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

II. Ouchterlony and Oakley Technics*

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Plates 30 to 33

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In the preceding report (1), it was shown that precipitating antibodies to streptolysin O concentrate were quite common in human sera, using the agar technic of Oudin (2). Although some sera showed several bands with this antigen, strong evidence was presented that the streptolysin O system was the most frequent in these tests.

With rabbit antisera to the streptolysin concentrate, up to four antigens could be found. The identity of the non-streptolysin bands could not be readily determined. Although the concentrates used were found to be contaminated with streptococcal desoxyribonuclease (3), and proteinase precursor (4), it was shown that the extra bands in the rabbit sera were apparently not due to these systems using the Oudin methods. The preparations were found to be essentially free of some of the other well characterized antigens or toxins produced by these organisms.

Because of the inherent advantage and simplicity of the technic described by Ouchterlony (5–7), the above findings were further explored with this method. Special attention could thus be directed toward the attempted identification of the non-antistreptolysin O systems found in individual human sera. Extensive study was made of sera from rheumatic fever patients, and these observations comprise a large portion of the following report. Because of the suprising multiplicity of antigen-antibody systems found, a few tests were made with Oakley's agar precipitin technic (8) for confirmation of this complexity.

The application of agar precipitin technics for the study of human antibodies has thus far been only fragmentary. One test has been reported by Schiott (9), and four by Wodehouse (10). Both investigators were studying human allergies.

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Materials and Methods

The description of the streptolysin O concentrates, some of the other test antigens, assay methods, and animal sera used, are presented in the foregoing report (1). In addition, the following antigen mixtures were employed.

A sonic vibrated extract of the streptococcal cells (C203S) was prepared with the Raytheon magnetostriction oscillator (9 Kc) at 150 volts output for 30 minutes with water cooling. Approximately 10 ml. of centrifuged packed cells was suspended in 25 ml. of 0.85 per cent NaCl solution. The vibrated suspension was spun to clarify at 20,000 R.P.M. for 1 hour in the Spinco preparative ultracentrifuge, with the No. 40 rotor.

Lyophilized preparations containing streptolysin "S" were made according to the technic described by Bernheimer (11). The powder thus obtained was apparently free of detectable streptolysin O, as the hemolytic activity was not affected by cysteine.

Samples of the second $(NH_4)_2SO_4$ precipitate obtained in the streptolysin purification procedure were used as "crude concentrate" in the following tests. They were approximately 45-fold concentrates of the crude supernates from the streptococcal growth.

The streptolysin O concentrate used here (H165-9) was about 99 per cent in the reversibly oxidized state. It contained approximately 14,000 hemolytic units/mg., 8,000 desoxyribonuclease units/mg. (12), and from 5 to 10 per cent by weight of proteinase precursor. It was free of detectable amounts of proteinase, streptokinase, hyaluronidase, "C" carbohydrate, streptolysin "S," and amylase. Tests for the latter enzyme utilized the technic of Crowley (13).

The human sera included a "non-rheumatic" group consisting of 35 sera from 35 patients with clinical diagnoses including hypertensive cardiovascular disease, cirrhosis, malignancy, diabetes, and toxemia of pregnancy, etc. The ages of these patients ranged from 31 to 62, averaging about 40 years. The acute rheumatic fever group included 49 sera from 33 patients at various stages of a well defined acute attack of this disease,[1] while the inactive rheumatic group consisted of 17 sera from 15 patients during convalescence from an acute attack. These bleedings were obtained 1 to 14 months following apparent cessation of rheumatic activity. Nine of the latter sera were from 9 patients also bled during the acute illness. The criterion used for rheumatic activity was elevation of the sedimentation rate, a generally accepted indication of continuing disease (14). The ages of the entire rheumatic group varied from 5 to 38, averaging about 14 years.

The Ouchterlony technic (6) was modified as follows: bacto-agar (Difco) was dissolved at 0.6 per cent by boiling in 0.85 per cent NaCl. When the solution was cooled to about 55°C., solid sodium barbital (veronal) was added to a final concentration of M/25. The pH was then adjusted to 7.4 with concentrated HCl using a glass electrode. 25 ml. of the agar was poured into unscratched sterile Petri dishes, and allowed to solidify on a levelled surface. The plates were then chilled in the refrigerator for several hours to overnight, sterile blotter discs being placed under the top lids to absorb moisture. They were placed over a pattern drawn on paper, and holes cut into the agar in the appropriate places with sterile cork borers. The centrally placed hole was the larger in most tests, averaging about 11 mm. in diameter. Surrounding the central well, a series of 6 smaller wells were placed in a circle equidistantly, each having a diameter of about 6 mm. The distance from the center of the plate (and the middle well) to the centers of the peripheral wells was 20 mm. The plugs of agar were easily removed intact with a very small spatula. The plates were allowed to stand in the refrigerator for at least an hour up to overnight, to allow excess fluid to seep into the wells. This fluid was removed, and 0.1 to 0.15 ml. of serum was placed in the central well, followed by 0.05 to 0.1 ml. of antigen solu-

[1] Many of these patients were included in a large cooperative rheumatic fever study, and the diagnoses were clearly established according to the criteria used in that project. The records and sera were kindly made available by Dr. E. Fischel and Dr. C. W. Frank.

tions in the peripheral wells. The plates were then placed in the refrigerator and the precipitin bands read at intervals starting from 3 days. Readings were made at room temperature as quickly as possible with a small fluorescent light and dark background, care being taken not to get too close to the light source. It may be recalled that with the Ouchterlony technic, antigen-antibody bands due to the same system will join when they occur appropriately near each other ("reaction of identity"). When the bands are due to distinct antigen-antibody reactions, they cross (7).

In many of the early studies with human sera, a troublesome complication arose in the development of opacity of varying intensity surrounding the serum wells. The effect was even worse when the buffer was omitted from the agar, and the turbidity often extended into and obscured the fainter bands. This problem was never observed to a serious degree with rabbit or guinea pig serum. It was found after much trial that addition of glycine to the molten agar prior to neutralization greatly alleviated and often prevented this difficulty. The concentration of glycine most frequently used in later tests was one molar, and the agar concentration was increased to 0.7 per cent. Even higher levels of glycine were shown not to significantly interfere with the development of precipitin bands.

The agar precipitin technic of Oakley and Fulthorpe (8) was used with only slight modifications. The same size tubes were used as in the previous study with the Oudin technic (1), and the agar concentration was reduced. The serum agar layer consisted of equal volumes of undiluted serum and of 0.5 per cent agar in 0.85 per cent NaCl, and the intermediate agar layer contained 0.5 per cent agar (0.2 ml.). The intermediate layer in which most of the precipitin bands developed was usually about 20 mm. long. The volume of antigen solution layered over this was usually 0.2 to 0.3 ml.

The antistreptococcal desoxyribonuclease tests were carried out as described by McCarty (15). The highly polymerized beef thymus desoxyribonucleic acid was prepared by the method of Zamenhof (16). Tests for streptococcal ribonuclease activity were carried out by the method of McCarty (3).

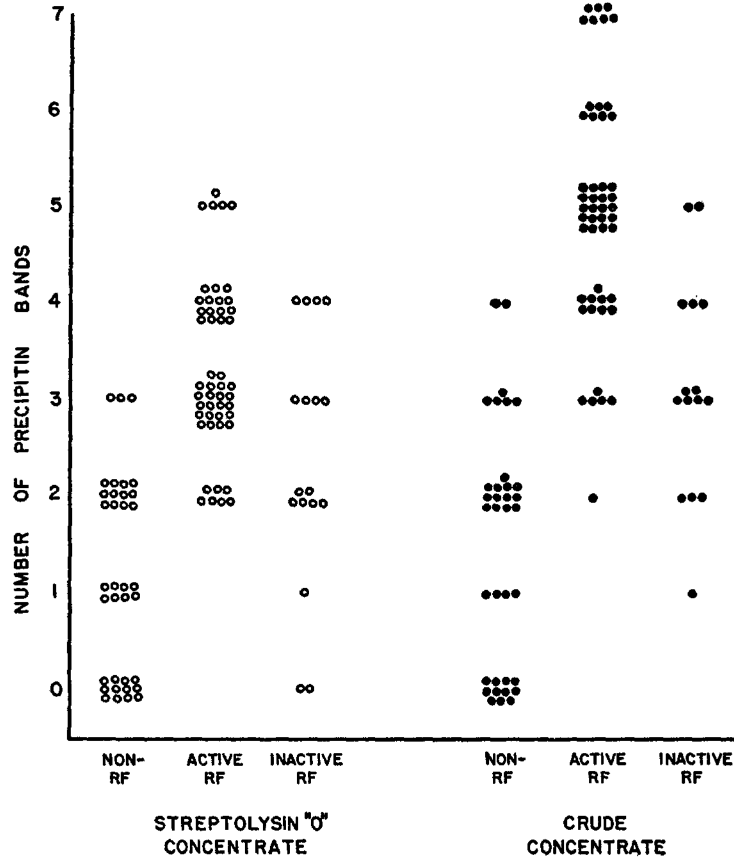
RESULTS

Tests with Ouchterlony Technic:

Human Sera.—From 0 to 5 bands were found with various human sera in tests with streptolysin O concentrate. When several lines were seen, one was usually quite intense, the others moderately to very delicate. The types of results that were obtained are exemplified in Fig. 1. It may be mentioned that the sharpness of the lines also showed considerable variation. Many were quite crisp and clearly defined, while others were somewhat fuzzy in outline. In the early stages of development, the separation of the bands was usually maximally defined. The heaviest lines were apparent first, (as early as 3 days); faint ones often took several more days to make their appearance. The degree of separation of the bands when fully developed (1 to 2 weeks) was quite variable, as can be seen from the examples shown throughout the report.

In tests with crude concentrates of the streptococcal culture supernates, from 0 to 7 bands were seen. Examples of those are shown in Fig. 2. In these patterns, when several bands were seen, from 1 to 3 of them might be quite intense. Most commonly, in plates with four or more bands, two were quite strong, as compared with the remainder.

The numbers of bands seen with these two streptococcal preparations were compared in three groups of human sera available. It may be seen in Text-Fig. 1 that patients with acute rheumatic fever showed many more bands



TEXT-FIG. 1. Numbers of antigen-antibody bands found in individual human sera with streptolysin O concentrate (H165-9), and crude concentrated streptococcal supernate (H246-4). Each point represents a single serum.

Non-RF, sera of non-rheumatic patients.

Active RF, sera of patients with acute rheumatic fever.

Inactive RF, sera of patients during convalescence from rheumatic fever.

than the non-rheumatic patients with both of these antigen preparations. It may also be mentioned that the bands were usually much more intense in the rheumatic sera than in the non-rheumatic. The inactive rheumatic group showed a trend to a decrease in numbers of the bands. That this was real is indicated in the example shown in Fig. 3, where two bleedings from the same

patient are compared. The first (3 *a*) shows the patterns resulting with serum obtained 3 weeks after a streptococcal infection, and 1 week after the onset of his first acute rheumatic fever attack (patient J. H.). The disease lasted 5 months, and the second serum shown (3 *b*) was obtained 7 months after the initial upper respiratory infection. The disappearance of some of the faint bands, and the decrease in intensity of the heavy bands seen in the acute illness is apparent. Similar results were noted in other patients when such pairs of sera were available. In some instances, detectable antibodies seemed to persist for as long as 1½ years following the initial upper respiratory infection.

It is of interest that two patients in the non-rheumatic group had recorded in their histories the occurrence of an upper respiratory infection ("cold") 3 and 6 weeks prior to the time of the blood sample. Both of these showed three bands each with the streptolysin and crude concentrates. In addition, one 51 year old man ill with hyperthyroidism, had had a history of childhood rheumatic fever, and subsequent mitral stenosis with decompensation. There was no mention in the hospital records of recent upper respiratory infection or rheumatic activity at the time of bleeding, but he showed three bands with the streptolysin concentrate and four with the crude preparation. None of the other "non-rheumatic" patients had histories of upper respiratory infection just prior to the time of bleeding, nor did they reveal histories of old rheumatic fever in their clinic records.

An examination of many of the sera was also made with the following streptococcal products; crystalline proteinase precursor and proteinase, C carbohydrate, M extract of the C203S cells, streptokinase-streptodornase (desoxyribonuclease, DNAse) mixture from Group C streptococcus (varidase-Lederle), and sonically vibrated extracts of C203S cells. Typical samples of the findings are shown in Fig. 4. It was generally true, as seen there, that bands were found much less frequently with these materials. In addition, the precipitin lines noted with these preparations were not usually intense, and they were often indistinct in outline. The incidence of bands with these antigen mixtures and the human sera are shown in Table I. The greater frequency, and greater numbers of bands seen with the sera of active rheumatic patients is again apparent. The surprising infrequency of bands with the sonic vibrated cell extracts must be stressed, however. Such extracts are known to contain surface antigens such as M and T proteins, as well as intracellular constituents such as nucleoproteins, C carbohydrate, and other less well characterized components (17, 18). Yet 16 of the 35 acute rheumatic sera showed no detectable antibody to any of these components, and 14 of the group showed only one. In a number of instances, sera showed 4 or 5 bands with the streptolysin O concentrate and none with the cell extract. Also to be emphasized is the rarity of precipitins to crystalline proteinase in these patients. Although bands to proteinase precursor were somewhat more frequent, it seems probable that the latter material is contaminated with at least one substance to which

antibodies may develop in humans, since two bands occurred with this preparation on several occasions.

Human Gamma Globulin.—Because of the unexpected frequency and number of antistreptococcal antibodies in the non-rheumatic sera, a study of pooled normal human gamma globulin was carried out. The preparations used[2]

TABLE I

Precipitin Bands with Human Sera; Ouchterlony Technic with Other Streptococcal Antigen Preparations

Antigen	Sera	No. tested	No. of bands				
			0	1	2	3	4
Streptokinase-streptodornase (varidase-Lederle) Group C	Non-RF	34	19	9	4	2	0
	Active RF	49	0	8	13	19	9
	Inactive RF	17	3	3	7	3	1
Sonic vibrated streptococcal cell extract	Non-RF	13	8	4	1	0	0
	Active RF	35	16	14	4	1	0
	Inactive RF	11	6	5	0	0	0
Crystalline proteinase precursor	Non-RF	6	4	1	1	0	0
	Active RF	36	20	14	2	0	0
	Inactive RF	9	7	0	2	0	0
Crystalline proteinase	Non-RF	6	6	0	0	0	0
	Active RF	49	41	8	0	0	0
	Inactive RF	15	15	0	0	0	0
C carbohydrate	Non-RF	34	34	0	0	0	0
	Active RF	36	34	2	0	0	0
	Inactive RF	10	9	1	0	0	0
M extract	Non-RF	6	5	1	0	0	0
	Active RF	34	27	7	0	0	0
	Inactive RF	9	9	0	0	0	0

were the same employed for the prophylaxis of measles and poliomyelitis. They contained 16 per cent gamma globulin in 0.3 M glycine solution.

Although it was expected that a few bands might be found when tests with various streptococcal antigens were carried out, the intensity and numbers of lines found were most surprising. Against the streptolysin O concentrate, 4 bands were frequently seen, while with the crude concentrates 7 bands were the rule under optimum conditions. Against group C streptokinase-strepto-

[2] The authors are deeply grateful to the American Red Cross for donation of this material.

dornase, four bands were usually noted, and two each were found with the sonic vibrated C203S cell extract, and one preparation of erythrogenic toxin. One band was seen with purified C carbohydrate, while none was ever noted with M extract, proteinase precursor, proteinase or streptolysin S preparation. Typical results are shown in Fig. 5. With the crude concentrate, two bands were always quite intense, while the remainder were of varying degrees of optical density. As a check on the validity of these findings, a number of other antigens or potential antigens were examined. Of these, crude concentrated *Staphylococcus aureus* filtrate showed four heavy bands, while diphtheria toxin preparation (New York City Department of Health, 300 MLD/ml.) and pneumococcal type II polysaccharide[3] (0.8 mg./ml.) showed one band each, both of the latter being moderately faint. None of these joined the bands formed with crude streptococcal concentrate when this was in an adjacent well. The preparations that failed to show any bands included streptococcal hyaluronic acid, *Clostridium novyi* toxin (10 mg./ml.), *Cl. septicum* toxin (10 mg./ml.), *Cl. perfringens* toxin (10 mg./ml.), old tuberculin (undiluted), *Shigella paradysenteriae* type III somatic antigen (1 mg./ml.), concentrated short ragweed pollen extract, long ragweed pollen extract, and three virus preparations, all high titered chorioallantoic fluid harvests; influenza virus type A, Newcastle virus, and herpes simplex virus.

Four lots of gamma globulin were tested and all showed similar results. These lots were fractionated from bleedings obtained in the winter months.

Rabbit Antisera to Streptolysin O Concentrates.—Up to four bands were seen with rabbit antisera to streptolysin O in tests with homologous antigen. It was typical of rabbit antisera, however, that the bands would rapidly spread in thickness, and interpretations would become rather difficult, if not impossible. At 3 or 4 days the bands were usually most clearly defined. In Fig. 6 *a* is shown the pattern resulting with a potent rabbit serum and several of the streptococcal antigens. It was generally true, as shown in this figure, that the rabbit sera showed one band with proteinase and its precursor, two bands with sonic vibrated extract, and one or two bands with varidase. The band formed with proteinase, proteinase precursor, and one of the streptolysin bands always joined. No bands were ever seen with rabbit or guinea pig antisera and C carbohydrate.

In Fig. 6 *b* is shown the result of a similar test with rabbit antiserum against crystalline proteinase precursor.[4] Two bands were seen with this

[3] The authors are indebted to Dr. H. Piersma of the Lederle Laboratories for the pneumococcal polysaccharide, Dr. J. MacLennan for the clostridial toxins (partially purified), Dr. S. Ellison for the virus preparations, Miss R. Pauli for the pollen extracts, and Dr. S. Beiser for the staphylococcal filtrate.

[4] The authors are especially indebted to Dr. S. Elliott for his kindness in supplying this serum and other preparations.

antiserum and the crude streptococcal concentrate as well as the streptolysin preparation. In addition, it is important to point out the heavy joining of the band formed with proteinase precursor and proteinase. A strong suggestion of the reaction of partial identity (7) is seen with this system as indicated by the spur seen in the band in the lower part of the figure. The joining of the heavy band with all of the preparations tested in Fig. 6 *b*, (except the varidase) indicates their contamination with this system. Because of the joining between the proteinase and the precursor bands with rabbit antiprecursor serum, bands seen in human sera with the proteinase or precursor were considered to be due to antibodies against these products only when bands were seen with both, and when these bands joined, or joined with the same band of the crude concentrate. Using these criteria, antibody to proteinase probably occurred only in 5 of 43 rheumatic sera adequately tested (5 of 26 patients).

Attempted Identification of Bands Seen with Human Sera and Gamma Globulin. It was pointed out above that the streptolysin O concentrate was contaminated with appreciable amounts of desoxyribonuclease, and precursor of proteinase. Since it was free of detectable hyaluronidase, streptokinase, C carbohydrate and streptolysin S, and amylase, it is reasonable to assume that antibodies to these latter materials were not involved in the three to five bands frequently seen with the streptolysin concentrate and the human sera tested.

The streptolysin O concentrate (H165-9) was found to contain a trace of ribonuclease. However, on the basis of comparative tests with crystalline pancreatic ribonuclease (Nutritional Biochemicals, Cleveland), 1 mg. of streptolysin concentrate was equivalent to 0.000,06 mg. of the crystalline enzyme. If the streptococcal ribonuclease possessed similar enzymatic activity, it seems likely that antibody to this contaminant was not involved in the bands found. Two other streptolysin concentrates as well as the crude concentrates also showed only traces of ribonuclease activity.

It seems probable that one of the bands with the streptolysin O concentrate was usually due to the streptolysin O system. In the previous paper (1), strong evidence was presented that precipitating antibody to streptolysin O very commonly occurs, even in low titered antistreptolysin human sera. In addition, in the Ouchterlony tests, when several bands were noted with the streptolysin preparation, one was usually appreciably more intensive than the others (*e.g.* see Figs. 1 *c*, 1 *d*, and 1 *f*; 4 *b* and 4 *e*). This band usually joined a similarly heavy band seen in the crude concentrate, which was also quite rich in streptolysin O. It was never seen to join any of the bands seen with the sonic vibrated extracts, the varidase mixture, proteinase, proteinase precursor, C carbohydrate, or M extract, or erythrogenic toxin when these occurred and were in a well adjacent to the streptolysin concentrates. None of these preparations contained a significant amount of streptolysin activity. Finally, the identity of one of the heavy bands produced by gamma globulin and a number of human sera with the streptolysin O-antistreptolysin O system was confirmed below in tests with a rather specific antistreptolysin horse serum obtained from an independent source. On the basis of these data, therefore, it may be

assumed that one of the bands usually seen with the concentrate is the streptolysin system itself.

It seems clear in the majority of instances, that none of the other bands seen with the streptolysin O concentrate and the human sera was due to the proteinase precursor system. With many sera, three to five bands were seen with the streptolysin concentrate, whereas proteinase or proteinase precursor in an adjacent well showed no reaction whatever. In the uncommon instances in which antibody against proteinase precursor (or proteinase) seemed to be present, one of the bands seen with streptolysin did show the joining of one line characteristic of the "reaction of identity." When this occurred, the band closest to the serum well joined with the proteinase or precursor band. The same was found with the rabbit antistreptolysin serum (see Fig. 6), and indicates a *relatively* low concentration of antibody in both instances, since the concentration of precursor in the streptolysin well was only about 0.05 to 0.1 mg./ml.

In many instances, one band of the group C streptokinase-streptodornase (varidase) did join with one band of the streptolysin concentrate, and similarly with one band of the crude concentrate. This is clearly exemplified in Figs. 3 *b*, 4 *f*, and 4 *g*. Sera that showed few and faint bands, usually revealed them against the crude and streptolysin concentrates only. Fig. 4 *f* therefore, is one of the unusual instances in which the only detectable antibody present seemed to be directed against this antigen possessed in common by varidase, crude and streptolysin concentrates. Although it was first suspected that this band could possibly be due to the desoxyribonuclease-antidesoxyribonuclease system, further tests have shed considerable doubt on this attractive hypothesis. A series of human sera was assayed for antistreptococcal desoxyribonuclease and the results compared with the presence or absence of the precipitin band common between varidase and the streptolysin and crude concentrates. These data are shown in Table II. It is quite clear that no correlation was found between the presence or absence of the band shared by the three preparations, and the antidesoxyribonuclease titers. Many sera were devoid of anti-enzyme activity, and yet showed the band. In addition, two sera showed significant anti-DNAse against the group A enzyme, but failed to show the common band. It is of great interest that no correlation was found between the anti-enzyme titers of the sera against the group C and group A DNAse. Since the "challenge" doses used in the anti-enzyme tests were adjusted so that the same amount of DNAse activity was added to the serum dilutions, this strongly suggests antigenic differences between the two enzymes, and indicates that response to the group A enzyme is more frequent in human beings.

Tests with Erythrogenic Toxin.—It was indicated in the previous paper that erythrogenic toxin was absent from the streptolysin concentrate. The evidence upon which this was based

included observations here by the Ouchterlony technic with human gamma globulin, streptolysin O, and crude concentrates as well as tests with erythrogenic antitoxin prepared in the horse and these antigens.

It was found that human gamma globulin would show one early heavy band with purified erythrogenic toxin (Figs. 7 *a* and 7 *c*). This band did *not* join with any of the 4 bands formed in an adjacent well with streptolysin O concentrate. However, the heavy band against erythrogenic toxin did join with one of the two heavy bands usually formed with the crude concentrate. This same band (common between the erythrogenic toxin and the crude concentrate) is the

TABLE II

Antidesoxyribonuclease Titers of Human Sera in Relation to an Antigen-Antibody Band Common to Streptolysin Concentrate, Varidase and Crude Streptococcal Supernate Concentrate*

No. of sera‡	No. of patients	Antidesoxyribonuclease titers *vs.*		One band joining between lysin conc., varidase, crude conc.
		Group C DNAse	Group A DNAse§	
12	11	<10	<10	+
8	6	<10	—	+
5	5	<10	10–20	+
4	4	<10	40–80	+
2	2	<10	160–320	+
4	4	10–20	10–20	+
2	2	10–20	40–80	+
2	2	10–20	160–320	+
1	1	160	320	+
3	3	10–80	—	+
6	6	<10	<10	0
2	2	<10	40–80	0
1	1	>80	—	0

* Streptokinase-desoxyribonuclease of Group C streptococci (varidase-Lederle).

‡ Most of these sera showed 3 to 5 bands with the streptolysin concentrate, and 3 to 7 bands with the crude streptococcal supernate concentrate. 52 sera from 39 patients were examined.

§ Streptolysin concentrate (H165-9), contaminated with streptococcal desoxyribonuclease was used as the DNAse challenge in the assay.

one which always crossed all the bands in the streptolysin O concentrate when the crude concentrate was placed adjacent to it (Figs. 5 *b* and 7 *a*). The band common to the crude concentrate and erythrogenic toxin, with human gamma globulin, was the heavy one closest to the serum well, while the heavy band farthest from the serum well always joined with the heaviest band of the streptolysin concentrate, and was apparently due to the streptolysin O system.

In tests with antiserum against erythrogenic toxin prepared in the horse, these observations were largely confirmed. An early intense band appeared with each of two purified erythrogenic toxin preparations[5]. These bands joined with one heavy band found with crude streptococcal

[5] These preparations, kindly supplied by Dr. A. Stock, are fractions 62-67-E and 62-67-P as reported in reference 19.

concentrate, but did not join the two faint bands seen with the streptolysin concentrate. It seems likely that the early heavy bands were due to the erythrogenic toxin system. These results are represented in Fig. 7 *b*.

Tests with C Carbohydrate.—In a number of tests with human gamma globulin, one precipitin band was seen with C carbohydrate (see Fig. 5 *c*). This band always crossed all of the bands produced with the streptolysin and crude concentrates. This anti-C band always joined one of the two bands seen with the sonic-vibrated extract. It seems fair to assume, therefore, that the crude and streptolysin concentrates are free of C carbohydrate, while the sonic vibrated extracts contain appreciable amounts of this material. In support of this was the finding that streptococcal grouping serum (group A)[6] showed only one joining band with sonic vibrated extract and C carbohydrate, and nothing against the other antigens tested here.

Joining of Human, Rabbit, and Guinea Pig Antibody.—It was of interest to see whether information obtained with animal antisera could be transposed to the results seen with the human sera. For this reason, agar precipitin plates were prepared with the small antigen well placed centrally and the larger serum wells placed peripherally. Either streptolysin or crude concentrate was placed in the central well, while the rabbit, guinea pig, or human sera were added in alternate peripheral wells. The type of results obtained are shown in Fig. 8. It is quite clear that rabbit, guinea pig, and human antibody react similarly in the agar precipitin tests, and show the joining of bands characteristic of the reaction of identity. It is therefore of importance that some guinea pig and rabbit antistreptolysin sera of low antihemolytic titer which revealed only one band with streptolysin concentrate showed joining of this one band with one heavy band seen with human sera or gamma globulin and streptolysin concentrate (Fig. 8 *a*). Further tests were carried out with an antistreptolysin standard serum obtained from the Wellcome Laboratories, Beckenham, England. This was partially purified horse antibody, and had a titer of 180 Todd units/ml. Streptolysin and crude concentrates only showed one joining band with this serum, while proteinase, proteinase precursor, varidase and sonic-vibrated extract revealed no bands at all. In tests such as shown in Fig. 8 *e*, when the standard horse antistreptolysin was alternated with human sera or gamma globulin, the heavy band suspected of being antistreptolysin O system always joined with the horse antibody band.

It may be noted that the heavy and diffuse band seen with the animal antisera closest to the serum well (Figs. 8 *a* to 8 *d*) does not often join with any of the bands produced by the human sera. (An exception to this is seen with serum H4 in Figs. 8 *c* and 8 *d*). Strong evidence presented above has indicated that this diffuse band with the animal antisera was due to the proteinase precursor antigen-antibody systems. Serum H4 (Figs. 8 *c* and 8 *d*) had previously shown a joining band between streptolysin O concentrate, proteinase

[6] Lederle product.

precursor, and proteinase. This further supports the earlier data indicating the low frequency of antibody response in human beings to this enzyme.

Also shown in Fig. 8 is the frequency of occurrence of human antibody to components of these concentrates, to which antibodies were not apparently present in the animal antisera.

Effect of Glycine on "Non-Specific" Serum Agar Turbidity.—As was indicated above, in tests with human serum, an early troublesome complication was the development of varying degrees of precipitation about the serum agar wells (*e.g.* see Figs. 4 *a* to 4 *e*). This complication never arose in tests with the purified human gamma globulin. In fact, with these preparations, the agar surrounding the globulin well was even clarified to a considerable degree (see Fig. 5). It is apparent, therefore, that these precipitations were not due to human antibody directed against substances in the agar.

Although these non-specific effects were decreased to some degree upon the addition of M/25 veronal in the agar at pH 7.4, only with the inclusion of glycine was the effect markedly reduced. This is clearly illustrated in Fig. 9, which reveals results with one human serum with and without glycine at one molar concentration in the agar. Coincident with the decrease of this non-specific agar turbidity it seems likely that faint bands close to the serum wells may be made visible, as is indicated in Fig. 9.

A few experiments were carried out to determine the effect of heating the streptolysin O concentrate prior to its use in the Ouchterlony plates. A sample of H165-9 was heated to 56, 65, 80, and 100°C. for 30 minutes. These fractions were tested along with the unheated control against three potent rabbit antisera. It was found that heating at 56° abolished one heavy band of the four seen with these sera and the control solution. This band was the one closest to the antigen well and was suspected of being due to the streptolysin O-ASO system on the basis of evidence presented above. This finding thus supports the previous data, since the biological activity of streptolysin is well known to be irreversibly destroyed at 56° for 30 minutes. Heating at 65° abolished one other band, while the remaining two were unaffected by exposure to 80 or 100°C. Splitting of the bands did not occur with these heated fractions.

Several tests were made with various concentrations of the antigens used here and human gamma globulin. Lower concentrations of the antigens than the ones generally used resulted only in a decrease of the numbers and intensities of the bands found. In no instance was inhibition of band formation obtained because of excess antigen.

Tests with the Oakley Technic.—A limited number of tests with the Oakley technic confirmed the multiplicity of the bands found. The bands would be of varying thickness, from 0.5 to as much as 7 or 8 mm., and of widely varying intensities. Because of the overlaps that sometimes occurred, it was often difficult to precisely determine the number of bands produced. In addition,

the bands appeared much later than with the Ouchterlony tests, the maximal effects being seen at 2 or 3 weeks. It is quite likely that this could be substantially reduced by altering the volumes of the intermediate agar layer, or by other variations of technic. In Fig. 10 are shown the results obtained with one serum from a rheumatic fever patient, and one potent rabbit antiserum. It may be noted that this rheumatic serum showed at least 7 bands with the crude concentrate, 3 with varidase, and 3 with streptolysin concentrate. The same number of bands were seen with this serum on the Ouchterlony plates. On the basis of similar tests with 12 rheumatic sera and 5 rabbit antisera, it was not uncommon to find a few more bands with the Oakley technic than with the petri plate method. The multiplicity of antigen-antibody systems were almost always completely confirmed, however.

Lastly, it may be mentioned that capillary precipitin tests were carried out with a number of non-rheumatic sera and streptolysin O concentrate. The precipitates that were observed were very delicate, and usually appeared after overnight at 4°C. A rather good correlation was observed between the results of these tests and Ouchterlony agar precipitins. In general, the latter seemed slightly more sensitive and much more informative.

DISCUSSION

The data presented here demonstrate that rheumatic fever patients develop antibodies to a large number of antigens present in crude concentrates of streptococcal culture supernates. Such patients, in addition, frequently showed antibodies to three to five antigens present in a partially purified streptolysin O concentrate. One of these bands is most probably due to the streptolysin O antistreptolysin O system. The evidence in favor of this includes the following:—

(*a*) The relatively high potency of these preparations with regard to streptolysin.

(*b*) The previous observations by the Oudin technic which indicated the frequency of precipitating antibody to this toxin in human sera.

(*c*) In sera with multiple bands, the heaviest of these would usually join a similar heavy band produced with the crude concentrate, which was also rich in streptolysin O; the heavy band never joined bands formed with the other streptococcal antigens, which were devoid of streptolysin O or only contained negligible amounts.

(*d*) The heavy band (described in (*c*)) usually joined a similar heavy band in rabbit or guinea pig antistreptolysin sera, produced by immunization with the streptolysin concentrates. In a number of instances, this joining occurred when the animal antisera were weak in antihemolytic titer and showed only one band. (See Fig. 8). The latter was almost certainly antistreptolysin O.

(*e*) An antistreptolysin O antibody produced in the horse by immunization with preparations derived from another streptococcal strain (Richards) showed only a single band (joining) with the streptolysin concentrate and with the crude concen-

trate. No bands were produced with the other preparations studied here. This standard antistreptolysin antibody, which was therefore rather highly specific, joined with one heavy band produced with a number of human sera and streptolysin concentrate.

Although it thus seemed almost certain that antistreptolysin O accounts for one of the bands produced against the streptolysin concentrate with these human sera, the identity of the other two to four bands is not clear. Although the concentrates were shown to be contaminated with proteinase precursor and streptococcal desoxyribonuclease it was demonstrated that, in most cases, none of the extra bands was due to the proteinase or proteinase precursor systems.

It was found that one of the streptolysin bands commonly joined one of the bands produced with the streptokinase-streptococcal desoxyribonuclease mixture of Group C streptococci (varidase-Lederle). The possibility that this band was due to the DNAse–anti-DNAse system seems unlikely however, in view of the rather complete lack of correlation of anti-enzyme titers and the presence or absence of this joining band. In addition, many of the sera tested for anti-enzyme activity showed none against the group C enzyme, but varying titers (usually low) against the group A DNAse. If this band were due to the desoxyribonuclease system, it would be necessary to assume that common precipitins to the group A and C DNAse were quite frequent, but that the anti-enzyme activity against group C DNAse was usually low or negligible, while that against the group A DNAse was quite variable. In support of the unlikelihood that this band was due to the DNAse system is an observation by McCarty with high titered antidesoxyribonuclease sera of rheumatic fever patients (20). When these were tested with highly purified preparations of streptococcal desoxyribonuclease, only negligible amounts of precipitates were obtained over a wide range of concentrations. McCarty's finding enhances the possibility that the band found in the present study, which was common to varidase and streptolysin O concentrates, was due to the same contaminant in both preparations.

It seems clear that the extra bands found with *individual* human sera and the streptolysin concentrates are not due to the C carbohydrate, nor *usually* to any of intracellular or surface components present in sonic vibrated streptococcal cell extracts. The absence of streptokinase and hyaluronidase from the concentrate strongly indicate that these systems are not involved. The non-antigenicity of streptolysin S, (21, 22) the absence of bands in tests of a preparation of this material with human gamma globulin, and the absence of this hemolysin from the streptolysin O concentrates, eliminate this substance from consideration. The tests carried out with partially purified erythrogenic toxin and antitoxin indicated the absence of erythrogenic toxin from the streptolysin concentrate, but its presence in the crude concentrate. Thus, none of the extra bands found with streptolysin O was probable due to this toxin. The absence

of amylase, and the small degree of contamination of the concentrate with ribonuclease most probably eliminates these enzymes. The findings, therefore, strongly suggest that the streptolysin O concentrates are contaminated with several substances (3 to 4) to which antibodies are commonly formed in human disease, and which have been heretofore unidentified. As further purifications of the streptolysin O are carried out, the fractions will all be studied with a view to the eventual isolation of these other antigens. When separations are achieved, a study of the biological and enzymatic properties of the "new" components will be made. It is possible, though perhaps unlikely, that some of these antigens may represent incompletely formed known products (such as hyaluronidase, etc.) which are non-active enzymatically or biologically, but are able to react with antibody.

It is necessary to include a few words concerning the reality of the bands found, and the lack of artifacts in the results. On the whole, most studies with Ouchterlony's technic have indicated that single bands represent different systems (6, 7, 23). In some instances, multiple bands to an apparent single antigen might be formed when the antigen was partially damaged as by heat treatment (24). In addition, the possibility has been suggested by Jennings (38) that multiple bands may be due to a single antigen if the antigen possesses several determinant groups. However, in most instances described here, such effects seem unlikely for the following reasons:—

(*a*) The various bands with one serum and one concentrate might all be very different in appearance; some being heavy and crisp, others being faint and somewhat fuzzy. This was frequently true of two very close bands.

(*b*) Not infrequently, when two very similar and close bands were seen, one of them might join a band produced in an adjacent well, and the other continue straight out (see Fig. 5).

(*c*) Not infrequently, two bands close to each other, and somewhat similar, would diverge quite widely at the edges of the bands.

(*d*) In many instances, bands could be differentiated by their dissimilar joining behavior with various antigens in adjacent wells.

(*e*) The number of bands against a given product with different human sera varied by increments of one. If single antigens were producing multiple bands, one might expect sera with one or two bands to a given preparation to be rare.

(*f*) In those instances in which a band was tentatively identified with a given antigen-antibody system (*e.g.*, streptolycin O, erythroganic toxin, "c" carbohydrate, and proteinase or proteinase precursor), only a single band was observed in each case.

(*g*) In a few tests with rabbit antisera, heating the streptolysin concentrate resulted only in a reduction of the numbers of bands. Splitting of bands was not observed.

(*h*) In general, the multiplicity of bands was confirmed by the Oakley technic.

It seems fair to assume therefore, that most of the results obtained here do actually represent antigen-antibody systems to distinct immunological entities.

It is intriguing to note that the majority of the bands found with human sera seemed directed against extracellular components of the streptococcal growth. Very few were found with the sonic vibrated cellular extracts. These, of course, have been shown by other investigators to contain a number of surface and intracellular substances. The multiplicity of extracellular antigens to which antibodies are formed in human beings is in good support of the suggestion made by McCarty that the changes in gamma globulin in rheumatic fever probably largely represent changes in antibody levels (25).

The observations made with human pooled gamma globulin are significant for several reasons. They confirm quite conclusively the antibody nature of the bands found with individual human sera. They also clearly demonstrate that the turbidities surrounding the serum agar wells with individual human sera are not due to antibody directed against substances in the agar, but to some non-antibody components of the serum. Most important, perhaps, are the epidemiological implications of the results. In spite of reports indicating that streptococcal infections cause a variable, but often rather low percentage of upper respiratory disease in human populations (26, 27), these findings suggest that such infections may be remarkably frequent. In addition, they suggest that many of the antigens produced by these organisms are quite commonly synthesized "*in vivo*" in human clinical or subclinical streptococcal infections. Since the antibody levels in uncomplicated streptococcal infections do not seem to remain elevated for unusually prolonged periods, (28–31), the bands found with the gamma globulin imply continued high level of infection in the general population. The regular presence of C precipitins in the globulin and their frequent absence in the individual serum tests may be due to a quantitatively low or infrequent antibody response to this substance in different individuals. It is to be noted that the C precipitin band was often quite close to the globulin well, indicating a *relatively* low concentration of antibody. The infrequent antibody response in individual sera to this carbohydrate (or extremely small response) is in general agreement with older observations (18, 32). It is of interest that these pools of human gamma globulin showed at least four bands with the crude staphylococcal filtrate concentrate and only one rather faint band each with pneumococcal type II polysaccharide, and with a crude diphtheria toxin preparation. Further studies along this line with more adequate and varied antigen preparations could be quite informative epidemiologically.

The comparatively small percentage of human beings who seem to develop antibody to proteinase and proteinase precursor is of some significance with respect to recent findings by Kellner and Robertson (33). These investigators found focal areas of myocardial necrosis in experimental animals upon injection of large doses of proteinase, and suggested the possible significance of these findings in rheumatic fever. In our studies, the failure to find antibody to proteinase more frequently in rheumatic patients suggests failure of human "*in vivo*" production of signifi-

cant amounts of this material. This is especially so, since in rabbits immunized with the streptolysin concentrate contaminated with proteinase precursor, the high degree of antigenicity of this latter substance was indicated. Thus, 6 of 6 rabbits which received cysteine-reduced streptolysin concentrate containing an estimated total dose of 0.045 mg. up to 0.080 mg. of proteinase precursor, developed antibodies to both precursor and proteinase. One of two rabbits receiving reversibly oxidized streptolysin concentrate containing an estimated 0.10 mg. of precursor, also showed antibody to both precursor and proteinase. These immunization courses lasted over a period of several months. Should the same high degree of antigenicity obtain in the human being, the detection of precipitins to the proteinase in only a comparatively small percentage of the rheumatic fever patients studied here may thus cast doubt upon the significance of this enzyme in the pathogenesis of the rheumatic process.

It is of much interest that antibodies from several species form bands in the Ouchterlony plates that join each other as readily as do precipitin bands from the same species. This confirms independent observations of a similar nature by Ouchterlony and Bjorklund (34). It thus makes possible the use of specific antisera prepared in animals for the identification of the precipitin bands found in human sera. Such animal sera may be prepared in abundance, absorbed with necessary heterologous antigens, and kept in stock for this purpose. Alternatively, such animal sera may at times be made effectively specific for these tests by mere dilution. The identification of the human precipitin bands may also be approached by tests with various antigen preparations from the same organism. Ultimately, the availability of highly purified products from the microorganism will serve as excellent cross-checks with the specific animal antibody in these identification procedures.

The significance of these findings in relation to the pathogenesis of rheumatic fever cannot yet be assessed, although the differences between the non-rheumatic and rheumatic groups studied here are clear. A careful comparison of similar patient groups of post-streptococcal non-rheumatic sera with rheumatic sera will be necessary. Such a study is being planned. It is conceivable that some of the apparently new antigens which have been described here may have a rather direct bearing on the pathogenesis of this disease.

The data presented here also point to a fresh approach to the study of infectious disease processes in man. Heretofore, the pathogenic agent was examined *in vitro* for the production of substances of biological activity. If and when such substances were found, the human patient was then examined to determine whether antibody to these particular products (toxin, capsule or surface antigen, enzyme, etc.) increased in titer during convalescence. The introduction of the agar precipitin technic now enables us to estimate the total number of distinct antibody responses of the human patient with a given disease. Tests should be made with a variety of antigen mixtures derived from the pathogen by a number of technics, including "*in vivo*" produced extracts

(35). Chemical fractionation of each of these antigens can then be carried out, the fractionation procedures being followed by agar precipitin tests with human sera from convalescent patients. Once purification of each antigen is achieved, an estimate of its biological significance can be assayed. A start has been made with reference to streptococcal disease (as related to rheumatic fever) in this study, but the potential use of this approach in staphylococcal infections, tuberculosis, brucellosis, and viral diseases for examples, would seem strongly indicated. The preliminary results obtained with human gamma globulin and staphylococcal filtrate point to the fruitfulness of this method generally. This approach may also be informative in a study of acute glomerulonephritis, in which certain streptococcal strains seem to have a unique ability to damage the kidneys of human patients (36, 37). It is conceivable that comparison of streptococcal culture filtrates, etc. from nephritogenic and non-nephritogenic strains with sera from nephritic patients may demonstrate a significant toxin produced "*in vivo*" by the nephritogenic strains and not the others.

SUMMARY

It has been shown by agar precipitin tests (Ouchterlony and Oakley) that human sera may contain from 0 to 5 antibodies against antigens present in a partially purified streptolysin O preparation, and from 0 to 7 antibodies against antigens in a crude ammonium sulfate concentrate of the streptococcal culture supernate used. These antigens were prepared from a Group A hemolytic streptococcus (strain C203S).

Strong evidence was presented suggesting that some of the bands seen with streptolysin O concentrate represented antibody reponses to streptococcal antigens heretofore undescribed.

Tests were also carried out with other streptococcal antigens, including streptokinase-desoxyribonuclease mixture from Group C streptococci (varidase-Lederle), crystalline proteinase, proteinase precursor, C carbohydrate, and sonic vibrated streptococcal cell extracts (group A, C203S). Fewer bands were seen with these preparations, and with some they were quite uncommon. The observations indicated that the predominating antibody responses in human streptococcal infections were to extracellular products of the micro-organisms, and only very slightly and infrequently to intracellular antigens.

The human sera studied included sera from patients with active or convalescent rheumatic fever, and non-rheumatic subjects suffering from a variety of illnesses. As was expected, the rheumatic subjects showed antibody responses to many more of the antigens present in these preparations than did the non-rheumatic group.

Pooled normal human gamma globulin was found to contain many of the antibodies found in potent human sera. This finding confirmed the antigen-

antibody nature of the bands seen with individual sera. The epidemiological significance of these findings with gamma globulin was briefly discussed.

It was found that rabbit, guinea pig, and human antibody precipitin bands join quite readily in the Ouchterlony tests. This finding adds another tool for the identification of the precipitin bands found with human sera.

Evidence was obtained which indicated differing immunological specificities of two samples of streptococcal desoxyribonuclease, one from Group A, the other from a Group C streptococcus.

The value of these technics as representing a new approach to the study of human infectious disease was discussed.

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EXPLANATION OF PLATES

PLATE 30

FIG. 1. Agar precipitin bands with human and rabbit sera, tested against streptolysin O concentrate (H165-9) 1 mg./ml. Antigen well above, serum well below.

Fig. 1 *a*, human, non-rheumatic (P. K.), 8 days development.

Fig. 1 *b*, human, acute rheumatic (I. S.), 8 days development.

Fig. 1 *c*, human, acute rheumatic (E. E.), 21 days development.

Fig. 1 *d*, human, acute rheumatic (J. H.), 8 days development.

Fig. 1 *e*, human, acute rheumatic (M. P.), 8 days development.

Fig. 1 *f*, human, acute rheumatic (A. S.), 6 days development.

Fig. 1 *g*, rabbit, immunized with reduced streptolysin concentrate, 9 days development.

FIG. 2. Agar precipitin bands with human sera, tested against crude concentrated streptococcal supernate (H246-4). Antigen well above, serum well below × 1.5.

Fig. 2 *a*, acute rheumatic (P. N.) 8 days.

Fig. 2 *b*, acute rheumatic (P. H.), 6 days.

Fig. 2 *c*, inactive rheumatic (A. G.) 6 days.

Fig. 2 *d*, acute rheumatic (W. F.) 6 days.

Fig. 2 *e*, acute rheumatic (I. S.) 8 days.

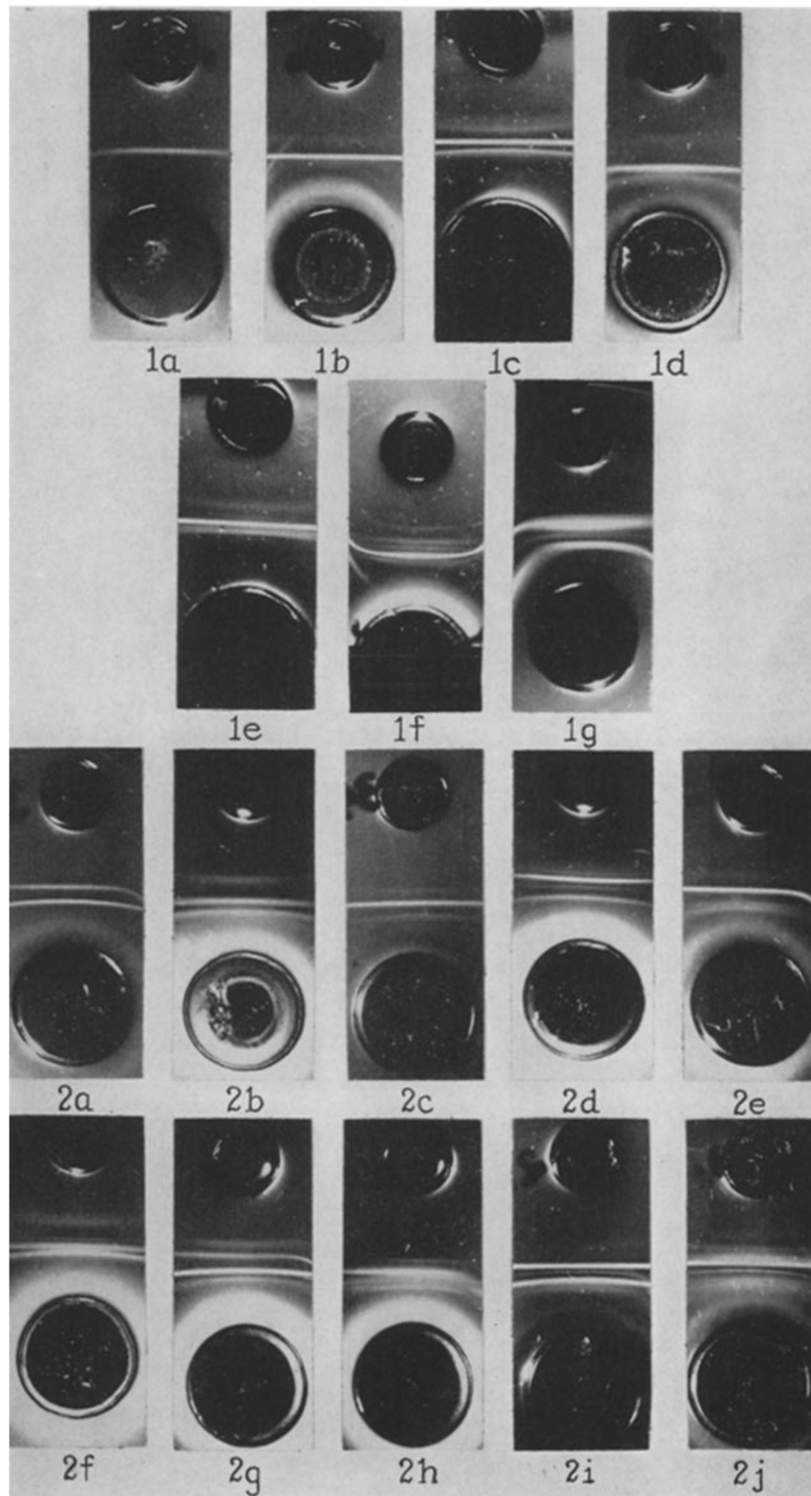
Fig. 2 *f*, acute rheumatic (V. W.) 8 days.

Fig. 2 *g*, acute rheumatic (P. McL.) 6 days.

Fig. 2, *h*, acute rheumatic (F. C.) 6 days.

Fig. 2 *i*, acute rheumatic (P. M.) 8 days.

Fig. 2 *j*, acute rheumatic (A. S.) 5 days.



(Halbert *et al.*: Precipitin analysis and streptococcal infections)

Plate 31

FIG. 3. Tests with acute and convalescent sera from one rheumatic fever patient (J. H., 23, white male). × 1.

Fig. 3 *a*, Serum obtained 1 week after onset of first rheumatic attack, and 3 weeks after a streptococcal infection (6 day development).

Fig. 3 *b*, Serum obtained 7 months after the onset of the rheumatic fever, and 2 months after the sedimentation rate returned to normal (19 day development).

The antigens tested are:

L, streptolysin O concentrate, H165-9, 1 mg./ml.

CR, crude concentrated streptococcal supernate (H246-4).

V, varidase (group C streptokinase-streptodornase, 1 mg./ml).

C, streptococcal C carbohydrate (Group A).

SV, sonic vibrated streptococcal cell extract (Group A).

M, streptococcal cell extract, prepared as for M protein purification.

P, crystalline streptococcal proteinase 1 mg./ml.

FIG. 4. Typical results with several human sera and various streptococcal products. × 1.

Sera

Fig. 4 *a*, acute rheumatic (A. D.).

Fig. 4 *b*, acute rheumatic (A. Se.).

Fig. 4 *c*, acute rheumatic (A. So.).

Fig. 4, *d*, acute rheumatic (P. H.).

Fig. 4 *e*, acute rheumatic (V. W.)

Fig. 4 *f*, non-rheumatic (C. F.).

Fig. 4 *g*, acute rheumatic (J. H.).

Antigens

L, streptolysin O concentrate H165-9.

SV, sonic vibrated streptococcal cell extract.

C, streptococcal C carbohydrate (Group A).

N, 0.85 per cent NaCl.

V, varidase, 1 mg./ml.

CR, crude concentrate streptococcal supernate.

M, M extract from streptococcal cells.

P, crystalline proteinase (Elliott).

PP, crystalline proteinase precursor (Elliott).

FIG. 5. Tests with pooled normal human gamma globulin. × 1.

Fig. 5 *a*, lot 179-4 (8 days).

Fig. 5 *b*, lot 255-1 (5 days).

Fig. 5 *c*, lot RC-20C (5 days).

Antigens

L, streptolysin O concentrate (H165-9, 1 mg./ml.)

SV, sonic vibrated cell extract.

CR, crude concentrate streptococcal supernate.

V, varidase, 1 mg./ml.

N, 0.85 per cent NaCl.

ET, erythrogenic toxin concentrate (stock No. ST-E-14).

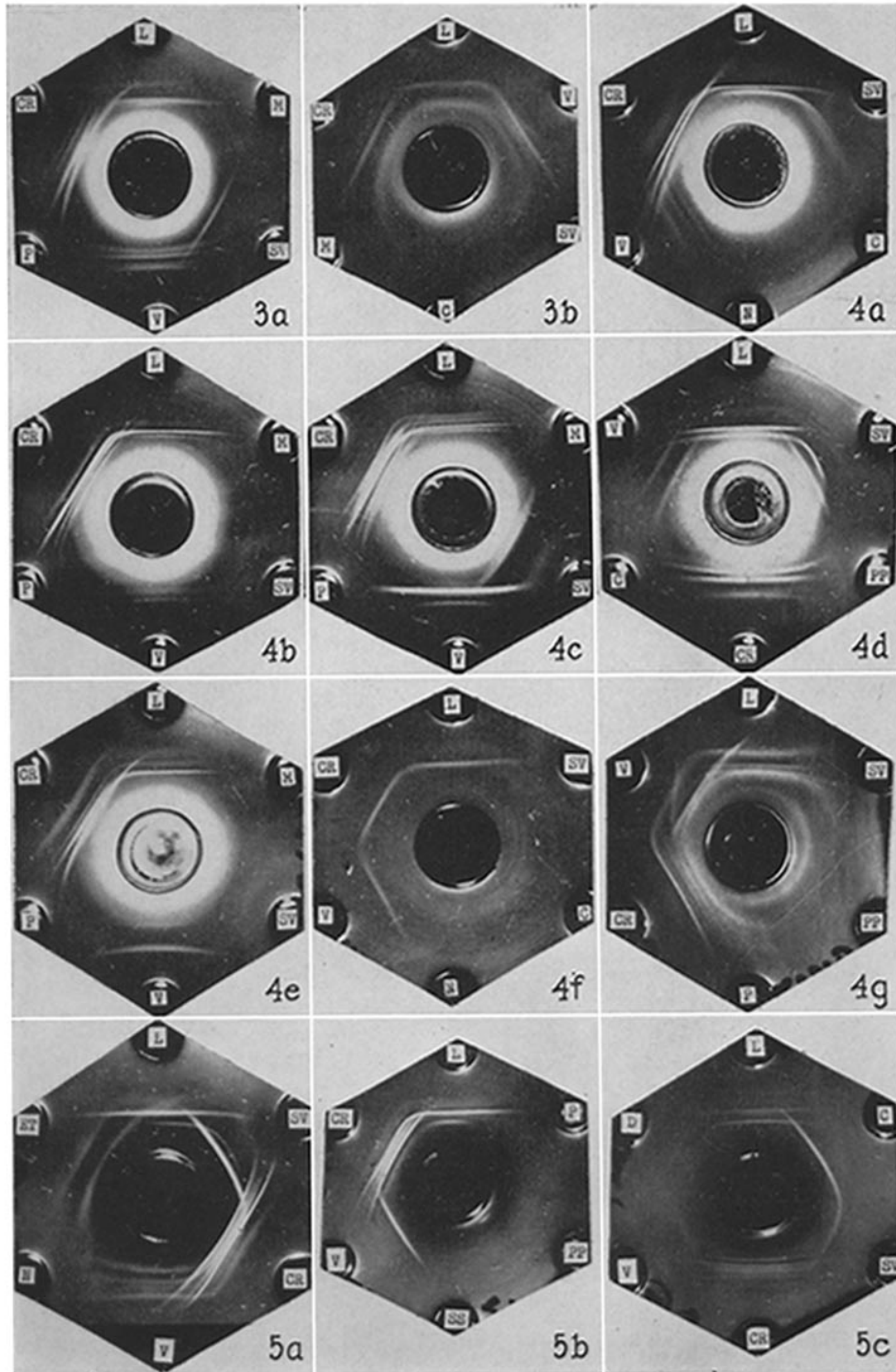
P, crystalline proteinase (Elliott).

PP, crystalline proteinase precursor.

SS, streptolysin S preparation.

C, streptococcal C carbohydrate Group A (0.1 mg./ml.).

D, streptodornase (Lederle) 25,000 units/ml.



(Halbert *et al.*: Precipitin analysis and streptococcal infections)

PLATE 32

FIG. 6. Rabbit antisera tested against various streptococcal products. Fig. 6 *a*, immunized with reduced streptolysin "O" concentrate. Fig. 6 *b*, immunized with crystalline proteinase precursor (Elliott). × 1.

Antigens

L, streptolysin O concentrate (H165-9, 1 mg./ml.).
CR, crude concentrated streptococcal supernate.
P, crystalline proteinase (Elliott).
PP, crystalline proteinase precursor (Elliott).
V, varidase, 1 mg./ml.
SV, sonic vibrated cell extract.

FIG. 7. Tests indicating the absence of erythrogenic toxin from the streptolysin concentrate, and its presence in the crude concentrate. × 1.
Fig. 7 *a*, normal pooled human gamma globulin (6 days).
Fig. 7 *b*, erythrogenic antitoxin, horse (Lederle) (3 days).
Fig. 7 *c*, normal pooled human gamma globulin (6 days).

Antigens

L, streptolysin O concentrate (H165-9, 1 mg./ml.).
CR, crude concentrated streptococcal supernate.
ET1, purified erythrogenic toxin (Stock, 62-67-E).
ET2, purified erythrogenic toxin (Stock, 62-67-P).
ET3, partially purified erythrogenic toxin (Stock, ST-E14)
M, M extract from streptococcal cells.
SV, sonic vibrated cell extract.
V, varidase (1 mg./ml.).

FIG. 8. Joining of antibodies derived from human, rabbit, guinea pig and horse sera. × 1.

Test Antigens (Central Well)

For Figs. 8 *a* and 8 *c*, streptolysin O concentrate (H165-9, 1 mg./ml.).
Figs. 8 *b*, 8 *d*, and 8 *e*, crude concentrated streptococcal supernate.

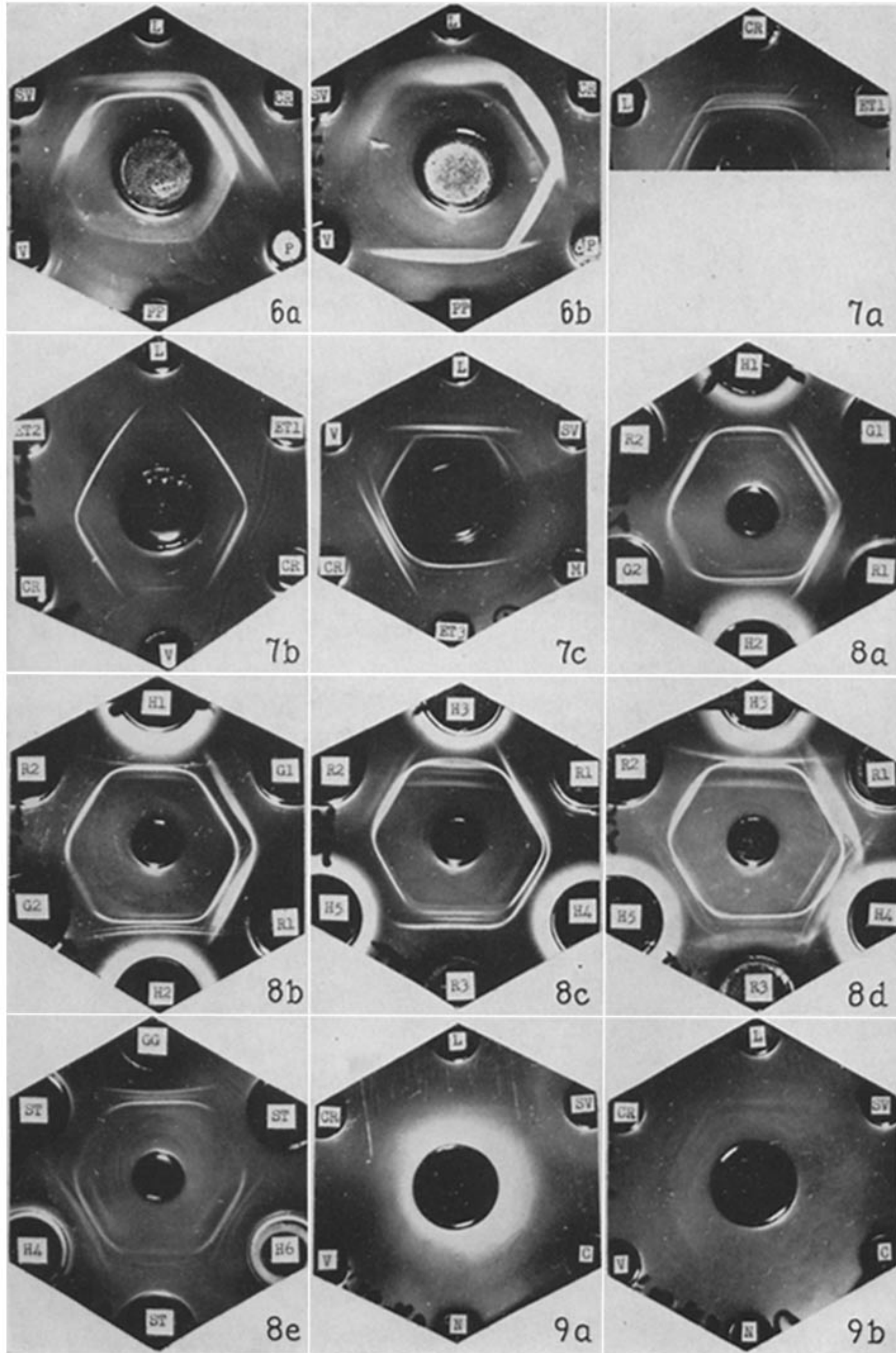
Sera

H1, *H2*, etc., different human sera.
R1, *R2*, etc., different rabbit antisera.
G*1*, G*2*, different guinea pig antisera.
ST, standard horse antistreptolysin O (Wellcome).
GG, normal pooled human gamma globulin.

FIG. 9. Comparative tests with serum of a non-rheumatic individual (R. D.) in agar with and without glycine. × 1.
Fig. 9 *a*, 0.6 per cent bacto-agar, M/25 veronal, pH 7.4 (12 days).
Fig. 9 *b*, 0.7 per cent bacto-agar, M/25 veronal, M/1 glycine, pH 7.4 (12 days).

Antigens

L, streptolysin O concentrate (H165-9, 1 mg./ml.)
SV, sonic vibrated cell extract.
C, streptococcal C carbohydrate (Group A).
N, 0.85 per cent NaCl.
V, Varidase, 1 mg./ml.
CR, crude concentrated streptococcal supernate.



(Halbert *et al.*: Precipitin analysis and streptococcal infections)

PLATE 33

FIG. 10. Tests with Oakley's technic and several streptococcal preparations. × 2.5.

Sera

Fig. 10 *a*, human, acute rheumatic, R. B. (4 weeks development).

Fig. 10 *b*, rabbit, immunized with reduced streptolysin O concentrate (6 weeks development).

Antigens

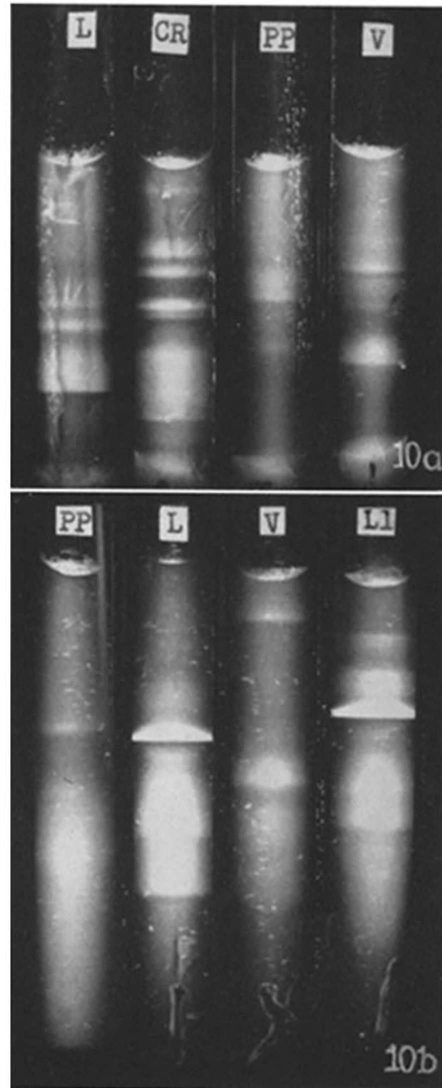
L, streptolysin O concentrate (H165-9, 1 mg./ml.).

L1, streptolysin O concentrate (H96-13, 1 mg./ml.).

CR, crude concentrated streptococcal supernate.

PP, crystalline proteinase precursor (1 mg./ml.).

V, varidase, 1 mg./ml.



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