


FIG. 2. Tear immunoelectropherogram showing unlysed *Micrococcus* in the shape of the dissociated lysozyme (L) precipitin band surrounded by a halo of lysed *Micrococcus*.

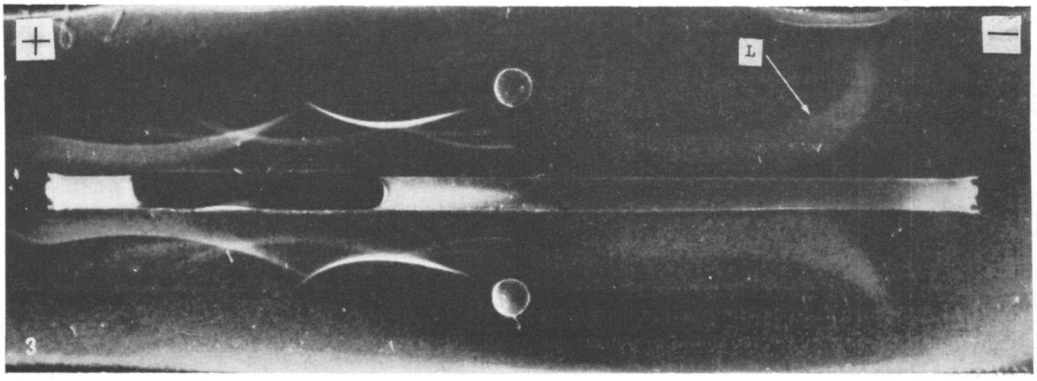
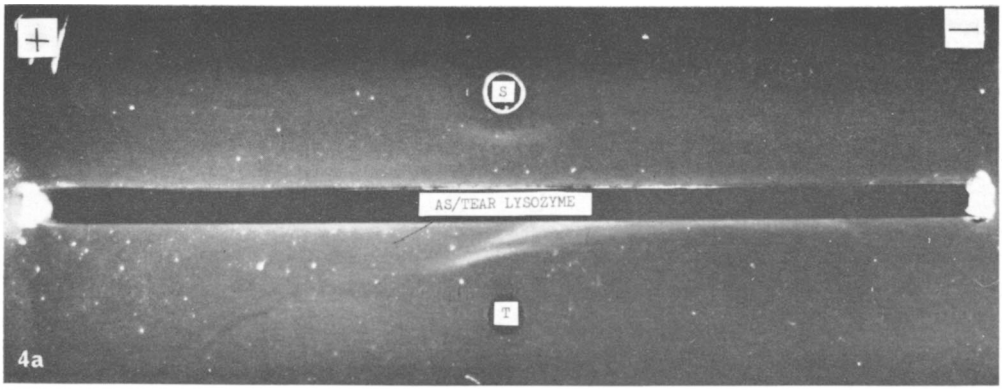


FIG. 3. The turbid arc is not specific Ag–Ab precipitate; it is the remnant of *Micrococcus* in the overlay protected from the lysozyme (L) by reassociation of the enzyme with Ab following dissociation. This has a uniform and diffuse appearance in contrast to the dense, much finer appearance of Ag–Ab precipitates.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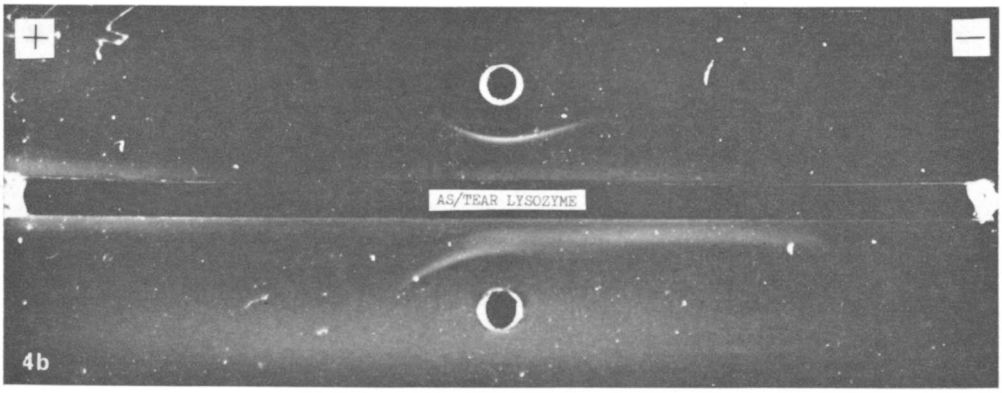
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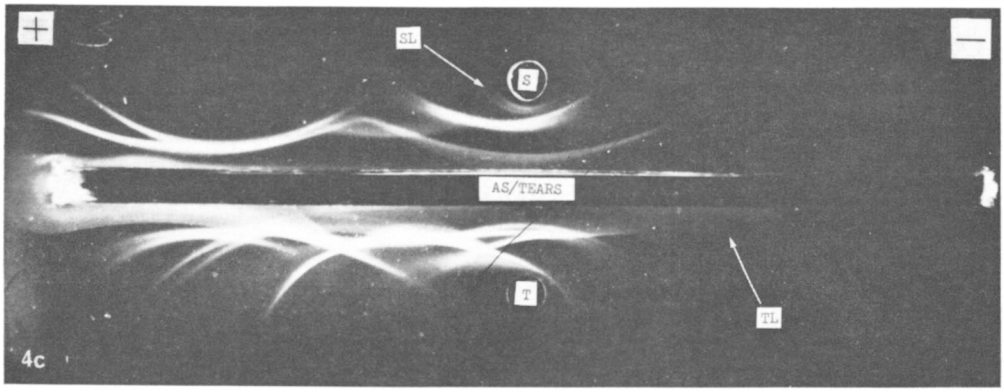
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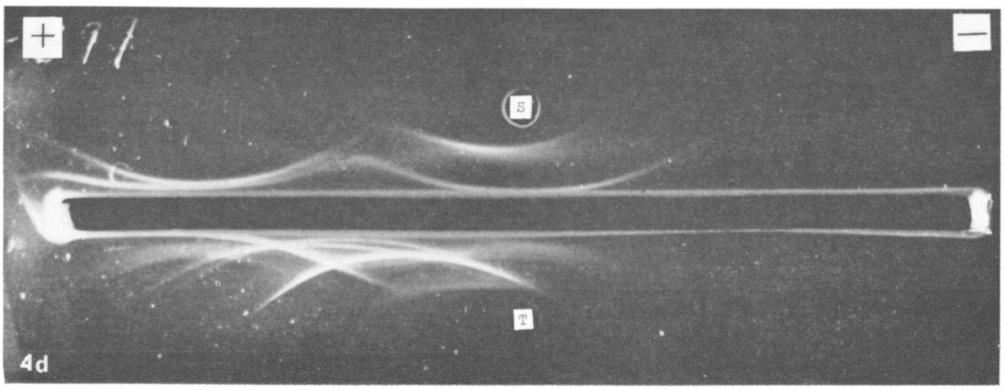
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FIG. 4. Immunoelectropherograms of the following: (a) Saliva (S) and tears (T) as developed with As/tear lysozyme demonstrating cross-reaction of the two lysozymes but different electrophoretic mobilities. (b) Tears mixed with saliva 1:16 top and 1:1 bottom developed with As/tear lysozyme. (c) Saliva (S) and tears (T) developed with As/tears. Note location of salivary lysozyme (SL) compared to tear lysozyme (TL). (d) Saliva (S) and tears (T) developed with As/tears, absorbed with purified tear lysozyme. Note the loss of salivary and tear lysozyme arcs compared to (c).

luted tears, and following 7 more days they were bled.

Electrophoresis. This was according to Schneidegger's micromethod (8). 2.5 ml of 1.5% SeaKem agarose (agar was not used because it complexes with lysozyme), dissolved in either barbital-acetate or barbital-hydrochloride buffer (pH 8.6 and ionic strength 0.025) was poured on each microscope slide pre-dipped in 0.2% agarose. Electrode vessels contained the same buffer as used on the slide but at double strength. Electrophoresis was usually applied at between 7–10 V/cm until a thiazine red marker (9) had moved 5 cm from its origin near the cathode.

Micrococcus-in-agarose suspension. Dried *M. lysodeikticus* cells were suspended in a small amount of physiologic phosphate buffer (pH 7.4) and added to 0.5% agarose dissolved in a pressure cooker in the same buffer and then cooled to 56%. One mg of bacteria was used/ml of buffered agarose. This suspension was used either as an overlay or as an underlay to detect enzymatic activity of lysozyme dissociated by acid, and as an absorbant for excess unprecipitated lysozyme as described below.

Experiments and Results. The tear lysozyme precipitin arc was identified immunoelectrophoretically by direct enzymatic activity as follows:

Tears were electrophoresed from a center well. After electrophoresis, troughs alongside the fractionated tears were filled with antiserum to tears, developed at 4° for 3 days in a humid atmosphere, and washed by soaking for several days in physiologic phosphate buffer of pH 7.4. To remove as much free unprecipitated enzyme as possible, the slide then was overlaid with a *Micrococcus*-in-agarose suspension and incubated at 4° for 7 days. The overlay acted as an absorbent for lysozyme. Then the overlay was removed and the slide was electrodialyzed across its width for 75 min at 50 V and 50 mA using phosphate buffer (pH 7.4) of ionic strength 0.05. A cathodal precipitin line, presumed to be lysozyme, next was dissociated by applying a piece of filter paper, dipped in 1.0 *N* HCl, to the surface of the agarose directly over the precipitin band for 1 min, upon which, this band disappeared. The filter paper was removed, and the slide was laid face down on a flat 0.6-mm thick *Micrococcus*-in-agarose suspension covered immediately beforehand with hot 0.5% agarose.

After 16 hr in a humid chamber at 4°, the slide showed a turbid area of unlysed *Micrococcus*, the approximate size and shape of the original precipitin band; and this was surrounded by a clear halo in the underlay due to lysis by acid-dissociated lysozyme (Fig. 2). Lack of lysis in this zone's central area of lysis presumably was due to local recombination and neutralization of lysozyme with its antibody after removal of the acid-impregnated filter paper.

Figure 3 shows results from a confirming experiment with an overlay of *Micrococcus* suspension in which lysis was allowed to continue until all the *Micrococcus* had been lysed except where specifically protected by the free antibody to lysozyme.

Tear lysozyme was relatively easy to detect and was next used to identify saliva lysozyme. Cross reactivity of saliva and tear lysozyme was confirmed and the location of the saliva lysozyme was determined by immunoelectrophoresis of tears and saliva with antiserum to tear lysozyme used in a trough between the respective tear and saliva. In subsequent immunodiffusion development at 4° for 3 days, the trough was recharged with antiserum twice because of low precipitin titer to tear lysozyme. Figure 4a shows that saliva lysozyme is near the origin, but tear

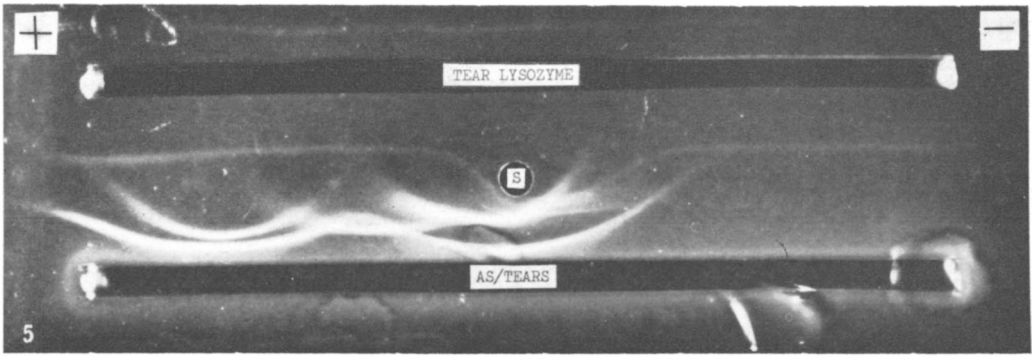

FIG. 5. Reaction of identity (Osserman technique) between tear lysozyme and lysozyme in saliva. Saliva (S) was electrophoresed and reacted simultaneously with As/tears and tear lysozyme. Note straight precipitin band deviating toward antiserum in area of salivary lysozyme.

lysozyme extends from origin to the more cathodal region. Thus, salival lysozyme is less cathodal, perhaps due to a "carrier"-lysozyme complex. A blip in the tear lysozyme arc near the origin suggests the presence of the same carrier in tears as in saliva but in lower relative proportion to the lysozyme concentration in tears.

Additional evidence for the lysozyme-"carrier" complex stems from immunoelectrophoresis of two mixtures of saliva and tears. They were mixed 1:1 and 1:16, electrophoresed as described above, and developed with As/tear lysozyme. The 1:1 mixture produced a gull-wing type of precipitin band, the anodal arc around the origin presumably being the carrier-complexed lysozyme and the cathodal arc free lysozyme. In the 1:16 mixture all the lysozyme appears to have been bound by carrier, for only the anodal portion arc developed (Fig. 4b).

Figure 4c shows an immunoelectropherogram of both saliva and tears developed with As/tears. Saliva produced 4 major bands near the origin. Repeating this experiment with As/tears absorbed with purified tear lysozyme caused loss of the arc nearest the origin in the saliva immunoelectropherogram, (Fig. 4d), suggesting that this arc nearest the origin (cf. Fig. 4c) is saliva lysozyme. The effectiveness of specific absorption is confirmed by the absence of the tear lysozyme precipitin band in the tear immunoelectropherogram. Absorption was accomplished by filling the trough with tear lysozyme for 1 hr following fractionation and then replacing the residual fluid with As/tears. Normal rabbit serum produced no precipitin arcs against electrophoresed saliva or tears, thus ruling out nonspecific precipitation of lysozyme by rabbit serum constituents.

Figure 5 shows the location of salivary lysozyme as confirmed by Osserman's technique (10) using purified tear lysozyme. Saliva was electrophoresed from a center well. Troughs on either side of the well were then filled with purified tear lysozyme on one side, and antiserum to tears in the trough on the opposite side of the fractionated saliva. After development for 3 days at 4°, the immunoelectropherogram was washed in distilled H_2O for 48 hr and then soaked in 0.05% cadmium acetate in distilled H_2O to intensify the precipitin lines before photographing (11).

To confirm the relative mobility of lysozyme in saliva, 0.1% egg lysozyme (Worthington), 10% human serum, 10% tears, and undiluted saliva and parotid fluid were electrophoresed simultaneously in agarose. The saliva origin was recharged three times and the parotid fluid origin twice before electrophoresis to increase the amount of protein in the sample. Following electrophoresis, *Micrococcus* suspension in agarose was poured over the electrophoresed slide and held at 4°. After 2 weeks, bacterial lysis in the overlay indicated saliva lysozyme to be located in the area demonstrated by the Osserman technique [Fig. 7 of Ref. (10)] and truly to differ in mobility from tear lysozyme (Fig. 6).

Discussion. Quantitative changes of ly-

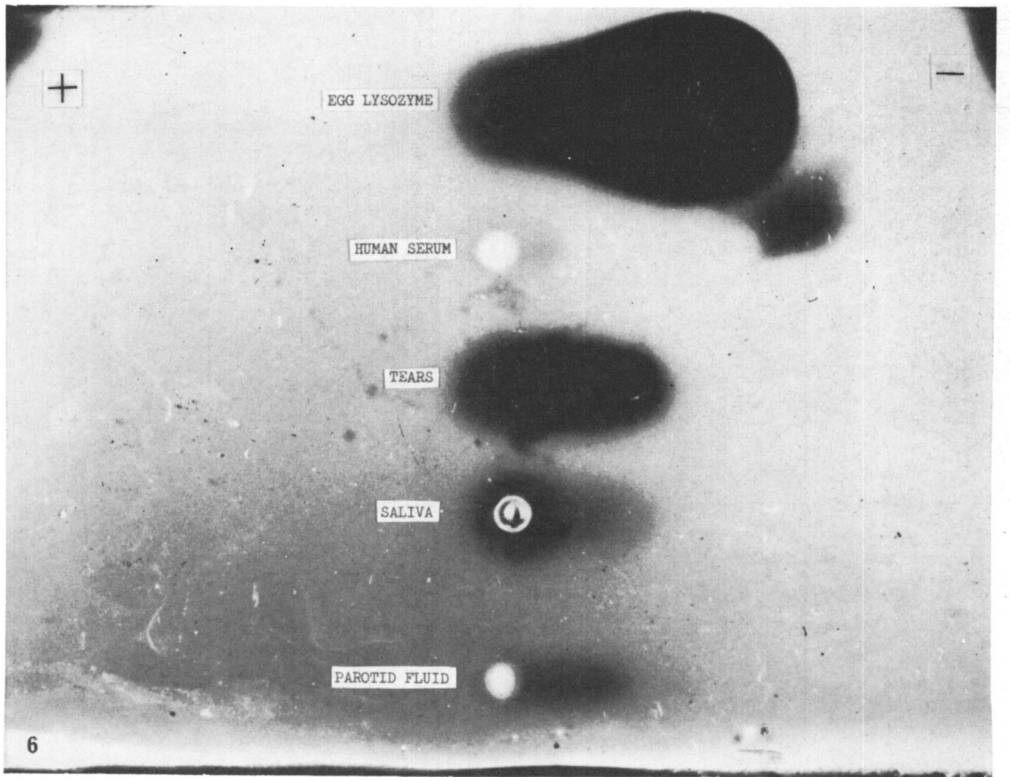

FIG. 6. Comparative simultaneous agarose electrophoresis of egg lysozyme, human serum, tears, saliva, and parotid fluid showing enzymatic activity by lysis of *Micrococcus*.

sozyme levels in body fluids can reflect a disease process. For instance, increased levels are seen in the serum and urine in monocytic and monomyelocytic leukemia, in the gastric juice with peptic ulcers, and in the stools and colonic mucus of patients with ulcerative colitis and regional enteritis (12). It is reasonable to infer that changes in saliva lysozyme could also be used as an indicator for pathology. Thus, methods for identifying and studying this cathodic protein in saliva and other secretions are important.

Body tissue and secretion lysozymes are generally thought to be identical. Our data show that tear and salivary lysozyme cross react antigenically, but that they differ electrophoretically because lysozyme is complexed with a carrier. Tear lysozyme also will complex with this carrier, but in tears the lysozyme–carrier ratio is so large that most of the lysozyme is uncomplexed and therefore migrates farther toward the cathode than saliva lysozyme, virtually all of which is carrier complexed.

Our findings that Ag–Ab complexing completely neutralized lysozyme, and that the enzyme was reactivated following dissociation provided a novel dual way of detecting both enzymatic activity and specific neutralization in association with a particular arc in the immunoelectropherogram.

Summary. Saliva lysozyme was identified in immunoelectropherograms by its relationship with tear lysozyme which itself was identified directly by enzymatic activity. Lysozymes in saliva and tears appear to differ in electrophoretic mobility because of quantity-dependent interactions with an electrophoretically more anodic carrier. They cross react antigenically.

The author gratefully acknowledges the guidance and assistance of Dr. Alfred J. Crowle.

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Received Dec. 16, 1970. P.S.E.B.M., 1971, Vol. 137.