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

IV. Further Observations on the Purification of Group A Extracellular Antigens

By S. P. HALBERT, M.D., and T. AUERBACH

(From the Department of Microbiology, College of Physicians and Surgeons, Columbia University, New York)

Plates 23 and 24

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It is well known that streptococci can cause a variety of infections which are sometimes followed by non-invasive sequelae, *e.g.*, rheumatic fever, chorea, etc.

The factors involved in the production of these postinfection complications, and their mechanisms are not clearly understood. In view of the specificity of these sequelae relative to streptococcal disease, it seems likely that one or more particular products of the organisms are etiologically involved in some way. It has long been clear that a number of streptococcal substances are released in the tissues during the course of human infection, as evidenced by antibody rises at the time of convalescence (1). However, these bacterial products have only been discovered on a hit-or-miss basis until recently. The development of precipitin analysis in agar (2, 3) has furnished a tool for determining the total number of antibody responses following streptococcal infection in man, and thereby the total number of antigenic components released *in vivo*. Such studies carried out in this laboratory several years ago (4, 5) demonstrated that the numbers of streptococcal components which reach antibody-forming sites in significant amounts during human infections were surprisingly large. In addition, unexpectedly, most of the antigens thus detected were apparently of extracellular origin, and represented secretion products of the microorganisms. Very few antibody responses were found against the bacterial cellular extracts prepared in a variety of ways. Furthermore, pooled gamma globulin from normal human adults proved to be extraordinarily rich in these anti-streptococcal antibodies, thus indicating very frequent and widespread streptococcal disease in the population at large. Confirmatory results have been obtained by other groups (*e.g.*, references 6, 7).

Since it is of primary importance to clarify the identity and possible pathological significance of these streptococcal factors thus shown to be secreted *in vivo* in humans, an extended program was begun to purify and characterize these antigens. Early re-

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sults (8) demonstrated the effective separation of some of these by a combination of continuous flow electrophoresis and column chromatography on calcium phosphate gels. These observations revealed that the number of *in vivo* produced streptococcal extracellular antigens totalled at least twelve, as compared to seven detectable with the crude extract using the Ouchterlony technic. Five of these components were well isolated from the others, and four were rather firmly identified with known streptococcal antigens or toxins.

It is the purpose of the present report to describe the extension of such attempts to unravel these complex mixtures with a view to the eventual study of the possible biological significance of each of the isolated streptococcal antigens or toxins. In the previous communication, electrophoretic fractions closest to the anode and cathode were further separated by column chromatography. In this report, the more complex mixtures in electrophoretic drip points between the two extremes were further fractionated, and the data revealed an even greater complexity of antigen-antibody systems than that previously detected. In addition, some fractions were rechromatographed either on calcium phosphate gels or on cellulose-derived adsorbents.

Materials and Methods

The crude culture supernate concentrates were obtained from the C203S strain of group A hemolytic streptococci and were prepared according to the conditions described previously (8). Details of the continuous flow electrophoresis and calcium phosphate column chromatography are also recorded in the earlier report, as are the gel precipitin methods and various assay methods utilized.

The calcium phosphate (hydroxyapatite) prepared according to the technic of Tiselius *et al.* (9) had proven to be somewhat variable in packing and flow-rates properties. It was found that the first $CaPO_4$ precipitation was of great importance in obtaining reproducible large particle final suspensions. This was accomplished by adding the original solutions of 0.5 M $CaCl_2$ and Na_2HPO_4 simultaneously by means of a twin-tubed peristaltic pump which regulated the input of both solutions simultaneously. As recommended by Tiselius, slow dropwise additions were necessary and the rate was adjusted so that approximately 3 to 4 liters of each solution were added in a 24 hour period. The drops were added close together and the precipitating mixture of brushite was stirred gently with a magnetic stirrer. In this way, three to four times the described quantity of hydroxyapatite could be conveniently and rapidly prepared reproducibly. These suspensions proved to yield very sturdy columns, with extremely rapid flow rates and similar adsorption properties to those previously found. With them, as much as 10 gm. of some protein mixtures could be rapidly processed with four buffer changes in 1 to 1½ days on a column 8 cm. wide by 11 cm. high. All procedures were carried out at 4°–6°C.

Preparations of erythrogenic toxin concentrates were obtained through the kindness of Dr. A. Stock (Pittsburgh), Dr. L. Hansen (Göteborg, Sweden), and Dr. E. Soru (Bucharest, Roumania). Those of Drs. Stock and Soru were prepared from the NY5 strain of Group A streptococcus while the other was obtained from different organisms. The former two were purified according to Stock's procedure (10), while the latter was processed by another technic (11).

The DEAE and CM cellulose were the commercial products available from the Brown Com-

pany (Berlin, New Hampshire) type 20. The pooled normal human gamma globulin was a 16 per cent solution as furnished by the American Red Cross.[1] Several Squibb (New York) preparations were used throughout in these tests (lot 270–2, 286-6, 321-1). All were obtained from plasma pools of bleedings made in the late winter or early spring, and all appeared to show identical reactions.

The milk-clotting assay for proteinase activity was slightly modified from the procedure described by Elliott (12). The buffer consisted of a mixture of 0.15 M NaCl and 0.15 M sodium phosphate, pH 7.0 and activation of proteinase precursor to proteinase was carried out by incubating the test solution with an equal volume of neutralized 0.1 M *l*-cysteine-HCl (Nutritional Biochemicals, Cleveland) at 37°C. for 30 minutes. The thioglycollate was supplied as the analytical grade mercaptoacetic acid (Eastman) after neutralization with NaOH, since stable powdered sodium thioglycollate is not available. Powdered skimmed milk was used (Pet Milk Company, St. Louis). Serial 2X dilutions were made by the dropping volume technic (0.4 ml. volume), and an equal volume of the homogenized milk suspension was added. Incubation at 37°C. for 2 hours was followed by room temperature overnight. Under these conditions, the end-points were satisfactorily reproducible, and 1 unit of proteinase was designated as that amount of test protein required to clot 0.4 ml. of milk suspension. The test was carried out in duplicate, one series not being activated. This enabled an estimation of the percentage of active enzyme as well as of precursor. The highly purified crystalline proteinase precursor described below revealed 1580 units of milk-clotting activity/mg. protein, of which 100 units (6 per cent) were apparently already in the activated proteinase state.

The desoxyribonuclease assay used was that described by McCarty (13). With sufficient attention to detail adequate reproducibility could be established. One unit of DNAse activity was defined as that amount required to depolymerize 400 μg. of beef thymus DNA at 37°C. in 30 minutes.

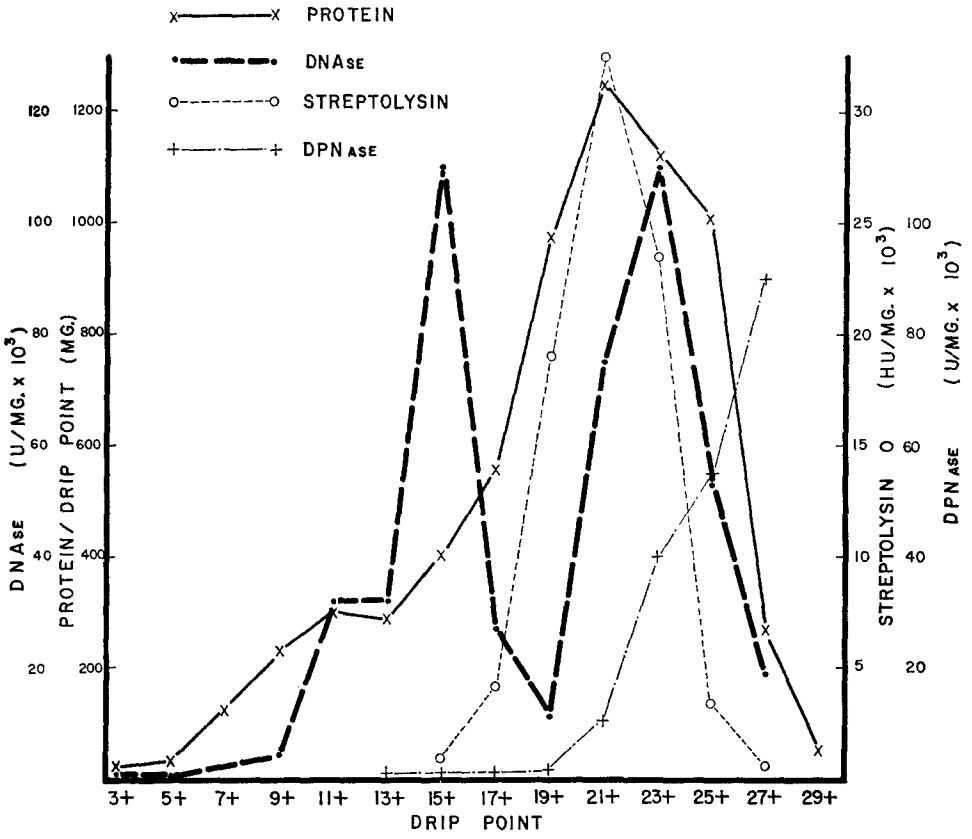
RESULTS

In the previous paper dealing with this subject (8), $CaPO_4$ fractionation of the antigen-containing electrophoretic drip point migrating most rapidly to the anode (11+, see Text-fig. 1) revealed predominantly one antigenic component. This eluted from the column as a small protein peak in the elution step from 0.05 M to 0.1 M sodium phosphate buffer at pH 6.8. For purposes of identification it was called the "far left" fraction. It has been found to contain roughly $\frac{1}{10}$ of the desoxyribonucleic acid depolymerase activity of the highly purified desoxyribonuclease B (DNAse B) isolated previously, but was distinct immunologically from this antigen, as well as from C carbohydrate, streptolysin "O," diphosphopyridine nucleotidase (DPNase), and proteinase precursor. In addition, it showed no streptokinase activity when tested with whole human plasma (14). The possible identity of this component with another of the DNAses reported by Wannamaker (15) remains to be seen.

Incidentally, two distinct peaks of DNA depolymerase activity were found in the streptococcal electrophoretic fractions reported previously (8), in confirmation of Wannamaker's observations (15). These are represented in Text-

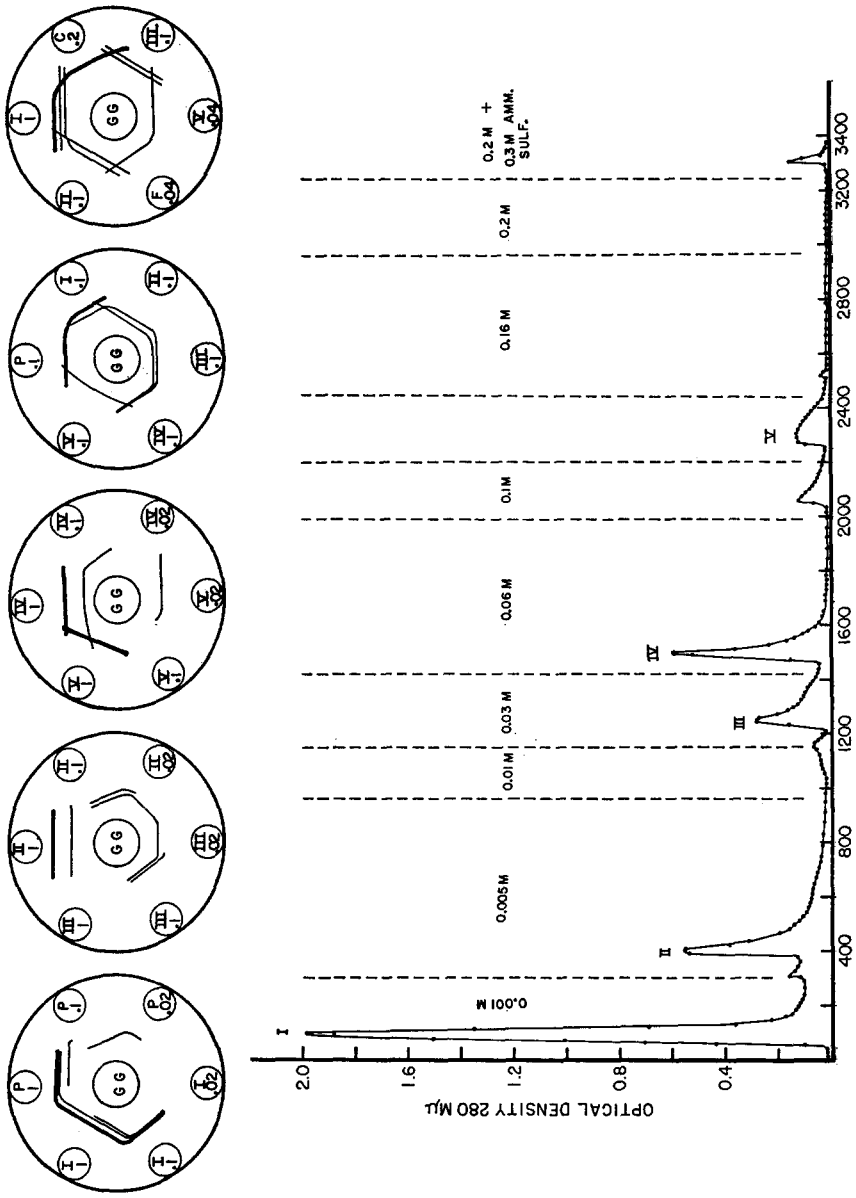
[1] The authors are deeply grateful to Dr. J. N. Ashworth of the American Red Cross for making these gamma globulin preparations available.

fig. 1, along with the peaks of streptolysin "O," and diphosphopyridine-nucleotidase activities. These latter separate in a manner quite similar to that reported by Carlson, Kellner *et al.* (16).



TEXT-FIG. 1. Results of continuous flow electrophoresis of crude group A extracellular antigens, analyzed for desoxyribonuclease, streptolysin "O," and diphosphopyridine-nucleotidase activities.

$CaPO_4$ Chromatography of Electrophoretic Fraction 15+.—When the electrophoretic drip point fraction (15+) was chromatographed on calcium phosphate, the results are depicted in Text-fig. 2. It is to be noted that significantly less protein eluted at low salt concentration than was found in the chromatogram of drip point 11+, and that the antigen contents of the chromatographic fractions were much greater (see reference 8). Tracings of the gel precipitation assays are depicted on the upper part of the chart where serial dilutions of each fraction (milligrams protein/milliliter) were tested against normal human gamma globulin.



TEXT-FIG. 2. Chromatographic separation of electrophoretic drip point 15+ on hydroxyapatite column. A total of 425 mg. of protein in 50 ml. of 0.001 M sodium phosphate buffer pH 6.8, was applied to a column 4.0 cm. wide by 3.3 cm. high. Stepwise elution with increasing phosphate buffer concentrations, pH 6.8, were carried out as indicated. For the final stepwise elution at high salt concentrations, ammonium sulfate was added because of the limited solubility of the phosphate at cold room temperatures. Agar precipitin assays are indicated in the tracings drawn above the chart, normal human gamma globulin being placed in the central well, and the fraction dilutions peripherally. Numbers refer to concentration in mg./ml. P, sample applied to the column. C, cellular "C" carbohydrate. F, "Far left" chromatographic fraction.

Several points should be stressed. Appreciable amounts of three antigens remained unadsorbed (fraction I). One of these (closest to the antigen well) showed the "reaction of identity" with the single band produced with a cellular C carbohydrate preparation obtained by the hot formamide extraction method of Fuller (17), as seen in the upper right diagram. The second band forming with the unadsorbed fraction showed "reactions of non-identity" with the C carbohydrate band, and with the components of fraction II, which eluted at 0.005 M sodium phosphate pH 6.8. The innermost of the bands produced by the unabsorbed fraction did merge in an "identity" reaction with one component of the fraction II. It was seen in fractionation of adjacent drip points that the former two components are always unadsorbed on columns prepared in this way, while the innermost antigen may or may not be loosely adsorbed.

For reasons to be indicated, the component with C carbohydrate specificity was designated C carbohydrate–protein complex, while the second unadsorbed antigen was called the non-C unadsorbed component. The latter was shown to be immunologically related to a similar unadsorbed fraction derived from a group C streptococcal strain. These data will be described in a subsequent report.

It may be noted in Text-fig. 2 that fractions II and III eluting at 0.005 M and 0.03 M phosphate respectively are very similar in their immunological specificities. Both contain appreciable amounts of two components which are completely dissolved by antigen excess effects at 1 mg./ml., and one of the fractions is still strongly detectable at 0.02 mg./ml. As will be shown, considerable interest was given to these antigens as one of them proved to show reactions of identity with a component present in high concentrations in purified erythrogenic toxin preparations obtained from three different sources. In addition, these two antigens proved "identical" with the two components designated "antigen excess" fractions in the reference system reactions between crude streptococcal concentrates and human gamma globulin (see reference 8). Fraction II did differ from fraction III in containing small amounts of two other components detectable only at the highest concentration tested.

Fraction IV eluting at 0.06 M contained smaller amounts of these antigen excess components (see diagram at upper right), while fraction V contained very high concentrations of an antigen distinct from any of the others eluted up to this point. It also revealed abolition of the band at the higher protein concentration. Except for small amounts of still another component present in fraction V at 1 mg./ml., it thus represents a rather thoroughly isolated immunological component. When tested adjacent to a well containing the "far left component" described above, it proved to be the same. The elution step in which it desorbed from the calcium phosphate agreed with that obtained previously for this latter antigen.

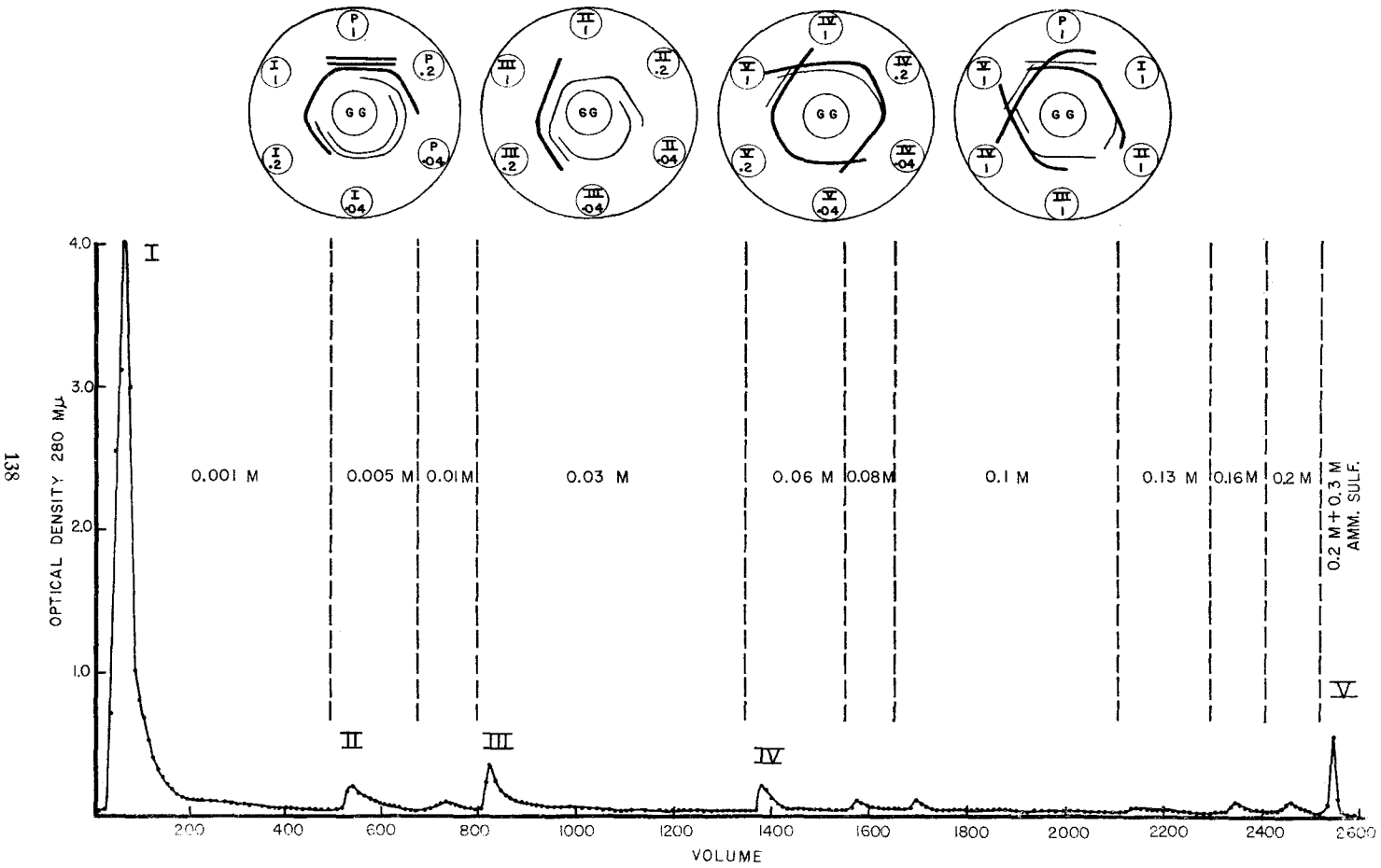
Lastly, it may be pointed out that a very small protein peak was obtained at

the highest salt concentration whereas none at all was observed in the chromatograms of the two adjacent drip point fractions toward the anode. Insufficient amounts of this peak were available for testing.

$CaPO_4$ Chromatography of Electrophoretic Fraction 17+.—When the next electrophoretic fraction (towards the cathode), 17+ was chromatographed, the elution patterns and precipitin assays obtained are depicted in Text-fig. 3. Several facts should be pointed out. In contrast to the chromatogram of the adjacent drip point (15+), a larger proportion of the protein remained unadsorbed. The unadsorbed fraction I, also revealed three antigenic components, and the band closest to the antigen well proved to give an "identity reaction" with C carbohydrate. However, the other two antigens present in the unadsorbed peak, were shown to be the same as those eluting in the fractions II and III. These proved to be the same two "antigen excess" components seen in the previous chromatogram. The unadsorbed fraction here, therefore, did *not* contain the non-C-unadsorbed component.

The column was 3 cm. wide x 2.8 cm. high, and 420 mg. protein was applied to it. The tendency of the "antigen excess" components to appear in large concentrations in the unadsorbed peak, may possibly be due to this relatively heavy "loading", 21.2 mg. protein being applied to each ml. of column. In contrast, the previous chromatogram of electrophoretic fraction 15+, and the subsequent ones were loaded at approximately 10 to 11 mg. protein/ml. of column. Fraction III contained one component in addition to the "antigen excess" ones, and this was further eluted in a high degree of concentration in peak IV. The identity of this latter component is at present unknown, but it was found to be distinct immunologically from proteinase precursor, C protein complex, non-C-unadsorbed antigen, DNAse B, streptolysin "O," DPNase, and the far left component. It proved identical with one of the components eluting in a similar stepwise buffer increase during chromatography of the next adjacent drip point (19+) (see Text-fig. 4). Its lack of relationship to the "antigen excess" factors was also shown in other tests, as well as in tests adjacent to original crude concentrates. It failed to show streptokinase activity, and showed only traces of DNAse depolymerase activity. It also proved immunologically unrelated to the five antigens detected with the group C streptokinase-streptodornase concentrate (varidase, Lederle, lot 165A). It was, therefore, assumed to be another antigen which had been largely separated from the complex mixture in the crude concentrate.

An appreciable protein peak was obtained in the elution at high salt concentration, unlike the results in the previous chromatograms. This fraction, V, contained a large concentration of one antigen, and a small concentration of a second. The predominant component revealed the "reaction of identity" with the band produced with highly purified streptolysin "O" obtained previously (8) from a group C streptococcal strain. This is shown in the photograph of



TEXT-FIG. 3. Chromatographic separation of electrophoretic drip point 17+ on hydroxyapatite column. 420 mg. protein in 19.7 ml. of 0.001 M phosphate buffer pH 6.8 was applied to a column 3.0 cm. wide by 2.8 cm. high. Elution carried out as previously described. P, sample applied to the column.

Fig. 1. In agreement with the immunological finding, this peak showed a streptolysin potency of about 70,000 hemolytic unit/mg. protein, while the other peaks (I–IV) showed titers of < 100 HU/mg. The sample fed on the column had a potency of 4,100 HU/mg.

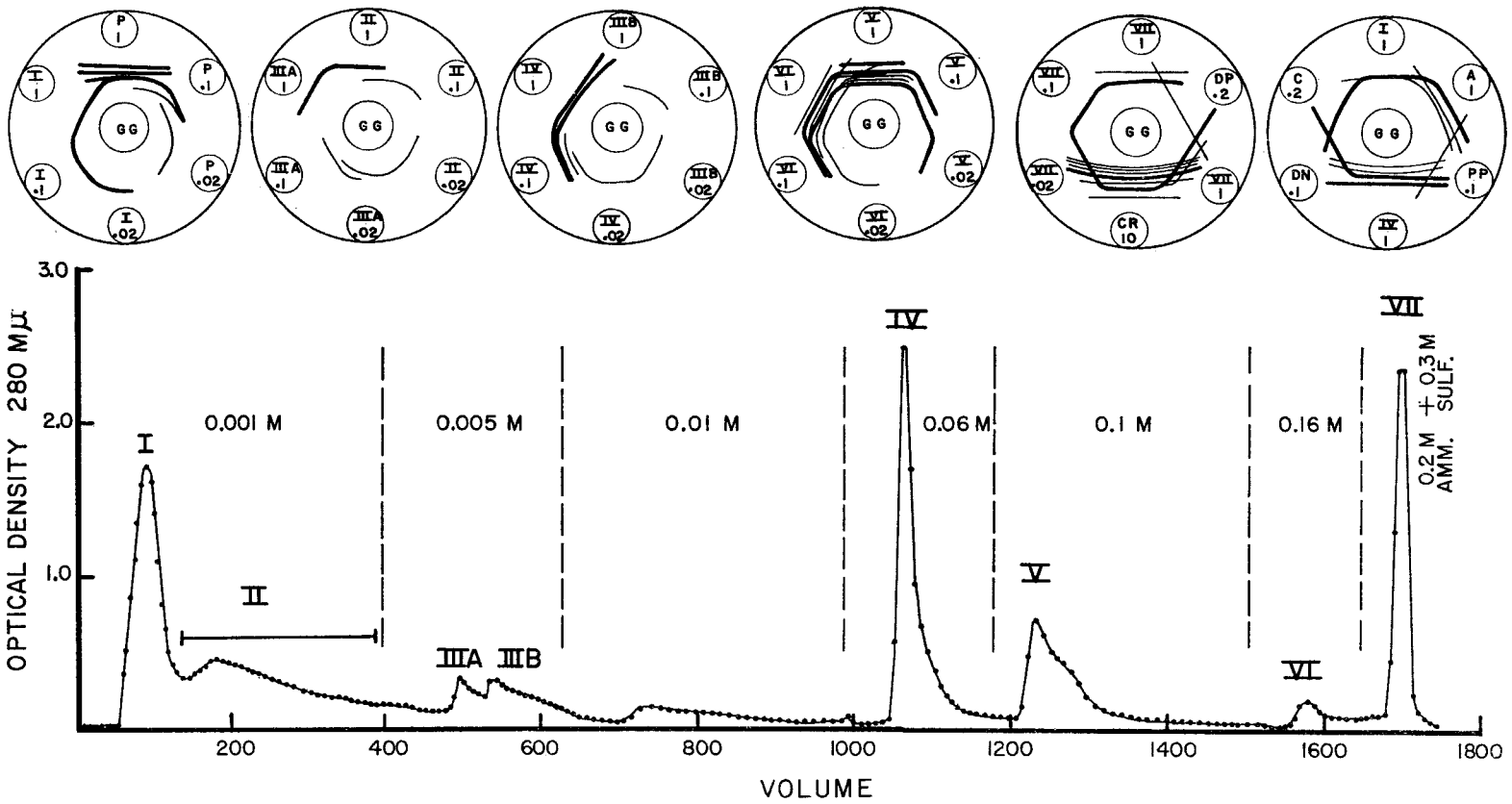
Chromatography of Electrophoretic Drip Point 19.—When the next electrophoretic fraction (19+) was chromatographed on $CaPO_4$, the resulting pattern was obtained (see Text-fig. 4).

Several points are worthy of comment. In this test, the unadsorbed material eluted in two distinct peaks (I and II), and it is of considerable interest that these were distinct immunologically. The sharp large unadsorbed peak proved to have the specificity of C carbohydrate (see diagram upper right), and it assayed to rather high dilutions as an apparent single band. It may be pointed out here that the dilutions were based on protein estimations indicated by ultraviolet absorption at 280/260 mμ wave length. The ratio of optical densities at these wave lengths was 1.27. Spectral analysis revealed a single absorption peak at 277 mμ, and a single minimum at 260 mμ. Absorption rose to very high levels at 230 to 235 mμ, and decreasing absorption was found above 277 mμ to very low levels at 350 mμ. Similar spectra were found for two other such fractions unadsorbed on calcium phosphate, and all were thus typical of proteins containing some aromatic amino acids. Total nitrogen, ninhydrin, hexosamine, and rhamnose estimations indicated that roughly 80 per cent of the sample was protein and the latter two compounds accounted for most of the remainder. These data will be reported in detail elsewhere. Since all the fractions were harvested by precipitation in saturated ammonium sulfate, and the various steps up to this point of chromatography involved at least five such precipitations and repeated dialysis, it seems very likely that this antigen probably represents a rather firm complex between the group specific carbohydrate and protein containing aromatic amino acids. Formamide-extracted or enzymatically prepared C carbohydrate have been found to be devoid of absorption peaks at 275 to 280 mμ (18, 19).

When fraction I was assayed adjacent to the unadsorbed fraction of the previous chromatogram (see Text-fig. 2) and C carbohydrates (upper right diagram), it may be noted that small amounts of another antigen could be detected in this fraction. Without such tests, this second component, which merges with one of the "antigen excess" factors would be readily missed by overlapping.

Fraction II proved to be very rich in the "antigen excess" components, and these were shown to give the "reactions of identity" with similar fractions from chromatograms of adjacent electrophoretic drip points. Fractions III A and III B also were rich in these antigens, and this agreed with the previous finding that these substances adsorb loosely onto the gel, and elute over a wide range of low salt concentrations.

Fraction IV, the relatively high crisp peak eluting between 0.01 and 0.06 M



TEXT-FIG. 4. Chromatographic separation of electrophoretic drip point 19+ on hydroxyapatite column. 520 mg. protein in 47.1 ml. was applied to a column 4.0 cm. wide by 3.8 cm. high. Elution carried out as previously described. P, sample applied to column. In upper right diagram,

DP, DPNase chromatographic fraction. A, unadsorbed peak 1 of Text-fig. 2. PP, crystalline proteinase precursor. DN, DNAse B chromatographic fraction. CR, crude culture supernate concentrate.

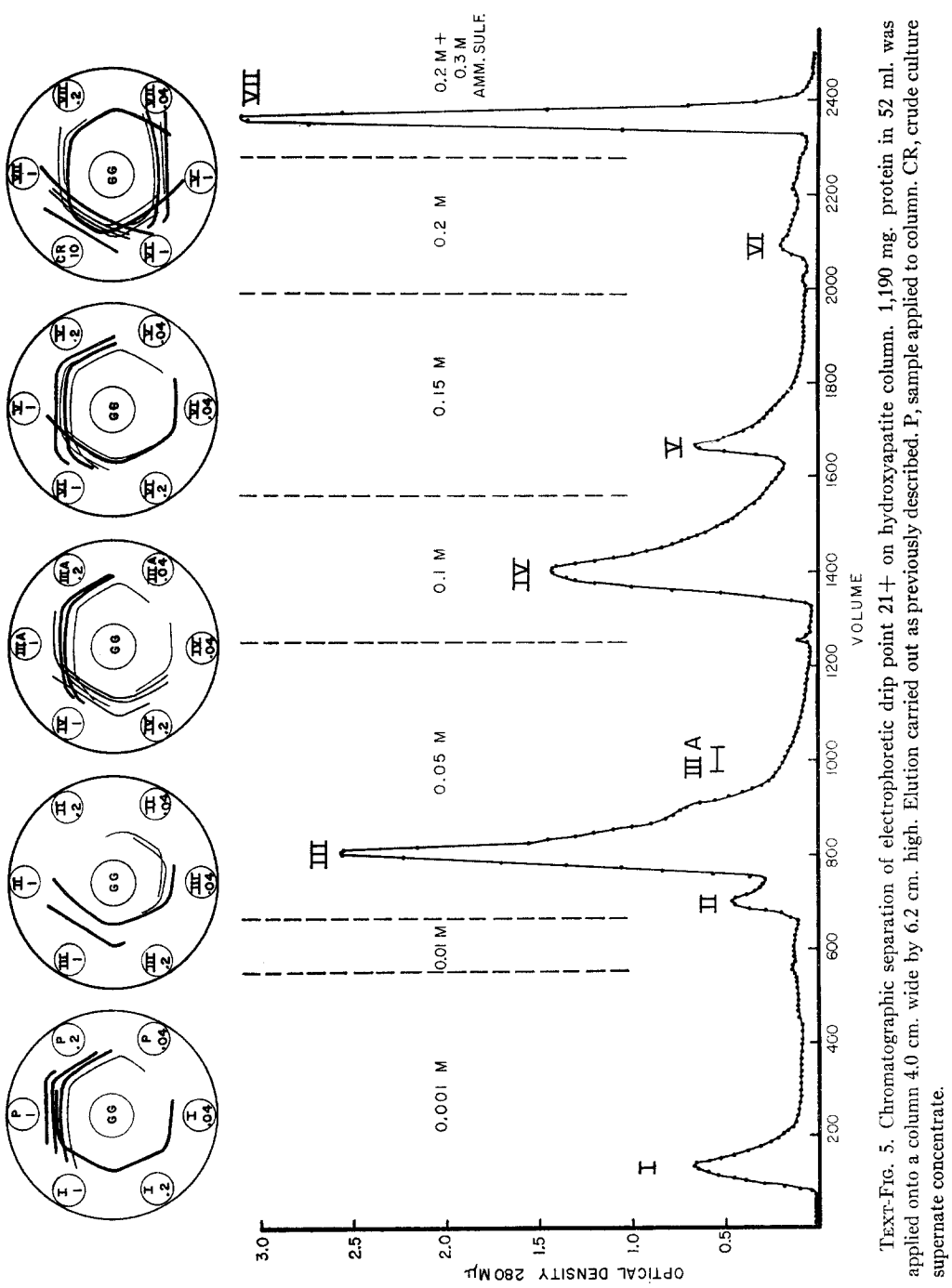
phosphate concentration still contained the "antigen excess" factors, but had added appreciable quantities of two other compounds. One of these proved to be DNAse B (see diagram upper right); the other was the same component eluting at similar salt concentrations in the chromatogram of the adjacent drip point (see Text-fig. 5).

Fractions V and VI proved to be extremely complex mixtures. Other tests revealed that none of the components present in these two peaks were detectable in fraction IV. In addition, it is clear that two or three antigens present in peak VI were absent from peak V. One of these (the one represented in VI by the band closest to the serum well) was in relatively high concentration. Similarly, the component of fraction V producing the band closest to the antibody well seemed to be present in highest concentration. The identities of the antigens in peak V and VI are unknown, but it was clear on the basis of non-identity reactions that most of them represented components distinct from those eluted at lower or higher salt concentrations. In the reference system of crude concentrate and gamma globulin (8), the heavy band seen closest to the serum well with peak V merged with the band closest to the *antigen* well indicating that this component had been highly concentrated.

The peak eluting at 0.2 M phosphate plus 0.3 M amonium sulfate was largely one antigen. It showed the "reaction of identity" with the band produced by highly purified streptolysin "O," as well as with the previously identified streptolysin "O" band seen in the standard reference system with the crude concentrate. The other component seen in peak VII only at 1 mg./ml. was shown to be distinct from the other antigens eluted up to this point as well as from DPNase, and proteinase precursor.

Chromatography of Drip Point 21+.—In the chromatogram of this electrophoretic fraction, shown in Text-fig. 5, it may be seen that the unadsorbed fraction represented a much smaller percentage of the total sample than in the separation of adjacent drip points described above. The band formed with this component also merged with the "C" carbohydrate band, and proved to contain a trace of one of the "antigen excess" components, as had the similar fraction in Text-fig. 3. Chromatography of the adjacent drip point toward the cathode (23+) showed only very small quantities of this unadsorbed antigen (C protein complex), while none was detected in processing drip point 25+ (see 8).

Peak II revealed only the "antigen excess" fractions in very high concentrations, when checked adjacent to the previous peak containing these substances. Fraction III also contained an abundance of these components, but in addition it showed appreciable quantities of another antigen titrating to 0.04 mg./ml. On the basis of the eluting buffer concentration, it was suspected to represent the DNAse B system, and this proved to be the case when a purified preparation of this enzyme (8) was placed in an adjacent well. It is of some interest that



TEXT-FIG. 5. Chromatographic separation of electrophoretic drip point 21+ on hydroxyapatite column. 1,190 mg. protein in 52 ml. was applied onto a column 4.0 cm. wide by 6.2 cm. high. Elution carried out as previously described. P, sample applied to column. CR, crude culture supernate concentrate.

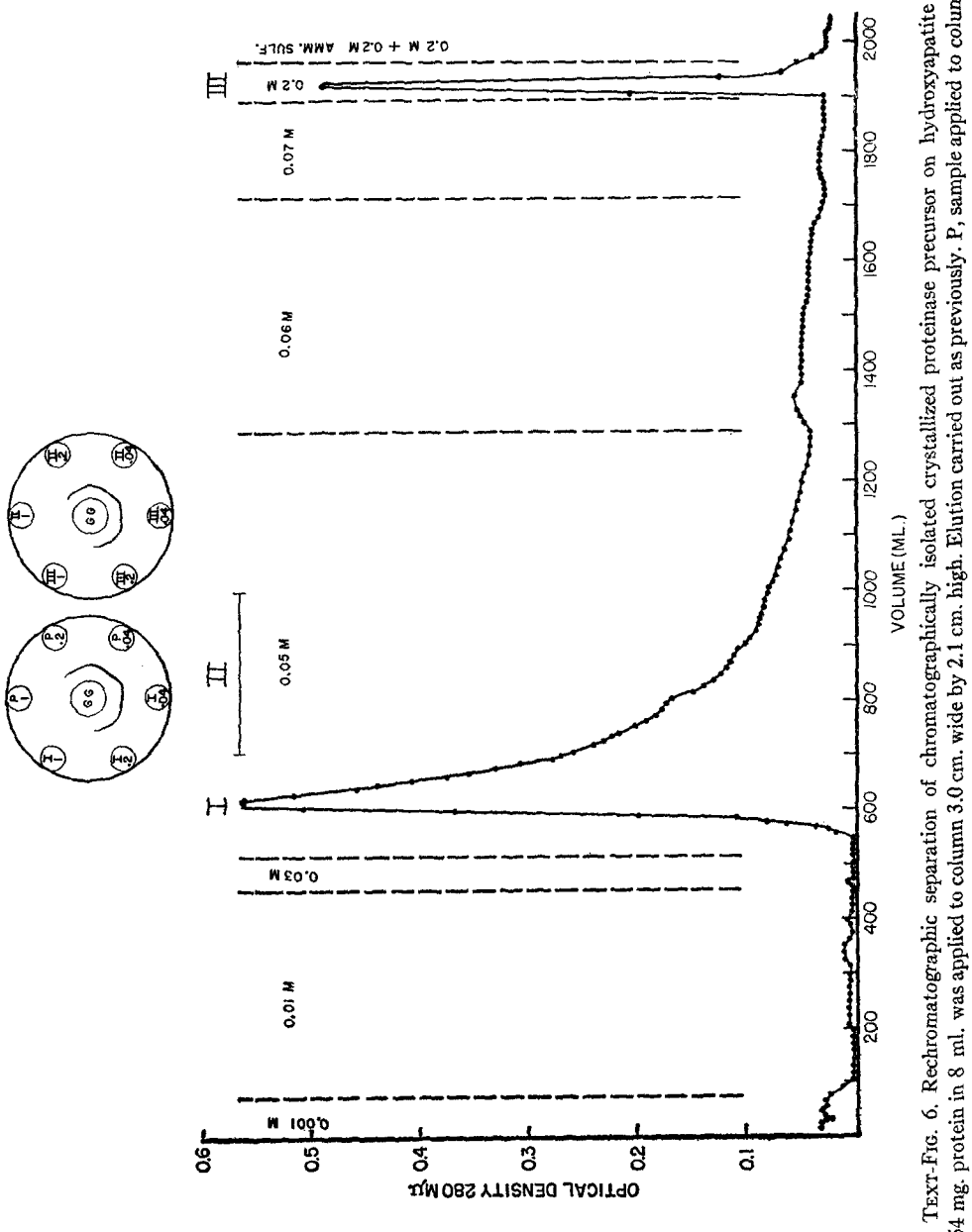
the proportions of these antigens were quite different in the tailing part of this peak, shown by the harvested antigens in fraction III A.

Fraction IV revealed three, perhaps four components not represented in the previous eluates, one being in high concentration and titrating to 0.04 mg./ml. This latter system was shown to represent the proteinase precursor system by the "reaction of identity" with this highly purified component (8). This component was also present as the innermost band of peak V, but was absent from fractions VI and VII. The immunological findings were confirmed by proteinase precursor assay, the fraction IV revealing 316 units of milk-clotting activity/mg. protein while fractions 1, III, and VII showed less than 2.5 units/mg.

Peak V contained four antigens, but it was shown that three of these were apparently the same as those found in peak IV. On the other hand, the small peak VI and the larger peak VII, showed three components, two of which were distinct from all the others thus far eluted in this run. The principal component in each was streptolysin "O" as judged by its "reaction of identity" with purified streptolysin and with the crude concentrate reference system. This tendency for streptolysin "O" to elute at several steps with high salt concentrations was also previously observed with streptolysin "O" of group C streptococcal origin (8). The identity of one of the components present in relatively low concentrations in fractions VI and VII is not known, while the other appears to be DPNase immunologically.

Chromatography of Drip Point 23+.—This fraction revealed a very small peak of unadsorbed protein which was rich in the C protein complex, while the extremely small amount of material eluting at the 0.001 M to 0.01 M step contained a high concentration of one of the antigen excess fractions. As expected, the appreciable quantity eluting at 0.03 M was quite rich in DNAse B. The very large peak eluting between 0.03 M and 0.15 M was rich in proteinase precursor, and showed moderate quantities of three of the antigens found in peak IV of Text-fig. 5. At eluting buffers above 0.2 M, large concentrations of streptolysin "O" were desorbed, along with appreciable amounts of two other components. Elutions were carried out at 0.2 M, 0.22 M, and 0.2 M + 0.2 M ammonium sulfate. It was in the latter step that the bulk of the streptolysin was eluted, the former two peaks being rather low in protein yield. A pool of all of the eluates above 0.2 M revealed a streptolysin potency of 160,000 units/mg. protein, while all of the peaks below this buffer concentration showed less than 100 units/mg. protein. The electrophoretic drip point fraction fed on to the column had a potency of 23,500 hemolytic units/mg.

Rechromatography of Crystalline Proteinase Precursor on Calcium Phosphate.—In the previous report dealing with this subject (8), calcium phosphate chromatography of an electrophoretic drip point (25+) adjacent to the above was described. It was shown there that the large protein peak eluting in the step from 0.03 M to 0.06 M phosphate consisted of apparently clean proteinase precursor which could be readily crystallized. This fraction was redissolved in

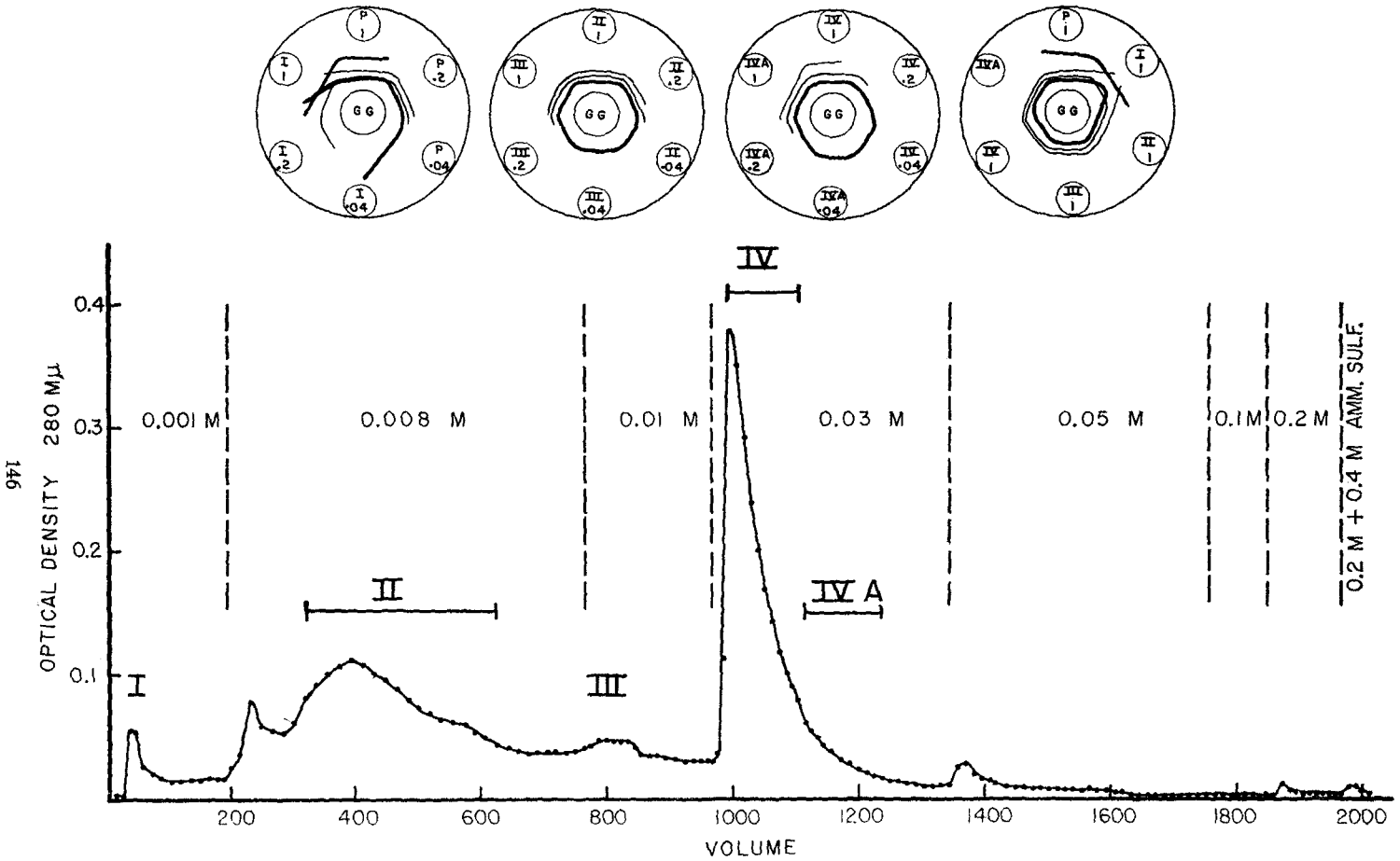


TEXT-FIG. 6. Rechromatographic separation of chromatographically isolated crystallized proteinase precursor on hydroxyapatite column. 154 mg. protein in 8 ml. was applied to column 3.0 cm. wide by 2.1 cm. high. Elution carried out as previously. P, sample applied to column.

0.001 M phosphate pH 6.8, dialyzed thoroughly against this buffer, and rechromatographed on calcium phosphate. The results are shown in Text-fig. 6. It may be seen that the bulk of the protein eluted at the same stepwise increase of buffer concentration. The two fractions representing this peak (I and II) were both seen to contain only one detectable antigen, dissolved by antigen excess effects at 1 mg./ml. The fraction fed onto the column behaved similarly. It may be seen that small increments of buffer concentration (0.06 M and 0.07 M) did not cause appreciable desorption of the trailing portion of the peak, whereas a sharp stepwise increase in phosphate concentration apparently caused complete elution of this residual material. A pool of fractions I and II was readily crystallized as previously described (8). It is of some interest that this crystalline preparation showed milk-clotting precursor potency of 1580 units/mg., while only 100 units/mg. were revealed without preliminary cysteine activation, indicating that approximately only 6 per cent was already transformed to the proteinase state. In contrast, fraction III showed milk-clotting activity at 1580 units/mg. and 790 units/mg. with and without cysteine pretreatment respectively, indicating that this fraction was about 50 per cent in the proteinase state. It is quite conceivable, therefore, that precursor and proteinase elute at slightly different salt concentrations from the gel, although both seem immunologically the same with naturally occurring human antibody, since "spurs" in these reactions have not been seen.

Rechromatography of DNAse B on Calcium Phosphate.—The chromatographic peak eluting in the step from 0.01 M to 0.03 M phosphate in the fractionation of electrophoretic fraction 23+ (see above) was found to be predominantly DNAse B, contaminated with smaller amounts of three other antigens, two of which were the "antigen excess" components. This fraction was rechromatographed on a smaller column, with the results shown in Text-fig. 7. The heavy *inner* band seen in the precipitin results in all the tracings above the chart was shown to represent the DNAse B system.

In this refractionation, the sharpest protein peak eluted in the expected buffer step (0.01 M to 0.03 M), but smaller quantities also eluted at some lower salt concentrations. Significantly, the protein eluting with these latter buffers (0.008 M and 0.01 M) revealed an abundance of DNAse B immunologically as well as enzymatically. In a number of previous chromatograms of *electrophoretic drip points*, DNAse B always failed to elute below 0.03 M phosphate. The finding here that considerable amounts desorb at lower salt concentrations suggests that some structural changes in the molecule may have occurred during its harvest by ammonium sulfate precipitation from the previous chromatographic run. Enzyme determinations revealed that peak IV had an average potency of 810,000 DNAse units/mg. protein, while peak II showed 510,000 units/mg. and the fraction fed on the column 570,000 units/mg. Also of interest is the failure to completely separate the "antigen excess" components from the



TEXT-FIG. 7. Rechromatography on hydroxyapatite of DNAse containing peaks from chromatogram of electrophoretic drip point 21+ The fraction had eluted in the step from 0.01 M to 0.03 M phosphate buffer. 89 mg. protein in 14.2 ml. was applied to a column 3.0 cm. wide by 1.8 cm. high. Elution carried out as previously described. P, sample applied to column.

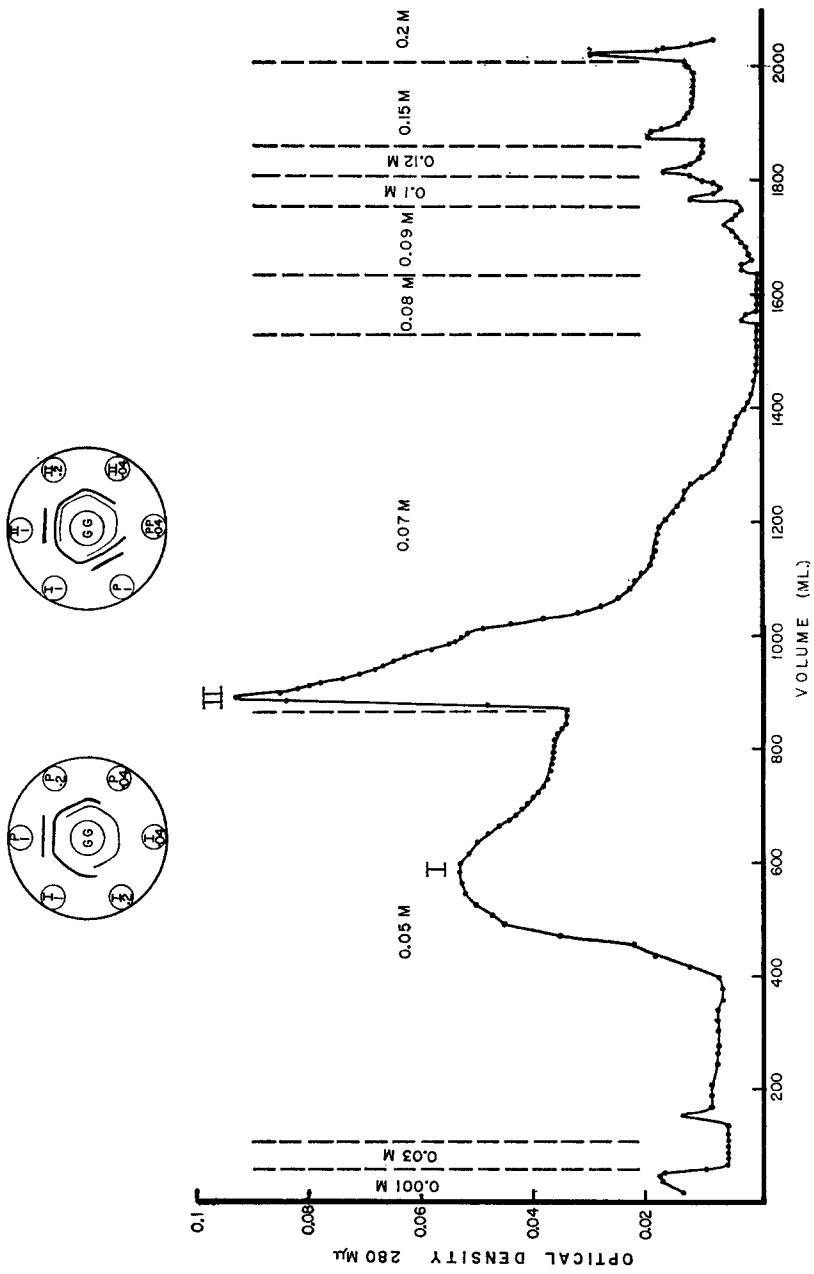
main DNAse peak (IV). These two antigens appeared to be richest in peak II, but were appreciably present in III, IV, and IV A.

In the previous report (8), it was found that the adjacent electrophoretic drip point (25+) yielded apparently uncontaminated DNAse B on chromatography in the elution step 0.01 M to 0.03 M. However, rechromatography on calcium phosphate of a sample of this latter DNAse B fraction revealed the same pattern as above. A low flat peak eluted at 0.005 M, no significant peak was seen at 0.01 M, and a crisp high protein peak desorbed at 0.03 M. In this instance, the fraction fed onto the column revealed only DNAse B immunologically with human gamma globulin; no other antigenic components were present in detectable amounts. The similarity between the rechromatography pattern obtained with this apparently clean fraction, and with that in Text-fig. 7 lends further support to the suspicion that some alteration of the molecule had taken place.

It is also worth pointing out that no significant protein peaks eluted at salt concentrations above 0.03 M. This is in striking contrast to the situation found above with proteinase precursor, and some of the other antigens, and indicates that this enzyme does not show the troublesome trailing on the downslope of the peak.

Rechromatography on Calcium Phosphate of the Mixture of Antigens Eluting Between 0.06 M and 0.15 M Phosphate (Chromatogram of Electrophoretic Drip Point 25+).—In the previous report dealing with this subject (8), drip point 25+ revealed a sharp elution peak (IV) in the step from 0.06 M to 0.15 M when chromatographed on $CaPO_4$. It was found that this fraction was rich in proteinase precursor, derived from the trailing portion of the main precursor peak. In addition, the above peak contained an abundance of a second antigen, which proved to be the same as one of the antigens seen in the complex mixtures eluting at the same buffer concentrations in peak V of Text-fig. 4, and peak V of Text-fig. 5. In the hope of separating the proteinase precursor from the other principle antigen, the above fraction was rechromatographed on $CaPO_4$. A very low loading was used, 50 mg. of protein being applied to a column 3 cm. wide by 1.8 cm. high. The results are revealed in Text-fig. 8. It may be seen that peak II is relatively enriched in the non-proteinase precursor component (see upper right precipitin tracings), whereas peak I is predominantly precursor. It is conceivable in spite of the tendency of the precursor to trail in elution, that if adequate amounts of material were available, repeated rechromatograms would finally achieve satisfactory separations.

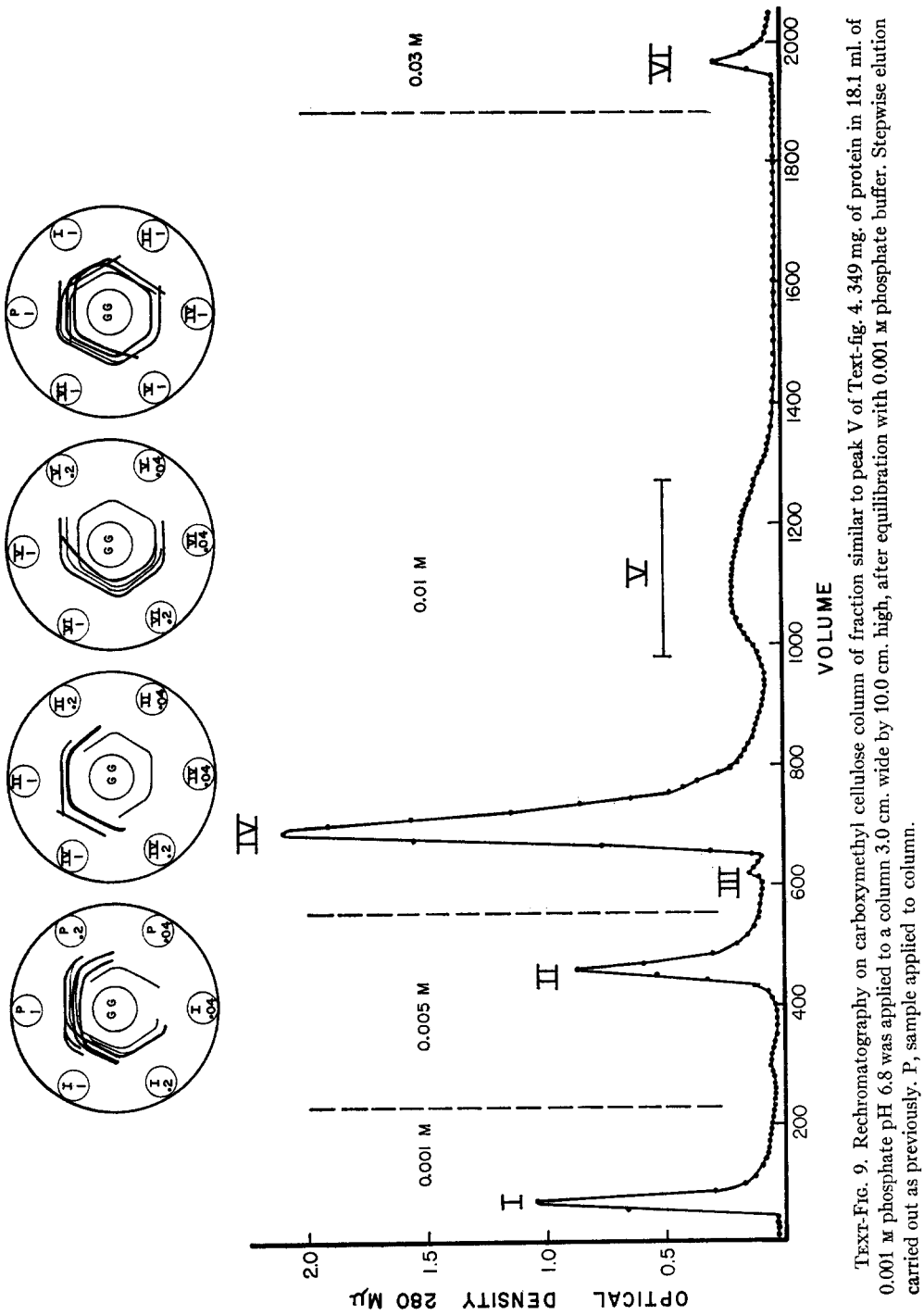
Carboxymethyl Cellulose Rechromatography of the Mixture of Antigens Eluting Between 0.03 M and 0.15 M of Electrophoretic Fraction 23+.—The development of cellulose derivatives useful for the column chromatography separation of proteins by Sober and Peterson (20) prompted the attempt to separate some of the streptococcal antigen mixtures with these. A fraction similar to the



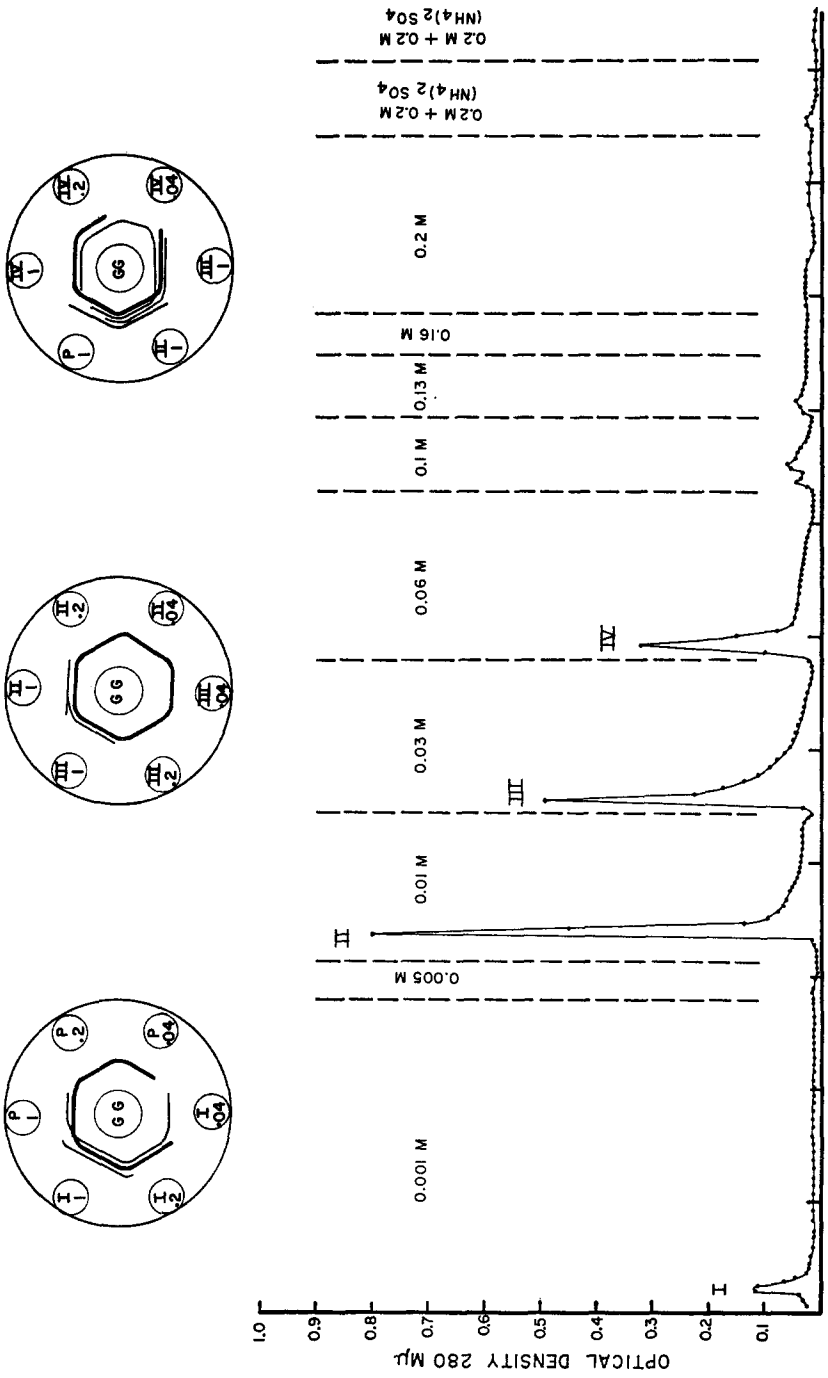
TEXT-FIG. 8. Rechromatography on hydroxyapatite of elution peak in the step 0.06 M to 0.15 M phosphate, in the calcium phosphate chromatography of electrophoretic fraction 25+. It contained proteinase precursor and two other antigens. 50.4 mg. protein in 7.2 ml. was applied to a column 3.0 cm. wide by 1.8 cm. high. Elution carried out as previously described. P, sample applied to the column. PP, crystalline proteinase precursor.

above (Text-fig. 7) containing proteinase precursor and at least 4 to 5 additional antigens as in peak V Text-fig. 4 and peak V of Text-fig. 5 was studied. The carboxymethyl cellulose column was prepared as recommended (20), but stepwise elution was carried out as indicated in Text-fig. 9. The impressive differences in the protein peaks, and the crispness of the elution patterns unfortunately did not represent good antigen separations, as seen in the precipitin diagrams above. Elutions with higher salt concentrations were carried out and no other protein eluted even at concentrations of 0.2 M phosphate plus 0.4 M ammonium sulfate, except for a very small peak in the step from 0.1 M to 0.13 M. It may be seen, however, that the unadsorbed material (1) was relatively enriched in two components, while fractions II, III, IV, and V were relatively rich in one component which was shown to be proteinase precursor immunologically and enzymatically. Peak VI was relatively enriched in two other components. This data shows clearly, however, that carboxymethyl cellulose used under these conditions does not offer much hope for separations of the mixture of antigens eluting from calcium phosphate between 0.03 M and 0.15 M phosphate concentration. In addition, it graphically demonstrates the danger of relying on different peaks seen in chromatographic patterns as the only significant index of the separations achieved. This is also brought out in the two other rechromatograms reported below.

Rechromatography of Streptolysin "O" Peaks on Carboxymethyl Cellulose.— The bulk of the protein eluting at 0.2 M phosphate or greater in the chromatogram of electrophoretic drip point 23+ above, was pooled, and thoroughly dialyzed against 0.001 M phosphate buffer. This fraction, rich in streptolysin "O" (160,000 HU/mg.), contained at least two other antigens at 1 mg./ml. The sample (64.5 mg./13.1 ml.) was applied to a carboxymethyl cellulose column 2.3 cm. wide by 7.3 cm. high. The chromatogram is shown in Text-fig. 10. It may be seen that a number of well defined protein peaks were obtained. As in the preceding chromatogram with this adsorbent, however, it may be noted that the antigen separations achieved are not too satisfactory. The heavy band in each fraction was identified as streptolysin "O" immunologically. It was found that a pool of fractions II and III showed 225,000 HU/mg., while fraction IV contained 49,000 HU/mg. As in the previous chromatogram *relative* enrichment of some of the antigens was achieved. Peak 1 contained considerably higher concentrations of one antigen closest to the peripheral well in the preliminary sample (P in the upper left diagram). Peak IV, on the other hand, was quite enriched in another component (upper right diagram). This latter antigen was demonstrated immunologically to be the enzyme DPNase. The relatively enriched component in peak I was not identified but was found to be distinct from proteinase precursor as well as DPNase immunologically. Another point which may be mentioned here, is the tendency of hidden overlapped bands to be separated when placed adjacent to a well rich



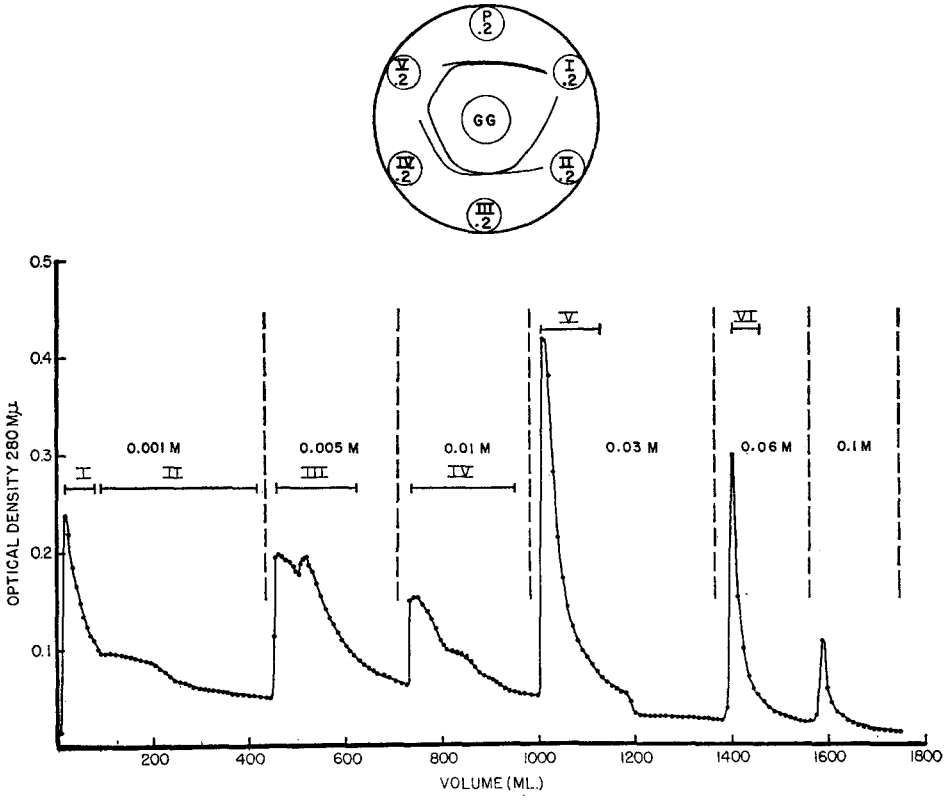
TEXT-FIG. 9. Rechromatography on carboxymethyl cellulose column of fraction similar to peak V of Text-fig. 4. 349 mg. of protein in 18.1 ml. of 0.001 M phosphate pH 6.8 was applied to a column 3.0 cm. wide by 10.0 cm. high, after equilibration with 0.001 M phosphate buffer. Stepwise elution carried out as previously. P, sample applied to column.



TEXT-FIG. 10. Rechromatography on carboxymethyl cellulose column of streptolysin "O" chromatographic fraction derived from electrophoretic fraction 23+. 64.5 mg. protein in 13.1 ml. was applied to a column 2.3 cm. wide by 7.3 cm. high, after equilibration in 0.001 M phosphate. Stepwise elution carried out as previously. P, sample applied to column.

in one of the components; *e.g.* compare between II and III on the center plate, and the same reactions when adjacent to IV as on the right. Similar effects may be seen in some of the other results shown above, and have been reported by others.

Rechromatography of "Antigen Excess" Components on DEAE Cellulose.—It was found that the two "antigen excess" fractions repeatedly tended to show



TEXT-FIG. 11. Rechromatography on *DEAE cellulose* of "antigen excess" fraction mixture, similar to peak III of Text-fig. 3. 92 mg. of protein in 7.1 ml. of 0.001 M phosphate, pH 6.8, was applied to a column 2.0 cm. wide by 4.5 cm. high, after equilibration in 0.001 M phosphate. Stepwise elution was carried out as indicated. P, sample applied to the column.

similar elution properties, and attempts were made to demonstrate their separability by rechromatography. A number of trials with such fractions were carried out on long thin calcium phosphate columns, with or without gradient elutions. The results were not too satisfactory, although slight differences in their elution properties were indicated. For that reason, a DEAE cellulose column was employed using a fraction similar to peak III, Text-fig. 3, with the

results shown in the Text-fig. 11. Although complete assays are not shown in the precipitin diagrams, the only two antigens seen in the assay results were demonstrated in other tests to be the "antigen excess" substances. It may be seen that they are separable to some extent at low salt concentrations, and that the second hump of unadsorbed protein represents one of these antigens in relatively clean form. In fact, peak II showed only one component when assayed at 1.0, 0.2, and 0.04 mg./ml. The subsequent peaks at higher salt concentrations showed varying mixture of the two antigens, but the immunological distinctness of these two substances was convincingly demonstrated in these tests.

Possible Immunological Relationship of One of the "Antigen Excess" Components and Scarlet Fever Toxin.—Three preparations of scarlet fever toxin obtained from widely different sources, different streptococcal strains, and under different growth conditions were each compared and assayed immunologically with the antigens described above. It was found that each of the erythrogenic toxin concentrates were rich in one of the "antigen excess" components. The fraction of Dr. A. Stock (No. 101-4C, 1,000 Lf/mg. protein) described some years ago (10), revealed at least three components on precipitin analysis with human gamma globulin, one being in highest concentration (Fig. 2 *a*). The fraction obtained from Dr. Hanson revealed only one component on precipitin assay (2 *b*), while the concentrate from Dr. Soru showed two components (2 *c*). The difference in end-points should be noted, that of Stock's preparation being most potent. When the fractions were tested against each other, they were shown to give merging bands of "identity", and to also merge with the innermost band of the "antigen excess" fractions. (See Figs. 2 *d*, 2 *e*, and 2 *g*). The reactions with Soru's preparation are not well seen in the photograph (2 *d*) but in other plates at higher concentration the two bands resulting were found to merge completely with both the "antigen excess" fractions described here.

Interestingly, it was possible to identify the other two components detected in Stock's concentrate. The heavy band closest to the antigen well assaying to 0.33 mg./ml. proved "identical" with the band produced by DNAse B. These reactions are shown in Fig. 2 *f*. The erythrogenic toxin used here was in high enough concentration to cause almost complete inhibition of the two other precipitin bands of Fig. 2 *a*. The middle band of the three found with this preparation assayed to 0.11 mg./ml., and it showed a smooth merging with the band formed by proteinase precursor (Fig. 2 *g*). The immunological detection of proteinase precursor related antigen in Stock's concentrate was confirmed by enzyme assay. It was found that 40 milk-clotting units/mg. protein were present, and all seemed transformed to the proteinase state. This correlated roughly with the endpoint seen by immunological assay. Incidentally, it may be seen that the two "antigen excess" mixtures tested in Fig. 2 *g* have

reversed concentrations of these components. Stock's erythrogenic toxin preparation is apparently devoid of the "antigen excess" fraction usually farther from the serum well.

Preliminary tests by rabbit intradermal inoculation in this laboratory failed to demonstrate erythrogenic toxin activity of a fraction rich in the innermost "antigen excess" component (peak III of Text-fig. 4), or of Hanson's scarlatinal toxin concentrate. For this reason, samples of three chromatographic fractions rich in this component (II of Text-fig. 4, II of Text-fig. 5, and another similar fraction) were sent to Dr. Stock,[2] who kindly titrated the skin toxin potency in a rabbit known to be a good "reactor." Only about 50,000 skin test doses/mg. were found with these fractions, although all were as rich in the antigen under discussion as was Dr. Stock's concentrate. The failure to find abundant erythrogenic toxin activity is puzzling and perhaps suggests that the antigen secreted by the C203S strain is deficient in the toxin moiety of this molecule.

Biological Activity of C Protein Complex.—Recent intriguing studies by Schwab, Cromartie, *et al.* (21) have demonstrated that peculiar recurrent nodular skin lesions in rabbits were produced by sonic extracts of streptococcal cells. These investigators have presented evidence that the active substance is a complex of C carbohydrate and protein derived from the cell walls, both portions of the agent being necessary for the development of lesions. In addition, they showed that only relatively large particulate fractions were effective. Because of these findings, it was felt of interest to test the C protein complex described above (I of Text-fig. 5, and I of Text-fig. 4), intradermally in rabbits. Although suggestive injection site nodules developed in two rabbits, no such similar lesions were found in three other animals. One rabbit received 5 mg. of one of the fractions intravenously, and suffered no apparent ill effects. Samples of these two fractions were kindly tested by Dr. Schwab[2] in his laboratory, and no significant skin lesions were found. These fractions had been clarified by centrifugal forces of 60,000 *G* and more at several stages during the course of preparation, the supernates being harvested. It is possible, therefore, that they represent the same component described by Schwab *et al.*, but in a smaller particle size.

DISCUSSION

The results presented here emphasize several facts. Perhaps the most important is the clear demonstration of the remarkable complexity of the group A hemolytic streptococcus. It is worth stressing again that the precipitating systems detected here were produced by human antibodies occurring as a

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[2] The authors are grateful to Dr. Stock for carrying out this assay, and to Dr. Schwab for testing the C carbohydrate protein complex in rabbits.

result of *natural subclinical or clinical infections*. The multiplicity of the antibodies demonstrates that these large numbers of streptococcal extracellular products are secreted in human tissues during the course of infection.

The results of the chromatographic separations of the rather more complex electrophoretic fractions reported here, indicate that an even greater number of extracellular products are secreted by the microorganisms than was previously thought. Indeed, by adding the distinct antigen-antibody systems found above, the total of such *in vivo*-produced antigens numbers at least fifteen to sixteen as compared to the twelve previously estimated on the basis of the electrophoretic data. That the large number is a closer approximation to the truth is indicated by recent immunoelectrophoretic observations. These tests, to be reported in detail elsewhere, were made possible when certain technical difficulties were overcome due to the high concentration of human gamma globulin used. Suffice to say at present, when the crude C203S streptococcal culture supernate is separated by immunoelectrophoresis, at least *20* distinct antigen-antibody systems were detectable with normal pooled human gamma globulin.

It is intriguing to note that this strain of hemolytic streptococcus was apparently isolated from a case of scarlet fever about 25 years ago (22) and has been passed from laboratory to laboratory (*e.g.* reference 23). It still continues to synthesize in broth all these extracellular antigens which are secreted in the tissues of human beings currently infected with other streptococci. In studies to be reported soon, it has been found that a group C streptococcal strain, used in England for the commercial production of streptokinase and streptodornase, produces at least eight antigens for which human gamma globulin has corresponding antibodies. All of these are apparently related to or identical with group A C203S antigens detected above.

It is at once apparent that the problems involved in achieving complete purification of individual components from such complex mixtures for the study of their biological properties, is a much more imposing problem than appeared at first. In spite of this, the methods described in this report and the previous one (8) have indicated that much progress has been made along these lines. Up to the present, a total of nine group A extracellular antigens have been well separated from all the others, either to a large extent or completely, within limitations of the sensitive methods used. Often the quantities reaching this state are rather small, especially since the peripheral electrophoretic drip points were most likely to yield the cleanest fractions. Of these nine, the identities of five have been fairly well established as streptolysin "O," DPNase, DNAse B, proteinase precursor, and C carbohydrate-protein complex. Suggestive evidence has related a sixth to erythrogenic toxin, and the identity of the other three is not known. Since there seem to be more antigens than there are known extracellular streptococcal products, it is apparent that some of

these must represent heretofore unknown substances, perhaps of considerable significance biologically.

Similar antigen components in adjacent electrophoretic fractions seem to elute from calcium phosphate columns at the same elution steps of buffer concentration increases. In addition, rechromatography on calcium phosphate demonstrated that the *bulk* of those fractions elute at the same buffer step as originally. Possible deteriorative changes, as indicated by minor secondary peaks, in the rechromatographic patterns were pointed out. Furthermore, attempts to separate some of the complex mixtures eluted as single peaks from the hydroxyapatite, on CM cellulose or DEAE cellulose, resulted in excellent protein elution patterns which were only comparatively poorly reflected in the separations of immunological components. Further studies along these lines with still other adsorbents, and other conditions are necessary, but must await adequately large amounts of potent crude concentrates. Detailed and unequivocal biological observations with immunologically purified preparations will require substantial quantities of the isolated fractions. It is conceivable that strains may be found producing especially large yields of one or another of these components, so that their isolations may thus be facilitated. A thorough analysis by these methods of the antigen-producing potentialities of large numbers of different strains of different types, and from distinct disease patterns, is also certainly indicated.

SUMMARY

Studies on the purification of group A streptococcal extracellular antigens detectable with naturally occurring human antibodies from normal individuals have been extended. It has been shown that streptococcal electrophoretic fractions intermediate between the most rapidly migrating components are quite complex. In the calcium phosphate chromatography of adjacent electrophoretic fractions, particular antigenic components desorbed at similar buffer elution steps.

It is clear from the results obtained that substantially more extracellular antigens than the twelve heretofore recognized are secreted in human beings during infection, as judged by their detection with human antibodies. The precise number is not yet known, but is probably greater than 16.

Of the nine components which thus far have been separated rather well from the others, four were previously identified as streptolysin "O," diphosphopyridine nucleotidase, proteinase precursor, and desoxyribonuclease B. The accumulated data substantiated these previous identifications.

The identity of a fifth antigen has been made as a possible complex of C carbohydrate and protein. Tentative evidence for the relationship of a sixth component to scarlet fever toxin has been presented.

It has been shown that rechromatography of crystalline proteinase precursor

and desoxyribonuclease B on calcium phosphate columns resulted in elution principally at the expected stepwise increase in buffer concentration.

Attempts to isolate antigens present as mixtures in some calcium phosphate chromatographic peaks, by rechromatography on DEAE or CM cellulose columns resulted in only limited further purifications.

The authors wish to express their appreciation for the capable technical assistance of Miss Suzanne Lucille Keatinge.

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EXPLANATION OF PLATES

PLATE 23

FIG. 1. Immunological identification of fraction V of Text-fig. 3 with streptolysin "O." Human gamma globulin in central well. × 1.3.

LY, highly purified group C streptolysin "O."

DP, diphosphopyridine nucleotidase, C203S.

DN, desoxyribonuclease B, C203S.

C, C carbohydrate, C203S, formamide extract.

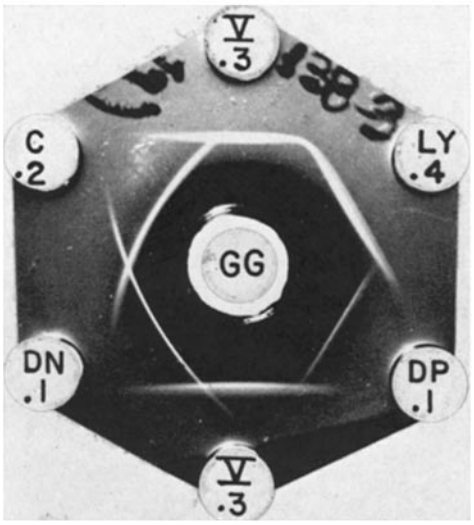

FIG. 1

(Halbert and Auerbach: Precipitin analysis and streptococcal infections. IV)

PLATE 24

FIG. 2. Immunological tests with various scarlet fever toxin preparations, and fractions containing the "antigen excess" components. GG, normal pooled human gamma globulin. Numbers represent mg. protein/ml.

FIG. 2 *a*. Assay of erythrogenic toxin concentrate No. 101-4C (ST) obtained from Dr. A. Stock.

FIG. 2 *b*. Assay of erythrogenic toxin concentrate obtained from Dr. L. Hanson (H).

FIG. 2 *c*. Assay of erythrogenic toxin concentrate obtained from Dr. E. Soru (RU).

FIG. 2 *d*. Immunological comparison of the erythrogenic toxin preparations and three different "antigen excess" containing chromatographic fractions.

III-2, peak III of Text-fig. 2.

III-3, peak III of Text-fig. 3.

II-4, peak II of Text-fig. 4.

FIG. 2 *e*. Immunological comparison of erythrogenic toxin preparation of Dr. Hanson with various chromatographic fractions.

DN, DNAse B.

L, group C streptolysin "O."

DP, DPNase, group A.

II-4, "antigen excess" peak II of Test-fig. 4.

FIG. 2 *f*. Identification of a "minor" immunological component in Stock's erythrogenic toxin concentrate with DNAse B.

DN, DNAse B preparation used above in 2 *e*.

DN′, Rechromatographed DNAse B, peak IV of Text-fig. 7.

CR, crude culture supernate concentrate.

FIG. 2 *g*. Identification of the second "minor" immunological component in Stock's erythrogenic toxin concentrate with proteinase system.

IV-5, peak IV of Text-fig. 5.

PP, crystalline-proteinase precursor.

II-5, "antigen excess" fraction peak II of Text-fig. 5.

III-3, "antigen excess" fraction peak III of Text-fig. 3.

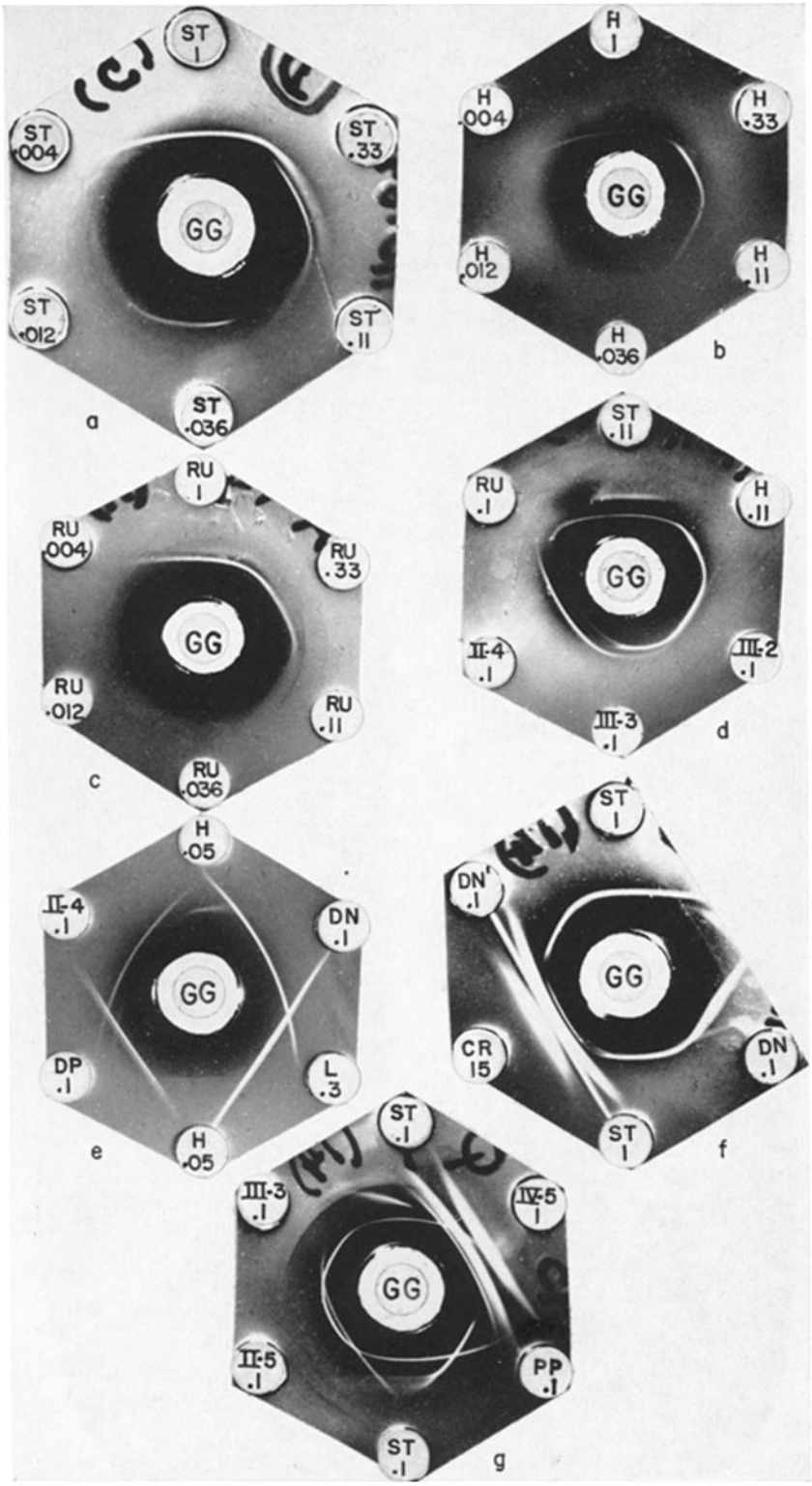

FIG. 2

(Halbert and Auerbach: Precipitin analysis and streptococcal infections. IV)