

THE USE OF PRECIPITIN ANALYSIS IN AGAR FOR THE STUDY OF
HUMAN STREPTOCOCCAL INFECTIONS*

III. THE PURIFICATION OF SOME OF THE ANTIGENS DETECTED BY
THESE METHODS

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PLATES 29 TO 32

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In order to understand the pathogenesis of any infectious process, it is obviously necessary to have knowledge of the substances produced in the tissues by the micro-organisms which may play active roles in the disease. In the past, such substances have been detected by much effort, or by chance. When they have been discovered, evidence regarding the production of such materials *in vivo* during the infection has often been obtained by examining human patients for antibody response to these products. It has been pointed out previously (1-3) that the use of precipitin analysis in agar furnishes a valuable tool for estimating the total number of antibody responses which occur as a result of infection. This in turn, of course, reflects the total number of antigens or toxins secreted by the infecting agent *in vivo*. The use of *human* antibodies automatically restricts our studies to those antigens formed *in vivo*, and prevents diversion of interest to substances produced by the micro-organism in cultures alone. The use of these precipitin technics also furnishes the tool for following the isolation, purification, and eventual characterization of each of the antigens so detected. It is the purpose of the present report to describe the use of this approach for the separation and analysis of streptococcal antigens detected with human antibodies.

Human sera have revealed a surprisingly large number of extracellular antigenic components to be present in a Group A streptococcal culture concentrate (1-3). Pooled gamma globulin samples from normal individuals were also very

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rich in antibodies to these streptococcal products. As the ultimate goal here was the isolation of all the components detected immunologically, it was of importance to use technics which tend to sort out individual proteins in complex mixtures. Two such methods have been developed and refined in recent years; continuous flow electrophoresis (4) and column chromatography on calcium phosphate preparations (5). These methods have been utilized jointly in the studies reported here on the purification of the extracellular streptococcal antigens detected with human sera. Their value is pointed up by the fact that thus far five of the components found have already been separated in an apparently high state of purity.

Materials and Methods

Culture Concentrates.—The Group A *Streptococcus pyogenes* strain C203S employed in the previous studies (1) was used for most of the observations here. The organisms were grown in 5-gallon pyrex carboys containing 11.5 liters of the following composition:

8,130 ml.	proteose peptone No. 3 tryptic digest dialysate
1,100 “	asparagine mixture
1,100 “	H ₂ O
440 “	8.4 per cent NaHCO ₃
690 “	50 per cent glucose

The first three components were autoclaved, and the latter two were added aseptically after Seitz filtration. The dialysate base was prepared by adding 3,240 gm. of proteose peptone No. 3 (Difco) to 18 liters of H₂O at 37°C., and adding to this 18 gm. of bacto-trypsin (1:250, Difco) in a heavy suspension. Digestion was allowed to proceed for 1 hour, then the digest was placed in large dialysis tubing, and the filled sacs put into 31.8 liters of H₂O. Dialysis was allowed to proceed in the cold (4–6°C.) for 7 to 8 days, with daily agitation, after which time the dialysate was harvested and used in the preparation of the medium. The asparagine mixture contained the following components:

Bacto-asparagine (Difco)	25 gm.
Sodium citrate·2 H ₂ O	9 “
KH ₂ PO ₄	18 “
Bacto yeast extract	20 “
Phenol red	100 mg.
H ₂ O	1000 ml.

Seed bottles of the same medium, consisting of 10 to 15 per cent of the volume of the bulk medium used, were inoculated from lyophilized vials during the evening. The carboys were inoculated with the contents of the seed bottles in the morning, and the air space was flushed with nitrogen. During incubation at 37°C., the pH was maintained at 7.0–7.5 as judged by the color of the phenol red, with repeated additions of 5 N NaOH. The total incubation period was usually about 11 to 13 hours, and during this time for 20 such large scale growths, an average of 855 ml. of 5 N NaOH was required per carboy of medium to maintain the pH. After chilling overnight at 4°C., the cells were centrifuged off in the cold, and to the supernate was added solid (NH₄)₂SO₄ to 85 per cent saturation (full saturation was assumed to be 72 gm. per 100 ml. of solution). After several days in the cold, the scum was scooped off the surface, dissolved in water, and dialyzed till largely sulfate-free. The sedimented precipitate was also collected, centrifuged, and dialyzed. The two solutions were pooled and reprecipitated with solid (NH₄)₂SO₄ to a concentration of 75 per cent saturation in the cold for several days. The precipitate was collected in the centrifuge, dialyzed thoroughly till sulfate-free, against dis-

tiled water adjusted to pH 6.5-7.0. The variably tan solution was then clarified in the Spinco preparative centrifuge using the No. 20 rotor at 12,000 r.p.m. for a half-hour. The clear solution was then lyophilized from the frozen state. This represented the crude concentrate which was the starting material for further purification. In some experiments, the supernates obtained from the crude concentrates after removing the precipitates thrown down at pH 4.3, were also used as starting material. This precipitation removed the bulk of the streptolysin "O" from the solution, but left behind many of the antigens detected with human gamma globulin (1, 2).

The crude concentrate of the Group C strain of streptococcus was a commercial product kindly donated by the Wellcome Research Laboratories¹ (Beckenham, England), and represented crude streptokinase-streptodornase. It was in the form of a lyophilized ammonium sulfate precipitate from which the organisms had been removed, and was very rich in streptolysin "O."

Continuous Flow Electrophoresis.—The continuous flow electrophoresis apparatus used was the Spinco Model CP (Beckman Instruments, Inc., Belmont, California), which is a modification of the apparatus described by Durrum (8). All the separations were carried out in a cold room at about 4-6°C. with the anode on the left. After a number of preliminary trials, sodium acetate buffer was most frequently used at pH 6.0 and ionic strength (μ) 0.06. The samples to be applied to the curtain were adjusted at 2 to 6 per cent protein concentration, and thoroughly dialyzed against the buffer used for the electrophoresis. They were all then thoroughly clarified by centrifugation in the cold at high speeds, and occasionally by filtration through coarse Seitz filter pads under pressure. The rate of flow of buffer through the backwash siphons which rinse the electrode chambers was slowed down so that the buffer drained from the system amounted to about 5 to 6 liters per day. Since some of the runs were prolonged (up to 14 days), and since some of the drip point fractions contained fairly low concentrations of protein which could deteriorate during this time, the following arrangement was used for collecting the fractions. Dialyzing tubing (Visking, $3\frac{3}{32}$ inches inflated diameter) was tied firmly around drip point spouts 1 and 2, 3 and 4, etc. The free end was knotted, and the entire series of sacs receiving the drip point fractions was placed in a saturated solution of $(\text{NH}_4)_2\text{SO}_4$ adjusted to pH 6.5 to 6.8 by the addition of a small amount of solid Na_2HPO_4 . An ample excess of solid ammonium sulfate was maintained in this bath, while excess solution that formed was removed daily. In this way, the fractions were both rapidly concentrated and simultaneously precipitated as they were obtained. No definite evidence of deterioration of the fractions was ever noted, and they were always freely soluble in water after centrifugation. With buffers of μ 0.06, usually about 300 to 350 volts were applied across the curtain and a current of 60 to 70 milliamperes was achieved. With some fractions, it was possible to use acetate buffer of lower ionic strength (0.02), in which case 400 to 450 volts were applied with a current of 30 to 33 milliamperes. With many of the fractions, attempts to use buffer of this low ionic strength resulted in distortion of the separations, with irregular deposits of material on the curtain. The cause of these effects was not clear. All the drip point fractions thus obtained were stored at 4°C. as the ammonium sulfate precipitates. They seemed to be stable under these conditions. The curtains were thoroughly dried in the horizontal position at 130-140°C. for about half an hour. After inspection in a darkened room with ultraviolet light of peak wave length about 250 m μ for the presence of fluorescing bands, they were stained with amido black 10B solution as described by Grassmann (9).

In early tests with the continuous flow electrophoretic separation of these components, it was found that hyaluronic acid which was present in the crude Group A concentrates to varying amounts, appeared to interfere to some extent with the resolution. It was demon-

¹ The author is deeply grateful to Dr. H. Proom of the Wellcome Research Laboratories for his generosity in supplying this material and horse antistreptococcal serum.

strated that addition of protamine sulfate (Nutritional Biochemicals, Cleveland) would remove this polysaccharide, without significantly affecting the content of antigens detected immunologically. When hyaluronic acid was found to be present, the protamine was added in very slight excess as an aqueous solution at pH 6.7, to an aqueous solution of the crude concentrate at the same pH. The light tan to white precipitates which formed were thoroughly removed by centrifugation, and the supernate was then exhaustively dialyzed against the buffer being used for the electrophoretic run. It was of some importance that the sample being fed on the curtain be crystal clear, and not be viscous, as these factors tended to distort the patterns of separations. Eleven large scale or moderate runs were carried out with various crude concentrates and essentially similar results were obtained in all.

Calcium Phosphate Chromatography.—The chromatography of various electrophoretic drip point fractions was carried out in the cold (4–6°C.) according to the recommendations of Tiselius *et al.* (5). The samples were all dissolved in 0.001 M sodium phosphate buffer at pH 6.8, and dialyzed thoroughly against this buffer till sulfate-free. Solutions containing 5 to 50 mg. protein/ml. were loaded onto the column by allowing them to flow gently through fine polyethylene tubing onto the top of the column under a layer of 0.001 M phosphate buffer. The columns used were supported on a 2 to 4 mm. layer of glass wool on coarse sintered glass disks fitted into a glass joint. In our experience, short columns seemed to be as effective as long ones, and often they were prepared only as high as they were wide. The widths used ranged from 10 to 40 mm. With proteins that were adsorbed, it was found that as much as 16 mg. of protein could be applied to each milliliter of final packed calcium phosphate gel. Because of this high capacity, quantities of protein as large as a gram or more could be conveniently processed. The fractions were collected in a fraction collector (either constant volume or constant time interval) and the individual tubes were examined in a spectrophotometer at 280 $m\mu$ wave length. Elutions were carried out with stepwise increases in concentration of sodium phosphate buffer at pH 6.8, until 0.2 M was reached. At this point, approaching the limit of solubility of the phosphate at cold room temperatures, further salt increases were made by the inclusion of $(\text{NH}_4)_2\text{SO}_4$ in various concentrations to 0.2 M phosphate. The pH throughout was maintained at 6.8. When peaks of 280 $m\mu$ absorbing materials were found, a pool was made of the tube contents containing the bulk of the peak. The pool was concentrated (and the proteins precipitated) by placing it into small dialyzing tubing, and immersing it in saturated $(\text{NH}_4)_2\text{SO}_4$ solution in the cold, with frequent stirring. As much as a threefold decrease in volume would occur in the course of several hours. The various precipitates thus obtained were stored as such in the cold.

One troublesome difficulty was occasionally encountered with these calcium phosphate columns. After settling and packing, and sometimes even after the sample had been applied, the top of the column would separate to a small extent from the vessel wall in one or more areas of varying sizes. Although these looked quite disturbing, chromatographic runs made with such columns showed peaks and general patterns which seemed to reveal no effect on the resolution or character of the curves obtained. They would, of course, have been quite troublesome had multiple peaks been obtained at each stepwise elution. All of the purification procedures were carried out in the cold, at about 5°C.

Assays.—The protein estimations were made by ultraviolet absorption measurements at 280 and 260 $m\mu$ wave lengths in 1 cm. silica cells, and the concentrations estimated according to the formula: $1.45 D_{280} - 0.75 D_{260} = \text{mg. protein/ml.}$ (6).

The streptolysin determination was carried out according to the method previously described (1). The hemolytic units thus obtained were compared with the hemolytic units used by Dr. A. Bernheimer, by an exchange of samples between the two groups (7). As expected by estimation of the relative concentrations of erythrocytes used, length of incubation, etc., one unit used by the Columbia Laboratory represented approximately 1.5 times more streptolysin than did one Bernheimer unit.

The agar precipitin technic was a modification of the Ouchterlony method (2, 10). Bacto-agar (Difco) was used at 0.7 per cent concentration in 0.85 per cent NaCl, and it contained 0.04 M veronal as buffer and 0.3 M glycine. The pH was adjusted so that the cooled gel was at pH 7.4. Development of the precipitin bands was allowed to proceed at 4–6°C., and readings and photography were done at room temperature. To stabilize the components, all dilutions for the agar precipitin assays were made with a 1 mg./ml. solution of bovine serum albumin (Armour) in 0.05 M phosphate buffer at pH 6.8.

The proteinase and proteinase precursor assays were done by the milk clotting technic as used by Elliott (11). The diphosphopyridine nucleotidase (DPNase) assays were carried out by the Carlson modification (12) of the technic described by Kaplan (13). This streptococcal enzyme, recently discovered by Bernheimer, Carlson, Kellner *et al.* (28–30) has been investigated in some detail by them. The "C" carbohydrate was prepared by the hot formamide extraction technic of Fuller (14).

The pooled normal human gamma globulin was obtained through the American Red Cross.² Most of the study was carried out with one lot (270-2) prepared by E. R. Squibb and Sons, which was obtained from the plasmas of donors during the late winter months of 1954.

RESULTS

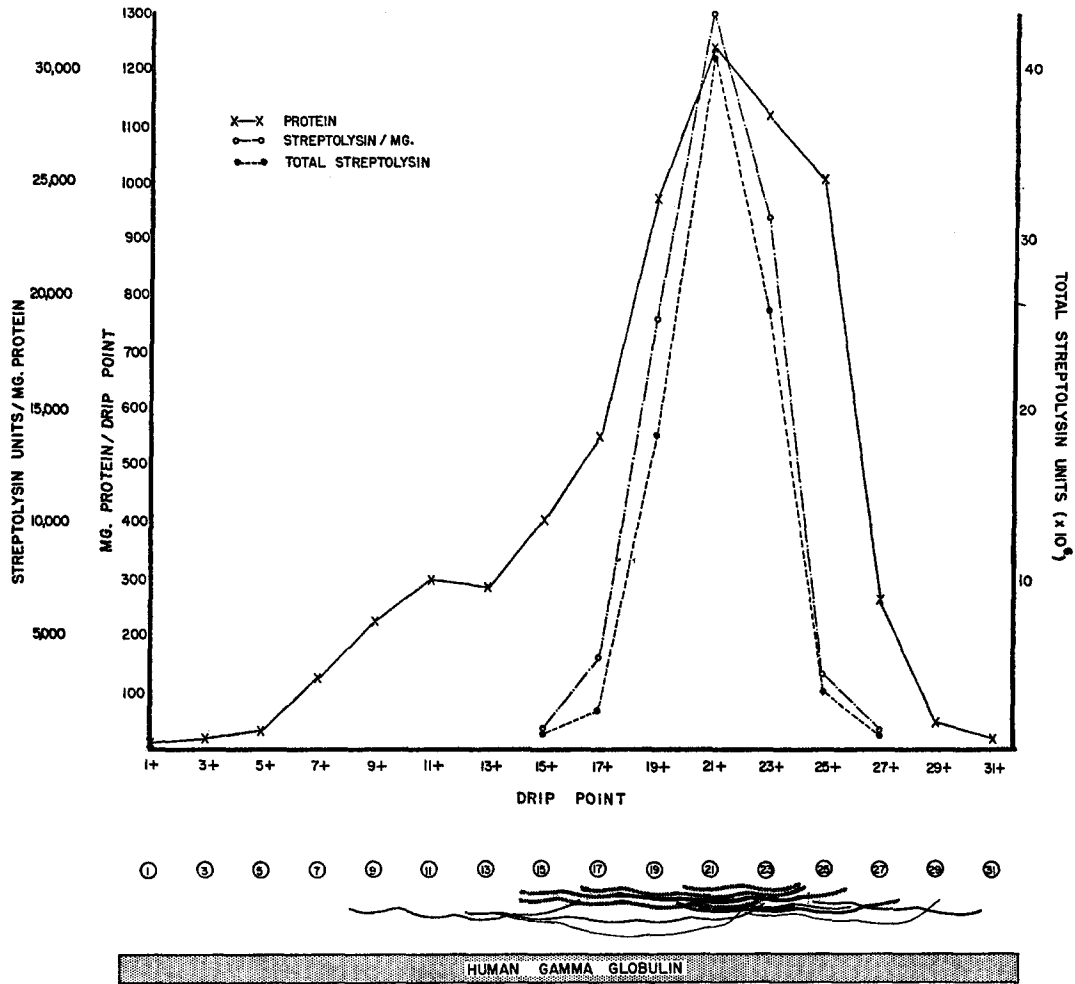
Continuous Flow Electrophoresis.—The results of a typical run, described below, characterizes much of the experience obtained with this method.

A total of 19.6 gm. of lyophilized crude concentrate of the culture supernate obtained from the growth of about 100 liters of C203S streptococci was dissolved in 280 ml. water (70 mg./ml.). The pH was adjusted to 6.7 with 1 N NaOH, and the solution placed in an ice bath. To this was added with stirring, 130 ml. of protamine sulfate solution in water at 24 mg./ml., pH 6.7, clarified by filtering while warm, and kept at 37°C. The resultant precipitate was allowed to stand for 5 minutes in an ice bath, and then was centrifuged off in the Spinco preparative centrifuge at 25,000 R.P.M. in the No. 30 rotor for half an hour. The clear supernate was concentrated to about half volume by pervaporation in the cold, care being taken to avoid air spaces in the dialyzing sac. The contents were then repeatedly dialyzed against sodium acetate buffer pH 6.0, ionic strength 0.06. The sample was finally centrifuged again and the clear supernate applied to the curtain as a 3 per cent protein solution. That volume of sample which could not be placed in the sample reservoir of the electrophoresis apparatus was stored frozen.

The voltage applied across the curtain was 310 volts, and the current obtained varied between 68 and 70 milliamperes. The 3 cm. diameter sample reservoir was used, and fresh sample had to be added roughly every 4 days. Approximately 14.9 ml. of sample (450 mg. protein) was fed per day above drip point 19+ for a total of 14 days. During this time about 6.3 gm. of protein were processed. The drip points, of course, were received into dialyzing sacs immersed in saturated $(\text{NH}_4)_2\text{SO}_4$ so that they were accumulated as precipitated suspensions. The total protein yields of each drip point are shown in Text-fig. 1, which also records the recovery of streptolysin "O." The potency of the crude concentrate applied to the curtain was about 7,900 hemolytic units/mg. protein, and it can be seen that the drip point peaks of streptolysin revealed about 32,000 units/mg. As in similar runs, practically all of the activity was recovered.

² The author is deeply indebted to Dr. J. N. Ashworth for his generous helpfulness and cooperation in this regard.

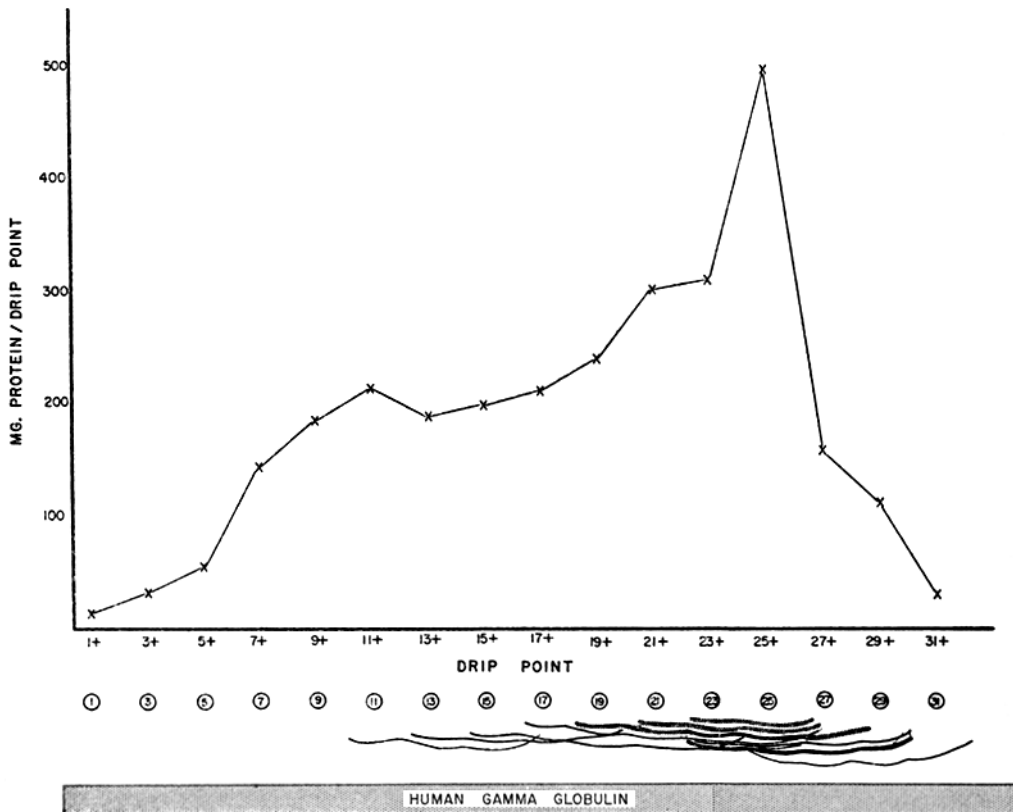
Samples of the drip point fractions were centrifuged and the precipitates dissolved in 0.05 M phosphate at pH 6.8. They were assayed for their content of precipitating antigens in a concentration of 1 mg. protein/ml. in wells adjacent



TEXT-FIG. 1. Results of continuous flow electrophoretic separation of Group A streptococcal extracellular antigens, strain C203S. Acetate buffer pH 6.0, μ 0.06 was used, with 310 volts and 68 to 70 milliamperes applied across the curtain. The drip point precipitin assays were carried out using a protein concentration of 1 mg./ml. in the antigen wells.

to each other against human gamma globulin. In Text-fig. 1 is also shown a reconstruction of the rough sorting out of the antigens that were found. Photographs of the plates from which the drawings were made are shown in Fig. 1.

In addition to this, it proved necessary to titrate each of these fractions on agar precipitin plates. Their content of a particular antigen could be roughly estimated in this way, and precipitin bands of some of the antigens were completely inhibited in antigen excess which obtained with protein concentrations of 1 mg./ml. The types of results obtained are shown in Fig. 2.



TEXT-FIG. 2. Results of continuous flow electrophoretic separation of Group A streptococcal extracellular antigens, strain C203S. The sample fed consisted of the crude concentrate from which a large portion of the streptolysin "O" had been removed by precipitation at pH 4.3 in the cold. The buffer used for the electrophoresis was sodium acetate pH 6.0, μ 0.02; 450 volts and 30 to 32 milliamperes were applied across the curtain. The drip point precipitin assays were carried out using a protein concentration of 1 mg./ml. in the antigen wells.

It can be seen that antigen excess band inhibitions were observed with a number of drip point fractions (17+–25+). In general, thus, it was found that much overlapping of the separation of the antigens occurred with the electrophoretic technic, but that it furnished a very useful rough sorting out.

In several instances, streptolysin "O" was largely removed from the crude

Group A concentrates by adjusting the pH to 4.3 in the cold, and quickly centrifuging the precipitates (1). The supernates still contained an abundance of the other streptococcal antigens, and these were processed by continuous flow electrophoresis. In two instances, with such preparations, they could be run at low ionic strength, μ 0.02 with acetate buffer at pH 6.0. Text-fig. 2 shows the total protein contents of the drip points, and the reconstruction of the agar precipitin results obtained in one such experiment. In this instance, the duration of the processing was short, only 3 days, but 1.25 gm. of protein was fed daily as an 8.8 per cent solution. It was felt that the low ionic strength may have contributed to the high processing rate in allowing larger voltages to be used, with subsequent enabling of increased sample feed and buffer flushing rates.

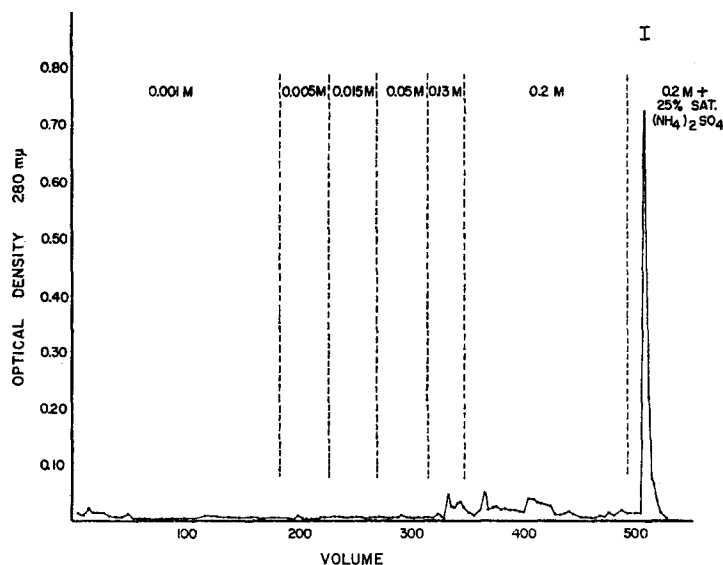
It was originally planned to proceed in the purification attempts by recycling each drip point fraction under slower flow rates, with lower ionic strengths and higher voltages so that each fraction could then be further spread and resolved. In a number of these trials quite erratic patterns resulted. The stained curtain in one such run is shown in Fig. 5, in which the streptolysin "O" peak drip points from the electrophoresis of a crude concentrate were recycled. Lyophilized drip point fractions were dissolved at 1.7 per cent protein concentration in acetate buffer at pH 6.05, μ 0.02, and the recycling attempt was made with this buffer using a very slow curtain flushing rate to allow the components a long exposure to the electrical field. The sample feed was adjusted so that 1.8 ml. was fed per day (31 mg. protein/day). Although it was apparent that the components appeared to start spreading well over a large segment of the curtain, some event in the middle of the curtain caused a serious distortion in the resolution over the lower half. The same sort of occurrence developed in a number of instances, but in one recycling attempt at higher pH and low ionic strength when a very small sample feed rate was used, the pattern of resolution seemed satisfactory. The stained curtain from this run is shown in Fig. 6. In this run, the streptolysin "O" peak from the electrophoresis of crude concentrate was used at 20.7 mg. protein/ml. in sodium phosphate buffer of μ 0.02, at pH 7.5. Approximately 35.2 mg. protein was fed/day in a short term run. In spite of apparent good resolution and separation seen on the curtain, considerable overlapping of the antigens present was still found on precipitin analysis, although the components were now spread from drip points 7 through 31. Because of these problems encountered with electrophoretic recycling attempts, most of the subsequent efforts were applied to calcium phosphate chromatographic separations of the individual drip points, with highly successful results.

Calcium Phosphate Chromatography.—

Fractionation of drip point 29+, Text-fig. 1:

The far right electrophoretic fraction obtained in Text-fig. 1 and Fig. 1 (29+) was dissolved in 0.001 M sodium phosphate buffer at pH 6.8, dialyzed against this buffer until thoroughly

sulfate-free, and 1.7 ml. of a solution containing 6 mg. protein/ml. (10.2 mg. total) was applied to a column 20 mm. wide by 30 mm. high. The concentration of buffer was increased stepwise as shown in Text-fig. 3, with the eluates revealing the recorded optical densities at 280 μ . It can be seen that no significant amount of protein was eluted till the eluant was increased from 0.2 M phosphate to 0.2 M phosphate containing 25 per cent saturated $(\text{NH}_4)_2\text{SO}_4$ (assuming 72 gm. $(\text{NH}_4)_2\text{SO}_4/100$ ml. solvent is 100 per cent saturation). Precipitin analysis of this fraction with human gamma globulin revealed that the principal component could be diluted to 12 $\mu\text{g./ml.}$ with persistence of the band. It was also found that a faint component became evident on longer development. This fraction disappeared upon dilution beyond 330 $\mu\text{g./ml.}$ (See Fig. 3.)



TEXT-FIG. 3. Calcium phosphate chromatography of electrophoretic drip point 29+ of Text-fig. 1. A total of 10.2 mg. of protein in 0.001 M phosphate buffer at pH 6.8 was loaded onto a column 30 mm. wide by 22 mm. high. Stepwise elution was carried out as indicated.

If the "minor" component when pure could be expected to titrate to extinction at roughly the same concentration as the principal one, it may be estimated that the latter is about 95 per cent free of any of the other streptococcal antigens. That this may be justified will be indicated below in separations of other antigens.

It was found that this chromatographic fraction revealed a diphosphopyridine nucleotidase (DPNase) potency of 1,900,000 units/mg. protein, which indicated very strongly that the major component was DPNase itself. That this is most likely is supported by the finding that this "major" band joins in a "reaction of identity" with the band occurring with a group "C" fraction which could be purified in a similar way. (See Figs. 10 *d* and 10 *e*.) The latter also migrated to the right of the streptolysin "O" in the electrophoretic separations

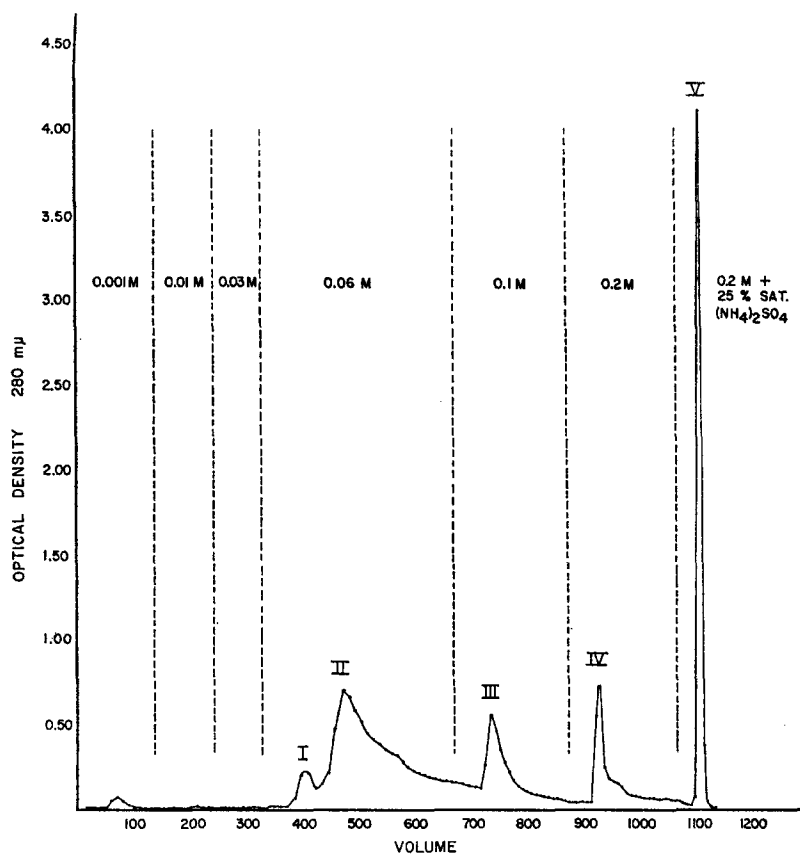
(see Text-fig. 7) and also was only eluted in the elution step from 0.2 M phosphate to 0.2 M phosphate with 25 per cent saturation of $(\text{NH}_4)_2\text{SO}_4$. The DPNase content of the processed group C component was also very high, 1,700,000 units/mg. protein.

Fractionation of drip point 27, Text-fig. 1: When the drip point fraction next to the far right in Text-fig. 1 and Fig. 1 (27+) was processed in a calcium phosphate column in a similar way, the following chromatogram was obtained (Text-fig. 4). In this run, a total of 223 mg. of protein in 22.5 ml. of 0.001 M phosphate buffer, pH 6.8 was applied to a column 30 mm. wide by 60 mm. high. It may be seen that essentially all of the protein was adsorbed, and that none was eluted until 0.06 M was applied, when a double peak was obtained. Other peaks were obtained as indicated. When these peaks were precipitated with ammonium sulfate, and then assayed by precipitin analysis with human gamma globulin, the results obtained are shown in Fig. 4. It may be seen that the starting material applied to the column showed three bands, one of which was absent in the 1 mg./ml. well owing to antigen excess, but which was seen in the 0.2 and 0.04 mg./ml. wells. Of the other components in the starting sample, one titrated to 0.2 mg./ml., and the one closest to the antigen well was found only in the 1 mg./ml. well. It may be noted that it was the antigen causing this latter band which was present in very high concentration in the first peak at 0.06 M. This component was thus concentrated at least 25-fold.

The second peak of the 0.06 M eluate proved to contain only one immunological entity, which assayed to 0.04 mg./ml., and which was inhibited by antigen excess at 1 mg./ml. (Fig. 4 b). By adjusting this fraction to high protein concentration in 0.1 M phosphate buffer at pH 8.0, and slowly adding saturated $(\text{NH}_4)_2\text{SO}_4$ adjusted to pH 8.0 at room temperature to the first faint turbidity, this developed as a silken sheen. Upon placing this tube in a water bath at 37°C. a heavy crop of crystals developed within 5 to 15 minutes. In appearance, the crystals were identical with the long needles of proteinase precursor described by Elliott (15), and they proved to reveal a comparable degree of milk clotting activity upon conversion to proteinase. It seems most likely, therefore, that the 2nd component eluted at 0.06 M is highly purified proteinase precursor and that the band seen with gamma globulin is actually due to this streptococcal product and antibody reacting with it. In further agreement with the identification of this system, is the finding that this band showed the "reaction of identity" with a crystalline proteinase precursor preparation previously obtained from Dr. S. Elliott, from a different strain of Group A streptococcus. Moreover, the Group C concentrate did not possess this component immunologically, nor did it reveal proteinase or precursor activity. It is clear now that failure to detect this precipitin system in previous studies with human gamma globulin (2) was probably due to use of too high a concentration of this component (0.5 to 1.0 mg./ml.) as this is apparently well within the zone of antigen excess

in which the precipitin band is prevented. These concentrations had been originally chosen on the basis of tests with rabbit antisera to the proteinase precursor, and were, therefore, quite misleading.

The eluates at 0.1 M (III) revealed four components and the 0.2 M eluate (IV) revealed two bands. In appropriate tests it was shown that the component



TEXT-FIG. 4. Calcium phosphate chromatography of the electrophoretic drip point 27+ of Text-fig. 1. A total of 223 mg. of protein in 22.5 ml. of 0.001 M phosphate buffer at pH 6.8 was applied to a column 30 mm. wide by 60 mm. high. Stepwise elution was carried out as indicated.

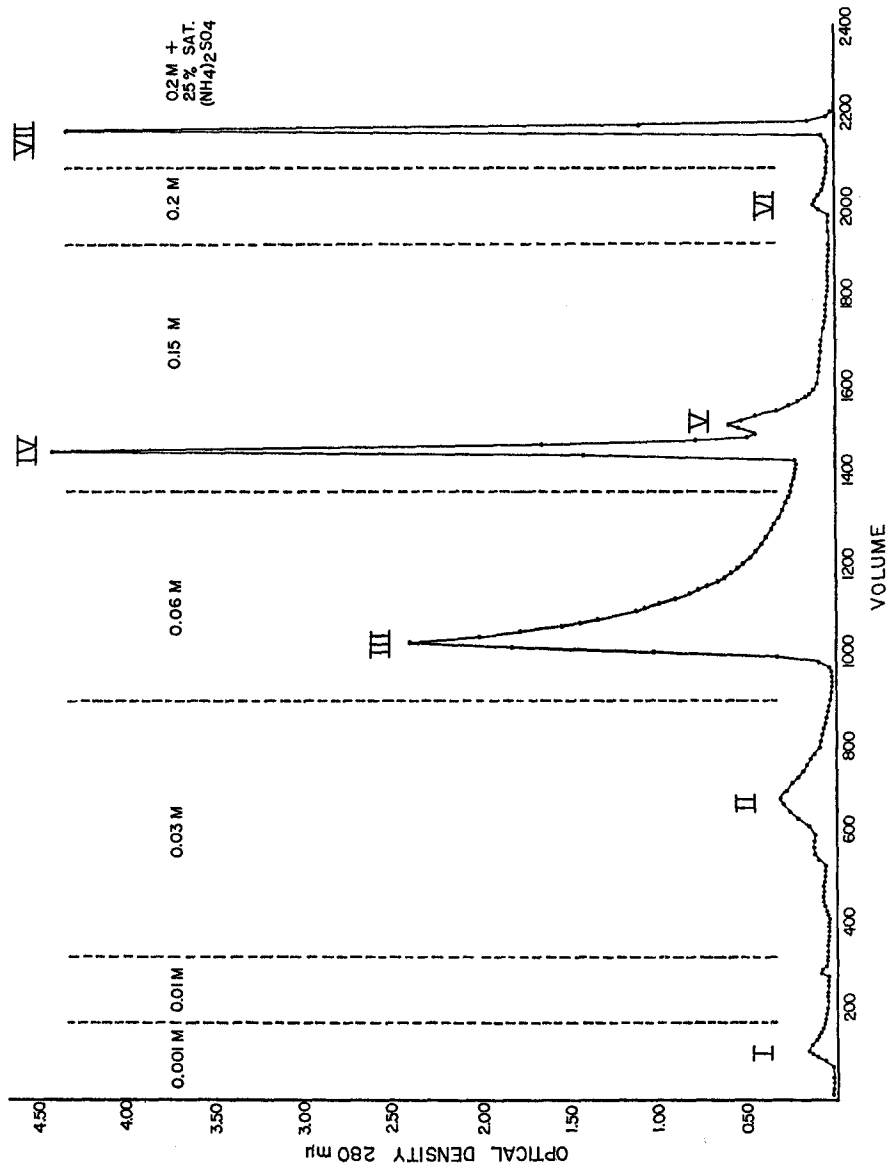
still present in the 0.04 mg./ml. well in both of these fractions was "identical" with the proteinase precursor. The fraction in the latter eluate (IV) which was revealed only in the 1 mg./ml. well proved to be distinct from the three non-precursor components in the previous eluate. This persistence of the proteinase precursor in these two latter eluates points up one of the very serious problems in calcium phosphate chromatography, that of tailing of the peaks.

Finally, the eluate obtained in the next step, 0.2 M + 25 per cent saturated $(\text{NH}_4)_2\text{SO}_4$, revealed two components. The principal component assayed to at least 0.04 mg./ml. (Fig. 4 c), and was distinct from proteinase precursor, as shown in Fig. 4 d. It proved "identical" immunologically with the factor found to be eluted at the same elution step in the chromatography of the principal antigen component in drip point 29+, shown in Text-fig. 3 and Fig. 3. As expected, it also revealed an extremely high potency of DPNase activity (1,550,000 units/mg. protein),³ in further support of its identification. The factor present in low concentration (1 mg./ml. only) in the DPNase fraction was "identical" with the low concentration component found in the 0.2 M eluate.

Fractionation of drip point 25+, Text-fig. 1: The next drip point (25+) of Text-figs. 1 and Fig. 1 was also chromatographed, with the results shown in Text-fig. 5 and Fig. 7. A column 40 mm. wide by 60 mm. high was used, and 813 mg. of sample was applied to it in 43 ml. of 0.001 M phosphate buffer pH 6.8. The sample applied to the column revealed five components, one being inhibited by antigen excess at 1 mg./ml. (Fig. 7 a). The small amount of unadsorbed protein was inactive immunologically. The delayed small peak which occurred with 0.03 M appeared to be homogeneous immunologically and could be diluted as far as 12 $\mu\text{g.}/\text{ml.}$ with persistence of the band (Fig. 7 d). At high concentrations it shows widening and sometimes striations after 5 to 6 days of development, which it is felt is most likely due to antigen excess effects (16, 17). The most important evidence for this is the fact that the striations do not reveal "reactions of identity" with any of the other bands in the same sample applied to the column; *e.g.*, see Figs. 7 h and 7 i). Should these striations be due to antigen-antibody systems other than the main component of the 0.03 M eluate, one would expect them to show such "reactions of identity." This 0.03 M component did show "reactions of identity" with similar fractions from two other analogous chromatographic runs, and in these also, the antigen was eluted after some lag following the addition of the 0.03 M buffer. This component also showed the "reaction of identity" with the "major" antigen of the *first* 0.06 M peak of Text-fig. 4. It seems likely that its failure to elute there at 0.03 M was due to an inadequate length of elution time with this buffer. When tested appropriately, this antigen does join that band found with crude concentrates which earlier evidence (2) had suggested was due to erythrogenic toxin-antitoxin system (see Figs. 7 i and 11 b). In passing, it may be noted that high concentrations of the DPNase fractions also produced striation effects, but "identity" joining reactions of these also indicate that only a single system is involved (see Figs. 10 d and 10 e).

The 0.06 M eluate proved immunologically "identical" with the 0.06 M eluate (II) of Text-fig. 4, could be readily crystallized and revealed proteinase pre-

³ The potency of this DPNase fraction was kindly checked and confirmed by Dr. A. Bernheimer.



TEXT-FIG. 5. Calcium phosphate chromatography of electrophoretic drip point 25+ of Text-fig. 1. A total of 813 mg. of protein was applied to a column 40 mm. wide by 60 mm. high, as a 1.9 per cent solution in 0.001 M phosphate buffer at pH 6.8. Stepwise elution was carried out as indicated.

cursor activity comparable to that component. It, therefore, also represents quite highly purified proteinase precursor.

The double peaks found with 0.15 M elution showed the presence of two further components in appreciable amounts, other than precursor which was still abundantly present.

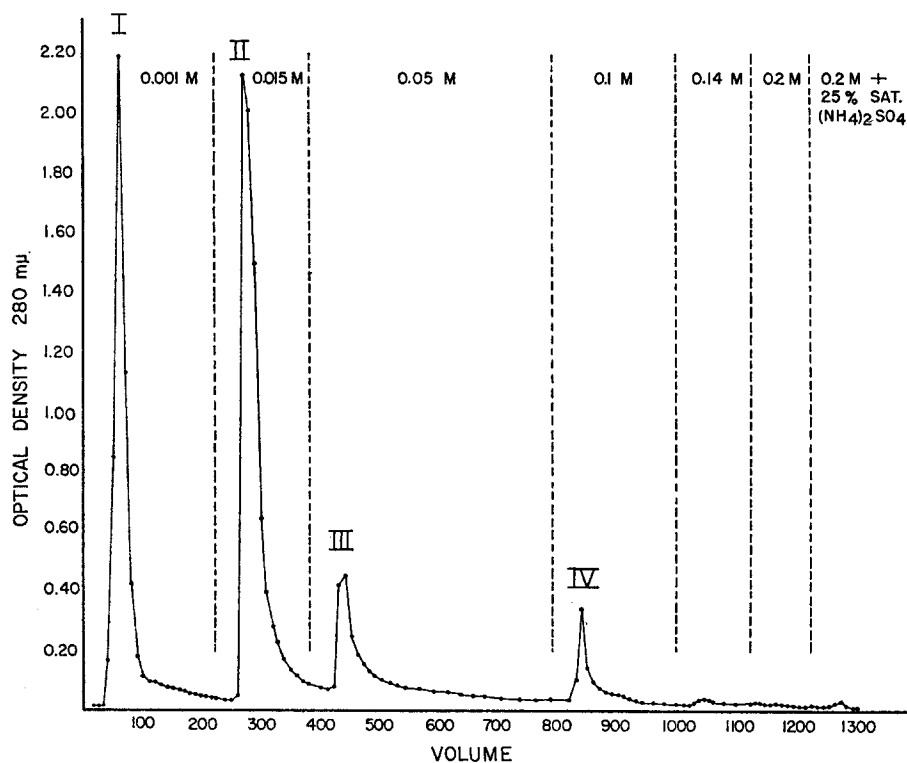
The extremely minute amount of protein eluted at 0.2 M revealed at least one additional component, while the sharply peaked fraction obtained with 0.2 M + 25 per cent saturated $(\text{NH}_4)_2\text{SO}_4$ revealed one component which titrated to 0.04 mg./ml. This antigen showed the "reactions of identity" with the DPNase fraction shown in Fig. 4 c. As expected, it revealed a high concentration of this enzyme (840,000 u/mg.). The factor which persisted to 0.2 mg./ml. in this high salt eluate revealed the "reaction of identity" with the streptolysin "O" anti-streptolysin band previously identified (2) in the crude concentrate (see Figs. 11 a and 11 b, and 7 i).

Data presented below indicated that the streptolysin "O" from Group C streptococcal culture concentrates eluted from calcium phosphate in the same range of salt concentration.

Fractionation of drip point 11+, Text-fig. 1: The antigen which migrated farthest to the left on the electrophoretic run of Text-fig. 1 has also been chromatographed on calcium phosphate columns. The results of this separation are shown in Text-fig. 6. Drip point 11+ was applied in 0.001 M phosphate at pH 6.8 to a column 30 mm. wide by 65 mm. high. The total amount of protein applied was 268 mg. as a solution containing 9.6 mg. protein/ml. As can be seen in Fig. 7, a large portion of the fraction was unadsorbed, and large amounts were eluted at 0.015 M, and 0.05 M phosphate concentrations. The principal antigenic component of this electrophoretic fraction was eluted, however, at 0.1 M as a small peak. Immunologically, the faint band persisted to the 10 $\mu\text{g.}/\text{ml.}$ well, as shown in Fig. 8. The other eluates only revealed very small concentrations of other antigens. In Fig. 8 b is also included a similar fraction (A) at 50 $\mu\text{g.}/\text{ml.}$ obtained previously from another electrophoretic and chromatographic run. In addition to revealing an immunological "reaction of identity," it showed excellent agreement with the above component in its position on the electrophoretic curtain and also had eluted from the calcium phosphate column with the stepwise increase in phosphate concentration from 0.05 to 0.1 M. This fraction (A) depicted in Fig. 8 b had also been rechromatographed on calcium phosphate, and again had eluted with this same stepwise increase of phosphate concentration. The identity of this component has not been determined as yet, but immunologically it is distinct from the bands caused by proteinase precursor, streptolysin "O," DPNase, erythrotoxic toxin (?), and C carbohydrate. In addition, it has been shown to be distinct from the antigens which eluted in Text-fig. 5 with the 0.15 M phosphate buffer. Further quantities of it are being processed, with a view to determining whether it represents a heretofore known

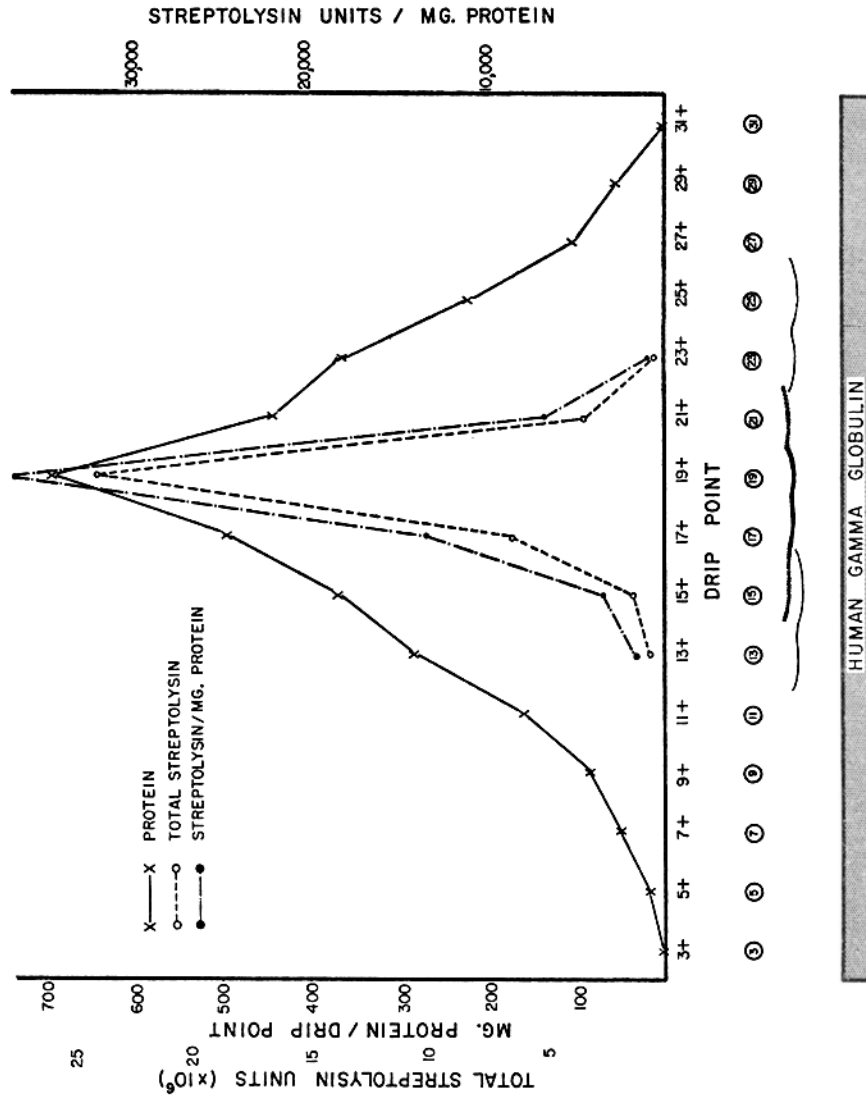
or unknown streptococcal product. It is of interest that antibody to this component is present in somewhat higher concentration in a horse antiserum to another streptococcal strain (see Fig. 12 *b*). The single band with this horse antiserum revealed the "reaction of identity" with the above band.

Streptolysin "O" from Group C streptococci: The crude concentrate from the growth of the Group C streptococcus revealed about 2,000 streptolysin "O"



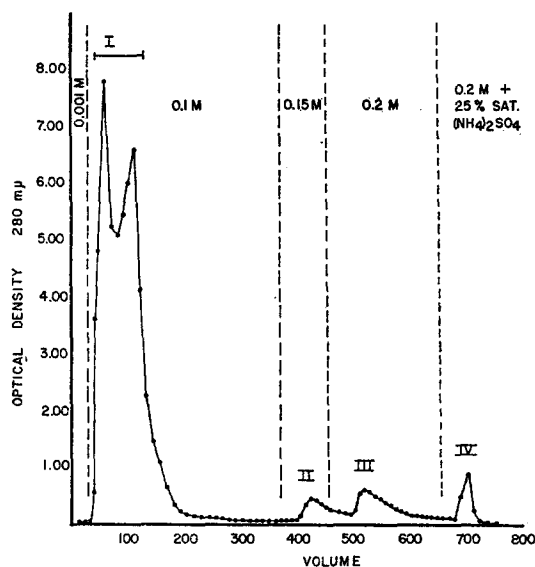
TEXT-FIG. 6. Calcium phosphate chromatography of electrophoretic drip point 11+ of Text-fig. 1. A total of 201 mg. of protein in 0.001 M phosphate buffer at pH 6.8 was loaded onto a column 30 mm. wide by 50 mm. high. Stepwise elution was carried out as indicated.

units/mg. protein, but when dissolved in the cold in acetate buffer at 0.06 ionic strength pH 6.0, yielded a very viscous solution which could not be applied to the electrophoresis curtain satisfactorily. It was found that much of the viscous material could be removed by precipitation at 35 per cent saturated ammonium sulfate, and that the bulk of the streptolysin could be recovered in the 35 to 67 per cent saturation cut. This latter precipitate was dialyzed thoroughly against the acetate buffer, and any insoluble material was discarded. The resulting clear pale tan solution contained about 8,200 streptolysin units/mg.



TEXT-FIG. 7. Results of continuous flow electrophoretic separation of Group C streptococcal extracellular antigens. Acetate buffer, pH 6.0, μ 0.06 was used with 300 volts and 66 to 68 milliamperes applied across the curtain. The drip point precipitin assays were carried out using a protein concentration of 1 mg./ml. in the antigen wells.

protein, and it was applied above the drip point 17+ to the curtain as a 3.74 per cent protein solution in the μ 0.06 acetate buffer. The run lasted for 8 days, with a daily feed of 494 mg. protein (total 3.96 gm.), at 300 volts and 66 to 68 milliamperes of current. The resulting protein separations and streptolysin recoveries are shown in Text-fig. 7. It may be seen that the peak of streptolysin "O" was recovered at drip point 19+, with a potency of about 35,000 units/mg. The total yield of activity was apparently quantitative within the limits of assay uncertainty. The precipitin assays are reconstructed in Text-

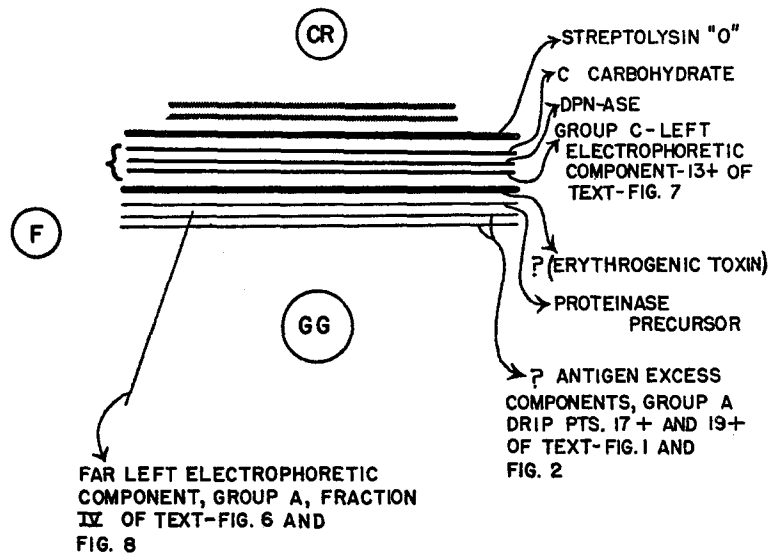


TEXT-FIG. 8. Calcium phosphate chromatography of streptolysin "O" electrophoretic drip point 19+ of Text-fig. 7. A total of 657 mg. of protein was applied to a column 40 mm. wide by 25 mm. high, as a 2.53 per cent solution in 0.001 M phosphate buffer pH 6.8. Stepwise elution was carried out as indicated.

fig. 7, and reveal many fewer antigens present in these protein concentrations which react with human gamma globulin than did the Group A concentrate. The antigen which is present from drip points 15+ through 21+ correlates with the streptolysin content and proved "identical" with the streptolysin "O" band of crude Group A concentrate.

Preliminary tests of Group "C" streptolysin peaks revealed that the streptolysin was well adsorbed onto CaPO_4 columns, and eluted only with high salt concentrations. In order to work rapidly, therefore, the high potency electrophoretic fractions were applied to columns after dialysis against 0.001 M phosphate buffer at pH 6.8, and then eluted overnight with 0.1 M buffer. The next day, elutions with the higher salt concentrations were carried out to finish the run with the results such as are shown in Text-fig. 8. Peaks I and II showed

streptolysin titers of 150 and 22,900 units/mg. protein respectively, but the streptolysin "O" was principally eluted in small peaks at 0.2 and 0.2 M containing 25 per cent saturated $(\text{NH}_4)_2\text{SO}_4$. The high potency of these fractions (207,000 and 156,000 HU/mg. protein, respectively) was correlated with a high precipitin titer in agar. In Fig. 9 a single band from 1 mg./ml. to 12 $\mu\text{g.}/\text{ml.}$ was found with human gamma globulin. A total of 657 mg. protein had been applied to this column which was 40 mm. wide by 25 mm. high, the sample being in a 25.3 mg./ml. solution. A total of 24 million units were applied (35,000 HU/mg.),



TEXT-FIG. 9. Diagrammatic representation of Figs. 11 a to 11 h

and roughly 16 million units were recovered. These streptolysin potency figures are based upon assays performed in England. However, repeat assays carried out 6 months later in the United States revealed much lower values, about 100,000 units/mg. The cause for the discrepancy is not as yet clear, but it is conceivable that the rabbit erythrocytes were more sensitive there, or that deterioration had occurred in storage. This is being investigated. The single precipitin band found with both the high salt eluates also joined the band previously identified as the streptolysin "O"-anti-streptolysin "O" band with crude Group A concentrate, so the identity of this band can now be accepted as thoroughly established. Two other chromatographic separations of Group "C" streptolysin "O" electrophoretic fractions showed practically identical results as in Text-fig. 8, with very similar precipitin and streptolysin activities.

As indicated above, the streptococcal Group "C" component which migrated electrophoretically to the right of the streptolysin "O" in Text-fig. 7, could

be tentatively identified as DPNase on the basis of its chromatographic behavior, "reaction of identity" with the Group A antigen (see Fig. 10 *d*) and on its content of enzyme activity. The identity of the component which migrates to the left of the streptolysin "O" has not yet been looked into.

In order to conclusively demonstrate that the bands of the highly purified components were distinct from each other, they were set up in agar plates in wells adjacent to each other. A series of such plates are shown in Fig. 10. It can be seen that the DPNase, proteinase precursor, streptolysin "O," erythrotoxic toxin (?), C carbohydrate, and far left component are immunologically clearly distinct. The failure of the DPNase to completely cross the streptolysin concentrate (Fig. 10 *b*) was correlated with a small contamination of this fraction with DPNase (6,600 units/mg). In addition, several other components not so highly separated were similarly tested and it can be seen that many of these are immunologically different from the above and each other. It seems certain from these observations that 12, and probably 13 or 14 antigens are produced by this particular strain of Group A streptococcus which are liberated in humans during the course of infection.

It was also of some importance to identify the bands formed with crude Group A concentrate and the various components separated electrophoretically and chromatographically. Such attempts to "map" the antigenic patterns formed with crude concentrates are shown in Fig. 11. It is to be noted that the pattern formed by the crude material at 10 mg. dry weight/ml. (6.7 mg. protein/ml.) is typified by two dense bands, one apparent band between these, two fainter bands closer to the antigen well, and three or four bands in antigen excess which appear late in development, or on dilution of the crude. It is clear now that the *heavy* band closest to the antigen well is the streptolysin "O" system, while the *heavy* band closest to the gamma globulin well was previously suspected to be due to the erythrotoxic toxin (?) system. The proteinase precursor system represents the first of the bands in antigen excess. The C carbohydrate band is one of the at least three which are now known to overlap in the area between the two heavy bands, while the DPNase system probably comprises another one of these. The third in this overlap area is represented by the left electrophoretic Group C component. The component which migrates to the far left which has been substantially isolated, does not appear to show a "reaction of identity" with any of the readily visible bands of the crude, and this must be masked there by overlapping because of its faintness. The progress made thus far in tentatively identifying and separating these bands are shown more clearly in Text-fig. 9 in a diagrammatic representation.

As a further check on the immunological homogeneity of the fractions that have been isolated, tests were carried out with horse antiserum to a Group A streptococcus culture filtrate (strain Richards) which is available commercially from the Wellcome Research Laboratories. This lyophilized serum,

when reconstituted to 2,000 Todd antistreptolysin "O" units/ml., revealed a number of bands with the C203S crude concentrate at 10 mg./ml. Much overlapping of rather broad bands was seen (Fig. 12 *a*) so that the minimum number of systems cannot be stated at present, but may be as many as 8. When the streptolysin, erythrogenic toxin (?), and far left components were tested against this serum, only one band was seen with each; no bands were noted when crystalline proteinase precursor or "C" carbohydrate was used. In Fig. 12 *b* the results of cross-testing this serum and human gamma globulin are seen. Although not too clearly visualized in the photograph, the far left component (F) showed joining of the faint band seen with human gamma globulin and the heavier band formed with the horse antiserum. The greater intensity of the precipitate and its position clearly indicate that the horse serum possessed a much higher antibody concentration to this component than the gamma globulin. However, the fact that the fractions tested revealed only one or no bands with the horse antiserum strongly implies their relative freedom from the other antigens present in the crude concentrate.

DISCUSSION

These observations demonstrate that the use of precipitin analysis in agar does in fact represent a valuable new approach to the study of pathogenesis of infectious diseases. When human sera or antibodies are used, as here, it may again be stressed that each antigen detected almost certainly must have been produced by the micro-organisms in the tissues during human infections. For a complete analysis of the extracellular antigenic armamentarium of the organisms *in vivo* (including possible toxic products), it will be necessary to test culture filtrates from a number of strains to determine the total capacity of the species. Other sources of antigens such as experimental exudates produced *in vivo* should also be examined, as some antigens may possibly be produced *in vivo* and not in culture media. One test of this with a Type 12 streptococcus revealed fewer antigens in the exudates than were found in the culture filtrates, but the possibility remains a real one and should be checked for completeness. Although pooled human gamma globulin from normal individuals was used here for the test antibody solution, it would be much preferable to use pooled sera from convalescent patients. Indeed, this was made possible only because of the wide prevalence of subclinical or clinical streptococcal infections. In other diseases, convalescent sera would be essential. It is conceivable that other antigens may be detected with pooled sera from patients with streptococcal diseases, such as rheumatic fever, for example.

That this approach is beginning to yield fruit with other human infections is evidenced by reports on the use of agar precipitin analyses in human staphylococcal infections (18), toxoplasmosis (19, 20), amebiasis (21), adenovirus infections (22), and poliomyelitis (23). That such studies may prove quite profit-

able in other virus infections is suggested by the excellent report of Gispen on agar precipitin analysis of the pox viruses (24). Although he did not use human antibodies, the rabbit sera used were produced by the injection of living virus suspensions of rabbit origin, while the test antigens consisted of clarified homogenates of infected duck chorioallantoic membranes. As many as six distinct soluble antigens were found with some systems. This points up the possibility that other viruses may produce a number of distinct soluble antigens, and that the approach used here may be of value in separating and characterizing them chemically and biologically. That several distinct viral antigens may be produced during influenza virus infection has been suggested in recent reports (*e.g.*, 27).

A start has now been made in the formidable task of applying these technics for the purification of the streptococcal products thus implied to be produced *in vivo* in humans during infection. It is quite clear from these findings thus far, that the two technics employed here for sorting out the proteins in complex mixtures are admirably suited for use together in this work. Three known streptococcal extracellular antigens have already been separated almost completely from the others, and in all likelihood in a very high state of purity. It also may be stressed that two of them were isolated first, and then identified. The proteinase precursor so isolated could be very readily crystallized. Another component (Text-fig. 6) has been completely separated from the others, and is known to be distinct from the streptolysin "O," proteinase precursor, erythrogenic toxin (?), C carbohydrate, and DPNase. When more material is available, it will be examined further to see whether it represents one of the other known streptococcal products or is heretofore undescribed. Should the latter prove to be the case, it will be studied for its toxicity, biochemical properties, and behavior, as well as its possible role in the infectious process. It seems quite probable that continuing efforts will fairly readily result in isolation of the other components visualized with human gamma globulin. For those antigens which fail to separate from the calcium phosphate column even with close stepwise increase of buffer concentration, it is planned to resort to the cellulose ion-exchange derivatives, as reported by Peterson and Sober (25, 26). A few trials suggest the usefulness of these adsorbents in such cases. Very large amounts of protein have been loaded onto some of the calcium phosphate columns used here. It is quite possible that some may have been overloaded with subsequent displacement effects (5), but the limits of the capacity of calcium phosphate for these materials are now being explored.

The production of proteinase precursor by the C203S strain grown under these conditions is of interest. Elliott (31) reported the failure of streptococci to produce appreciable amounts of this enzyme precursor or active enzyme when the pH of the medium was maintained above 7.0. The optimum range for this purpose was found by him to be pH 5.5 to 6.5. The detection here of precursor

production in a medium maintained above pH 7.0 could be due to a strain difference, or to the choice of medium used. Elliott had found much variation in the amounts of enzyme liberated when different sources of peptones were employed in the broth (32). He had previously tested several crude streptolysin "O" concentrates prepared in this laboratory with antisera against precursor or proteinase, and had found only the former in concentrations up to 5 to 10 per cent by weight (2).

Some of these antigens revealed complete inhibition of the precipitin band with antigen excess, while others revealed spreading and striations under these conditions. These findings are in agreement with other investigators using animal sera (16, 17). That the striations which occur with high concentrations of the erythrogenic toxin (?) do not represent contaminating antigens is indicated above. It is quite clear from these data, however, that those separated bands seen early in development, and those seen with lower concentrations of antigens (below 0.1 mg./ml.) almost certainly represent distinct antigen-antibody systems. The findings presented here should largely lay to rest fears that many of these bands seen are artefacts produced by a single system. If not too much reliance is placed on the late development patterns, one may rather safely be guided as to the minimum number of systems involved. Indeed, overlapping may frequently occur, and such has been found to be the case here. Previously in our studies, crude Group A streptococcal concentrates revealed only a minimum of 7 systems with human gamma globulin, while it is apparent now that at least 12, and possibly 13 or 14 antigens are involved, each of which shows crisp reactions of non-identity from the others when adequately separated from them.

The degree of purity of the antigens thus far isolated may be questioned briefly. Each of the five has been found to reveal its precipitin band when diluted to at least 12 μ g. protein/ml. in the antigen wells. Any such preparation which reveals only one band at 1 mg. protein/ml. in a well, therefore, probably contains less than 1 per cent of the others as contaminants, if overlapping can be demonstrated to be absent. Since the antigen wells used here hold only about 0.1 ml. of solution, at their precipitin end points only about 1 μ g. of protein was present in each well. Because this does approach the limits of sensitivity of this technic, it supports the impression that these fractions are in all likelihood very highly purified. In addition, the extremely high functional activity of the DPNase is in accord with this. It can be calculated that 1 mg. of fraction I of Text-fig. 3 can destroy 12.6 gm. of DPN in $7\frac{1}{2}$ minutes at 37°C. The very ready crystallizability of the immunologically clean proteinase precursor is also in agreement with this possibility. Finally, the findings with the horse antistreptococcal serum are in support of this (see Fig. 12). The possibility that these preparations may be contaminated with significant amounts of non-antigenic substances, or substances to which humans had not produced antibodies, is

definitely real. More stringent criteria for chemical homogeneity will necessarily await preparation of large enough quantities of the materials.

The author is deeply grateful to Dr. W. E. van Heyningen for his guidance, and help in many aspects of this work, and for his kindness in the generous use of his laboratory during the stay at Oxford. Thanks are also gladly given to Dr. A. Woodin for many stimulating discussions concerning the work. The technical assistance of Miss P. Womar and T. Auerbach is gratefully acknowledged.

SUMMARY

As evidenced by precipitin analysis with pooled human gamma globulin, at least 12 distinct antigens were produced in cultures by one strain of Group A streptococcus (C203S). It was suggested on this basis, that these antigens were produced *in vivo* during human infections.

By the combined use of continuous flow electrophoresis on paper curtains, and column chromatography with calcium phosphate gels, five of these have been isolated in a probable high state of purity. One of the components was obtained from culture filtrates of a Group C streptococcal strain.

Three of the purified antigens have been tentatively identified as streptolysin "O", diphosphopyridinenucleotidase, and proteinase precursor. The latter could be very readily crystallized, and appears "identical" with that described by Elliott. The DPNase was of extremely high potency, 1 mg. being capable of destroying 12.6 gm. of DPN in $7\frac{1}{2}$ minutes at 37°C .

The identity of the other two components is uncertain as yet. They are distinct from each other and the above products immunologically, and are not related to the "C" carbohydrate.

The applicability of these methods for the analysis of infectious diseases generally was discussed.

Addendum.—The component previously suspected to be erythrotoxic toxin (e.g. II, of Text-fig. 5) has recently been assayed for desoxyribonuclease (DNase) activity. Extremely high potencies have been observed, about 1 to 1.5 milligrams being capable of depolymerizing 400 gm. of calf thymus desoxyribonucleic acid (DNA) in 30 minutes at 37°C ., using the alcohol precipitation technic of McCarty (33). By comparison, highly purified group C streptococcal DNase⁴ required 7 mg., and 1 X crystallized bovine pancreatic DNase (Nutritional Biochemicals, Cleveland) required 4 mg. for the same quantity of DNase activity.

A lyophilized sample of one such fraction (as II, of Text-fig. 5) was assayed by Dr. L. W. Wannamaker⁵ who confirmed the potency of this enzymatic activity. He found it to be immunologically related to DNase B, one of the several distinct DNases

⁴ Thanks are given to Dr. F. Ablondi, of the Lederle Laboratories, Pearl River, New York, for the donation of these lyophilized preparations.

⁵ The author is very grateful to Dr. Wannamaker and Dr. Stock for their cooperation in carrying out these tests.

he has currently shown is produced by Group A hemolytic streptococci (34). The same material was assayed by Dr. A. Stock for erythrotoxic potency. Although he observed only about 500,000 skin test doses/mg. by the rabbit intradermal assay, by flocculation test he found about 3,100 Lf units/mg. with a high titered scarlatinal antitoxin. The most potent erythrotoxic toxin he had previously isolated was of the order of magnitude of 100,000,000 to 150,000,000 skin test doses/mg., and only 2,100 Lf/mg. (35). Because of this discrepancy in the flocculation and skin test titers, the relationship of the above fraction to erythrotoxic toxin is still uncertain. It seems reasonable, however, to interpret Stock's findings as due to a second flocculation zone, unrelated to erythrotoxic toxin. The above component, therefore, can be tentatively identified at this time as DNase B.

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EXPLANATION OF PLATES

PLATE 28

FIG. 1. Precipitin tests with drip points obtained in continuous flow electrophoretic run of Text-fig. 1. All drip point fractions were dissolved in 0.05 M phosphate buffer pH 6.8 at 1 mg./ml. protein concentration. Normal pooled human gamma globulin used in the trench well.

FIG. 2. Precipitin assays of electrophoretic drip point fractions obtained in Text-fig. 1. All antigen dilutions were made in bovine serum albumin, 1 mg./ml., in 0.05 M phosphate buffer pH 6.8. Development at 4°C. for 6 days, except Figs. 2 *a* and 2 *b*, which were photographed at 10 days. Figures refer to the drip point fraction and its concentration in milligrams protein/milliliter. GG is normal pooled human gamma globulin.

FIG. 3. Agar precipitin assay of fraction I obtained at high salt concentration in calcium phosphate chromatography (Text-fig. 3) of electrophoretic fraction 29+ of Text-fig. 1.

FIG. 3 *a*. After 5 days' development.

FIG. 3 *b*. After 8 days' development.

The figures refer to the concentration of protein in milligrams/milliliter while GG is normal pooled human gamma globulin.

FIG. 4. Agar precipitin assays of the chromatographic fractions obtained in Text-fig. 4, which were derived from electrophoretic drip point 27+ of Text-fig. 1.

I, II, III, etc. represent the chromatographic fractions.

1, .2, .04 represent the protein concentrations, milligrams/milliliter.

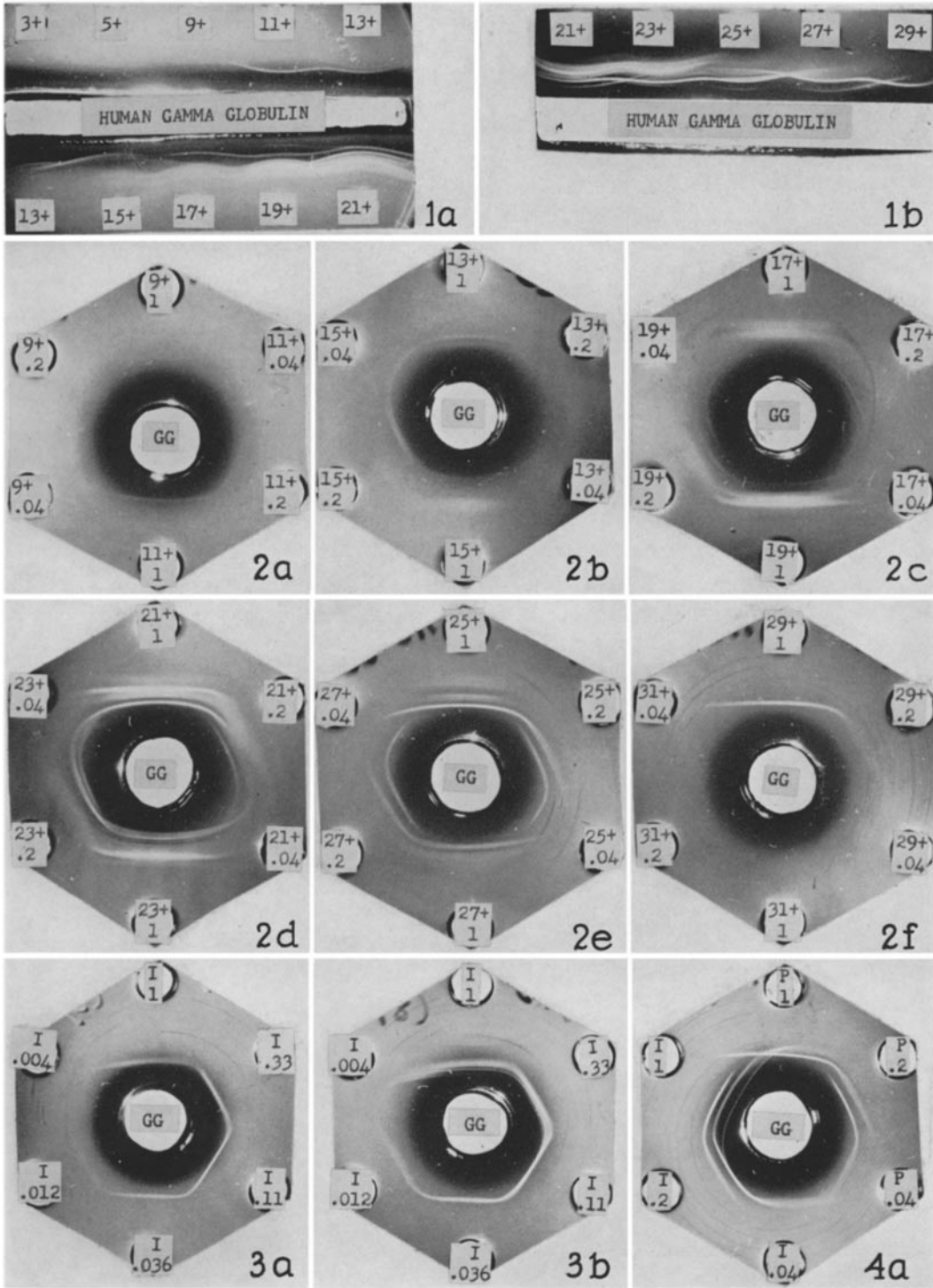
P is the sample applied to the column, drip point 27+.

A is the 0.03 M eluate of the chromatogram from drip point 23+.

B is the 0.2 M + 25 per cent saturated (NH₄)₂SO₄ eluate from drip point 23+.

C is the antigen found in electrophoretic drip point 11+ of Text-fig. 1 (see Fig. 2).

GG is normal pooled human gamma globulin and development proceeded for 6 days at 4°C. This was not long enough for fraction C component to be visualized.

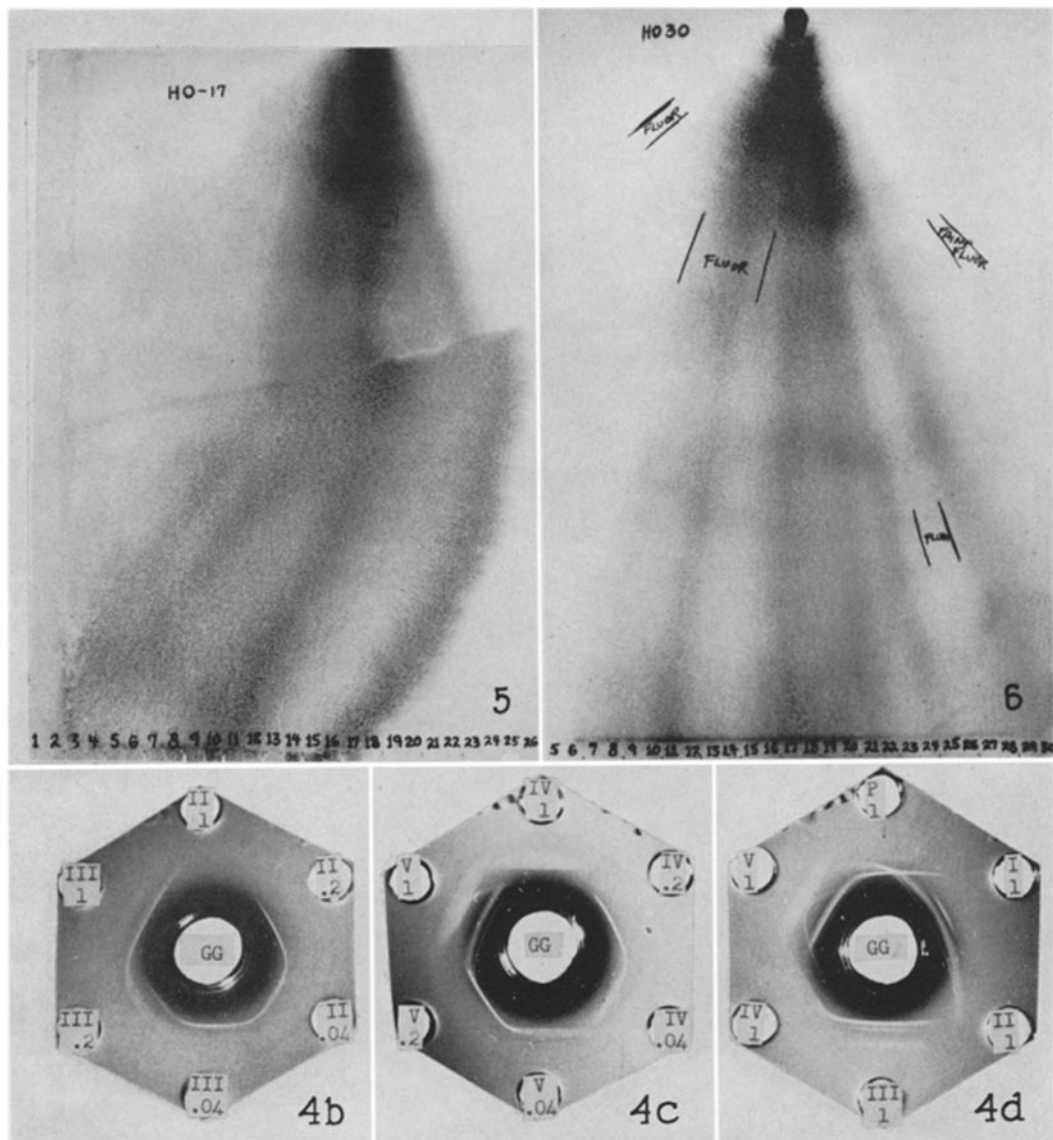


(Halbert: Precipitin analysis and streptococcal infections. III)

PLATE 29

FIG. 5. Attempted recycling of streptolysin "O" peak drip points, similar to those obtained in Text-fig. 1. In this above run, acetate buffer pH 6.05, μ 0.02 was used, with 430 volts and 31 to 33 milliamperes applied across the curtain. Very slow sample feed and buffer flushing rates were employed.

FIG. 6. Attempted recycling of streptolysin "O" peak drip points, similar to those obtained in Text-fig. 1. In the above run, sodium phosphate buffer, pH 7.5, μ 0.02 was used with 430 volts and 20 to 24 milliamperes applied across the curtain. Very slow sample feed and buffer flushing rates were employed.



(Halbert: Precipitin analysis and streptococcal infections. III)

PLATE 30

FIG. 7. Agar precipitin assays of the chromatographic fractions obtained in Text-fig. 5, which were derived from electrophoretic drip point 25+ of Text-fig. 1. Developed for 6 days, except Fig. 7 *c* which represents the same plate as Fig. 7 *d*, as seen at 3 days.

I, II, III, etc. represent the chromatographic fractions.

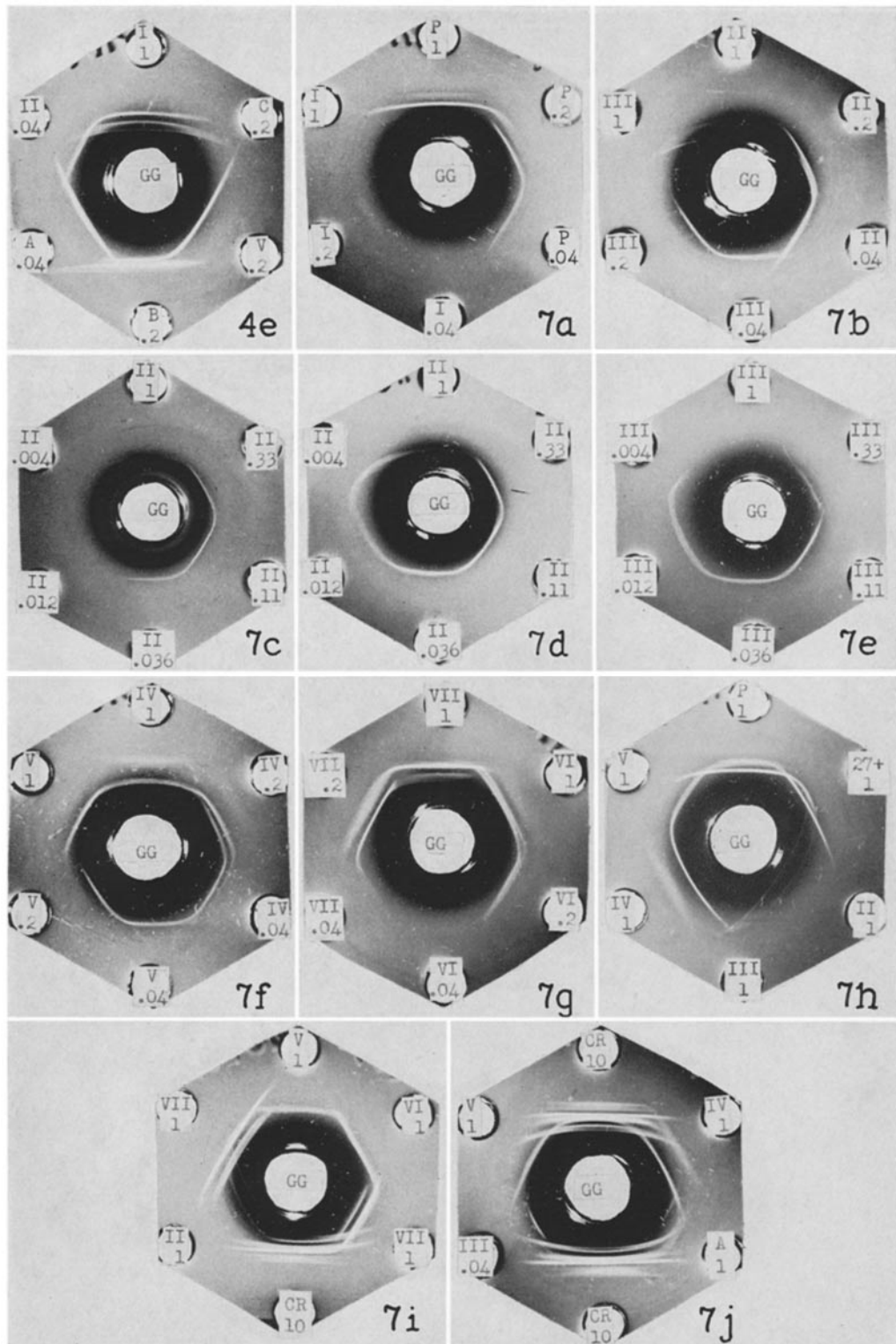
1, .2, .04, etc., represent the protein concentrations, milligrams/milliliter.

P is the sample applied to the column, drip point 25+.

CR is the crude concentrate, at 10 mg./ml. dry weight.

A is the 0.01 M eluate obtained in the chromatogram of drip point 23+.

27+ is the drip point fraction 27+ of Text-fig. 1.



(Halbert: Precipitin analysis and streptococcal infections. III)

PLATE 31

FIG. 8. Agar precipitin assays of chromatographic fraction IV obtained in Text-fig. 6, which was derived from the electrophoretic drip point 11+ of Text-fig. 1. 1, .2, etc. represent protein concentrations, milligrams/milliliter. Development 12 days.

A represents a similar fraction obtained from a different electrophoretic and chromatographic run.

FIG. 9. Agar precipitin assay of chromatographic fraction III of Text-fig. 8, which was derived from Group C streptococcal electrophoretic fraction 19+ of Text-fig. 7. 1, .33, etc., refer to protein concentrations, milligrams/milliliter.

FIG. 10. Agar precipitin demonstration of non-identity of the fractions isolated in highly purified form, as well as other components. All plates were developed 8 days, except 15 days for Figs. 10 a and 10 b.

L, streptolysin "O," fraction III of Text-fig. 8 and Fig. 9.

P, far left electrophoretic component, chromatographic fraction IV of Text-fig. 6 and Fig. 8.

ET?, erythrogenic toxin?, chromatographic fraction II of Text-fig. 5 and Fig. 7.

C, group A formamide cellular extract rich in "C" carbohydrate.

D, diphosphopyridine nucleotidase, chromatographic fraction V of Text-fig. 4 and Fig. 4.

PP, crystalline proteinase precursor, chromatographic fraction III of Text-fig. 5 and Fig. 7.

A, chromatographic fraction IV of Text-fig. 5 and Fig. 7. At this concentration only the two "major" components are visualized (see Fig. 7 *f*).

G, electrophoretic drip point 13+ from group C streptococcal concentrate shown in Text-fig. 7.

DC, electrophoretic drip point 23+ from group C streptococcal concentrate shown in Text-fig. 7.

H, chromatographic fraction analogous to chromatographic fraction IV of Text-fig. 5 and Fig. 7, obtained in previous runs.

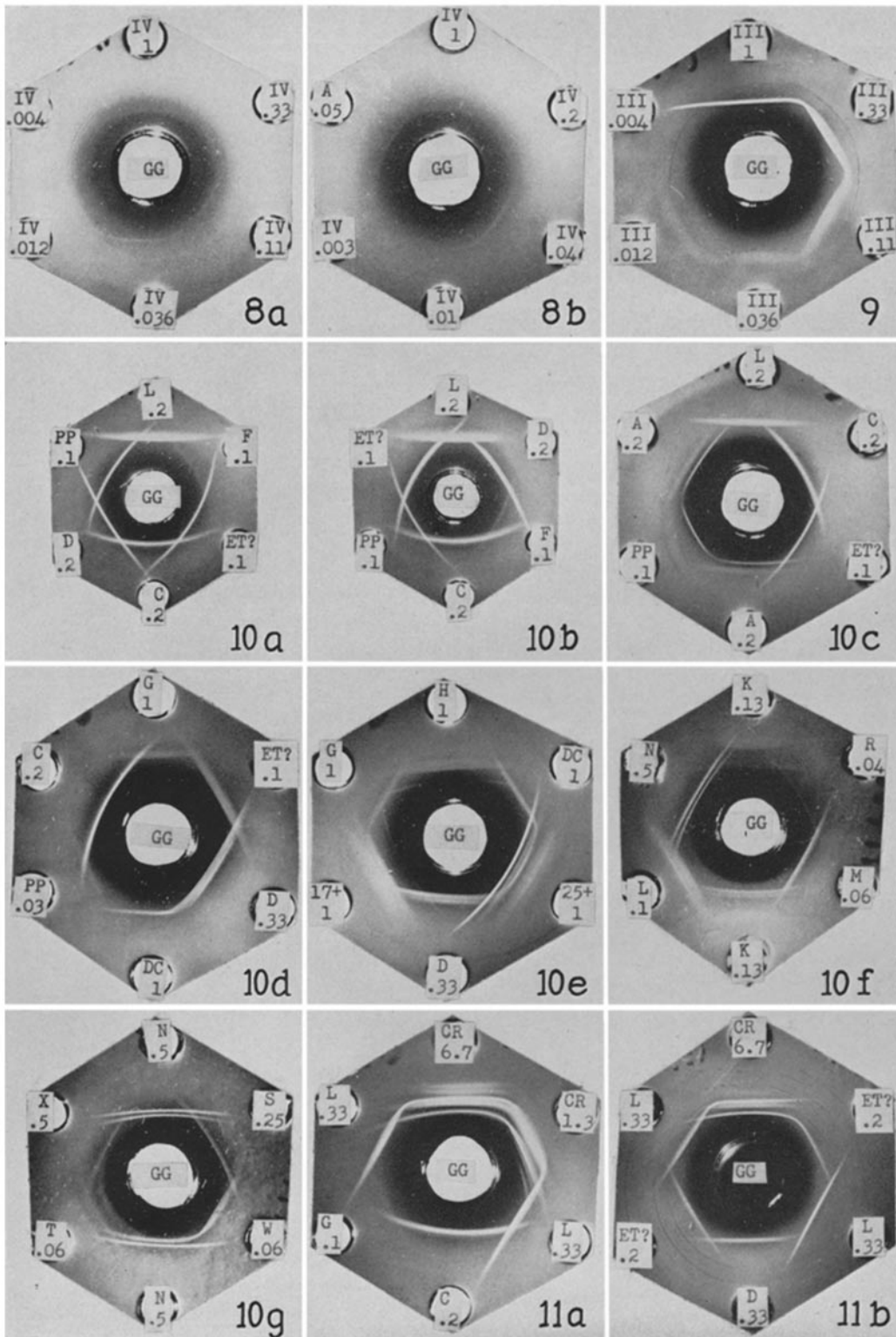
25+, electrophoretic drip point 25+ of group A streptococcal concentrate shown in Text-fig. 1 and Fig. 2.

17+, electrophoretic drip point 17+ from Text-fig. 1 and Fig. 2.

K, electrophoretic drip point analogous to fraction 19+ of Text-fig. 1 and Fig. 2.

<i>M</i> ,	"	"	"	"	"	"	25+	"	"	"	"
<i>N</i> ,	"	"	"	"	"	"	27+	"	"	"	"
<i>R</i> ,	"	"	"	"	"	"	15+	"	"	"	"
<i>S</i> ,	"	"	"	"	"	"	13+	"	"	"	"
<i>T</i> ,	"	"	"	"	"	"	21+	"	"	"	"
<i>W</i> ,	"	"	"	"	"	"	25+	"	"	"	"
<i>X</i> ,	"	"	"	"	"	"	11+	"	"	"	"

FIGS. 11 *a* and 11 *b*. For legend see following plate.



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FIG. 11. Attempts to "map" bands found with crude concentrates, using isolated or partially isolated fractions. Development in Figs. 11 *b*, 11 *d*, and 11 *e* was 4½ days, while the rest were developed for 8 days. Numbers represent milligrams protein/milliliter.

CR, crude concentrate of Group A culture supernate.

L, streptolysin "O" Group A electrophoretic and chromatographic fraction III of Fig. 9.

C, Group A, C carbohydrate.

G, Group A electrophoretic fraction analogous to drip point 15+ of Text-fig. 1 and Fig. 2.

ET?, erythrotoxic toxin (?), fraction II of Fig. 7.

D, diphosphopyridine nucleotidase, fraction I of Text-fig. 3 and Fig. 3.

H, Group C electrophoretic drip point 13+ of Text-fig. 7.

K, " " " " " 23+ " "

LP, chemically purified streptolysin "O" concentrates, as in reference 1.

PP, proteinase precursor, fraction III of Text-fig. 5 and Fig. 7.

M, Group A electrophoretic fraction analogous to drip point 17+ of Text-fig. 1 and Fig. 2.

N, Group A electrophoretic fraction analogous to drip point 13+ of Text-fig. 1 and Fig. 2.

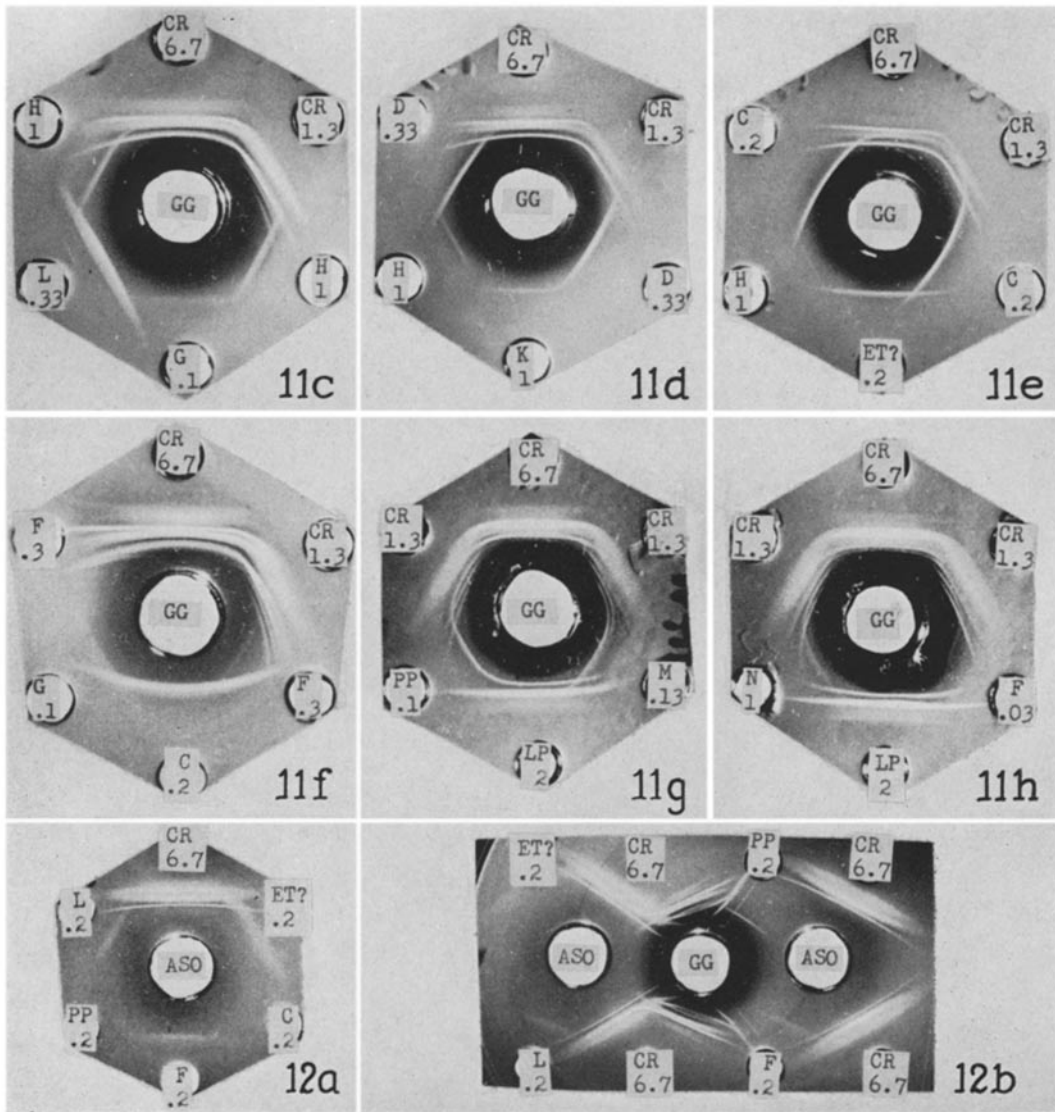
F, far left electrophoretic Group A antigen, same as fraction IV of Text-fig. 6 and Fig. 8.

FIG. 12. Precipitin tests of some of the highly purified fractions using standard horse anti-streptococcal antibody in high concentration. Developed 7 days.

ASO = horse antiserum to Richards strain of Group A streptococcal filtrates.

Lyophilized serum dissolved so that there was 2,000 Todd antistreptolysin units/milliliter.

For remainder of code letters, see Fig. 10.



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