

## ANTI-DNA ANTIBODIES IN HYPERIMMUNIZED RABBITS\*

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Precipitating and/or complement (C')-fixing antibodies to deoxyribonucleic acid (DNA) have been observed in the following circumstances: (a) naturally occurring in the sera of some humans with systemic lupus erythematosus (SLE) (1-3), (b) immunization of rabbits with T-even phages of *Escherichia coli* (4), (c) immunization of rabbits with purine and pyrimidine haptenic groups (5-7), (d) immunization of rabbits with heat-denatured DNA complexed to methylated bovine serum albumin (8), and (e) in rabbits hyperimmunized with Gram-negative bacteria (9). This report concerns the latter method of inducing anti-DNA antibodies.

As previously reported from this laboratory, prolonged immunization of rabbits with formalin-killed Gram-negative bacteria resulted in the development of anti-gamma globulin antibodies that closely resembled human rheumatoid factors (10, 11). The sera of these rabbits were screened for anti-DNA antibodies by the method of Wasserman and Levine (12) and 4 out of 28 demonstrated anti-DNA reactivity at serum dilutions greater than 1:100. Studies of the development and characterization of this factor will be described.

### *Materials and Methods*

All serological procedures involving complement fixation were conducted in chemically clean glassware and utilized sera that had been heated at 56°C for 30 minutes.

Complement fixation method of Wasserman and Levine (12). In contrast to the usual complement fixation procedures, equal quantities of antiserum (dilutions between 1:100 and 1:200) were incubated with varying amounts of antigen in the presence of complement (C')

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for 18 hours at 0°C in an ice bath. The per cent of complement fixation was then determined by optical densities of the supernatants after immune hemolysis.

Pooled guinea pig serum in dilutions between 1:100 and 1:200 was used as the source of complement.

The antigens utilized for C' fixation were: (a) Calf thymus DNA, salmon sperm DNA, and yeast RNA from the Mann Research Laboratories, New York. (b) Pneumococcal DNA prepared according to the method of Hotchkiss (13) (kindly supplied by Dr. S. M. Beiser). (c) Rabbit bone marrow, rabbit thymus, and *E. coli* DNA prepared according to the method of Marmur (14). (d) Rabbit reticulocyte RNA, kindly supplied by Dr. Edward Burka (prepared by the method of Kirby, reference 15).

DNA preparations were denatured by heating at 100°C for 10 minutes followed by rapid dilution with ice cold isosavers solution.

DNA concentration was estimated by absorption at 260  $\mu$  in a Beckman model DU spectrophotometer.

Studies of inhibition of DNA complement fixation by nucleotides and nucleosides were performed by incubating the reactants of the systems with graded amounts of the nucleotides and nucleosides for 1 hour at 0°C before adding a quantity of denatured DNA which by itself produced approximately 50 per cent fixation of complement. Reagents (obtained from Mann Research Laboratories) included deoxycytidine hydrochloride, adenylic acid (mixed isomers 2' and 3'), deoxycytidylic acid, cytidylic acid (mixed isomers 2' and 3'), and adenosine.

Crystalline pancreatic DNase (Worthington Biochemical Corp., Freehold, New Jersey) was utilized for treatment of DNA preparations. For digestion of 2 mg of DNA, 0.1 mg of DNase was added in a neutral buffer containing 0.003 M MgSO<sub>4</sub> and incubation at 37°C continued for 1 hour.

Procedures for (a) the preparation of bacteria, (b) injection of rabbits, (c) agglutination tests, (d) absorption experiments, (e) density gradient centrifugation analyses, and (f) nitrogen estimations are contained in a previous report (10).

## RESULTS

Sera of 28 hyperimmunized rabbits were examined for anti-DNA factors and 4 were positive at a dilution of 1:100 or greater. This C' fixation reaction is illustrated in Fig. 1. Serum 1-34, diluted 1:180, effectively fixed complement when incubated with less than 1  $\mu$ g denatured DNA. Figs. 1 and 2 illustrate the reactivity of serum 1-34 with preparations of DNA obtained from salmon sperm, calf thymus, rabbit thymus, rabbit bone marrow, and pneumococci. Dilutions of serum which reacted strongly with denatured DNA gave little or no detectable reaction with comparable amounts of native DNA as demonstrated in Fig. 3. Prior treatment of the DNA with DNase resulted in loss of its reactivity (Fig. 4). Yeast RNA did not react with anti-DNA antisera. Variable results were obtained using rabbit reticulocyte RNA. Reactions were usually negative but occasional incomplete fixation of C' was encountered with amounts of RNA that were 20-fold greater than amounts of DNA that gave 100 per cent C' fixation.

Fig. 5 summarizes studies which compared the reactivity of preparations of DNA from calf thymus, salmon sperm, and *E. coli* with the serum (E 15) of a rabbit hyperimmunized with *E. coli*. The *E. coli* DNA was less effective in

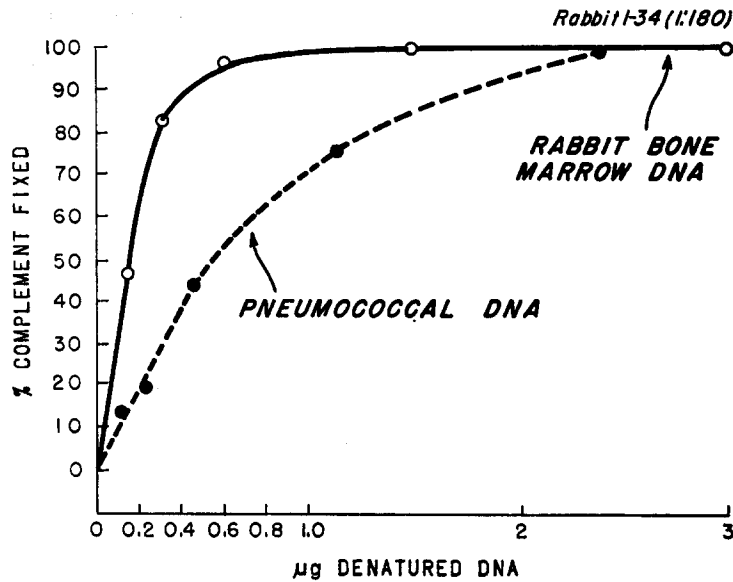


FIG. 1. DNA C' fixation with hyperimmune rabbit serum and denatured DNA of pneumococcal and rabbit origins.

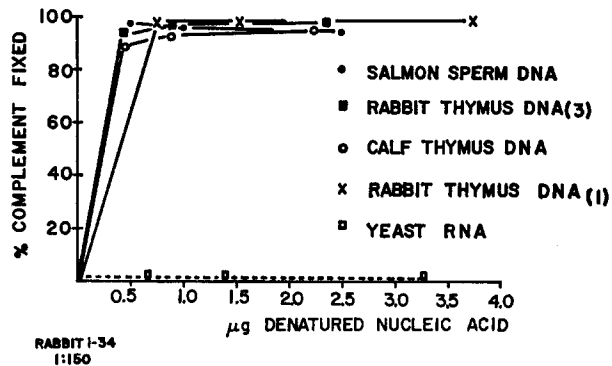


FIG. 2. DNA C' fixation. Comparison of reactivities of various DNA preparations and yeast RNA.

fixing C', 10 µg resulting in only 50 per cent fixation while 1 to 2 µg of salmon sperm and calf thymus DNA caused 100 per cent fixation of complement. Fig. 6 illustrates the effect of absorbing an anti-DNA antiserum with whole *E. coli*. Although there was a marked reduction of the C' fixation reaction (using rabbit DNA), coincident to absorption of antibacterial antibodies, there was residual activity directed against rabbit DNA.

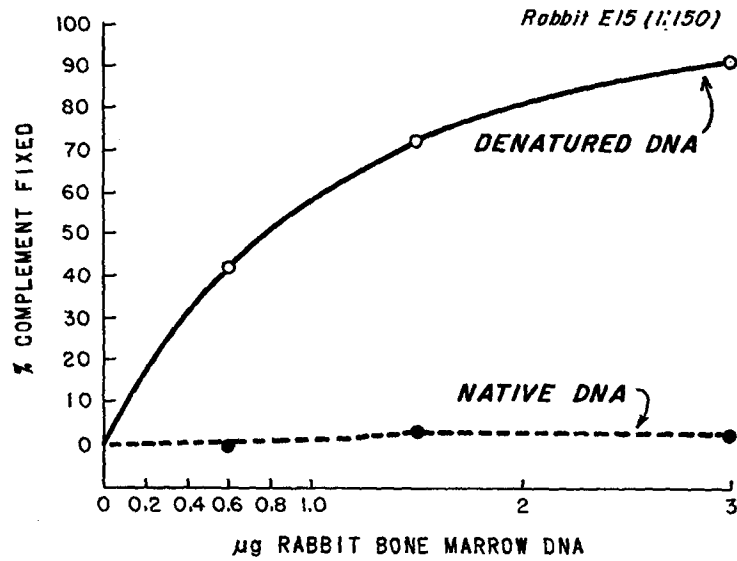


FIG. 3. DNA C' fixation, using denatured and native rabbit bone marrow DNA.

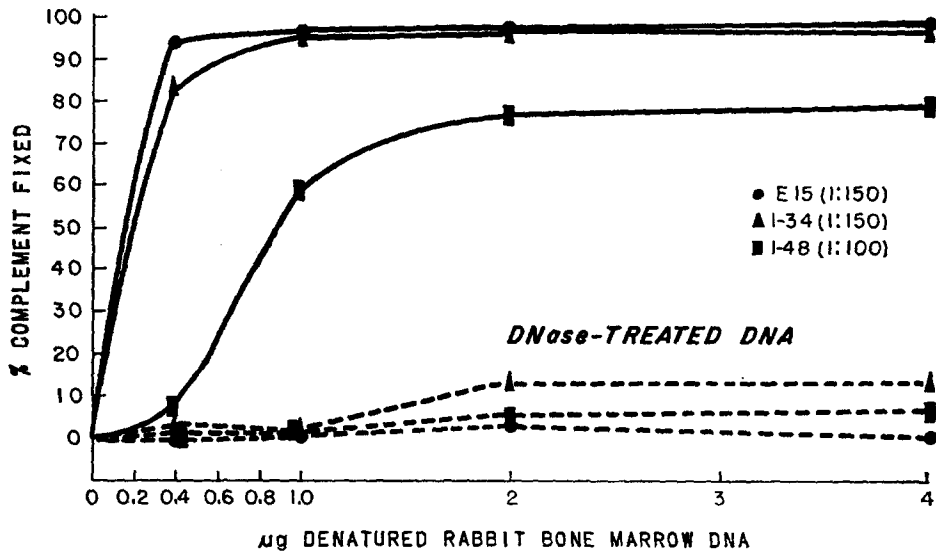


FIG. 4. DNA C' fixation. Effect of DNase treatment of rabbit bone marrow DNA.

Fig. 7 illustrates the inhibition of DNA C' fixation by adenylic acid. In other experiments, when approximately 50 per cent C' fixation was obtained in the control (no inhibitor), adenylic acid gave virtually complete inhibition at 0.1  $\mu$ molar concentration and adenosine and other nucleotides and nucleosides gave significant but incomplete inhibition at 1.0  $\mu$ molar concentrations.

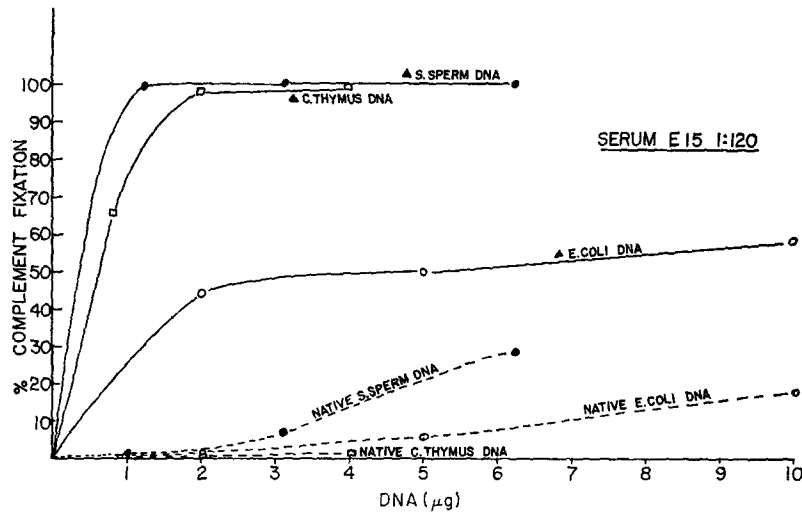


FIG. 5. DNA C' fixation by the serum of a rabbit hyperimmunized with *E. coli*. Comparison of the reactivity of native and heat-denatured ( $\blacktriangle$ ) DNA of *E. coli*, bovine (c.), and salmon (s.) sperm origins.

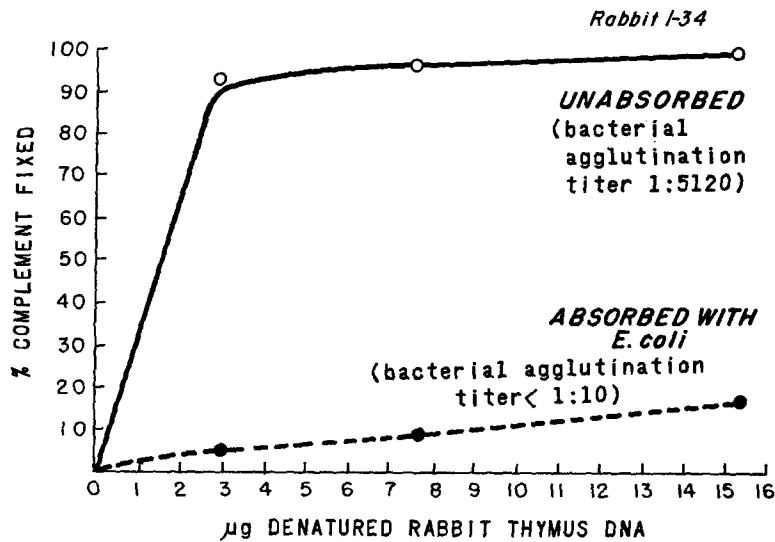


FIG. 6. DNA C' fixation. Effect of absorption of antiserum with intact *E. coli*.

Figs. 8 and 9 illustrate the development in 2 animals of the anti-DNA and anti-gamma globulin factors. Although both factors were detected relatively late in the course of the immunization, there did not seem to be a significant interrelationship of the two factors. In rabbit 1-34 (Fig. 8), the anti-DNA

antibody preceded the development of rheumatoid factor and fixed more C' at 20 weeks than at 16 weeks whereas the titer of rheumatoid factor decreased from 1:640 to 1:80 during the same interval. The serum of E 15 (Fig. 9) contained significant quantities of anti-gamma globulin factor (titer 1:160) at 18 weeks but did not effectively fix C' with DNA until 42 weeks of immunization. Again, during the latter stages of immunization, there was a tendency

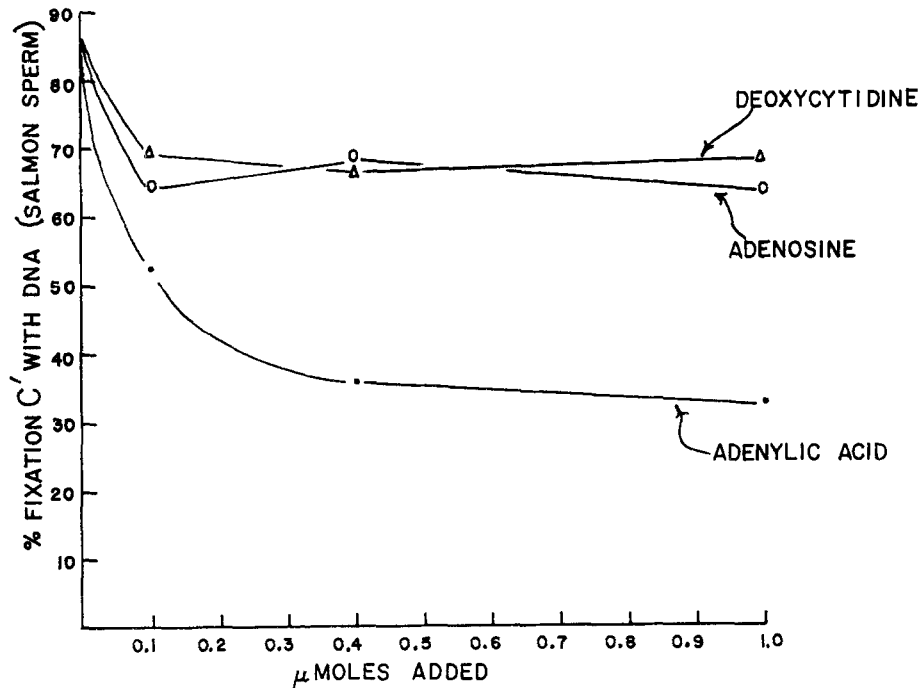


FIG. 7. Inhibition of DNA C' fixation by adenylic acid (serum E 15).

for anti-gamma globulin reactivity to diminish while anti-DNA reactivity increased.

Density gradient centrifugation analyses of sera of rabbit E 15 (Fig. 10) and rabbit 1-34 (Fig. 11) were similar. The rheumatoid factor-like activity sedimented to the bottom of the tube while the major part of the antibacterial agglutinin activity and the anti-DNA reactivity was located in the middle of the tube. Some of the anti-DNA reactivity, however, which penetrated to the bottom of the gradient (more readily noted in Fig. 11), probably represented high molecular weight anti-DNA antibodies.

#### DISCUSSION

Data have been presented demonstrating the presence of anti-DNA reactivity in the sera of 4 rabbits hyperimmunized with killed Gram-negative

bacteria. The specificity of the serological reactivity for denatured DNA is a feature observed in the other experimental models (4-8) and with sera of some patients with SLE (2). The requirement of denatured (single strand) DNA and the demonstration of inhibition by adenylic acid (Fig. 7) suggests that specificity is directed primarily against base constituents of the DNA. The comparable reactivity obtained with DNA derived from various sources suggests

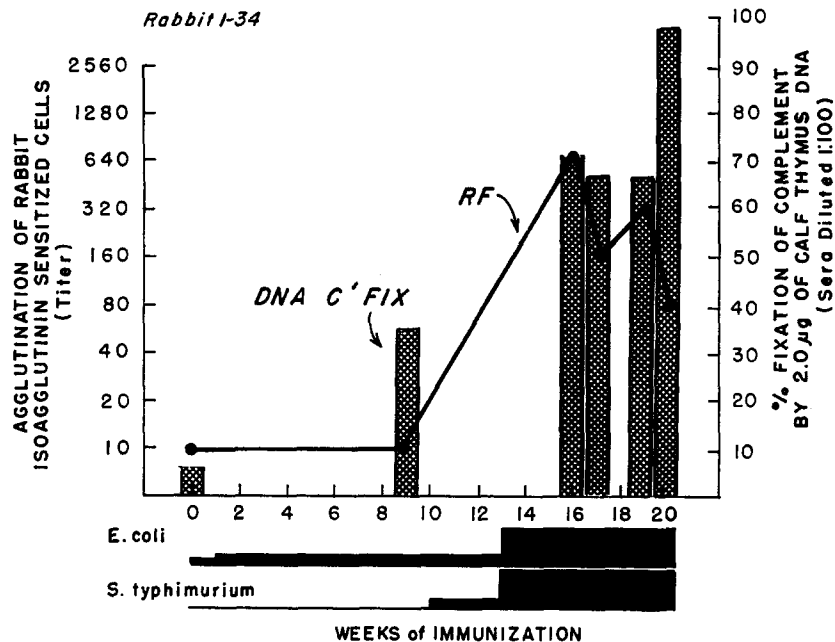


FIG. 8. Development of anti-DNA antibodies and anti-gamma globulin antibodies (*RF*) in a rabbit (No. 1-34) immunized with *E. coli* and *Salmonella typhimurium*. The lower horizontal bars indicate relative increases in the doses of bacterial antigens.

that the stimulus for its induction is not an antigenically unique DNA. (In studies of antisera to T-even coliphages, the antibody did not react with DNA from sources other than T-even phages and was found to be specific for a glucosylated pyrimidine grouping on phage DNA, reference 16.) The demonstrations that DNase treatment of DNA destroyed its reactivity and that inhibition of C' fixation was accomplished with nucleic acid constituents offer confirmation that specificity was directed against DNA rather than a trace contaminating antigen.

In the present studies, the stimulus for production of anti-DNA factors is not known. Two general possibilities exist: (a) antibodies specific for bacterial DNA develop and cross-react with other DNA's,<sup>1</sup> (b) host DNA is altered in

<sup>1</sup> Efforts to obtain anti-DNA antibodies by injection of animals with purified DNA have been uniformly unsuccessful.

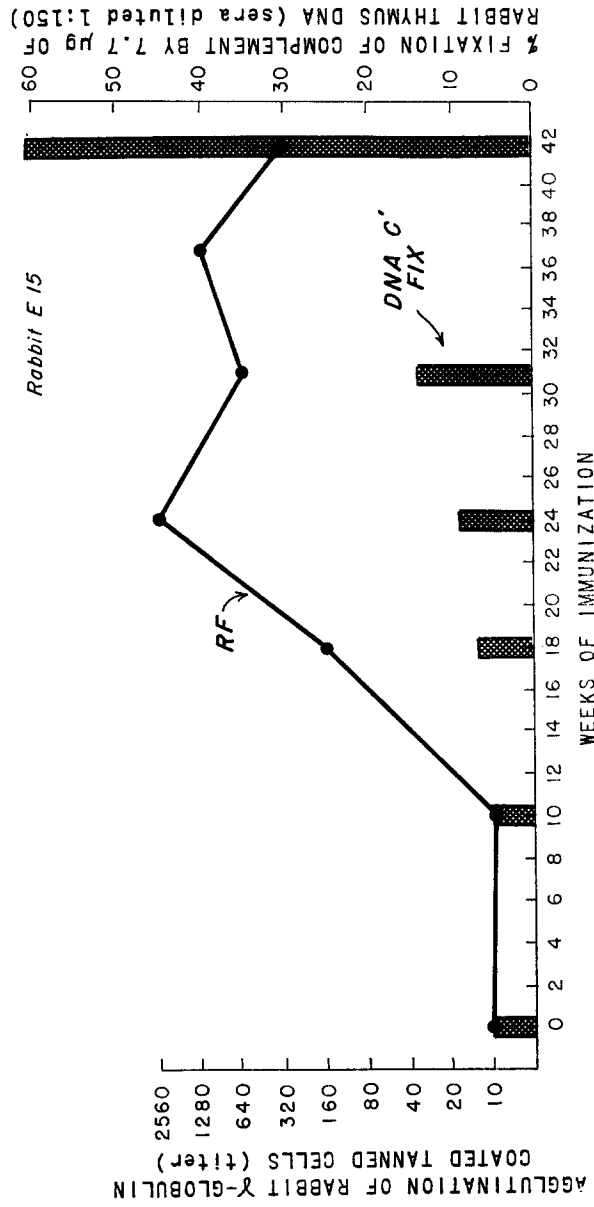


Fig. 9. Development of anti-DNA antibodies and anti-gamma globulin antibodies (RF) in a rabbit (No. E. 15) immunized with *E. coli*.



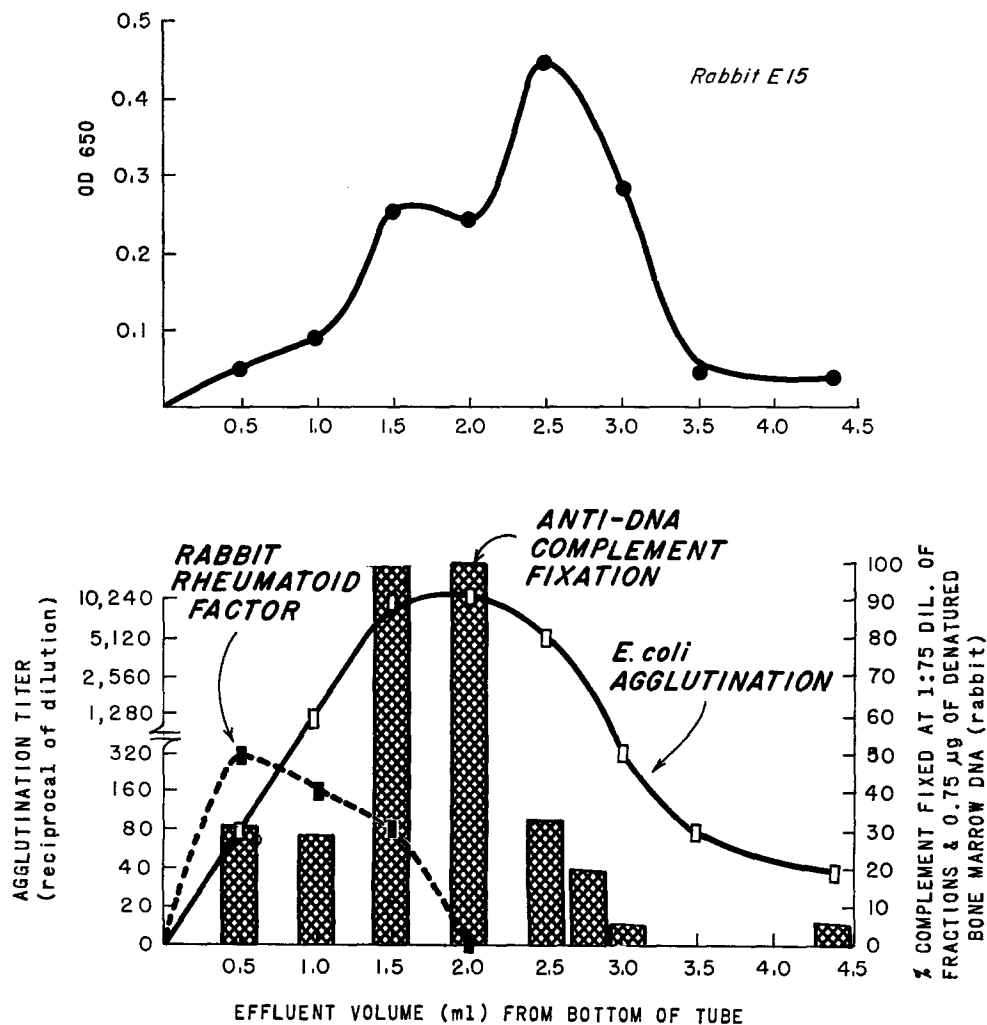


FIG. 10. Separation of anti-DNA antibodies, antibacterial agglutinins, and anti-gamma globulin antibodies (rabbit rheumatoid factor) by zone centrifugation. (Protein concentrations are indicated in the upper part of the figure.) Rabbit E 15.

some way, as a consequence of hyperimmunization, so that it becomes an immunogenic stimulus to the host. Although the data herein reported are not conclusive, they tend to support the latter possibility. Mammalian and salmon sperm DNA's were superior to *E. coli* DNA in fixation of  $C'$  (Fig. 5), and, although exhaustive absorption of sera with whole *E. coli* removed the bulk of anti-DNA activity, there was residual activity against rabbit DNA (Fig. 6).

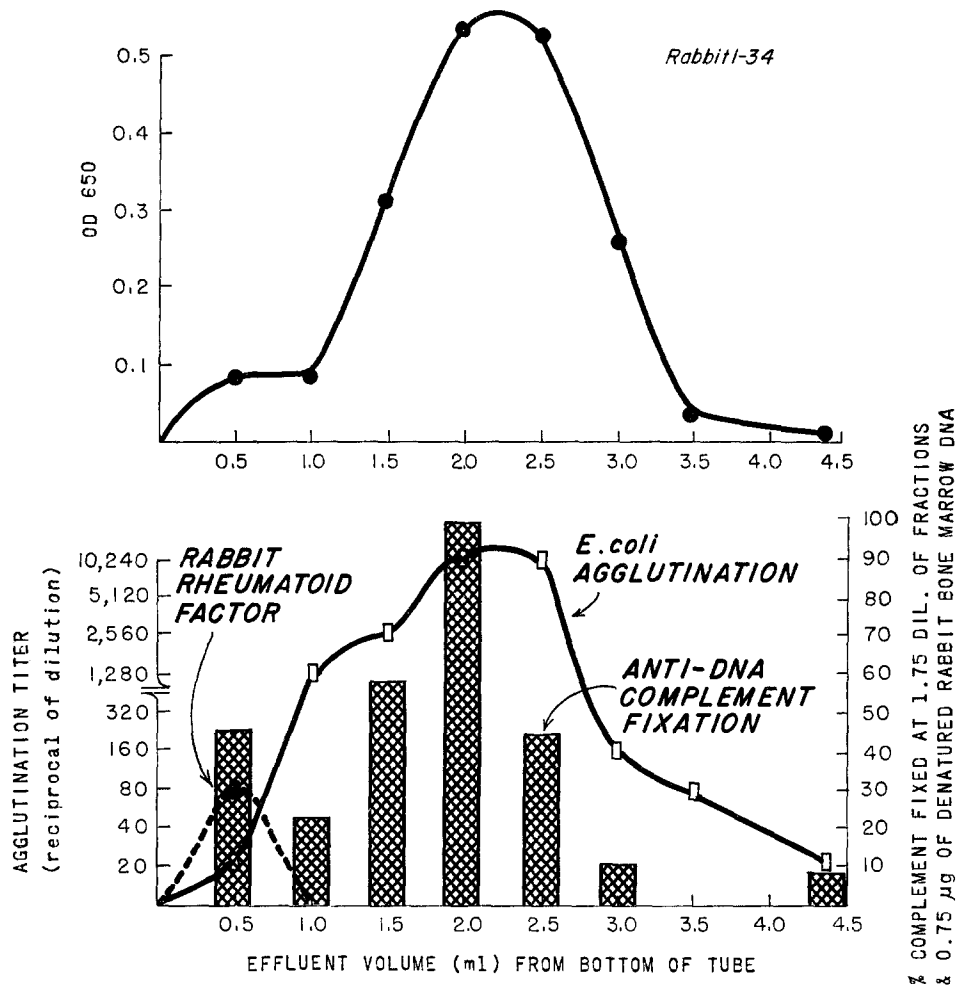


FIG. 11. Separation of anti-DNA antibodies, antibacterial agglutinins, and anti-gamma globulin antibodies (rabbit rheumatoid factor) by zone centrifugation. (Protein concentrations are indicated in the upper part of the figure.) Rabbit 1-34.

All 4 rabbits demonstrating anti-DNA activity were immunized with a Gram-negative organism (*E. coli*, *Salmonella typhimurium*, or both). The same immunization procedure was used in a group of 7 rabbits with a Gram-positive organism, *Bacillus subtilis*. Anti-DNA activity could not be detected after 6 months' immunization when anti-gamma globulin titers were high. Although this difference may have been a chance occurrence, it is possible that products of Gram-negative bacteria are required for the development of anti-DNA

factors. Host differences may also be important. A minority of rabbits developed anti-DNA antibodies. Of possible significance was the observation that one animal which developed anti-gamma globulin factor but not anti-DNA antibodies exhibited proteinuria and early amyloid disease at autopsy; while another rabbit given an identical course of immunization developed both anti-DNA and anti-gamma globulin antibodies and in the absence of gross proteinuria, demonstrated proliferative glomerulitis.

In addition to complement-fixing anti-DNA antibodies, the sera of patients with SLE show other types of antinuclear reactivity. Serological activity has been demonstrated against intact nuclei (17, 18), nucleohistones (19), phosphate buffer extract (19), and against DNA by precipitin (21, 21), agglutination (22), and passive cutaneous anaphylactic (PCA) (23) techniques. Although they were not studied extensively, the rabbit sera which gave positive  $C'$  fixation tests with DNA did not react in a  $PCA^2$  system nor did they react with nuclei of calf thymus or rat testis cells by immunofluorescence. The evidence that anti-DNA factors herein described are antibodies rests heavily on the technique of  $C'$  fixation. The failure to demonstrate the reaction by other immunological techniques, *i.e.* immunofluorescence, precipitation, and PCA, may reflect quantitative features of the techniques or it may result from a qualitatively unique antibody. Several hyperimmune sera were examined with a calf thymus DNA-bentonite flocculation test by Dr. Benjamin Sturgill at the National Institutes of Health (24). A close correlation between  $C'$  fixation and flocculation titers was evident and, in addition, significant DNA-bentonite flocculation titers were obtained with some hyperimmune sera which failed to fix  $C'$  at dilutions of 1:100 (25).

We have already alluded to the lack of a consistent interrelationship of the anti-gamma globulin and anti-DNA factors (Figs. 8 and 9). Although all animals develop anti-gamma globulin factor after a period of several weeks of hyperimmunization, the anti-DNA antibodies were detected in a minority of animals (the  $C'$  fixation method used precludes testing sera in dilution below 1:100 because of anticomplementary properties of the sera). Density gradient analyses disclosed a distinct difference in their molecular size although a small portion of the anti-DNA factors appeared to be macromolecular.

The biological significance of the present studies remains in doubt. Although hyperimmunization of rabbits results in varied pathological changes, *i.e.* amyloidosis and glomerulitis, there is no direct evidence that either anti-DNA or anti-gamma globulin factors mediate these changes (26). It is of interest, however, that something as simple and reproducible as hyperimmunization with bacterial antigens can induce serological factors that resemble those associated with the human connective tissue syndromes.

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<sup>2</sup> The authors are indebted to Dr. Zoltan Ovary for assistance in performing PCA studies.

## SUMMARY

Complement-fixing anti-DNA antibodies were detected in a minority of sera of rabbits hyperimmunized with killed Gram-negative bacteria. The C'-fixing property of DNA was lost after DNase treatment. Preferential reactivity with denatured DNA was observed. The antisera reacted with DNA preparations derived from rabbit bone marrow and thymus, calf thymus, pneumococci, salmon sperm, and *Escherichia coli*. *E. coli* DNA was less effective than preparations of mammalian and salmon sperm DNA in fixation of C'. Inhibition of DNA C' fixation by nucleotides and nucleosides was observed. The bulk of anti-DNA activity was associated with the low molecular weight antibody fraction.

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