STUDIES ON THE BACTERIOPHAGES OF HEMOLYTIC STREPTOCOCCI

II. Antigens Released from the Streptococcal Cell Wall by a Phage-Associated Lysin

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In the first paper of this series, attention was directed to a bacteriolytic factor in phage lysates of Group C streptococci which lyses Groups A and C hemolytic streptococci and their isolated cell walls (1), an observation also reported by Maxted (2). Furthermore, it was found that lysis is accompanied by the release into solution of cell wall components with the specific serologic reactivity of type-specific M protein and group-specific C substance. These preliminary studies suggested that this phage-associated lysin might be a particularly useful agent for isolation and purification of M protein and C carbohydrate. M protein is ordinarily extracted from streptococcal cells by boiling at pH 2, and considerable purification of this type of material has been achieved (3). However, this extraction procedure has limitations, since the antigen is not quantitatively removed from the cell and, in addition, the drastic conditions required may result in some degradation. The cell wall-dissolving enzymes from *Streptomyces albus* have been usefully employed for the isolation of the C carbohydrate (4), but unfortunately this material contains a highly active proteolytic enzyme which destroys M protein. Thus, it is unsuitable for the extraction of M antigen from the cell wall.

The present investigation was undertaken to devise methods for obtaining phage-associated lysin for the disruption of cell walls of hemolytic streptococci. The studies reported here indicate that the group carbohydrate released by this process is chemically and immunologically similar to the carbohydrate obtained by the *Streptomyces albus* enzyme. M substance has been identified in the protein fraction released from the cell wall by the lysin, and partially purified material has been obtained.

Phage-associated bacteriolytic substances have been reported in phage lysates of *Escherichia* (5), *Staphylococcus* (6), *Klebsiella pneumoniae* (7), and *Bacillus megatherium* (8). Little is known, however, of the cell wall components released by these substances. The present study has special interest since two streptococcal antigens have been identified as split products of cell walls lysed by phage-associated lysin.

Materials and Methods

Strains of Streptococci.—The Group A streptococcal strains T12/36, S43/100, and S43/137; and the Group C strains H46A and 26RP66 were from The Rockefeller Institute collection. Group C strain 4540 was obtained from W. R. Maxted.

Bacteriophage.—Bacteriophage labelled C1 was obtained from W. R. Maxted. This phage is virulent for most Group C hemolytic streptococci. Stock lysates of this strain were prepared with strain 4540 grown in Todd-Hewitt broth.

Todd-Hewitt Broth.—This beef heart infusion was prepared by a modification of the original procedure (9).

Preparation of Agar Plates.—Agar plates for the assay of bacteriophage were prepared as previously described (1), except that proteose peptone–beef heart infusion agar was used instead of a dialysate medium. The proteose peptone–beef heart infusion agar is usually satisfactory for the demonstration of phage plaques of Group C hemolytic streptococci, but in general, it does not support sufficient growth of Group A streptococci for the formation of Group A phage plaques.

Proteose Peptone–Beef Heart Infusion Agar.—This infusion agar when combined with defibrinated sheep or rabbit blood is used at The Rockefeller Institute for growing hemolytic streptococci. It is prepared as follows.

The infusion is prepared from freshly ground beef hearts which have been stripped of all fat. 1050 ml. of cold tap water is added to each pound of meat, and any remaining fat which floats to the surface is removed. The mixture is infused overnight at 4°C., heated to 85°C. for one-half hour, and filtered through coarse filter paper (Eaton and Dikeman No. 615). To each liter of infusion 10 gm. of proteose peptone and 5 gm. of NaCl are added. This mixture is brought to a boil and adjusted to pH 7.8 with NaOH. Boiling is continued for 15 minutes, and agar[1] is added to make a final concentration of 1.2 per cent. This is then heated in the Arnold sterilizer for 1 hour and the precipitate which forms is removed by filtering the infusion agar through three layers of Red Cross absorbent cotton.[2] The filtered and clear medium is autoclaved at 116°C. for one-half hour.

Dialysate Broth.—This broth is prepared as the previously described medium C (1), except that Pfanstiehl peptone, R. I. is used. Broth prepared with this peptone does not form a precipitate when brought to 0.8 saturation with ammonium sulfate. Prior to use the broth is combined with an equal volume of sterile distilled water, and 2 gm. NaH_2CO_3 per liter of diluted broth is added. The NaH_2CO_3 is first dissolved in a small volume of broth and sterilized by filtration through a Coors P3 filter.

Plating and Counting Bacteriophage.—The soft agar layer method (10) of plating phage and streptococci was used to demonstrate plaque-forming particles. Streptococcal strain 26RP66 was used for the plating of group C phage.

Serological Identification.—The serological identification of the strains used in these experiments was checked with the capillary tube precipitin test (11) using streptococcal grouping and typing rabbit antisera. The same method was employed to identify C substance and M protein in the purified preparations and the fractions separated by zone electrophoresis.

Preparation of Cell Walls.—The cell walls were prepared by the method of Salton (12) in which streptococci are disrupted in a Mickle disintegrator. The cell walls, following separation from the intracellular material, were washed with distilled water until free of debris, and then lyophilized.

Preparation of Phage-Associated Lysin.—The partially purified lysin is isolated from a phage lysate of Group C streptococci. The lysate is prepared by seeding dialysate broth with a

[1] Baltimore Biological Laboratories.

[2] Johnson and Johnson, New Brunswick, New Jersey.

sufficiently heavy inoculum of strain 26RP66 so that after 3 to 4 hours the culture reaches an optical density of 0.16 to 0.18 on measurement in the Coleman Jr. spectrophotometer at a wavelength of 650 mμ using a cuvette measuring 19 × 105 mm. Enough phage lysate of strain 26RP66 is added to the culture so that lysis occurs within 1 hour. Following lysis the lysate is immediately chilled in an ice bath, brought to 0.5 saturation with ammonium sulfate, and stored for 3 days at 4°C. The fine precipitate is then collected by centrifugation for 30 minutes in the Spinco centrifuge at 10,000 R.P.M. with the 20S head. This material is taken up in cold saline, the insoluble portion removed by centrifugation, and the supernatant reprecipitated at 0.5 saturation with ammonium sulfate. This precipitate is collected by suction filtration through a filter bed of potato starch. The starch layer is removed and the precipitate redissolved by rinsing several times with cold saline. This material is dialyzed for 18 hours against 20 volumes of saline buffered between pH 7 and 7.4. Bacteriophage and much other contaminating material are removed by two cycles of ultracentrifugation for 2 hours at 30,000 R.P.M. in the Spinco centrifuge with the 40S head. The supernatant of the second centrifugation, clear and almost colorless, contains the lysin, and represents a hundredfold concentration of the crude lysate. The lysin may be stored in the lyophilized state. Prior to use it must be activated with neutralized thioglycolic acid (2).

Preparation of the Streptomyces albus Enzyme.—This material, kindly supplied by Dr. M. McCarty, was prepared by methods previously described (4).

Preparation of Cell Wall Carbohydrate.—The carbohydrate was prepared from cell walls by both the *S. albus* enzyme and the phage-associated lysin. Aliquots of the same lot of cell walls were extracted separately by each enzyme so that a direct comparison could be made of the carbohydrates prepared by the two methods.

Digestion with the *S. albus* enzyme was by the method previously described (13). Lysis of cell walls with phage-associated lysin was carried out in saline, buffered with phosphate at pH 7. To this mixture one-tenth volume of a neutralized 2 per cent thioglycolic acid solution and a few drops of chloroform were added. It was incubated for 12 hours at 37°C.

The carbohydrate was isolated from both enzyme digests of the cell walls by the same method (13). The preparations were then treated with Dowex[3] 2 × 10, 200 to 400 mesh, medium porosity anion exchange resin, and Dowex[3] 50 × 8, 200 to 400 mesh, medium porosity cation exchange resin, as suggested by McCarty (14).

Analytical Methods.—Methylpentose was determined by the method of Dische and Shettles (15). Quantitative hexosamine determinations were done by a modification of the Elson and Morgan procedure (16). In this method hydrolysis is carried out at 100°C. for 4 hours. While this period of hydrolysis has proved satisfactory for the analysis of Group A carbohydrate, it was found that hydrolysis for 12 hours was required to give a reliable estimate of glucosamine in Group C carbohydrate. Total nitrogen was determined by the procedure of Koch and McMeekin (17).

Electrophoretic Methods.—Electrophoretic separation of the carbohydrates was performed by the method described by Kunkel (18), modified in most instances, however, by using polyvinyl chloride for the supporting medium (19). Sodium borate buffer, $\mu = 1$, pH 9, and glycine buffer, $\mu = 1$, pH 9 were used (20). The potential gradient was 8.9 volts/cm., and the temperature 4°C. The duration of electrophoresis was 24 hours for Group A carbohydrate and 36 hours for Group C carbohydrate. The block was cut into centimeter sections and each fraction eluted with 2 cc. of 0.05 N HCl in saline. The distribution of carbohydrate in the fractions was determined both serologically and by chemical analysis for rhamnose.

Separation of the protein fraction obtained by phage lysin digestion of the cell walls was carried out on a starch supporting medium, with a potential gradient of 8.9 volts/cm., at 4°C. for 24 hours. The glycine buffer employed for the carbohydrate analysis was used. The centi-

[3] Dow Chemical Company, Midland, Michigan.

meter sections of the block were eluted with 2 cc. of saline and protein assayed by a modified Folin procedure (21). The distribution of M protein was established serologically.

Ultracentrifugal Methods.—The Spinco model E machine was used for analytical ultracentrifugation. The carbohydrates were dissolved in distilled water at a concentration of 5 mg. per ml., centrifuged at 52,640 R.P.M. and a temperature of 20°C., and the schlieren patterns were analyzed by methods described by Trautman (22).

Precipitin Analysis.—Quantitative precipitin tests on the carbohydrate preparations were done by a spectrophotometric procedure in which the antibody content of redissolved precipitate is determined by measurement of the optical density at 287 mμ in the Beckman quartz spectrophotometer (13).

EXPERIMENTAL

Production and Purification of Lysin.—While almost all fresh phage lysates of Group C hemolytic streptococci contain the phage-associated lysin, the production appears to be enhanced under certain conditions. The highest yield of lysin with a given streptococcal strain is obtained when a culture in the logarithmic phase of growth is seeded with sufficient phage so that lysis occurs within 1 hour. The lysates of different Group C strains show considerable variation in lytic activity, and a strain was selected for production which gives a good yield of lysin.

Purification of the lysin from the lysate is complicated by the presence of intracellular material released by lysis, since much of this protein also precipitates out with ammonium sulfate. However, additional contamination of the lysin with constituents of the medium is avoided by preparing the lysates in a dialysate broth which does not yield precipitates with ammonium sulfate. The precipitate of lysin obtained at 0.5 saturation with ammonium sulfate is further purified with the removal of bacteriophage and much contaminating material by ultracentrifugation.

In Table I are recorded the results of ultracentrifugation of several lots of lysin. The supernatant fluid of the first centrifugation was recentrifuged, and the phage titer and lytic activity of both supernatants compared with the initial material. As indicated in Table I, the two cycles of centrifugation resulted in a 5 log decrease in bacteriophage in each experiment, but in two cases this was associated with only a twofold decrease in lytic activity. This clearly indicates that a lysin preparation retains lytic activity following the removal of bacteriophage.

Lysis of Cell Walls.—When a suspension of cell walls of hemolytic streptococci is mixed with reduced lysin, a reduction in the optical density of the mixture indicates lysis of cell walls has occurred (1). The cell walls of strain T12, for example, are readily lysed with a rapid decrease in the optical density until the residual turbidity is about 10 per cent of the initial value. This represents insoluble material which may be either fragments of cell wall or intracellular material which was not removed from the cell walls because of insufficient washing. There is considerable variability in the lytic susceptibility of cell walls from various streptococcal strains, and in general the strains of Group A

streptococci are much more susceptible to lysis than Group C strains. However, under optimal conditions and with the addition of sufficient lysin the turbidity experiments indicate most of the cell wall is lysed, and this has been confirmed by serological and chemical analysis.

A suspension of streptococcal cell walls was mixed with sufficient lysin to ensure maximum lysis in 12 hours at 37°C. The residue in the digestion mixture was collected by centrifugation, a sample saved for a rhamnose determination, and the remainder was treated with the *S. albus* enzyme. Group precipitin tests with absorbed rabbit antisera were performed on the supernatant fluid obtained with the lysin and the *S. albus* enzyme digest of the residue. Both were tested for the presence of rhamnose.

TABLE I

Removal of Phage Particles from Lysin by Ultracentrifugation

Fraction	Lot 1		Lot 2		Lot 3	
	Lytic activity	Phage plaque count	Lytic activity	Phage plaque count	Lytic activity	Phage plaque count
Non-centrifuged lysin	1024	$10^{10.2}$	512	10^{11}	1024	$10^{11.8}$
Supernatant from first centrifugation	512	$10^{6.9}$	256	$10^{8.6}$	512	10^{8}
Supernatant from second centrifugation	512	$10^{4.4}$	256	$10^{6.4}$	256	$10^{6.5}$

The lytic activity is expressed as the reciprocal of the lysin dilution which cleared a standard culture of strain T12/36 in 1 hour at 37° C. Centrifugation was carried out in a Spinco centrifuge with a 40S head, at 30,000 R.P.M. for 2 hours.

In Table II are recorded the group precipitin reactions and rhamnose values on the supernatant fluids and the residues from centrifuged lysin digests for several cell wall preparations. The supernatants give a particularly strong group precipitin reaction, while the residues give a weak reaction. Since all of the cell wall rhamnose is located in the C substance (4), the percentage of the total rhamnose which is in solution is an accurate estimate of the dissolved carbohydrate. The rhamnose values indicate that almost all the carbohydrate of both Groups A and C streptococci is released from the cell wall by the phage lysin.

The supernatant fluids for the two Group A strains listed in Table II gave a strong type-specific precipitin reaction with homologous antisera, although weak cross-reactions occurred with a few heterologous antisera. This suggests that the type-specific M protein is also liberated from the cell wall by the lysin. However, since no quantitative method for releasing M protein from the cell wall is known, it is difficult to estimate either serologically or chemically the percentage of the total cell wall M protein recovered in the supernatant fluids.

Cell Wall Carbohydrate.—While the carbohydrate chemically isolated from

the lysin digests of cell walls gave a group-specific precipitin reaction, other criteria were employed to demonstrate that the released carbohydrate was streptococcal C substance. The characteristics of this antigen have been defined in experiments which employed the carbohydrate extracted by the *S. albus* enzyme (4, 23, 13). Therefore the carbohydrate chemically isolated from cell walls digested with phage-associated lysin has been compared with that obtained from standard *S. albus* enzyme digests by several different analytical and immunological procedures. Hereafter, for convenience, the carbohydrates are referred to by the following symbols: carbohydrate S.E. indicates the material purified from cell walls digested with the *S. albus* enzyme, and carbohydrate P.L. refers to that obtained with phage-associated lysin.

TABLE II

Group-Specific Carbohydrate and Rhamnose Released from the Cell Wall by Phage-Associated Lysin

Serological and chemical analysis	Lysin digest of cell walls	Streptococcal strains			
		Group A		Group C	
		S43/100	T12/36	26RP66	H46A
Streptococcal Group precipitin reaction	Supernatant	++++	++++	++++	++++
	Residue	±	±	±	±
		Per cent of the total cell wall rhamnose			
Rhamnose	Supernatant	98	96	99	99
	Residue	2	4	1	1

The cell wall suspensions, following digestion by the lysin, were centrifuged. The supernatants and the sedimented residues, after digestion with the *S. albus* enzyme, were tested with streptococcal grouping antisera. The precipitin reaction was graded ± to 4+.

Chemical Analysis of the Carbohydrate.—Rhamnose and glucosamine have been identified as the principal sugars in the hemolytic streptococcal cell wall carbohydrate (4). While the ratio of rhamnose to glucosamine approaches 2 in the case of Group A carbohydrate, some strain variation exists in the percentages of rhamnose and hexosamine. Therefore, for the purpose of comparison, both carbohydrates S.E. and P.L. were isolated from the same lot of cell walls. The chemical data on preparations from two Group A strains and two Group C strains are given in Table III. Although the carbohydrates P.L. of both Groups A and C have somewhat higher nitrogen values than carbohydrates S.E., the rhamnose and glucosamine values are similar with ratios approximately 2 for Group A and close to one for Group C. In spite of the higher nitrogen values of the P.L. preparations, which may be due to protein impurities, the chemical composition of carbohydrates S.E. and P.L. are similar.

Zone Electrophoresis of the Carbohydrate.—A further comparison of the two carbohydrate preparations was obtained by zone electrophoresis. The separation of Group A carbohydrate in a typical experiment is given in Figs. 1 and 2. In both figures are recorded the results of the simultaneous electrophoresis of carbohydrates S.E. and P.L. in borate buffer. Fig. 1 demonstrates the distribution of the carbohydrates as determined by analysis of the fractions for rhamnose. Both preparations migrated slowly to the cathode. The serological reactivity of the fractions when tested with rabbit grouping antisera is shown in Fig. 2, and a comparison of Figs. 1 and 2 shows that the reactivity of the fractions with homologous antisera parallels the rhamnose value. Of some interest, however, is the separation of Group A carbohydrate into immunologi-

TABLE III

Chemical Composition of Streptococcal Group Carbohydrates Prepared with S. albus *Enzyme and Phage Lysin*

	Preparation	Total Nitrogen	Rhamnose	Glucosamine	Ratio Rhamnose: Glucosamine
		per cent	*per cent*	*per cent*	
Group A carbohydrate	S.E.	3.4	46.1	24.2	1.9
	P.L.	4.1	46.7	22	2.1
Group C carbohydrate	S.E.	3.7	37	30.5	1.2
	P.L.	5.2	32	25	1.3

The letters S.E. refer to carbohydrate prepared with the *S. albus* enzyme, and the letters P.L. refer to the carbohydrate prepared with the phage-associated lysin.

cally heterogeneous fractions. The carbohydrate which migrated most rapidly toward the cathode reacts with both Group A and Group A variant antistreptococcal sera (23). The material distributed nearest the origin, on the other hand, reacts only with the Group A antistreptococcal serum. The carbohydrates released from the cell wall with both enzymes showed this immunologic heterogeneity.

In Figs. 3 and 4 are recorded similar data obtained by the simultaneous electrophoretic separation of Group C carbohydrates S.E. and P.L. These materials migrate to the anode under the same conditions in which the Group A carbohydrate migrates to the cathode. Carbohydrate S.E. migrates somewhat more rapidly than carbohydrate P.L., however, both preparations have been separated into immunologically heterogeneous fractions. The carbohydrate which remains near the origin reacts with both Group C and Group A variant antistreptococcal sera, while the material which migrates to the anode reacts only with homologous antiserum.

Ultracentrifugal Studies.—Fig. 5 compares the schlieren patterns obtained by ultracentrifuge analysis of carbohydrates S.E. and P.L. prepared from Group A strain S43/100.[4] The dispersion which occurred probably reflects the relatively low molecular weight of these materials. However, the curves of

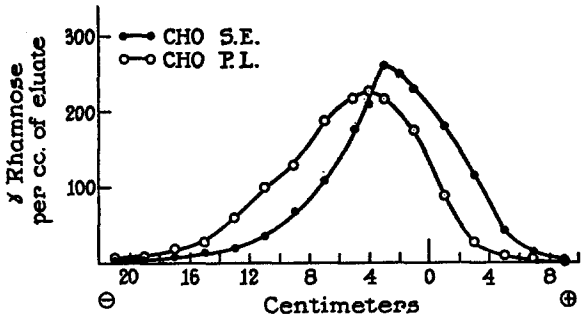

FIG. 1. Electrophoretic distribution of streptococcal Group A carbohydrate prepared with *S. albus* enzyme (S.E.) and phage-associated lysin (P.L.). The carbohydrate was prepared from strain S43/100. Borate buffer $\mu = 0.1$, pH 9, temperature 4°C., 24 hours at 400 volts. Both materials were placed on the block at the point indicated by zero.

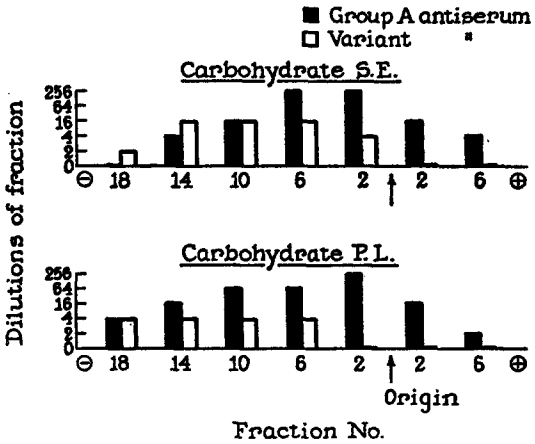

FIG. 2. Serological reactivity of the electrophoretic fractions of Group A carbohydrate. The highest dilutions which gave a precipitin reaction with grouping antisera are recorded.

both preparations are remarkably symmetrical, and the S values are nearly identical.

Quantitative Precipitin Analysis.—Additional information concerning the serological reactivity of carbohydrates S.E. and P.L. was obtained by quantitative precipitin analysis. In Fig. 6, the results with Group A carbohydrates

[4] The author gratefully acknowledges the assistance of Dr. E. Franklin who performed these analyses.

S.E. and P.L. and Group A antiserum are presented. This shows that both preparations precipitate the same amount of antibody in the range of antibody excess, while in the regions of maximum precipitation and antigen excess a somewhat greater amount of antibody is precipitated by the carbohydrate S.E. This is probably referable to the fact that the Group A antiserum used in these tests contains a slight trace of antibody to the variant carbohydrate,

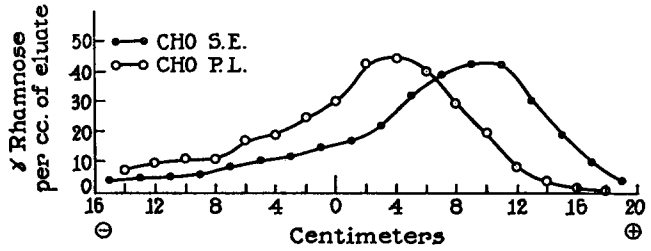

FIG. 3. Electrophoretic distribution of streptococcal Group C carbohydrate prepared with *S. albus* enzyme (S.E.) and phage-associated lysin (P.L.). The carbohydrate was prepared from strain 26RP66. Borate buffer $\mu = 0.1$, pH 9, temperature 4°C., 36 hours at 400 volts. Both materials were placed on the block at the point indicated by zero.

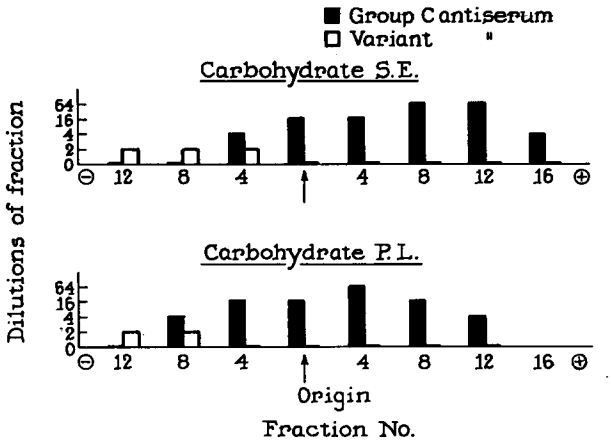

FIG. 4. Serological reactivity of the electrophoretic fractions of Group C carbohydrate. The highest dilutions which gave a precipitin reaction with grouping antisera are recorded.

and the *S. albus* enzyme preparation gives a stronger cross-reaction with variant antiserum than the carbohydrate P.L.

Quantitative precipitin experiments with Group C carbohydrates S.E. and P.L. and Group C antiserum are shown in Fig. 7. Both preparations precipitate approximately the same amount of antibody, but again it is to be noted that carbohydrate S.E. precipitates a slightly greater amount of antibody in the region of antigen excess than carbohydrate P.L. As in the case of Group A,

this result is probably referable to the fact that Group C antiserum contains a slight trace of Group A variant antibody, and the Group C carbohydrate

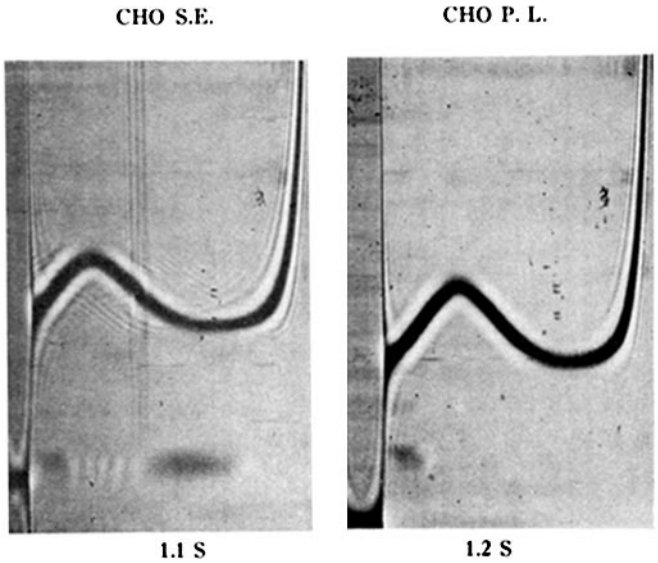

FIG. 5. Schlieren patterns obtained by ultracentrifuge analysis of carbohydrates S.E. and P.L. For CHO S.E. $S_{20.w}$ = 1.1 S, for CHO P.L. $S_{20.w}$ = 1.2 S.

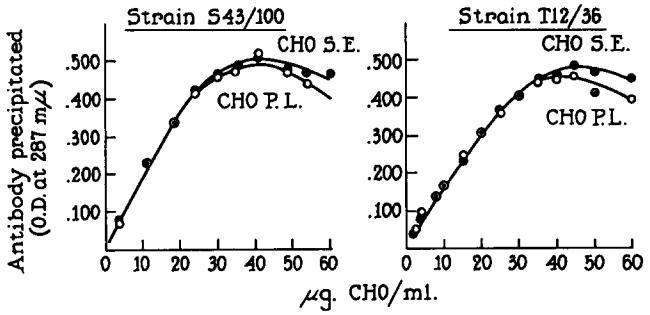

FIG. 6. Quantitative precipitin analysis of the carbohydrates S.E. and P.L. of Group A hemolytic streptococci with homologous antiserum.

S.E. gives a somewhat stronger cross-reaction with variant antiserum than carbohydrate P.L.

Phage Inactivation by Carbohydrate.—A further comparison of Group C carbohydrate prepared by the two methods was made possible by the fact that the carbohydrate of Group C streptococci specifically inactivates Group C bacteriophage (1).

For phage inactivation experiments 2.0 mg. of carbohydrates S.E. and P.L. were dissolved in tubes containing 0.9 ml. of dialysate broth. A control tube contained broth without carbohydrate. To each tube 0.1 ml. of a C1 phage suspension was added. The mixtures were incubated at 37°C. and sampled at zero time and 10 minute intervals. The surviving phage in the samples were counted by the soft agar layer technique.

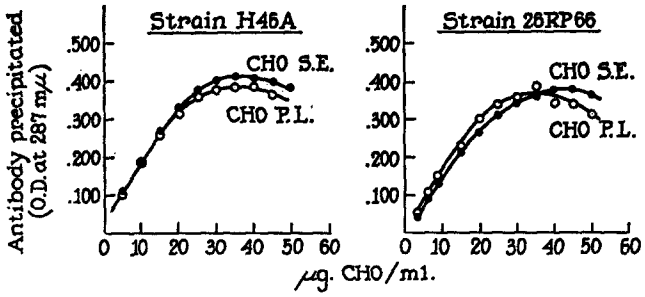

FIG. 7. Quantitative precipitin analysis of the carbohydrates S.E. and P.L. of Group C hemolytic streptococci with homologous antiserum.

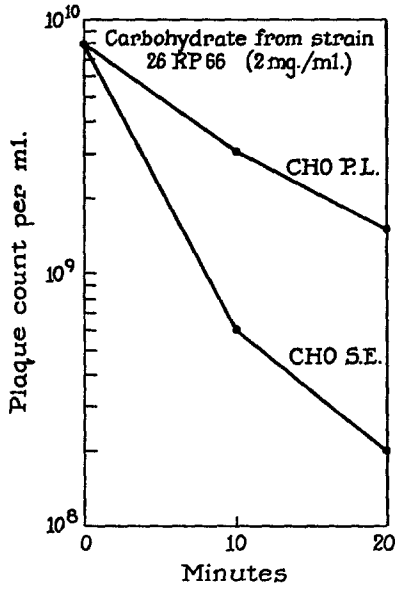

FIG. 8. Inactivation of C1 phage with carbohydrates S.E. and P.L. of Group C hemolytic streptococci.

In Fig. 8 the results of incubating C1 phage with the two carbohydrate preparations are seen. Both materials inactivated the phage, but the rate of inactivation was greater with the carbohydrate S.E. However, since these

kinetic studies do not necessarily reflect the phage-combining capacity of the carbohydrates, inactivation experiments were performed in which varying concentrations of phage were incubated with a constant amount of carbohydrate.

One-tenth ml. of various decreasing phage dilutions was added to three series of tubes. To the first series was added 0.2 mg. carbohydrate S.E. in 0.9 ml. of dialysate broth, and to the second series 0.2 mg. carbohydrate P.L. in 0.9 ml. of dialysate broth. Dialysate broth was added to the third series which served as the control. Each tube, after the addition of the broth, was incubated for exactly 30 minutes at 37°C., and the surviving phage counted.

The results of this type of experiment are shown in Fig. 9 *a*. A comparison of the two carbohydrates from these data is facilitated by estimating the phage

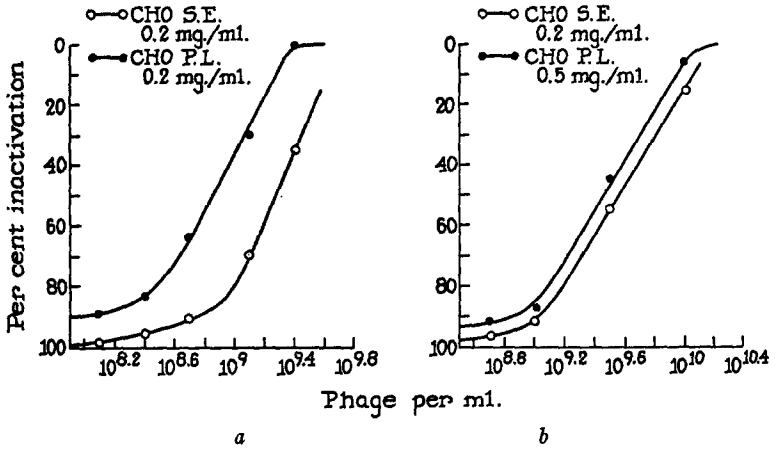

FIG. 9. Per cent inactivation of C1 phage by the carbohydrates S.E. and P.L. of Group C streptococci. The surviving phage were counted after incubation at 37°C. for 30 minutes.

concentrations which are 50 per cent inactivated by both preparations. Under the conditions of the present experiment 50 per cent of $10^{9.2}$ phage per ml. were inactivated by carbohydrate S.E., while carbohydrate P.L. inactivated 50 per cent of the phage at the somewhat lower concentration of $10^{8.8}$ per ml. Thus the carbohydrate S.E. inactivated 2½ times the number of phage as were inactivated by the carbohydrate P.L. In the per cent inactivation experiment recorded in Fig. 9 *b*, the concentration of the carbohydrate P.L. was increased 2½ times to 0.5 mg. per ml., while the concentration of carbohydrate S.E. was unchanged at 0.2 mg. per ml. Under these conditions the per cent inactivation curves for both carbohydrates are almost superimposed. While these data suggest that a somewhat greater number of phage are inactivated by Group C carbohydrate S. E. than by carbohydrate P. L., it should be emphasized that both enzyme preparations specifically inactivate group C bacteriophage.

The foregoing data on chemical composition, physical chemical properties, immunological reactivity, and phage inactivation, indicate that streptococcal carbohydrate prepared by the *S. albus* enzyme is similar to the carbohydrate prepared by the phage-associated lysin.

Identification of M Protein.—M protein was recovered from the lysin digests of Group A cell walls by the following procedure.

The streptococci of strain S43/137 from 21 liters of Todd-Hewitt broth were collected in the Sharples centrifuge. This strain was Group A Type 6 and was known to have a high titer of M content after recent passage through mice. The cell walls were isolated and then lysed with phage-associated lysin. The digest was centrifuged, the insoluble material discarded, and the supernatant was brought to 0.6 saturation with ammonium sulfate, a procedure also employed by Lancefield and Perlmann (3) for the isolation of M antigen of Type 1 streptococci. The precipitate which contains very little of the group carbohydrate was collected by centrifugation, taken up in saline, and the insoluble material removed by centrifugation. The supernatant was reprecipitated at 0.6 saturation with ammonium sulfate, and the precipitate collected on a filter cel pad and washed from the pad with saline. The insoluble material was removed from this protein solution by centrifugation, and the supernatant was concentrated in the cold to 2 cc. in a collodion bag (Schleicher and Schuell Co.) under vacuum and at the same time dialyzed against glycine buffer. Electrophoretic separation of the concentrate was carried out on a starch medium in glycine buffer.

The crude lysin digest of the Type 6 cell walls gave a strongly positive type-specific precipitin reaction with homologous absorbed anti-serum and weakly positive cross-reactions with several absorbed heterologous antisera. The protein which was precipitated at 0.6 saturation with ammonium sulfate, however, gave a reaction with Type 6 antiserum and no cross-reactions with the heterologous antisera. No M protein could be detected in the protein which was precipitated above 0.6 saturation.

The distribution of protein following zone electrophoresis of the M preparation is illustrated in Fig. 10. The Folin reaction on the fractions indicates that a major protein peak travelled slowly to the cathode. Only those fractions within this peak gave a type-specific precipitin reaction with absorbed homologous rabbit antiserum. The fraction located 4 cm. to the left of the origin, when diluted 1:40, still gave a type-specific precipitin reaction. In other experiments for which a more potent type-specific antiserum was available, a 1:100 dilution of this fraction gave a type-specific precipitin reaction. It should be emphasized that this protein retained serological activity following boiling with HCl at pH 2, but this was lost after brief exposure to 10 μg. of trypsin per ml. These two properties are characteristic of the M antigen (3).

Fig. 10 also demonstrates that the bulk of the serologically detectable carbohydrate remaining in the M protein preparation following electrophoresis was located between the origin and the anode. The fractions of this region contained rhamnose and gave a strong group-specific precipitin reaction with Group A antiserum. The movement of free carbohydrate here is quite different from that in the experiments depicted in Figs. 1 and 2, since glycine buffer

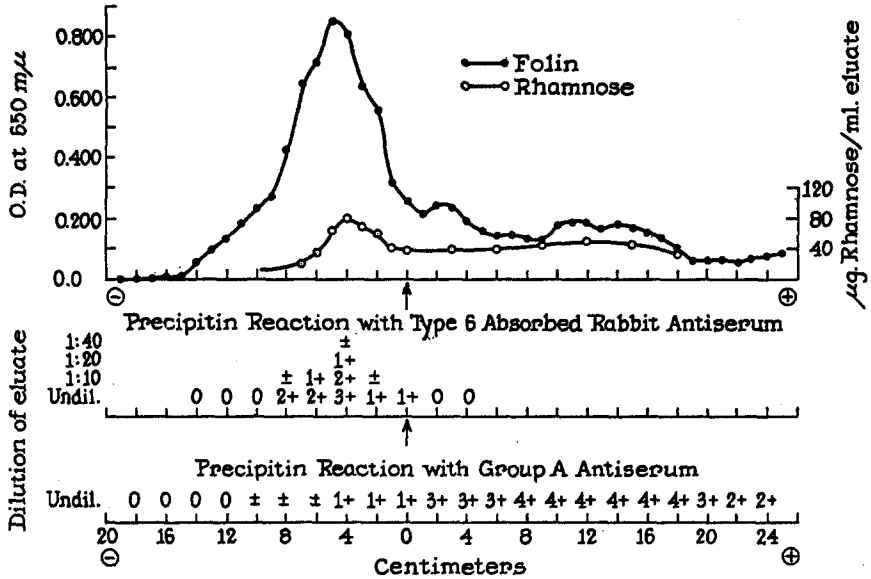

FIG. 10. Electrophoretic distribution of Group A Type 6 cell wall protein released by phage-associated lysin. Glycine buffer pH 8.8, $\mu = 1$, temperature 4°C., 24 hours at 400 volts. The protein was placed on the block at zero. Each fraction was eluted with 2 ml. of saline. The Folin reaction was determined with 0.05 ml. of eluate and rhamnose with 0.2 ml. of eluate.

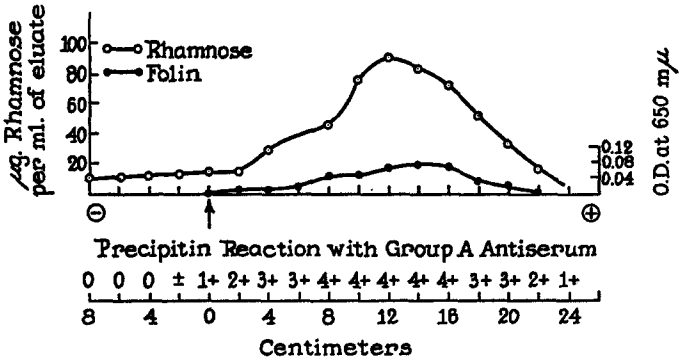

FIG. 11. Electrophoretic distribution of streptococcal Group A carbohydrate prepared from strain S43/100 with phage-associated lysin. The procedure was carried out on a potato starch supporting medium, with glycine buffer $\mu = 0.1$, pH 9, temperature 4°C., for 24 hours at 400 volts. Rhamnose and the Folin reaction were determined on 0.2 cc. samples of the eluates.

rather than borate buffer was employed. This is illustrated in Fig. 11 which shows the distribution of Group A carbohydrate P.L. following electrophoresis in glycine buffer under conditions otherwise identical with those employed when borate buffer was used. Carbohydrate was detected serologically

and chemically by analysis for rhamnose. The broad asymmetrical peak of carbohydrate was located 12 cm. from the origin toward the anode, and no significant carbohydrate was detected between the origin and the cathode.

While the fractions of the major protein peak of the M preparation illustrated in Fig. 10 reacted only slightly with Group A antiserum, analysis revealed that this material contained appreciable rhamnose and glucosamine in the usual proportion of Group A carbohydrate. These facts suggest that C substance, which is bound to protein, migrates to the cathode and the serological reactivity is masked by the protein. Various procedures were utilized, therefore, in an attempt to separate the carbohydrate from the protein. In Table IV are recorded the precipitin reactions of the fractions following treatment with proteolytic enzymes and boiling at pH 2. After either type of treatment group-specific carbohydrate becomes readily detectable serologically in

TABLE IV

Group A Precipitin Tests on Electrophoretic Fractions after Treatment with Enzymes and HCl

Treatment of fraction	Final dilution of eluates 1:10											
Control	0	0	0	0	0	0	±	±				
HCl pH 2	0	0	0	±	1+	1+	2+	2+				
S. albus enzyme	0	0	±	1+	2+	2+	2+	2+				
Trypsin	0	±	1+	2+	2+	3+	3+	3+				
	10	8		6		4		2	↑		2	
	⊖ ..... Centimeters ..... ⊕											

The final dilution of the eluates following hydrolysis and neutralization was 1:10. The strength of the precipitin reaction in the capillary tubes is graded from ± to 4+.

those fractions containing rhamnose and glucosamine. However, it should be noted that only 2 per cent of the total cell wall carbohydrate extracted by phage-associated lysin is bound to protein.

From these preliminary experiments it is clear that the M protein preparations, although surprisingly homogeneous on electrophoresis, are, nevertheless, mixtures containing some group-specific carbohydrate as well as proteins other than M antigen.

DISCUSSION

Phage-associated lysins have been described in the lysates of several different bacteria. In the case of hemolytic streptococci this lysin acts on intact organisms as well as the isolated cell walls, and the reaction is not dependent upon the presence of the phage particle. Little is known, however, of the lytic mechanisms of these lysins. Since a knowledge of the split products is important for an understanding of the lytic process, this paper has described the mate-

rial released from the streptococcal cell wall. Hemolytic streptococci are particularly useful for such a study since previous work has identified several cell wall antigens, including the group-specific carbohydrate (4) and the M protein (24). In the experiments reported here, both of these antigens were isolated from the cell wall digests obtained with phage-associated lysin. The observations on the M protein are preliminary, but the carbohydrate was shown to be chemically and immunologically similar to the material isolated with *S. albus* enzyme. The analytical values for the carbohydrate obtained by both enzymes are essentially in agreement with the findings of McCarty (4) for the chemical composition of group-specific carbohydrate. It is of some interest that a nearly identical carbohydrate can be extracted from the cell wall by two entirely different enzymes. Although cell wall fractions following digestion with phage-associated lysins for other bacterial systems have not been extensively studied, non-dialyzable reducing sugar appeared following lysis of *B. megatherium* cell walls (8).

Hemolytic streptococci are not affected by lysozyme, but the action of this enzyme on certain other bacteria has similarities to lysis of streptococci by phage-associated lysin. It is clear that the substrate for both enzymes is the bacterial cell wall. Incubation of the cell walls of *Micrococcus lysodeikticus*, *Sarcina lutea*, and *Bacillus megatherium* with lysozyme results in complete dissolution and the release of complex dialyzable and nondialyzable substances (25). The latter material comprises 50 to 70 per cent of the cell wall and on ultracentrifugal analysis has a value from $1.15S_{20}$ to $1.5S_{20}$. Split products with similar values were reported here following lysis of streptococcal cell walls with *S. albus* enzyme and phage-associated lysin. The dialyzable component resulting from lysozymic action appears to be a disaccharide complex of two different amino sugars (26). While a small amount of dialyzable carbohydrate has been detected following the lysis of streptococcal cell walls, this material has the serological reactivity of streptococcal group carbohydrate (27).

Several lines of evidence indicate that the phage-associated lysin, as produced within infected bacteria, may be responsible for subsequent lysis of the culture. Certainly the description of these enzymes for several different bacteria suggests a general phenomenon. In instances in which the process has been carefully investigated, the lysin has not been detected within the cell prior to phage infection. However, after a virulent infection, and during the latent period up to the time of lysis, an increasing amount of lysin accumulates within the cell (8). Finally, it has been shown that lysis of isolated Group C cell walls by purified lysin and lysis of a Group C streptococcal culture following a virulent infection are accompanied by release of cell wall carbohydrate which retains serologic reactivity (1). Such a similarity of split products suggests a common lytic process.

Of particular interest is the electrophoretic separation of a protein fraction,

released from the cell walls of Type 6 streptococci by phage-associated lysin, which reacts with type-specific antiserum. In view of the few purification procedures employed, this preparation, while remarkably homogeneous in the electrophoretic pattern, may contain proteins other than the M antigen. Some group-specific carbohydrate remained in the preparation which appeared to be bound to protein.

Two characteristic properties of the M protein as isolated by Lancefield and Perlmann from Type 1 streptococci are its antigenic stability with boiling at pH 2, and its sensitivity to the action of trypsin (3). The protein isolated from the phage-associated lysin digest of cell walls also has these properties. Experiments are now in progress to demonstrate that the protein isolated here is antigenic, inducing the formation in rabbits of type-specific precipitins and protective antibodies. The need for elucidating the characteristics of M protein is obvious, since the evidence strongly suggests that it contributes to the virulence of Group A hemolytic streptococci in natural infections of man (28–31), and in experimental infections of animals (32–35).

SUMMARY

The lysis of cell walls of hemolytic streptococci by a phage-associated lysin has been described. A method is presented for preparing the lysin from Group C streptococcal phage lysates.

Following lysis almost all of the cell wall carbohydrate is recovered in solution. This material has the serological reactivity, physical-chemical properties, and values for nitrogen, rhamnose, and glucosamine similar to those of the carbohydrate isolated from the cell walls by the *Streptomyces albus* enzyme. Group C carbohydrate isolated by either enzyme inactivates Group C bacteriophage.

The protein liberated by the lysin from Group A Type 6 cell walls gives a type-specific precipitin reaction with homologous rabbit antiserum. Preliminary data are presented on the ammonium sulfate fractionation and the electrophoretic separation of a protein fraction with the serological reactivity of M protein.

BIBLIOGRAPHY

1. Krause, R. M., Studies on bacteriophages of hemolytic streptococci. I. Factors influencing the interaction of phage and susceptible host cell, *J. Exp. Med.*, 1957, **106,** 365.
2. Maxted, W. R., The active agent in nascent phage lysis of streptococci, *J. Gen. Microbiol.*, 1957, **16,** 584.
3. Lancefield, R. C., and Perlmann, G. E., Preparation and properties of type-specific M antigen isolated from Group A, Type 1 hemolytic streptococcus, *J. Exp. Med.*, 1952, **96,** 71.
4. McCarty, M., The lysis of Group A hemolytic streptococci by extracellular

enzymes of *Streptomyces albus*. I. Production and fractionation of the lytic enzymes, *J. Exp. Med.*, 1952, **96**, 555. II. Nature of the cellular substrate attacked by the lytic enzymes, *J. Exp. Med.*, 1952, **96**, 569.

5. Sertic, V., Untersuchungen über einen Lysinzonen bildenden Bakteriophagen, *Zentr. Bakteriol. Parasitenk. Abt., 2*, 1929, **110**, 125.
6. Ralston, D. J., Lieberman, M., Baer, B., and Krueger, A. P., Staphylococcal virolysin, a phage-induced lysin; its differentiation from the autolysin of normal cells, *J. Gen. Physiol.*, 1957, **40**, 791.
7. Humphries, J. C., Enzymic activity of bacteriophage-culture lysates. I. A capsule lysin active against *Klebsiella pneumoniae*, type A, *J. Bact.*, 1948, **56**, 683.
8. Murphy, J. S., A phage-associated enzyme of *Bacillus megaterium* which destroys the bacterial cell wall, *Virology*, 1957, **4**, 563.
9. Todd, E. W., and Hewitt, L. F., A new culture medium for the production of antigenic streptococcal haemolysin, *J. Path. and Bact.*, 1932, **35**, 973.
10. Gratia, A., Numerical relations between lysogenic bacteria and particles of bacteriophage, *Ann. Inst. Pasteur*, 1936, **57**, 657.
11. Swift, H. F., Wilson, A. T., and Lancefield, R. C., Typing Group A hemolytic streptococci by M precipitin reactions in capillary pipettes, *J. Exp. Med.*, 1943, **78**, 127.
12. Salton, M. R. J., and Horne, R. W., Studies of the bacterial cell wall. II. Methods of preparation and some properties of cell walls, *Biochim. et. Biophysica Acta*, 1951, **7**, 177.
13. McCarty, M., Variation in the group-specific carbohydrate of Group A streptococci. II. Studies on the chemical basis for serological specificity of the carbohydrates, *J. Exp. Med.*, 1956, **104**, 629.
14. McCarty, M., personal communication.
15. Dische, Z., and Shettles, L. B., A specific color reaction of methylpentoses and a spectrophotometric micromethod for their determination, *J. Biol. Chem.*, 1948, **175**, 595.
16. Elson, L. A., and Morgan, W. T. J., A colorimetric method for the determination of glucosamine and chondrosamine, *Biochem. J.*, 1933, **27**, 1824.
17. Koch, F. C., and McMeekin, T. L., A new direct nesslerization micro-Kjeldahl method and a modification of the Nessler-Folin reagent for ammonia, *J. Am. Chem. Soc.*, 1924, **46**, 2066.
18. Kunkel, H. G., Zone electrophoresis, *Methods Biochem. Anal., New York, Interscience Publishers*, 1954, **1**, 141.
19. Muller-Eberhard, H. J., and Kunkel, H. G., The carbohydrate of γ-globulin and myeloma proteins, *J. Exp. Med.*, 1956, **104**, 253.
20. Clark, W. M., The Determination of Hydrogen Ions, Baltimore, Williams and Wilkins Co., 1928, 3rd edition.
21. Kunkel, H. G., and Tiselius, A., Electrophoresis of proteins on filter paper, *J. Gen. Physiol.*, 1951, **35**, 89.
22. Trautman, R., Operating and comparating procedures facilitating schlieren pattern analysis in analytical ultracentrifugation, *J. Physic. Chem.*, 1956, **60**, 1211.

23. McCarty, M., and Lancefield, R. C., Variation in the group-specific carbohydrate of Group A streptococci. I. Immunochemical studies on the carbohydrates of variant strains, *J. Exp. Med.*, 1955, **102,** 11.
24. Salton, M. R. J., The nature of the cell walls of some Gram-positive and Gram-negative bacteria, *Biochim. et Biophysica Acta*, 1952, **9,** 334.
25. Salton, M. R. J., The properties of lysozyme and its action on microorganisms, *Bact. Rev.*, 1957, **21,** 82.
26. Salton, M. R. J., The action of lysozyme on cell walls of some lysozyme-sensitive bacteria, *Biochim. et Biophysica Acta*, 1956, **22,** 495.
27. Krause, R. M., data to be published.
28. Kuttner, A. G., and Lenert, T. F., The occurrence of bacteriostatic properties in the blood of patients after recovery from streptococcal pharyngitis, *J. Clin. Inv.*, 1944, **23,** 151.
29. Rothbard, S. J., Bacteriostatic effect of human sera on Group A streptococci. I. Type-specific antibodies in sera of patients convalescing from Group A streptococcal pharyngitis, *J. Exp. Med.*, 1945, **82,** 93. II. Comparative bacteriostatic effect of normal whole blood from different animal species in the presence of human convalescent sera, *J. Exp. Med.*, 1945, **83,** 107. III. Interference with bacteriostatic activity by blockage of the leukocytes, *J. Exp. Med.*, 1945, **82,** 119.
30. Rothbard, S., and Watson, R. F., Variation occurring in Group A streptococci during human infection. Progressive loss of M substance correlated with increasing susceptibility to bacteriostasis, *J. Exp. Med.*, 1948, **87,** 521.
31. Rothbard, S., Watson, R. F., Swift, H. F., and Wilson, A. T., Bacteriologic and immunologic studies on patients with hemolytic streptococcic infections as related to rheumatic fever, *Arch. Int. Med.*, 1948, **82,** 229.
32. Lancefield, R. C., Specific relationship of cell composition to biological activity of hemolytic streptococci, *Harvey Lectures*, 1940–41, **36,** 251.
33. Watson, R. F., and Lancefield, R. C., Studies on the antigenic composition of group A hemolytic streptococci. III. Types with serologically identical M but distinct T antigens: Types 10 and 12, *J. Exp. Med.*, 1944, **79,** 89.
34. Rothbard, S. J., Protective effect of hyaluronidase and type-specific anti-M-serum on experimental Group A streptococcus infections in mice, *J. Exp. Med*, 1948, **88,** 325.
35. Rothbard, S., and Swift, H. F., Type-specific protection and immunity following intranasal inoculation of monkeys with Group A hemolytic streptococci, *J. Exp. Med.*, 1946, **84,** 127.