STUDIES OF THE MUCIN-CLOT PREVENTION TEST FOR THE DETERMINATION OF THE ANTIHYALURONIDASE TITRE OF HUMAN SERUM [1]

By ROBERT W. QUINN [2]

(From the Section of Preventive Medicine, Yale University School of Medicine, New Haven)

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INTRODUCTION

The presence in human serum of an inhibitory substance which is capable of neutralizing an enzyme elaborated by a strain of hemolytic streptococcus recently has been demonstrated by Friou and Wenner (1). They further showed that the amount of inhibitory substance in sera from patients with rheumatic fever was greater than in sera from patients early in the course of uncomplicated hemolytic streptococcal infections or from normal individuals. These workers (1) employed the mucin-clot prevention test (M.C.P.) described by McClean (2) which was based on the observations of Seastone (3) and Meyer and Palmer (4) that hyaluronic acid mixed with serum protein in the presence of acetic acid gives a characteristic clot.

A brief historical background of the work preceding that of Friou and Wenner begins in 1928 with the observation of Duran-Reynals (5, 6) that vaccinal infection of the rabbit was considerably enhanced when the virus was injected into the skin along with an aqueous extract of rabbit, guinea pig or rat testicle. McClean (7) and Hoffman and Duran-Reynals (8, 9) in 1930 described independently the ability of testicular extracts to increase tissue permeability. These extracts were subsequently termed "spreading factors." Extensive studies by Duran-Reynals later showed that spreading factors were present in extracts of certain invasive bacteria (10), poisonous insects and snake venoms (11). Meyer *et al.* (12) found similar so-called enzymes in autolysates of a rough (R) type II pneumococcus. Enzymes of the same type were subsequently obtained from the bovine ciliary body and iris (13), from certain strains of Group A hemolytic streptococcus and spleen (14, 15), and from *Clostridium welchii* (10, 16). It remained for Chain and Duthie (17, 18) in 1939 to show the relationship between these enzymes (hyaluronidases) and the spreading factor and to correlate the phenomenon of spreading in animal tissues with an enzymatic effect on the hyaluronic acid of connective tissue.

Recently studies dealing with the composition of connective tissue has received deserved attention. In 1934 Meyer and Palmer (19) reported the occurrence in vitreous humor of cattle eyes of a mucopolysaccharide consisting of equimolecular concentrations of acetylglucoseamine and glucuronic acid. They named this substance hyaluronic acid. In the same year Bensley (20) reported on histological observations of connective tissue. The distribution of ground substance was found to be the same in the umbilical cord, intima and media of blood vessels, connective tissue of lower vertebrates, gastric mucous membranes, and in general in all reticular and embryonic tissues. Meyer *et al.* have since extracted hyaluronic acid from aqueous humor of cattle (21), Wharton's jelly (12), and synovial fluid (22). It has also been extracted from rabbit and pig skin (23, 24), rabbit fascia (16) and the capsules of certain strains of hemolytic streptococci (14, 25).

Duran-Reynals (26) in 1932 reported experiments which showed that the spreading factor of testicle was neutralized *in vitro* by an antiserum against homologous testicle extract. McClean (27) and McClean and Hale (28) have demonstrated that sera prepared against the so-called spreading factor, neutralizes the diffusing, viscosity reducing, and hydrolytic activity of *Clostridium welchii* and vibrion septique. They also demonstrated antihyaluronidase activity against the homologous enzyme in rabbits immunized with hyaluronidase from Group A, type 4 and Group C, type 7 streptococci.

These studies and work now in progress (29) indicate that streptococcal hyaluronidase is anti-

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[2] Milbank Memorial Fund Fellow, 1946–47.

genic in rabbits and that antisera can be produced against this enzyme. There are, however, some different opinions concerning the specificity of these antienzymes. Haas (30) states that there is an enzyme (antinvasin I) in normal blood plasma of all mammals, birds, and fish investigated by him, which, by destroying hyaluronidase, acts as an anti-invasive catalyst. According to Haas this substance destroys hyaluronidases derived from a number of different sources. Following their recent studies, Hechter and Scully (31) cast some doubt in the biological significance of the enzymatic theory for the mechanism of invasion proposed by Haas.

The purpose of this paper is to describe studies of the method of antihyaluronidase determination by use of the mucin-clot prevention test (M.C.P.), first used by Robertson, Ropes, and Bauer (16) and later standardized by McClean (2).

MATERIALS

Enzyme:

The enzyme used in these tests was obtained from a strain of Group A, type 4 streptococcus hemolyticus. 1000 ml. of beef heart infusion broth enriched with 10% normal calf serum was seeded with 25 ml. of a 12-hour beef heart infusion broth culture of Group A, type 4 hemolytic streptococcus and incubated at 37° C. for 36 hours. The bacterial cells were separated from the supernatant fluid by centrifugation and the supernatant fluid which contained the enzyme was then filtered through a Seitz filter and the enzyme content titrated. This supernatant fluid was kept frozen at −70° C. in 40-ml. lusteroid tubes until ready for use as enzyme. Two lots of enzyme, A and B, were prepared; the enzyme content of each lot was found to be similar.

Substrate:

Potassium hyaluronate was prepared from human umbilical cords according to the method of McClean *et al.* (30). The cords were washed free of blood and stored in acetone in the refrigerator until ready for use. The cords were then ground in a meat grinder. To 500 grams of the minced cords were added 1000 ml. of water and the mixture allowed to stand for 24 hours in the refrigerator. The mixture was then squeezed through four thicknesses of surgical gauze. The mince was again extracted with 1000 ml. of water. The pooled extracts were centrifuged at 2200 RPM for 30 minutes. The thick, grayish-pink viscous solution was adjusted to a pH between 9 and 10 with potassium hydroxide. One and one-fourth volumes of cold methyl alcohol saturated with potassium acetate were added slowly to these pooled extracts which had previously been cooled in the refrigerator. The potassium hyaluronate which was precipitated as a characteristic asbestos-like clot was washed several times with cold methanol, then with cold ether and finally dried *in vacuo* over phosphorous pentoxide. It was then ground into a fine powder and stored at room temperature until used. It was found that a greater yield could be obtained during each extraction if the acetone in which the umbilical cords were kept was changed at least once. A 0.15 per cent solution of potassium hyaluronate in distilled water was made up in 100-cc. volume and stored in the refrigerator at −4° C. This solution was used within three to four days and was sufficient to test 40 sera. No preservative was employed. Solution of the powder was facilitated by placing the flask in an incubator at 37° C. for two to three hours with occasional shaking. It is advisable to make up several grams of potassium hyaluronate and use the same hyaluronate for all experiments rather than use hyaluronate extracted at different times, since the strength of the hyaluronate may vary with each extraction and thus introduce another variable into the test. McClean (2) showed that the apparent potency of enzyme is inversely proportional to the concentration of the substrate mixture. Although different preparations of potassium hyaluronate were used in these experiments the enzyme titre did not change more than one two-fold dilution when the same enzyme was tested against substrate containing potassium hyaluronate extracted at different times. The potassium hyaluronate was standardized by testing the unknown preparation of potassium hyaluronate with a constant amount of purified hyaluronidase (bull's testicle, Schering). The clotting ability of different hyaluronate solutions used in the substrate was adjusted to give the same end point with a standard amount of hyaluronidase by dissolving the proper weight of potassium hyaluronate in water. This amount was usually 1.5 mgm. per ml. of water.

Serum:

The blood used in the study of normal individuals and patients was drawn as aseptically as possible. The serum was separated from the clot within 24 hours in most instances, and stored in lusteroid tubes at −70° C.

Normal horse serum (Lederle) was used in the substrate. Potassium hyaluronate will not form a clot on the addition of acetic acid unless blood serum is also present. Normal rabbit serum and calf serum were tried with equally good results. None of these sera contained antihyaluronidase when tested by the mucin-clot prevention test.

Preparation of substrate mixture:

In each tube in the test, 1 ml. of substrate was used. The total volume was made up in the following proportions:

Substance	Ratio
Potassium hyaluronate 0.15% in distilled water	1
Normal Horse Serum 1 : 10 dilution in Normal saline	1
Distilled water	2

To avoid waste and diminished clot-forming ability of the substrate it was found best to make up only enough substrate for each day's testing.

EXPERIMENTAL METHODS

The mucin-clot prevention test was used as a test for hyaluronidase and antihyaluronidase. The method used is a modification of that described by McClean, Rogers, and Williams (32) which was employed by Friou and Wenner (1). The serum being tested rather than the enzyme was the variable factor in the test. Serum dilutions were made in two-fold decrements and each serum was tested against a constant amount of hyaluronidase.

A. *Test for hyaluronidase*

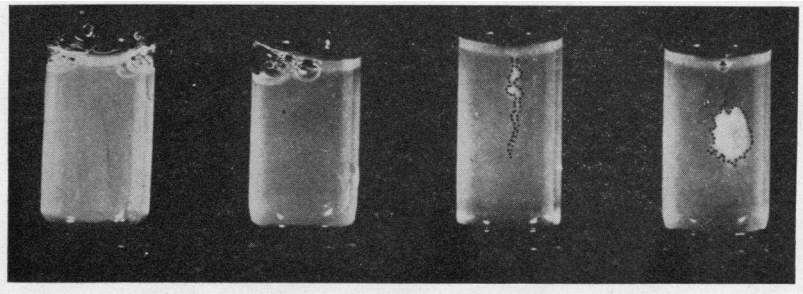
1. Serial two-fold dilutions of the enzyme being tested were made in distilled water; 0.5 ml. to each tube.
2. 0.5 ml. distilled water was added to each tube.
3. The racks of tubes were placed in an ice bath for five minutes and then 1.0 ml. of substrate was added to each tube. (An automatic pipette greatly facilitates the speed and accuracy of adding solutions to the tubes.)
4. The racks were placed in a water bath at 37° C. for 20 minutes.
5. The racks were then removed and placed in an ice bath for five minutes.
6. Finally 0.2 ml. of 2 N acetic acid was added to each tube beginning with the greatest dilution and proceeding to lesser dilution and the tubes were shaken lightly.

Figure 1 shows an enzyme titration and the determination of enzyme units. Frequent titration of the enzyme revealed it to be quite stable. Its titre did not change in a 14-month period.

Occasionally when a tube is near the end point of the enzyme, shreds will form. In these studies only a fully formed clot was counted as +. Shreds were counted as 0. A photograph of four tubes of a completed ten-tube test for hyaluronidase overemphasizes the turbidity of the solution. In the actual test turbidity is much less noticeable and the clots are seen more clearly. Following the lead of McClean, Rogers, and Williams (32) results were expressed in terms of the original dilution of enzyme in 1.0 ml. and the subsequent dilution with substrate mixture was disregarded. Data in Figure 1, where a clot is first seen in the tube in which the final enzyme dilution before addition of substrate is 1 : 2048, are interpreted to mean that a 1 : 1024 dilution of the enzyme being tested is just enough to prevent the formation of a clot in the substrate on the addition of 0.2 cc. of 2 N acetic acid. The amount of enzyme in the last tube (next to the end point) has been arbitrarily designated as 1 unit. 16 units of this particular enzyme would be a 1 : 32 initial dilution or 1 : 64 final dilution.

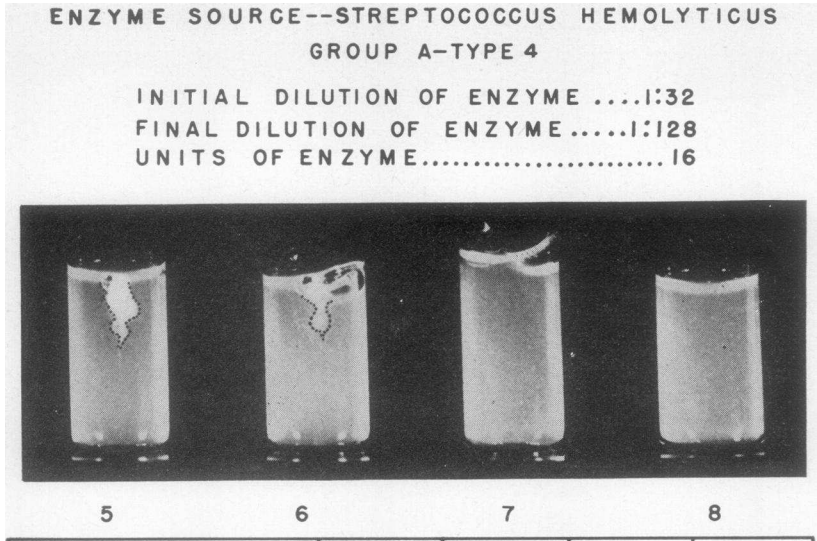
B. *Test for antihyaluronidase:*

1. Serial two-fold dilutions of the patient's serum were made in distilled water, 0.5 ml. to each tube. It was found that the end point of most sera would fall within a ten-tube range if the first tube contained a 1 : 32 dilution of the serum under test. Occasionally the antihyaluronidase titre of a serum will be too low or too high to fall within this ten-tube range. Then, a further titration must be done using lower or higher dilutions of serum



SERUM TUBE	7	8	9	10
INITIAL DILUTION OF ENZYME	1:256	1:512	1:1024	1:2048
FINAL DILUTION OF ENZYME	1:512	1:1024	1:2048	1:4096
CLOT FORMATION	0	0	+	+
UNITS OF ENZYME	2	1		

FIG. 1. SAMPLE TITRATION OF HYALURONIDASE



SERUM TUBE	5	6	7	8
INITIAL DILUTION OF SERUM	1:512	1:1024	1:2048	1:4096
FINAL DILUTION OF SERUM	1:1024	1:2048	1:4096	1:8192
CLOT FORMATION	+	+	0	0

FIG. 2. TITRATION OF A SERUM FOR ANTIHYALURONIDASE

as the case may be. Usually 12 to 20 sera were tested at a time.

2. 0.5 ml. of a predetermined dilution of enzyme was added to each of ten tubes and the racks were allowed to stand at room temperature for 15 minutes. The enzyme dilution was adjusted so that the same amount of enzyme was used in each test. Most of the sera were tested against 16 units (0.5 ml. of an initial dilution of 1:32) of enzyme. Occasionally 32, 64 or 128 units of enzyme were used.

3. The racks were then placed in an ice bath and allowed to remain there for five minutes.

4. 1.0 ml. of substrate was then added to each tube and the racks were placed in a water bath at 37° C. for 20 minutes.

5. The racks of tubes were then removed and placed in the ice bath for five minutes.

6. 0.2 ml. of 2 N acetic acid was finally added to each tube beginning with the greatest dilution and proceeding to the lesser dilution and the tubes shaken gently. (Figure 2 shows the results of the determination of the antienzyme titre of a serum.) The first tube in which a clot appeared reading from greater to lesser dilution was established as the end point of the serum antienzyme titre.

The serum represented in Figure 2 was capable of inhibiting 16 units of streptococcal hyaluronidase in a final dilution of 1:2048. The antihyaluronidase titre of this serum therefore is 1:2048 when tested against 16 units of enzyme. Here again the photograph overemphasizes the turbidity of the solution. Precedent for not including the volume of the substrate mixture in calculating the antihyaluronidase titre can be found in the work of McClean (2).

7. Along with the unknown sera two known sera were tested. These sera served as controls. It is important to make serum dilutions with fresh serum each day. If sera are previously diluted and stored in an ice box the antienzyme titre decreases markedly within a few days.

RESULTS

The length of time of incubation of the enzyme-substrate mixture at 37° C. was 20 minutes in all the tests for the determination of hyaluronidase. However, hydrolysis of the substrate by the enzyme continues rapidly for at least an hour according to McClean's (2) work. In Table I is shown the results of incubating the enzyme-substrate mixture for variable periods of time. The hyaluronidase titre obtained was the same after five- and 20-minute incubation periods but the titre was slightly higher after 60 minutes of incubation.

The two lots of hyaluronidase used throughout these experiments were of almost equal strength.

TABLE I

The effect of incubation time on the enzyme strength
Test for Hyaluronidase
Source of Enzyme: Streptococcus Hemolyticus
Group A—Type 4

Time of incubation of enzyme-substrate mixture	Final enzyme titre
5 *minutes*	1:1024
20 *minutes*	1:1024
60 *minutes*	1:2048

The results of duplicate titrations of enzyme performed on different dates are shown in Table II and they suggest that a more accurate titration of the enzyme can be made if the enzyme dilutions are made in increments of 50 or 100 in each successive tube, *e.g.*, 1 : 50, 1 : 100, 1 : 150, 1 : 200, etc. rather than in serial two-fold dilutions.

TABLE II

Methods of dilution of enzyme to determine its strength
Tests for Hyaluronidase in Duplicate
Source of Enzyme: Streptococcus Hemolyticus
Group A—Type 4

Date of tests	Final enzyme titre		
	Increment of 50	Increment of 100	Serial two-fold dilution
17 June 1947			
1st Test	1:900	1:800	1:1024
2nd Test	1:800	1:600	1:1024
18 June 1947			
1st Test	1:900	1:600	1:1024
2nd Test		1:600	1:1024
1 July 1947			
1st Test	1:800	1:800	1:1024
2nd Test	1:800	1:800	1:1024

The enzyme preparations did not lose strength when stored in a refrigerator at − 4° C. or in the frozen state at − 70° C. Titrations of the same enzyme several months apart revealed the same titre.

Apparently the reaction between the inhibitory substance in serum and the streptococcus enzyme was nearly completed within five minutes. Table III shows the results of a test to demonstrate the effect of the length of time of incubation of the serum-enzyme mixture on the antihyaluronidase titre. Incubation of the mixture for 190 minutes resulted in a four-fold increase in the final antihyaluronidase titre as compared to the titre obtained after only five minutes' incubation. There was no difference in the antihyaluronidase titre obtained after incubation periods of five, 15, and 20 minutes.

TABLE III

Test for antihyaluronidase to demonstrate the effect of length of the time of incubation of the serum-enzyme mixture on the antienzyme titre
Enzyme Source: Group A, Type 4 Hemolytic Streptococcus 64 Units

Time of reaction between serum and Enzyme at room temp.	Serum antienzyme titre
5 *minutes*	1:128
15 *minutes*	1:128
20 *minutes*	1:128
190 *minutes*	1:512

In order to determine the effect of different storage temperatures on the antihyaluronidase titre of sera, samples of sera were stored at − 70° C., − 4° C., and at room temperature. There was no apparent loss of titre in sera kept frozen at − 70° C., but in serum stored at room temperature the antihyaluronidase titre decreased considerably. The titre of serum stored at − 4° C. decreased slightly (Table IV).

TABLE IV

Effect of storage temperature and time on the antihyaluronidase titre of sera

Serum	Storage temperature and time			
	− 70 c. 13 Mo. antihyaluronidase titre	− 4 c. 2 Mo. antihyaluronidase titre	Room Temp. 2 Mo. antihyaluronidase titre	Original antihyaluronidase titre
A	1:32768	1:16384	1:8192	1:32768
B	1:1024	1:512	1:256	1:1024

To determine the limits of experimental error for the test for antihyaluronidase, four sera, and normal human serum gamma globulin [3] were used as controls and were tested along with patients' sera. The results of these titrations on different days are tabulated in Table V. The greatest variation in antihyaluronidase titre of any of the control sera was a two-tube (or four-fold) difference. These results would seem to establish the limits of experimental error for the test sera as two two-

[3] Prepared by the Department of Physical Chemistry, Harvard Medical School, in cooperation with the Antitoxin and Vaccine Laboratory, Mass. Dept. of Public Health, from blood collected by American Red Cross for the Committee on Medical Research, Office of Scientific Research and Development.

TABLE V

Serial tests for antihyaluronidase of four control samples of serum and serum gamma globulin

Date of test	Control serum enzyme titre				
	Frick	Aldrich	Raymond	Faracles	Serum gamma globulin
12/26/46	1:65,536				
3/18/47			1:1024		
3/26/47			1:1024		
3/21/47			1:1024		
			1:1024		
3/31/47		1:1024			
4/ 7/47	1:16,384	1:1024		1:1024	1:32,768
4/12/47	1:16,384	1:4096		1:1024	1:32,768
4/14/47	1:16,384	1:2048		1:1024	1:32,768
4/16/47		1:2048		1:512	1:32,768
4/17/47		1:2048			
4/21/47		1:2048		1:512	1:16,384
4/23/47					1:16,384
4/29/47	1:32,768	1:4096			
	1:65,536	1:4096			
5/ 2/47	1:65,536	1:1024			
5/ 7/47		1:1024			
5/ 8/47	1:32,768				
5/ 9/47	1:16,384	1:1024			
5/15/47	1:65,536	1:4096			
5/19/47	1:32,768	1:2048			
5/23/47	1:32,768	1:1024			
	1:32,768	1:1024			
5/24/47			1:2048		
5/26/47	1:16,384	1:1024			
5/27/47	1:32,768	1:1024			
7/ 4/47	1:32,768	1:1024			

fold dilutions. It is important to make serum dilutions with fresh serum each day because if sera are previously diluted and stored in a refrigerator, the antienzyme titre decreases markedly within a few days.

In order to determine the effect of different strengths of hyaluronidase on the antihyaluronidase titre of sera, 14 sera were tested against different enzyme strengths. Early in the study, sera were tested abainst 64 or 128 units of enzyme. Later, all sera were tested against 16 units of enzyme. Serial two-fold dilutions of serum were tested against 128, 64, 32, 16, 8 and 4 units of enzyme, respectively. The results of some of these titrations are plotted in Figure 3. In general a linear relationship was observed when antienzyme titre was plotted against enzyme units. If a linear relationship were always observed, theoretically a given serum dilution would inhibit 128 units of enzyme, a two-fold greater dilution of serum would inhibit 64 units of enzyme, and so on. But such a relationship was not always observed, particularly in sera with very high antienzyme titres. In sera with lower antienzyme titres the linear relationship held, particularly in the range between 64 to 16 units of enzyme. The antihyaluronidase titres of sera tested against 64 or 128 units of enzyme were extrapolated in terms of 16 units of enzyme. Four of the sera as shown in Figure 3 (M.M., R.L., L. and D.A.) had antihyaluronidase titres which fell along a perfect straight line when plotted against units of enzyme within the range between 64 to 16 units. In only two instances (F. and D.A.) did the observed antihyaluronidase titre differ more than a two-fold dilution from the predicted antihyaluronidase titre.

Employing the (M.C.P.) method for testing

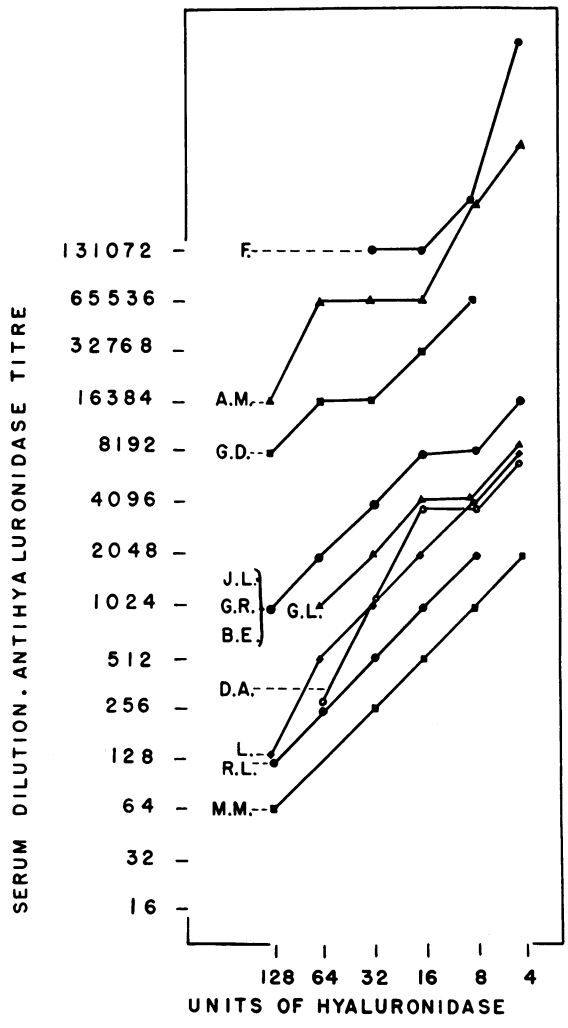


FIG. 3. RESULTS OF TESTS OF SERIAL TWO-FOLD DILUTIONS OF SERUM AGAINST SERIAL TWO-FOLD DILUTIONS OF STREPTOCOCCUS GROUP A, TYPE 4 HYALURONIDASE

antihyaluronidase, 16 sera from ten individuals were tested against hyaluronidase obtained from a strain of Group A, type 4 hemolytic streptococcus, a strain of staphylococcus aureus, and against purified bull's testicle hyaluronidase.[4] The sera tested all showed the presence of an inhibitory substance againt hyaluronidase from the hemolytic streptococcus, little or no antienzyme against the staphylococcus enzyme and no inhibition of the bull's testicle enzyme. These results are similar to those reported by Friou and Wenner (1). However, using the viscosity reducing method described by Haas (30) eight of the sera exhibited the presence of an inhibitory substance against enzyme from bull's testicle (Schering).[5] These same eight sera contained no antienzyme against staphylococcus or bull's testicle (Schering) enzyme when tested according to the mucin-clot prevention test of McClean *et al.* (32) but they did exhibit antienzyme against streptococcus enzyme in considerable amounts. Neither normal calf serum nor normal horse serum contained any demonstrable antienzyme against hyaluronidase from any of the above sources when tested with the mucin-clot prevention test.

Twenty-three sera in all including normal horse serum and normal calf serum were tested by the viscosity reducing method and all were found to contain a substance which would inhibit the hydrolysis of hyaluronic acid by hyaluronidase from bull's testicle. This substance has been termed antinvasin I by Haas (30). The substance which will prevent the action of hyaluronidase in the mucin-clot prevention test has been termed antihyaluronidase.

DISCUSSION

Setting up the mucin-clot prevention test for antihyaluronidase so that the serum being tested rather than the enzyme is the variable factor, has increased the efficiency of the test as a measure of antihyaluronidase activity. Previously when the enzyme was the variable factor, some sera would inhibit undiluted enzyme thereby making an end point unobtainable. The method described in this study will always enable one to obtain an end point of antihyaluronidase titre. Two-fold serum dilutions rather than three-fold enzyme dilutions increase the accuracy of the test. It is believed that the accuracy of the test can be increased further by using standardized preparations of hyaluronidase and potassium hyaluronate.

The linear relationship which was observed after plotting the results of testing sera diluted serially in two-fold decrements against serial dilutions of beta hemolytic streptococcal enzyme in two-fold decrements seems to make it possible to extrapolate the antihyaluronidase titres of sera tested against 64 or 128 units of enzyme to the titre which would be predicted if the sera were tested against 16 units of enzyme.

When a few sera were tested by means of the viscosity reducing method they exhibited the property of inhibiting the viscosity reducing action of hyaluronidase from bull's testicle (Schering). However, when the same sera were tested by the mucin-clot prevention test the property of inhibition of clot production by hyaluronidase from bull's testicle (Schering) was not present nor was there any appreciable inhibition of hyaluronidase from staphylococci. These sera did, however, exhibit significant inhibition of hyaluronidase from a strain of Group A, type 4 hemolytic streptococcus. The reasons for these discrepancies are not clear but from the work of McClean (2) it would appear that viscosimetry and the mucin-clot prevention test measure the activity of the same agent but that destruction of the clotting power of the substrate occurs earlier than any appreciable fall in viscosity. Possibly a longer incubation period of serum-enzyme mixtures will lessen the differences between the two methods in measuring antihyaluronidase activity. Studies are in progress in an effort to clarify the significance of these differences.

SUMMARY AND CONCLUSIONS

1. Slight modifications of methods for the determination of the hyaluronidase activity of certain strains of hemolytic streptococci and staphylococci as well as hyaluronidase extracted from testes have been described.

2. A modification in the method of determining the antihyaluronidase titre of sera by means of the mucin-clot prevention test whereby the serum

[4] The purified bull's testicle hyaluronidase was furnished through the courtesy of Dr. Erwin Schwenk of Schering Corporation, Bloomfield, N. J.

[5] This method was not used routinely because it is so much more time-consuming than the M.C.P. test.

rather than the enzyme is the variable factor were made. The limits of error of this method at present is about two two-fold dilutions, but it is believed that the accuracy can be increased.

3. Serial two-fold dilutions of serum tested against serial two-fold dilutions of streptococcal hyaluronidase, in general, exhibited a definite relationship; *i.e.:* if a serum dilution of 1 : 512 would inhibit 128 units of enzyme, the same serum diluted to 1 : 1024 would inhibit 64 units of enzyme, etc. This linear relationship was most constant when sera were tested against hyaluronidase strength within the range between 64 to 16 units.

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