

## STUDIES ON A LIPOPROTEINASE OF GROUP A STREPTOCOCCI\*

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A streptococcal enzyme which causes opalescence in serum was described by Ward and Rudd in 1938. These authors found that the enzyme was produced by many types of group A streptococci, but not by streptococci belonging to other groups. Seitz filtrates of serum broth cultures of active strains produced opalescence in rabbit, mouse, or human sera. These authors postulated that the enzyme altered serum protein but found no evidence to substantiate their theory (1).

The studies reported in this paper were undertaken to determine the nature of the alteration in serum responsible for the development of opalescence. It was found that streptococci produce an enzyme, designated a lipoproteinase, which acts upon  $\alpha_1$ -lipoproteins and splits them into lipid and protein moieties.

### *Methods and Materials*

*Paper Electrophoresis.*—Electrophoretic patterns were prepared by a modification of the method described by Kunkel and Tiselius (2) using a buffer consisting of 9.83 gm. of sodium barbital and 6.5 gm. of sodium acetate per liter, adjusted to pH 8.8 with HCl (3). A double thickness of Whatman 1 filter paper was used for all patterns. When electrophoresis was completed the papers were dried at 90–100°C. The upper sheet was stained with Sudan black to show the lipids (3), the lower with an alcoholic solution of bromphenol blue to show the proteins (2).

*Sudan Black Stain.*—1.5 gm. of Sudan black B was suspended in 100 ml. of 70 per cent alcohol. After standing for several days the solution was filtered to remove any undissolved dye and diluted 1-1 with 70 per cent alcohol before use. The dried patterns were immersed in this solution for 15 minutes and then differentiated in 50 per cent alcohol. No attempt was made to remove all stain from the paper.

*Chemical Analyses.*—Non-protein nitrogen was determined by the method of Koch and McMeekin described in reference 4, phosphorus by the method of Fiske and Subbarow described in reference 4, total lipids by the method of Bragdon (5), cholesterol by the method of Bloor (6)(7). A micro Kjeldahl method was used for total nitrogen determination (8). A ninhydrin method was employed to detect protein breakdown (9). Carotenes were determined by the method of Lewis, Bodansky, and Haig (10). The protein content of enzyme

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preparations was determined by flocculation with 3 per cent sulfosalicylic acid. The turbidity was measured in a Klett-Summerson colorimeter with a No. 42 blue filter and compared with that of a standard of diluted serum, the protein concentration of which was determined by a Kjeldahl method.

*Media.*—Brain heart infusion (Difco), to which 50 ml./liter of a buffer-glucose solution (11) was added, was used to grow cultures for enzyme preparations.

*Group A Streptococci.*—A strain of group A type 4 isolated from a case of streptococcal pharyngitis and a stock Griffith strain of type 13 were used in most experiments.

#### EXPERIMENTAL

##### *Electrophoretic Studies of Enzyme Action*

In initial experiments, human and horse sera<sup>1</sup> were treated with crude enzyme preparations consisting of suspensions of killed organisms.

Merthiolate in a final concentration of 1-2000 was added to 100 ml. of an 18 hour broth culture. After refrigeration overnight the cells were recovered by centrifugation, washed with physiological saline, and resuspended in 10 ml. of saline.

Protein patterns of the sera were prepared by free electrophoresis in a Perkin-Elmer Tiselius electrophoresis apparatus (8). When patterns of these sera made before and after enzyme treatment were compared there appeared to be no alteration in the protein fractions. Since the opalescence observed in sera following enzyme action resembled that seen in lipemic sera, paper electrophoretic patterns were made and stained with Sudan black to show the lipids.

Clear cut changes were seen in the lipid patterns. Fig. 1 shows lipid patterns of horse, human, and dog sera before and after enzyme treatment. The lipid portion of the  $\alpha_1$ -lipoprotein has virtually disappeared. The lipid which originally travelled with this fraction may be seen at the origin or in the area of the  $\beta$ -lipoproteins. Similar results were obtained with sera from rabbits, sheep, and chickens. The major aim of these studies has been to characterize the nature of this effect on the  $\alpha_1$ -lipoprotein.

##### *Preparation of Enzyme Solutions*

It was found in preliminary experiments that the enzyme which splits the  $\alpha_1$ -lipoprotein is attached to the bacterial cell and may be demonstrated in living organisms and in washed organisms which have been killed with merthiolate, penicillin, or aureomycin. It is also present in organisms which have been killed with cold acetone and rapidly dried. A trace of enzyme activity is found in washed formalin-killed cultures. The enzyme is not present in serum-free broth culture filtrates nor can it be extracted from the bacterial cell with buffer solutions covering a wide pH range. It may be extracted from the bacterial cell with serum, either by growing the organisms in serum broth or by

<sup>1</sup>Normal horse serum was generously supplied by the Bureau of Laboratories of the Department of Health of the City of New York.

shaking living or dead organisms with serum. The possibility that albumin might be the substance in serum responsible for the extraction of the enzyme was investigated. Washed organisms, both living and dead, were shaken with a solution of crystalline bovine albumin.<sup>2</sup> A weak enzyme solution was obtained when the albumin was dissolved in a buffer solution of low molarity. When albumin dissolved in a molar phosphate buffer at pH 7.0 was used for extraction a stronger enzyme preparation was obtained. However, these preparations were not satisfactory for studies on the mode of action of the enzyme

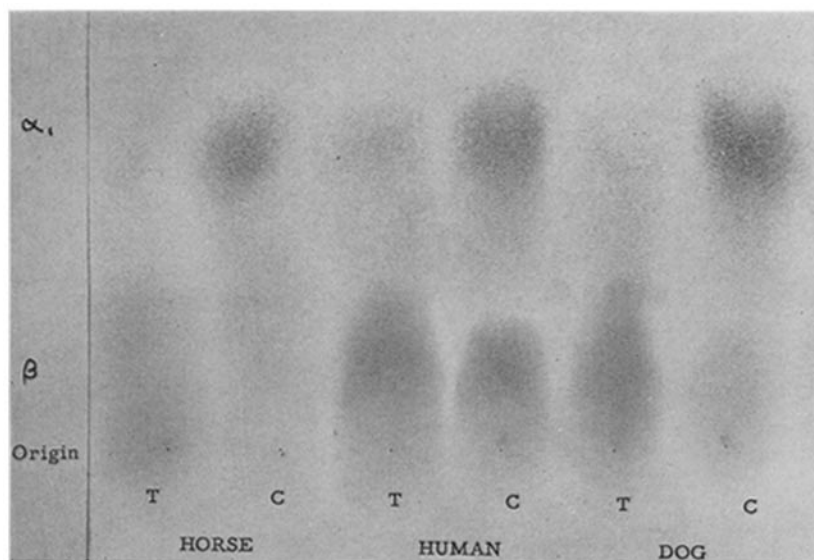


FIG. 1. Lipid patterns of horse, human, and dog sera showing the effect of the enzyme upon  $\alpha_1$ -lipoprotein. T, enzyme treated serum; C, untreated serum.

owing to the presence of albumin and the high concentration of phosphorus. Since urea often affects the solubility of proteins, extraction with 40 per cent urea was tried. The following method was found to yield satisfactory enzyme solutions.

4 liters of broth was inoculated with 800 ml. of a young culture of a stock strain of type 13 and incubated overnight. The cells were recovered by centrifugation and washed twice with physiological saline. The packed washed organisms were extracted with 100 ml. of 40 per cent urea in molar sodium acetate adjusted to pH 7.0 with molar acetic acid. Extraction was carried out by mechanical stirring for one-half hour in an ice bath followed by overnight refrigeration. The resultant gray suspension was centrifuged the following day at 4000 R.P.M. in an angle centrifuge at 4°C. The enzyme could be demonstrated in the water-clear supernatant.

<sup>2</sup> Bovine albumin powder, Armour Laboratories.

Attempts were made to determine the optimum volume of 40 per cent urea to be used in extraction of the enzyme. Although it was found that the total yield could be increased by the use of larger volumes of urea solution the yield was not proportionate to the volume of solvent and there was a disproportionate increase in the amount of extraneous protein material extracted.

The water-clear urea extract was concentrated by dialysis against 20 per cent dextran (12)<sup>3</sup> in M/10 sodium acetate adjusted to pH 7.0 with M/10 acetic acid. When the sacs had collapsed and contained only a grayish white sediment they were dialyzed against M/10 sodium acetate in physiological saline at pH 7.0 until all traces of urea were removed. The material was then centrifuged at high speed in the cold and the water-clear enzyme solution was stored in a deep freeze.

The grayish white sediment was tested and found to contain enzyme as well as extraneous protein material indicating that enzyme was precipitated from solution. Efforts to dissolve the sediment in the sodium acetate saline solution used for dialysis yielded a very weak enzyme suggesting that the solubility of the enzyme was low. In order to minimize the loss by precipitation during concentration, the sac was removed from the dextran solution while it still contained fluid material and was dialyzed against sodium acetate in saline until all traces of urea were removed. This alteration in the method of concentration proved unsatisfactory. It led to increased protein values without an accompanying rise in enzyme activity. The loss in enzyme activity resulting from maximum concentration appeared to be compensated for by a higher ratio of enzyme activity to protein content. The protein concentration of satisfactory enzyme preparations was 13 mg. per cent or less.

#### *Studies of the Action of Enzyme on Horse Serum*

*Results of Enzyme Titrations.*—A typical titration of a urea-extracted enzyme against a horse serum substrate is shown in Fig. 2.

2 ml. of normal horse serum was added to 0.5 ml. of undiluted enzyme and to a series of tubes containing 0.5 ml. of twofold serial dilutions of the enzyme in M/10 sodium acetate adjusted to pH 7.0. A tube containing 0.5 ml. of sodium acetate and 2 ml. of horse serum served as a control. After 2 hours' incubation in a water bath at 37°C. paper electrophoretic patterns were made and the development of opalescence measured. These measurements were made by diluting 1.0 ml. of the material with 4.0 ml. of physiological saline and reading in a Klett-Summerson colorimeter with a No. 54 green filter against a distilled water blank. The final readings represent the value obtained by subtracting the reading for the control from the readings for the treated sera. Incubation was continued for another 24 hours and a second series of electrophoretic patterns were prepared and final determinations of opalescence made. When the effect of refrigeration was to be studied the 2-hour readings were omitted. The test was stored in the refrigerator and observed after 48 hours. It was found that shorter periods of refrigeration led to inconsistent results.

<sup>3</sup> Dextran was donated by the R. K. Laros Company.

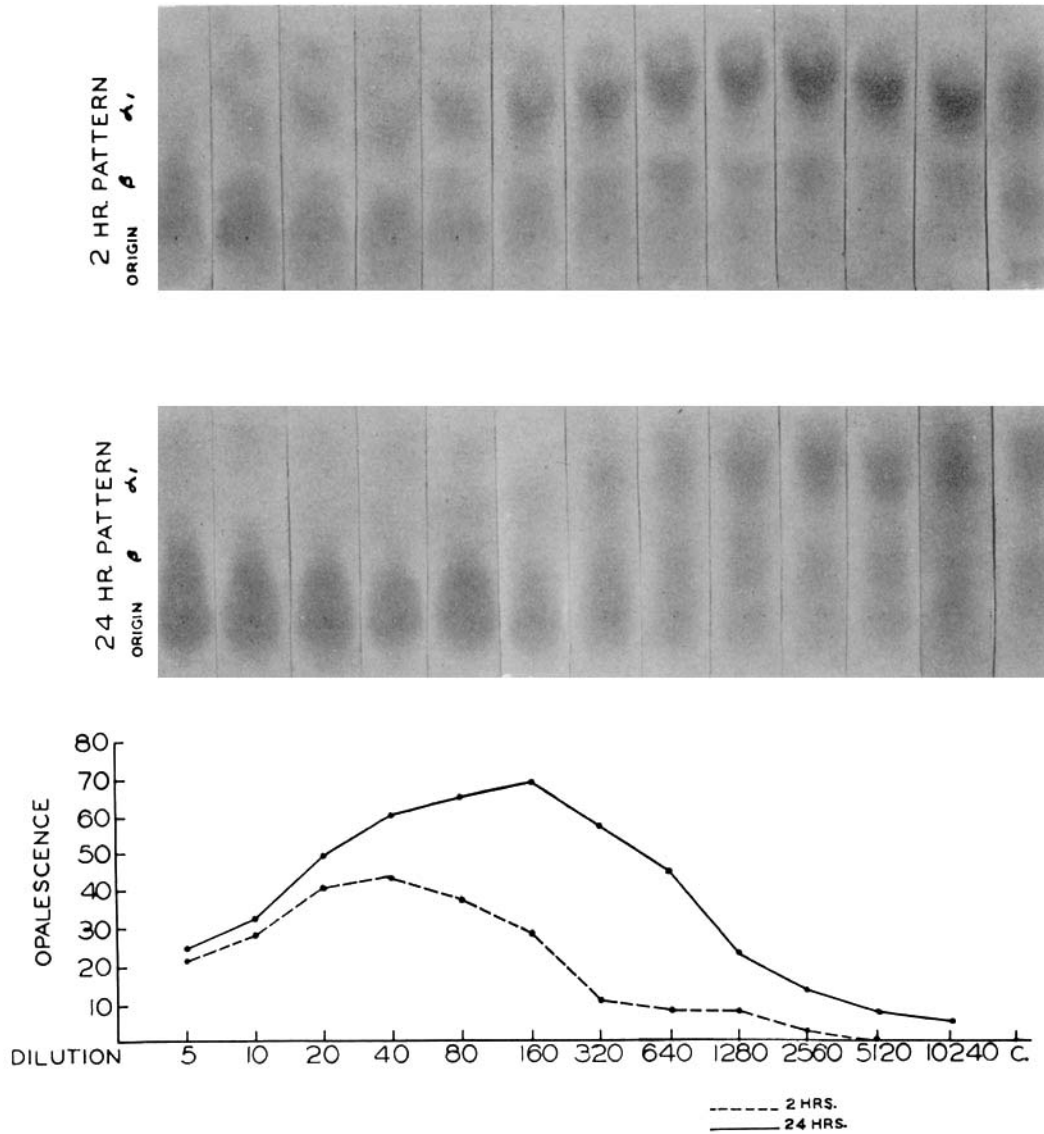


FIG. 2. Results of enzyme titration with horse serum. The patterns, stained with Sudan black, show the effect of the enzyme upon the  $\alpha_1$ -lipoprotein and the curves the development of opalescence at 2 and 24 hours.

It may be seen in Fig. 2 that after 2 hours' incubation the maximum split in the  $\alpha_1$ -lipoprotein occurred in the 1-5 dilution of enzyme and that the maximum opalescence was observed in the 1-40 dilution. This phenomenon is

even more marked at 24 hours. In the 1-5 through 1-40 dilutions only a portion of the lipid remained attached to the globulin. In the subsequent dilutions there were increasing amounts of unaltered lipoprotein and decreasing amounts of freed lipid at the origin. A comparison of the 2- and 24-hour curves representing the development of opalescence shows that there was little increase in opalescence in the 1-5 and 1-10 dilutions upon prolonged incubation, while the opalescence was markedly increased in subsequent dilutions reaching its maximum in the 1-160 dilution. These results indicate that there is no direct correlation between the degree of opalescence and the splitting of the lipoprotein. The maximum opalescence was observed at enzyme concentrations below that which caused maximum splitting of the lipoprotein, and at higher dilution of the enzyme there was marked opalescence unaccompanied by significant alteration of the lipoprotein as evidenced by the electrophoretic pattern. If the tubes were refrigerated after 24 hours' incubation, a layer of fat rose to the surface of the tubes containing the 1-160 through 1-10,240 dilutions. This suggests that the substances which produced the opalescence observed in these tubes differ from those which produced the opalescence observed in the lower dilutions.

When an enzyme preparation was titrated against sera from a number of different horses the results were not identical. The curves representing the development of opalescence showed marked variations. The increase in opalescence from the first to the fifth tube might be marked or negligible. The thickness of the layer of fatty material which rose to the surface during refrigeration also varied. While the titres in relation to maximum splitting action remained essentially constant, the amount of unaltered  $\alpha_1$ -lipoprotein varied. These findings suggest that the degree of opalescence, size of the fatty layer, and percentage of lipoprotein which can be split are all influenced at least in part by factors in the serum. Variations of the location of the freed lipid are seen in Fig. 1. The freed lipids are either in the area of the  $\beta$ -globulins or at the origin. This distribution appears to be a factor which is not species-specific. In some horse sera a portion of the freed lipid appeared to travel with the  $\beta$ -globulins. This may represent a phenomena similar to and yet at the same time the reverse of the clearing observed by Anfinsen and his coworkers (13).

*Chemical Analyses of Enzyme-Treated Sera.*—In order to determine what portion of the  $\alpha_1$ -lipoprotein was acted on by the enzyme human and horse sera were analyzed before and after enzyme treatment. No changes were observed in the total and non-protein nitrogen, in the inorganic, acid-soluble and organic phosphorus, or in the total lipids. Changes in the ratio of free to esterified cholesterol were observed only in fresh sera which had not been inactivated and appeared to be due to serum cholesterol esterase.

*Studies to Detect the Presence of Lecithinase.*—Since organic phosphorus

values are not altered by lecithinases which produce lysolecithin (14), the possibility that the enzyme preparations contained this type of lecithinase was tested. When washed red cells were added to treated serum no hemolysis occurred, indicating that lysolecithin was not a product of enzyme action. As a further test to eliminate the possibility that the opalescence might be due to a lecithinase, the enzyme was tested against a substrate of pure lecithin in a Warburg apparatus. The lecithin was not affected.

*Studies to Detect the Presence of a Lipase or Esterase.*—The method used to determine total lipids was not believed to be sufficiently sensitive to detect splitting of neutral fats into fatty acids and glycerol since the percentage of carbon in the glycerol molecule is about equal to the percentage of error of the test. Therefore, a number of enzyme preparations were tested for lipase or esterase activity against  $\beta$ -naphthylacetate according to the method of Seligman and Nachlas (15). Some enzyme preparations were found to be capable of splitting  $\beta$ -naphthyl acetate. However, there was no correlation between the ability to split this material and the ability to split lipoprotein, and the pH of optimum activity of the enzyme which splits  $\beta$ -naphthylacetate is 3.5 which on the basis of preliminary tests appears to be well below that at which the lipoprotein is split. Furthermore, this enzyme is active in the absence of albumin.

#### *Studies of the Action of the Enzyme on Horse Serum Fractions*

*Preparation of Horse Serum Fraction.*—Since the substrate of the enzyme appeared to be the  $\alpha_1$ -lipoprotein, a fraction of horse serum was prepared following the method of Macheboeuf (16). However, the serum used was several months old and the material appeared to become denatured before complete purification was obtained. The method was altered in order to maintain a higher pH and to reduce the number of precipitations necessary for the elimination of albumin.

500 hundred ml. of a saturated solution of ammonium sulfate was added slowly with stirring to 500 ml. of horse serum. After standing at room temperature several hours or in the refrigerator overnight, the material was filtered through a layer of filter cel in a Buchner filter. The clear filtrate was warmed to 35°C. in a water bath and adjusted to pH 4.5 with  $N/1$  and  $N/10$  HCl. After centrifugation the supernatant was discarded and the sediment suspended in physiological saline. Dilution of the ammonium sulfate caused a large portion of the albumin to go into solution. The material was again centrifuged and the sediment washed with physiological saline. After a third washing most of the albumin had been removed and the sediment had a butter-like consistency. It could not be readily resuspended in saline to remove the remaining albumin and ammonium sulfate. The sediment was dissolved by stirring with 10 ml. of  $N/1$  sodium hydroxide. After the addition of 40 ml. of saline the material was warmed to 35°C. in a water bath and the pH adjusted to 4.8 with  $N/10$  HCl. and again centrifuged, the supernatant discarded, and 10 ml. of  $N/1$  sodium hydroxide added to the sediment. The resulting gelatinous material was broken up with a glass stirring rod, 40 ml. of physiological saline added, and the material refrigerated overnight. The following

day if any undissolved material remained, the mixture was stirred until complete solution was obtained. The pH was then adjusted to 7.0 with N/10 HCl. This material was tested for the presence of sulfate with 5 per cent barium chloride and for albumin by electrophoresis. If the fraction was found to be impure by either of these tests it was again precipitated at pH 4.8.

Electrophoretic patterns of horse serum and of this fraction are shown in Fig. 3. Protein stain showed that the fraction contained  $\alpha_2$ -globulins as well

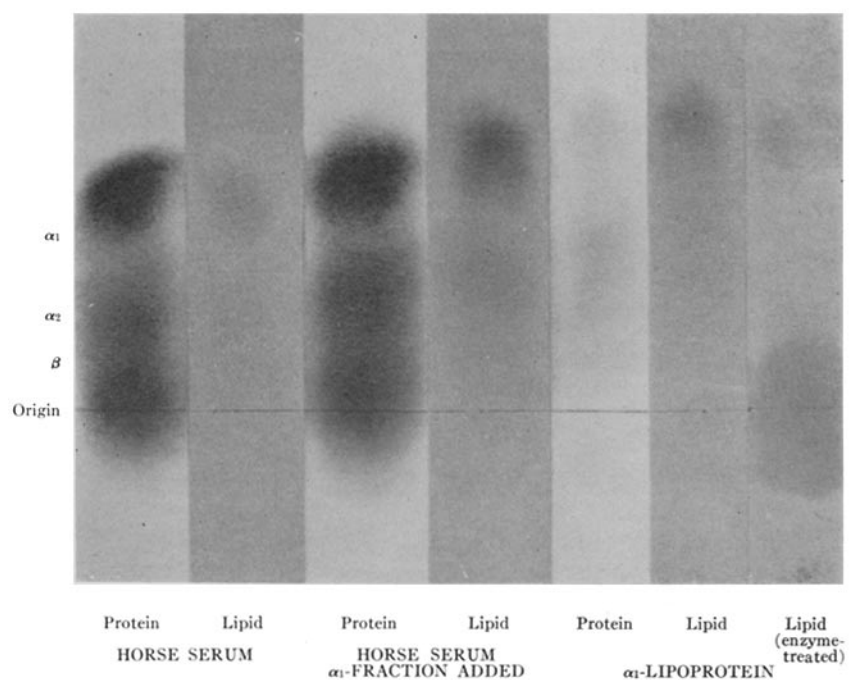


FIG. 3. Patterns of horse serum and an  $\alpha_1$ -lipoprotein fraction of horse serum stained with bromphenol blue and Sudan black.

as  $\alpha_1$ -globulins. The isoelectric point of these two fractions appeared to be too near one another to allow for fractional precipitation without a large decrease in the yield. The fact that  $\alpha_1$ -lipoproteins moved faster in the fraction than in horse serum suggested denaturation. However, when the fraction was added to horse serum all of the lipids in the serum travelled further suggesting that the forward movement was a factor of concentration rather than denaturation.

*Action of the Enzyme on Horse Serum Fraction.*—When a potent enzyme was added to the  $\alpha_1$ -lipoprotein fraction described above and the mixture incubated as long as 24 hours, no opalescence developed and electrophoretic



patterns showed that the material was unaltered. Since preliminary tests on the fraction before removal of all albumin had been satisfactory, albumin was added to the enzyme substrate mixture. Enzyme activity was restored. The last two strips in Fig. 3 show the effect of the enzyme upon the substrate in the presence of albumin. Horse, human,<sup>4</sup> and bovine albumin all proved satisfactory for activation. Since lipases are activated by both albumin and sodium oleate, the latter was tried as a substitute for albumin but failed to activate the enzyme.

*Chemical Analyses of Horse Serum Fraction.*—Several horse serum  $\alpha_1$ -lipoprotein preparations were analyzed. The ratio between phosphatids and cholesterol varied; however, all preparations appeared to be equally affected by the enzyme. The fraction was analyzed before and after enzyme treatment for total and non-protein nitrogen, organic, inorganic, and acid-soluble phosphorus, total lipids, cholesterol, cholesterol esters, and carotenes. In addition to these analyses a ninhydrin method was used to detect any breakdown in the proteins to amino acids or peptides. The results of these tests before and after enzyme treatment were identical.

#### *Preliminary Studies of Enzyme Activators and Inhibitors*

In order to determine whether calcium ions were necessary for enzyme activity the sodium salt of ethylenediaminetetraacetic acid (EDTA) was added to serum before enzyme treatment. The action of the enzyme on this serum was compared with that which occurs in the presence of normal serum calcium. Enzyme activity is slightly depressed when the serum calcium is bound with EDTA. Conversely the addition of calcium to enzyme titrations increases enzyme action, indicating that calcium increases but is not necessary for enzyme action.

#### *Antigenicity of Enzyme*

A number of human sera were encountered which were not affected by the enzyme. The addition of these sera to horse serum-enzyme mixtures prevented the splitting of the lipoprotein. A sample of human  $\gamma$ -globulin was also found to prevent alteration in the serum lipoprotein. In order to test the antigenicity of the enzyme, rabbits were immunized with filtrates of an enzyme producing strain grown in rabbit serum. Sera from these rabbits prevented enzyme action indicating that the enzyme is antigenic. When a method has been found for standardizing the enzyme on a unit basis the antibody titres of a series of human sera will be determined.

<sup>4</sup>The horse albumin was a crude product obtained during the preparation of the lipoprotein fraction. Normal serum albumin (human) was supplied by The American National Red Cross.

## DISCUSSION

These studies were undertaken to determine the nature of what was thought to be a single enzyme elaborated by some types of group A streptococci and producing opalescence in serum. The results suggest that the development of opalescence is due to a number of reactions some of which are initiated by enzymes attached to the bacterial cell and others by enzymes or other factors present in serum. The primary action appears to be a splitting of the  $\alpha_1$ -lipoprotein without alteration of any of its known constituents (17). The enzyme responsible for this reaction has been called a lipoproteinase. Subsequent investigation may show that this is a misnomer and that the true substrate is a less complex molecule. It is hoped that this enzyme may be a useful tool in demonstrating the nature of the protein lipid linkage in the lipoprotein molecule.

## SUMMARY

The opalescence produced in serum by group A streptococci has been investigated.

The development of opalescence is shown to be initiated by an enzyme attached to the bacterial cell which acts upon the  $\alpha_1$ -lipoprotein fraction of serum liberating the lipids from the protein.

This enzyme has been termed a lipoproteinase.

Evidence is presented which suggests that the degree of opalescence which develops following lipoproteinase activity is influenced not only by factors attached to the bacterial cell but also by substances present in serum.

The lipoproteinase is antigenic and many human sera contain specific antibodies which inhibit the action of the enzyme.

The author is indebted to Miss Patricia Norton for the free electrophoretic patterns and nitrogen determinations, to Mrs. Audrey Boyer for cholesterol and protein determinations, and to Dr. Walton B. Geiger for the Warburg studies on lecithin, the studies on lipase and esterase activity, and for his advice and encouragement throughout this investigation.

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