THE INHIBITION OF STREPTOCOCCAL DESOXYRIBONUCLEASE BY RABBIT AND HUMAN ANTISERA

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The fact that group A hemolytic streptococci produce an extracellular desoxyribonuclease in appreciable amounts has recently been encountered independently in two different laboratories (1, 2). The production of this enzyme appears to be a relatively constant property of these organisms, as indicated by the fact that it is elaborated by a wide variety of strains derived from different sources and with diverse biological properties. The possible significance of desoxyribonuclease in the pathogenesis of streptococcal disease is not known, although it seems certain that it does not act as a primary toxic agent.

In the case of other extracellular products of group A streptococci—such as streptokinase, streptolysin O, and hyaluronidase—it has been established that antibodies directed against them are highly effective in inhibiting their biological activity. The present investigation was undertaken to determine whether streptococcal desoxyribonuclease is similarly inhibited by its antibody and whether this antibody is commonly formed by human patients subsequent to streptococcal infections. It has previously been shown that rabbit antiserum against bovine pancreatic desoxyribonuclease effectively inhibits this enzyme (3). An opportunity was thus provided for a comparison of the antibody inhibition of two enzymes having similar action but prepared from widely different sources.

Materials and Methods

Preparation of Streptococcal Desoxyribonuclease.—The enzyme was prepared in a highly active form from supernates of cultures of a strain of group A hemolytic streptococcus. The procedure used in the preparation of the material used in most of this study is described in the following:

Fifteen liters of neopeptone dialysate broth (4) was inoculated with strain H105 and incubated at 37° for 20 hours. The cells were removed by centrifugation in a Sharples centrifuge and the slightly turbid supernate was brought to 0.4 saturation with ammonium sulfate by the addition of 243 gm. of solid salt per liter of supernate. One-tenth per cent each of filter cel and hyflo super cel were added and the suspension filtered with suction. The clear filtrate was brought to 0.8 saturation by the addition of 281 gm. of ammonium sulfate per liter of filtrate and the resulting precipitate recovered by filtration. The solution of this precipitate in 100 cc. distilled water was dark brown in color and contained practically all of the original desoxyribonuclease activity. Further fractionation was carried out with ammonium sulfate The solution was brought to 0.4 saturation and a precipitate formed which contained most

of the colored material but only about 15 per cent of the total activity. The bulk of the activity was recovered by bringing the 0.4 saturated supernate to 0.5 saturation. The precipitate so formed was recovered by filtration, dissolved in a small volume of water, dialyzed against distilled water, and dried *in vacuo* from the frozen state. The yield was 169 mg. of dried material with an activity of 25,000 viscosity units per mg. which is approximately 2.5 times that of the amorphous desoxyribonuclease prepared from beef pancreas by the method previously described (3). It did not contain measurable amounts of streptokinase or streptococcal proteinase.

Some increase in the initial production of enzyme can be obtained by the addition of excess glucose and growth factors after overnight incubation and continued growth of the culture with neutralization of the acid formed. However, with the strain used, the increase has never been more than twofold, which is small compared to the ten- to thirtyfold increase in streptokinase concentration which occurs at the same time. The results reported by Christensen (5) using a strain of group C also indicate a disparity between the production of streptokinase and desoxyribonuclease under these conditions, although the increase in desoxyribonuclease appears to be greater than that obtained with group A strains in this laboratory.

Recovery of Streptococcal Desoxyribonuclease from Streptokinase Preparation.—As observed by Tillett, Sherry, and Christensen (1), streptokinase purified by the method of Christensen contains relatively large amounts of desoxyribonuclease. Christensen (5) has described methods for partial separation of the desoxyribonuclease from streptokinase preparations from group C strains, and analogous results have been obtained independently in this laboratory in the fractionation of group A streptokinase.

Streptokinase purified by a modification of Christensen's procedure (6) was subjected to further fractionation with ammonium sulfate. Immediately following protamine precipitation, a concentrated solution of streptokinase was brought to 0.5 saturation by the addition of an equal volume of saturated $(NH_4)_2SO_4$. The streptokinase was precipitated almost quantitatively, and subsequent precipitation of the supernate at 0.8 saturation yielded a fraction containing 60 per cent of the desoxyribonuclease and only 0.01 per cent of the streptokinase of the original material. The specific activity of the desoxyribonuclease so obtained is essentially the same as that of the preparation described above.

Preparation of Substrate.—The desoxyribonucleate used as substrate was obtained by deproteinization of calf thymus nucleohistone prepared according to the method of Mirsky and Pollister (7). Deproteinization by the chloroform method requires numerous repetitions of the process and is especially cumbersome if the preparation of moderately large amounts of material is attempted. A method has been devised to facilitate the removal of protein which is based on the procedure used for the separation of pneumococcal nucleic acid and pneumococcal polysaccharides by the use of $CaCl_2$ and ethyl alcohol (8). The amount of chloroform treatment required is greatly reduced.

The solution of nucleohistone in M NaCl is mixed with an equal volume of 0.9 M $CaCl_2$. The volume of the solution is measured and 0.2 volume of ethyl alcohol added with stirring. The desoxyribonucleate separates out as a fibrous precipitate which is removed and washed twice in a solution containing $CaCl_2$ and alcohol in the same concentration as that from which it was precipitated. The bulk of the protein remains in solution. The fibrous precipitate is redissolved in M NaCl and final deproteinization carried out by the chloroform method. Variations are experienced from lot to lot in the ease with which the last fraction of protein is removed. In some cases it is difficult to obtain nucleate which gives water-clear solutions and negative qualitative tests for protein.

Rabbit Antisera against Streptococcal Desoxyribonuclease.—The enzyme was injected intraperitoneally into rabbits in the form of an alum precipitate. Aqueous solutions of the enzyme were mixed with an equal volume of 10 per cent aluminum potassium sulfate and the solution

brought to neutrality with N NaOH to precipitate the alum. The rabbits received weekly doses of 2, 2, 10, and 10 mg. of the enzyme purified according to the method described above, and blood was obtained for serum 5 days after the last dose.

Human Sera.—The human sera were those from an epidemic of scarlet fever occurring at the Great Lakes Naval Training Center from February, to May, 1946.[1] In a previous study, the antistreptokinase and antistreptolysin 0 titers of these sera had been determined (9). In addition, antihyaluronidase levels of certain of these sera have been reported by Friou (10).

Measurement of Inhibition of Desoxyribonuclease.—(*a*) *Viscosimetric test.*—With minor modifications, the viscosimetric test is the same as that used in the measurement of the inhibition of pancreatic desoxyribonuclease by rabbit antisera (3). A stock substrate solution was prepared containing 0.1 per cent sodium desoxyribonucleate and 0.005 M $MgSO_4$ in 0.025 veronal buffer, pH 7.5. The enzyme and serum dilutions were prepared in neopeptone dialysate broth to insure stability. Although dilute solutions of the enzyme are unstable in buffer or gelatin, neopeptone broth solutions with enzyme concentrations as low as 0.02 μg./ml. show no loss of activity during incubation. Equal volumes of the appropriate enzyme and serum dilution were mixed, incubated at 37° for 30 minutes, and 0.5 ml. of the mixture was added to 4.5 ml. of the substrate solution in an Ostwald viscosimeter for measurement of residual enzyme activity.

(*b*) *Alcohol Precipitation Test.*—The viscosimetric method is a relatively precise method for the measurement of desoxyribonuclease activity, but it has the disadvantage of being laborious and time-consuming. It is not adaptable to the titration of the antidesoxyribonuclease activity of a large number of sera such as those obtained by serial bleedings from patients with streptococcal infections. The following method, analogous in some respects to the mucin-clot prevention test for the measurement of anti-hyaluronidase (11), was devised for this purpose. The test is dependent upon the fact that unchanged sodium desoxyribonucleate when precipitated by alcohol forms a floating fibrous mass, and that after action of the enzyme only a light flocculent precipitate is formed.

Serial twofold dilutions of the sera, beginning at 1:10, were prepared in broth, and 0.25 ml. of the dilutions was mixed in pyrex test tubes (10 × 100 mm.) with 0.25 ml. of a solution of streptococcal nuclease containing 0.2 μg./ml. After 30 minutes' incubation at 37°C., 0.5 ml. of the substrate solution, prepared as above, was added to each tube and incubation continued for 30 minutes. One ml. of ethyl alcohol was added to each tube at the end of the second incubation period and the tubes were examined for the presence of floating fibrous precipitate. The end point was defined as the highest serum dilution which prevented enzymatic degradation of the substrate so that a definite fibrious precipitate was formed upon the addition of alcohol. The final enzyme concentration in the reaction mixture (0.05 μg./ml.) is the equivalent of 5 units of enzyme in the viscosimetric system and is approximately four times the amount required to cause degradation of the desoxyribonucleate to a point where no fibrous alcohol precipitate is formed under the conditions of the test.

As in the case of other similar titrations dependent upon a twofold serial dilution system, the titers are reproducible within one tube. Consequently, in attempting to determine whether a rise in antibody has occurred, a fourfold shift in titer is considered a significant change.

Interfering Action of Serum Nuclease.—Since mammalian sera contain measurable amounts of desoxyribonuclease, it was necessary to take this fact into consideration in devising procedures in which serum was employed for its inhibitory action on streptococcal desoxyribonuclease. In the case of rabbit sera, which contain somewhat more of the enzyme than human sera, the action of the serum enzyme was detectable in the viscosimetric test at serum con-

[1] Sera and cultures on these patients were collected during a cooperative project with the United States Naval Medical Research Unit No. 4. See reference 9.

centrations employed in the inhibition test. Heating of the diluted serum at 65° C. for 30 minutes destroys the enzyme without measurable effect on the antibody, and consequently this procedure was employed in all instances where dilutions of the serum of less than 1:50 were involved. The concentration of nuclease in human serum is too low in the vast majority of cases to be a factor at the dilution employed in this study.

EXPERIMENTAL

Inhibition of Streptococcal Desoxyribonuclease by Rabbit Antisera.—In comparison with certain of the sera obtained from patients following streptococcal infections, the sera from rabbits injected with the purified enzyme did not possess high inhibitory activity. However, tests on sera of the two rabbits giving the best response showed that by the viscosimetric test 1.0 cc. of serum was capable of almost complete inhibition of 5 units of enzyme and better than 95 per cent inhibition of 50 units of enzyme. By the alcohol precipitation technique an end point was obtained in both cases at a serum dilution of 1:640. There is no real discrepancy between the results obtained by the two techniques, since they measure inhibition somewhat differently. By the viscosimetric technique it is feasible only to measure rather high degrees of inhibition, while by the alcohol precipitation technique a relatively small inhibitory effect would be reflected by a preservation of part of the substrate in a form that would give a fibrous precipitate.

The specificity of rabbit antibody is of some interest. Desoxyribonuclease from beef pancreas, which, in so far as has been determined, is an enzyme analogous to the streptococcal enzyme in its action on desoxyribonucleic acid and in the optimal environmental conditions required, is entirely unaffected by the presence of rabbit antiserum against streptococcal nuclease. Similarly, antibody prepared in the same manner using pancreatic desoxyribonuclease as antigen has no inhibitory action on streptococcal nuclease. These relationships are illustrated in part in Fig. 1. It will be observed that the activity of approximately 1.5 units of streptococcal desoxyribonuclease is almost completely inhibited by homologous antiserum in a final dilution of 1:25, while the same serum is without effect on a similar concentration of pancreatic desoxyribonuclease. Repeated tests using varying concentrations of enzyme and serum in an attempt to increase the sensitivity of the measurements failed to reveal even a slight degree of cross-reactivity; *i.e.*, in no case did the effect of heterologous antiserum vary significantly from the effect of normal serum.

These results have some theoretical interest since they indicate that in this case the configuration of the enzyme proteins which determines enzymatic specificity is distinct from the configuration conferring antigenic specificity. The action of the two enzymes appears to be identical, but this identity in enzyme action is not reflected by cross-reactivity in the serological studies. The same phenomenon has been observed with more favorable material by

Krebs and Najjar (12). These workers used crystalline preparations of *d*-glyceraldelyde-3 phosphate dehydrogenase from rabbit muscle and from yeast, and thus they were dealing with highly purified enzymes. In addition, the relative simplicity of the substrate and the enzyme reaction rendered it more certain that the enzyme action was identical in both cases. They were able

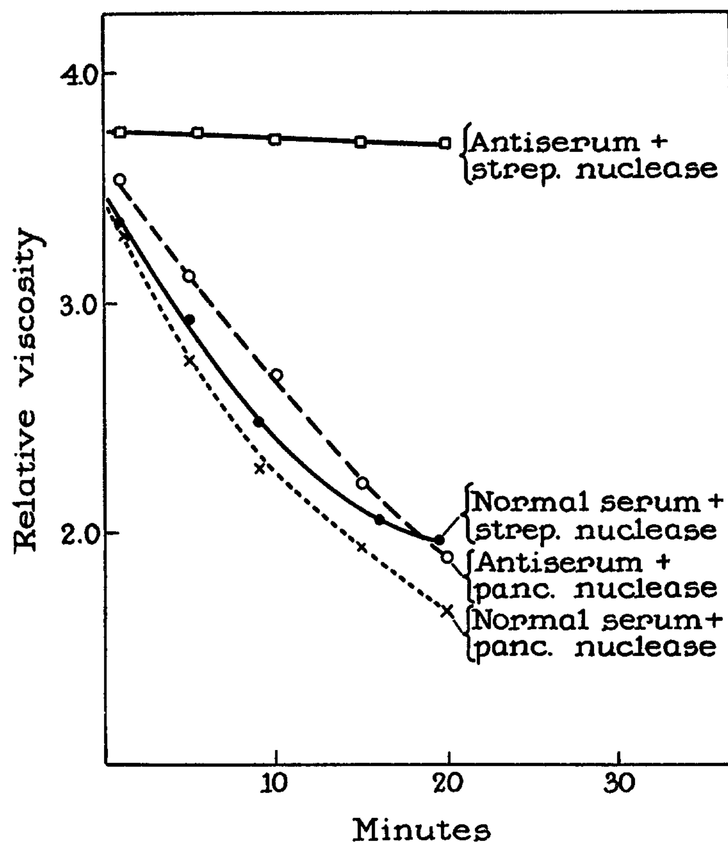

Fig. 1. Effect of rabbit antiserum to streptococcal desoxyribonuclease on the activity of streptococcal and pancreatic desoxyribonucleases.

to show that rabbit antiserum against yeast enzyme caused a marked degree of inhibition of the homologous enzyme but did not affect the activity of the rabbit muscle enzyme.

The specificity of rabbit antibody against streptococcal desoxyribonuclease has been further demonstrated with respect to its action on other extracellular products of hemolytic streptococci. For example, the activity of streptokinase and streptococcal hyaluronidase is not affected by the antiserum.

Inhibition by Human Sera.—In approaching the question of whether the human subject produces antibody to streptococcal desoxyribonuclease following streptococcal infections, initial studies were made employing the viscosimetric test. It was found that although a high degree of variability existed in the apparent antibody response of various individuals following scarlet fever, the development of antibody against this enzyme was pronounced in some cases.

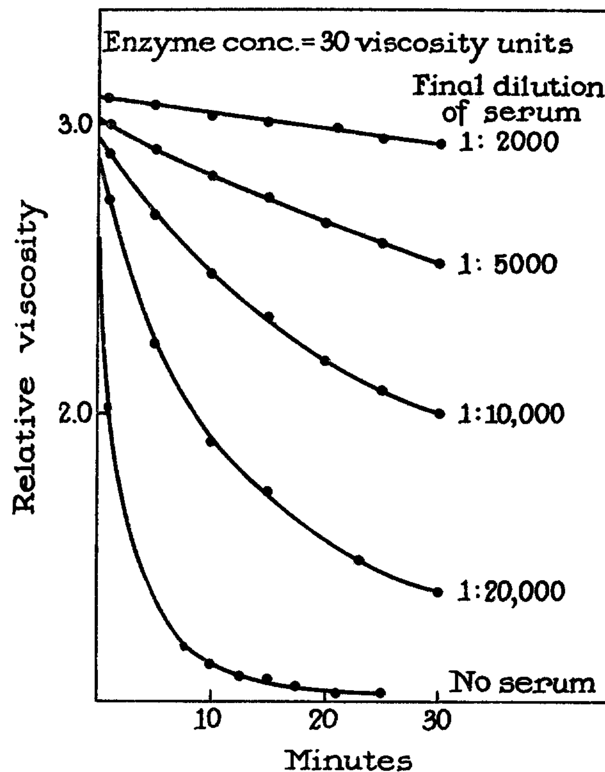

FIG. 2. Effect of human serum (obtained from patient 4 weeks after onset of scarlet fever) on streptococcal desoxyribonuclease. Constant enzyme concentration tested against varying dilutions of serum.

An example of one of the latter cases is illustrated in Fig. 2. Serum obtained from this patient at the onset of scarlet fever was totally devoid of the capacity to inhibit the enzyme, and subsequent bleedings taken at weekly intervals showed a progressive increase in this property. The serum used in the experiment illustrated in Fig. 2 was obtained 4 weeks after onset of the infection and represents the peak of antibody response. The inhibitory effect of the serum is further emphasized by the fact that a relatively high concentration of enzyme

for the viscosimetric test—*i.e.*, 0.25 μg./ml. or 30 units—was employed. Fig. 2 shows that even at a final serum dilution of 1:20,000, the serum causes a highly significant inhibition of the enzyme (90 per cent) and that with increasing concentration of serum up to 1:2000 complete inhibition (99.6 per cent) is approached.

The specificity of human antibody to streptococcal desoxyribonuclease is comparable to that of the rabbit antisera. Sera with high concentration of antibody, such as the one illustrated in Fig. 2, have no inhibitory action on pancreatic desoxyribonuclease or on a sample of crude desoxyribonuclease prepared from Pneumococcus (Type IIR).

The frequency with which patients respond by formation of antibody to desoxyribonuclease and the relative magnitudes of the responses were determined by applying the alcohol precipitation test to the sera of 90 patients from an epidemic of scarlet fever at the Great Lakes Naval Training Center. The antibody response of these same patients to streptokinase and streptolysin O had previously been determined, making possible a comparison of the antidesoxyribonuclease data with those of two more commonly studied streptococcal antibodies (9).

The results of the antinuclease determination show certain interesting differences from those obtained in the case of the other antibodies.

1. The percentage of patients showing a significant response to desoxyribonuclease (38 per cent) was definitely lower than in the case of streptokinase (61 per cent) and streptolysin O (70 per cent). Thirty-four of the 90 patients (33 per cent) did not have measurable amounts of antibody to desoxyribonuclease by this relatively sensitive test at any time during the period in which sera were obtained. It would appear, therefore, that human beings respond by antibody formation to this extracellular antigen less frequently than to certain others, despite the fact that desoxyribonuclease production is a rather constant property of hemolytic streptococci. Strains isolated from patients in this epidemic were among those tested for production of the enzyme *in vitro*, and all these elaborated the enzyme in an amount comparable to that of the various stock laboratory strains.

2. The values obtained for antidesoxyribonuclease titers are scattered over a wider range than in the case of the other two antibodies. Fifty-three of the 90 patients had no demonstrable antinuclease at the time of onset of the scarlet fever and while many of these patients showed no subsequent rise others had increases in antibody up to as high as a serum dilution of 1:10,240. In the study of the antistreptokinase and antistreptolysin O titers it was shown that the mean titers of those patients who developed rheumatic fever subsequent to scarlet fever were significantly higher than those of the patients with uncomplicated scarlet fever. The spread of the antidesoxyribonuclease titers renders a comparable use of mean titers valueless in this small series of cases,

and it can only be said that analysis of the data suggests that a comparable relationship holds in this case; *i.e.*, that the rheumatic subjects *on the average* have a greater antibody response to desoxyribonuclease.

3. The large number of patients showing no demonstrable antinuclease at the time of onset of scarlet fever is at variance with the fact that the great majority of the patients had appreciable antistreptokinase and antistreptolysin O titers at this time, presumably as the result of previous experience with the hemolytic streptococcus. An explanation which suggests itself for this finding is that antidesoxyribonuclease may be less stable and thus disappear more rapidly from the body than the other two antibodies. However, no support for this explanation could be obtained from antibody studies in a few instances in which sera were available for many months following the scarlet fever. The rate of decrease of antidesoxyribonuclease titer in these cases was comparable to that of antistreptokinase.

TABLE I

Antidesoxyribonuclease Titers of Twenty-Three Patients with Scarlet Fever Followed by Rheumatic Fever

Case No.	Antidesoxyribonuclease titer				
	Time after onset of scarlet fever, *wks.*				
	0	1	2	3	4–6
13	0	40	1280	10,240	10,240
16	320				640
23	0		0	0	0
30	10			10	20
51	80			80	80
52	0			20	320
65	0			40	0
88	10			80	80
92	20			40	20
120	0			80	640
234	160			320	160
247	80			160	160
251	0			10,240	10,240
61	20			1280	5120
42	0			0	0
45	0			0	0
181	40			80	80
175	40			40	20
164	0			0	0
173	0			640	640
217	0			0	0
337	40			320	40
44	0			0	0

Certain of the facts summarized above are illustrated in Table I, in which

the titers of antidesoxyribonuclease (as reciprocals of the serum dilution) are given for one group of the 90 patients. This group (designated group V in

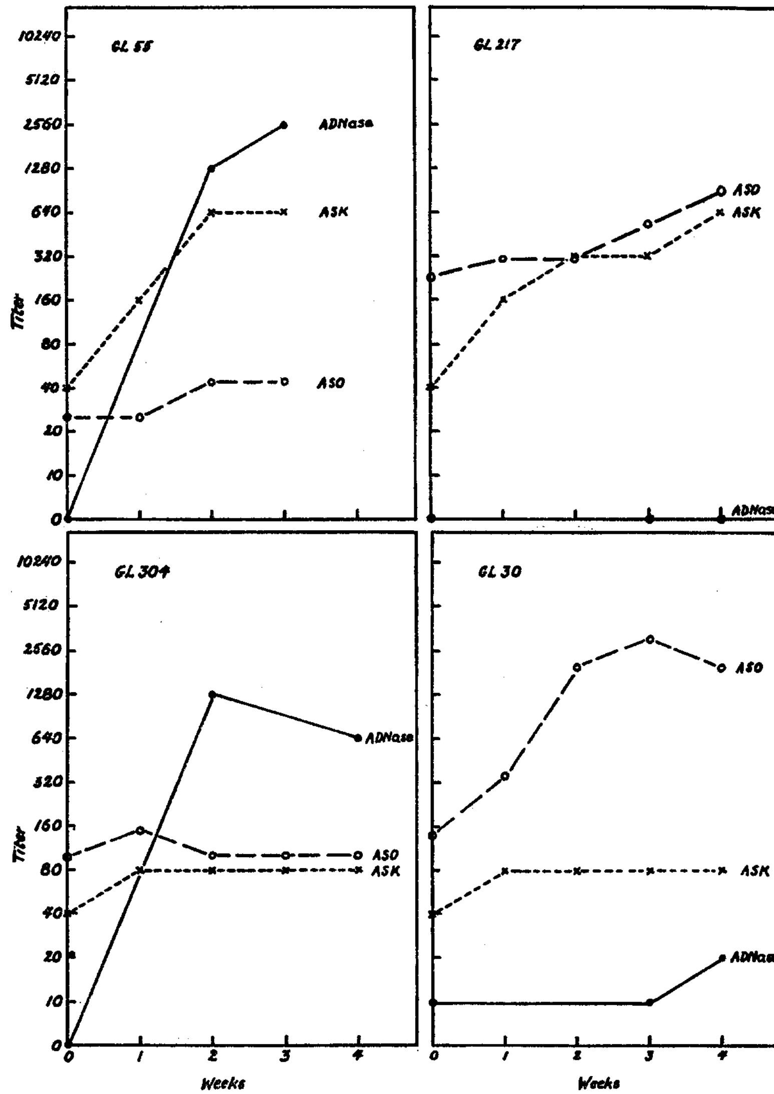

FIG. 3. Comparison of antidesoxyribonuclease response with antistreptokinase and antistreptolysin 0 titers in four patients following scarlet fever. ASK= antistreptokinase, ASO-antistreptolysin 0, and ADNase= antidesoxyribonuclease.

reference 9) represents those patients who developed rheumatic fever as a sequela of the scarlet fever. The case numbers are given so that direct comparison can be made with the data recorded in reference 9.

Observations previously made concerning the suppressive effect of penicillin therapy on antibody response to extracellular antigens of the streptococcus are confirmed by the antidesoxyribonuclease data. In a group of 17 patients who received "successful" penicillin therapy as indicated by the prompt and permanent disappearance of hemlytic streptococci from the nasopharynx, none had significant rises in antistreptokinase and there were no rises as great as fourfold in the antistreptolysin O titer. Similarly only one of the 17 patients showed a significant rise in antidesoxyribonuclease titer.

Independent Variation of Antibody Response to Different Antigens.—Inspection of the antibody data from the 90 scarlet fever patients indicates that practically all possible combinations of antibody response to the three extracellular antigens occurred. This fact is illustrated by the results in four of these cases which are charted in Fig. 3. It will be noted that in case 55 excellent responses occurred to streptokinase and desoxyribonuclease, while there was no significant increase in antistreptolysin O. In case 217 there was no response to nuclease but a good response to the other two antigens, and conversely in case 304 there was a response only to nuclease. An example of a patient showing a significant response only to streptolysin O is provided by case 30. In so far as *in vitro* tests can be relied on, it has been shown in the case of at least two of these antigens—streptokinase and desoxyribonuclease—that the variations are not attributable to quantitative differences in the amount of antigen produced by the strains. No study was made of the relative *in vitro* production of streptolysin O. The most reasonable assumption is that the variations represent individual differences in the ability of the host to respond by the formation of antibody to a given streptococcal protein. From the theoretical point of view it is of interest that the quantitative differences in antibody response do not merely reflect a general characteristic of the host that applies to all antigens of the streptococcal cell, but they are specifically determined also by the nature of the individual antigen.

SUMMARY

Rabbit antisera against partially purified streptococcal desoxyribonuclease inhibit the action of the enzyme on its substrate. The activity of pancreatic desoxyribonuclease is not affected by these antisera. Similarly antibody against pancreatic nuclease does not inhibit the streptococcal enzyme.

Certain patients develop inhibitory antibody to streptococcal desoxyribonuclease following streptococcal infections, occasionally in very high titer, although the proportion of patients showing an antibody response appears to be lower than in the case of streptokinase and streptolysin O.

The pattern of antibody response to desoxyribonuclease has been compared to that of streptokinase and streptolysin O in a group of ninety patients from an epidemic of scarlet fever.

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