streptococci, and with sera of human subjects. 2. Evidence was obtained that complement-fixing antigenic material in such preparations was not identical with the complement-fixing fractions obtained from streptococcal cells, and complement fixation was probably not due to any of a number of the extracellular enzyme-antigens which can be found in streptococcal culture supernates.

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Gel-Precipitation of Streptococcal Culture Supernates with Sera of Patients With Rheumatic Fever and Streptococcal Infection.* (21933)

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In the literature dealing with antigenic substances produced by the hemolytic streptococcus in liquid cultures the emphasis has been almost exclusively on those antigens which have some biologic activity, such as toxicity or enzymatic function. In present studies the reactions of tissues of the rheumatic host to streptococcal antigens are explored. These studies are concerned not only with streptococcal antigens which have known enzymatic function, but also with those which are demonstrable only by their ability to give rise to antibody formation and to combine with those antibodies in mass reactions such as complement-fixation or precipitation. Production and concentration of macromolecular materials produced in streptococcal culture, and their reaction in complement-fixation tests with sera of injected rabbits and human subjects are described in the preceding paper (1). The complement fixation test does not, however, distinguish between one or more immunologic systems in solution (except in quite special circumstances of wide difference of concentrations of reagents relative to their specific activity). For the purposes of these studies methods of greater resolution were necessary to examine the antigenic materials produced in streptococcal culture. Accordingly, such materials were studied by gel-precipitin tests against rabbit and human sera. During such studies differences were noted in the numbers of antibodies present in sera of various groups of human subjects, in sufficient concentration to be observed. These will be presented here.

Methods and materials. 1. *Antigenic material.* Streptococci of Lancefield group A (Type 4, Strain H_{44}) were cultivated for 18 hours in the synthetic medium described by Bernheimer *et al.*(2). Supernates of centrifugation of such cultures were concentrated by precipitation with ammonium sulfate (at 80% saturation) in the presence of a cellulose fiber filter aid, Solka Floc. The extract of the resulting sediment was collected, suitably

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dialyzed and desiccated from the frozen state. The dry material was weighed and solutions of this material were used as described below. This material will be referred to as streptococcal CSA (culture supernate antigens).

2. *Sera.* Rabbits were given injections of CSA in either of two forms: weekly injections of approximately 5 mg CSA precipitated with alum, or tri-weekly injections, each 2.5 mg of the material in solution. Sera were obtained after a 4-week initial course of such injections and then, after successive 2-week courses of such injections, separated by one-week intervals. Human sera were obtained from 3 sources. Sera of presumably normal human subjects were obtained from outdated refrigerated bloods through the courtesy of the Philadelphia Serum Exchange. Sera of patients with scarlet fever were obtained from patients with this disease at the Philadelphia General Hospital at the beginning and end of their 3-week period of hospitalization, through the courtesy of Dr. A. C. LaBoccetta. A later specimen was obtained from a number of these patients approximately 6 weeks after the onset of streptococcal infection. Sera of patients with rheumatic fever were obtained from patients with a definite diagnosis of active rheumatic infection who were studied in the wards of the Philadelphia General Hospital, the Hospital of the University of Pennsylvania and the Children's Hospital of Philadelphia. Sera were obtained at the beginning of hospitalization and at 2-month intervals thereafter. Sera of patients with inactive rheumatic heart disease were obtained from patients at the Rheumatic Fever Clinic of the Philadelphia General Hospital. These patients were known to have had rheumatic fever at some earlier time (at least 2 years earlier) from study in the wards of above hospitals. 3. *The gel-precipitin tests. Preparation of agar.* Agar was prepared by immersing commercially available agar (Difco) in several volumes of cold distilled water, and incubating at refrigerator temperature. After the agar had settled, the water was decanted and the sediment resuspended in fresh water. After 5 such changes, swelling of the agar began and the washing was terminated. The sediment was now collected by centrifugation and lyophilized. It was now white in color.

Technic of the tests. The following 2 procedures were employed: Single diffusion in 1 dimension: The procedure was described by Oudin, in which antigens diffuse from an overlying solution into a serum-agar gel(3). Double diffusion in 1 dimension: This method, described by Oakley(4), was used with modifications suggested by Preer(5). The technic involves 3 layers: antigen solution, an agar solution, and serum, from above downwards. In this procedure the antigens and antibodies each diffuse into the agar column. Bands of turbidity occur in the agar where any given antigen-antibody system is within the zone of equivalence, with a sufficiently high concentration of the antibody to cause visible precipitation. Soft glass tubes, acid cleaned, of 3 mm internal diameter and 10 cm length, were sealed at one end, lined with 0.3% washed agar solution, described by Oudin(3), and marked on the outside at 2, 3, and 5 cm from the bottom. Serum was added to the tube to the 2 cm mark (0.13-0.14 cc of serum). Bubbles were removed if they formed. Agar, in 0.3% solution containing merthiolate 1:10,000, which had been heated and then maintained at 48-52°C, was added slowly over the serum layer up to the 3 cm mark. The tubes were allowed to stand 15-20 minutes at room temperature to ensure complete solidification of the agar. The antigen solution, generally used at optimum concentration of 3 mg/cc and containing 1:10,000 merthiolate, was added up to the 5 cm mark. The tubes were sealed with adhesive tape, placed in a glass jar or desiccator, and stored in a cabinet at room temperature. In the majority of sera tested, the maximum number of bands was present on the third day. The test was read in a darkened room with the tube held against a fluorescent back light. The number of bands was determined, and a brief physical description of each band noted. Three grades of relative density of bands could be readily differentiated: C—clear, distinct, dense band, with sharp boundaries; F—faint, distinct, band with sharp boundaries, but not dense; VF—very faint band with clear boundaries. These gradations of density reflect relative concentrations of the respec-

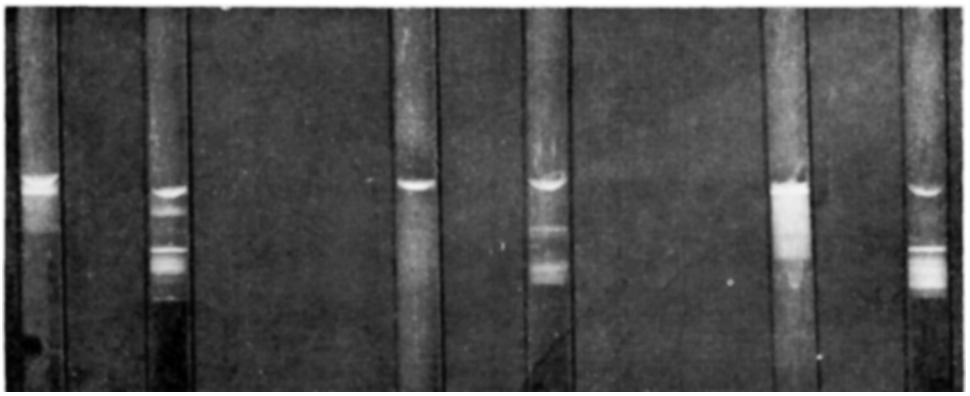

FIG. 1. Photograph of gel-precipitin tubes of 3 sera tested against concentrates of streptococcal culture supernate. Each pair of tubes is derived from a single serum, tested by single diffusion in the tube on the left and by double diffusion on the right. In all cases the meniscus constitutes the bottom of the antigen column. Note the greater resolution and the lower position (relative to the meniscus) of the bands in the case of the tubes set up by double diffusion (9,8,10).

tive antibodies at the level of the band, since the amount of specific precipitate reflects largely concentration of antibody. All bands not otherwise marked were of the dense type.

Fig. 1 shows gel-precipitin tubes of 3 sera tested by both single and double diffusion, on the third day of the test.

Results. 1. *Gel-precipitin tests with sera of rabbits injected with streptococcal CSA.* Rabbits given such courses of injections showed considerable variation in antibody response. Of those injected with alum-precipitated material only a few developed anti-CSA complement-fixation titers greater than 32, or more than 2 or 3 bands in the gel-precipitin tests, even after a number of courses of injection. Of the rabbits injected with the solution of CSA a higher percentage developed complement-fixation titers above 100 after the first 2 or 3 courses of injections, and at least 3 gel-precipitin bands within that time. Examination of the sera through the period of successive courses of injection with the CSA showed a general sequence of increasing complement-fixation titers and numbers of gel-precipitin bands against CSA from the pre-injection levels (CF titer below the threshold of measurement, and no gel-precipitin bands), although a number of instances of disparity occurred. Thus, one animal showed only 2 very faint bands and a complement-fixation titer of 128 after the first course of injections, whereas another of that group at the same stage showed 3 bands by gel-precipitin with a complement-fixation titer of only 32. Of the entire group of animals, the best antibody response obtained corresponded to 5 bands in gel-precipitin tests and complement-fixation titers of 192-256.

2. *Tests with sera of presumably normal human adults.* Eighteen such sera were collected from the group of normal men. These subjects were approved as blood donors by recent medical history and superficial physical examination. Of 10 such sera chosen at random for gel-precipitin tests against streptococcal CSA, 2 showed 2 bands of precipitate, 7 showed 1 band and 1 showed none. Eight other sera were selected from a larger number on the basis of antistreptolysin and antihyaluronidase titers which were relatively high for the normal population (AH, 16-192; ASL, 64-256). Of these sera 1 showed 3 bands in the gel-precipitin test, 3 showed 2 bands, and 4 showed 1 band. No association was apparent between the level of the titers of the 2 neutralizing antibodies and the number of bands in the gel-precipitin test. Samples of 7 lots of gamma globulin prepared from pools of normal human sera, made available through the courtesy of the American Red Cross, were similarly tested. Of these,

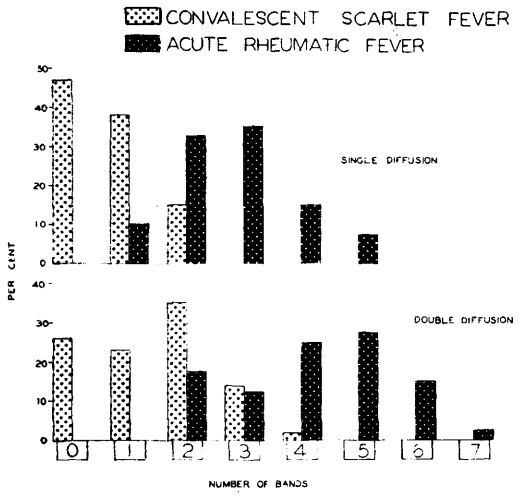

FIG. 2. Percentage distribution of sera of patients with acute rheumatic fever, and convalescent from scarlet fever, respectively, according to number of bands produced in tests with streptococcal culture supernates, by single-diffusion and double-diffusion technics.

1 sample yielded 2 bands, 5 gave 1 band, and 1 gave none. These tests were done by the single-diffusion method.

3. *Sera of patients convalescent from scarlet fever and patients with acute rheumatic fever.* A group of sera obtained from patients convalescent from scarlet fever were tested for antibodies to antigens present in the streptococcal CSA. A total of 65 such sera were tested by the double-diffusion technic, 34 of which were also tested by single diffusion. The number of bands which appeared by the third day ranged from 0-2 in the case of the tests by single-diffusion and 0-4 by double diffusion. Sera which were obtained from 40 patients with acute rheumatic fever were also tested. Using the single diffusion technic the number of bands which developed ranged between 1 and 5, and in tests by double diffusion the number of bands ranged between 2 and 7. The sera tested in each group were distributed according to the number of bands which appeared by each method, and percentages calculated. Fig. 2 presents the data obtained. A larger percentage of sera from patients with acute rheumatic fever developed relatively greater number of bands within this distribution in tests, by both technics. Of one of the sera of a patient convalescent from scarlet fever a larger specimen was available. Globulins were precipitated from a portion of this serum by adding an equal volume of a saturated solution of ammonium sulfate. The sediment was redissolved, and the solution dialyzed overnight and then brought to a volume equal to that of the original portion of serum. This solution yielded the same number of gel-precipitin bands as did the original serum.

4. *Sera of patients with scarlet fever at various times relative to the onset of the disease.* Of the sera of patients with scarlet fever a number were chosen whose convalescent sera had developed from 2-4 bands, for a comparison of the number of bands present early in the disease, at convalescence (3 weeks), and 6 weeks after the onset of symptoms. These were examined in a single test by double diffusion. The results are summarized in Table I. In all cases there was an increase in the number or intensity of the bands from the acute to the convalescent stage of this disease. In some cases, where bands were already present in the acute serum specimen, this difference was smaller. The relatively high antistreptolysin-O titers in some of these acute specimens would suggest, however, that some of these sera may have been obtained after the disease had been present long enough for some antibody response to have occurred. (It should be noted that the acute serum is obtained at the time of admission to the hospital, which is usually some days after the onset of symptoms. Also, the notation of day of disease on which the serum is obtained depended on the medical history provided by

TABLE I. Number of Gel-Precipitin Bands Produced by Sera from Patients with Scarlet Fever at Various Times in Relation to Onset of the Disease, Tested with Streptococcal CSA. (8 patients.)

Early serum specimen		No. of bands		
Day after onset of symptoms	Antistreptolysin titer	Acute	3-wk	6-wk
3	12	0	2	2 + 1F
3	256	1	2	2
4	64	0	4	1 + 2F
2	384	3F	3	2F
2	96	0	2	2
4	128	3F	3	2 + 1F
4	64	1VF	2	2
4	256	2	2 + 1F	3

TABLE II. Number of Gel-Precipitin Bands Produced by Sera of Patients with Acute Rheumatic Fever Observed over Periods Ranging from 4-18 Months. (18 patients.)

Acute serum	Months after onset					
	2	4	6	8	12	18
1 + 1F	3	2	2	1 + 1F	1 + 1F	1 + 1F
5F	1 + 1VF	1	1			1
4	2	1 + 1F	1 + 1VF	1F	1F	
5	1 + 1F	1F	1F	1VF	1VF	
3	2	2F			1 + 1F	
4	1 + 2F	2VF			1VF	
3	1 + 2F				1 + 1F	
7	1 + 1F	3F	2	1 + 1F		
5	1 + 1F	2	2	1 + 1VF		
3	2	1 + 1F	1F + 1VF	1F + 1VF		
5	2	3		2 + 1VF		
3	1 + 1F	1 + 1F	1			
3	2	2	2			
4 + 2VF	6	5	3 + 2VF			
6	3	2				
5	1 + 1VF	1				
6	3 + 3F	3				
5 + 1F	4F					

a parent.) In the sera obtained 6 weeks after the onset of symptoms no increase in the number of bands was found over the 3-week serum specimen, and occasionally a decrease in the number of bands was noted.

5. *Sera of patients with rheumatic fever at different stages of the disease.* A group of patients with rheumatic fever was studied over the period from acute disease to quiescence. For each patient, sera which were obtained in the acute stage and 2, 4, 6, 8, 12 and 18 months later were tested by double diffusion. There was a definite decrease in the number of bands, from the period of acute rheumatic fever to inactive rheumatic fever. In many instances some decrease in the number of bands was already evident 2 months after the acute attack. Table II summarizes the findings obtained in 18 patients with acute rheumatic fever followed for 4 to 18 months. Sera obtained in an out-patient clinic from 12 persons with inactive rheumatic fever were tested by the double-diffusion technic. Of these, 1 serum showed 3 bands; 3 sera 2 bands; 5 sera 1 band; and 1 serum no bands.

Discussion. Comparison of data obtained by single-diffusion (Oudin) and double-diffusion technics. In all groups of sera, both by single and double diffusion, the latter technic resulted in more bands, for the reason that in the double-diffusion method the concentration of any antibody varies over a continuous range which extends to zero, whereas in the single-diffusion method the concentration of an antibody is never below a given fraction of its concentration in the serum.† A technical advantage of the double-diffusion technic is that it permits the use of turbid sera, or sera which have become turbid through freezing and thawing, since the observation of bands of precipitate is not made in the zone which contains serum. *Number of gel-precipitation bands obtained in various groups of sera tested with streptococcal culture supernate concentrates.* The data presented above indicate that there are a considerable number of antigenic components in concentrates of streptococcal culture supernates prepared for these studies. As many as 7 bands have been found in the case of single sera and the total number of antigenic species may be greater than that for 3 reasons. (1). Within any single tube the number of bands is a minimum

† In the case of single diffusion the concentration of antibody at the interface soon becomes one-half its concentration in the serum-agar column (i.e., one quarter its concentration in the original serum); concentration of antibody thus varies within the agar column from one-quarter to one-half its concentration in serum. In the case of the double-diffusion technic the concentration of any antibody within the agar column varies from zero, at the antigen interface, to one-half its concentration in the original serum, at the serum interface.

of the number of antigen-antibody systems present because the difference between rates of diffusion of 2 antigenic species may be too small for the limit of visual resolution of the corresponding bands; (2). identification of the bands with respective antigens is not possible at present, and we do not know that antigens from the same group of 7 are involved in all the bands observed in various sera; (3). there may be antigens to which the antibodies are not present in the rabbit or human sera in sufficient concentration to produce visible precipitate. Evidence that these bands are produced by precipitation of streptococcal antigens with homologous antibodies is offered by the fact that they appeared *de novo* in sera of rabbits injected with the streptococcal CSA, and by the time relation of numbers of bands found in the sera of patients with scarlet fever and rheumatic fever to the times of onset of the diseases.

The relationship of any of these antigens to the known extracellular enzymes of the hemolytic streptococcus is not known. There have been two reports of gel-precipitin studies of concentrates of streptococcal culture filtrates which had been partially purified with respect to a biologically active agent. Jennings(6) found 8 separate precipitin systems in preparations of streptococcal erythrogenic toxin tested by the Oudin technic against horse antitoxin, and identified one of these as the toxin itself. Recently Halbert *et al.* examined preparations of streptococcal hemolysin-O by using human and rabbit sera in the same technic and found as many as 4 bands. Here, again, evidence was presented that one of these was hemolysin, the other bands being due to other antigens, which had not been separated from the biologically active one for which partial purification had been made(7). Using a crude concentrate of streptococcal culture filtrate in which no separation of macromolecules had been attempted, these authors found as many as 7 bands in the sera of patients with acute rheumatic fever, and no more than 4 in sera of patients with miscellaneous diseases other than rheumatic fever (8). The data presented here on the numbers of bands obtainable in gel-precipitin tests between sera in acute rheumatic fever and crude streptococcal culture supernate concentrates thus agrees with those of Halbert *et al.*

Difference between distributions of gel-precipitation band numbers in sera of patients with acute rheumatic fever and those convalescent from scarlet fever. The presence of a band in gel precipitation reflects the presence of an antibody in a concentration above the threshold required to produce a visible precipitate. Accordingly, the differences in band numbers found between these 2 groups of patients reflect higher concentrations of serum antibodies to streptococcal antigens in acute rheumatic fever than in convalescence from an uncomplicated streptococcal infection. It is not possible at present to compare antibody concentration to the individual antigens contained in these preparations, since there is no way of identifying the bands due to the respective antigen-antibody systems. This suggestion of higher concentrations of antibodies to streptococcal antigens in acute rheumatic fever, in comparison with streptococcal infection, agrees with observations by various workers in the case of a number of antibodies to streptococcal antigens (neutralizing antibodies to extracellular enzymes and complement-fixing antibodies to somatic nucleoproteins)(9,10).

No explanation can be suggested for the difference in range of distribution of serum antibody concentration to streptococcal antigens in these two diseases. Data are available on only one aspect of the contact between streptococcal antigens and the tissues of the rheumatic host which might result in such a difference—the greater interval of time than 3 weeks which might elapse between the onset of a foregoing streptococcal infection and admission to a hospital for rheumatic fever of many of these patients. That mere duration of contact between antigens and host tissues is not the causative factor is suggested by the fact that in the case of the individual patients with scarlet fever tested at both 3- and 6-week intervals after the onset of the disease there was no increase in the number or intensity of bands at the 6-week interval, in comparison with the 3-week, but rather a decrease in the intensity of some of these.

Summary. 1. Precipitin tests in semi-solid

agar (gel-precipitin) have been carried out between concentrates of streptococcal culture supernates and sera of various sources. Two technics of diffusion in 1 dimension (tube) were used: the single-diffusion technic of Oudin, and a double-diffusion technic. 2. Sera of rabbits previously injected with streptococcal culture supernates showed increasing numbers of bands as the course of injections progressed. Sera of such rabbits showed as many as 5 bands when tested with such concentrates, indicating that this was the minimum number of antigen-antibody systems present. 3. Sera of patients with acute rheumatic fever and of patients convalescent from an acute streptococcal infection (scarlet fever) were also tested by this technic against concentrates of streptococcal culture supernates. The group with rheumatic fever showed a greater number of bands (a range of 2-7) than did the group with scarlet fever (0-4), when tested by the double-diffusion technic. The corresponding ranges found with the single-diffusion technic were 1-5 and 0-2, respectively. In a sampling of sera from convalescents from scarlet fever the number of bands was found to be no greater 6 weeks after the onset of the infection than 3 weeks after the onset.

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Teratogenic Effects of *Lathyrus odoratus* Seeds on Development and Regeneration of Vertebrate Limbs.* (21934)

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Diets containing *Lathyrus odoratus* seeds fed to young rats produce characteristic defects of mesodermal structures(1). Chemical analysis of extracts from the seeds points to the β-aminopropionitrile (BAPN) as the active fraction of the teratogenic principle(2-5). However other aminonitriles(5) and β-mercaptoethylamine(6) when given to rats have been reported to produce similar lesions. The Lathyrus poisons gain in interest through the similarity of the induced lesions with certain skeletal and vascular diseases in man, such as slipped epiphyses, degenerative arthritis, and dissecting aneurism of the aorta. Therefore the use of aminonitriles in animal research seemed to offer an opportunity for the elucidation of these human diseases.

We reported(7) that BAPN at a concentration of 2 μl/liter of aquarium water produces characteristic deformities in Xenopus larvae. The high sensitivity of Xenopus larvae suggests their use for the detection and quantitative evaluation of lathyrism-inducing agents. The effect of BAPN appears to be highly specific, affecting particularly certain rapidly developing mesodermal tissues. In view of the similarity between the processes of embryogenesis and of regeneration, the studies were extended also on the restitution of amputated legs in the newt, *Diemictylus viridescens*.

Methods. In a *first series* of experiments

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