STUDIES ON STREPTOCOCCAL HYALURONIDASE
AND ANTIHYALURONIDASE

I. THE DEVELOPMENT IN VITRO OF STREPTOCOCCAL (GROUP C) HYALURONIDASE, ITS
ISOLATION AND USE AS AN ANTIGEN IN RABBITS

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The uniformity with which high streptococcal (group A) antihyaluronidase titer occurs in
rheumatic fever (Quinn, 1950; Harris and Harris, 1949a, 1949b; Harris et al., 1949; Stoppelman,
1951; Friou and Wenner, 1947; and Salman et al., 1950; 1951) and its reported occurrence in
cases of rheumatoid arthritis (Quinn, 1950; Faber, 1953a, 1953b), in glomerulonephritis (Faber,
1953a, 1953b) and scarlet fever (Friou, 1950) suggest the importance of experimental studies
on the antigenicity of streptococcal hyaluronidase and its histological localization. Therefore we
have undertaken to investigate the development of antibody in rabbits and the histological locali-
ization of the injected enzyme in mice using the Coons fluorescent antibody technique (Coons
et al., 1941, 1942, 1951; Coons, 1952; Hill et al., 1950; Kaplan et al., 1950). The present report
deals with the preparation of partially purified streptococcal (group C) hyaluronidase, its use
as an antigen in rabbits and the course of de-
velopment of antibody.

MATERIALS AND METHODS
The Streptococcal strain and culture methods.
Since it has been shown that various strains of
streptococci differ markedly in the production of
hyaluronidase (Harris et al., 1949; Stoppelman,
1951; Russell and Sherwood, 1949), a strain was
selected which could be depended upon, under
controlled conditions, to produce hyaluronidase
in the culture medium in high concentration.
This strain was received from Dr. H. J. Rogers
and is a subculture of a strain referred to by him
as "group C, Lancefield Type 7, Griffiths" or
briefly "Streptococcus C7" (Rogers, 1948).

This strain has been cultured in a modified Todd-Hewitt broth (Todd and Hewitt, 1932)
containing 2.5 per cent sodium β-glycerophos-
phate, 0.25 per cent glucose and 0.02 per cent hyaluronic acid at pH 7.8. In addition, we have
further modified the Todd-Hewitt medium as
described by Swift (1948) by the substitution of
ground horse meat in place of the fresh beef
heart.

For the study of the growth rate of Strepto-
coccus C7 strain and the development of hy-
aluronidase in the culture medium used, 10 ml
of medium was plated with 0.05 ml of an 18-
hour culture. Cultures were grown at 37 C and
growth was measured by the increase in turbidity
at successive intervals of time. After shaking,
turbidities were read by means of a Coleman
spectrophotometer (Model 6A) at a wavelength
of 600 μ. The growth rate was represented by
the mean turbidity value from quadruplicate
tubes of cultures of increasing age growing in
modified Todd-Hewitt medium both without
hyaluronic acid (medium A) and with the addi-
tion of 0.02 per cent hyaluronic acid (me-
dium B).

Assay of culture medium for hyaluronidase.
After three days’ incubation the filtrate from
Streptococcus C7 cultures containing 0.02 per
cent hyaluronic acid was diluted 50 per cent and
assayed for hyaluronidase, and the results were
compared with filtrates from culture medium to
which no hyaluronic acid had been added. Assays
were also made of cultures of various ages to de-
termine the rate of production of the enzyme.
Assays for the presence of hyaluronidase were
carried out using a turbidimetric method (Tolks-
dorf et al., 1947; Emmart and Longley, 1954)

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modified from the original turbidity reduction technique described by Kass and Seastone (1944). This test for the hydrolytic activity of hyaluronidase upon chondroitin sulfate, hyaluronic acid and its sulfate esters (Meyer and Palmer, 1936; Meyer et al., 1941) is based upon the observation that the enzyme in increasing concentration inhibits progressively the precipitation of the substrate in the presence of albumin in acid medium. The turbidity of the test solution therefore decreases with increase in concentration of enzyme. Since streptococcal hyaluronidase is reported to be inactive against chondroitin sulfate (Meyer and Rapport, 1952) hyaluronic acid has been used uniformly as the substrate.

The turbidities of the aliquots of media A and B in serial dilution were compared with those of bovine hyaluronidase dissolved in modified Todd-Hewitt medium. In these assays one lot of bovine hyaluronidase having a potency of 1400 turbidity reducing units was prepared at a concentration of 30 μg (42 turbidity reducing units) per ml of culture medium adjusted to pH 5.5. This enzymatic preparation was used for the establishment of a curve of enzymatic activity which served as a standard of reference for the estimation of the enzymatic potency of the crude filtrates from the streptococcal cultures. When assaying the potency of partially purified streptococcal enzyme, the bovine enzyme used as a standard was dissolved in sodium phosphate-citric acid buffer solution at pH 5.5. In calculating the potency of enzyme in either the crude filtrate or the partially purified enzyme, comparisons were made at the turbidity value of 0.2 which approximated the midpoint of the straight line portion of the hydrolysis curve.

In these assays aliquots of the enzyme were added to a series of tubes in increments from 0.2 ml to 1.0 ml. To the initial tube containing no enzyme, 1.0 ml of buffer at pH 7.2 was added and the volume of the rest of the series of tubes was adjusted to 1 ml with the same buffer. (When assaying the potency of the enzyme in crude culture filtrate, medium was substituted for the buffer.) One ml of substrate solution containing 0.5 mg of hyaluronic acid in sodium phosphate-citric acid buffer at pH 5.5 was then added, and the mixture was incubated for 30 minutes at 37 C. The tubes were then chilled in a 4 C water bath for 3 minutes to delay further enzyme action and 10 ml of 0.1 per cent solution of bovine albumin (Armour Fraction V) in sodium acetate buffer at pH 3.7 was added; the tubes were shaken gently and the turbidity values were obtained immediately.

In preparing streptococcal enzymes for assay 0.1 per cent albumin was added to stabilize the enzyme (Freeman et al., 1949). These materials were freshly prepared for each set of experiments and kept on ice when not in use.

Methods of isolation of streptococcal C7 hyaluronidase. In an attempt to isolate streptococcal hyaluronidase in a partially purified but stable form we have made use of the method originally described by Freeman et al. (1949) and Freeman and Webster (1952) for testicular hyaluronidase and further modified by Webster et al. (1954) for the isolation and fractionation of bacterial hyaluronidase from cultures of Clostridium perfringens. Using one ammonium sulfate precipitation, followed by three ethanolic fractionsations at −5 C, Webster was able to obtain preparations of hyaluronidase with a 100-fold purification. The success of the method depends principally upon control of pH values, temperature and the molarity of the buffers used, especially during the primary stages of fractionation. In isolating hyaluronidase from cultures of Streptococcus C7 we have followed this technique in simplified form.

Preparation and assay of crude extraction of culture medium. For the inoculation of cultures of Streptococcus C7 to be used for enzyme isolation, aliquots of 10 ml of an 18-hr-old culture were added to 15 250 ml volumes of the modified Todd-Hewitt medium containing 0.02 per cent potassium hyaluronate and incubated at 37 C for 72 hours. At the end of this period all volumes were pooled and an aliquot of approximately 250 ml was removed for assay. The pH of the remaining 3500 ml was adjusted to pH 9.0 with 5 N NaOH and stored at 5 C for 48 hours.

The aliquot for assay was filtered through Seitz S1 filters, one part set aside for freeze-drying and for nitrogen assay and a second part was diluted 50 per cent in 0.02 m sodium phosphate buffer at pH 7.2. This was assayed for potency by the turbidity reduction technique and the potency was calculated against bovine enzyme of known turbidity reducing units.

Ammonium sulfate fractionation. After the remaining 3500 ml of the culture medium had settled, the supernatant fluid was pipetted off.
and centrifuged at zero degrees at 3000 rpm for 30 minutes. The supernatant fluid was filtered and 22.8 g of ammonium sulfate were added to each 100 ml of filtrate, to give 0.3 saturation. The mixture was gently stirred and left to precipitate overnight at 5 C, then centrifuged at +4 C and the precipitate discarded and the supernatant fluid saved. A second ammonium sulfate fractionation at 0.7 saturation was similarly carried out but here the supernatant fluid was discarded and the precipitate saved and dissolved in 700 ml of 0.02 M sodium veronal buffer pH 9.0, containing 0.45 per cent NaCl and 1-10,000 merthiolate.

Ethanol fractionation. To this 700 ml of the crude enzyme in veronal buffer, sufficient absolute ethanol, precooled to -20 C, was added to give 0.1 M fraction of ethanol. After 24 hours at -8 C the preparation was centrifuged at the same temperature and the precipitate taken up in 148 ml of 0.02 M phosphate buffer at pH 7.2. This was dialyzed against cold running tap water until free of sulfate ions. Sufficient 0.2 M phosphate buffer was added to adjust the solution to 0.02 M concentration, the nitrogen determinations were obtained, and the preparation was assayed for enzyme potency.

The second ethanol fractionation was similarly carried out but at a molarity of 0.2 ethanol. In this fraction also the nitrogen value was obtained and a part assayed for potency.

A third and fourth ethanolic precipitation was carried out by the addition of 0.3 and 0.4 M concentration of ethanol but neither fractionation was found to yield enzyme activity. In the fourth ethanol fractionation an inactive brown pigment was removed which raised the turbidity readings during assay.

Calculations of unitage. From dry weights per ml and nitrogen values (Kjeldahl) of the unpurified filtrate and each of the enzyme preparations from the first and second ethanol fractions, along with the turbidity reducing units per ml it was possible to calculate the expected recovery in units per mg, and the loss of potency during the process of purification. From these data the units of activity were calculated in terms of units per mg of protein.

When preparations of the first and second ethanol fractions were lyophilized there was little difference between the activity of the two owing to a loss in potency during the drying process. The two fractions were therefore pooled, assayed in vitro and used as an antigen in rabbits.

Method for developing antihyaluronidase in rabbit sera. In order to determine the optimum amount of antigen needed to produce sufficient antibody in rabbit serum for assay by the turbidimetric method, four white New Zealand rabbits, numbered 060, 061, 062, and 063 were inoculated in the ear vein with 0.31, 0.62, 1.25, and 2.50 mg, respectively, of streptococcal hyaluronidase dissolved in standard phosphate buffer at pH 7.2. These doses were given daily for four successive days, interrupted for three days and resumed on the following day. Thirty-two doses were administered covering an interval of eight weeks and totalling 10, 20, 40 and 80 mg of streptococcal hyaluronidase per rabbit. The maximum daily dose of 2.5 mg representing 21.9 turbidity reducing units was believed to be well below the toxic level for rabbits, which tolerate intravenous injections of bovine hyaluronidase as high as 100 to 200 mg (800 units per mg) per kg of body weight (Seifter, 1950).

The experimental rabbits as well as three control rabbits were bled at approximately weekly intervals and the sera heated to 56 C for 30 minutes to inactivate the nonspecific hyaluronidase inhibitors which are present in many sera (Dorfman, 1950; Fulton et al., 1950; Hadidian and Pirie, 1948; Dorfman et al., 1948). All rabbit sera were assayed before antigen injections and following the beginning of injections at approximately weekly intervals for eight weeks.

Method of assay of the sera. Aliquots in volumes of 0.5 ml of the sera were incubated for 20 minutes at room temperature with 0.5 ml of enzyme solution containing 2.1 mg of streptococcal enzyme in sodium phosphate-albumin solution at pH 7.2. This amount of enzyme was found to have a marked hydrolytic effect on 0.5 mg of hyaluronic acid (figure 7). One ml of hyaluronic acid solution (0.5 mg per ml) was added and the tubes were incubated at 37 C. After the addition of 10 ml of albumin, turbidity readings were obtained in the usual manner. Since the initial turbidities of the serum samples differed from time to time, a correction for this was made by obtaining the difference in turbidity of tubes containing serum with enzyme and the control tubes containing the same serum with buffer. These differences were plotted as “delta turbidity” against the
increase in time concurrent with the antigen injections.

After assays of the sera in all four rabbits indicated that sufficient antibody was present to inhibit 2.1 mg of enzyme, the sera of all four immunized rabbits were assayed in twofold dilution to obtain the antibody titer. In calculating the turbidity readings an aliquot of serum without enzyme was employed as a control and the corrected turbidities were plotted as $\Delta$. At the midpoint of the titration curves, a perpendicular was dropped to the abscissa, and the reciprocal of the nearest dilution to the perpendicular on the abscissa was designated as the titer of the particular antiserum.

The sera of the rabbits with the highest titers were fractionated with cold half-saturated ammonium sulfate, centrifuged, the supernatant material decanted, and the precipitate taken up in cold water and dialyzed against saline at 4 C until free of ammonium sulfate. This fraction was then assayed concurrently with the globulin from normal serum similarly prepared. Assays of globulin fractions were carried out by micro-methods in which the total volume of the test comprised 2 ml. In these latter tests 0.1 ml of a solution containing 2.1 mg of streptococcal hyaluronidase was added in 0.1 ml volume to each tube, the globulin added in serial dilution and the volumes adjusted to 0.5 ml. After 20 minutes at room temperature, 0.5 mg of substrate in 0.5-ml volume was added and the tubes were shaken and incubated at 37 C for 30 minutes. One ml of albumin solution (0.13 per cent) was then added and the turbidity reducing units readings were obtained as described.

RESULTS

Growth of the bacterial strain. Rogers (1945) has shown that the production of hyaluronidase by strain "Streptococcus C7" may be modified considerably by the culture medium and that good growth may not necessarily be accompanied by good hyaluronidase production. Since he used the complex Bernheimer culture medium (Bernheimer et al., 1942), replicate experiments have been carried out to determine both growth and enzyme production of the strain in the modified Todd-Hewitt medium.

In figure 1 the curves represent the mean growth from four tubes in one such experiment. The growth of the cultures, as expressed in increasing turbidities, is plotted against increase in time over a 10-hour period. After 3 hours, the organisms multiplied rapidly for 8 hours until turbidity values reached a plateau between 0.4 and 0.5. During this period the pH of the medium fell from 7.8 to 7.2. A slight clumping of the organisms occurs approximately 12 hours after the period of rapid growth ceased. This was accompanied by a slight fall in turbidity after which there is no change over a 3-day period. There is also little further change in pH; at the end of 8 days this value is 7.0. At maximum growth, the mean turbidity of cultures containing 0.2 per cent hyaluronic acid was only slightly higher than those lacking hyaluronic acid. The results tend to agree with the observations of Rogers (1945) that hyaluronic acid in 0.2 per cent concentration (10 times more concentrated than in our experiments) had little, if any, effect upon rate of growth of Streptococcus C7.

Development of hyaluronidase in culture medium as affected by the addition of hyaluronic acid. A comparison of the data from assays of 20-hour-old culture filtrates with (B medium) and without (A medium) hyaluronic acid showed that in all cultures to which hyaluronic acid had been added there was an increase in hyaluronidase production. In figure 2 these data are presented and a comparison is made of the potency of the hyaluronidase of these crude preparations with bovine hyaluronidase. At the optical density of 0.2 $\text{m}_{\mu}$, 0.45 ml of bovine hyaluronidase,
which had a potency of 18.9 TRU, had equivalent turbidity reducing units in 0.55 ml of the control medium (A) and in 0.17 ml of filtrate (B). Since the filtrates A and B had been diluted 50 per cent before assaying, the turbidity reducing units were calculated as 108 per ml for solution A and 222 for solution B. This indicates an approximate increase of 100 per cent in hyaluronidase production by the addition of 0.02 per cent hyaluronic acid to the culture medium.

These results are similar to the earlier research of McClean and Hale (1941), McClean (1943), Rogers (1946) and Pike (1948), who have shown that the addition of hyaluronic acid to cultures of C. perfringens and to cultures of certain strains of streptococci in various media resulted in an increase in hyaluronidase production. Sellers (1949), working with strains of Streptococcus agalactiae and using 0.2 per cent hyaluronic acid, was unable to obtain similar results. The inherent differences in hyaluronidase production by streptococci belonging to various groups are well recognized (Russell and Sherwood, 1949; Wenner et al., 1951). It has also been shown by Sallman et al. (1951) that diverse streptococcal strains differ widely in their ability to utilize hyaluronic acid, some showing rapid oxygen uptake only in the presence of enzymatic cleavage products of hyaluronic acid.

**Development of enzyme with age of culture.** In addition to the assays of the enzyme in 20-hour cultures, assays were also carried out in serial dilution at 65, 72 and 192 hours after inoculation. By comparison with the standard curve of bovine hyaluronidase described above, these had 378, 756 and 945 turbidity reducing units per ml of culture medium. These data suggested that in volumes of 10 ml of the modified Todd-Hewitt medium the most rapid production of the enzymes occurred during the 2nd and 3rd days following inoculation, and after the point of maximum growth had been attained. Since cultures of 72 hours in our experiments showed high production of the enzyme, this interval was used for further studies in enzymatic production.

**The purification of streptococcal hyaluronidase.** The estimation of the potency of the enzyme preparations at successive steps in purification was based upon the assay of turbidity reducing units per ml of solution as shown in figure 3. Secondly, the increase in potency as expressed in turbidity reducing units was correlated with loss in solids, determined by dry weight per ml, and with mg of protein calculated on the basis of mg of nitrogen per ml of solution.

The procedure followed in the isolation of streptococcal hyaluronidase is shown diagrammatically in figure 4. As shown in this diagram the pooled filtrates had a potency of 108 turbidity reducing units per ml. Before fractionation the pH of this material was adjusted to 9.0 to stabilize the enzyme during the two ammonium sulfate fractionations. This procedure had been found satisfactory in inhibiting loss of potency in the purification of hyaluronidase from filtrates of C. perfringens cultures (Webster et al., 1954). In an attempt to prevent further loss of activity during fractionation with ammonium sulfate the customary dialyses were omitted until after the first ethanolic fractionation (fig. 4).
**Figure 4.** Method of extracting streptococcal hyaluronidase from culture medium.

After this latter procedure, the precipitate, dissolved in 0.02 M phosphate buffer, was dialyzed against water until the solution within the sac was free of ammonium sulfate. After adjustment of the volume as described, assays showed the first ethanolic fraction to have 100 turbidity reducing units per ml and 10.8 turbidity reducing units per mg of solids. This fractionation was
accompanied by a loss of 71 per cent in solids and a 3-fold increase in potency. Since most of the salts and other impurities were removed after the first ethanolic fractionation the turbidity reducing units per mg of protein were increased from 5.2 in the starting material to 96.3 after this extraction (table 1). Subsequently, a 0.2 M ethanolic fractionation of the supernatant fluid remaining from the first ethanol fraction yielded a preparation having 133 turbidity reducing units per ml with a 50 per cent reduction in solids and 86.0 turbidity reducing units per mg of protein.

A 0.3 and 0.4 M ethanolic fractionation similarly carried out upon the remaining supernatant fluids yielded preparations with 0.725 and 1.14 mg of nitrogen per ml but with no enzymatic activity. Since the last two fractions served only to indicate that all activity had been removed by the first two ethanolic fractionations, these were discarded.

It was desirable to express potency of the enzyme in terms of turbidity reducing units per mg, therefore each of the first two ethanolic fractions was lyophilized and the total weight was obtained. When these two fractions were pooled and the dried enzyme assayed, a potency of 8.75 turbidity reducing units per mg of solids was obtained. When this value was correlated with protein present, the dried enzyme was found to have 43 turbidity reducing units per mg of protein (table 1). This represented 50 per cent loss in activity during lyophilization.

The total recovered turbidity reducing units of the lyophilized enzyme represented 4.7 per cent of the amount calculated to be present in the crude culture filtrate. The purified solids recovered were 2.04 g; this was 2 per cent of the total 108.5 g calculated on the basis of dry weight in 1 ml samples of the starting material (table 1). The preparation when kept at 5 C has proved to be stable after a year in storage.

It has been observed that the removal of protein during purification with ethanol (Freeman and Webster, 1952), by chromatographic fractionation (Rogers, 1948) or by heat (Emmart and Longley, 1954) tends to lessen the stability of the enzyme. Since stability of the dried preparation was essential for our research further steps in fractionation were for the present not undertaken.

**Antibody to streptococcal hyaluronidase.** The development of antibody to streptococcal group C hyaluronidase in the sera of inoculated rabbits as expressed by turbidity reduction units is shown in comparison with that of sera from

### TABLE 1

**Purification of streptococcal "C" hyaluronidase**

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<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
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<td></td>
<td>TRU/ml *1 Assay</td>
<td>Total Solid g</td>
<td>Mg/ml Dry wt</td>
<td>TRU/mg A C = D</td>
<td>Mg of N per ml in Kjeldahl</td>
<td>TRU per mg of N A F = E</td>
<td>TRU per mg of Protein F G =</td>
<td>Total TRU</td>
<td>% TRU Recovered</td>
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<td>I. Starting material</td>
<td>108</td>
<td>108.5</td>
<td>31</td>
<td>3.48</td>
<td>3.29</td>
<td>32.8</td>
<td>5.2</td>
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<td></td>
<td>C X 3500 ml</td>
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<td>II. First ethanol fractionation. Total</td>
<td>100</td>
<td>1.353</td>
<td>9.1</td>
<td>10.80</td>
<td>0.155</td>
<td>602.0</td>
<td>96.3</td>
<td>14,000</td>
<td>3.8</td>
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<td>vol before drying, 148 ml</td>
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<td></td>
<td>(B)</td>
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<td>(148) Calculated</td>
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<td>III. Second ethanol fractionation. Total</td>
<td>133</td>
<td>0.690</td>
<td>4.6</td>
<td>28.90</td>
<td>0.345</td>
<td>538.0</td>
<td>86.0</td>
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<tr>
<td>IV. Frozen-dried and pooled ethanol fractions I and II</td>
<td>35.75</td>
<td>2.043</td>
<td>4.2</td>
<td>8.75</td>
<td>0.134</td>
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<td>43.0</td>
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TRU = Turbidity reducing units.

*1, 2 = Standardized against bovine hyaluronidase (1 mg = 1400 TRU).
control rabbits in figure 5. In this chart the turbidity values for hyaluronidase activity are plotted against time, the latter representing successive weekly periods of inoculation over a total of 8 weeks. With rise in antibody in successive serum samples, the hydrolytic activity of enzyme upon the substrate is increasingly inhibited. Expressed as “Δ turbidity” this inhibition of the enzyme is indicated by a fall in the slope of the curve. The sera of rabbits 063 and 062 receiving four doses of 2.50 and 1.25 mg weekly were the first to show the development of antibody. After four weeks rabbits 061 and 060, which received 0.63- and 0.31-mg doses, also showed marked antibody development. The respective dosages were continued for four weeks longer, after which the sera in 50 per cent dilution of all four animals had sufficient antibody to markedly inhibit 2.1 mg of hyaluronidase. The assays of the sera of normal rabbits 064, 065 and 066, which were carried out concurrently, showed no such consistent change with increase in time (figure 5).

Titer of the antisera. Eight weeks after commencement of intravenous injections of the antigen, all rabbits were again bled and the sera were assayed in twofold serial dilution. The rise in the slope of the curves in figure 6 is associated with the serial dilution of the antibody. The titer of the serum from the rabbit which received the lowest dose (0.31 mg) was readily distinguished from the serum of the rabbit receiving 2.5 mg and from those receiving the intermediate doses but the difference in the titer of the sera of the rabbits receiving 0.63 and 1.25 was obscure. By the end of eight weeks the experimental rabbits had received total doses of 10, 20, 40 and 80 mg and had titers of 16, 32, and 128 respectively. None of the rabbits showed any evidence of toxicity from the doses given.

Inhibiting action of the globulin fractions of antisera to streptococcal hyaluronidase. To correlate the antihyaluronidase activity with the globulin fraction of the sera, a comparison was made between the inhibiting action of globulin fractions of sera of sensitized and normal rabbits. Concurrently an assay of the hydrolysis of the substrate, hyaluronic acid, by the enzyme was also carried out. In figure 7 (A) the hydrolysis of 0.5 mg of hyaluronic acid by increasing concentration of the streptococcal hyaluronidase is represented by the mean values of four experiments. The resulting curve served as a reference for comparison with enzyme hydrolysis in the presence of antisera. At a concentration of 2.1 mg of enzyme, precipitation of the substrate

Figure 5. Development of homologous antihyaluronidase sera in rabbits immunized with streptococcal (C7) hyaluronidase. Δ = turbidity (sera plus buffer) minus turbidity (sera plus enzyme).

Figure 6. Titer of antisera of rabbits receiving intravenous inoculations of streptococcal hyaluronidase after 8 weeks' immunization.

Figure 7. Antihyaluronidase activity of globulin fractions of three preparations of antisera as compared with normal globulin. A, Hydrolysis of hyaluronic acid by enzyme. B, Inhibition of the hydrolysis with 2.1 mg of enzyme by increasing concentrations of antiglobulin.
with its accompanying turbidity was markedly inhibited. This concentration was therefore selected to test the action of the antihyaluronidase globulin.

In figure 7 (B) the curves represent three experiments in which the activity of the enzyme is progressively inhibited with increase in concentration of antibody to hyaluronidase. The variation in the curves is due to obtaining globulins from bleedings at three different times after cessation of immunization. As shown, globulin from sera of normal rabbits has no such inhibitory effect.

Retention of antigen and anamnestic reaction. After the first eight weeks of successive inoculations further inoculations of enzyme were discontinued for four months. At the end of this period the rabbits that had formerly received doses of 1.25 and 2.50 mg were again bled and the sera again assayed for antibody. These data indicated that a marked decline in antibody had occurred; rabbit 063, which had an original titer of 128, now had a titer of 8; and rabbit 062, which had an original titer of 32, now had a titer of 2. Although the titer of the sera had declined significantly over the four-month rest period, sufficient was retained to be readily measurable by the turbidimetric reduction technique.

After this rest period of four months, single weekly inoculations were resumed in doses of 2 mg per week in the rabbits with high titer. The rise in titer which followed was determined by weekly assays of the sera. In the first period of inoculation rabbit 063 had received a total of 80 mg in 32 injections in eight weeks with a resulting titer of 128. During the second period of inoculation, in which 12 mg were administered over a six-week period, the titer rose from 8 to 2048. In rabbit 062, which originally had received 40 mg with a resulting titer of 32, had, after the rest period, a serum titer of 2. After the secondary injection of a total of 12 mg, a titer of 62 was obtained.

This rapid rise of antibody following a second course of injections of "C₇-streptococcal hyaluronidase" in rabbits with persistent low titers is consistent with the usual anamnestic antibody response from diverse antigens (Harris and Harris, 1949b; Willis, 1928; Baldwin and Gardner, 1921; Boyd, 1943) and conforms in particular with the results obtained by McClean (1943) using hyaluronidase from group C strain 7 streptococcus as an antigen.

DISCUSSION

The data presented above from serum assays covering a period of nine months indicate that the hyaluronidase from group C strain 7 streptococcus, prepared as described, is highly antigenic in the rabbit, and that the resulting antibody persists at lowered levels for many weeks after cessation of injections.

The earlier researches of McClean (1943) and Hobby et al. (1941) also have shown that antisera to partially purified hyaluronidase from group A streptococcus can be produced experimentally in the rabbit. McClean (1943) observed that the immune response of antisera of A and C group hyaluronidase was specific. More recently Wenner et al. (1951), using crude bacterial filtrates of streptococcal cultures of groups A, B, C, and G, have immunized rabbits by both intravenous and intramuscular injections and have obtained antisera to hyaluronidase in two to six weeks. They were able to show that groups A and B produce antisera distinct from groups C and G. It is suggested that with purification of the antigen further distinctions may be possible.

Our data give additional support to the original observation of McClean (1943) that rabbits immunized with group C hyaluronidase maintain in the sera detectable antibody for many weeks after cessation of injections. Whether the antibody to group C hyaluronidase or the antigen persists in host tissues is a debatable point. Further evidence must await the completion of future studies in the histological localization of the injected antigen. The success in detecting injected antigens is dependent not only upon the presence of the antigen but upon the potency of the antisera. Our studies in assaying the antisera in vitro by the turbidimetric reduction technique have shown that it is possible to determine potency of the antisera and by this method assay the titer of sera before use in studies involving the preparation of fluorescent antibody conjugates.

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**SUMMARY**

The present report has shown that:

(1) A modification of the Todd-Hewitt medium supplemented with β-glycerophosphate 2.5 per cent, glucose 0.25 per cent and hyaluronic acid 0.02 per cent can be substituted for the Bernheimer medium used by Rogers in the cultivation of streptococcus group C strain 7 without impairing the production of hyaluronidase by the organism.

(2) Under these conditions and following the period of rapid growth the strain is capable of producing hyaluronidase in high concentration.

(3) This enzyme has been isolated in a partially purified and lyophilized form which has maintained its potency for a year or longer.

(4) It has been demonstrated by assay of sera of immunized rabbits that the enzyme prepared as described is antigenic. Following the intravenous administrations of total doses of 80 mg, high titers are attained. Upon cessation of injections these titers fall slowly, but are readily measurable after four months. A rapid anamnestic antibody rise, exceeding the original titer, occurs on resumption of antigen injection.

(5) These data indicate that the administration of the enzyme produces specific antibody which affords means for studying the histological localization and retention of hyaluronidase within tissues of the experimental animal.

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