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

I. Oudin Technic*

By SEYMOUR P. HALBERT, M.D., LOIS SWICK, and CONSTANCE SONN

(From the Departments of Ophthalmology and Microbiology, Columbia University, College of Physicians and Surgeons, and the Institute of Ophthalmology, New York)

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The antistreptolysin O activity of human sera has been under investigation for many years (1–3). It has been generally assumed that this activity is due to antibody because of the changes in its titer that occur during disease and because antisera of this sort are so readily produced in animals by immunization procedures. High antistreptolysin O activity of human gamma globulin also has been reported (4, 5). In addition, the ready absorption of antistreptolysin activity of serum with streptolysin O containing preparations supports this view (6). Although cholesterol is capable of inhibiting streptolysin O in very low concentrations, the failure to affect antistreptolysin activity of some human sera by extraction with fat solvents at low temperatures (4, 6, 7) has also suggested the antibody nature of antistreptolysin O in human sera. However, several non-specific inhibitors are known to occur. In acute hepatitis sera, high levels of antistreptolysin O have been found which were clearly of non-antibody character (6, 7). The antistreptolysin O agent of human pleural exudate fluids has also been shown to be a non-antibody inhibitor (6). Rosendal and Bernheimer have shown that mice given sublethal doses of streptolysin O revealed a non-antibody inhibitor in the circulation (8). Stollerman (9) has recently shown that hyperlipemia in rabbits will result in non-specific antistreptolysin O activity when the cholesterol-phospholipid ratios are within a certain range. Robinson (5) has reported the development of non-specific antistreptolysin O activity in rabbits severely ill with Group A streptococcal infection. In addition, it may be recalled that acid or alkali treatment of human sera may result in striking increases in antistreptolysin O, presumably due to the effect on lipoprotein complexes (6). The above findings thus raise the question of the frequency and significance of these non-specific factors in the antistreptolysin O activity of sera from the population at large.

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The investigations recorded here were carried out during the course of studies on the biological effects of streptolysin O concentrates. They strongly indicate that even the low titers of antistreptolysin O activity in human sera of individuals without an obvious recent streptococcal illness are associated with precipitating antibody. In addition, this and the following study emphasize the general value of the agar precipitin technics in analyzing the antigens or "toxins" produced "*in vivo*" during the course of human illness.

Materials and Methods

***Antigens*:**

The streptolysin O concentrates were prepared by modifications of the method of Herbert and Todd (12). The C203S[1] strain of Group A hemolytic streptococcus was grown in a medium of the following composition; in grams/liter:

Proteose-peptone No. 3 (Difco), trypsin-digested	40.0
Asparagine (Difco)	2.5
Sodium citrate $2H_2O$	0.9
KH_2PO_4	1.8
Yeast extract (bacto)	2.0
Phenol red	0.01
Glucose	30.0
$NaHCO_3$	3.0

The last two ingredients were added aseptically as Seitz-filtered solutions. The proteose-peptone No. 3 digest was prepared by aseptically adding 1 volume of sterile bacto-trypsin solution (Difco) in 0.8 per cent saline (1 gm./liter) to 10 volumes of a 6 per cent solution of autoclaved proteose-peptone. This was incubated at 37°C. for 48 to 72 hours, then autoclaved for 1 hour, and filtered.

The inoculum consisted of the overnight growth of the C203S strain in the same medium in $\frac{1}{40}$ of the bulk volume. After 4 to 5 hours of incubation at 37°C., neutralization with 5 N NaOH was usually required. The acid that was formed was repeatedly neutralized until none was further produced. From 55 to 65 ml. of alkali per liter of medium was usually required. The pH was never allowed to drop below 6.8 during the active growth. Incubation for 13 to 14 hours was the general rule, at which time the cultures were put into the cold room. They were centrifuged the next day, and to the supernate was added solid $(NH_4)_2SO_4$ to 75 per cent saturation (assuming 72 gm./100 ml. equals full saturation). This was stored at 4°C. for 2 days, the bulk of the precipitate collecting as a surface scum. This was scooped off, washed lightly with 75 per cent saturated $(NH_4)_2SO_4$, dissolved in water and dialyzed in the cold with distilled water until sulfate-free. This solution was reprecipitated by the addition of solid $(NH_4)_2SO_4$ to 66 per cent saturation. The centrifuged sediment, after 3 days at 4°C. was dissolved in water and dialyzed thoroughly. The solution was spun at high speeds to clarify, and while cold the pH was adjusted to 4.3 with N/1 HCl. (This pH was found in several preliminary tests to be the level of greatest insolubility of the streptolysin O under these circumstances.) After 40 minutes in an ice bath, the precipitate was centrifuged in the cold. It was dissolved in M/10 phosphate at about pH 7.0 and to it was added saturated $(NH_4)_2SO_4$ to a final concentration of 60 per cent saturation. After 2 days in the cold room, the precipitate was centrifuged, dissolved in water, and thoroughly dialyzed. Absorption was then carried out on freshly pre-

[1] The authors are grateful to Dr. A. Bernheimer for a transplant of this culture.

cipitated calcium phosphate by the addition of 1/10 volume of M/2 phosphate buffer at pH 7.2, followed by the rapid addition of 1/25 volume of M/1 calcium acetate, maintaining the pH at 6.5. The gelatinous precipitate was collected after 1 hour in the ice bath, and eluted 6 to 8 times with small volumes of 25 per cent saturated $(NH_4)_2SO_4$. The pooled eluates were dialyzed until sulfate-free, and the absorption-elution repeated. The final solution was lyophilized, and was usually a very light tan, fluffy powder. The streptolysin O potency ranged from about 12,000 to 20,000 H.U./mg. All of the preparations proved to be largely in the reversibly oxidized non-hemolytic state (98 to 99+ per cent).

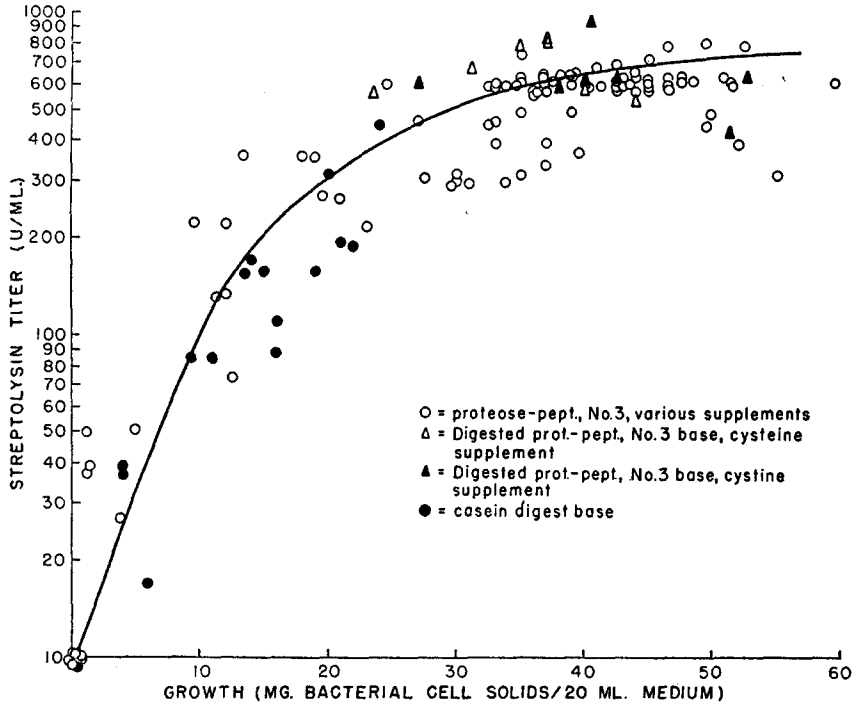

FIG. 1. The relationship of streptolysin O production to the yield of bacterial cell solids, with the C203S strain of beta hemolytic streptococcus grown in modifications of the medium described.

In preliminary tests leading to the choices of the medium listed above for streptolysin production, the titer was found to be closely related to the yield of the bacterial cell solids. This relationship is shown in Fig. 1. In these tests, various concentrations of the ingredients listed were examined, as well as numerous supplements to the basic medium. These latter included reducing agents such as cysteine and glutathione, as well as cystine, numerous amino acids, and vitamins, etc. A few tests with tryptic digested casein (N-Z amine, Sheffield Farms Co. Inc., New York) were also made which showed similar results. When the supplements improved the streptolysin titer, they seemed to do so in relation to the increased bacterial cell yield. In no instance was there found good growth with poor streptolysin formation. These results are unlike those found by Slade and Knox (13) in this respect. However, only one

streptococcal strain was examined in each case and the conditions were not exactly similar in the two studies.

Other Test Antigen Concentrates and Preparations:

Streptokinase-Streptodornase[2].—This mixture was obtained as the lyophilized commercial product, varidase, Lederle, lot No. 7-1089-232A. The contents of several vials were dissolved in water, and dialyzed thoroughly in the cold. After spinning to clear, the solution was lyophilized. The estimated streptokinase potency was 21,000 units/mg., and the streptodornase potency was 4,000 units/mg., in terms of the Lederle units.

Crystalline Proteinase and Proteinase Precursor.—These preparations were lyophilized, once crystallized, products. A moderate percentage of the powders were insoluble, but this did not seem to interfere with the reactions.

Erythrogenic Toxin.—This material was a partially purified concentrate prepared by growth of the NY5 strain of beta hemolytic streptococcus. It contained about 1400 flocculating units/ml.

M Protein.—This was prepared by the technic of Lancefield (14) from the C203S cells harvested after the streptolysin production. The final lyophilized white powder was partially insoluble.

C Carbohydrate.—The technic of Fuller (15) was used, utilizing formamide extraction of the C203S cells from streptolysin harvest.

Hyaluronic Acid.—This was obtained as a by-product of the growth of the C203S cells in recent large scale growth using the dialysate of the above medium, following the suggestion of Stock (31). This material, obtained by alcohol precipitation from the fluid supernates after $(NH_4)_2SO_4$ precipitation of the streptolysin O, was further purified by chloroform deproteinization, and repeated alcohol precipitation in the presence of sodium acetate. The highly viscous product was kindly studied by Dr. K. Meyer,[3] and found to be an extremely homogenous high molecular species, of great purity and stability.

Streptolysin O Assay:

The diluent used in most of these assays consisted of a 0.5% solution of bovine serum albumin (Armour fraction V) in a mixture of equal parts of 0.15 M NaCl, and of 0.15 M Na_2HPO_4-NaH_2PO_4 solution, with a final pH of 6.7. Serial twofold dilutions of the test materials were prepared in a volume of 0.8 ml. To these tubes were added 0.4 ml. of a neutralized *l*-cysteine-HCl solution containing 1 mg./0.4 ml. After shaking, and activation at 37°C. for 10 minutes, 0.4 ml. of a 4 per cent thoroughly washed rabbit red blood cell suspension in saline phosphate at pH 6.7 was added to each tube with a Cornwall automatic pipetting unit (Becton, Dickinson and Co., Rutherford, New Jersey). The tubes were incubated at 37°C. for 1 hour, being shaken at 0, 30, and 60 minutes. After storage in the refrigerator overnight, 1 ml. aliquots of the supernates showing partial hemolysis were diluted 1:4 with saline phosphate solution, and read in a Klett-Summerson photoelectric comparator. A green filter was used with maximum light transmission at 5250 Ångström units. A pooled sample from 5 or 6 tubes in the assay showing complete hemolysis was read in the same way. From the results, 50 per cent hemolysis end points were estimated. 1 hemolytic unit (H.U.) was considered that amount of preparation which would cause 50 per cent hemolysis of 1.6 ml. of 1 per cent red cell suspension.

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[2] The authors are deeply indebted to the following for supplies of streptococcal antigen concentrates: crystalline proteinase and proteinase precursor, Dr. S. D. Elliott; erythrogenic toxin, Dr. A. Stock; streptokinase-streptodornase, Dr. H. Peirsma, Lederle Laboratories.

[3] The authors are deeply indebted to Dr. Meyer for his assistance and suggestions concerning the hyaluronic acid preparation.

Earlier assays, which were not as reproducible as later ones, were carried out by activation of the samples with cysteine prior to dilution, and in the absence of bovine serum albumin. In agreement with a recent report (10), the albumin seemed to act as a stabilizing agent.

Sera:

After preimmunization bleedings, 19 albino female rabbits (2.5 to 3 kilos) were immunized with a streptolysin O concentrate. The total doses ranged from 1.5 to 4.5 mg. of a preparation with a potency of about 14,000 H.U./mg. The injections were given in one to three courses of six doses each at 2 to 3 day intervals with rests of 1 to 2 months between courses. Most of the injections were given intravenously, although for a number of animals one course was by other routes. Thirteen of the rabbits received the concentrates reduced to activity with cysteine; the remaining six were given the 98+ per cent reversibly oxidized form. Bleedings were made 1 week after the last dose of each course of injections, and the sera were stored aseptically at 4°C.

Guinea pigs (albino female 300 to 350 gm.) were given three courses of injections of the same streptolysin O concentrate intraperitoneally, in a total dose of 3.2 mg. Each course consisted of 4 to 6 doses on alternate days. 1 week after the last dose of each course, sample groups of animals were exsanguinated by cardiac puncture, and the serum collected. Twenty guinea pigs received the cysteine-reduced concentrate, and 19 the reversibly oxidized form. As controls, the sera of 10 uninoculated guinea pigs from the same shipment were collected. In addition, a smaller group of control animals received the same courses of injections of saline-phosphate buffer, or bovine serum albumin solution (total dose of 98 mg). Samples of these were bled at the same time as the streptolysin-immunized group.

Human sera (136) were obtained from the routine Diagnostic Chemistry Laboratory at the Presbyterian Hospital, New York, from 136 patients with a wide variety of illnesses including hypertensive cardiovascular disease, malignancy, thyroid disturbances, nephritis, uncomplicated surgical problems, etc. Two of these had had childhood rheumatic fever with no sequelae, while 4 had had reported upper respiratory infections a short while prior to the sampling. The ages of these patients ranged from 19 to 80, averaging about 40. Sera from patients with rheumatic fever had been stored in the deep freeze for varying lengths of time.[4] Only seven of these were tested by the Oudin technic.

Antistreptolysin Assay (ASO):

The sera to be tested were usually diluted serially by 2× starting with 1:10 in a volume of 0.4 ml. To these tubes were added 0.4 ml. of a reduced streptolysin O concentrate. (The one most frequently used had a median activity of 14,000 H.U./mg., and the test dose contained 2 μg. or 30 H.U./0.4 ml. This solution had been prepared in the bovine serum albumin diluent containing 4 mg./ml. of neutralized *l*-cysteine-HCl, and allowed to activate at room temperature for 10 minutes.)

The streptolysin O–serum dilution mixtures were shaken, and incubated at 37°C. for 30 minutes, at which time 0.8 ml. of thoroughly washed 2 per cent rabbit red cell suspensions were added to each tube. The tubes were incubated at 37°C. for 1 hour and 50 per cent hemolysis levels determined, as with the streptolysin O assays.

Simultaneously with the ASO assay of unknown sera, an assay under identical conditions was carried out in duplicate with an antistreptolysin O standard containing 180 Todd antistreptolysin units/ml. (Wellcome Laboratories, Beckenham England). From the results of the test with the standard, the ASO titers of the unknown sera could be directly calculated. The dilution of standard ASO revealing 50 per cent hemolysis with the usual test dose of

[4] These sera were kindly supplied by Dr. E. Fischel.

streptolysin, showed surprising reproducibility from one assay to the next, (*e.g.* 35 of 45 assays with this system showed 50 per cent hemolysis at dilutions from 1:54 to 1:56). All assays of the unknown sera were carried out on at least two different occasions, and only values agreeing within ±10 per cent were accepted.

Agar Precipitin (Oudin):

Essentially the modification of Munoz and Becker (11) was used with pyrex tubes of 4 mm. inner diameter and 12 cm. in length. These were lined with 1 per cent aqueous bacto-agar (Difco) and dried. The serum agar consisted of 50 per cent serum containing 1 part of serum and 1 part of 0.5 per cent agar in 0.15 M NaCl which had been previously adjusted to pH 7.5 and clarified. After cooling, the antigen solutions were overlayed on the agar, usually about 0.3 to 0.4 ml. of each being used. One preparation of streptolysin O (H107-10) was used throughout these tests in a concentration of 1 mg./ml. in 0.85 per cent NaCl. This material showed an average potency of 14,000 H.U./mg., and the solution was not reduced. From the time of addition of the antigen, the tubes were kept chilled. Development was allowed to take place in the refrigerator and readings of the leading edges of precipitin bands were made with the tubes immersed in an ice bath, under low magnifications with a centimeter rule. Distances could be estimated to ±0.3 mm. Occasionally, with very faint bands, readings could best be made when the tubes were placed against diffused light with a dark background without magnification. The optical density of each band was roughly graded by visual estimation.

RESULTS

Agar Precipitin Tests:

All preimmunization and control sera from the rabbits and guinea pigs showed no precipitin bands, and they did not reveal any detectable antistreptolysin O activity. The rabbit antisera obtained after the first course of immunization frequently showed only one band, while those obtained after the total series of injections showed up to four bands. The migration of these bands all followed a straight line relationship with the square root of time (in minutes), as shown first by Oudin (16). The results with several sample sera at different stages of immunization are shown in Fig. 2. The migration rates ($\text{mm.}/\sqrt{\text{time (min.)}}$) can be readily calculated from the data. That these rates were fairly reproducible can be seen by the results of the repeat tests carried out at different times (Table I). It may also be noted that the oxidation-reduction state of the immunizing antigen apparently did not affect the antigenicity of the preparation, either with regard to the agar precipitins or the antistreptolysin response. This was generally true of all the rabbit and guinea pig antisera tested.

Of the 143 human sera tested with the same streptolysin concentrate under the same conditions, 94 showed migrating bands. Of these 68 showed one, 23 showed two, and 3 showed three such bands. As with the rabbit antisera, these also revealed the straight line relation between the migrations and the square root of time, indicating the antigen-antibody nature of the bands. Samples are shown in Fig. 3.

Several tests were carried out using preparations derived from uninoculated medium as test antigens for controls. Several potent rabbit antisera and a few positive human sera were examined. The uninoculated medium antigens included whole medium, an ammonium sulfate

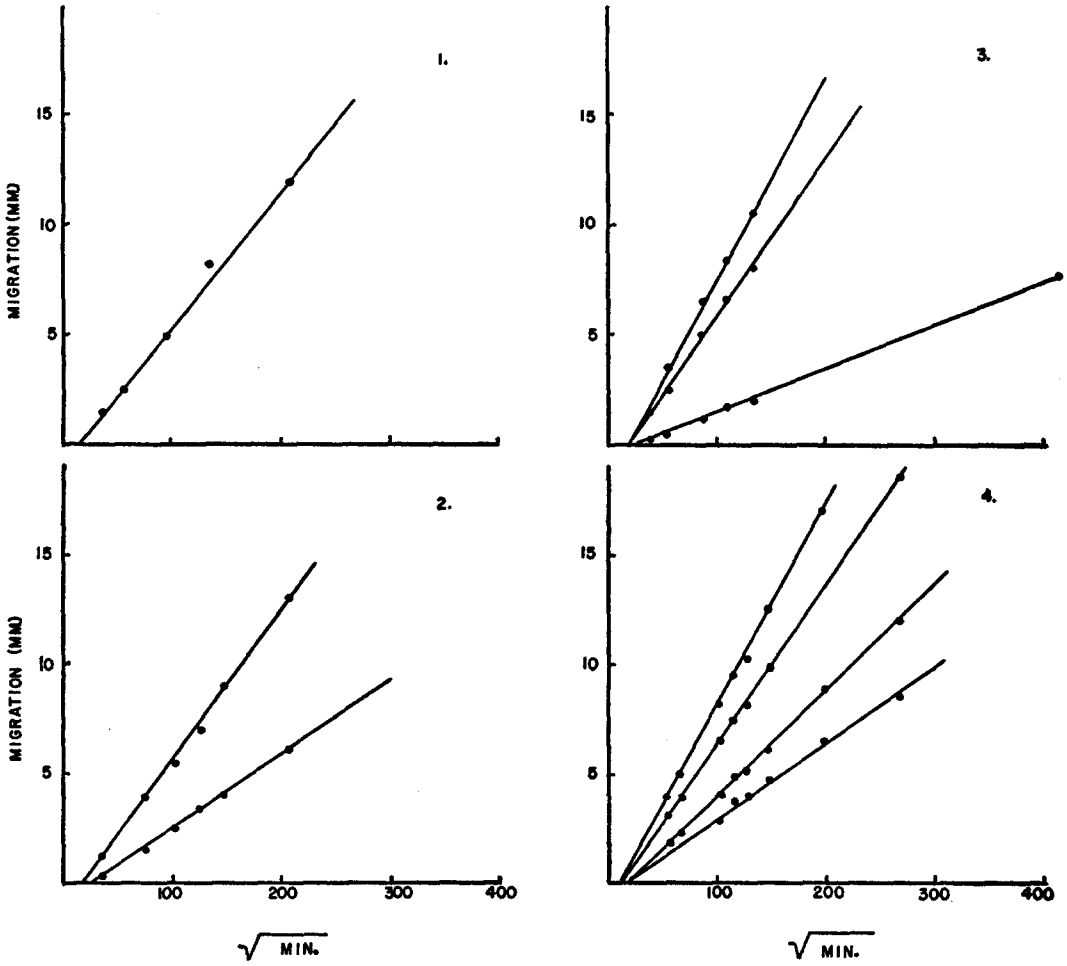

FIG. 2. Migration of precipitin bands with various rabbit sera and streptolysin O concentrate at 1 mg./ml. (H107-10, reversibly oxidized). 1, 2, and 4 were from rabbits immunized with cysteine reduced antigen, 3 was from a rabbit immunized with reversibly oxidized antigen.

precipitate of it, and a fraction obtained by the same purification steps used for streptolysin concentration. The yield in the latter was quite small, about 3 mg. of solid being obtained from 12.2 liters of uninoculated broth. This is in sharp contrast to a yield of 400 to 500 mg. that might be expected from a similar volume of broth harvested after streptococcal growth. At any rate, all the control tests failed to show any precipitins, and it is clear that the observations

TABLE I

Repeat Determinations of Migration Rates of Bands Produced with Rabbit Antistreptolysin O Sera and Streptolysin O Concentrate (H107-10, 1 mg./cc.)

Serum	Migration rates of band				Date of agar pptn. test	ASO‡	Redox state of immunizing antigen
	H + *	H	F	VF			
	mm./√min.	*mm./√min.*	*mm./√min.*	*mm./√min.*			
H111-2D	—	0.040	0.083	—	3/12/53	340	Red.
	—	0.039	0.079	—	9/ 9/53		
H111-3D	—	—	0.090	—	3/10/53	30	Ox.
	—	—	0.088	—	9/ 9/53		
	—	—	0.081	—	9/23/53		
	—	—	0.086	—	9/30/53		
H124-5A	—	0.046	—	0.058	3/10/53	180	Red.
	—	0.045	—	0.059	3/12/53		
	—	0.048	—	0.056	3/30/53		
H124-6A	—	0.037	0.074	—	3/10/53	175	Red.
	—	0.033	0.068	—	3/24/53		
	—	0.035	0.075	—	4/ 6/53		
	—	0.036	0.070	—	4/17/53		
	—	0.035	0.075	—	5/ 4/53		
	—	0.026	0.068	0.086	5/18/53		
H124-2B	—	0.052	0.076	0.100	5/ 4/53	180	Red.
	0.038	0.049	0.073	0.093	7/14/53		
	0.040	0.046	0.073	0.092	9/23/53		
	0.032	0.046	0.077	0.089	9/30/53		
H124-3B	—	0.053	0.094	—	5/ 4/53	180	Red.
	—	0.054	0.093	—	7/14/53		
	—	0.055	0.085	—	9/23/53		
	—	0.052	0.070	—	9/30/53		
H124-5B	—	0.059	0.087	—	5/ 4/53	190	Red.
	—	0.055	0.074	—	6/11/53		
H146-11A	—	0.031	0.057	0.132	4/ 6/53	180	Ox.
	—	0.030	0.049	0.150	9/23/53		

* The bands were roughly graded on the intensity of the precipitate opacity. H+, very strong; H, heavy; F, faint; VF, very faint.

‡ Antistreptolysin O titer of the sera (Todd units/milliliter).

described here are not complicated by contaminating antigens present in the uninoculated medium. This did prove to be a problem in recent studies by Jennings with erythrogenic toxin concentrates and animal antisera to them (19, 20).

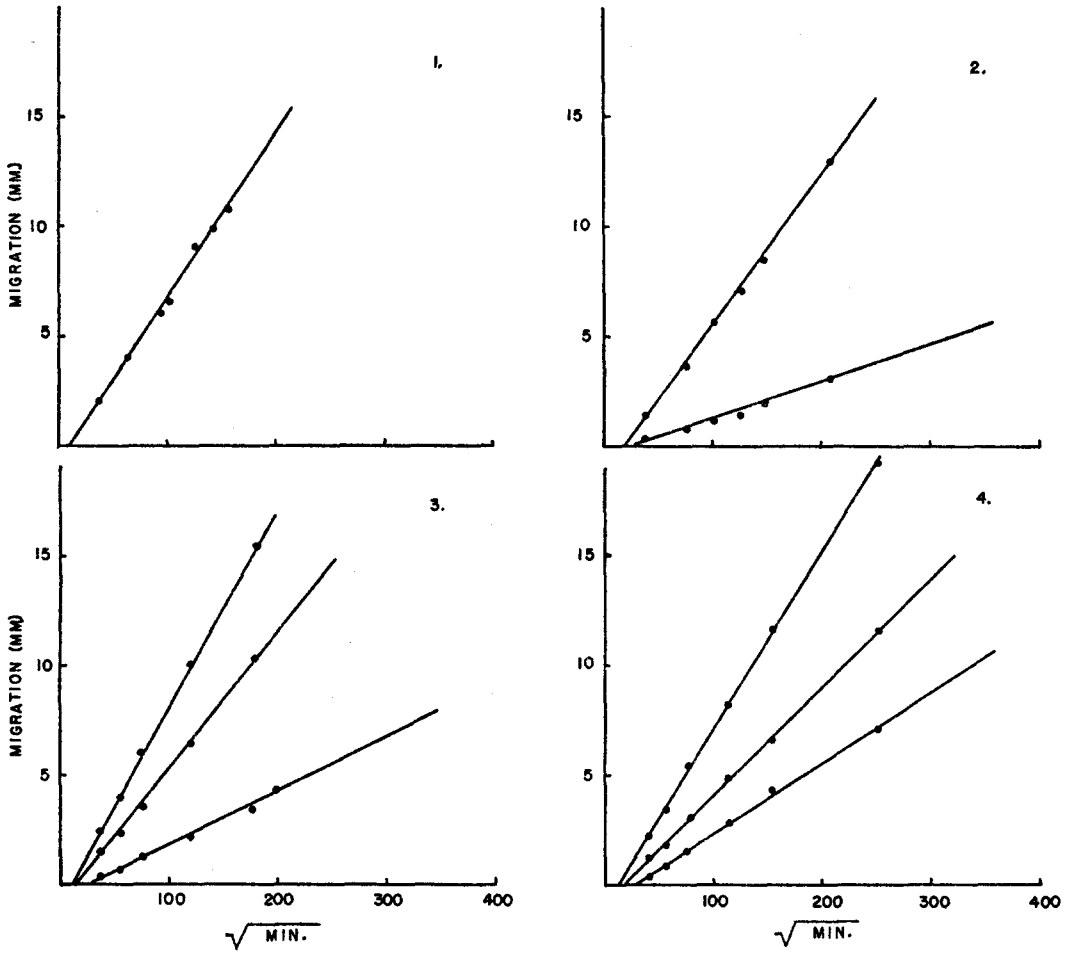

FIG. 3. Migration of precipitin bands with four human sera and streptolysin O concentrate at 1 mg./ml. (H107-10, reversibly oxidized).

Relation between ASO Titers and Migration Rates.—Oudin (16), and Becker (21) have shown that with constant antigen concentration, the migration rate increases with decreasing antibody levels. The relationship between the logarithm of the antibody concentration and the migration rate is a linear one. In order to obtain supportive evidence regarding the identity of the bands

noted with streptolysin O concentrates, the relationship between the antistreptolysin titers and migration rates was examined in some detail. In Fig. 4 are shown the results of these observations with rabbit and guinea pig antisera. About one-third of these sera showed only one band, the remainder showing several. Of the latter, the point recorded for each was that obtained with the heaviest, most slowly moving band. This choice, based on the assumption

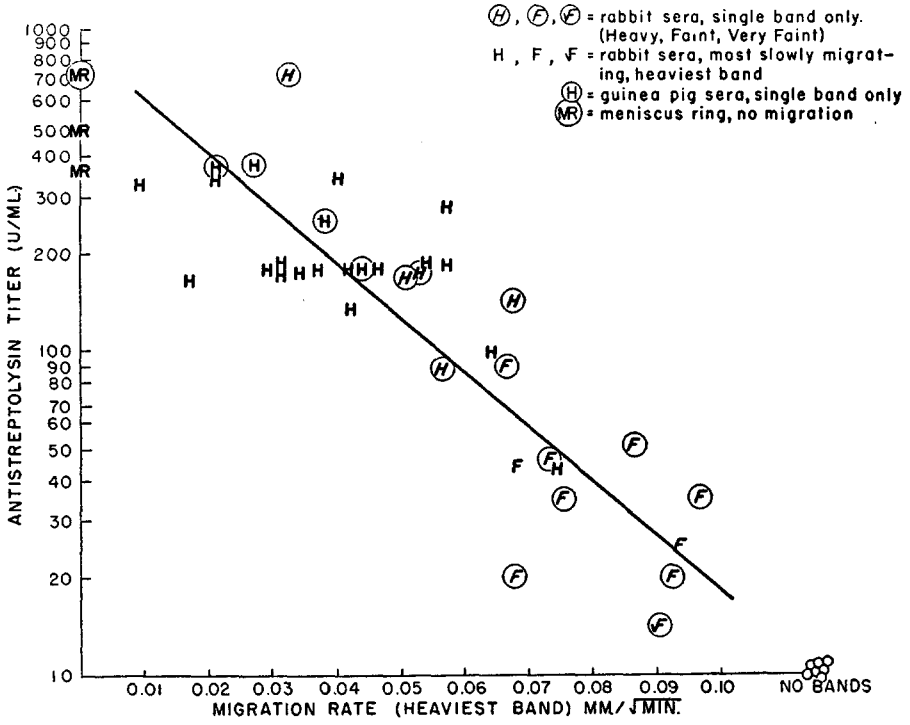

FIG. 4. Relation of the migration rates of individual rabbit and guinea pig antistreptolysin O sera (with streptolysin O concentrate), and the antistreptolysin titers (Todd units/milliliter)

that the concentrates were quite rich in streptolysin and that antibody to it would predominate, seems borne out in the straight line relation found. It may be noted that there was also a general correlation between the optical density of the bands, and the antistreptolysin titer. It seems quite likely, therefore, that the streptolysin O-ASO system is that seen in sera with one band, and is the heaviest, most slowly moving where multiple bands were observed. It is surprising that the relationship is so evident since the antistreptolysin assay entails a sizable experimental error. In addition, Becker has shown serious discrepancies in using the Oudin technic for quantitative esti-

mation of antibody, by measurement of migration rates at constant antigen concentration with a highly purified antigen (21).

The human sera migration rates were similarly related to the antistreptolysin titers. The points recorded include the 68 sera showing one band only. With 10 sera showing multiple bands, the heaviest, most slowly migrating one was recorded. The remaining 16 points were chosen of the 2 or 3 bands ob-

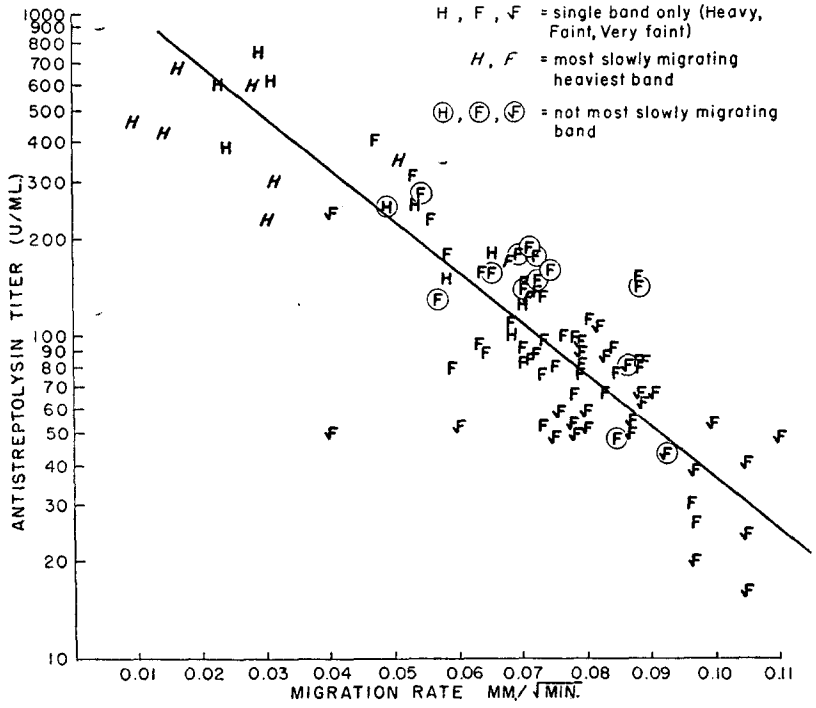

FIG. 5. Relation of the migration rates with streptolysin O concentrate of human sera, and the antistreptolysin titers (Todd units/milliliter).

served, to best fit the other 78 values. It may be seen (Fig. 5) that the data are similar to those found with the rabbit and guinea pig antisera. As with them, there was a general correlation between the faintness or heaviness of the bands, and the antistreptolysin titers. On the basis of these results, it seems quite likely that in human sera showing a single band, it was usually produced by the streptolysin O system. Of those sera showing several bands, the heaviest band was probably due to this system in about one-third of the cases, and one of the lighter bands due to this system in the remainder.

Of the 49 human sera which failed to show any precipitins, 28 showed anti-

streptolysin titers below 40, 17 titers between 41 and 65, and four showed titers between 66 and 85. Although one would have expected to see bands with some of these, it must be pointed out that a number of human sera proved difficult to examine because of turbidity of greater or lesser degree that developed upon dilution with the agar. This proved especially troublesome with the rheumatic fever sera available, and only seven of these sera could be adequately tested with the Oudin technic. In addition, the very faint bands seen with low ASO titered sera were quite delicate and could easily be masked by even a slight opacity of the serum agar. It may be noted, however, that all sera showing ASO titers above 85, showed one or more migrating bands. It seems quite likely therefore that most of the low titered sera failed to show a band because of lack of antistreptolysin antibody. The remaining low titered sera may have had the precipitins masked by the turbidity of the serum-agar gel. It is curious that this problem never arose with the rabbit antisera.

A few tests were carried out using purified human gamma globulin preparations.[5] This material was the same used for measles and poliomyelitis prophylaxis and consisted of a 16 per cent solution of pooled globulin. It was found to have an antistreptolysin O titer of about 1,500 units/ml. Migrating bands were readily seen, although turbidity of the serum agar was quite troublesome. In 1:4 and 1:16 dilutions of the gamma globulin in agar, one band was noted in each instance. The migration rates of these were 0.034 and 0.068 respectively. These data fit well with the other observations shown in Fig. 5.

Attempts at Identification of Non-Antistreptolysin O Bands.—The first step in this direction consisted of an examination of the streptolysin concentrate for the presence of known antigenic products of beta hemolytic streptococci.

Hyaluronidase was shown to be completely absent from several preparations by Dr. K. Meyer,[6] using the technic he has described (22).

Streptokinase was found to be absent in 2 streptolysin preparations, and present in concentrations of 15 and 60 units/mg. in two others. The last was the one used in most of the Oudin tests reported here. However, no differences in the numbers of precipitin bands were found in comparative tests with rabbit and human sera with the latter concentrate, and with one free of streptokinase. In addition, it may be pointed out that the purest streptokinase thus far recorded has shown a potency of about 300,000 units/mg. protein (23). The largest contamination by this substance of the streptolysin concentrates therefore represents only 0.0002 mg./mg., and it is almost certain that this system did not enter the picture.

Examination for the presence of C carbohydrate with grouping serum for group A beta hemolytic streptococci revealed this substance to be absent. Control tests showed that this serum reacted satisfactorily with the purified C preparation.

Erythrogenic toxin, M and T antigens were not searched for specifically, but evidence indicating their absence from the streptolysin concentrates will be presented in the succeeding paper.

[5] The human gamma globulin was kindly supplied by the American Red Cross.

[6] The authors are indebted to Dr. Meyer for his assistance in carrying out these tests.

Streptolysin S was absent from the concentrates, since over 99 per cent of the agent was in the reversibly oxidized inactive state, requiring reduction for activation. Streptolysin S, of course, does not show such behavior (24). In addition, all of the hemolytic activity of the streptolysin O concentrates could be readily inhibited by cholesterol. This has been shown not to be true of streptolysin S (25, 26).

Streptococcal desoxyribonuclease (streptodornase) was present in significant amounts in all the preparations, however. The concentrate used in the ASO and agar precipitin tests contained about 8,000 Christensen units/mg. (27). Other similar fractions contained from 4,000 to 8,000 units/mg.

Streptococcal proteinase[7] was found by Dr. Elliott to be absent from three different concentrates. However, proteinase precursor was present in appreciable amounts. The fraction used for the Oudin agar precipitin tests (H107-10) contained approximately 4 per cent by weight as *roughly* indicated by serological methods. The two other fractions contained about 0.1 per cent and 5 to 10 per cent respectively.

It thus appeared that the streptolysin O concentrates were significantly contaminated with only two of the well characterized streptococcal products, streptodornase and proteinase precursor.

Agar Precipitin Tests Using Rabbit and Human Sera, with Other Streptococcal Preparations.—Before carrying out more elaborate tests for the identification of the non-antistreptolysin O bands produced with the streptolysin O concentrate, a number of rabbit and human sera were examined for the presence of antibody to other streptococcal preparations. Samples of the results are shown in Table II. A total of 13 rabbit antisera, and 24 human antisera were thus tested. One rabbit and 10 human sera showed only one band with the streptolysin concentrate. Of these, the rabbit sera and 8 of the 10 human sera showed no reactions with any of the other preparations. This finding further supports the identification of the principal single system as that due to streptolysin O. Cross-reactions were most common with the streptokinase-streptodornase mixture. Many of the rabbit sera showed single heavy bands with both streptococcal proteinase and proteinase precursor, and 3 of the 24 human sera showed a band with the proteinase. None of the human sera showed anti "C," or antistreptococcal hyaluronate antibody. Precipitin bands with erythrogenic toxin preparations were rather frequent, but lack of material prevented more extensive tests.

An attempt was next made to see if any of the non-streptolysin bands seen with potent rabbit antisera could be identified by some of the methods suggested by Oudin (16). One technic involved the development of bands, followed by a replacement of the antigen layer with heterologous antigens. Continued movement of any particular band would indicate its presence in the homologous antigen.

A series of observations of this nature was made with rabbit antisera.

[7] The authors are grateful to Dr. Elliott for his kindness in carrying out these tests, and for supplying samples of proteinase precursor antiserum.

Bands with homologous streptolysin concentrate (1 mg./ml.) were allowed to develop for 8 days in replicate tubes. At this time, the antigen layers were withdrawn, the residue gently rinsed with cold saline, and replaced with heterologous antigen or saline as control. An example of the type of results

TABLE II

Agar Precipitin Bands Using Rabbit and Human Sera with Various Streptococcal Antigens

Serum	Agar precipitin bands with						
	Streptolysin O*	Varidase	Proteinase	Proteinase precursor	C carbohydrate	Erythrogenic toxin	M extract
Rabbit							
H111-5D	H‡	O	O	O	O	O	O
H146-12A	H+, F+	F+	O	O	O	O	?
H124-2B	H, F+, F	F	H	H	O	H	O
H146-11A	H, H−, F+, F	H, H−, F	O	O	O	F	O
H124-7C	H+, H, H−, F−	F, F−	H	H	O	O	O
Human							
R3-12	H	O	O	O	O	O	—
R8-90	H−	O	O	O	O	—	—
R10-165	F+	O	O	O	O	—	—
R7-36	H, F	F	O	—	O	—	—
R10-171	F+, F	O	O	—	O	—	—
R15-163	F+, F	O	F	—	O	—	—
R14-166	F+, F	F	O	—	O	—	—
R8-88	H, F+, F	H−, F	O	—	O	—	—
Streptococcus group A serum (anti-C)	O	O	O	O	H	O	O

* All the test antigens were used in a concentration of 1 mg./ml. in 0.85 per cent NaCl, except for the C carbohydrate, and erythrogenic toxin. This streptolysin concentrate contained 5 to 10 per cent proteinase precursor, and about 8,000 streptodornase units/mg.

‡ The bands are recorded as follows: H+, very heavy; H, heavy, etc. and — signifies not done. The heavier the band, the slower the migration, in these tests.

obtained are shown in Fig. 6. It may be seen that the three bands ceased to migrate upon addition of the replacement proteinase precursor (0.3 mg./ml.) or saline. With the former, it is clear that a new band appeared and migrated rapidly through the old. Similar results were seen when proteinase (0.3 mg./ml.) was used as replacement antigen. It thus seems likely that none of the original bands seen were due to this system, and that proteinase precursor was not present in the streptolysin in concentrations high enough to produce visible migration in the precipitin test. Similar tests suggested that

one of the rapidly moving bands found with homologous streptolysin was due to an antigen present in the streptokinase-streptodornase solution (1 mg./ml.) when this was used as replacement antigen. On the whole, however, this technic proved too difficult with the rather complex mixtures involved here. Similarly,

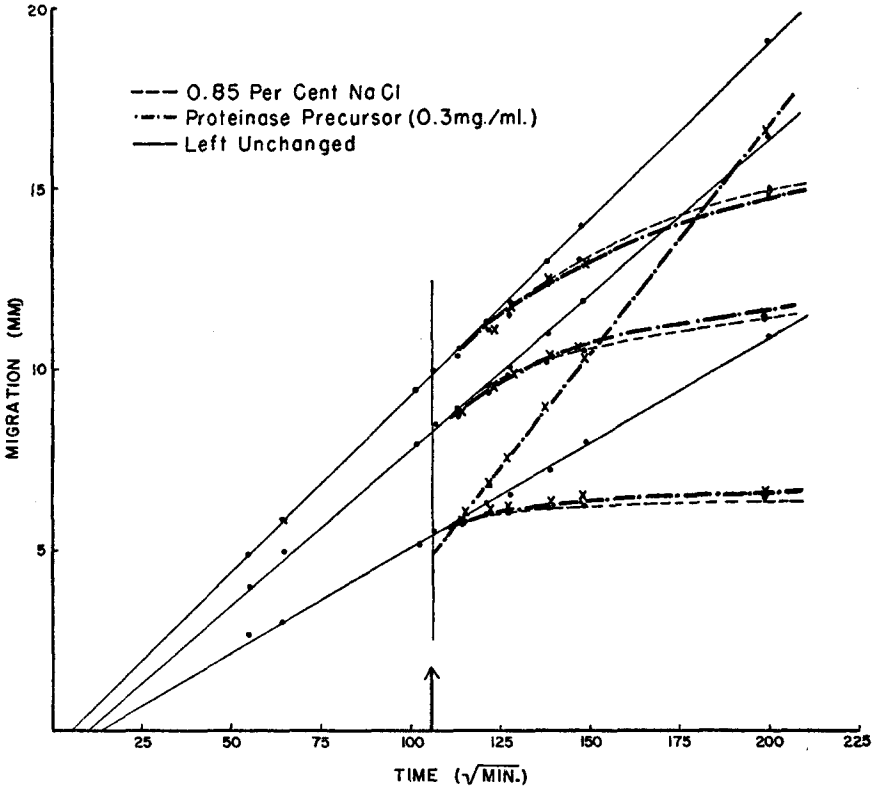

FIG. 6. Attempt to identify bands produced with rabbit antiserum and streptolysin O concentrate. At the time indicated by the arrow the antigen layer of one tube each was replaced with material as noted in the key.

the use of mixed antigen solutions was unsatisfactory in attempting to identify the secondary bands found with the streptolysin. Because of the volumes required, tests of this nature could not be done with the human sera available.

Attempts to Demonstrate Differences in Specificity of Oxidized and Reduced Streptolysin O.—It was felt that the rabbit antisera and the concentrate afforded an excellent opportunity to determine any possible differences in immunological specificity of the oxidized and reduced form of streptolysin O. Such differences have been found with lens protein (28), urease (29), and keratin (30). Accordingly, a series of tests were set up with rabbit sera

using as antigen layer either the 98+ per cent oxidized streptolysin (1 mg./ml.), or the same preparation reduced with 10 mg./ml. of neutralized *l*-cysteine HCl. Regardless of whether antiserum against the oxidized or the reduced form was used, typical bands developed only in the tubes with the oxidized form as test antigen in the majority of instances. In the tubes containing reduced streptolysin either no bands whatever developed, or one faint one at the most.

In addition, bands were allowed to develop with the test antigen in oxidized form (using anti-oxidized or anti-reduced sera) in the typical way for several days. At this time, the antigen layer was withdrawn, the tube rinsed gently with saline, and the antigen replaced with a *l*-cysteine–HCl solution (10 mg./ml., neutralized). Although the entire procedure was carried out in an ice bath, the precipitates began to dissolve within 30 minutes. The dissolving effect proceeded so that after several hours as much as 5 mm. of precipitate would be involved, and after overnight in the refrigerator as much as 10 to 20 mm. would have disappeared. A number of oxidizing or reducing compounds were tested, and only those capable of activating the streptolysin were capable of dissolving or preventing the antigen-antibody bands.

Control tests with other antigen-antibody systems such as bovine serum albumin, hen egg albumin, type II pneumococcal polysaccharide showed no significant differences when the test antigen was added with or without cysteine, or other reducing agents. In other control tests however, these unrelated systems were allowed to develop using as test antigen a mixture of the homologous antigens (*e.g.* bovine serum albumin), and oxidized streptolysin O solution. After the bands had migrated for several days, the test antigen mixtures were withdrawn and replaced with a cysteine solution. The unrelated precipitates did dissolve on these occasions. Further control tests of the latter type (homologous antigen and streptolysin O) showed that replacement of the streptolysin concentrate with streptococcal proteinase, proteinase precursor, or papain resulted in similar effects, although they were not as rapidly apparent in these instances. Inclusion of trypsin or chymotrypsin in these control homologous antigen solutions revealed no effect on the agar precipitates before or after the test antigens were replaced by cysteine.

It thus appears likely that the dissolving effects obtained with the reduced streptolysin O system were due to the presence of proteinase precursor as a contaminant. It is surprising that the proteolytic effects are so rapidly apparent at low temperature, and are produced by such low concentrations of enzyme. It is also surprising that two streptolysin preparations with 0.1 per cent and 5 to 10 per cent contamination of proteinase precursor show the same degree of dissolving effects. However, it is clear that further purification of the streptolysin concentrates will obviously be necessary for satisfactory examination of the immunological specificity of this agent in the oxidized and reduced states.

DISCUSSION

These observations clearly indicate that even low levels of antistreptolysin O activity of human sera are usually due to precipitating antibody and not the various non-specific inhibitors that have been described. The evidence for this consists in the correlation of migration rates of the bands found with the antistreptolysin titer, the correlation of the intensity of the bands with the ASO titer, and the similarity of these results with those seen in the animal sera in which antibody was clearly involved. In additional support, are the findings of the high antistreptolysin titer and migration rates of heavy bands with purified human gamma globulin preparation which fit well into the relationship.

These studies also reveal that the streptolysin concentrates used here are contaminated with at least three other antigens. It is apparent that antibodies to some of the latter are produced in the human subject. The following report will present evidence strongly indicating that some of these have been hitherto unknown, and do not seem to be related to any of the presently known products or "toxins" of the beta hemolytic streptococcus.

The lyophilized streptolysin concentrates were almost completely in the reversibly oxidized state. Antisera to the oxidized or reduced form prepared in rabbits and guinea pigs behaved similarly with regard to antistreptolysin O activity (antihemolytic), and precipitin formation. This confirms a previous report by Bernheimer (17), and extensive observations with the related pneumococcal hemolysin (18). Attempts to demonstrate differences in the immunological specificity of the oxidized or reduced form were unsuccessful, apparently because of the presence of proteinase precursor as a contaminant in these preparations.

SUMMARY

Using a highly concentrated and partially purified streptolysin O preparation, migrating agar precipitins have been found in 94 of 143 human sera from patients with a variety of diseases. Most of those showing no bands, had very low antistreptolysin titers.

A correlation was found between the migration rates of these bands and the antistreptolysin titer. A strong trend toward a straight line relationship was apparent when the ASO titers were plotted on a logarithmic scale. In addition, a roughly positive correlation was found between the intensity of these bands and the antistreptolysin O titers. The finding of high levels of antistreptolysin O activity and slowly migrating heavy bands in normal pooled human gamma globulin supported the above observations.

Very similar results were obtained with rabbit and guinea pig sera after immunization with the streptolysin O concentrates.

The data strongly indicate that antistreptolysin O activity in human sera is generally due to precipitating antibody, and that non-specific inhibitors are not usually involved, even with low titered sera.

Rabbit and guinea pig antisera to the oxidized inactive and to the reduced active forms of streptolysin O showed no obvious differences. Attempts to demonstrate immunological differences between the two states of streptolysin were apparently complicated by proteolysis, due to contamination of the concentrates with proteinase precursor.

BIBLIOGRAPHY

1. Rantz, L. A., Randall, E., and Rantz, H. H., *Am. J. Med.*, 1948, **5**, 3.
2. Mote, J. R., and Jones, T. D., *J. Immunol.*, 1941, **41**, 35.
3. Coburn, A. F. and Pauli, R. H., *J. Clin. Inv.*, 1935, **14**, 769.
4. Stollerman, G., Bernheimer, A. W. and MacLeod, C. M., *J. Clin. Inv.*, 1950, **29**, 1636.
5. Robinson, J. J., *J. Immunol.*, 1951, **66**, 661.
6. Packalen, T., *J. Bact.*, 1947, **56**, 143.
7. Oker-Blom, N., Nikkila, E., and Kalaja, T., *Ann. Med. Exp. et Biol. Fenniae*, Helsinki, 1950, **28**, 125.
8. Rosendal, K., and Bernheimer, A. W., unpublished observations quoted by Bernheimer, A. W. *in* Streptococcal Infections, (M. McCarty, editor), New York, Columbia University Press, 1954, 25.
9. Stollerman, G. H., *J. Clin. Inv.*, 1953, **32**, 607.
10. Gillen, A., and Feldman, H. A., *Fed. Proc.*, 1954, **13**, 494.
11. Munoz, J., and Becker, E. E., *J. Immunol.*, 1950, **65**, 47.
12. Herbert, D., and Todd, E. W., *Biochem. J.*, 1941, **35**, 1124.
13. Slade, H. D., and Knox, G. A., *J. Bact.*, 1950, **60**, 301.
14. Lancefield, R., and Perlman, G., *J. Exp. Med.*, 1952, **96**, 71.
15. Fuller, A. T., *Brit. J. Exp. Path.*, 1938, **19**, 134.
16. Oudin, J., *Methods Med. Research*, 1952, **5**, 335.
17. Bernheimer, A. W., *in* Streptococcal Infections, (M. McCarty, editor), New York, Columbia University Press, 1954, 19.
18. Neill, J. M., Fleming, W. L., and Gaspari, E. L., *J. Exp. Med.*, 1927, **46**, 735.
19. Jennings, R. K., *J. Immunol.*, 1953, **70**, 181.
20. Jennings, R. K., *J. Bact.*, 1954, **67**, 559.
21. Becker, E. E., *Fed. Proc.*, 1953, **12**, 717.
22. Meyer, K., *Physiol. Rev.*, 1947, **28**, 335.
23. Christensen, L. R., *in* Streptococcal Infections, (M. McCarty, editor), New York, Columbia University Press, 1954, 39.
24. Todd, E. W., *J. Path. and Bact.*, 1938, **47**, 423.
25. Hewitt, L. F. and Todd, E. W., *J. Path. and Bact.*, 1939, **49**, 45.
26. Cinader, B., and Pillemer, L., *J. Exp. Med.*, 1950, **92**, 219.
27. Christensen, L. R., *J. Clin. Inv.*, 1949, **28**, 163.
28. Ecker, E. E., and Pillemer, L., *J. Exp. Med.*, 1940, **71**, 585.
29. Pillemer, L., Ecker, E., Myers, V. C., and Muntwyler, E., *J. Biol. Chem*, 1938, **123**, 365.
30. Pillemer, L., Ecker, E., and Wells, J. R., *J. Exp. Med.*, 1939, **69**, 191.
31. Stock, A. H., *J. Biol. Chem.*, 1942, **142**, 777.