STUDIES ON STREPTOCOCCUS PYOGENES

IV. Effect of Whole Cells, M and C Antigens, and Other Components of the Cell on Adrenalectomized Rats*

By HUTTON D. SLADE‡, Ph.D., and YOSHITAMI KIMURA§, M.D.

(*From the Department of Microbiology, Northwestern University Medical School, Chicago*)

(Received for publication, March 5, 1959)

Knowledge of the biological and chemical properties of the molecular components of the Group A streptococcal cell should provide valuable information pertaining to the pathogenesis of streptococcal infections. Previous work in this laboratory has shown that exposure of streptococcal cells to sonic oscillation resulted in the release from the cell of macromolecular components containing serologically active C and M antigens (1) and enzymes concerned in carbohydrate and amino acid metabolism (2). These extracts are well suited for the fractionation and purification of components of the streptococcal cell (3, 4).

The possibility that components of the Group A streptococcus possess toxicity for laboratory animals is a subject of considerable interest. The information available however, is very limited. Preliminary studies (5) had indicated that heat-killed streptococci, as well as living cells, and purified preparations of M protein caused death in adrenalectomized rats. The normal rat was highly resistant. The present report will summarize the effect of various macromolecular components isolated from Group A streptococci and other bacterial species upon the adrenalectomized rat.

Materials and Methods

Test Animals.—Normal and adrenalectomized Sprague-Dawley white female rats weighing between 180 and 200 gm. were used. They were fed a diet of Purina checkers and were adrenalectomized bilaterally under ether anesthesia. The rats were maintained on 1.0 per cent NaCl drinking water for 8 to 10 days before use. This period is sufficient for recovery from the operative procedure.

All materials, other than bacterial cells, were tested by injection into the tail vein of the rat. The dose varied between 0.5 and 1.0 ml. depending upon the weight of the animal.

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* Supported in part by the National Heart Institute (H-3709), United States Public Health Service, the Office of Naval Research (Nonr-1228(09)), and the Chicago Heart Association.

‡ Established Investigator of the American Heart Association.

§ Fulbright grantee on leave of absence from the Department of Bacteriology, Nippon Medical School, Tokyo, Japan.

Culture of Cells.—For animal inoculation stock cultures on blood agar slants of the challenging strains were transferred to 10 ml. brain-heart infusion broth (Difco) and the latter incubated for 6 hours at 37°C. Ten ml. of the young cultures was then pipetted into 1 liter of brain-heart infusion broth and incubated at 37°C. for 18 hours. The cells were removed by centrifugation at 10,000 *G* for 10 minutes, and washed twice with sterilized 0.01 M phosphate buffer (pH 7.0) containing 0.85 per cent saline. The cells were resuspended in phosphate-saline to an optical density (O.D.) at 550 mμ of 0.65 (equivalent to 25.2×10^9 cells/ml.). These suspensions were further diluted before injection. All suspensions were injected into the peritoneal cavity.

For the large scale culture of bacterial cells, brain-heart infusion broth plus supplement (6) was used. The inoculum was prepared as described above. The cells were harvested by centrifugation as above and washed 3 times before use.

The designation 'm^-' following a strain number indicates a culture which synthesizes little or no M protein. The parent strain from which it originated formed normal quantities of M protein and is designated by 'm^+'. These cultures as well as T28 were obtained through the kindness of Dr. R. C. Lancefield.

Heat-Killed Cells.—Heat-killed cells were obtained by suspension in 0.01 M phosphate (pH 7.0) at 75°C. for 30 minutes. Each suspension was tested for viable cells before use.

Extraction Procedures.—Sonic Oscillation for C Antigen.—Forty ml. water containing 2 gm. (dry weight) whole cells was oscillated in a 10 kc. Raytheon instrument for 0.25 hours. The output current was set at approximately 1.25 amperes with the power control at maximum setting. The temperature during the process was held at 2–5°C. by a circulating alcohol-water mixture from a refrigerated bath.

The oscillated suspension was centrifuged at 20,000 *G* for 30 minutes at 3°C., the supernatant removed and the residue washed with 1/2 volume distilled water. The residue was resuspended in 20 ml. distilled water and oscillated again for 1.25 hours. This treatment liberates most of the C substance into the supernatant whereas the M protein remains in the residue. The supernatant was designated S.25–1.5 and the residue S 1.5R. The two fractions were dialyzed for 18 hours, frozen at -60°C., and lyophilized.

Sonic Oscillation for M Protein.—Fraction S 1.5R was suspended in water to a concentration of 10 mg./ml. and oscillated for an additional 2.5 hours. The supernatant was removed after centrifugation for 30 minutes at 20,000 *G* and the residue washed once with ½ volume water. The two were combined, dialyzed, frozen, and lyophilized. This fraction contains most of the M protein of the whole cell. It was numbered S 1.5–4.

Acid Extraction of M Protein and C Substance.—Ten mg. lyophilized whole cells was extracted at pH 2–2.5 according to the procedure of Lancefield (7). The acid extract of the cells was adjusted to pH 7.5 and centrifuged at 10,000 *G* for 10 minutes. The neutralized extract was then acidified to pH 2.5 with HCl, and the precipitate removed by centrifugation at 10,000 *G* for 10 minutes. The precipitate was used for the fractionation of M protein and the supernatant for C substance.

Chemical Purification.—M Protein.—Each of the extracts was treated as follows: 10 mg. was dissolved in 0.1 M phosphate (pH 8.0) and digested with crystalline ribonuclease (0.01 mg.) for 5 hours at 37°C. The solution was then dialyzed against 0.01 M phosphate (pH 8.0) for 2 days at 4°C., and fractionated with $(NH_4)_2SO_4$ at pH 7–8. Most of the M protein precipitated at 0.33 to 0.60 saturation (7). The precipitate was removed by centrifugation, dialyzed against distilled water, frozen, and lyophilized.

The following designations have been given to these fractions: sonic extract—S 1.5–4 AF_2, acid extract—ACE.AF_2.

All preparations reacted strongly in the precipitain test (8) against specific adsorbed antisera, and a one-plus reaction was obtained when the protein was diluted to 5 to 10 μg./ml.

No reactions with anti-M sera of a number of other types were observed. The type-specific reactivity of the preparations was destroyed by trypsin.

The M preparations contained between 0 and 0.3 per cent rhamnose (1) and hexosamine (9) when tested at a level of 2 mg./ml. Traces of C antigen were found to be present as shown by a weak positive reaction with group A antisera. These traces were removed by adsorption. Ten mg. M protein was dissolved in 1 ml. 0.05 M phosphate pH 8 and 20 mg. lyophilized antiserum was added. The solution was incubated at 37°C. for 3 hours, centrifuged to remove a trace of precipitate which formed during the neutralization period, and lyophilized. Three mg. of this material was equivalent to 1 mg. M protein. Precipitin tests for C antigen were negative.

Chemical Purification.—C Substance.—Sonic extract S.25–1.5 was digested with ribonuclease and fractionated with $(NH_4)_2SO_4$ as given above for M protein. One mg. of the lyophilized preparation was dissolved in 1.0 ml. 0.1 M phosphate (pH 8.0) and 1 ml. 0.85 per cent NaCl was added. A few grains of Na acetate were added, followed by 8 ml. 95 per cent ethyl alcohol. The solution was held overnight at 4°C., centrifuged at 10,000 *G* for 10 minutes and the supernatant was discarded. The precipitate was suspended in distilled water, frozen, and lyophilized. It was designated AF_2.AP.

The C antigen extracted by HCl was purified in the following manner:—the supernatant was neutralized to pH 7.5 with N NaOH and concentrated to about $\frac{1}{10}$ volume under a vacuum and dialyzed overnight against distilled water. Three volumes of 95 per cent ethyl alcohol were added and held overnight at 4°C. The precipitate was removed by centrifugation and discarded. Three volumes of alcohol was added to the supernatant, and the precipitate removed, dialyzed, and lyophilized as above. Preparations made in this way were designated as C-9.

All preparations were positive in the precipitin test against group A antiserum when diluted to 10 to 20 μg./ml. No reaction was found for M protein when tested against adsorbed antiserum specific for the type of Group A streptococcus from which the C antigen was prepared. The preparations contained between 15 and 25 per cent protein, 20 and 30 per cent rhamnose, and 18 and 20 per cent hexosamine.

Chemical Purification.—R Antigen.—The procedure used for the extraction of this substance was the same as that used for M protein. All preparations were obtained from sonic extracts S 1.5–4 and all were tested by injection into the tail vein of the rat.

Antisera.—All the antisera employed, except as noted otherwise, was obtained from the United States Public Health Service.

Chemical Determinations.—Protein and rhamnose were determined by methods previously described (1) and hexosamine by the procedure of Palmer *et al.* (9).

RESULTS

Effect of Living Streptocci upon the Normal Rat and the Adrenalectomized Rat.—Table I shows that the normal rat is resistant to large numbers of living mouse-virulent Group A streptococci. No marked clinical signs of infection were seen in this group during the incubation period (maximum 5 days). Those animals autopsied at 24 hours showed in only a few cases positive cultures on blood agar from the liver and spleen. Following this period no other positive cultures could be obtained.

In sharp contrast to the normal rat, the adrenalectomized rat is readily susceptible to Group A streptococcal infection. Positive cultures were consist-

ently obtained from these animals. The progress of the infection is characterized by bristling of the hair, edema, and scratching of the nose, general weakness, severe dyspnea, and spasms. Death usually occurred within 24 hours. Similar results were obtained with several other strains.

Table I shows that a mouse-avirulent strain of Group A streptococcus which had lost the ability to synthesize M protein, was able to cause death of the adrenalectomized rat. The lethal dose however, was 30-fold (based on cell numbers) that of the virulent strain.

The large numbers of both virulent and avirulent cocci required to kill the

TABLE I

Effect of Viable Group A Streptococci on the Normal and Adrenalectomized Rat

Group of rats	Strain of streptococcus	No. of cells injected	D/T*
Normal	$T3m^+$, Type 3, mouse virulent‡	5.3×10^{10} (broth culture concentrated 10 ×)	0/3
"	" "	5.3×10^{9} (undiluted broth culture)	0/3
Adrenalectomized	" "	5.3×10^{8} (10^{-1} dil.)	4/4
"	" "	5.3×10^{7} (10^{-2} dil.)	1/4
"	" "	5.3×10^{6} (10^{-3} dil.)	0/4
Normal	$S23m^-$, Type 14, mouse avirulent	9.6×10^{10}	0/3
Adrenalectomized	" "	19.2×10^{9}	3/3
"	" "	9.6×10^{9}	2/3
"	" "	4.8×10^{9}	1/3

* D/T = No. animals died/No. animals tested.

‡ Cells were virulent for mice in a dilution of 10^{-6} to 10^{-7} (0.5 ml. intraperitoneal injection).

adrenalectomized rat indicated that perhaps the lethal effect was due in large measure to the toxicity of components of the streptococcal cell.

Effect of Cells Other Than Group A.—Viable suspensions of a Group D streptococcus and *Staphylococcus aureus* were also tested in the adrenalectomized rat. Table II shows that these species were also toxic, however a comparable lethal dose would probably require $40 \times 10^{3} - 40 \times 10^{4}$ more cells than that required with the Group A streptococcus.

Effect of Heat-Killed Cells.—In order to determine the effect of non-viable Group A cocci upon the rat, cell suspensions were sterilized before injection by exposure to 75°C. The injection of these cells from several strains of Group A streptococcus resulted in death of the adrenalectomized rats. Approximately 100-fold more cells (8 times on dry weight basis) were required than living cells. These results demonstrated that the death of the adrenalectomized rat

was due primarily to a toxic component of the streptococcus. The 8-fold difference in dose between dead and living cells was probably overcome by the rapid multiplication of cocci in the animal.

Effect of Miscellaneous Materials.—It was considered advisable at this stage

TABLE II

Effect of Viable Group D Streptococci and Staphylococci on Adrenalectomized Rat

Group of rat		No. of cells injected	D/T
Normal	Streptococcus Group D, strain D10	19.2×10^{10}	0/6
Adrenalectomized	" "	19.2×10^{10}	1/6
Normal	*Staphylococcus aureus*, strain 71	19.2×10^{10}	0/5
Adrenalectomized	" "	19.2×10^{10}	3/5
"	" "	19.2×10^{9}	0/4

TABLE III

Effect of M Protein from Group A Streptococci on the Adrenalectomized Rat

Serological type	Strain	Method of extraction	Quantity injected (per 100 gm. rat)	D/T
			mg.	
14	$S23m^+$	Sonic oscillation	1	6/6
19	N19	" "	1	6/6
6	$S43m^+$	" "	1	3/3
3	C203	" "	1	3/3
1	$T1m^+$	HCl at 100°C.	1	0/3
3	$T3m^+$	" " "	1	1/4
6	$S43m^+$	" " "	1	2/7
12	12RN	" " "	1	0/3
14	$S23m^+$	" " "	1	0/3
19	N19	" " "	1	0/3
19	N19	" " "	2	0/2
19	N19	" " "	4	0/2

to check the toxicity to the adrenalectomized rat of various proteins not found in bacterial cells. Difco peptone (100 mg.), crystalline bovine (25 mg.) and egg albumins (25 mg.), and normal rabbit serum (100 mg.) possessed no toxicity upon intravenous injection. These results clearly indicated that the toxic effect of the Group A streptococcus was due to components which were peculiar to the bacterial cell. Also it seemed likely that the cell components were active in small quantities. Attention was first directed to the M protein.

Effect of M Protein.—Table III illustrates that M protein prepared by sonic

oscillation from 4 serological types of Group A streptococcus was toxic to the adrenalectomized rat in all the animals tested. On the other hand, the M protein prepared by extraction in 0.1 N HCl at 100°C. from 6 serological types was comparatively non-toxic. In the case of strain N19, the dose was increased 4-fold without effect.

M protein is known to be destroyed by exposure to trypsin. Each of the sonic preparations (listed in Table III) was treated with trypsin for 2 hours at 37°C. Fifty μg. enzyme was used per 2 mg. M preparation in 1 ml. 0.05 M phosphate pH 8.0. In each case the antigen possessed no toxicity after tryptic digestion. The rats showed no physical disturbance following the injection. Controls in each case demonstrated that the serological activity had been destroyed by the trypsin.

TABLE IV

Effect of Anti-M and Anti-C Rabbit Sera on the Toxicity of M and C Preparations to Adrenalectomized Rats

Toxic preparations M protein from		Serum	D/T
Strain	Type		
$S23m^+$	14	Anti-14	0/3
N19	19	Anti-19	0/3
$S23m^+$	14	Anti-3	3/3
$S23m^+$	14	Anti-26	3/3
N19	19	Anti-6	3/3
N19	19	Anti-12	3/3
C substance from strain			
$T3m^+$		Anti-A	0/3
$S23m^+$		Anti-A	0/3
$S23m^-$		Anti-A	0/3
$T3m^+$		Anti-B	3/3
$S23m^+$		Anti-C	3/3
$S23m^-$		Anti-D	3/3

1 mg. of M or C substance injected in each case per 100 gm. rat.

It was of considerable interest to determine whether the toxicity of the M preparations was affected by incubation with specific adsorbed antisera. Two mg. M protein in 1 ml. 0.85 per cent saline was mixed with lyophilized homologous antiserum equivalent to 1 ml. whole serum and held 3 hours at 37°C. This quantity of antiserum was found sufficient to neutralize the serological activity of the M preparation in the precipitin test. Table IV shows that neither the $S23m^+$ nor the N19 preparation after incubation with antibody caused death

in the adrenalectomized rat. No toxic symptoms were noted in any of the animals.

It was of interest also to determine the effect of heterologous antisera on the toxicity of the preparations. Table IV shows that the toxicity of the Type 14 preparation was not neutralized by either Type 3 or 26 antiserum. Also, the toxicity of the Type 19 preparation was not affected after incubation with either Type 6 or 12 antiserum. The quantity of antiserum used in each case is the same as that given above. All the animals died and in approximately the same time as the controls. It is apparent that the toxic effect of the M protein was in each case neutralized only by homologous antibody.

The *in vitro* neutralization of the toxic effect by homologous antiserum raised the question as to whether a similar effect could be demonstrated *in vivo*. Anti-

TABLE V

Effect of Components Other Than M and C Antigens of Group A Streptococci on the Adrenalectomized Rat

Group	Serological type	Strain	Material injected	Method of extraction	D/T
A	28	T28	R antigen	Sonic oscillation	0/3
A	28	T28	" "	HCl at 100°C.	0/3
B	—	Lewis	Cell protein	Sonic oscillation	0/3
D	—	D10	" "	" "	0/3
A	19	N19	Component 4	" "	0/3
A	19	B19	" "	" "	0/3

1 mg. of material injected in each case per 100 gm. rat.

serum (0.2 ml./100 gm. rat) was injected intravenously into the adrenalectomized rat 18 hours before the injection of 1 mg. M protein/100 gm. rat. The preparation from strain S23m was used. Three animals were tested and in each case no deaths occurred. No signs of distress in the animals were noticed.

Effect of R Antigen.—Some strains of Type 28 streptococci are known to be unable to synthesize M antigen (10). It was therefore of great interest to prepare protein from one of these strains by the method used for the extraction and chemical purification of M protein, and to test its toxicity in the adrenalectomized rat. The material was precipitated and purified from protein extracted from the cell by hot acid or sonic oscillation and was found to be the R antigen (10). The identification was based upon reaction with Type 28 anti-R serum, and resistance to digestion by trypsin. Traces of C antigen were removed by the procedure given above. Table V shows that neither of the R antigen preparations caused death of the rats.

Effect of Component 4.—A nucleoprotein has been isolated from several strains of Group A streptococci and has been characterized by physical and

chemical means (11). The preparations do not react in the precipitin test with either homologous C or M antiserum. Preparations from two type 19 strains were tested in the adrenalectomized rat. No toxic effect was evident in either case and none of the animals died (Table V).

Effect of Components from B and D Streptococci.—Protein components were extracted from strains of Groups B and D streptococci and purified by the methods used for isolation of M protein. Each was tested in the adrenalectomized rat at a level of 1 mg./100 gm. rat. Neither material was toxic (Table V).

Effect of C Substance.—In order to determine whether components other than M protein were toxic to the adrenalectomized rat, heat-killed cells were

TABLE VI

Effect of C Substance from Group A Streptococci on the Adrenalectomized Rat

Streptococcal			Method of extraction	Quantity injected (per 100 gm. rat)	D/T
Group	Type	Strain			
				mg	
A	3	T3m+	Sonic oscillation	1	6/6
A	14	S23m+	" "	1	6/6
A	14	S23m−	" "	1	6/6
A	19	N19	" "	1	2/3
B	—	Lewis	" "	1	3/3
C	—	H861	" "	1	3/3
D	—	D10	" "	1	3/3
A	19	N19	HCl at 100°C.	1	0/3
A	19	N19	" " "	2	0/3
A	19	N19	" " "	4	0/2
A	14	S23m+	" " "	1	0/3

treated with trypsin or *Streptomyces albus* enzyme. The latter is also known to destroy the M protein (12). The results indicated that such cells still possessed toxicity to the adrenalectomized rat. Attention was then directed to the group-specific C substance.

Table VI compares the effect upon the adrenalectomized rat of C substance released from the Group A streptococcal cell by 0.1 N HCl at 100°C. and that released from the cell by sonic oscillation. It can be seen that the material released by oscillation is practically 100 per cent fatal in all groups of animals tested at a level of 1 mg. per 100 gm. rat. Approximately one-half the animals died at a level of 500 μg./100 gm. rat. It should be noted that the m^+ and m^- strains of S23 produced equally potent C substance as judged by their action on the adrenalectomized rat.

In contrast to the material released by oscillation, the material extracted by hot acid, although of equal serological activity, was not toxic to the adrenalec-

tomized rat. Quantities as high as 4 mg./100 gm. rat were not effective. No signs of distress were noted in the animals.

In addition, it can be seen in Table VI that the sonic-extracted C substance from streptococcal Groups B, C, and D was toxic to the adrenalectomized rat. The dose was the same as the material extracted from Group A cells.

It was of considerable interest to determine whether the toxicity of the C preparations was neutralized by group-specific antisera. The lyophilized material equivalent to 1 ml. Group A antiserum was dissolved in 1 ml. of an 0.85 per cent saline solution containing 4 mg. C antigen. This quantity of antiserum was found by step-wise addition to be sufficient to neutralize all the C substance present. The mixture was held at 37°C. for 3 hours to allow antigen-antibody combination to occur. Table IV shows that each of the C substances prepared from Group A strains were no longer toxic to the adrenalectomized rat after incubation with Group A antiserum. No deaths and no abnormal symptoms were noted in the animals.

In accord with these results it was found that the toxicity to the adrenalectomized rat of the C preparations from Group A streptococci was not removed following incubation with either Group B, C, or D specific antisera (Table IV). The toxic effect thus appears to be a property of the C substance, and neutralization will occur only with homologous antiserum.

C antigen is not destroyed by the action of trypsin, therefore the toxicity of the above preparations should remain unchanged after digestion by the enzyme. Preparations S23m$^+$ and S23m$^-$ were digested at 37°C. for 3 hours (2 mg. antigen in 0.06 M phosphate pH 7.5 containing 50 μg. crystalle trypsin). No reduction in the toxicity of either preparation following digestion was seen.

DISCUSSION

It is well known that the toxicity of dead bacterial cells is much greater to the adrenalectomized rat than to the normal rat.

This was first demonstrated in 1924 with staphylococcus and a non-hemolytic streptococcus (13). Recent evidence has indicated that the toxic effect in part is related to the carbohydrate content of the material under test. Dextran and ovomucoid (14) and the endotoxin of *Escherichia coli* (15) are toxic to the adrenalectomized rat. The former contain large quantities of acetylglucosamine, mannose, galactose, or glucose (14). *E. coli* endotoxin is a lipopolysaccharide (16). Also, normal animals (rabbits) have been shown to develop a connective tissue lesion upon injection of a Group A streptococcal extract containing rhamnose, hexosamine, and protein (17). In addition, the streptococcal endotoxin which can cause inflammation in rabbits is considered to be similar to that of *E. coli* endotoxin (18). The toxic effect of the C preparations tested in this study is very likely related to their content of carbohydrate. Two mg. of either of the sugars present in the cell wall of the Group A streptococcus (rhamnose and *N*-acetylglucosamine) was found to have no toxic effect.

The results presented here show that the M protein of the Group A streptococcus also is toxic to the adrenalectomized rat. This is the first indication that a

bacterial protein material could be toxic under these conditions and is the first toxic property of the M protein to be described. No information is available as to the relationship of toxicity to chemical composition.

The toxic activity of the preparations used in these studies is considered to be a property of the M protein and C substance rather than that of contaminating materials. All preparations were highly purified as judged by serological activity. The toxic activity of each was neutralized by homologous group and type-specific antiserum but not by heterologous antiserum. In addition, it has been shown that other cellular components such as the R antigen and a nucleoprotein of the Group A streptococcus, as well as proteins prepared from Groups B and D streptococci were all non-toxic to the adrenalectomized rat. Staphylococcal coagulase,[1] (coagulase-positive in 0.01 μg. and tested at 5 mg./100 gm. adrenalectomized rat) also possessed no toxic activity to the animal.

The present results demonstrate that the toxic activity of the C and M preparations can be separated from the serological activity of the same material. Serological properties are stable to 0.1 N HCl at 100°C. whereas the toxic properties are not. These two activities are possibly functions of different parts of the molecule. The sites of the serological and toxic functions however, are likely to be close together. Rabbit antibody to the whole cell is shown to neutralize both functions. The antibody is "complete" in this respect.

M protein extracted from the cell by acid is a poor antigen (7). It would be of interest to determine to what extent the toxicity of the M protein preparations used in this study is associated with ability to stimulate the formation of antibodies.

The C and M preparations used in this study have been tested in the normal rat. Ten times the quantity of either material required to kill the adrenalectomized rat had no effect in the normal animal. Larger quantities of material have not been tested. It did not seem worth while to employ the limited quantities of purified material for this type of experiment. It is clear that the streptococcal C and M preparations are comparatively non-toxic to the normal rat. In contrast, *E. coli* endotoxin which is toxic to the adrenalectomized rat at a dose of 0.25 mg. is also toxic to the normal rat at a dose of 2 to 3 mg. (15).

SUMMARY

Heat-killed cells of Group A streptococci caused death of the adrenalectomized rat. While the adrenalectomized rat readily succumbed to intraperitoneal infection with living cells, death was due primarily to toxicity. The normal rat was highly resistant under either condition.

For studies on the toxic materials, the cells of numerous serological types of group A streptococci, and of a Group B and a Group D streptococcus, were

[1] Kindly supplied by Dr. Richard D. Ekstedt of this department.

extracted with 0.1 N HCl at 100°C. or by sonic oscillation. The extracts, containing macromolecular components, were subjected to chemical fractionation and purification.

C substance and M protein of Group A streptococci released from the cell by sonic oscillation were toxic to the adrenalectomized rat in quantities of 1 mg./100 gm. rat. Death usually occurred within 2 hours. On the other hand, C substance and M protein released from the cell with HCl at 100°C. were relatively non-toxic to the adrenalectomized rat. The sonic-extracted C substance of streptococcal Groups B, C, and D was also toxic.

The toxic property of the C and M preparations was neutralized *in vitro* in each case by group and type-specific rabbit antiserum. Heterologous antiserum was without effect. Adrenalectomized rats which received homologous antiserum 18 hours before challenge were also resistant to the toxicity of the C and M preparations.

Trypsin destroyed the toxic effect of the M protein preparations and was without effect on the toxicity of the C substance.

The R antigen and a nucleoprotein component of Group A streptococci, preparations of protein from Groups B and D streptococci, and coagulase from *Staphylococcus aureus* were all found to be essentially non-toxicic for the adrenalectomized rat. Large quantities of peptone, crystalline albumin, and rabbit serum were also without effect.

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