STUDIES ON THE ANTIGENIC COMPOSITION OF GROUP A HEMOLYTIC STREPTOCOCCI

I. Effects of Proteolytic Enzymes on Streptococcal Cells

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Exposure of suspensions of group A hemolytic streptococci to the action of trypsin has recently been employed by several investigators in an attempt to eliminate spontaneous agglutination (1–4). The behavior of cultures so treated is similar in many ways to the reactions characteristic of glossy variants (5, 6): both suspensions are diffuse and are extremely susceptible to agglutination by non-type-specific antibodies; both may fail to agglutinate in homologous type-specific antisera. It has been previously shown that glossy strains differ from matt in that they contain little or no M substance (6). It seemed possible, therefore, that the changes induced by enzyme treatment might be the result of the destruction of the type-specific M component of the cell.

The specific antigenic composition of the cell has been shown to be referable to at least two antigens designated as M and T (7), respectively. The protein, M, is specific for each individual type, and is related to virulence of the organism and to protection with type-specific antiserum. The M substance is present in matt variants, but degraded glossy strains contain little or none of this antigen. Matt strains are usually agglutinated type-specifically by M antibodies of homologous type, whereas glossy strains fail to agglutinate in anti-M serum. A precipitin technique is available for measuring the presence of M antigen in cell-free extracts (8, 9). The T antigen may have the same type distribution as the M antigen, but the same or related T substances are in some instances found in more than one type (10, 11). As far as is now known, the T antigen is unrelated to virulence, and T antibodies have no protective value. Most matt, as well as glossy, variants contain T antigens and are agglutinated by T antibodies. The chemical nature of the T substance is still unknown; and methods for extracting and demonstrating this antigen are not available at the present time.

The experiments reported in this paper are chiefly concerned with the effect of proteolytic enzymes on the M and T antigens of the streptococcal cell.

Materials and Method

Strains of Hemolytic Streptococci Employed.—The strains studied were group A hemolytic streptococci of known serological type and comprised a series of cultures,

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some of which were obtained directly from Griffith and others isolated in this country were also classified by the same investigator by means of slide agglutination (12). The type classification was confirmed by us in some instances by mouse protection tests. The particular strains studied were selected because they induced the formation in rabbits of type-specific M antibodies in high titer (Table I).

Preparation of M Extract.—Crude extracts, prepared by heating streptococci with HCl as previously described (8) contained both group-specific carbohydrate, C, and type-specific protein, M. The type-specific M substance in these bacterial extracts was separated from the group-specific C substance by fractional precipitation with ethyl alcohol. Usually two precipitations resulted in complete separation of M and C substances. The precipitate containing the M substance was dissolved in physiological NaCl solution.

TABLE I

Group A Strains Employed

Type	Strain	Original source and designation
1	T 1	Griffith, strain SF 130/2
3	D 58	Colebrook, strain Richards
6	S 43	Dochez, Avery, and Lancefield, strain S 43
14	C 630	Uncertain
17	J 17 E	Coburn, strain R 9
18	J 17 C	" " R 20
23	T 23	Griffith, " Barts 102
26	T 26	" " Withers
28	T 28	" " Small
29	D 23	" " Coggins
30	D 24	" " Quinn

Preparation of the Group-Specific Carbohydrate, C.—The crude extract containing both M and C substances, was used in some experiments to test antisera for their content of C antibody. Usually, however, a stock solution of C polysaccharide, prepared according to Fuller's formamide method, was employed (13).

The methods for immunization of rabbits, absorption of sera, and for agglutination, precipitin, and protection tests were similar to those previously described (7).

Preparation of Enzyme-Treated Suspensions.—

1. Enzyme Solutions.—10 mg. of crystalline chymotrypsin[1] were dissolved in 7 cc. of phosphate buffer, pH 8, and added to the bacterial sediment obtained by centrifuging ¾ liter of culture. Fairchild's trypsin and pepsin were used in final concentration of 1 per cent, pH 8 and pH 3, respectively. The enzyme solutions were sterilized by filtration through Chamberland filters.

2. Cultures.—The growth from 1½ liters of 18 hour broth culture was collected by centrifugation, suspended in physiological salt solution, and then divided into two

[1] The crystalline chymotrypsin was supplied through the kindness of Dr. M. Kunitz.

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equal portions: one was treated with enzyme and the other served as an untreated control. Enzyme solution was added so that the final volume in each case was 17 cc. The untreated control suspension was made up to the same volume either with heat-inactivated enzyme or with phosphate buffer.

The enzyme-treated suspensions and controls were incubated at 37°C. for 2 hours. Samples were removed to test the effect of the enzyme on the bacteria and the remainder in each case was heated for one hour in a water bath at 56°C. in order to kill the organisms and to inactivate the enzyme. The heat-killed cultures were diluted with saline to one-quarter the volume of the original broth culture and used for immunizing rabbits.

EXPERIMENTAL

Cultures of streptococci, types 17, 18, 26, 28, 29, and 30, were each treated with chymotrypsin. Cultures of types 1, 3, 6, 14, and 23 were treated with trypsin. A strain of type 28 was treated with pepsin. The antigenic composition, viability, and virulence of enzyme-treated and untreated streptococci were then compared.

In testing the effect of different enzymes, it was found that whenever an enzyme acted upon the M substance in cell-free extracts, it was also capable of destroying this antigen in the intact cell. The selection of enzymes suitable for particular experiments was facilitated by this means.

A. Effects of Proteolytic Enzymes on the Antigenic Components of These Streptococci.—

1. M Precipitin Tests on Extracts Prepared from Enzyme-Treated Streptococci.—At the end of the period of treatment with enzyme, a part of each suspension, equivalent to the growth from 200 cc. of original broth culture, was removed and heated in a boiling water bath for 10 minutes to inactivate the enzyme. The centrifuged bacterial sediments were then extracted to prepare crude M solutions. Using homologous and heterologous type-specific anti-M sera, these extracts were tested by means of the precipitin reaction for the presence of M substance. Table II shows an example, using a type 1 strain, of the effect of proteolytic enzymes on the M substance of living group A hemolytic streptococci. The extract of this culture, which had been treated with active enzyme, showed no trace of M substance although the extract from the control showed the usual well marked type-specific reaction; this indicated that trypsin had destroyed or inactivated the M antigen in the living bacterial cell. A similar result was obtained when heat-killed cultures were treated with proteolytic enzyme. A summary of M precipitin reactions of all the cultures used in these experiments except type 28 is recorded in Table VI and shows that in every instance the M substance was destroyed.

The action of proteolytic enzymes on living bacteria was also tested by growing the microorganisms in the presence of active enzyme. Streptococci were grown 16 hours in broth to which 0.05 per cent trypsin or chymotrypsin had

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TABLE II

Effect of Proteolytic Enzymes on M Substance of Group A Hemolytic Streptococci
M-Precipitin Reactions

M extracts from cultures of	Type 1 anti-M serum	Heterologous type anti-M sera
1. Type 1, enzyme-treated	−	−
2. " 1, grown in broth + enzyme	−	−
3. " 1 control, untreated	+++	−
4. Subculture of (1) in plain broth	+++	−
5. " " (2) " " "	+++	−
6. " " (3) " " "	+++	−

The technique used for the precipitin reaction was that previously described (7).
− indicates a negative reaction in all dilutions tested.
The degree of reaction is indicated on a + to ++++ scale. The reading recorded is a summary of the results of a series of dilutions.

TABLE III

Effect of Proteolytic Enzymes on Agglutinability of Group A Hemolytic Streptococci

Cultures used in agglutination reaction	Antisera*											
	Type 6 anti-M				Type 6 anti-T				Heterologous type (non-type-specific)			
Strain S 43, type 6												
1. Untreated	++++	++++	+++	++	++++	++++	+++	++	±	±	−	−
2. Trypsin-treated, 2 hrs.	−	−	−	−	++++	++++	+++	+++	++++	++++	++++	+++
3. " 48 "	−	−	−	−	+	+	±	±	++++	++++	++++	++++

See footnotes, Table II.
* Final serum dilutions 1:40, 160, 640, 2560.

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been added. In spite of the fact that good growth was obtained, extracts of the sedimented bacteria failed to yield M substance. Control cultures grown in broth containing heat-inactivated enzyme, on the other hand, produced the usual amount of M antigen. When enzyme-treated cultures were transferred to broth containing no enzyme, as shown in Table II (4–6), M substance was produced. The ability of the streptococcus to form M antigen was therefore not destroyed.

2. Agglutination Reactions of Enzyme-Treated Streptococci.—Both living and dead cultures of numerous types were exposed to enzymes and then tested to ascertain whether they retained their ability to agglutinate in type-specific anti-M sera and in homologous anti-T sera. Changes in their susceptibility to non-type-specific agglutinins were also tested. A protocol of one of these experiments is recorded in Table III. Type 6 antisera were absorbed as previously described (7) so that they contained only those antibodies indicated in the table. The heterologous type serum employed was known from previous tests to have a high content of non-type-specific antibodies. The untreated type 6 culture used for the agglutination reactions contained both M and T antigens and agglutinated well in the homologous anti-M and anti-T sera but only slightly in the heterologous serum. After 2 hours' exposure to trypsin, however, this culture was no longer agglutinated by M antibodies but was still agglutinated by homologous T antibodies. A marked agglutination in non-type-specific antiserum was observed with the culture at this time and persisted when T agglutination, as well as M agglutination, had disappeared after 48 hours' treatment with trypsin. The susceptibility of enzyme-treated streptococci to non-type-specific agglutinins was a constant and prominent finding.

3. Antigenicity of Enzyme-Treated Streptococci.—Although no M substance was demonstrable in enzyme-treated streptococci or in extracts prepared from them, it seemed possible that the injection of such suspensions into rabbits might, nevertheless, evoke an anti-M immune response. Rabbits were therefore immunized with suspensions of bacteria which were heat-killed immediately after exposure to proteolytic enzymes. The sera of these animals were tested for antibody content by several techniques.

(*a*) *Precipitins.*—All sera were tested for the following precipitins: type-specific anti-M, group-specific anti-C, and non-type-specific precipitins active against heterologous M extracts. None of the sera obtained at different intervals from the rabbits immunized with enzyme-treated cultures gave reactions with either the homologous or heterologous M extracts. In the corresponding sera of the control animals, however, type-specific anti-M precipitins were present. In most instances the content of antibodies for the group-specific carbohydrate, C, was greater in sera of rabbits injected with suspensions heat-killed after exposure to proteolytic enzyme than in those serving as controls. These findings are illustrated in Table IV.

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TABLE IV

Failure of Rabbits Immunized with Enzyme-Treated Cultures to Develop Anti-M Precipitins and Protective Antibodies

Immunological reactions	Rabbits immunized with Type 14 streptococci												Control No serum
	Enzyme treated culture						Untreated culture						
	Rabbit R51-53			Rabbit R51-54			Rabbit R51-51			Rabbit R51-52			
	Length of immunization, *days*												
	30	45	60	30	45	60	30	45	60	30	45	60	
Precipitin test with													
Type 14 M extract	–	–	–	–	–	–	++	++	++	++	++	++	–
" 6 " "	–	–	–	–	–	–	–	–	–	–	–	–	–
" 19 " "	–	–	–	–	–	–	–	–	–	–	–	–	–
"Purified" C (group antigen)	+++	++	++	++	+	–	++	+	+	+	–	–	–
Passive protection in mice against strain C 630/26/2 *cc.*													
10^{-1}	D1	D1	D1	D1	D1	D1	S	D1	D1	S	D1	D1	D1
10^{-2}	D1	D1	D1	D1	D1	D1	S	D1	D1	S	D1	D1	D1
10^{-3}	D1	D1	D1	D1	D1	D1	S	D1	D1	S	D1	S	D1
10^{-4}	D1	D1	D1	D1	D1	D1	S	S	S	S	S	D9	D1
10^{-5}	D1	D1	D1	S	D1	D1	D3	S	D7	S	S	S	D1
10^{-6}	D1	D1	S	D3	D1	D1	S	S	S	S	S	S	D1
10^{-7}	S	D1	D1	S	D1	D4	S	S	S	S	S	S	D2
10^{-8}	S	S	D3	S	D1	S	S	S	S	S	S	S	D7

– indicates a negative reaction in all dilutions tested.

The degree of reaction is indicated on a + to ++++ scale. The reading recorded is a summary of the results of a series of dilutions.

S indicates survival.

D indicates death on day indicated by numeral.

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(*b*) *Protective Antibodies.*—The presence of protective antibodies in some of these sera is also shown in Table IV. In contrast to the control animals, rabbits immunized with enzyme-treated cultures which had been heat-killed after exposure to the enzyme failed to develop protective antibodies.

(*c*) *Agglutinins.*—All sera were tested for agglutinins, as well as precipitins. Table V shows that rabbits immunized with enzyme-treated cultures developed anti-T but no anti-M agglutinins. A protocol illustrating these results, with a type 26 strain as an example, is presented. Since matt strains contain both

TABLE V

Presence of Anti-T Agglutinins in the Sera of Rabbits Immunized with Enzyme-Treated Cultures

Immune rabbit sera, type 26	Type 26 variants used for agglutination reactions	
	Glossy (T antigen)	Matt (M and T antigens)
A. *Rabbit R51-94* immunized with trypsin-treated matt culture, type 26 (anti-T)		
1. Unabsorbed	++++	++++
2. Absorbed with strain of heterologous type	++++	++++
3. Absorbed with type 26 glossy strain	−	−
4. Absorbed with type 26 matt strain	−	−
B. *Rabbit R51-91* immunized with untreated matt culture, type 26 (anti-M and anti-T)		
1. Unabsorbed	++++	++++
2. Absorbed with strain of heterologous type	++++	++++
3. Absorbed with type 26 glossy strain	−	++++
4. Absorbed with type 26 matt strain	−	−

The reading recorded in each case is a summary of the reactions obtained in antiserum in the following dilutions: 1:20, 40, 80, 160, 320, 640, 1280.

The technique used for agglutination reactions was that previously described (7).

M and T antigens, matt as well as glossy variants were agglutinated by the sera of these animals. Absorption with either matt or glossy cultures removed all anti-T agglutinins, and these sera then failed to agglutinate both matt and glossy strains. Absorption with streptococci of heterologous types had no effect on the T antibodies.

Sera of rabbits immunized with untreated cultures contained both anti-M and anti-T agglutinins. Absorption with a glossy variant removed the anti-T agglutinins, but left the M antibodies. Matt strains were, therefore, still agglutinated by a serum absorbed in this way. Absorption with a matt variant removed both anti-T and anti-M agglutinins.

The foregoing serological and immunological tests indicate that the M substance is destroyed in the living or dead streptococcal cell by short ex-

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posure to the action of proteolytic enzymes, but the T substance remains active unless the bacteria are subjected to more prolonged treatment.

B. Effect of Proteolytic Enzyme on Viability of Streptococci.—The survival of streptococci after treatment with proteolytic enzymes was compared with the

TABLE VI

Reactions of Bacteria from Control and Enzyme-Treated Cultures

Type		1		6		14		17		18		23		26		29		30	
Treatment of culture		Enzyme-treated	Control	Enzyme-treated	Control	Enzyme-treated	Control	Enzyme-treated	Control	Enzyme-treated	Control	Enzyme-treated	Control	Enzyme-treated	Control	Enzyme-treated	Control	Enzyme-treated	Control
M precipitin reactions with homologous type anti-M serum		−	++	−	+++	−	+++	−	+++	−	+++	−	++	−	++	−	++	−	+++
Colony counts	*cc.*																		
Dilution plates	10^{-6}	145	53	32	15	110	42	497	312	930	532	23	6	633	645	274	325	91	90
from each preparation	10^{-7}	12	10	6	1	10	1	35	30	119	58	3	0	83	63	23	43	12	4
	10^{-8}	5	1	1	0	1	0	5	2	9	3	1	0	8	9	5	2	1	0
Virulence tests	*cc*																		
	10^{-1}	D1	D1	D1	D1	D1	D1					D8	D2						
	10^{-2}	D1	D1	D2	S	D1	D2					D2	D2						
	10^{-3}	S	D2	D2	S	D2	D2					D1	D1						
In mice (31 hr. cultures)	10^{-4}	D2	S	S	S	D3	S					D8	S						
	10^{-5}	S	D3	S	S	D4	S					S	S						
	10^{-6}	S	D3	S	S	S	S					S	S						
	10^{-7}	S	S	S	S	S	S					S	S						
	10^{-8}	S	S	S	S	S	S					S	S						

The type 3 experiment was performed at a different time. Colony counts and virulence tests were not made.

Virulence tests were performed by the technique previously described.

S indicates survival.

D indicates death on day indicated by numeral.

— indicates a negative reaction in all dilutions tested.

The degree of reaction is indicated on a + to ++++ scale. The reading recorded is a summary of the results of a series of dilutions.

survival of bacteria in control cultures by plating dilutions of the respective cultures. The counts of colonies which grew in these plates (Table VI) indicate that the enzyme had no bactericidal effect. In several instances, preparations of enzyme-treated bacteria showed a higher colony count than the untreated controls. The significance of this increase is not known but may possibly have been due to breaking of the chains of streptococci into smaller units.

C. Effect of Proteolytic Enzymes on Virulence of Streptococci.—The virulence of enzyme-treated streptococci was compared with that of control untreated

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cultures of the following types: 1, 6, 14, and 23. 12 to 16 hour cultures of these strains usually killed mice in dilutions of 10^{-6} to 10^{-8} cc. The experiment recorded in Table VI was performed with the same dilutions prepared for colony counts. No difference in virulence was observed between control cultures, which contained M substance, and enzyme-treated cultures, which were devoid of M substance at the time of injection, although the virulence of all four cultures was lower than usual. This decreased virulence was probably related to

TABLE VII

Effect of Trypsin Treatment on Virulence of Group A Hemolytic Streptococci
Virulence Tests in Mice with 22 Hour Cultures of Type 1 Strains

Dose	Virulent matt strain		Avirulent glossy strain
	Trypsin-treated	Control	Untreated
Injected *cc.*			
10^{-1}	D1	D1	D1
10^{-2}	D1	D1	S
10^{-3}	D1	D1	S
10^{-4}	D1	D1	S
10^{-5}	D1	D1	S
10^{-6}	D2	D1	S
10^{-7}	S	D2	
10^{-8}	D3	S	
		Colony counts	
Plated *cc.*			
10^{-6}	561	411	314
10^{-7}	52	34	28
10^{-8}	7	5	7

Virulence tests were performed by the technique previously described.
S indicates survival.
D indicates death on day indicated by numeral.

the fact that the cultures were 31 hours old at the time of injection and had been subjected to washing and other manipulations.

In other experiments where the time required for the various procedures was materially shorter, the virulence of the enzyme-treated and control cultures remained uniformly high. The results of one such experiment are shown in Table VII. A glossy variant is included for comparison. In this experiment the cultures were 22 hours old when inoculated into mice. The high virulence of this strain, even though at the time of injection the culture contained no M substance, was probably due to the ability of streptococci to form M antigen while growing in the peritoneal cavity of the mouse. In spite of the fact that neither the glossy variant nor the enzyme-treated culture contained M antigen

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when injected, the contrast in virulence was striking. The glossy strain was unable to produce M substance in any environment. Subcultures from the heart's blood of a mouse dying following the intraperitoneal injection of a large dose of this culture showed only negligible amounts of M substance. On

TABLE VIII

Summary of Reactions of a Type 28 Strain after Treatment with Proteolytic Enzymes

Effect of enzyme treatment		Type 28 culture (strain T 28)			
		Pepsin-treated	Control (pH 2.5)	Chymotrypsin-treated	Control (pH 8.0)
	cc.				
Colony counts					
Dilution plates (from cultures	10^{-6}	Not done		274	287
used to immunize rabbits)	10^{-7}	(Incubation at pH 2.5		33	26
	10^{-8}	killed cultures)		4	4
M-precipitin reactions (with extracts of suspensions used to immunize rabbits) in type 28 anti-M serum		−	+++	+++	+++
Agglutinability (of suspensions used to immunize rabbits)					
1. In type-specific type 28 anti-M serum		−	++++	++++	++++
2. In heterologous serum containing non-type-specific antibodies		++++	−	−	−
Antigenicity					
* Antibodies formed in rabbits (after immunization with the cultures tested above)					
1. Anti-M precipitins		−	++++	++++	++++
2. Anti-M agglutinins		−	++++	++++	++++
3. Non-type-specific agglutinins		++++	+++	+	+
4. Group specific anti-C precipitins		++++	++	±	±

See footnotes, Tables II and III.

* No T antibodies were formed in response to immunization of rabbits with this strain (14).

the contrary, subcultures made in a similar manner from mice dying after the injection of trypsin-treated streptococci produced M substance in quantities comparable to the original culture. These findings indicate that, although the M antigen was destroyed by the enzyme, the streptococci still retained their capacity to produce this substance. The results are in accord with previous observations that virulence for mice is associated with the production of M substance.

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D. Susceptibility of Type 28 M Antigen to Pepsin but Not to Trypsin or Chymotrypsin.—In the course of these studies it was found that the M antigen of type 28 (and possibly type 44 also) differed from the M substances of all other types studied in being destroyed by pepsin but not by trypsin or chymotrypsin. The M substances of the other types were equally susceptible to the action of all three of these enzymes.

The difference in the effect of pepsin and chymotrypsin on type 28 M antigen is illustrated in Table VIII. The experiments summarized in this table show that the action of pepsin on type 28 streptococci is comparable to that of chymotrypsin, trypsin, or pepsin on cultures of other types. The table also shows that chymotrypsin had no effect on type 28.

DISCUSSION

In studying the effect of certain proteolytic enzymes on group A hemolytic streptococci, it was observed that the action of these enzymes removed the M substance from both living and dead cells. With the exception of type 28 and possibly of provisional type 44, trypsin, chymotrypsin, and pepsin were equally effective. The M substance of type 28 was unaffected by trypsin and chymotrypsin but was destroyed by pepsin.

The proteolytic enzymes were found to have no bactericidal effect. Furthermore, streptococci exposed to enzymes did not lose the ability to produce M substance when subcultured in broth containing no proteolytic enzymes, nor was their virulence for mice diminished. Since it is doubtful whether enzymes present in the external environment ever penetrate into the living cells, it may be assumed that M substance is located at the periphery of the bacterial cell. These results are similar to those obtained by Avery and Dubos with a specific enzyme, derived from a soil bacillus, which acts on the capsular polysaccharide of Type III Pneumococcus (15).

The complexity of the antigenic composition of the streptococcal cell is well known. A new method for analyzing some of the antigenic components of these microorganisms is provided by exposing them to proteolytic enzymes for varying periods. Thus a relatively short exposure is sufficient to destroy the M substance. The T antigen is more resistant but also disappears after long contact. The antigens of unknown nature responsible for the development of non-type-specific agglutinins appear to be unaffected by proteolytic enzymes. The group-specific carbohydrate, C, a polysaccharide, also remains intact.

It seems probable that the destruction of the M substance is fairly complete, since the injection into rabbits of suspensions prepared from cultures heat-killed immediately after exposure to the enzyme fails to stimulate the production of M antibodies. Usually the production of anti-T agglutinins, however, is increased and also the formation of group-specific anti-C precipitins and of non-type-specific agglutinins. Thus this antigenic response is similar to that

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obtained with glossy strains. Enzyme-treated suspensions also resemble glossy variants in that both are extremely susceptible to agglutination by non-type-specific agglutinins.

No evidence was obtained to suggest that T antibodies induced by immunization with enzyme-treated streptococci differ from those formed in animals immunized with untreated cultures. This method of obtaining anti-T agglutinating serum without M antibodies is reliable and more widely applicable than immunizing with glossy strains. Its use has proved helpful in analyzing the antigenic composition of group A hemolytic streptococci, and in explaining some of the difficulties encountered in the serological differentiation of these bacteria.

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SUMMARY

1. Proteolytic enzymes destroy the type-specific M antigen of group A hemolytic streptococci not only when the M substance is contained in cell-free extracts but also when it is a component of the living cell.

2. The injection of enzyme-treated cultures into rabbits fails to induce the formation of M antibodies, but does result in the production of T antibodies and, therefore, provides a method of preparing anti-T sera free from M antibodies.

3. Exposure to these enzymes does not kill the bacteria. Virulence and the ability to form M substance are restored on subculture and animal inoculation.

4. The study of the effect of proteolytic enzymes on group A hemolytic streptococci provides new techniques for analyzing the antigenic composition of these microorganisms.

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