THE PROPERTIES OF T ANTIGENS EXTRACTED FROM GROUP A HEMOLYTIC STREPTOCOCCI

By REBECCA C. LANCEFIELD, PH.D., AND VINCENT P. DOLE, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

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Two separable and distinct antigens, the so called M and T substances, have been found to participate in the serological reactions of group A hemolytic streptococci (1–7). The current serological classification into specific types is based upon the M antigens without regard to the presence in the same cells of T antigens, which often give rise to cross-reactions (3, 6–8). Earlier failure to recognize this fact often confused the interpretation of the cross-agglutination frequently observed between strains of different specific types.

In previous work the various combinations of M and T antigens which occur in group A streptococci of different types have been studied (3, 6, 7). Although a distinct M antigen occurs in each type,[1] the same or a closely related T antigen may be common to more than one type. Several series of types with related T antigens have been studied in detail (7). In other instances cross-agglutination reactions between different types have been observed which are probably also referable to T antigens, although the antigenic relationships have not been analyzed. Thus, in general, M and T antigens vary independently between different types but they sometimes parallel each other in distribution, for example, in types 2 and 6.[2]

In strains of any given type, a T antigen may be either present or absent (3, 5). This antigen is usually a very stable antigenic component and, under laboratory conditions, its loss has been observed in only a single instance (3). At the present time it is not known whether the strains which on first isolation are devoid of T substance once contained this component and then lost it, or whether these particular streptococci always lacked this antigen.

[1] Originally types 10 and 12 were classified separately (9). Subsequent work has shown that strains designated in this way contain distinct T antigens but a serologically identical M antigen (6). In view of this finding all such strains are now classified in this laboratory as type 12 in order to conform to the scheme used for other types. The designation of type 12 was chosen instead of type 10 because except for one endemic focus in Rumania (10), the latter type has been uncommon.

[2] Occasional cross-agglutination reactions between group A strains and strains of other serological groups have been observed. The most marked of these occur between group A, type 2, and group C, Griffith's original type 21 strains. This cross-reaction has been observed in this and in other laboratories (personal communications from Dr. E. W. Todd and Dr. S. D. Elliott).

The presence or absence of an M antigen is, on the other hand, a highly variable factor in streptococci. Strains easily lose this antigen. This loss is usually accompanied by a change in colony form from the matt to the glossy variant which is devoid of the M component (11, 12). The T antigen, if present in the original matt variant, also occurs in the glossy derivative, even though the M antigen has disappeared (1). By serial animal passage glossy variants may be reverted to the matt form; and the matt variant then regains the ability to synthesize the same type-specific M substance which characterized the parent strain (12).

M antigens are easily obtained in soluble form (13), but T antigens have not hitherto been obtained in extracts from streptococci; hence they were recognized only from an analysis of the agglutination reactions ascribed to them. In the present report a method is described for the isolation of the T antigen present in type 1 strains of group A hemolytic streptococci. Although this substance resembles the M antigen, in that both are proteins, the following experiments show that the T antigen is qualitatively different in certain of its chemical, physical, and serological properties.

Methods

Serological Techniques.—Agglutinin and precipitin techniques have been previously described (1). In the present experiments it was found that the results of precipitin tests performed with the capillary tube method were essentially the same as those obtained in parallel titrations carried out in the usual manner with larger volumes of extract and antiserum in small test tubes. Accordingly, the more convenient and economical capillary tube technique was used in most precipitin tests (14).

Preparation of Antisera.—Anterisera containing T antibodies were prepared as previously described (4), by immunizing rabbits with heat-killed streptococci in which the M antigen had previously been destroyed by 2 hour digestion of the cells with pepsin or trypsin; hence no M antibodies were formed. Antibodies other than the homologous anti-T precipitins and agglutinins were removed by selective absorption of the immune sera with heterologous strains known to contain no T antigens serologically related to that present in the cells used for immunization. Antisera so prepared are for convenience referred to as anti-T sera. In some cases the preliminary digestion of the bacteria used for immunization was omitted, and the M antibodies were removed by absorption with a strain of homologous type which contained M but lacked T antigen.

EXPERIMENTAL

While investigating the effects of proteolytic enzymes on intact living or heat-killed streptococci, we found that digestion of the organisms for 2 hours or

less with pepsin or trypsin sufficed to destroy the M antigen, but apparently did not effect the T antigen (4). When, however, the digestion was prolonged for 3 or more hours, the T content of the cells was reduced, as evidenced by progressive loss and final disappearance of agglutinability of the streptococci in antisera containing T antibodies. At the same time, the T antigen was found in the supernatant fluid, as shown by precipitin reactions. It was thus apparent that the loss of T antigen from the cells during prolonged digestion was due to liberation of the substance without subsequent destruction. This behavior is in contrast to that of the M antigen which is destroyed by the enzyme after release from the bacterial cell. It was, in fact, later found that the T antigen in soluble form was resistant to prolonged digestion with pepsin, trypsin, chymotrypsin, ribonuclease, and the papain-like proteinase (15) elaborated by certain strains of group A streptococci. These phenomena are illustrated by the following experiments.

Release of T Precipitinogen into the Supernatant Fluid during Digestion of Streptococcal Cells.—A number of different group A strains were digested with pepsin. These included type 1 matt variants, containing both M and T antigens or M antigen alone, and glossy derivatives of certain strains, containing T antigen alone. In addition, the type 3 strain (C203 matt), which was previously shown to contain T antigen characteristic of type 1 as well as the M and T antigens of type 3, was included, and as heterologous controls, two glossy variants (strains T3 and T17).

The washed bacterial cells from 1.5 liters of 18 hour broth cultures were suspended in 15 cc. of 0.85 per cent NaCl solution to which sufficient N/1 HCl was added to adjust the reaction to pH 2.5. To one-half of the suspension was added an equal volume of active 2 per cent commercial pepsin, pH 2.5. To the other half, which served as a control, was added an equal volume of the same pepsin solution previously inactivated by heat. After 18 hours' incubation at 37°C., the suspensions were neutralized with N/1 NaOH and the bacteria removed by centrifugation. The supernatant fluids were tested with type 1 antiserum prepared and absorbed, as described under "Methods," so that it contained only T antibodies.

The antigenic composition of the strains employed is given in Table I, together with the results of the precipitin reactions with type 1 antiserum and control normal rabbit serum. All the strains known from previous agglutination studies to contain type 1 T antigen yielded, on proteolytic digestion, a soluble precipitinogen which was reactive in type 1 antisera containing T antibodies; none of the other strains gave a positive reaction. It is noteworthy that a parallelism existed between the agglutinin reactions of the intact cells, which had been attributed to T antigens, and the precipitin reactions of the T substance obtained from the same cells by proteolytic digestion.

Duplicate cultures were similarly digested with trypsin at pH 8. In precipitin tests the supernatant fluids of the digestion mixtures gave the same results as those obtained in the experiments in which pepsin was employed.

The bacterial cells during treatment with pepsin or trypsin gradually lost their agglutinability by T antibodies, as is shown in the following experiment.

Disappearance of T Agglutinogen from Streptococcal Cells during Prolonged Peptic Digestion.—

An 18 hour broth culture of the type 1 matt strain (T1/79/4), which contained M and T antigens, was digested with pepsin as described above. At intervals during incubation at 37°C., 1 cc. samples were removed, neutralized with N/1 NaOH, and centrifuged. After resuspension in fresh broth, the bacteria were tested for agglutination by an antiserum which was known to contain only T antibodies; therefore any agglutination observed could be attributed to persistence of the T antigen in the bacterial cells.

TABLE I

Release of T Precipitinogen from the Bacterial Cells by Proteolytic Digestion of Group A Streptococci

Serum	Precipitin reactions with extracts prepared by peptic digestion of group A streptococcal cells*						
	Type 1 strains				Type 3 strains		Type 17 strain
	T1 matt (M1, T1)	T1 glossy (T1)	S118 (M1, T1)	S.F. 130 (M1)	C203 (M3, T3, T1)	T3 glossy (T3)	(T15–17–19–23–30–47‡
Type 1 anti-T serum...	+++	+++	+++	−	+++	−	−
Normal rabbit serum...	−	−	−	−	−	−	−

The antigenic composition of each strain with respect to M and T antigens is given in parenthesis.

For convenience, glossy strains are recorded as belonging to the type from which the variant was derived. The type 1 "anti-T" serum was antistreptococcal rabbit serum specifically absorbed so that it contained only T antibodies.

* Appropriate tests showed that none of these extracts contained M antigens, due to their destruction by proteolytic digestion.

‡ This designation indicates that strain T17 glossy contains the T antigen common to strains of types 15, 17, 19, 23, 30, and 47.

Control suspensions treated with heated pepsin gave negative precipitin reactions.

In all tables positive results of precipitin reactions are recorded on a + to ++++ scale; negative results are indicated by −.

As indicated in Table II, the agglutinability was unchanged after 1½ hours' digestion, although after 21 hours it was substantially reduced, and after 72 hours was completely lost.

Pepsin and trypsin both gave similar results in the digestion of other strains. With either enzyme some variation occurred in the rate of loss of T antigen, dependent on the enzyme concentration and on the particular strain employed. In general, digestion of streptococci for 2 hours did not demonstrably affect T agglutination of these cells, although after 18 hours agglutinability was greatly reduced or lost.

Occurrence of the Same or Closely Related T Antigens in Different Specific Types as Determined by Precipitin Reactions.—In order to study further the parallelism between the agglutinin reactions of intact streptococci referable to T antigens and the precipitin reactions of the T substance obtained from these cells, 12 strains, representing 12 different specific types, were selected and, on the basis of previously determined agglutination reactions, were divided into two series. Each series was characterized by strains which shared in common serologically related T antigens but individually possessed an M antigen distinctive for the specific type to which each belonged. The specific types represented by the strains used in this experiment are shown in Table III.

TABLE II

Progressive Loss of T Agglutinogen from the Bacterial Cells during Prolonged Peptic Digestion of Group A Streptococci

Peptic digestion of a type 1 matt culture														
Sample tested		Agglutination reactions												
hrs.	Preparation	Type 1 anti-T serum							Normal rabbit serum					
		1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
1½	Digested	+++	++++	++++	+++	+++	+++	+++	–	–	–	–	–	–	–
	Control	+++	++++	++++	++++	++++	++++	+++	–	–	–	–	–	–	–
21	Digested	+++	++	+	+	+	+	+	–	–	–	–	–	–	–
	Control	++++	++++	++++	++++	++++	++++	+++	–	–	–	–	–	–	–
72	Digested	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Control	++++	++++	++++	++++	++++	+++	+++	–	–	–	–	–	–	–

The type 1 anti-T serum was antistreptococcal rabbit serum specifically absorbed so that it contained only T antibodies. Final serum dilutions of immune and normal rabbit sera are indicated in the table.

In all tables positive results of agglutinin reactions are recorded on a + to ++++ scale; negative results are indicated by –.

Broth cultures of each of the 12 strains were grown in 3 liter lots for 48 hours at 22°C. The bacterial cells of each strain were separated by centrifugation and resuspended in 5 cc. of a buffered solution, pH 8, containing 1 per cent commercial trypsin. Digestion was carried out for 4 hours at 37°C., after which the supernatant fluids containing the T substance were removed by centrifugation and concentrated by precipitation at pH 3.5. The precipitates were dissolved in 1 cc. of phosphate buffer, pH 7, and tested by the capillary pipette precipitin technique against antistreptococcal rabbit sera from which all except T antibodies had been removed by absorption methods previously described.

The findings recorded in Table III show that cross-precipitin reactions occurred within each series of cultures representing 6 distinct and serologically specific types. Furthermore, these cross-precipitin reactions occurred only

among those strains belonging to types previously demonstrated by agglutination to possess common or closely related T antigens. The specificity of these precipitin reactions was further shown by the failure of T extracts prepared from strains in one series to react with T antisera against strains belonging to the other. Similar results were obtained when the same extracts were tested by the complement-fixation reaction.

Effect of Streptococcal Proteinase upon the T Antigen.—Since the proteinase elaborated by group A streptococci under certain conditions had been shown by

TABLE III

Occurrence of the Same or Closely Related T Antigens in Different Specific Types as Determined by Precipitin Reactions

		Precipitin reactions						
Anti-T serum prepared with strain from:		Extracts from strains of types:						
		15	17	19	23	30	47	46
	Types							
Types with related T antigen	15	++	++	+±	+++	+++	++++	−
	17	+++	+++	+++	+++	+++	++++	−
	19	++	+±	+±	+++	+++	+++	−
	23	+++	+±	+	+++	++±	+++	−
	30	++	++	++	+++	+++	+++	−
	47	+++	++	+++	+++	+++	++++	−
Types with related T antigen	4	−	−	−	−	−	−	+++
	24	−	−	−	−	−	−	+
	26	−	−	−	−	−	−	+++
	28	−	−	−	−	−	−	++
	29	−	−	−	−	−	−	+++
	46	−	−	−	−	−	−	+++

The absorbed antistreptococcal rabbit sera contained only T antibodies except the type 47 antiserum which also contained M antibodies.

Extracts were prepared by proteolytic digestion of streptococcal cells.

Suitable control tests with normal rabbit serum were all negative.

Elliott (15) to destroy one of the protein antigens, the M substance, formed by the same cells, the possibility was considered that this enzyme might have a similar action on the T antigens of these organisms. In the preceding experiment it was found necessary to concentrate the cellular extracts obtained by the proteolytic digestion in order to obtain the T antigens in sufficient amount for precipitin studies. This was true of all T antigens except that derived from type 1 strains. The type 1 antigen was easily obtained in much larger quantity by the same method of digestion which failed to yield large amounts from strains of other types. It is not yet known why smaller yields are obtained from cer-

tain strains, but it was thought possible that the streptococcal proteinase might destroy the T antigen either during growth of the streptococci or after the release of T substance from the cell body. Except for type 1, there was not much T antigen obtained irrespective of whether the organisms were grown in the presence or absence of active proteinase. None of the procedures known to retard or inhibit the action of the streptococcal proteinase affected the yield of T antigen: Growth of the cultures at 22°C., a temperature at which the activity of the enzyme is greatly reduced, or growth in media unsuitable for proteinase production, such as neopeptone broth or broth containing iodoacetic acid in a concentration inhibitory to the streptococcal proteinase, had no influence on the amount of T antigen recovered in extracts. Moreover, strains which normally produce little or no proteinase were no better sources of extractable T antigen than strains which produced large amounts of proteinase.

On the other hand, procedures which insured the presence of active proteinase during growth of the streptococci also failed to influence the yield of T substance. As already noted, the use of strains which produced large amounts of proteinase, as well as the use of special media which favored the production of the enzyme, or even the addition of active proteinase to the culture medium were without effect on the T antigen obtained.

In all these experiments with the active proteinase, the concentration of the enzyme was lower than that found optimal for release of T antigen from the streptococcal cell, and this accounted for the fact that streptococci grown in the presence of active proteinase still contained T antigen.

Other Methods of Extracting T Antigen.—Attempts were made to improve the yield of T antigen by finding better means of extraction. None, however, has met with success. Disintegration of the cells in a ball mill prior to digestion did not increase the yield. No appreciable amount of antigen was extracted with diethylene glycol, pyridine, high concentrations of salt, desoxycholate, or by heating under various conditions of pH, time, and temperature. The rapid loss of T activity that occurs in heated solutions explains the ineffectiveness of the last method, as well as the complete absence of T antigen from M extracts as ordinarily prepared by heating cell suspensions at pH 2 in a boiling water bath.

By only one method other than proteolytic digestion has any detectable amount of T antigen been extracted. During repeated daily extractions of bacterial suspensions at pH 2.0 in a 37°C. water bath (a technique employed for extracting the M substance in its most antigenic form), it was observed that the T antigen began to appear in the supernatant fluid after 3 days, with a maximum yield at about the 7th or 8th day. The amount obtained, however, was less than that obtained by proteolytic digestion of the streptococcal cells.

Chemical and Physical Properties of T Antigen.—The T antigen used for chemical study was extracted from a type 1 strain which contained both M

and T antigens, or from the glossy derivative, which contained T but no M antigen. The T substance was separated from the cells by peptic digestion and purified by subsequent reprecipitations at pH 3.0–3.5, followed by further enzymatic digestion and electrophoretic separation (16). These steps are outlined in Table IV.

Although trypsin had been found to be as efficient as pepsin in releasing the antigen from the bacterial cells, the latter was used for most preparations as it is commercially available in purer form, and has the further advantage of low pH optimum which reduced the danger of bacterial contamination; furthermore, it was possible to inactivate the enzyme for serological reactions by readjusting

TABLE IV

Preparation of T Antigen

1. Bacteria from 64 liters of culture separated in Sharples centrifuge, resuspended in 500 cc. 0.85 per cent NaCl.
2. pH adjusted to 2.5 with N/1 HCl; 10 gm. commercial pepsin + 2 cc. chloroform added. Incubated 6–12 hrs. at 37°C. Precipitate formed.
3. N/1 NaOH added to bring pH to 7.0. Precipitate redissolved. Bacteria removed by centrifugation and filtration through Chamberland L3 filter. Stored in ice box.
4. Pool made from 10 lots prepared as above.
5. Active material precipitated at pH 3.5 by addition of N/1 HCl; inactive supernate discarded after centrifugation. Precipitate redissolved at pH 5.0 or pH 7.0. After 4 such precipitations, finally redissolved in 100 cc. phosphate buffer, pH 6.9.
6. 2 mg. crystalline ribonuclease + 0.5 cc. chloroform added; incubated 6 days at 37°C. Following this, 1.5 mg. crystalline trypsin added; incubated 4 days. 2.0 mg. crystalline chymotrypsin + 0.5 mg. trypsin added; incubated 1 day.
7. Active material precipitated twice at pH 3.5.
8. Dialyzed 18 hrs. against phosphate buffer (pH 7.0, μ 0.1), then separated electrophoretically in five consecutive runs. Separated material dialyzed, then frozen, and dried; total yield 55 mg. This material, pooled and analyzed electrophoretically at pH 7.0, was homogeneous by this test.

the pH to 7.0–7.8. Maximal release of T antigen was obtained in about 6 hours. However, since there was no apparent loss of T activity on prolongation of the digestion for as much as 4 days, overnight digestion was sometimes employed as a matter of convenience.

After digestion the mixture was neutralized to redissolve the antigen, because in unpurified preparations the antigen was precipitated at the acid reaction employed for the digestion of the cells, pH 2.5. The bacterial cells were then removed by centrifugation. An ultraviolet absorption curve[3] of the material at this stage indicated the presence of nucleic acid as well as of protein (Fig. 1). Since it had previously been found that the serological activity of T antigens

[3] Dr. S. Granick and Dr. A. E. Mirsky very kindly measured the absorption spectra on the Beckman spectrophotometer.

was unaffected by ribonuclease, the solution was digested 3 days with this crystalline enzyme (2 mg. for an estimated 500 to 1,000 mg. of extract material in 100 cc. volume). A second absorption curve showed reduction in the quantity of nucleic acid present.

Following concentration by reprecipitation at pH 3.5, a portion of the material was taken up as a 2 per cent solution in phosphate buffer (pH 7.00, μ 0.1) and dialyzed 48 hours against 2 liters of this buffer for electrophoresis. The electrophoretic pattern (Fig. 2) showed that the T protein comprised about 20 per cent of the total material, and moved as a well defined peak of the lowest

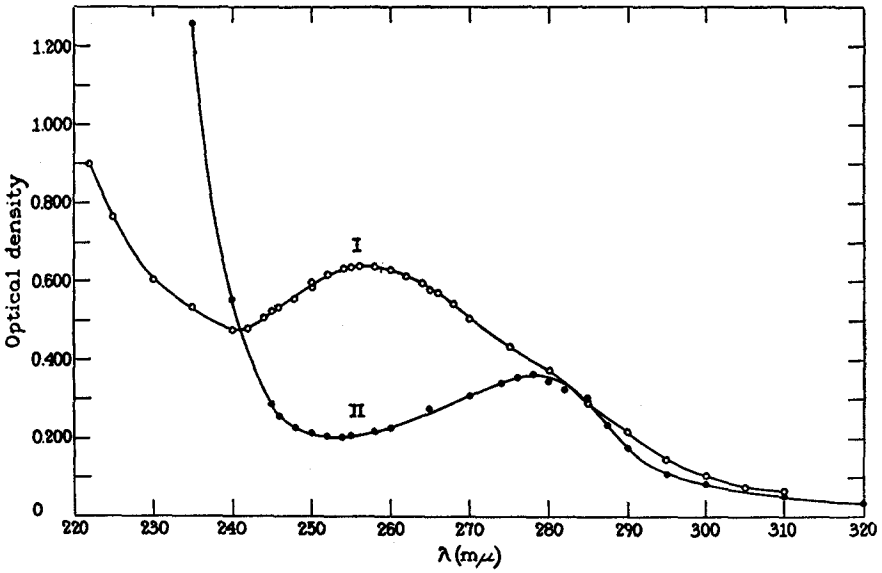

FIG. 1. Ultraviolet absorption spectra of T antigen preparation. Crude concentrate indicated by curve with open circle, the electrophoretic pattern of which is shown in Fig. 2. Electrophoretically separated fraction indicated by curve with closed circle; pattern shown in Fig. 3.

mobility. Closely adjacent to it was a larger peak, including about 75 per cent of the total, while moving in the lead was a small peak indicating a fast moving fraction probably composed of nucleic acid. Small portions, separated for identification, indicated not only that the slow moving peak carried the total T activity but also that it was free from a yellowish color and some serologically cross reacting material present in the whole extract. Electrophoretic separation of a useful quantity was, however, not practical at this stage, since the proximity of the central peak containing colored materials and inert proteins prevented the isolation of more than a small fraction of the T antigen after full resolution of the mixture.

Accordingly, other lots were subjected to further enzymatic digestion with ribonuclease, trypsin, and chymotrypsin. The most successful preparation (Table IV), a purified extract from the organisms grown in a total of 640 liters of medium, showed in electrophoresis that the protein adjacent to the peak representing the T substance had been satisfactorily removed. Repeated electrophoretic separations were made to recover T substance from this preparation: the unpurified main portion after each run was reconcentrated by precipitation at pH 3.5, then again dialyzed against the pH 7.00, μ 0.1 phosphate buffer.

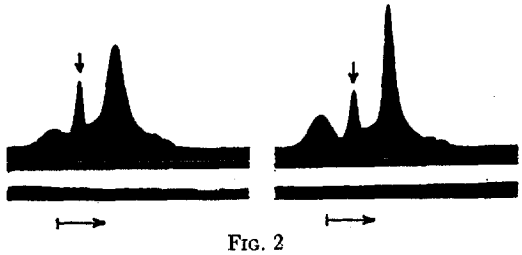

FIG. 2

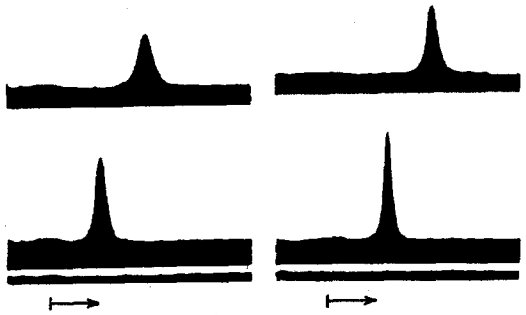

FIG. 3

FIGS. 2 (upper pair) and 3 (lower pair). Electrophoretic patterns of T antigen: original crude concentrate in upper pair and electrophoretically separated material in lower patterns. Vertical arrows indicate T antigen in the crude preparation. Horizontal arrows show the initial boundary and the direction of migration. Descending patterns are on the left; ascending, on the right.

A total of 5 such repeated separations yielded 55 mg. of purified T antigen. This purified material was electrophoretically homogeneous (Fig. 3) at pH 7.00, as would be expected from the method of preparation.

Ultraviolet absorption of the purified material, measured on the Beckman spectrophotometer,[3] showed a typical protein curve with a maximum at 378 mμ and a minimum at 254 mμ (Fig. 1).

Microchemical analysis of a portion after dialysis against distilled water gave N = 14.25 per cent, C = 50.11 per cent, H = 7.42 per cent, and ash = 1.90 per cent.

The isoelectric point was found to be about pH 4.50 by electrophoretic analyses of an unpurified preparation at various pH's shown in Fig. 4. With the cruder preparations it was not possible to obtain values for the mobility at pH's below 4.5 owing to the flocculation that occurred, apparently as a complex with nucleic acid. The purified T, obtained by electrophoretic separation, was soluble at least to the extent of a 2 per cent solution at pH 3.5, but was not available in sufficient quantity for these measurements.

Ultracentrifugal analysis, made on the purified material by Dr. Alexandre Rothen, showed that the material was heterogeneous. The sedimenting boundary was very broad but definitely separated into two major components. The faster one had a sedimentation constant of $S_{\text{medium}}^{25.4^\circ} \sim 6.4 \times 10^{-13}$. The slower one had a sedimentation constant of $S_{\text{medium}}^{25.4^\circ} \sim 5.1 \times 10^{-13}$. The

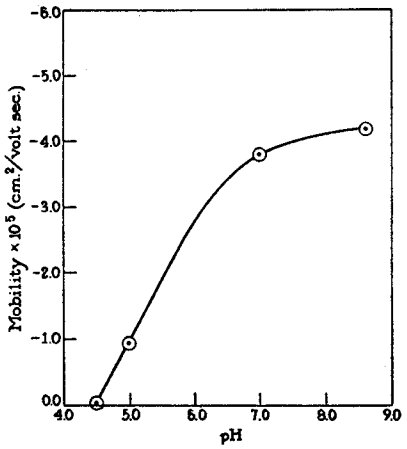

FIG. 4. Mobility of T antigen as a function of pH

experimental conditions were: speed, 57,000 R.P.M.; temperature, $t = 26.1^\circ$ before run, $t = 24.7^\circ$ after run; cell = 12 mm. depth.

Comparison of T and M Antigens with Respect to Solubility of Their Picrates.— The T antigen was tested for solubility of its picrate in 70 per cent acetone and in 70 per cent alcohol containing hydrochloric acid, according to the method used in the preparation of insulin (17, 18), in order to compare it with the corresponding solubility of the M antigen picrate. The latter antigen has been found to belong to the class of alcohol-soluble proteins by means of these reactions.

Accordingly, the picrate was prepared by adding picric acid crystals, in excess of the amount necessary for saturation, to a crude solution of T antigen, and then grinding the mixture in a mortar. The precipitate was separated from

the supernatant fluid after centrifugation and treated with 70 per cent acetone. All the T activity was found in the insoluble picrate, and none in the acetone-soluble fraction tested after removal of the acetone by vacuum distillation. This was in striking contrast to the behavior of M antigen treated in the same way. M antigen, like insulin, forms a picrate which is soluble in 70 per cent acetone. After removal of the acetone by vacuum distillation and complete reprecipitation of the M antigen by the addition of picric acid crystals, the picrate of M antigen, washed in ether to remove excess picric acid, is then soluble in 70 per cent alcohol containing hydrochloric acid. The M antigen is recovered by precipitation in cold absolute acetone. The final product is soluble in water and physiological salt solution, as well as in 80 per cent alcohol and in 80 per cent acetic acid.

In order to confirm this difference between these two classes of streptococcal antigens, a solution of T antigen of type 1 was mixed with an M antigen of another type; the two antigens were then separated by the difference in solubility of their picrates as outlined above. Although some of the M antigen remained with the T antigen in the original picrate, which was insoluble in 70 per cent acetone, most of it was recoverable in the acetone-soluble fraction by repeated extraction with 70 per cent acetone. The T antigen, on the contrary, remained throughout repeated extraction in the acetone-insoluble fraction.

Effect of Ultraviolet Radiation.—Solutions of T antigen in saline were exposed to ultraviolet radiation. In some experiments the purest preparation obtained by electrophoretic separation was employed; in other experiments crude extracts containing numerous cellular constituents in addition to the T substance were used. The other proteins used for comparison and as controls were M antigen from the same type 1 strain of group A hemolytic streptococcus from which T antigen was obtained, crystalline ovalbumin, and crystalline albumin from horse serum.[4]

The solutions were irradiated with the spiral quartz-mercury resonance radiation lamp described by Havens, Watson, Green, Lavin, and Smadel (19). The lamp operated at 30 milliamperes and 15,000 volts, obtained by a transformer from 110 volt alternating current. Eighty-five per cent of the energy radiated by the lamp is emitted as the 2537 Å mercury line. Under these conditions the lamp remains at about room temperature. The solutions were contained in quartz test tubes of approximately 6 mm. diameter, which were tightly closed by a tinfoil-covered rubber stopper. The tube was suspended in the center of the spiral. Samples of the irradiated solutions were removed at various intervals for serological tests, as shown in Table V.

The purified T solution lost its serological activity after 30 minutes' irradiation under these conditions; the crystalline ovalbumin, after 15 minutes; and the crystalline albumin from horse serum, which was the only one of these proteins

[4] We wish to thank Dr. George Lavin for his cordial cooperation in irradiating these solutions. We are indebted to Dr. Merrill W. Chase for supplying the crystalline horse serum albumin and its antiserum and also for the antiovalbumin serum.

TABLE V

Effect of Ultraviolet Radiation on Protein Antigens
Protective Action of Yeast Nucleic Acid

Antigen	Time of exposure to ultraviolet radiation (2537Å)	Precipitin reactions with homologous antiserum									
		Antigen dilutions									
	min.	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
1. Purified T* containing no demonstrable nucleic acid	0	++++	++++	++++	++++	+++	++±	++	+	+	−
	5	++±	+++	+++	++±	++	+±	±	−	−	−
	20	+±	++	++	+	±	−	−	−		
	30	−	−	−	−	−	−	−	−		
2. Crude T* containing approximately 5 mg./cc. ribonucleic acid	0	++++	++++	+++	+++	++	++	+±			
	90	++++	++++	+++	++±	++	++	+±			
3. Purified T after the addition of yeast nucleic acid 5 mg./cc.	0	++++	++++	++++	+++	++	+	±	−	−	−
	60	++++	++++	+++	+++	++	+	±	−	−	−
	120	++++	++++	+++	++	+±	+	±	−	−	−
4. Partially purified M*, 10 mg./cc. containing approximately 0.1 mg./cc. ribonucleic acid	0	++++	++++	++++	++++	++++	++++	+++	++±	++	+
	60	+±	+±	+±	+±	++	+±	±	−	−	−
	120	−	−	−	±	±	±	−	−	−	−
5. The same preparation of M, after the addition of yeast nucleic acid, 5 mg./cc.	0	++++	++++	++++	+++	+++	++±	++±	+±	+±	±
	60	++++	++++	++++	++±	++	+±	+±	+	±	−
	120	++±	++±	++	++	+±	+±	+	±	±	−
6. Crystalline ovalbumin, ‡ 1 mg./cc.	0	++++	++++	++++	+++	+++	++	+	±	−	−
	5	++++	+++±	+++	++	+					
	10	++±	++	±	−	−					
	15	±	−	−	−	−					
7. The same preparation of ovalbumin after the addition of yeast nucleic acid, 5 mg./cc.	0	++++	++++	++++	+++	+++	++	+			
	60	++++	++++	++++	+++	++±	+±	±			
	120	++++	++++	+++±	++	+±	±	−			
8. Crystalline albumin from horse serum 1 mg./cc.	0	++++	++++	++++	++++	++	+±	+	±	−	
	5	±	±	−	−	−	−	−	−	−	
9. The same preparation of horse serum albumin after the addition of yeast nucleic acid 5 mg./cc.	0	++++	++++	++++	++++	+++	++	+±	±	−	
	5	++++	++++	++++	+++	+++	++	+	−	−	
	60	++++	++++	++++	+++	++	+±	+±	±	−	
	120	++	++±	++±	+±	+	±	−	−	−	

* Prepared from group A, type 1 hemolytic streptococcus strain T1. The solution of T antigen used in (3) was of slightly different concentration from that used in (1) because not enough of the purified material was available for accurate weighing when it was necessary to prepare a fresh solution.

‡ Dr. Merrill W. Chase, who kindly supplied the anti-ovalbumin serum, has informed us that the anti-ovalbumin serum has no reactivity with denatured ovalbumin.

Controls with normal rabbit serum and with heterologous antisera were all negative.

coagulated by this exposure to ultraviolet radiation, lost its serological activity in 5 minutes or less. The partially purified M solution, on the other hand, still had some activity at the end of 1 hour, but none at the end of 2 hours. The crude solution of T, containing many impurities, was unaffected even after 90 minutes' irradiation.

Since crude preparations of T antigen were known to be rich in nucleic acid, the absorption maximum of which is at 2600 Å, titrations of the ribonucleic acid content of the various solutions were made, comparing the crude T and M solutions with dilutions of known strength of pure yeast nucleic acid. Bial's reagent was used for these estimations. The amount of ribonucleic acid in the crude T preparation, which was resistant to the effects of ultraviolet radiation, corresponded to about 5 mg./cc. of pure yeast nucleic acid. The partially purified M solution contained approximately 0.1 mg./cc. of ribonucleic acid. With diphenylamine reagent the tests for desoxyribonucleic acid were negative. Accordingly, a neutral solution of yeast nucleic acid in a concentration of 5 mg./cc. was added to the purified T solution which, with Bial's and with diphenylamine reagents respectively, had previously given negative reactions for ribonucleic acid and for desoxyribonucleic acid. In the same way, yeast nucleic acid was added to the other protein solutions. These preparations were then exposed to ultraviolet radiation in the same manner as before. The results are given in Table V.

The M and T antigens, as well as both crystalline albumins tested, withstood ultraviolet radiation for approximately 2 hours in the presence of 5 mg./cc. yeast nucleic acid. In an experiment not shown in the table, a mixture of the purified T antigen and the partially purified M antigen, containing 0.1 mg./cc. ribonucleic acid, was irradiated. Neither antigen was completely inactivated serologically in 1 hour, although both were destroyed in 2 hours. The low content of nucleic acid in the M preparation probably accounts for the fact that only partial protection from the effects of the ultraviolet radiation was observed. That the resistance of the crude T solution to ultraviolet radiation could have been due at least in part to the 5 mg./cc. ribonucleic acid which it contained as an impurity, was indicated by the high degree of protection conferred by the addition of a comparable amount of yeast nucleic acid to purified antigens, which alone were readily destroyed by ultraviolet radiation. The difference between the ultraviolet absorption of the crude and the purified T antigen preparations is shown by the absorption curves of Fig. 1.

As a result of the study of these effects of ultraviolet radiation, it appears that both the T antigen and the M antigen behave like other better known crystalline proteins when exposed to radiation of wavelength 2537Å.

Effect of Heating.—The effect of heat on the isolated T substance was of especial interest because of the known heat susceptibility of this substance when contained in the bacterial cell (3). Elliott has shown that T antigen is destroyed by heating bacterial suspensions at about pH 7.0 for 30 minutes in a boiling water bath; and our experiments have yielded the same result, particularly if the pH was maintained at either side of the neutral point, for example, pH 2.5 or pH 9.0.[5] In order to compare this with the effect of heat on T substance in solution, a concentrated, relatively crude preparation of T antigen was heated in solutions of different hydrogen ion concentrations and various ionic strengths.

[5] Some of these experiments are from unpublished data obtained in this laboratory by Dr. W. A. Stewart.

Buffers were prepared with ionic strengths ranging from 0.1 to 3.0 and with pH values of 3.5, 4.5, 7.0, and 8.5. Acetate buffers were used for the pH 3.5 and 4.5 series, phosphate for the pH 7.0 series, and diethylbarbiturate for the pH 8.5 series. A type 1 T preparation, partially purified by numerous reprecipitations at pH 3.0 and by digestion with crystalline ribonuclease, had been frozen and dried after dialysis against distilled water. A solution containing 20 mg./cc. of this material was prepared in distilled water and 0.1 cc. added to 0.9 cc. of each of the buffers and to 0.85 per cent NaCl solution as control for each series run on separate days. All tubes except the control tubes were immersed in boiling water for 10 minutes and then cooled immediately. At pH 7.0 and 8.5 the solutions at μ 1.0 were slightly opalescent after heating, and those at μ 2.0 and μ 3.0 had considerable precipitate which did not go back into solution after dialysis. All other solutions in these series remained clear. Precipitation occurred throughout the series at pH 3.5 and at pH 4.5, but this was reversed when the solutions were neutralized.

The results of the precipitin reactions with these heated and control solutions show that the T antigen was destroyed at pH 3.5 and 4.5 at every ionic strength tested (Table VI). At pH 7.0 and 8.5, T activity was little if at all impaired by this amount of heating unless the salt concentration was raised above μ 1.0.

Antigenicity of T in Solution.—Certain other properties of the T antigen in solution were studied. Since it is known from previous work (1, 3, 7) that T is one of the most active streptococcal antigens when intact bacteria are injected into rabbits, it was of interest to test the antigenicity of T in solution.

Two rabbits were immunized with a preparation of T antigen derived from a type 1 strain and purified by repeated precipitations at pH 3.5 and finally by prolonged digestion with ribonuclease. Electrophoretic analysis of a sample of this lot (Fig. 2) indicated that about 20 per cent of it represented the active material.

These animals each received intravenously a total of 55 mg. divided into 10 doses. Five milligram doses were given on each of the first 3 days; and anti-T agglutinins and precipitins were found in the serum of both rabbits 1 week after the first injection. Despite an increase in anti-T agglutinin and precipitin titers, which resulted from injecting 7 additional doses at intervals between the 7th and the 36th days, no antibodies capable of passively protecting mice against experimental intraperitoneal infection with type 1 streptococci were found in the serum obtained on the 45th day. The T antigen showed no toxicity for rabbits in the dosage employed.

Although insufficient purified material, separated electrophoretically, was available for immunization experiments with measured doses, a single rabbit was given a small amount of a very dilute solution. After 2 intravenous injections, this rabbit's serum contained anti-T agglutinins and precipitins, but no mouse-protective antibodies. No evidence of other antibody formation was observed in the sera of any of these rabbits, although they were tested for the presence of C antibodies, M antibodies, and non-type-specific agglutinins and precipitins. That the less purified antigen injected into the first 2 rabbits did not give rise to cross-reacting antibodies was probably due to the small dosage and relatively short period of immunization.

TABLE VI

Effect of Heating Solutions of T Antigen

Precipitin reactions with type 1 anti-T serum and heated and control solutions of T antigen

Ionic strength	pH 3.5				pH 4.5				pH 7.0					pH 8.5							
	1:4	1:8	1:16	1:32	1:4	1:8	1:16	1:32	1:2	1:4	1:8	1:16	1:32	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128
0.1	+	−	−	−	±	−	−	−	++++	+++	+++	++	±	++++	+++	+++	++	+±	+	±	±
0.2	−	−	−	−	±	−	−	−	++++	++++	++++	++	±	++++	++++	+++	++	+	±	±	±
0.5	−	−	−	−	±	−	−	−	++++	+++	++	+	±	++++	++++	+++	++	+	+	±	±
1.0	±	±	−	−	±	−	−	−	++	++	++	±	±	++++	+++	+++	++	+±	+	±	±
2.0	−	−	−	−	±	−	−	−	++	±	−	−	−	++++	+++	++	+	±	±	−	−
3.0	−	−	−	−	±	−	−	−	±	−	−	−	−	+++	++	++	+	±	±	±	−
Unheated control	+++	++	+±	±	+++	++	++	+	++++	++++	++++	±	±	++++	++++	++++	+++	++	+±	±	±

Absorbed antistreptococcal rabbit serum contained only T antibodies.

Stock T antigen was heated for 10 minutes at 100°C. in the buffer solutions of ionic strength and pH shown in the table. The dilutions of these preparations are indicated at the head of each column.

Control tests with normal rabbit serum were all negative.

TABLE VII

Specific Inhibition of T Agglutination by Solutions of T Antigens

T extract used for inhibition		Type 1 anti-T serum						Type 17 anti-T serum					
		Dilutions						Dilutions					
		1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:80	1:160	1:320	1:640	1:1,280	1:2,560
Type	*cc.*	*Agglutination reactions of type 1 culture*						*Agglutination reactions of type 17 culture*					
1	0.4	−	−	−	−	−	−	++++	++++	++++	++++	+	
	0.1	−	−	−	−	−	−	++++	++++	++++	++++	±	±
17	0.4	++++	++++	+++	+	+	±	−	−	−	−	−	−
	0.1	++++	++++	+++	++	+	+	++++	++++	+	−	−	−
None	−	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++

Extracts prepared by proteolytic digestion of types 1 and 17 streptococcal cells were mixed with absorbed antistreptococcal rabbit serum which contained only T antibodies. The mixtures were refrigerated for 16 hours, then incubated at 37°C. for 1 hour, after which the bacterial suspensions were added. Readings were made after 2 hours at 56°C. Controls with normal serum were negative.

Inhibition of T Agglutination by Solutions of T Antigen—Another interesting property of T antigens in soluble form is their ability to inhibit agglutination reactions due to T antibodies. Only crude extracts were available for inhibition tests, although some of these extracts were concentrated by precipitation of the active material with acid or with alcohol.

The method employed for the inhibition test was the following: Serial dilutions were prepared of antisera containing T antibodies against types 1 and 17 respectively. Normal rabbit serum was similarly diluted. To 0.1 cc. amounts of each dilution was added the T antigen, previously prepared by proteolytic digestion of type 1 and type 17 bacterial cells, in the following amounts: 0.4 cc. was added to one complete set of serial dilutions of each antiserum, and 0.1 cc. of antigen to another complete set. The volumes in all tubes were made up to 0.5 cc. with physiological NaCl solution; and another set of serum dilutions was included without added T antigen. The mixtures were given a preliminary period of 16 hours in the ice box and 1 hour's incubation at 37°C. to allow T antigen, if present in the solution, the opportunity of combining with T agglutinins in the antiserum. These mixtures were then tested for the presence of free antibody by adding to each tube 0.5 cc. of the appropriate type 1 or type 17 streptococci suspended in broth. The mixtures were incubated again for 2 hours at 56°C., then readings of agglutination were made.

The results, recorded in Table VII, show specific inhibition of agglutination by the homologous T extract in each case and only slight inhibition by T of the heterologous type. Although T antigen and antibody combined in the preliminary incubation period before the homologous streptococci were added, no visible precipitate was formed, because the serum dilution was too great. The type 17 extract inhibited agglutination of type 17 organisms with antiserum less completely than was the case with type 1 extract, and the homologous type 1 system. This observation is in general accord with the experience that the T antigen is obtained in extracts in much greater quantity from type 1 strains than from strains of other types.

The agglutination by T antibodies of strains of types 1, 3, 6, 14, and 17 was specifically inhibited in each case by homologous T solutions, which combined with these antibodies and thus prevented them from reacting with the homologous streptococci added later. On the other hand, similar proteolytic digests prepared from several strains known to lack T antigen were found incapable of inhibiting the T agglutination of T-containing strains of the respective types. In all cases the specificity of a solution of T antigen was the same as that of the T agglutinogen of the streptococci extracted. M extracts prepared by heating the streptococci with HCl did not inhibit T agglutination, a finding in agreement with the known heat sensitivity of T antigen.

In general, the inhibition reactions constituted a specific, though cumbersome, serological test for T substance in spite of the fact that only crude digests were employed. The purified material was not used in inhibition experiments on account of the large amounts required to perform inhibition tests.

DISCUSSION

The analysis of the antigenic composition of group A hemolytic streptococci by investigating the chemical nature and immunological properties of the cellular components has been the subject of continued study. Two general approaches had been employed in the investigations leading to the present work (20). Analysis of the antigens occurring in streptococci of diverse serological types and in variants of individual strains was accomplished at first by studying the antibodies evoked in animals immunized with these organisms. As a result of interpretations based on agglutination reactions and on agglutinin absorption, as well as on active and passive protection tests in mice, certain deductions were made with regard to the antigenic composition of group A hemolytic streptococci.

The second approach involved the isolation and chemical identification of the antigens actually responsible for the different serological and immunological reactions of the intact cells. This method of study has resulted in a better understanding of the interrelationships between the various cellular components than was possible either by serological analysis, using whole microorganisms, or by study of other biological characteristics of these cells. For example, in the group differentiation of hemolytic streptococci formerly based upon differences in source, pathogenicity for different animal species, cultural properties, and biochemical tests, the distinctions between groups were not always clear. Moreover, the use of serological methods in which the intact organisms were employed in agglutination reactions gave no indication of differentiation of streptococci into groups because antibodies to the so called C polysaccharide, on which substance it is now known that group specificity depends, do not ordinarily cause agglutination of streptococci. The explanation is unknown for the fact that streptococci do not agglutinate when mixed with homologous group antiserum even though under these conditions they absorb group antibodies specifically. It was not until group-specific C polysaccharides were isolated in soluble form that a satisfactory method of grouping streptococci was devised (21). By means of precipitin tests with these antigens more consistent results were obtained than with those based solely on biochemical and cultural reactions (22).

Similarly, chemical study of the antigens of group A hemolytic streptococci has resulted in identification of the type-specific protein, the so called M antigen, and has shown that this substance is not only responsible for the type-specific reactions but is also one of the factors intimately associated with virulence of the streptococcal cell (11–13). The specific antibody to this substance proved to be largely responsible for active and passive immunity (1, 23, 24). The probable localization of the M substance at the periphery of the cell was shown by the ease with which it could be removed from the living streptococcus by proteolytic digestion without otherwise injuring the microorganisms or

impairing their ability to elaborate more M antigen when growth was resumed in an environment free of active enzyme (4).

In early studies of the T antigen, it was thought that the M and T antigens had much the same distribution in the different serological types but that certain cross-agglutination reactions between types might, as in the case of strain C203, be due to an unusual distribution of T antigens (1, 2). As a result of this earlier work, both M and T antigens were considered to play a rôle in determining type-specific reactions. Later serological studies showed that the parallel occurrence of these two antigens has so far been observed in only two types. The distribution more frequently found was that a single or related T antigens occurred in several types, each of which was characterized by an individual M antigen (3, 7). The isolation of T antigen in soluble form from suitable strains of numerous types, and the investigation of certain of its chemical, physical, and serological properties has provided confirmation of the earlier serological analyses, which showed the presence of this substance in the streptococcal cell and its distribution in various types.

As shown in the present report, the T substance is separable from the cell by proteolytic digestion. The antigen thus isolated from a type 1 strain has been further purified by enzymatic digestion and electrophoretic separation, and characterized as a relatively pure protein, apparently free of nucleic acid. Although in this form the T substance is highly antigenic and serologically reactive and has the same specificity as when present in the intact cell, it is not known whether it exists in the cell originally as a protein free of nucleic acid or combined as a nucleoprotein. The chemical evidence available with regard to the M antigen suggests that it also may be obtained free of nucleic acid (24, 25). The M antigen belongs to the class of alcohol-soluble proteins, but the T antigen does not fall into this category.

Other characteristics in which M and T antigens differ are summarized in Table VIII. The chemical procedures used for the extraction of each one serve to destroy the other: M antigen is resistant to heat at low pH and can be extracted in soluble form from the intact cells by boiling them at pH 3, a procedure which rapidly destroys T antigen. The latter antigen is obtained from the cell by proteolytic digestion, apparently without destruction of the antigen. Although Elliott has shown that M antigen may also be released from the cell by proteolytic digestion (15), this antigen is quickly destroyed after separation from the cell unless the enzyme is dilute or the exposure of short duration (13). If streptococci are treated with proteolytic enzymes in the high concentration required to liberate T antigen completely, M antigen is destroyed so rapidly that it is not detected in solution. The serological activity of relatively purified preparations of M and T antigens is lost on short exposure to ultraviolet light, although these antigens are protected from this destruction when ribonucleic acid is present as an impurity.

In the past both of these antigens have been important factors in serological classification because each is active in the agglutination reactions widely used for differentiating specific types (26). Sometimes, however, the presence of an excess of one of these antigens apparently affects the other in such a way that

TABLE VIII

Comparison of Properties of M and T Antigens of Group A Hemolytic Streptococci

Method of differentiation	T antigens	M antigens
Method of extraction from the streptococcal cell	Proteolytic digestion	Heating at pH 2 to 3
Proteolytic enzymes	Resist digestion	Rapidly digested
Chemical composition	Protein, immunologically active substance prepared free of nucleic acid. Not an alcohol-soluble protein.	Protein (probably independent of nucleic acid): belongs to class of alcohol-soluble proteins.
Isoelectric point	Close to pH 4.50	Not known
Ultraviolet radiation	Serological activity destroyed.	Serological activity destroyed.
Heat	Labile	Stable
Occurrence	Constant component of a given strain, present in both matt and glossy variants.	Variable cellular component, constant in certain strains and readily lost in others. Regained on mouse passage of cultures. Present in matt variants only.
Specificity	One T antigen may be common to several types; another may be restricted to a single type.	Distinct M antigen for each type.*
Antigenicity and relationship to protection	Highly antigenic both in the intact cell and in solution. Antibodies not protective.	Moderately antigenic in the intact cell. Poorly antigenic in solution. Antibodies confer type-specific protection.
Relationship to virulence	None known. Present in both virulent and non-virulent forms.	One of the essential factors, but additional unknown factors sometimes necessary.

* See footnote 1.

the second phase of agglutination may not occur, even though the initial phase of antigen-antibody combination can be proved to have taken place (3, 5, 7). In recent work the T antigen has been found unsatisfactory as a basis for type classification since it apparently is unrelated to virulence or immunity, with both of which the M antigen is intimately associated. Since immunity to streptococcal infection appears to be type-specific and dependent upon antibodies directed against the M substance, it is obviously important in epidemio-

logical investigations to study matt variants which contain this antigen. The T antigens complicate the interpretation of agglutination reactions in such studies unless the distribution of T substances in various types is understood.

Since the T antigen is found in glossy variants after the loss of the M antigen which characterizes the matt form (1), it can sometimes be used as a "marker or tag" of strains occurring in persistent carriers at the end of an epidemic. Indeed, the T antigen, if present in a strain of streptococcus, is a stable component which is rarely lost. It is well known, on the other hand, that the M antigen is a variable component, decreasing or disappearing with loss of virulence. An avirulent strain can often regain this antigen by serial animal passage or by other methods which restore virulence (11). The presence of M substance in cultures of a given variant is at times conditioned by the presence or absence of the streptococcal proteinase described by Elliott, who showed that most glossy strains produce this enzyme (15). When strains become virulent after mouse passage, the active enzyme disappears from the cultures and the M antigen is again demonstrable. The T antigen appears to be unaffected by this enzyme.

The relationship of active immunity to the presence of M antigen in the cell and the lack of importance of T antigen in this respect has been emphasized by the recent work of Kuttner and Lenert and by that of Rothbard in studying the bacteriostatic and bactericidal action of patients' sera after infection with known types of group A hemolytic streptococci (27, 28). The same relationship of M and T antigens was found by Watson, Rothbard, and Swift to hold for the implantation of group A hemolytic streptocci in the nasopharynx of monkeys inoculated intranasally (29). Only matt variants containing the M antigen could be successfully established in this way. Following such implantation, the animals developed type-specific immunity for intranasal inoculation lasting several months to a year or more. The presence or absence of T antigen in the streptococci had no relationship either to the ability of streptococci to become established in the monkey's nasopharynx or to the resistance of this animal to later inoculation with other strains.

The available evidence derived from the present study on the T antigen, as well as previous studies of both M and T antigens, and the observations of other investigators on the relationship of M and T antigens to the development of immunity, indicate that the M antigen is of primary importance in this respect. Knowledge of the T antigen is nevertheless of value for certain phases of epidemiological investigations; further study is required to discover the significance of this component in the biological activities of the streptococcal cell.

SUMMARY

1. T antigens of group A hemolytic streptococci have been obtained in soluble form by digestion of the bacterial cells with pepsin or trypsin. Large

quantities of this antigen were readily extracted from type 1 strains, whereas only small amounts could be obtained from strains of other types.

2. The T antigen, prepared in this way from a type 1 strain, was partially purified by chemical precipitation and further enzymatic digestion. An active fraction, apparently protein in nature, was separated electrophoretically at pH 7.00. The separated material, pooled and analyzed at the same pH, gave only a single peak. The isoelectric point of this substance was about pH 4.50. An elementary analysis was obtained. Although the T antigen was resistant to digestion with proteolytic enzymes and ribonuclease, it was readily inactivated by heat, especially in acid media and in strong salt solutions. The serological activity of this purified T substance was lost after exposure to ultraviolet radiation.

3. Analysis by means of the ultracentrifuge showed that the material was polydisperse and therefore probably impure.

4. The soluble form of the T substance was active in the precipitin reaction, in the fixation of complement, in inhibition of T agglutination, and as an antigen when injected into rabbits. The antibodies produced did not protect mice against infection with virulent strains of hemolytic streptococci containing the same T antigen.

5. The immunological specificity of T antigen in soluble form is the same as that of the T antigen in the intact streptococcus from which it was derived

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