# THE CELLULAR ANTIGENS OF GROUP A STREPTOCOCCI

## IMMUNOELECTROPHORETIC STUDIES OF THE C, M, T, PGP, $E_4$, F, AND E ANTIGENS OF SEROTYPE 17 STREPTOCOCCI

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In addition to the precipitating antigens C, M, T, and R described by Lancefield (1) and polyglycerophosphate described by McCarty (2) it is likely that many other cellular components of Group A streptococci are capable of serving as antigens. The technic of immunoelectrophoresis appeared to offer an opportunity for discovering such substances. One problem in an investigation of this type lies in the difficulty of liberating the cellular antigens from the streptococcal cell in serologically active form for testing. Certain classical extracts, such as those made with acid, formamide, *Streptomyces albus* enzymes and others can be studied. Two recent reports from the Pasteur Institute (3, 4) have described the immunoelectrophoretic patterns of streptococci of serotypes 39 and 24, and have shown that acid or neutral extracts of these organisms have two or three antigenic constituents.

The present study is concerned with the immunoelectrophoretic characteristics of some of the cellular antigens of Group A streptococci and in particular with precipitating antigens found in various extracts of serotype 17 streptococci, including three antigens that have not been described before.

### *Methods and Materials*

*Strains.*—The streptococcal strains used in these studies were obtained from the collection maintained in this laboratory and from Dr. R. C. Lancefield. The strains were preserved in the dried state for long periods or in blood broth at refrigerator temperature for current study.

*Immunoelectrophoresis* was performed on microscope slides (usually 1½ × 3 inches) employing the technic of Grabar and Williams (5) as modified by Scheidegger (6). The matrix for electrophoresis and diffusion was 0.7 per cent Ionagar No. 2 in Grabar's barbital buffer, pH 8.2, $\mu = 0.05$ (7). Sodium azide in a final concentration of 0.02 per cent was used as a preservative. The same buffer was used in electrode vessels. Runs were usually made at room temperature, 16 ma per 1½ inch slide, for 25 minutes. Patterns were developed in a moist chamber in a constant temperature room (25°C) or in the refrigerator (approximately 4°C). Washed slides were dried and stained with thiazine red (8). The illustrations were made by placing the stained slides on Eastman Kodak F-5 projection paper, and exposing as for contact prints. In some illustrations visualization of the well has been aided by placing an India ink dot in the center of the well.

*Double Diffusion Tests* (Ouchterlony, reference 9) were performed in 0.7 per cent Ionagar

No. 2 in 0.85 per cent NaCl solution containing sodium azide as a preservative. The tests were performed on glass microscope slides and were developed, washed, stained, and photographed as described above for immunoelectrophoretic preparations.

*Extracts.*—Several types of extract have been used in this study, and to obtain the antigens in desired concentrations the extracts were diluted with saline solution (0.85 per cent NaCl in distilled water) or were concentrated by pervaporation in dialysis-tubing sacs, by flash evaporation, by negative-pressure dialysis in collodion sacs, or by combinations of these methods. The final extracts were clarified by centrifugation and, when necessary, were equilibrated across collodion or viscose membranes with Grabar's buffer or with saline solution.

*Crude acid extracts* were prepared according to the technic of Swift *et al.*, (10). *M extracts* were prepared by alcohol precipitation of crude extracts according to Lancefield (11). *10× M extracts* were made by taking up the final alcohol precipitate of M extracts in $\frac{1}{10}$th the usual volume of saline solution. *Acid (IP) extracts* were made by bringing crude acid extracts to pH 2.5 or 3.5 with 1 N HCl and allowing to stand in the refrigerator overnight. After centrifugation the precipitate was taken up in minimal amounts of 1 N or N/5 NaOH and saline solution to dissolve. *NaCl-56°C extracts* were made by suspending the wet, packed cells of an overnight culture in five volumes of saline solution and immersing the suspension in a 56°C water bath for 30 minutes. *Distilled water extracts* were made by suspending packed cells of overnight culture in distilled water at room temperature. *Buffer extracts* were made by suspending packed living or heat-killed cocci in M/15 or M/100 phosphate buffer of the desired pH, usually 7.4. After centrifuging the suspension, the supernatant fluid was dialyzed against several changes of distilled water and concentrated to the desired degree. Generally 25 to 100 ml of buffer extract was reduced to 0.25 to 1.0 ml for testing. *Trypsin extracts* were made by the method of Lancefield and Dole (12). *Phenol extracts* were made by the method of Moskowitz (13), in which packed wet streptococci were suspended in equal quantities of 0.85 per cent saline solution and approximately 95 per cent phenol solution, brought to pH 5.5, allowed to stand at room temperature 2 hours with occasional shaking, and centrifuged. The aqueous phase was drawn off and extracted three times with ethyl ether to remove excess phenol or the phenol was removed by dialysis. *Formamide extracts* were made by Fuller's procedure (14), and *Streptomyces albus enzyme extracts* by Maxted's procedure (15).

*Media.*—For routine purposes streptococci were grown in a commercially available modified Todd-Hewitt broth, sterilized by autoclaving (16). For special purposes streptococci were grown in the dialysate medium of Dole (17) as modified by Wannamaker and Krause (18) using the formula designed for Group C strains. This medium is designated in the text as DKW medium.

*Antisera* were made with vaccines of whole, heat-killed streptococcal cells. The immunization schedule followed was that of Lancefield, in which rabbits were inoculated the first 3 days of the week and rested the other 4. *Capillary precipitin tests* were performed by the technic of Swift, *et al.* (10).

Details of *bactericidal tests* and *ingestion tests* are given in earlier publications by Wiley and Wilson (19, 20).

*Materials.*—The authors are indebted to Dr. Maclyn McCarty for highly purified preparations of Group A carbohydrate and polyglycerophosphate, and to Dr. Willard Schmidt for another highly purified preparation of the Group A polysaccharide. Dr. Seymour Halbert kindly supplied us with a sample of his concentrated culture supernate of strain C203S (21).

## RESULTS

*The Group A Carbohydrate.*—Highly purified preparations of the polysaccharide of Group A streptococcus were obtained from Dr. McCarty and Dr. Schmidt. The starting material for these preparations was a lysate of the

cocci produced by *Streptomyces albus* enzymes. Immunoelectrophoretically this antigen was seen to have a slight mobility towards the anode (Fig. 1, top pattern) when allowance was made for shift of the origin towards the cathode by electroendosmosis. The arc formed with rabbit antibody was somewhat

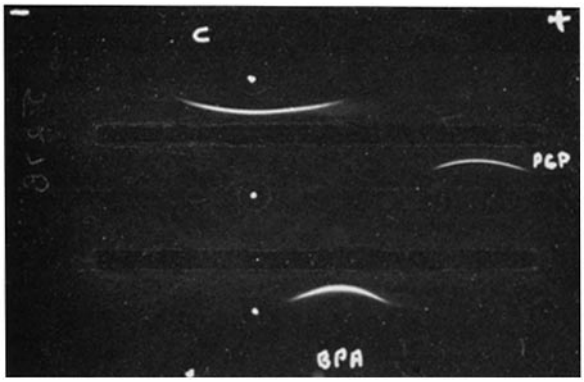


FIG. 1. Immunoelectrophoretic appearance of group A carbohydrate, polyglycerophosphate and bovine plasma albumin.

Top well: Group A carbohydrate, highly purified preparation (McCarty). Upper trough: serum containing antibodies for C and PGP. Middle well: polyglycerophosphate, highly purified preparation (McCarty). Lower trough: antiserum prepared against bovine plasma albumin. Bottom well: crystalline bovine plasma albumin.

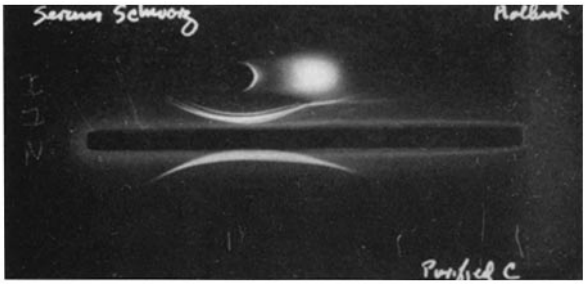

FIG. 2. Antibodies for Group A polysaccharide in serum of human patient.

Upper well: concentrated culture supernate of C203S (Group A, serotype 3) prepared by Dr. S. Halbert. Trough: serum Schwarz. Lower well: purified C of Group A (McCarty). The intense arc in the upper pattern is from the group carbohydrate, but the other arcs are unidentified.

flat and was usually located close to the serum trough. The same antigen tested with serum from a patient with acute streptococcal parotitis, otitis media, and septicemia[1] gave the arc shown in the lower pattern of Fig. 2.

[1] This patient was not treated with penicillin from the onset of symptoms until the development of septicemia 8 days later. We are indebted to Dr. Herman Rosenblum and Dr. Mark G. Cohen for the serum specimen, which was drawn on the 15th day of the patient's illness.

The electrophoretic mobility of C in crude formamide extract is faster than that in the purified, enzyme-extracted preparation, and the C in crude acid extract is faster still (Fig. 3). Although the differences in mobility are slight, they suggest that minor differences exist in the chemical structure of C prepared in different ways and subjected to different degrees of purification.

*Polyglycerophosphate (PGP) and* $E_4$.—Polyglycerophosphate, originally described in the streptococci by McCarty (2), has the fastest mobility of any component that has been encountered in streptococcal extracts in these studies (Fig. 1, center arc). The mobility of a purified preparation of PGP kindly

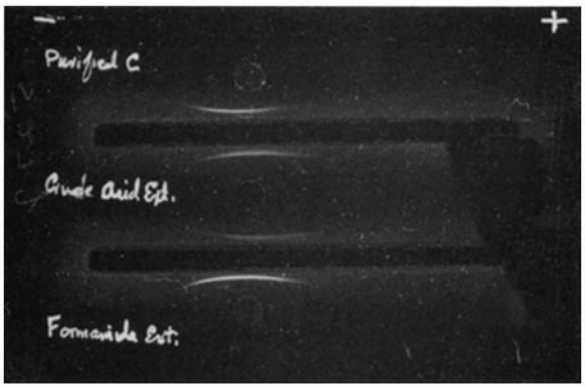


FIG. 3. Effect of type of preparation on location of arcs of Group A carbohydrate.

Top well: purified Group A carbohydrate (McCarty). Middle well: D205/37M, crude acid extract. Bottom well: D205/37M, formamide extract. Serum troughs: antiserum for S43G, containing anti-C.

supplied by Dr. McCarty is roughly twice that of bovine plasma albumin subjected to electrophoresis under the same conditions, (Fig. 1), and in fact, its mobility is so rapid that unless a short running time is used, the antigen may pass entirely off the slide.

The antigen we have designated $E_4$ was encountered when extracts of serotype 17 strains were tested in immunoelectrophoresis with certain heterologous antisera. Antibodies for $E_4$ are found in high concentration in certain bleedings of rabbits inoculated with AD540 (serotype 9), C95/60B (serotype 39), S43FL (serotype 6), and to a lesser extent with other strains. The antigen is found in extracts made with saline or distilled water at room temperature or 56°C (concentrated by pervaporation), fresh crude acid extracts and M extracts, the cytoplasmic fraction of cells disintegrated with glass beads, formamide extracts, extracts made with *Streptomyces albus* enzymes, and concentrated culture filtrates. It can be extracted free of many other cellular components by means of phenol, according to the method of Moskowitz (13) (see Methods

and Materials). The serological activity of $E_4$ declines with time in many of these extracts, so that, for example, although it is found in fresh M extracts, it is largely or completely absent from M extracts that have been stored for a few days at room temperature.

The non–serotype-specific nature of the $E_4$ antigen is shown by its reactivity with antisera prepared against strains of heterologous serotype, and by the

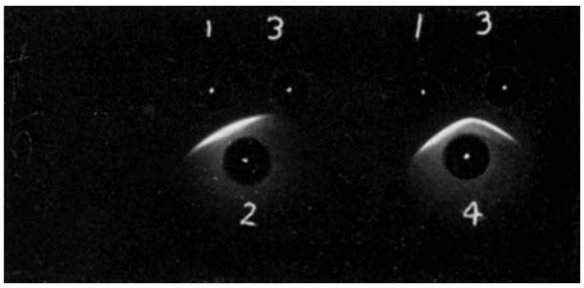


FIG. 4. Double diffusion tests showing immunological relationship of $E_4$ and PGP.
Wells: 1. D205/37M, phenol extract containing $E_4$. 2. Anti-$E_4$ serum. 3. Purified PGP (McCarty). 4. Anti-PGP serum.

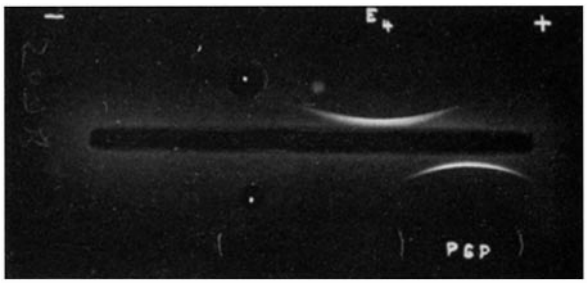


FIG. 5. $E_4$ and PGP react with anti-PGP serum.
Upper well: D205/37M, saline (0.85 per cent NaCl solution) extract containing $E_4$. Trough: antiserum for F108B-M containing anti-PGP. Lower well: purified PGP (McCarty).

reactivity of extracts made from strains of many types in Group A (all types that have been tested) and from Groups B, C, and G, (but not D or O), with the same antiserum containing antibodies for $E_4$.

There are two kinds of antiserum that react with $E_4$. In one kind (anti-PGP serum), the serum reacts in capillary precipitin tests and in immunoelectrophoresis with purified PGP (McCarty) as well as with $E_4$. In the other kind (anti-$E_4$ serum), the serum reacts with $E_4$ but does not react with PGP. In our experience all sera that react with PGP also react with $E_4$.

These reactions are readily demonstrated in double diffusion tests, using anti-PGP serum, in which the $E_4$ and PGP lines join without crossing or spur

formation, (Fig. 4, right). Conversely, anti-$E_4$ serum reacts with the $E_4$-containing extract, but not with PGP (Fig. 4, left).

When the anti-$E_4$ serum is tested in immunoelectrophoresis with an $E_4$-containing saline extract on one side and with PGP on the other, a single arc is formed for each extract, but these arcs are located at different distances from

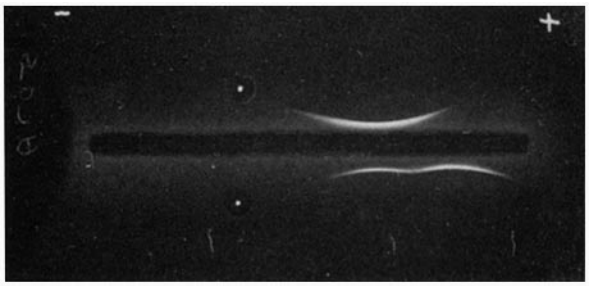

FIG. 6. When $E_1$ and PGP are present in the same extract they form two arcs.

Upper well: D205/37M, saline extract containing $E_4$. Trough: antiserum for F108B-M containing anti-PGP. Lower well: a mixture of the saline extract used in upper well and purified PGP (McCarty).

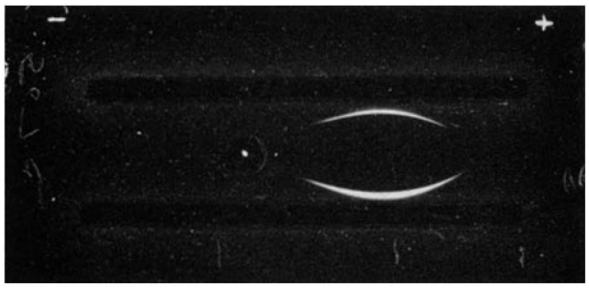

FIG. 7. $E_4$ reacting with its own antibody or with anti-PGP has the same mobility.

Upper trough: antiserum for AD540, containing anti-$E_4$, but no anti-PGP. Well: D205/37M, saline extract of living organisms made at room temperature. Lower trough: antiserum for AD563, containing antibodies for PGP.

the origins, $E_4$ being of slower mobility than PGP (Fig. 5). This indicates that the reacting antigens in the two extracts are physicochemically different. The singleness of the arcs indicates that in these preparations neither antigen is contaminated with significant amounts of the other. When an artificial mixture is made of the two antigen preparations and is tested against anti-PGP serum, a double arc is formed (Fig. 6, lower pattern) indicating that when a mixture of these antigens is present, this fact can be determined by immunoelectrophoresis. When the $E_4$-containing preparation is tested in immunoelectrophoresis with the two kinds of antisera in opposite troughs, arcs are

formed the same distance from the origin in each case, (Fig. 7), suggesting that the antibodies in both sera are reacting with the same antigen.

Thus, $E_4$ and PGP are physicochemically different because they have different electrophoretic mobilities. They are immunologically different because $E_4$ reacts with the two kinds of sera described above, whereas PGP reacts with only one kind. They are immunologically related because their precipitin arcs join in immunoelectrophoresis (Fig. 6) and in immunodiffusion (Fig. 4).

TABLE I

*Lack of Effect of Antibodies for $E_4$ in Bactericidal Tests with Human Blood and in Mouse Protection Tests*

A. Bactericidal tests

| Strain used for inoculaion | Serum | Antibodies | Results of bactericidal test inoculum, chain count | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 480,000 | 48,000 | 4,800 | 480 | 48 | 5 |
| D205/37M serotype 17 | Normal rabbit serum | | L | L | L | L | PL | TM |
| | R765 C95/60B | M of serotype 39, $E_4$ | L | L | L | L | PL | TM |
| | R831 S43/137 | No $E_4$ | L | L | L | L | PL | TM |
| | R661 D205/37M | M and F of serotype 17, no $E_4$ | PL | 96 | 17 | 0 | 0 | 0 |

B. Mouse protection tests

| Strain used for inoculation | Serum | Antibodies | $LD_{50}$* | $LD_{50}$ in chains | Protective Index* |
|---|---|---|---|---|---|
| | | | *ml* | | |
| D205/37M serotype 17 | None | | $10^{-8}$ | 5 | −5.6 |
| | Normal rabbit serum | | $10^{-7.27}$ | 28 | 1 |
| | R890 D205/37M | M, F, and E of serotype 17, no $E_4$ | $10^{-2}$ | 520,000 | 18,570 |
| | R765 C95/60B | M of serotype 39, $E_4$ | $10^{-8}$ | 5 | −5.6 |

* The $LD_{50}$ in ml and chains, and protective index were determined as in Table III.

The possible role of antibodies for $E_4$ in virulence phenomena was investigated by performing bactericidal tests and mouse virulence tests, using a highly potent anti-$E_4$ serum. The serum was prepared with a strain of different serological type (serotype 39) from that of the culture used to inoculate the blood or mice (serotype 17). Results are shown in Table I. Antibodies for $E_4$

conferred no bactericidal power on human blood nor did they have a protective effect in mouse virulence tests.

Although the chemical nature of the $E_4$ antigen is unknown, for operative purposes it may be defined as a non–serotype-specific antigen extractable from Group A and other streptococci that reacts with a serum selected to contain abundant antibody precipitating with it but not reacting with PGP, and lacking antibodies for the group carbohydrate or other recognized streptococcal antigens. Its electrophoretic mobility is faster than that of the Group A carbohydrate but slower than that of PGP.

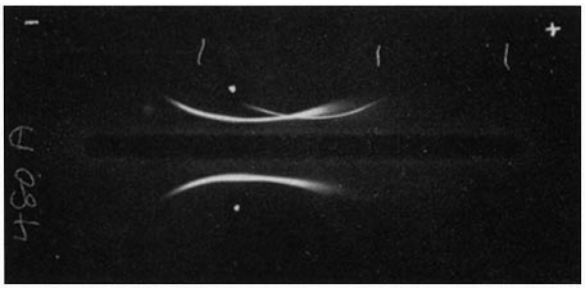


FIG. 8. Immunoelectrophoretic appearance of the fast and slowly migrating antigens in M extracts of serotype 17 strains.

Top well: D205/37M, 10× M extract. Trough: antiserum prepared with D205/37M. Bottom well: F108B-M, 10× M extract. The antiserum contained antibodies for both components and the D205/37M extract contained both antigens. The F108B-M extract contained only the slowly migrating antigen.

*The M and F Antigens of Serotype 17.*—Immunoelectrophoretic analysis of several Group A streptococcal serotypes indicated that M extracts in a number of instances reacted with homologous absorbed antisera to give more than one arc. The most impressive of these was serotype 17, where the pattern of strain D205/37M showed two arcs of good density (Fig. 8, top pattern), and this serotype was selected for more detailed study. The best patterns were produced when the extracts were about ten times more concentrated than the standard M extracts of Lancefield (11). One of the two antigens centered about the original well, whereas the other moved farther towards the anode. That they were immunologically distinct was shown by crossing of the lines of the arcs (Fig. 8, top pattern).

A survey of the serotype 17 strains at hand showed that most strains contained both antigens. One strain, however, was encountered (F108B-M) that had the slowly moving antigen but lacked the faster moving component (Fig. 8, lower pattern). By immunizing rabbits with this strain, it was possible to prepare sera that contained antibodies for the slowly moving component

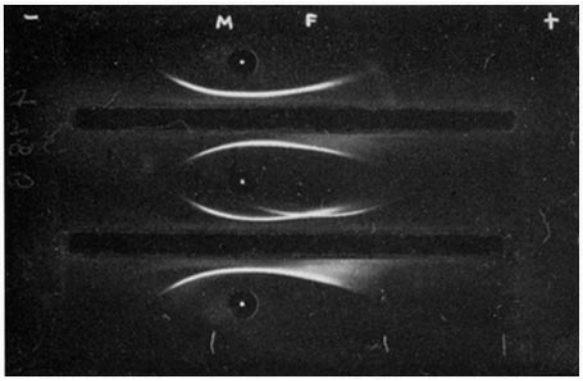


FIG. 9. Comparison of antigens in M extracts of F108B-M and D205/37M.

Top and bottom wells: M extract of F108B-M. Upper trough: antiserum for F108B-M, containing antibodies for slow component. Central well: M extract of D205/37M, containing slow and fast components. Lower trough: antiserum for D205/37M, containing antibodies for slow and fast components.

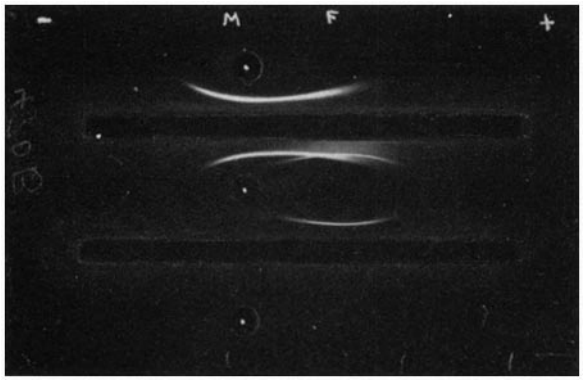


FIG. 10. Absorption of D205/37M antiserum with F108B-M organisms removes antibodies for the slowly migrating component but not for the fast moving component.

Top and bottom wells: F108B-M, 10× M extract. Upper trough: antiserum prepared with D205/37M, containing antibodies for both components. Central well: D205/37M, 10× M extracts, containing fast and slow antigens. Lower trough: same antiserum as in upper trough, but absorbed with suspension of F108B-M organisms. Only antibodies for the fast component remain.

without those for the fast moving component (Fig. 9, upper patterns). By absorbing antiserum prepared with strain D205/37M, containing antibodies for both components (Fig. 10, upper trough), with a suspension of F108B-M, a serum resulted that contained antibodies for the fast moving component but not for the slowly moving component (Fig. 10, lower trough). These two types of antisera made it possible to test the effect of antibodies for either one

TABLE II

*Bactericidal Tests*

*Bactericidal Effect of Antibodies for Slow (M) and Fast (F) Electrophoretic Components of Serotype 17*

| Strain used for inoculation | Serum | Antibody | Results of bactericidal test* | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Inoculum, chain count | | | | | |
| | | | 395,000 | 39,500 | 3,950 | 395 | 39 | 4 |
| D205/37M serotype 17 (M and F) | Normal rabbit serum | — | L | L | L | L | PL | 1036 |
| | R661 D205/37M unabsorbed | Serotype 17, M and F | 1640 | 68 | 8 | 0 | 0 | 0 |
| | R661 D205/37M absorbed 2 times F108B-M | Serotype 17, F | L | L | L | L | PL | 712 |
| | R453 S43/101 unabsorbed | Serotype 6 | L | L | L | L | L | TM |
| | | | Inoculum, chain count | | | | | |
| | | | 470,000 | 47,000 | 4,700 | 470 | 47 | 5 |
| D205/37M serotype 17 (M and F) | Normal rabbit serum | — | L | L | L | L | PL | 748 |
| | R663 F108B-M unabsorbed | Serotype 17, M | TM | 84 | 12 | 4 | 0 | 0 |
| | R453 S43/101 unabsorbed | Serotype 6 | L | L | L | L | L | 1072 |
| | | | Inoculum, chain count | | | | | |
| | | | 1,060,000 | 106,000 | 10,600 | 1,600 | 160 | 16 |
| F108B-M (M, no F) | Normal rabbit serum | — | L | L | L | L | L | PL |
| | R661 D205/37M unabsorbed | Serotype 17, M and F | PL | 604 | 64 | 0 | 0 | 0 |
| | R661 D205/37M absorbed 2 times F108B-M | Serotype 17, F | L | L | L | L | L | PL |
| | R453 S43/101 unabsorbed | Serotype 6 | L | L | L | L | L | PL |
| | | | Inoculum, chain count | | | | | |
| | | | 1,230,000 | 123,000 | 12,300 | 1,230 | 123 | 12 |
| F108B-M | Normal rabbit serum | — | L | L | L | L | L | TM |
| | R663 F108B-M unabsorbed | Serotype 17, M | TM | 596 | 40 | 4 | 0 | 0 |
| | R453 S43/101 unabsorbed | Serotype 6 | L | L | L | L | L | TM |

* Chain count after 3 hours' rotation. L, blood agar plate laked from streptococcal growth; PL, plate partially laked. TM, too many colonies in plate to count. (Figures signify calculated number of chains in total contents of rotated tube, determined by counting colonies on plate inoculated with ¼th content of tube.)

of the antigens in bactericidal and mouse protection tests, without interference from antibodies for the other antigen.

*Bactericidal and ingestion tests:* D205/37M grows very well in whole human blood. By means of the sera just described, having antibodies for the slow component or for the fast component, it should be determinable which of the two components is the M antigen, because antibodies for M confer bactericidal power on human blood (22, 23). The results of bactericidal tests with human blood to which these types of antisera have been added are shown in Table II. It is seen that the addition of unabsorbed antiserum made with D205/37M, containing antibodies for both components, enabled the blood to inhibit the growth of the homologous strain. A similar inhibitory effect was conferred on blood by adding serum containing antibodies for the slowly moving component. On the contrary, no such inhibitory effect was conferred by adding antibodies for the fast moving component. It was apparent, therefore, that the slowly moving component was the M antigen and that the fast moving component was a different kind of antigen. Hereafter, in this report, the two antigens will be called by the letters M and F, respectively.

Ingestion tests confirmed these results and showed that antibodies for M enabled the phagocytes to ingest otherwise uningestible cocci, whereas antibodies for F had no such effect.

*Long-chain tests:* Stollerman and Ekstedt (24) have shown that Group A streptococci that normally grow in short chains will form long chains when grown in the presence of anti-M serum. Using a modification of Stollerman and Ekstedt's technic, D205/37M was tested for long chaining in the presence of anti-M and anti-F sera, prepared as described above. Without serum the strain formed chains of one to four cocci. With anti-M serum the chains ranged from 10 to 25 cocci in length. With anti-F serum the chain length was no greater than in the serum-free control. Therefore, anti-F serum, unlike anti-M serum, does not cause long chaining of a strain containing both F and M antigens.

*Mouse protection studies:* To determine whether antibodies for the two antigens would be effective in protecting mice against otherwise lethal inocula of D205/37M, two mouse protection tests were performed. The results were similar in each experiment and have been combined for analysis, as presented in Table III. It is seen in section A of the table that the anti-M, and the anti-M plus anti-F antisera had a strong mouse protective effect against the homologous strain (four logs), but no protective effect against a strain of heterologous type, as shown in section B. The anti-F serum appeared also to have a protective effect for the homologous strains, but this was considerably less (two logs). In section B the anti-F serum, which was an absorbed serum, was seen to have a possible protective effect for the heterologous strain S43/137. This appeared to be a non-specific effect or artifact, for the unabsorbed serum did not show

TABLE III

*Mouse Protection Studies*

Effect of antibodies for M alone, F alone and M and F together

| Dilution | Average viable chain-count | No serum | Normal rabbit serum | Anti-M serum | Anti-F serum | Anti-M plus anti-F serum |
|---|---|---|---|---|---|---|
| *ml* | | | | | | |
| | | A. Mice inoculated with D205/37M (serotype 17) | | | | |
| $10^{-1}$ | 94,000,000 | | | 1/9 | | 1/9 |
| $10^{-2}$ | 9,400,000 | | | 2/8 | 1/9 | 2/8 |
| $10^{-3}$ | 940,000 | 0/5* | 0/10 | 5/5 | 3/7 | 6/4 |
| $10^{-4}$ | 94,000 | 0/5 | 0/10 | 8/2 | 3/7 | 5/5 |
| $10^{-5}$ | 9,400 | 0/5 | 1/9 | 7/3 | 4/6 | 7/3 |
| $10^{-6}$ | 940 | 0/5 | 1/9 | 9/1 | 7/3 | 8/2 |
| $10^{-7}$ | 94 | 0/5 | 3/7 | 10/0 | 8/2 | 8/2 |
| $10^{-8}$ | 9 | 2/3 | 7/3 | | 10/0 | |
| $LD_{50}$, ml‡ | | $10^{-8.00}$ | $10^{-7.17}$ | $10^{-8.11}$ | $10^{-5.00}$ | $10^{-8.22}$ |
| $LD_{50}$ in chains | | 9 | 63 | 720,000 | 9,400 | 459,000 |
| Protective index§ | | | 1 × | 11,400 × | 149 × | 7,300 × |
| | | B. Mice inoculated with S43/137 (serotype 6) | | | | |
| $10^{-5}$ | 2,830 | 1/4 | 1/9 | 0/10 | 3/7 | 1/9 |
| $10^{-6}$ | 283 | 0/5 | 0/10 | 2/8 | 4/6 | 2/8 |
| $10^{-7}$ | 28 | 2/3 | 4/6 | 5/5 | 7/3 | 5/5 |
| $10^{-8}$ | 3 | 4/1 | 7/3 | 7/3 | 9/1 | 7/3 |
| $LD_{50}$, ml | | $10^{-7.07}$ | $10^{-7.15}$ | $10^{-7.04}$ | $10^{-6.25}$ | $10^{-7.00}$ |
| $LD_{50}$ in chains | | 24 | 20 | 26 | 161 | 28 |
| Protective index | | | 1 × | 1.3 × | 8.0 × | 1.4 × |

| Dilution | Average viable chain count | No serum | Normal rabbit serum | S43 serum unabsorbed | S43 serum absorbed with F108B-M |
|---|---|---|---|---|---|
| *ml* | | | | | |
| | C. Mice inoculated with D205/37M | | | | |
| $10^{-4}$ | 36,400 | | | 0/5 | 1/4 |
| $10^{-5}$ | 3,640 | 0/5 | 0/5 | 0/5 | 1/4 |
| $10^{-6}$ | 364 | 0/5 | 0/5 | 3/2 | 4/1 |
| $10^{-7}$ | 36 | 0/5 | 2/3 | 1/4 | 3/2 |
| $10^{-8}$ | 3 | 4/1 | 4/1 | 3/2 | 5/0 |
| $LD_{50}$, ml | | $10^{-7.48}$ | $10^{-7.18}$ | $10^{-7.11}$ | $10^{-5.35}$ |
| $LD_{50}$ in chains | | 15 | 25 | 27 | 1609 |
| Protective index | | | 1 × | 1.1 × | 64 × |

* Ratio of mice surviving to mice dead, 14 days after inoculation.

‡ Reed and Muench (42).

§ Ratio of $LD_{50}$ in group given normal rabbit serum to $LD_{50}$ of mice given antiserum.

the effect shown by the absorbed serum. It was considered likely that in the process of absorbing the antiserum something responsible for this effect was released from the absorbing suspension of F108B-M into the serum. To investigate this possibility, the experiment presented in section C of Table III was performed. Here it is seen that absorption of a heterologous antiserum with F108B-M did, indeed, confer a moderate protective activity on it against D205/37M. The effect was about half that of D205/37M antiserum absorbed with the same strain. Thus, although the experimental data suggest that anti-F serum may have some mouse-protective effect, it is of a low order, and the major mouse-protective action lies in the slowly moving electrophoretic component, which we identify as the M antigen on the basis of these mouse protection tests and bactericidal tests with human blood.

*Effect of mouse passage on F antigen:* F108B-M, which had not been passed through mice, and possessed the M antigen but not the F antigen, was submitted to intraperitoneal passage through mice. After 11 passages the $LD_{50}$ of the strain decreased from $10^{-1.11}$ to $10^{-7.28}$ ml and associated with this increase in virulence was the appearance of the F antigen in M extracts of the passage strain. Using 10× M extracts in capillary precip'tin tests the unpassed strain failed to react with anti-F serum, whereas the mouse-passage strain gave a $+++\pm$ reaction with the same serum. Three other strains (554C, 4034B, and 4230B) having no F or at best a trace of that antigen before mouse passage increased in virulence and in F production following mouse passage. It is thus apparent that mouse passage of strains having little or no F antigen to begin with, led to increase in virulence associated with the appearance or increase in F antigen extractable from the cells. The association of the F antigen with mouse virulence is not invariable, however, for we have many unpassed strains in our collection that have abundant F antigen but low virulence for mice. In this respect, again, the F antigen resembles the M antigen.

The $F^-$ strains F108B-M and 554C were also serially passed through whole human blood ten times each. No appearance of the F antigen resulted from these passages. This is not surprising, for blood has little or no ability to inhibit the proliferation of the initial strains and no preferential selection of $F^+$ variants would be expected.

*Properties of the F antigen:* The F antigen, as noted above, is extracted from the streptococci by dilute acid. On repeated acid extraction, a small amount of F appears in the second extract, but the third is usually devoid of it. F is precipitated from neutralized acid extracts by three volumes of alcohol, by reducing pH to 3.5 or lower and by 0.6 saturation with ammonium sulfate. In these respects, it resembles the M antigen.

To determine the location of F in the cell, a suspension of D205/37M cocci was disintegrated with glass beads in the Mickle apparatus (25). An acid extract was made of the cell wall fraction, and the soluble cytoplasmic material

was concentrated for testing in precipitin reactions. F was not found in either fraction. Investigation of its fate revealed that it had been washed off the cell bodies during the three distilled water washes employed prior to disintegration, and that it could be recovered in concentrated form by reducing the volume of the pooled washes by flash evaporation or by pervaporation in viscose dialysis tubing (Fig. 11). Some $E_4$ was present in this type of preparation, but there was relatively little M and only traces of E and PGP.[2]

A further investigation of extraction of F by distilled water showed that it was removed from living and heat-killed cells with equal ease. Washing with 0.85 per cent saline solution, performed successively eight times, removed very little F from living cells and not much more from cells killed by exposure to 56°C for 20 minutes. In successive washing of cells with distilled water, very

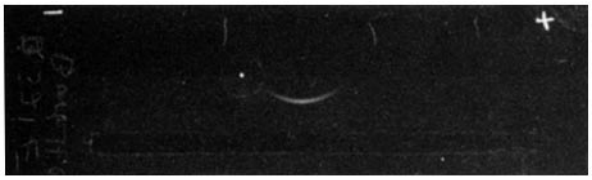


FIG. 11. F in distilled water extract of D205/37M.

Well: D205/37M, distilled water extract. Trough: antiserum for D205/37M, containing antibodies for E, M, and F. This preparation was given a shorter electrophoretic run than usual, which accounts for the compact form of the F arc compared to that seen in other figures.

little F was found in the first wash, most of it was present in the second and third washes, but traces were released even in the eighth wash. Acid extraction of cocci that had been washed with distilled water released too little F to be detected in precipitin tests. It is apparent therefore that washing with distilled water removes all or almost all of the F from the cell.

The effectiveness of the washing procedure was shown to be attributable chiefly to the pH of the suspension in the washing fluid. Cultures of D205/37M in modified Todd-Hewitt broth have a final pH of 5.9–6.3. In one representative experiment, when the cells were harvested by centrifugation and washed repeatedly with 0.85 per cent saline solution the wash waters maintained a pH of slightly below 6.0, varying in eight successive washes from pH 5.7–5.9. On switching to distilled water for the extraction, the first wash had a pH of 6.8 and subsequent washes ran around 7.2–7.4. The F first appeared in large quantity in the first wash that had a pH above 7.0. When M/100 phosphate buffer of pH 7.4 in 0.85 per cent saline solution was used to extract previously unwashed cells, F was released at a fairly uniform rate for three washings and in reduced amounts for another three washings. On shifting to distilled water for

[2] The nature of the E antigen will be described in later sections of this study.

the washing, F was released in moderate amounts for two additional washes. Buffer without the saline was equally good for extraction, but saline without buffer extracted very little F. F was also released from a heavy suspension of living cells of D205/37M placed in a well in a double diffusion test (Fig. 12).

The ease of extraction of F by simple washing with distilled water suggests that F is a superficial component of the cell. Is it located in the capsule? To answer this, packed living encapsulated cells of D205/37M were taken up in ten volumes of 1 per cent hyaluronidase (bovine testis) in 0.85 per cent saline solution, and incubated at 37°C for 10 minutes. By this time the cocci had lost their anatomical capsules, as determined with India ink preparations. The

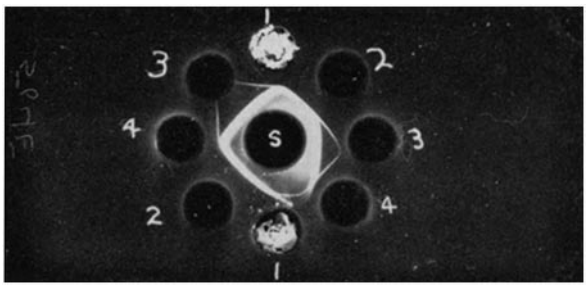


FIG. 12. Release of F from suspension of living cells of D205/37M placed in antigen well.

Central well: antiserum for D205/37M containing antibodies for E, M, and F. Antigen wells: 1. Living suspension of D205/37M. 2. 10× M extract of D205/37M containing E, M, and F. 3. 10× M extract of F108B-M, containing E and M. 4. Distilled water extract of D205/37M, containing F, but lacking E and M. The fine lines of the F precipitate show that F diffuses from wells 1, 2, and 4. M diffuses from wells 2 and 3. No separate line is seen for E.

suspension was centrifuged and the supernate concentrated 100 times by pervaporation following dialysis. Extracts of this type contained at best only a trace of F. On the other hand, the decapsulated cells yielded large amounts of F on making crude acid extracts in the usual way. It is thus apparent that most of the F associated with the cell is located under the capsule, but whether in the cell wall, cell membrane, or cytoplasm has not been determined.

The F antigen in M extracts is very stable. An extract prepared in 1951 from D205/36 and stored at refrigerator temperature still gives excellent precipitin reactions in capillaries or in immunoelectrophoresis in 1963. Preparations of F in distilled water are likewise stable for at least 2 years.

It has not been possible to separate F from M by acid precipitation at varied pH, by precipitation with various concentrations of ammonium sulfate or by passage through sephadex columns. A separation can be achieved by electrophoresis in agar, but the extensive overlapping of the two antigens limits recovery to a small anodal part of the F antigen spot. The best differential procedure encountered so far is that accomplished by distilled water extraction of intact cells, but such preparations are far from pure.

The knowledge that F is extracted from cocci by washing with distilled water at pH 7.4 or by buffer at that pH suggested that it might be released into the culture medium during growth if the medium were not allowed to become acid. With this possibility in mind, D205/37M was grown in DKW medium (see Methods and Materials). The pH of the growing cultures was monitored by means of a combined glass-calomel electrode. 5 N NaOH was added at intervals to maintain the pH between 7.4 and 7.8. At the termination of growth, the culture was centrifuged and part of the sedimented organisms were used to make a 10× M extract. The culture supernate was sterilized by filtration and

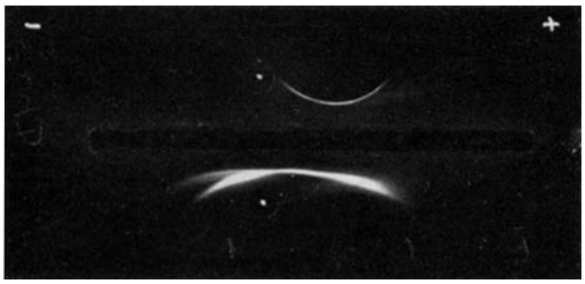


FIG. 13. F is largely released into medium when pH is kept at 7.4 or over during growth.

Upper well: culture filtrate of D205/37M grown in DKW medium kept at pH 7.4 or over during growth. Concentrated about 60 times. Trough: antiserum for D205/37M, containing antibodies for E, M, and F. Lower well: D205/37M, 10× M extract of sedimented organisms from the same culture that supplied the filtrate in upper well.

concentrated by dialysis and precipitation at pH 3.5. Immunoelectrophoretic analysis of these extracts showed that the concentrated culture filtrate gave a strong band from its F content, but contained very little M or E (Fig. 13, upper pattern).[2] In a complimentary way the 10× M extract of the cells from this culture had strong arcs for M and E but only a faint diffuse band for F (Fig. 13, lower pattern). Furthermore, absorption of serum containing antibodies for E, M, and F with living cells grown in the continuously alkaline medium removed anti-E and anti-M but not anti-F (Fig. 14).

A similar experiment was done in which the medium was kept at about pH 6.5, and not allowed to rise above pH 7.0. Both the culture filtrate and a 10× M extract of the sedimented organisms contained abundant F. By serial dilutions of these materials it was estimated that the amounts of F in the filtrate and extractable from the cells were approximately the same, whereas in the culture grown at pH 7.4 or over there was approximately 500 times more F in the filtrate than was extractable from the cells. Nevertheless, cells grown in the usual way, *i.e.* with pH of the medium falling from about 7.8 to about 6.0, retain enough F to yield, on acid extraction, a concentration of antigen readily demonstrable in immunoelectrophoresis.

These experiments indicate that under mildly alkaline conditions of growth, most of the F antigen leaves the cells and is found in the fluid environment.

The failure of F108B-M to release F on acid extraction suggested that it might already have been released to the medium during growth. With this in

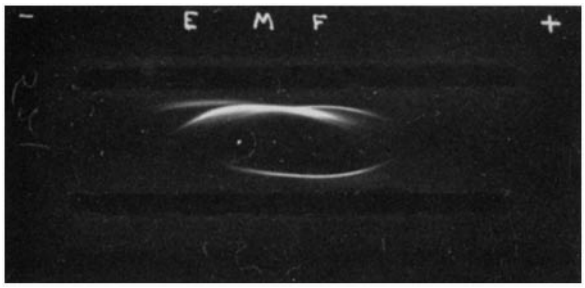


FIG. 14. Absorption by living cells of D205/37M, grown at maintained pH of 7.4 or higher removes anti-M and anti-E, but not anti-F, because F has been released to the medium during growth.

Upper trough: antiserum for D205/37M containing antibodies for E, M, and F. Well: 10× M extract of D205/37M, containing E, M, and F. Lower trough: same antiserum as in upper trough, but absorbed with suspension of living cells of D205/37M grown at pH maintained at 7.4 or above. This serum retains anti-F, but has lost anti-E and anti-M as a result of the absorption.

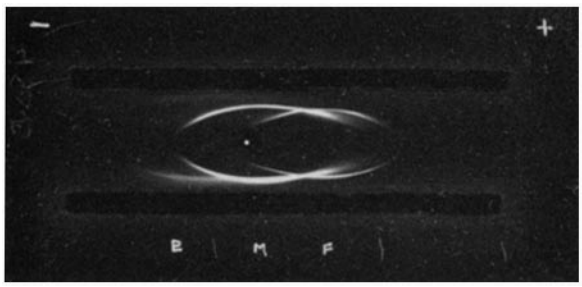


FIG. 15. Antibodies for the E antigen are present in some antisera but not in others.

Top trough: antiserum prepared with D205/37M, an early bleeding. Well: D205/37M, 10× M extract, containing E, M, and F antigens. Bottom trough: antiserum prepared with D205/37M, late bleeding of a different rabbit. A third arc (*E*) is shown.

mind, filtrates of cultures grown in the usual way in modified Todd-Hewitt broth and of cultures grown in DKW medium with the pH maintained at 7.3 or over were concentrated and examined for F. It was absent. The lack of F in F108B-M appears, therefore to arise from failure to produce the antigen, rather than from its release from the cells to the culture medium during growth.

A fuller characterization of F must await purification and chemical analysis of the antigen.

*The E Antigen.*—In most of the immunoelectrophoretic patterns described in the preceding sections, only two arcs were observed when M extracts of D205/37M reacted with homologous antiserum, as shown in Fig. 15, upper pattern. Later bleedings from some of the same rabbits, tested with the same extracts, showed a new arc located towards the cathode from the M antigen arc (Fig. 15, lower pattern). This was arbitrarily named the E antigen. F108B-M and other strains of serotype 17 also possessed the E antigen (Fig. 16).

In testing several types of extract for the presence of E, it was noticed that occasionally an M extract or an acid (IP) extract had little or none of this antigen, whereas M and F were in the usual concentration. Repeated extraction

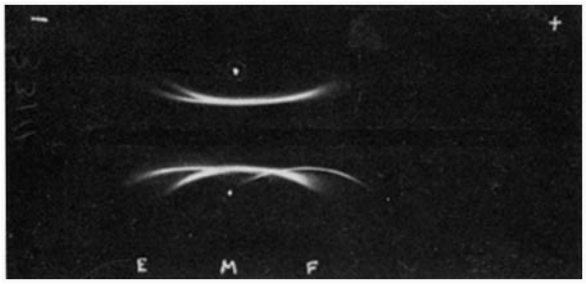


FIG. 16. F108B-M, as well as D205/37M, has the E antigen.
Top well: F108B-M, 10× M extract. Trough: antiserum for D205/37M, containing antibodies for E, M, and F. Bottom well: D205/37M, 10× M extract.

of D205/37M cells with dilute acid yielded larger amounts of E, whereas F decreased and finally disappeared. An extract of D205/37M, containing no demonstrable E (Fig. 17, upper pattern) was hydrolyzed by HCl at pH 2.0 for 30 minutes in a sealed tube in a boiling water bath. This treatment caused the appearance of E, a shift in the M arc towards the anode and broadening of the curvature of the F arc (Fig. 17, lower pattern). Acid hydrolysis of an extract of F108B-M selected to contain no E and, of course, lacking F, (Fig. 18, upper pattern) resulted in the appearance of E and again a slight shift of the M arc towards the anode (Fig. 18, lower pattern). This indicated that the E antigen was derived from the M antigen by acid hydrolysis and not from the F antigen.

The E antigen was shown to be serotype-specific because M extracts of heterologous strains did not react with anti-E serum to give an arc in the E position, because antibodies for E were not removed by absorption with a heterologous strain (S43/101) and because extracts containing E did not react with heterologous antisera to give an arc in the E position.

The fact that some sera with abundant anti-M do not react with E (Fig. 15, upper trough) is a clear indication that anti-M and anti-E are distinctly

different antibodies, and correspondingly M and E must be of different antigenic specificity. We are not confronted, then, with a situation in which two molecular species of different electrophoretic mobility (M and E) are reacting with the same antibody. Yet in some preparations (Figs. 17 and 18, upper troughs) M and E move together in electrophoresis and form a single arc with

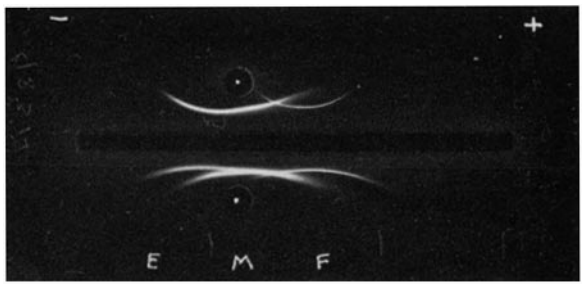


FIG. 17. Appearance of E following acid hydrolysis of M (D205/37M).

Top well: D205/37M, phosphate buffer pH 7.0 extract, concentrated. Trough: antiserum for D205/37M. Bottom well: the same extract as in top well, but brought to pH 2.0, immersed in boiling water bath 30 minutes, neutralized, and centrifuged.

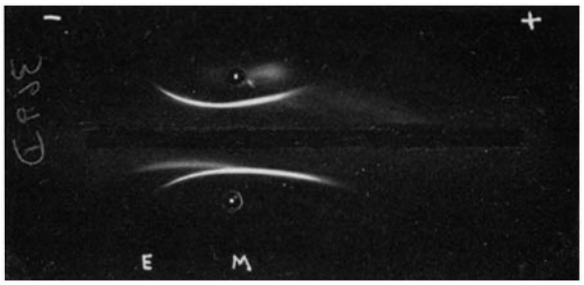


FIG. 18. Appearance of E following acid hydrolysis of M (F108B-M).

Top well: F108B-M, acid (IP) extract. Trough: antiserum prepared against D205/37M, containing antibodies for E, M, and F. Bottom well: same extract as in top well but brought to pH 2.0, immersed in boiling water bath 30 minutes, centrifuged, and neutralized.

their antibodies. This suggests that M and E in such preparations are in some sort of molecular union. Mild acid hydrolysis disrupts this union, allowing M and E to travel separately electrophoretically. These observations suggest that the serotype 17 M molecule possesses two antigenic determinants, M and E, that mild acid hydrolysis splits the molecule into fragments possessing one or the other of the determinants, and that these fragments migrate differently in the electrophoretic field and react with serologically distinct antibodies.

Another possibility is that E and M exist separately in the intact cell, but form a molecular complex during the process of extraction or after separation

from the cell. Further work will be required to determine which of these possibilities is correct.

The E antigen is found in simple saline extracts made at 56°C of heavy suspensions of D205/37M. The pH of such extracts is in the neighborhood of 6.0, which is also the final pH of the culture from which the organisms were harvested. It is conceivable that these mild conditions of heat and acidity might have led to the dissociation of E from M. A culture therefore was grown, using DKW medium (see Methods and Materials) which was kept from falling below pH 7.3 by frequent additions of 5 N NaOH. The harvested organisms were extracted three times with phosphate buffer, M/15, pH 7.0 at 100°C for

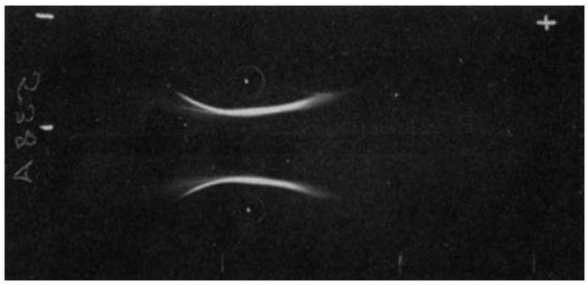


FIG. 19. A small amount of E is present in neutral extract of D205/37M grown at neutral pH.

Upper well: D205/37M (grown in DKW medium with maintained pH of 7.3 or over) phosphate buffer pH 7.0 extract. Trough: serum prepared with D205/37M, containing antibodies for E, M, and F. Lower well: same extract as in upper well, but diluted with equal volume of 0.85 per cent NaCl solution.

15 minutes according to the method of Cayeux (4). The extracts were pooled, concentrated by flash evaporation, precipitated by 0.6 saturation with ammonium sulfate, and dialyzed to remove the ammonium sulfate. Immunoelectrophoretic analysis showed that there was a small but definite amount of E in the buffer extract (Fig. 19). Since the pH of the culture and of the streptococci during extraction did not fall below 7.0, it is highly likely that the E present in the extract existed as such in the intact organisms. It could not have appeared as a result of environmental acidity occurring during growth or extraction. Furthermore, an aliquot of living cells grown in modified Todd-Hewitt broth under the same conditions of controlled pH and used as an absorbing suspension, removed antibodies for E as well as for M, indicating that the E antigenic determinant existed as such in cells not exposed to an acid environment (Fig. 14).

The E antigen is not found in detectable amounts in extracts made with distilled water, formamide, phenol, or active trypsin. It is rapidly rendered serologically inactive when crude acid extracts or M extracts are treated with

trypsin. E and M have not been successfully separated by precipitation of M extracts at various pH values or by ammonium sulfate precipitation.

Does E have any role in virulence, and do its antibodies have an effect in bactericidal and mouse protection tests? This question cannot be answered with certainty, chiefly because we have been unable to develop a serum having antibodies for E but not for M. All sera that give an arc for E also give an arc for M, the latter being always a stronger arc than that for E. Bactericidal tests with sera containing anti-E as well as anti-M showed little more effect than when antisera with anti-M but lacking anti-E were used. Mouse protection tests showed a greater effect from an anti-E plus anti-M serum than from anti-M serum alone, but since there is no way to be certain that the amounts

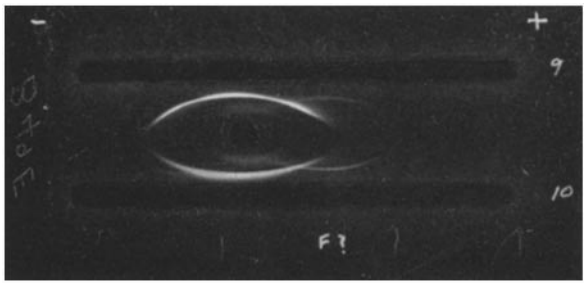


FIG. 20. S43MV has a weak arc in the F position.

Troughs: two bleedings from rabbits immunized with S43MV. Well: 10× M extract of S43MV. The heavy arc presumably is M, and the faint arc between M and the well is unidentified.

of anti-M in the two sera were the same (indeed, they were very probably different) it is impossible to know whether antibodies for E were in part or at all responsible for this difference.

The E antigen is present in extracts of strains that are of low virulence for mice as well as in strains that have been passed serially and have become highly virulent. In this respect, it resembles the M and F antigens.

*Occurrence of E, M, and F antigens:* Eighty-four type 17 strains have been studied. They were isolated between 1932 and 1945, and have been preserved in the dried state.[3] Two strains had received serial passage through mice (D205/37M and J17E/110) but the others had not. All the strains had the M antigen,

[3] It is of some interest to note that serotype 17 strains, which were among the commonest streptococci in military camps and in the civilian population during World War II (26) are now exceedingly rare. Personal communications from Dr. Elaine Updyke and Dr. M. T. Parker indicate that no serotype 17 strains have been sent to the streptococcal reference laboratories in Atlanta, Georgia, or Colindale, England, during the past 10 years. Dr. Lancefield recently isolated several serotype 17 strains in a military base in the United States (personal communication) but unfortunately these strains were not preserved.

and when suitable extracts were tested against suitable antisera in immunoelectrophoresis, all were shown to have the E antigen. Seventy-three of the 84 strains possessed the F antigen as well. This was demonstrated by testing 10× M extracts in capillary precipitin tests against a serum containing only antibodies for the F antigen and by immunoelectrophoresis using unabsorbed serum containing both anti-M and anti-F. Eight strains had the E and M antigens, but no trace of F antigen either by capillary precipitin tests or immunoelectrophoretic analysis. Three strains had small amounts of F antigen by capillary precipitin tests, but to demonstrate the antigen in immunoelectrophoresis it was necessary to use a highly concentrated extract prepared by precipitating crude acid extracts at pH 2.0 and taking up the precipitate in a minimal amount of saline solution. No strain was encountered that had the F antigen without also having M and E.

To determine whether the F antigen of serotype 17 occurs in other serotypes, M extracts of representative strains of all presently recognized serotypes were tested in capillary precipitin tests. All of these were negative except for a weak overnight reaction with serotypes 4, 12, and 15. These reactions were too weak to be demonstrated by immunoelectrophoresis. Absorption of anti-F serum with serotype 15 suspensions removed the antibodies responsible for the cross-reactions without affecting the antibodies for E, M, or F. Thus it appears that the immunologically specific F antigen of serotype 17 occurs only in strains of that serotype.

Immunoelectrophoretic studies have been made of M extracts and homologous antisera of strains of serotypes 1, 2, 3, 5, 6, 12, 14, 15, 18, 19, 24, 28, 39, and 51. It is hoped to report the findings of these studies in detail later. For present purposes suffice it to say that none of these show a strong arc in the same position as the arc of the F antigen of serotype 17. In M extracts of some serotype 6 strains, however, there is a rather weak arc having the same mobility as the F antigen of serotype 17, and this may be an F-type antigen in serotype 6 (Fig. 20). Unfortunately no variants have been encountered that lack the fast moving antigen, so that it has not been possible to assess the role of this component of serotype 6 organisms in virulence or to test for other biological properties.

The occurrence of an arc in the E position in serotypes other than 17 has not been encountered frequently. Some bleedings of rabbits immunized with serotype 6 strains had a suggestive band on the cathodal extremity of the M arc. Fig. 21, upper pattern, shows the arc formed when such a serum reacted with an extract that formed a single rather broad arc. The lower pattern shows the additional arc formed after the extract had been hydrolyzed with acid. This is quite similar to the release of E on hydrolysis of serotype 17 extracts, although in the case of serotype 6 the new arc did not move as far towards the cathode.

*The T Antigen.*—D205/37M, with which most of the present studies have

been performed, lacks a T antigen. Most other serotype 17 strains have a T antigen that is shared also with serotypes 15, 19, 23, 30, and 47 (27). Trypsin extracts for T according to the procedure of Lancefield and Dole (12) were made of the serotype 17 strains F108B-M and J17E/110, and of the serotype 47 strain C744/Rb4. On immunoelectrophoresis of these extracts with antiserum prepared with F108B-M the T arc was located close to the well of origin, having (when allowance was made for electroendosmosis) a slow mobility towards the anode (Fig. 22). A second, fainter arc was located farther to the anode. This arc was shown to be non-specific because it was also given by trypsin extracts of heterologous strains.

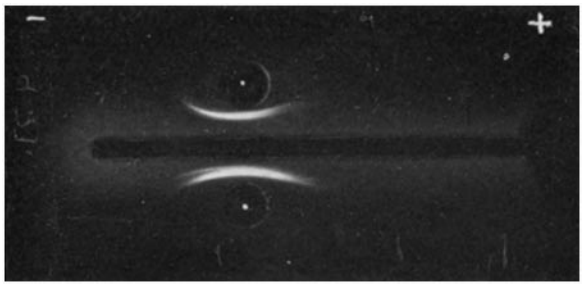


FIG. 21. Appearance of E on acid hydrolysis of M of S43/137.

Top well: S43/137, acid (IP) extract. Trough: antiserum prepared with S43FL. Bottom well: S43/137, acid (IP) extract brought to pH 2.0, immersed in boiling water bath 15 minutes, centrifuged, and neutralized. A second arc appears following acid hydrolysis. This new arc seems to be equivalent to E in serotype 17.

A large quantity of organisms is necessary to give a sufficiently concentrated trypsin extract to form a visible arc in immunoelectrophoresis, and even so the arc is faint. In position, this arc could be confused with the M arc. It differs, however, in its general appearance, and the extracts in which the two antigens are found are different. The T arc is a fine line that is nearly symmetrical and does not have the flattened, attenuated form, or the flared ends that are characteristic of the M arc. Trypsin extracts that contain T do not contain M, for the latter has been destroyed by the enzyme. Acid extracts and M extracts that contain M do not contain T, for the latter is destroyed by heat and acid. T is absent from, or present in too low concentration to be detected in, the other types of extract employed in this study. The T antigens of serotypes 1 and 2 form arcs similar in form and position to the T antigen of serotype 17. The secondary agglutinating antigen described in the 15, 17, 19, etc., series (27) has not been encountered in these studies.

*Other Antigens.*—Lancefield has described R antigens occurring in several serotypes of Group A streptococci (23). We have not studied these extensively

by immunoelectrophoresis, but preliminary examination of the R antigens of serotypes 3 and 28, using preparations and antisera kindly supplied by Dr. Lancefield, have shown that the position of the arcs of the R antigens in the two serotypes are quite different from each other, and have a faster mobility to the anode than the M antigens of those types. The R antigen has not been encountered in serotype 17 streptococci.

Freimer (28) has described immunoelectrophoretic and other characteristics of two antigenic substances present in streptococcal cell membranes.

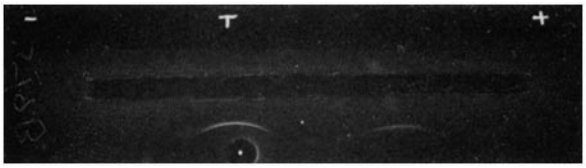


FIG. 22. T of serotype 17.

Trough: antiserum for F108B-M, containing anti-T. Well: trypsin extract of J17E/110 containing T. The minor arc on the anodal end of the slide is from an unidentified non–serotype-specific antigen.

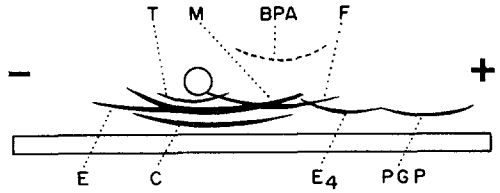


FIG. 23. Composite diagram of immunoelectrophoretic patterns of cellular antigens of Group A streptococcus, serotype 17. Bovine plasma albumin arc has been added for comparison.

We have observed a number of other precipitating cellular antigens in immunoelectrophoretic studies of serotype 17 streptococci using homologous and heterologous antisera prepared by immunizing with whole cells suspended in Freund's adjuvant, with cell walls or with cytoplasm released by disruption of cells in the Mickle disintegrator, or by testing other types of extracts; *e.g.*, material released into solution by phage-associated lysin or *Streptomyces albus* enzymes. These have been studied too little to justify presentation here, and are mentioned merely to avoid giving a false sense of simplicity concerning the antigenic structure of the serotype 17 cocci.

*Composite Diagram of Serotype 17 Cellular Antigens.* Fig. 23 gives a composite diagram of the immunoelectrophoretic appearance of the cellular antigens of serotype 17 streptococci. It was made by projecting several patterns from an enlarger, aligning the images of the antigen wells and serum troughs of succes-

sive slides and tracing the precipitin arcs. No single streptococcal extract contains all the illustrated antigens in optimal amounts and no single serum possesses all the antibodies concerned in the reactions shown.

## DISCUSSION

That crude acid extracts and M extracts may contain antigens other than the group carbohydrate and the M protein was shown early by Lancefield (29), who described non-serotype-specific cross reactions with heterologous antisera. Depending largely on classical immunological technics, particularly absorption of antisera with strains of varying antigenic composition, Lancefield also demonstrated that in certain serotypes (28 and 3) M extracts contained another class of protein antigen, the R antigen (23). McCarty has shown that M extracts contain polyglycerophosphate (2).

In view of the multiplicity of antigens demonstrated in extracts of Group A streptococci, as established by the investigations just enumerated, it is not surprising that complex patterns should be encountered when acid extracts, M extracts, and other types of extract are used in immunodiffusion and immunoelectrophoretic tests of Group A streptococci. So far only a few studies of the cellular antigens of streptococci have been made, using the newer technics. Pierce (30) studied Group A strains of three serotypes (12, 14 and 36) by agar double diffusion tests in tubes and described as many as ten zones of precipitate. Schmidt (31) found a single band in a type 19 strain when highly purified M substance was used, employing the Ouchterlony technic. Wahl, Cayeux, and Derlot (3) found three arcs in immunoelectrophoretic analysis of serotype 39 strains, and Cayeux (4) similarly found two arcs with serotype 24 strains. Freimer (28) has described two arcs in trypsin extracts of streptococcal cell membranes. Fox (32) has published a double diffusion pattern of serotype 14 streptococci in which one of three lines of precipitate was due to M of serotype 14. Jennings (33), Harris *et al.* (34), Halbert and Keatinge (21) and Grabar (35) have studied the extracellular products of Group A streptococci by agar diffusion or immunoelectrophoretic technics, It is likely that some of the cellular antigens were present in the culture filtrates, but of these only the group carbohydrate was identified.

In the studies presented here it has been possible to demonstrate the immunoelectrophoretic characteristics of seven cellular antigens found in serotype 17 streptococci. Four of these (E, M, F, and T) are serotype-specific or serotype-associated. One is group-specific (C) and the other two ($E_4$ and PGP) have a wide distribution among the groups.

Chief interest, of course, attaches to the M antigen because of its demonstrated relationship to virulence. It was hoped at the onset of this study that the appearance of the M arc in immunoelectrophoresis would be so characteristic as to allow a presumptive identification of M thereby, but this has not been borne out by experience. Not only does the exact electrophoretic mobility of the M antigen vary to some extent with the mode of extraction, but the oc-

currence of arcs formed by other antigens in positions close to that of the M arc (C and T, for example) makes such identification uncertain. Further, the electrophoretic mobility of the M of strains of various serotypes varies, within limits, rather widely, as we hope to show in later publications.

Advantage may be taken, however, of several further aids as adjuncts to immunoelectrophoresis. These include the use of absorbed sera, known to have antibodies for certain antigens and to lack others. Various types of extracts may be used, for some contain certain antigens and lack others. And finally, biological tests, such as the bactericidal test with human blood or the mouse protection test may be used to discover whether anti-M is present in the serum.

An antiserum may be absorbed with a suspension of a Group A strain of heterologous serotype, removing non-specific or non-serotype-associated antibodies (anti-C, anti-$E_4$, anti-PGP). Such antisera used in immunoelectrophoresis will restrict the possibilities in determining identity of the arcs. They may however, contain antibodies for certain type-associated antigens, such as T, R, and F as well as M. The electrophoretic mobility of F and of some of the R antigens distinguishes them from M. T may be differentiated by its absence from acid extracts, and by its presence in trypsin extracts (in which the M, of course, has been destroyed).

In those serotypes that have been most thoroughly studied, and in which variants are available that are known to lack one or another of the antigens, it is possible to prepare absorbed antisera that are specific for a single antigen, or that lack antibodies for a particular antigen. We have shown that antisera specific for the M of serotype 17 may be prepared by immunizing with strain F108B-M (which contains M but not F), and absorbing with a strain of heterologous serotype in Group A. Antiserum specific for the F antigen of serotype 17 may be prepared with D205/37M (which contains both M and F) and removing all antibodies except those for F by absorbing with F108B-M. Antisera for $E_4$ are obtained by selection of antisera that react with an extract containing $E_4$ but not with one containing PGP.

When purified antigens exist (C and PGP) it is possible to investigate immunological identity by placing the known and unknown or questionable antigens in adjacent wells and observing whether precipitate lines join, join with spur formation or cross, in double diffusion tests.

Thus by combining insights gained in immunoelectrophoresis using absorbed and unabsorbed sera, and using various types of extract and purified preparations of particular antigens when these are available, it is usually possible to identify a particular arc with a high degree of confidence.

Of the antigens just discussed, the E antigen is unique because it can be detected only by immunoelectrophoresis. This is because it has not been possible to prepare an anti-E serum that does not also have anti-M. One can, of course, obtain an anti-M serum without anti-E, since early bleedings of rabbits have

no anti-E, or have so little that it fails to form a visible arc in immunoelectrophoresis.

The possibility we have suggested that the M protein molecule may possess two antigenic determinants, M and E, is not without parallel. For example, Lapresle *et al.* (36) found that human serum albumin could be split into three components by a proteolytic enzyme from rabbit spleen, and these components had different electrophoretic mobilities and reacted with antibodies of different immunological specificities in immunoelectrophoresis. Grabar (37) has made similar observations on splitting of fibrinogen by plasmin. In both examples cited the unsplit native protein formed a single arc in immunoelectrophoresis, resembling our experience with certain preparations of M before acid hydrolysis (Figs. 17 and 18).

The drastic conditions customarily used to separate M from the streptococcal cell could easily result in severe changes in the native protein as it exists in the cell, and the separation of E from M might be one of these. Nevertheless, it is unlikely that E is simply an artifact of extraction, resulting from acid treatment of the cells, because some E is detected in neutral extracts of cells that have been grown in medium which has been maintained at a pH of 7.3 or over. Furthermore, antibodies for E are produced by whole-cell suspensions that have been treated only by resuspending in 0.85 per cent saline solution after centrifugation and heating at a temperature of 56°C for 30 minutes, and it is hence likely that E exists as such in the intact cells.

F appears to be a new class of streptococcal antigen, unlike any hitherto described. It differs in electrophoretic mobility from the other antigens described here. It differs by being liberated almost completely from the cells when the pH of the culture medium is maintained at pH 7.3 or over. It is extracted from cell suspensions more completely than other antigens by distilled water or phosphate buffer at pH 7.3 or over, at room temperature. It is digested by trypsin more readily than is T, and it is present in strains that do not have the recognized T of the 15, 17, 19, etc., series. It differs from C, $E_4$, and PGP by being digested with trypsin and by being destroyed by formamide extraction. Its antibodies do not confer protective power on human blood but may have a moderate mouse protective effect.

The fact that the F antigen is largely released to the medium when the environmental pH is maintained at 7.3 or above raises the question of whether it should be considered a cellular or extracellular product of the cocci. A somewhat similar circumstance is found with streptolysin S, which is firmly bound to the cells under ordinary conditions of growth in broth, but is released to the medium when serum (38) or nucleic acid fragments are present (39, 40). Some portion of certain unquestioned cellular antigens may be released to the culture medium during growth, as shown by Olarte (41) with respect to M and Halbert and Keatinge (21) with respect to C, but the quantity of these antigens released is relatively small compared to that remaining associated with the cells. It is

thus apparent that an antigen cannot be classified as cellular or extracellular merely because it is found in one location or the other, but its most characteristic site must be ascertained, and this may vary strikingly according to environmental conditions. For the purposes of this report F is considered to be a cellular antigen, although it is recognized that further study may require a change in this judgment.

SUMMARY

The immunoelectrophoretic characteristics of the known cellular antigens of serotype 17, Group A streptococci have been presented. These include C, M, T, and polyglycerophosphate. In addition, three hitherto undescribed antigens of serotype 17 have been encountered.

The F antigen occurs in most serotype 17 strains, has a faster electrophoretic mobility than M, appears in acid, distilled water, and other extracts of harvested cells, is released from the cells in large quantity into the culture medium during growth when the pH is maintained at 7.3 or over, is probably protein in nature, and may play a minor role in mouse virulence of serotype 17 strains. Its antibodies do not confer bactericidal power on human blood.

The E antigen is serotype-specific and is closely associated with the M antigen. The suggestion is made here that E is a part of the M molecule, acquiring independent electrophoretic mobility when separated from the rest of the M molecule by acid hydrolysis and carrying an antigenic determinant serologically distinct from the determinant on the rest of the M molecule. E can be recognized only by immunoelectrophoresis. Its role, if any, in virulence has not been established.

The third antigen, $E_4$, is a non–serotype-specific antigen found in most serological groups and types of hemolytic streptococci. It is serologically related to polyglycerophosphate, but its chemical nature has not been determined. It appears to be unrelated to virulence.

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