Isolation and Purification of Streptococcal Hyaluronic Acid. (25165)

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Since the first isolation of hyaluronic acid from strains of Groups A and C streptococci (1) numerous methods for extraction and purification of streptococcal hyaluronic acid have been reported(2-4). In view of possible significance of bacterial hyaluronic acid and its depolymerization products in certain physiological and medical problems(5-7), it was of interest to investigate procedures whereby large quantities of streptococcal hyaluronic acid may be prepared conveniently for these studies. Present methods for preparation and production of bacterial hyaluronic acid depend mainly on growth of the organism in complex organic culture media many of which contain a polysaccharide hydrolyzed by hyaluronidase(8,9). Moreover, purification technics are laborious, complicated and feasible for isolation of relatively small quantities of material. Our purpose is to present a simple semi-synthetic medium, developed with the purpose of providing conditions not only for optimum growth of *S. pyogenes* but also for formation of large quantities of hyaluronic acid. A rapid, reproducible method for isolation of partially purified hyaluronic acid from the filtrate of Group A streptococcus will also be described.

Materials and methods. Culturing methods. Streptococcus pyogenes Group A type 9 (T9/63/3), obtained from the Hospital of Rockefeller Institute, was used. The organism was maintained on blood agar containing 5% defibrinated sheep's blood. Actively growing seed cultures were prepared by transfer of growth from 24 hour stock slants to 10 ml of culture medium (described below) before transfer to 500 ml of similar broth which was then incubated for 18 hours at 37°C. The medium used consisted of casein hydrolysate (enzymatic) 50 g; $Na_2HPO_4 \cdot 12\ H_2O$, 11.2 g; KH_2PO_4, 0.96 g; $MgSO_4$, 0.04 g; Ca-d-pantothenate, 1 mg; pyridoxine HCl, 1 mg; riboflavin, 0.1 mg; distilled water, 1 liter. The medium was adjusted to pH 7.6-7.8 with 5N NaOH and sterilized by autoclaving at 120°C for 20 minutes. After sterilization, the medium was supplemented with glucose (sterilized by filtration through Seitz filter) to a final concentration of 10 mg/ml. Nine-liter Pyrex carboys containing 5 liters of medium were employed. Inoculation was made with 500 ml of seeding culture/5 liters medium. *Production of hyaluronic acid.* Hyaluronic acid was readily produced by this strain of streptococcus after 16-18 hours incubation at 37°C. The contents of the 9-liter carboys was heated in boiling-water bath for 1 hour, quickly cooled and formalin added with mixing to a final concentration of 2%. The bottles were stoppered with rubber stoppers and stored at room temperature. Crude hyaluronic acid broths treated in this manner may be stored at room temperature for at least 6 months with no loss in yield of final product. The crude broth, clarified by filtration through "Hyflo Super-Cel" (Johns Manville) was treated with 3 volumes of cold acetone. The highly viscous brown liquid phase which immediately separated was stored overnight in cold room (5°C). The supernatant fluid was decanted and the brown liquid phase was diluted with equal volume of distilled water. To 1 liter of viscous brown liquid phase extract, 250 ml of glacial acetic acid and 125 g of potassium acetate were added. The mixture was stirred 5 minutes and poured into 1,250 ml of cold 95% ethyl alcohol producing a white flocculent precipitate. After 30 minutes in ice bath, the precipitate was collected by filtration with suction through "Hyflo Super-Cel" and dissolved with distilled water. A second and third reprecipitation was performed in this manner. The final precipitate was washed with absolute ethyl alcohol, dissolved in distilled water, dialyzed against frequent changes of distilled water for 48 hr at 4°C and then freeze-dried. The yield of potassium hyaluronate from 5 liters of culture medium averaged 1.2 g. *Chemical analysis.* The purified

product gives negative tests for glycogen and sulfur. Nitrogen was determined by micro-Kjeldahl method on 35 preparations and values varied between 2.9 and 3.9%, average of 3.4%. Glucosamine was determined by the method of Elson and Morgan(10) as modified by Rimington(11). Glucosamine content for the mucopolysaccharide was 32.3%.*

Results. Rate of hyaluronic acid formation in broth. It has been demonstrated that streptococci which produce hyaluronic acid also elaborate hyaluronidase(12). Thus coexistence of the enzyme and its substrate in the same culture is apparently responsible for disappearance of hyaluronic acid from the broth culture on continued incubation. If this reasoning holds true, prevention of the reaction between enzyme and substrate ought to be possible by addition to the broth of a hyaluronidase inhibitor. Under these circumstances the inhibitor might prevent disappearance of hyaluronic acid in the broth culture that is otherwise certain to occur after the logarithmic growth phase ceases. It was, therefore, of interest to correlate hyaluronic acid production with age of organism as well as to determine the effect of hyaluronidase inhibitor on rate of hyaluronic acid formation in broth. An alginic acid sulfate preparation, previously found very effective inhibitor of hyaluronidase (unpublished observations), was used. Nine-liter carboys containing 5 liters of semi-synthetic stock medium with added alginic acid sulfate (0.1 mg/ml) were inoculated and incubated as described. Aliquots were removed at varying intervals and assayed for hyaluronic acid. Technics of sampling and measurement of hyaluronic acid concentration have been reported(13). The results shown in Fig. 1 demonstrated that rate of hyaluronic acid synthesis in casein hydrolysate broth without addition of alginic acid sulfate is maximal after first 18 hours of incubation. Continued incubation revealed a marked decrease in hyaluronic acid originally produced, resulting in its complete disappearance in 64 hours. However, hyaluronic acid levels in broth culture of *S. pyogenes* containing 0.1

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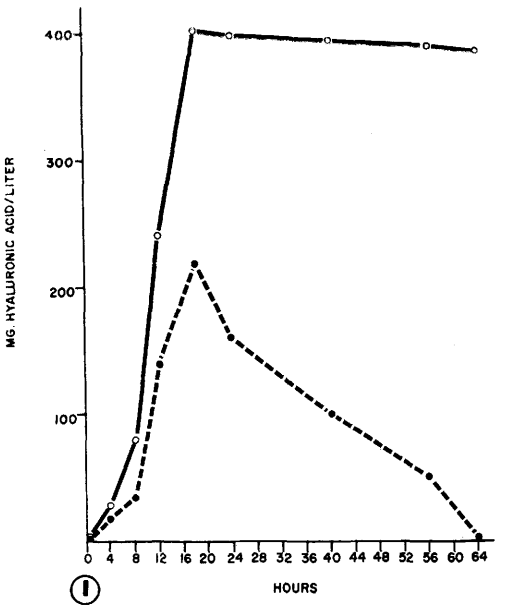

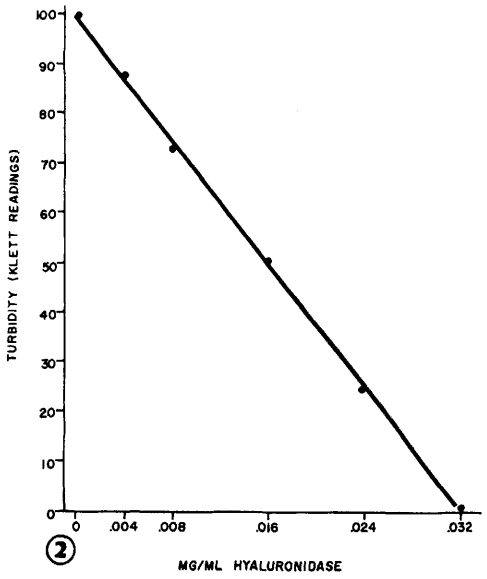

FIG. 1. Concentration of hyaluronic acid in growing culture of Group A *S. pyogenes* in semi-synthetic culture medium. ●—● = hyaluronic acid formed in absence of hyaluronidase inhibitor. ○—○ = hyaluronic acid formed in presence of hyaluronidase inhibitor.

FIG. 2. Depolymerization of partially purified streptococcal hyaluronic acid by purified bovine testicular hyaluronidase. The system consisted of 0.5 ml of 0.4 mg/ml hyaluronic acid and 0.5 ml of various dilutions of hyaluronidase. Incubated 30 min. at 37°C. Reaction stopped and turbidity developed by addition of 4 ml of 1:40 acidified horse serum, pH 3.1. Read in Klett-Summerson colorimeter; red filter No. 66.

mg/ml of alginic acid sulfate were stabilized on continued incubation and a significant increase in hyaluronic acid levels of broths containing hyaluronidase inhibitor was obtained. The curves (Fig. 1), are typical of those obtained with the mucopolysaccharide and are readily reproducible with every batch of material.

Depolymerization of purified hyaluronic acid by hyaluronidase. Depolymerization of partially purified streptococcal hyaluronic acid by purified bovine testicular hyaluronidase (1550 USP units/mg) was studied using turbidimetric assay method described previously(14). A turbidity value of 100 scale divisions on the Klett-Summerson colorimeter was produced by interaction of 0.2 mg of polysaccharide and acidified horse serum. A linear correlation was found between enzyme concentration and depolymerization of the polysaccharide substrate (Fig. 2).

Summary. 1. Hyaluronic acid was released from cells of a Group A *Streptococcus pyogenes* when the latter was grown in medium in which all constituents except casein hydrolysate were chemically defined. With this medium it has been possible to produce high yields of streptococcal hyaluronic acid following 16 to 18 hour incubation at 37°C. 2. Stability of hyaluronic acid in growing cultures of *S. pyogenes* following addition of a hyaluronidase inhibitor, alginic acid sulfate, to the medium, together with a significant increase in hyaluronic acid levels of the broths is further evidence that strains of streptococci which produce hyaluronic acid also produce hyaluronidase. 3. Isolation and partial purification of the bacterial mucopolysaccharide are described.

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Induction and Maintenance of Lactation in Rats by Electrical Stimulation of Uterine Cervix.*† (25166)

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Electrical stimulation of the uterine cervix can induce pseudopregnancy in sexually mature rats(1,2) and hasten sexual maturation in prepubertal rats(3). Presumably, these are elicited by nervous stimuli through sympathetic and hypothalamic pathways, resulting in release of FSH, LH and luteotropin by the anterior pituitary(2,3). There is also evidence that mechanical stimulation of the uterine cervix may elicit oxytocin and possibly vasopressin release from the posterior pituitary(4,5). These findings, as well as our recent observation that epinephrine, acetylcholine or serotonin can initiate and maintain

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