Precipitin Analysis of C-Reactive Protein by Gel Diffusion.* (22072)

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C-reactive protein (C-RP), an abnormal protein present in the "acute phase" serum, has been under investigation for the past several years. This substance, which forms a precipitate with the non-type specific C-polysaccharide of the pneumococcus in the presence of Ca^{++}(1), was found by electrophoresis, to migrate between the α_2 and β globulin fractions(2). The C-reactive protein was separated from the human serum(3) and was prepared in crystalline form from human serous fluids(2). A number of investigations have shown the presence of C-RP to be a very sensitive indicator of activity in rheumatic fever(4-6). Evidence has been presented that C-RP may be present in cancer(7) and in acute myocardial infarction(8). In rheumatoid arthritis and active tuberculosis, the C-RP is significantly correlated with seromucoid(9). Small amounts of this abnormal protein may be detected in human serum by a precipitin test utilizing specific antiserum from rabbits hyperimmunized with purified C-RP.

The present study was carried out during the course of studies on the biological properties of C-reactive proteins. With the use of agar gel diffusion technics it was possible to obtain quantitative and qualitative values of C-RP in the serum of seventy-one patients with a variety of illnesses.

Procedure. C-RP antiserum—Three New Zealand Red rabbits (2 kg) were injected intravenously, twice a week for 3 weeks, with 0.5 cc of a solution containing 1 mg/cc of crystalline C-RP. 1.5 cc of Freund's adjuvant(10) was incorporated with 1 mg of crystalline C-RP and injected subcutaneously into 2 separate abdominal sites at the time of the first intravenous injection. On the fourth week the rabbits were bled and the sera were preserved with merthiolate in a final concentration of 1:10,000. The *crystalline C-RP* was prepared from 2 liters of fluid obtained by thoracocentesis from a patient with Hodgkin's disease. The isolation and crystallization of C-RP was performed following the method described by Wood *et al.*(11). The total yield of crystallized material was 28 mg. *Human sera* were obtained from the serology laboratory at the Mount Sinai Hospital, Chicago, from 71 patients with a wide variety of illnesses. The age of the patients ranged from 12 to 74 years. *The quantitative precipitin technic* for the determination of antibodies in rabbit C-RP antisera was that described by Kabat and Mayer(12). The amount of antigen was added in slight excess to produce a total antibody precipitation and the total nitrogen precipitated was determined by the microkjeldahl technic. Each analysis was carried out in duplicate. Tests of the supernatant fluid showed that the dilution of crystalline C-RP reacted with antibodies in the region of antigen excess. The amount of antibody nitrogen precipitated at the equivalence zone from each rabbit antiserum was, respectively 0.652 mg, 0.615 mg, and 0.485 mg per cc of antiserum. *Agar precipitin (Oudin) technic*—The modification of Munoz and Becker(13) was employed. The serum agar contained 1 part of rabbit antiserum diluted 1:5, 1 part of 0.6% agar which had been previously adjusted to pH 7.5 and clarified in 0.15 M NaCl. After cooling, 0.4 ml of the antigenic solution was overlayed on the agar. Several dilutions of the C-RP solution in 0.15 M NaCl were used to determine the rate of penetration of the precipitating bands. The serial dilutions of this antigenic solution contained respectively 0.080, 0.040, 0.020, 0.010, 0.005 mg of crystalline C-RP per ml. *Agar precipitin (Ouchterlony) tech-*

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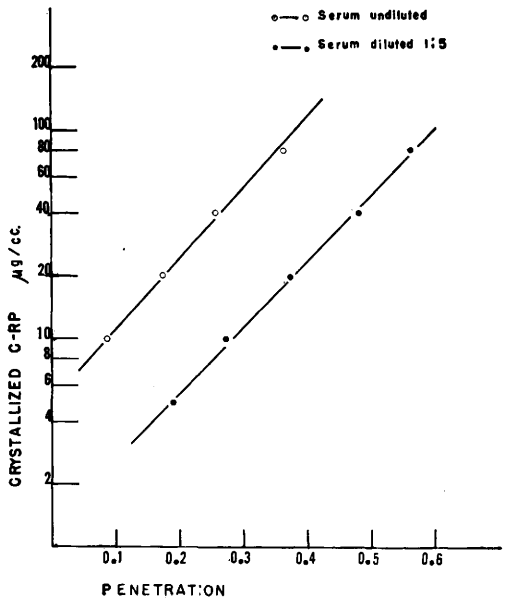

FIG. 1. Penetration values of serial dilutions of crystalline C-reactive protein into agar-serum. Serum contained 0.625 mg of antibody N/ml. Penetration values computed from formula: Penetration (P) = Distance (D)/$\sqrt{\text{Time (T)}}$.

nic—The Ouchterlony technic was used as modified by Halbert *et al.*(15). 0.15 ml of rabbit antiserum was placed in the central well, and 0.1 ml of patients sera in the peripheral wells. The plates were then placed in the refrigerator at 6°C and the precipitin bands read at intervals starting from 7 days. The final readings were done after 24 days.

Results. Crystalline C-RP obtained from the thoracocentesis fluid of a patient with Hodgkin's disease, showed precipitin bands migrating in antiserum-agar medium. The migration of the bands followed a straight line relationship with the square root of the time. The rate of penetration appeared to be directly proportional to the log of C-RP concentration and inversely proportional to the antibody content of the rabbit antiserum. Fig. 1 was obtained by plotting the penetration against the concentrations of crystalline C-RP (respectively, 0.08, 0.04, 0.02, 0.01, 0.005 mg/ml). The rabbit antiserum contained 0.652 mg of antibody nitrogen per cc and was used diluted 1:5 and undiluted.

With antisera containing a known concentration of antibodies, the penetration rates of the C-RP present in the blood of 16 patients with rheumatic fever were determined. The rates of penetration ranged between 0.652 and 0.100. The concentration of the protein was calculated by the use of the standard curve of penetration of the crystalline C-RP (Fig. 1), and was expressed in terms of mg of protein per cc of serum. The values ranged between 0.140 and 0.003 mg/ml.

We further studied the immunological reactions between C-RP and C-RP antiserum by the technic devised by Ouchterlony(16). When crystalline C-RP was allowed to diffuse against rabbit C-RP antiserum, 3 distinct bands appeared, one sharp and 2 faint. Increasing the concentration of the crystalline C-RP increased the distance between the precipitation bands and the antigen wells; but no variations in the shape of the lines of precipitation were noted. The sera of 71 pa-

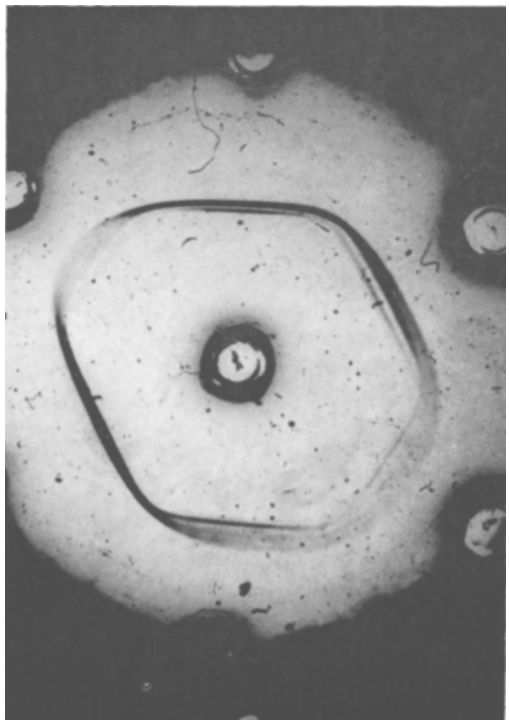

FIG. 2. Agar-serum precipitation bands with sera of patients with acute rheumatic fever tested against rabbit anti-crystalline C-RP serum. Undiluted rabbit antiserum in the central well. Human sera in the peripheral wells. Photographed after 24 days at 4°C.

tients with different diseases were studied with the Ouchterlony technic. From 0 to 3 bands of precipitation were formed in the agar medium. Sometimes it was difficult to define the exact number of the formed bands. However, more than 3 precipitation bands were never observed. When 2 or 3 lines were noted, one was normally quite intense and common to all the sera; others were moderately to very delicate. The sharpness of the bands also showed considerable differences. Some were clearly defined, others faint and diffuse. The heaviest common line was apparent first; the others took several more days to appear. The final configuration of the bands was present after 3 weeks (Fig. 2). No relationship was observed between the concentration of the C-RP in the blood (as determined by the penetration rate of the main band in the Oudin tubes) and the number and shape of the precipitation lines present in the agar plates.

Several controls confirmed that no cross reactions occurred between human normal sera and the rabbit C-RP antisera used. No bands appeared when sera containing high levels of C-RP were allowed to diffuse against pneumococcus C-polysaccharide.

Discussion. The total amount of C-RP that is present in the "acute phase serum" is very important in evaluating an inflammatory process. It is most helpful in those cases in which the concentration of the C-RP in the serum is low, and when other laboratory tests and clinical evidence of activity are equivocal (4,14). The use of the agar diffusion technic offers the possibility of evaluating with a high degree of accuracy very small amounts of the C-RP present in the serum.

Each precipitin band represents an antigen-antibody system (16-17), therefore, at least 3 antigens are present in the C-RP in its crystalline form. These 3 antigens are present in "acute phase sera" in different concentrations. The quantity of one antigen is not apparently related to the amount of the others. The possibility has been suggested by Jennings (18) that multiple bands represent several determinant groups of a single antigen. From the data reported here, both of these possibilities are likely.

The results obtained indicate that an inflammatory stimulus causes the appearance in the blood stream of varying amounts of C-RP which may give rise to differences in its antigenic components. Determination of the values of these components may be of importance in studying the progress of an inflammatory process.

Summary. 1. The rate of penetration of crystallized C-RP into agar-antiserum medium has been determined. 2. With this procedure, the C-reactive protein present in human serum of 16 patients with rheumatic fever was evaluated. Variations of less than 0.001 mg of C-RP per ml of serum were detectable. 3. By observing the pattern of the precipitin bands at least 3 antigenic components were found to be present in crystalline C-reactive protein. 4. In human serum, the C-RP present appeared to contain up to 3 antigenic components in different concentrations.

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Triazenes as Inhibitors of Mouse Sarcoma 180.* (22073)

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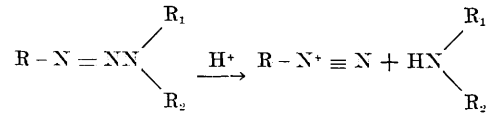
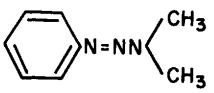
3,3-Dimethyl-1-phenyltriazene (Fig. 1) has recently been found to inhibit *in vivo* growth of the mouse sarcoma 180 (S-180). Tumor inhibitory activity has not previously been described for triazenes; thus a new biological activity is disclosed for derivatives containing the diazo moiety to which this activity might be ascribed. The triazenes are characterized by a high degree of lability in the presence of H-ions, resulting in the formation of a diazonium ion and an amine, thus:



The vigorous coupling activity of the diazonium ion is well known and this particular property of the triazenes ("diazoamines") has been utilized to advantage in the synthesis of azo dyes(1), for polymerization in the rubber industry(2), and for alkylation of dissimilar hydrocarbons in the synthesis of high-octane gasoline(3). It has heretofore been demonstrated that S-180 is sensitive to alkylating agents such as the mustard gases(4), triethylene melamine(5), and the polyfunctional phosphoramides(6). It therefore seemed reasonable to assume that a compound such as 3,3-dimethyl-1-phenyltriazene (hereafter to be referred to as "the triazene") could achieve systemic distribution intact and, at the cellular level, be subject to H-ion catalysis with liberation of benzenediazonium ion and dimethyl amine. The diazonium ion might then inhibit S-180 by reacting with elements essential for cell proliferation—a phenomenon postulated for other alkylating agents(7). This hypothesis will be examined below. It remains to be seen whether the triazenes are sufficiently selective for tumor cells over normal, rapidly proliferating tissue to be useful in higher mammals. In the mouse the therapeutic index is fairly low.



3,3-DIMETHYL-1-PHENYLTRIAZENE

FIG. 1.

Special emphasis will be given the triazene described above since it has proved so far to be the most interesting member of the series (Table I). A study of other diazoamines is continuing in an effort to establish a correlation between structure and tumor-inhibitory activity.

Materials and methods. Dimethyl-phenyltriazene† was synthesized by one of us (C. S. R., Jr.)(8). The compound is a pale, amber fluid with a specific gravity of 1.02.

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