cal Laboratory, for her cooperation in obtaining the marine specimens.

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Direct Localization and Visualization of Hyaluronate Lyase Activity by Agar Gel Electrophoresis.* (32063)

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Procedures for localization, visualization and identification of enzymatically active substances following electrophoresis have been described for several enzyme systems, *i.e.*, xanthine oxidase, lactic dehydrogenase, phosphatase, esterase, and glutamic oxaloacetic transaminase(1-7). All of these enzymes were derived from mammalian species. Electrophoretic fractionations of enzyme-containing material from these studies were performed with a variety of supporting matrices, such as paper strips, starch blocks, starch-agar gel, or cellulose acetate strips. Such electrophoretic preparations, exhibiting visualized enzyme activities, have been termed zymograms(2). In most cases, preparation of zymograms has required a complex and often time-consuming series of histochemical steps to localize the enzyme activity.

Electrophoretic localization and visualization studies with enzyme systems of microbial origin have only recently received attention in the literature. Some of these studies include analysis of bacterial coagulases(8), hemolysins(9), and deoxyribonucleases(10). This report is concerned with description of a rapid enzymophoretic technique for direct localization and visualization of hyaluronate lyase (hyaluronidase) derived from bacteria, as compared to hyaluronidase derived from mammalian testes.

Material and methods. Enzyme source. Staphylococcal hyaluronidase-rich concentrates were derived from a strain of *Staphylococcus aureus* (AEMC-1801) isolated in 1960 from an infected hospitalized patient (11). The organism was cultivated in brain heart infusion broth dialysate medium(11,12). Enzyme-rich concentrates were prepared by ammonium sulphate precipitation of 18-hour culture supernatants, essentially as described by Harris and Harris for preparation of streptococcal hyaluronidase(12). Concentrated staphylococcal supernatants contained 12 to 15 antigens as demonstrated by agar gel diffusion analysis with hyperimmune rabbit antiserum(11).

Testicular hyaluronidase samples were kindly supplied by Dr. George Warren, Wyeth Institute for Medical Research, Radnor, Pa. Streptococcal hyaluronidase-rich concentrates were prepared from a Group A hemolytic *Streptococcus pyogenes,* Type H-44, origi-

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nating from the laboratories of Dr. Carl Meyer, Columbia University, New York(12).

Substrate. Potassium hyaluronate was prepared from umbilical cords according to the method of Harris and Harris(12). An extract, prepared from 3 mg lyophilized potassium hyaluronate concentrate per ml distilled water, containing 10% sterile horse serum, constituted the reaction substrate for assay of hyaluronidase activity(12).

Enzyme assay. The mucin clot prevention (MCP) method of Robertson *et al*(13), as modified by McClean(14), was used for assay of hyaluronidase in culture supernates, and modified for enzymophoresis as described below. The assay depends on the observation that native non-depolymerized hyaluronic acid in acetic acid solution precipitates with protein to form a mucin clot. Following incubation of hyaluronate-protein complex with hyaluronidase, the quantity of the clot for a given amount of substrate is reduced and the character of the precipitate changes from fibrous, to flocculent, to amorphous, and finally to a clear solution at high enzyme concentration. Serial 2-fold dilutions of test material were prepared in 0.5 ml volumes phosphate buffered saline, pH 7.2. To each tube was added 0.2 ml substrate, which consisted of 3% potassium hyaluronic acid extract in distilled water plus 10% normal hourse serum. Final concentration of substrate in the system was 0.4 mg hyaluronate per ml of reaction mixture(12). The enzyme-substrate mixture was incubated for 20 minutes at 37°C in a water bath. The reaction was stopped by immersing the tubes in an ice bath for 5 minutes. To develop the "mucin clot," 0.2 ml 2 N acetic acid was added to each tube, followed by vigorous shaking. The number of units of enzyme activity (MCP unit) was expressed as the reciprocal of the highest dilution of test sample which completely prevented mucin clot formation.

Agar gel electrophoresis. Acid cleaned microscope slides, 1 × 3 inches, were covered to a 1 mm thickness with warm melted (48-52°) 1% Noble agar (Difco, Detroit, Mich.) prepared with barbital buffer, pH 8.6, ionic strength 0.025. Following solidification of the agar, 2 mm circular wells were cut in the gel. The wells were filled with test sample, usually 0.025 ml volumes, and electrophoresis was carried out either at room temperature or in the cold for 45-90 minutes at 250 volts, 15 to 20 milliamps. Barbital buffer, pH 8.6, ionic strength 0.1 was used for all separations. A Buchler model No. 26 chamber and power supply (Buchler Instrument Co., New York), and LKB immunoelectrophoresis accessories (LKB Produkter, Stockholm, Sweden) were used.

Enzyme localization and visualization. Following electrophoresis, 2 to 3 ml of warm agar-substrate mixture were layered directly over the agar on the electrophoretic slide. The mixture contained one part each hyaluronic acid-horse serum complex and melted 1% Noble agar maintained at 48 to 52°C. The agar-substrate overlay was allowed to solidify at room temperature, followed by incubation at 37°C in an air-circulating incubator for 36-60 minutes. The slides were then treated with 5 to 10 ml 2 N acetic acid for 2 to 5 minutes at room temperature. Excess acetic acid was decanted and hyaluronidase activity was visualized directly as a localized clear area against the background of precipitated hyaluronic acid substrate in the agar.

Results. In development of the assay for hyaluronidase activity following agar gel electrophoresis, the possibility that agar or barbital buffer would interfere or react in the MCP technique had to be tested. There was no detectable interference of the tube clot test with the reagents to be used in the agar assay. Tests to determine the feasibility of detecting hyaluronidase activity with concentrated supernatants incorporated in agar indicated that the MCP technique could be modified and adapted for enzyme localization under these conditions. Table I indicates results obtained using various concentrations of ammonium sulphate prepared hyaluronidase-rich concentrates from staphylococci. Enzyme activity was visualized after incubation of the agar slides with hyaluronic acid-horse serum-agar substrate overlay, followed by further treatment with 2 N acetic acid. Concentrated supernatants with enzyme activities (10^4-10^5 MCP units per ml) were found neces-

TABLE I. Hyaluronidase Activity Detected by Clearing of Hyaluronate-Horse Serum Substrate in Agar After Double Diffusion or Enzymophoresis of Graded Concentrations of Staphylococcal Enzyme Preparations. 0.025 ml samples assayed.

Dry wt culture supernate ($AmSO_4$ precipitate) (mg/ml)	Hyaluronidase titer (MCP units/ml)	Zone of clearing (diameter in mm)* 1 hr	2 hr	24 hr	Activity after electrophoresis (250 v, 20 ma)
50	163,840	6.9	8.0	10.3	++++
25	81,920	6.0	7.0	8.5	++++
12.5	40,960	5.0	6.5	7.3	+++
6.25	20,480	5.0	5.5	6.8	++
3.12	10,240	4.5	5.0	6.0	+
1.56	5,120	4.0	4.0	5.2	—
0.78	640	4.0	4.0	5.0	—
0.39	320	0	0	4.0	—
Saline	—	0	0	0	—

* 2 mm wells; 0.025 ml samples.

sary for optical visualization of depolymerization of substrate following electrophoresis in agar. Enzyme containing supernatants with an activity as low as 10^3 MCP units per ml was sufficient for visualization of activity following direct incubation of an enzyme-rich supernatant without electrophoresis on an agar slide for one or two hours. The discrepancy appeared to be due to the effect of dilution of enzyme through the agar during electrophoretic migration. The minimum incubation time at 37°C for visualization of activity on agar was 45 minutes.

Enzyme activity could be visualized following agar gel electrophoresis as an area of clarity in the substrate agar layer at the anodal portion of the slide. The size of the clearing due to enzyme activity was directly related to the concentration of sample applied (Fig. 1). When less than 10^3 MCP units were used, the zone of clearing was generally smaller than 2 mm in diameter. A concentration of 2×10^4 MCP units resulted in a zone of activity of 1 cm diameter or greater. Incorporation of antiserum to staphylococcal hyaluronidase, produced by hyperimmunization of rabbits with staphylococcal culture supernatants, prevented depolymerization of the substrate in the above reaction.

Hyaluronidase derived from streptococcus, staphylococcus strain 1801, or bovine testicles were compared enzymophoretically. Fig. 2 illustrates the localization of these catalytically similar enzymes following simultaneous electrophoresis in agar gel. The mobility of the mammalian enzyme differed from the bacterial hyaluronidases, under the conditions used, although the polarities of each were anodal. Testicular hyaluronidase activity migrated at a faster rate than did the microbial enzymes. When enzymophoresis was extended to 2 hours, or longer, the staphylococcal enzyme activity could be separated from the

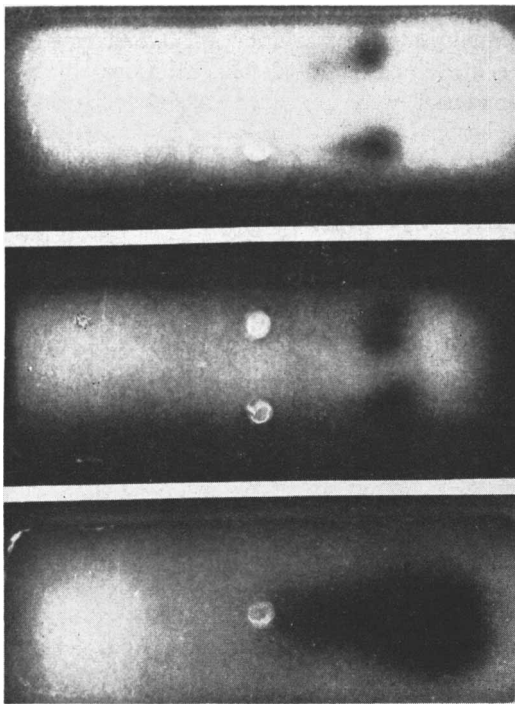

FIG. 1. Enzymoelectrophoresis of several concentrations of staphylococcal supernatant containing hyaluronidase activity. Electrophoresis performed for 40 min as described under *Methods*. From top to bottom wells contained 10,240, 20,480, 40,960, 81,920 and 163,840 MCP units/ml, respectively.

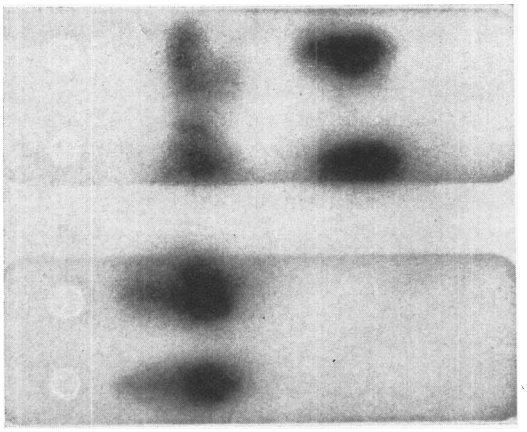

FIG. 2. Separation of testicular and bacterial hyaluronidases by enzymophoresis in agar gel for 40 min. Upper well contained a mixture of streptococcal and testicular hyaluronidase; well second from top contained staphylococcal and testicular hyaluronidase; well third from top contained streptococcal hyaluronidase, and lower well contained staphylococcal enzyme (64,000 MCP units of each enzyme preparation per well).

slower migrating streptococcal hyaluronidase activity (Fig. 3).

Discussion. Electrophoretic and enzymatic localization of mammalian enzyme activities in agar gel has been extensively studied by others(7). Most procedures require preparation of 2 separate agar slides, one for electrophoretic separation and another for detection of enzyme activity using appropriate substrate and co-enzymes. In a typical system(7), an agar slide containing test sample, following electrophoresis, is placed in contact with a substrate slide. Special precautions must be observed not to trap air bubbles between the slides, generally a troublesome problem. Duplicate slides, following incubation, are then transferred to a spectrophotometer modified for absorption scanning. Changes in optical density of either substrate or co-enzyme may then be recorded.

For this study, the general procedure used for enzymophoresis of mammalian enzyme systems has been modified and adopted for assay of staphylococcal hyaluronidase activity. Use of an agar-substrate indicator layer directly on an electrophoretic slide permitted rapid localization and visualization of enzyme activity, following initial agar gel electrophoresis of supernatants prepared from staphylococcal cultures. This procedure has also proven valuable for rapid screening and detection of hyaluronidase activity during purification and fractionation procedures(15). The patterns of clearing produced by hydrolysis of substrate in the agar overlay differs with increased stages of purity of enzyme. Crude hyaluronidase-containing preparations produce small, relatively "cloudy" zones of clearing, whereas purer enzyme preparations result in larger, well defined zones of clearing. In addition, purified preparations of hyaluronidase, obtained by gel-filtration with Sephadex, often migrate as several distinct areas, suggesting that hyaluronidase may exist in multiple molecular forms(15).

Hyaluronidase activity from streptococcal and bovine testicular extracts have also been localized and detected in this study following agar gel electrophoresis. Migration of these enzymes, with regard to polarity, was the same as that observed for the staphylococcal enzyme. However, individual mobilities differed. When all 3 enzyme preparations were pooled, in equal concentration, and eletrophoresed on agar, the bacterial enzyme activities had a mobility distinct from the testicular enzyme. These results suggest a method for the physicochemical differentiation between hyaluronidases from different sources.

Enzymophoretic methods for detection of bacterial deoxyribonuclease, lipase, phosphatase, staphylokinase, and alpha, beta, and delta hemolysins have also been developed (16). By correlating the enzymophoretic position of the above mentioned enzymes with that of hyaluronidase, it has been possible to map directly the relative electrophoretic migration in agar of these enzymes from a

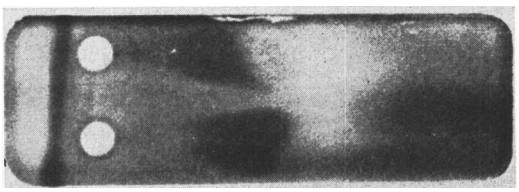

FIG. 3. Enzymophoresis of staphylococcal (lower) and streptococcal (upper) hyaluronidase for 90 min. 64,000 MCP units of each enzyme preparation was used.

single strain of micro-organism(17).

Summary. A rapid agar slide method for localization and visualization of hyaluronate lyase (hyaluronidase) has been described. This procedure is based on enzymophoretic techniques developed for localization of mammalian enzymes following agar gel electrophoresis. Differences in electrophoretic mobility between staphylococcal, streptococcal and bovine testicular hyaluronidase have been demonstrated. The use of enzymophoresis in agar for study of bacterial enzyme systems has been discussed as a valuable procedure for enzyme analysis during purification and characterization studies.

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Reutilization of Presumably Degraded Radioactive Label from a Chromosomal Fraction of Homologous and Heterologous Cells. (32064)

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Several authors have reported evidence for reutilization of DNA by cells in which it did not originate(1-7). Borenfreund and Bendich (1) labeled purified DNA, suspended it with HeLa cells and later recovered the label from HeLa DNA. Bensch *et al*(7) combined pure DNA with gelatin and studied its phagocytosis and digestion by cultured fibroblasts.

The purpose of this research was to develop the necessary methods and study the uptake of labeled DNA from isolated whole chromosomes by dividing cells of the same and different species.

Materials and methods. Labeling, cell synchronization and preparation of a chromosome suspension. The cell line employed was Chinese hamster fibroblast strain V37/A clone F, generously provided by Dr. J. Mendelsohn and Dr. N. P. Salzman. The cells were grown as monolayer cultures in modified Eagle's medium, according to Robbins and Marcus(8) supplemented with 10% fetal calf serum. Tritiated thymidine, 0.5 μc/cc (specific activity 14.4 curies/millimole) was added to each of 6 large Blake culture bottles freshly planted with 4×10^6 Chinese hamster fibroblasts. Five hours later, Colcemid was added to a final concentration of 0.02 gamma/cc. The cultures were harvested at 20 hours, or 15 hours after Colcemid was added, when 70-80% of the cells are in metaphase. At this time the medium was substituted with prewarmed 1% sodium citrate solution (37°C). The bottles were then inverted with the cell layer uppermost and shaken forcefully several times to dislodge the swollen metaphase cells