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Bruce Lamont Innis
*Yale University*

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Bruce Raymond Innis

Signature of Author

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Date

# THE INTERACTION OF C-REACTIVE PROTEIN WITH ENVELOPED VIRUSES

Bruce Lamont Innis

B.S. United States Military Academy, 1973

A thesis submitted to the Department of Epidemiology and Public Health in partial fulfillment of the requirements for the degree Doctor of Medicine

Yale University School of Medicine

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1977

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# THE INTERACTION OF C-REACTIVE PROTEIN WITH ENVELOPED VIRUSES

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## I. Introduction

It has been observed that titers of selected serum proteins rise and are elevated during the acute phase of many infectious diseases including those of viral origin (1). Why this occurs however, is not known in spite of active research. The best studied of these acute-phase reactants is C-reactive protein (CRP). It has been shown to interact with the complement system under a number of circumstances. This investigation grew from an observation made during the study of complement-mediated immune lysis of rubella virus. A serum sample with no detectable rubella hemagglutination inhibiting, complement-fixing or neutralizing antibody, but containing an elevated level of C-reactive protein effected lysis of rubella virus. A second serum sample from the same patient but collected at a time when CRP was not elevated failed to yield significant lysis (2). The present study was conceived to test the hypothesis that C-reactive protein as a component of the early humoral response to acute viral infection,acting alone or in concert with other serum components such as complement, can have a direct lytic effect on enveloped viruses.

Though much is known of the interaction of antibody and virus, less is understood about the interactions of virus and accessory factors such as the components of complement. Certainly complement acting in the presence of antibody has been implicated in humoral defense (3-10). Is it unreasonable to suspect that C-reactive protein, long

recognized as a harbinger of the inflammatory response, and an evolutionarily ancient protein (11), may also provide some measure of protection against overwhelming acute viral infection?

C-reactive protein was purified by affinity chromatography and added to purified virus preparations. Evidence for virus-CRP interaction was sought by 1) tests for virolysis, 2) complement fixation, 3) neutralization tests, and 4) electron microscopy.

## II. Literature Review

The following paragraphs present the early research on C-reactive protein in a chronological fashion, discuss the major work delineating its biochemical nature and review its biology as a component of the inflammatory response. In addition, an effort is made to present some data on the mechanisms of complement-effected neutralization and virolysis.

### Discovery and Association with Acute-Phase Sera

In 1930 Tillet & Francis (12) found that serum obtained from patients in the acute stage of lobar pneumonia yielded a precipitate in the presence of dilute solutions of the C-polysaccharide of Pneumococcus.

Precipitins for the C-polysaccharide were also found in the sera of patients with streptococcal infection and acute rheumatic fever.

Ash (13) extended this observation by demonstrating the precipitation reaction in patients acutely ill with both gram positive and gram negative infections. In all cases, the precipitation reaction subsided after the cessation of the acute stage. By 1940, Abernethy & Avery (14) had conclusively demonstrated that the "acute stage" reactant was a protein but not an immunoglobulin and that the presence of calcium ions was necessary for C-polysaccharide precipitation. In addition, MacLeod & Avery described a method of partial purification of the C-reactive protein (15) by ammonium or sodium sulfate precipitation and succeeded in raising rabbit antibody to CRP (16) which did not react with normal human or monkey sera but did precipitate human or monkey sera from infected individuals. McCarty succeeded in crystallizing the protein from human serous fluids in 1947 (17) and following the work of Lofstrom (18, 19), who found acute phase rabbit sera as well as human sera caused capsular swelling of certain strains of pneumococci, Anderson and McCarty crystallized the rabbit acute phase protein (20). The rabbit form was named Cx-reactive protein and was found to be remarkably similar to human CRP.

## Biochemical and Biophysical Characteristics

Physicochemical studies by Wood, McCarty and Slater (21) demonstrated that CRP migrates in the beta-globulin region in moving boundary electrophoresis. In contrast, by the method of zone electrophoresis on starch medium the protein migrates with the gamma-globulins. Its

isoelectric point is at pH 4.82 and its Svedberg coefficient is 7.5S. Gotschlich and Edelman (22) found CRP crystallized by the method of Wood et al. (21) to be an aggregate of probably identidal subunits held together by noncovalent interactions. Ultracentrifugation in 5M guanidine-HCl dissociates the protein into subunits with average molecular weight 24,300. 8M urea also dissociates the protein to subunits with a molecular weight of about 20,000; these subunits give rise to a single band in starch gel electrophoresis. They proposed that one of several forms of CRP was composed of six identical subunits each with an intrachain disulfide linkage and a total molecular weight of 129,000. In double immunodiffusion tests with CRP in serum, up to three lines of identical specificity have been observed indicating heterogeneity of size (23). In response to these observation, Kushner and Sommerville (24) determined the molecular weight of CRP as it occurs in serum by gel filtration and density gradient centrifugation. They estimated that the molecular weight lies between 135,000 and 140,000 consistent with a molecule composed of 6 subunits of approximate molecular weight 23,000.

## Characterization of Receptors for CRP

Gotschlich and Edelman (25) also studied the CRP-C-polysaccharide (CPS) precipitation reaction and found that it could be inhibited by phosphate monoesters. Indeed the precipitation inhibition mimicked the hapten inhibition phenomenon seen with antigen-antibody reactions.

It was suggested that each subunit of CRP could bind one molecule of phosphate monoester and that CPS precipitation occurred as a lattice of interacting molecules formed in a fashion similar to the interaction of antibody with polyvalent antigen. Further attempts to characterize the specificity of C-reactive protein by Volanakis and Kaplan (26) confirmed the inhibition of CRP-CPS precipitation by phosphate monoesters but demonstrated that choline phosphate is a markedly more active inhibitor of this reaction and thus possibly the major determinant for precipitation of CPS.

Though these reports present some understanding of the biochemistry of C-reactive protein, the literature echoes a common theme. That is, that the method of preparation of CRP shapes experimental results and at present there is disagreement on the native structure and state of CRP _in vivo_. This confusion naturally extends to the biology of CRP. Again, differing methods of purification and differing methods of measurement with widely varying accuracy and precision, confuse the literature and make interpretation a challenge.

## Biological Distribution

In spite of areas of controversy, it is undisputed that elevated levels of C-reactive protein have been found in the blood during virtually all diseases associated with active inflammation, particularly in patients with the rheumatoid diseases, acute infectious processes, post myocardial infarction or post surgery, advanced malignancy and

chronic infections (27-31). Less clear until recently was the incidence of measurable CRP during pregnancy, in cord blood, in the newborn and in apparently healthy adults.

CRP has been demonstrated in frequencies as high as 82 per cent during the latter part of pregnancy and at parturition, but it was rarely found in umbilical cord sera (32, 33). This has been interpreted as evidence against transplacental passage of maternal CRP. However, more than 50% of newborns in the first week of life showed the presence of CRP, many from day one of the newborn period with a rapid decline after day four of life (32, 34). Two-thirds of newborns with infection had CRP positive sera by the capillary precipitation test (34).

## Methods for Detection and Quantitation

Before more data on the clinical behavior of CRP are presented, the revealing study by Nilsson (35) which evaluated the efficacy of quantitative precipitation tests should be reviewed. Nilsson found that the capillary tube precipitation test was subject to occasional false positive results but more importantly, frequently failed to indicate the presence of CRP detectable by double diffusion in agar. However, he regarded neither method as more than semi-quantitative and he advanced single radial immunodiffusion as a simple quantitative method able to detect as little as one microgram CRP per milliliter. His research casts doubts as to the validity of many of the studies done utilizing simple capillary tube precipitation or double diffusion to detect and quantitate CRP. Most recently, Claus et al. (36)

in the single definitive study to date, developed a radioimmune assay for CRP and then proceeded to demonstrate that CRP is almost certainly a constituent of normal human serum which only increases substantially in concentration during inflammation. They found, by assaying sera from 153 healthy adult blood donors, normal adult levels of the protein ranging from 68 to 8,100 ng. per milliliter with a median value of 580 ng. per milliliter. No individual was found to be lacking CRP. Just as these results contradicted the numerous reports that CRP is not present in the sera of healthy adult normals, analysis of 24 samples of cord blood found a range of CRP concentrations between 10 and 350 ng. per milliliter demonstrating that there is indeed either transplacental passage or fetal synthesis of this protein. Also, in contrast to the healthy adult levels, CRP concentrations in the sera of 246 patients being evaluated for autoimmune disease ranged from 2,000 ng. per milliliter to 256,000 ng. per milliliter with a median of 13,000 ng. per milliliter, a dramatic and intriguing increase. This important work substantially revises some longstanding conceptions of the as yet undetermined function of CRP.

## Time Course of Elevation in Serum Concentration

Though the function of C-reactive protein is not known, more than three decades of experimentation have illuminated several aspects of its biological behavior. For instance the time course of the rise in CRP titer is well known. Crockson et al. (37) followed CRP levels pre

and post surgery in patients and showed CRP to be detectable within 24 hours and to reach a peak concentration in one to four days, with an average of forty-eight hours. Hokama et al. (38) also found a 24-hour CxRP[1] response in rabbits immunized with intravenous bovine serum albumin or human gamma globulin and in addition they noted a secondary response which was found to be attributable to the formation of intravascular antigen-antibody complexes. The duration of the period of net CxRP production and the amount of CxRP formed by rabbits in response to the administration of intravenous endotoxin was examined in a kinetic study by Yen-Watson and Kushner (39). The CxRP response was found to be directly related to endotoxin dose. Serum CxRP concentration increased exponentially after endotoxin administration with the increases most often noted immediately, although some animals had on the average a 6-hour lag time. The data were interpreted as suggesting that mediators inducing CxRP synthesis by the liver act in an all or none manner, the amount of CxRP formation depending on the duration of the mediating stimulus.

## Site of Synthesis

That the liver is a site of CRP synthesis was nicely worked out by Hurliman et al. (40). Monkey and rabbit liver tissue taken from animals stimulated with inflammation inducing agents and then incubated

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[1] CxRP is structurally and functionally analogous to CRP, but with different antigenic specificity.

in media containing $^{14}C$-lysine and $^{14}C$-isoleucine was found to synthesize $^{14}C$-labeled CRP and CxRP. No other tissue incorporated $^{14}$carbon into CRP. There was, in addition, no apparent correlation between CRP formation and immunoglobulin production.

## Localization at Sites of Inflammation

CxRP has also been identified by immunofluorescent staining in association with necrosis in the local inflammatory lesions produced by intramuscular injection of typhoid vaccine (41). The CxRP was detected both in serum and in tissue within eight hours of the injection and was only found in myofibrils undergoing necrotic change where it was localized particularly at the outer edge of these cells and in vacuoles in the sarcoplasm. CxRP was not detected in any peri-inflammatory tissue, in the polymorphonuclear leukocytes infiltrating the lesion or in the cellular elements in the draining lymph nodes. The relative number of myofibrils containing CxRP showed progressive increase from early lesions at 8 to 12 hours to older lesions at 48 hours, the end of the period of observation. The identical localization of CxRP was seen in animals rendered granulocytopenic by nitrogen mustard. Kushner et al. (42) observed identical histochemical localization of CxRP in rabbit myocardium rendered necrotic by coronary artery ligation. In addition, the protein was found in vascular lumens and interstitial spaces. Although these results were not originally interpreted as such, they provide reasonable support for the concept of localization of circulating CRP at the site of an inflammatory response.

## CRP's Role as an Opsonin

Early observations of the capsular swelling effect of CRP on the pneumococcus (43) and the ability of CRP, when added to plasma above centrifuged normal whole blood, to accelerate the rate at which leukocytes leave the buffy layer (44) prompted studies of the effect of C-reactive protein on phagocytosis and nonspecific immunity. Hokama et al. (45) noted enhancement of phagocytic activity of peripheral blood leukocytes when bacteria were pretreated or mixed with CRP prior to the addition of whole blood. This opsonizing effect was not seen when the CRP was added directly to heparinized whole blood, but subsequent work has shown that this negative result may have been due to the binding of CRP by heparin (25). In 1969, Ganrot and Kindmark (46) showed that highly purified CRP markedly stimulated the phagocytosis of Goffkya tetragena,a nonpathogenic bacterium, by human leukocytes and in the following year went on to demonstrate the same stimulation of in vitro phagocytosis of the human pathogens Streptococcus pneumoniae, Staphylococcus aureus, E. coli, and Klebsiella aerogenes (47). Kindmark further strengthened the evidence that CRP serves as a bacterial opsonin by demonstrating that pathogenic gram positive and gram negative bacteria absorbed C-reactive protein from sera taken from postoperative patients and that the protein could then be eluted by treatment of the bacteria with EDTA (48). Clearly then, the role of CRP as a nonspecific bacterial opsonin has been defined.

## Complement Consumption and Activation

Happily, the story does not end here, for in recent years other roles for C-reactive protein have been delineated. Kaplan and Volanakis (49) in 1974 demonstrated that the reaction of CRP positive sera or crystalline CRP with pneumococcal C-polysaccharide induced consumption of human complement and $C_3$ conversion in the absence of detectable antibody. All normal human sera tested permitted consumption of complement by CRP-CPS complexes, including sera from newborns with physiological hypogammaglobulinemia and sera previously adsorbed with pneumococcal cell walls to remove trace antibody to C-polysaccharide. The complement consumption reaction which consumed 80% or more of hemolytic complement $C_1$, $C_4$, and $C_2$, and 42 to 66% of $C_3$-$C_9$, was totally inhibited by phosphoryl choline, but not at all by N-acetyl galactosamine, the major determinant of CPS antibody specificity. CRP also reacted with phosphoryl choline, lecithin and sphingomyelin (when these were suspended with cholesterol as a lipid carrier) by complement fixation. Immunodiffusion of the washed complexes resulting from precipitation of CRP-positive sera with C-polysaccharide or lecithin gave strong lines of precipitation against anti-CRP, anti-$C_{1q}$ and anti-$C_3$, whereas immunoglobulins were absent or present only in trace quantities.

Subsequently Siegel et al. (50) found that cationic homopolymers of poly-L-lysine were able to activate complement via CRP and deplete complement components $C_1$ through $C_5$. The naturally occurring polycations, myelin basic proteins, rabbit leukocyte cationic proteins, and lysine and arginine rich histones, were also complement consuming

proteins react so with CRP is of special interest, since they accumulate at sites of injury and tissue destruction. Siegel speculates that this reactivity with CRP results in activation of complement at inflammatory sites, and in modulation of the ability of polycations to initiate mast cell (51) and platelet (52)activation, or in favoring of CRP binding at anionic cell surfaces. These above experiments with CRP-mediated complement consumption were studies of complement activation in the fluid phase. Osmond et al. (53) made an important contribution to this field when they coated erythrocytes with C-polysaccharide and demonstrated that C-reactive protein in reacting with them initiated the full biologic expression of complement activation-cell lysis and death.

## Interaction with the Cellular Elements of Blood

Contributing to the mounting evidence that C-reactive protein is perhaps both a modulator and effector in the inflammatory response is the discovery of the interactions between CRP and platelets and lymphocytes. Fiedel and Gewurz (54) found that the addition of small amounts of CRP (2-25 µg/ml) to an amount of aggregated human IgG which induced 50% maximal rate of platelet aggregation significantly reduced or abolished this response. Platelet viability remained intact and addition of large amounts of aggregated IgG reversed the CRP-induced inhibition.

Early studies of the effect of C-reactive protein on lymphocyte function were contradictory. Hornung and Fritchi (55) reported that

CRP concentrations of 10 micrograms per milliliter were mitogenic, whereas twice this concentration of CRP resulted in loss of lymphocyte viability and cell death. Hornung later reported that CRP in the presence of lymphocytes inhibited the growth of human melanoma cells in vitro (56). A conflicting report by Hokama (57) suggested that CRP was neither toxic or mitogenic by itself, but it inhibited the response to phytohemagglutinin. Additionally, this group found that in leprosy patients, CRP in autologous serum may in part be associated with the depression of $^3$H-thymidine incorporation into the DNA of leukocytes (58).

In an attempt to more systematically examine the effect of CRP on lymphocytes, Mortensen and Gewurz (59) found that CRP binds selectively to T lymphocytes, inhibits their ability to form spontaneous rosettes with sheep erythrocytes and inhibits their response to allogeneic cells in mixed lymphocyte culture reactions. CRP did not bind to B lymphocytes, nor did it alter B cell functions, such as binding to activated complement components and to the Fc portion of immunoglobulin, or mediating antibody-dependent cytotoxicity reactions. Furthermore, CRP failed to suppress PHA or concanavalin A-induced mitogenesis. Mortensen and Gewurz (60) further defined the effect of CRP on the response to allogeneic cells in a murine system by showing that the CRP binding was to theta bearing, extrathymic T cells, and that CRP specifically inhibited the proliferative response. Murine cytolytic lymphocytes, once generated were not affected by human

CRP. It was suggested that suppression of the mixed lymphocyte reaction was due either to a failure to initiate differentiation of cytotoxic lymphocyte precursors or to abrogation of their full differentiation. Further unpublished data by these workers indicates that CRP depresses both the blastogenic response and the elaboration of MIF by sensitized human peripheral lymphocytes to antigens. All of these data are consistent with the concept that CRP may serve some immuno-regulatory function limiting the cellular response during inflammation.

## Summary of the Data Supporting CRP's Role in Defense Against Bacteria

In summary, the cogent data gleaned from the literature which have some bearing on the "CRP as humoral defense" hypothesis are the following: C-reactive protein appears within hours of tissue injury and persists for several days. It localizes at sites of injury. It can bind to phosphate monoesters and phosphatidyl choline and activate complement via the classical pathway. When fixed to red blood cell membranes, it can activate complement components C1 through C9 with resulting cell lysis. There is evidence to suggest that it fixes to and enhances the phagocytosis of various types of bacteria.

## CRP and Viral Infections

It will be noted that there is no mention of virological studies with C-reactive protein. The field is apparently unexplored with only a single study by Parker et al. (61) who in 1962 reported that CRP was

"found" or elevated during viral infection much as it was in bacterial infection. They also mentioned the intriguing preliminary finding that partially purified CRP will neutralize the infectivity of poliovirus types 1 and 3 and will non-specifically fix complement when used with a wide variety of viral antigens. This work was not followed by later reports extending or confirming these observations.

Several questions now derive from the preceding two paragraphs: Can C-reactive protein bind to a viral envelope? Once bound, can CRP mediated complement activation neutralize or lyse the virion?

It is clear that enveloped viruses acquire their envelopes from the cell membrane through which they bud, the lipid and carbohydrate produced by the appropriate enzyme system of the host cell, the envelope proteins being virion specific (62). It is also clear that phosphatidyl choline is a relatively abundant phosphoglyceride in the plasma membrane of higher animals (63). If it is postulated that CRP can bind to the virion envelope with the subsequent activation of complement, what are the possible results?

## The Role of Complement in Virus Neutralization

The critical role of complement in viral neutralization has already been intimated in the introduction and a more detailed review is now in order. Lennette (64), Svehag (65), Way and Garwes (3), and Chappell et al. (66), have all reported on the ability of a serum

accessory factor or a heat labile serum factor to enhance the neutralization of virus. In 1968, Berry and Almeida (67) described the enhancement of neutralization of avian infectious bronchitis virus by complement (C) as seen with immune electronmicroscopy. Then Daniels et al. (5) demonstrated that the sequential addition of the first four functionally pure C' components could neutralize sensitized virus, offering more support to the hypothesis that C' neutralizes by piling up on the virion surface. In these experiments, in the presence of an optimal concentration of $C_1$, IgM sensitized herpes simplex virus was neutralized by $C_4$ and neutralization was increased by the addition of $C_2$ and $C_3$ when the concentration of $C_4$ was limiting. Radwan et al. (7-9) in some very neat experiments with sensitized equine arteritis virus demonstrated that C' does not act to stabilize the V-Ab complex but that it neutralizes it, most likely in a two step reaction, where initially $C_1 - C_3$ is bound, followed by the late acting components and virolysis. Radwan did show that the first four components of C'were sufficient to assure complete neutralization of sensitized virus in the absence of virolysis. This phenomenon was then shown to be true for sensitized polyoma (6) and sensitized Newcastle disease virus (68) as well.

Most recently sera with functional C' sources were found to inactivate and lyse avian, feline, murine and simian oncornaviruses apparently in the absence of antibody (4). All of these viruses are enveloped. Complement seemed to be the mediator of this lysis since

agammaglobulinemic sera containing less than 10 μg/ml IgG and no other Ig was able to lyse virus while $C_2$ and $C_4$ deficient sera were not. Subsequent C' component titrations indicated activation via the classical pathway.

Clearly complement plays an important part in the neutralization of virus coated with antibody; there are suggestions that complement also may act in the absence of antibody. Perhaps in this story is a role for CRP.

## III. Materials and Methods

### Rubella Virus

The virus is the Therien wild type rubella virus isolated in this laboratory in 1965 (69). It is grown in Vero monkey kidney cells in stationary or roller bottles. Details of the cell and virus propagation have been described previously by Leibhaber et al. (70). The virus was purified in one of two ways. The first method employed sucrose gradient centrifugation and has been described by Schluederberg et al. (2). The second method precipitated the virus from tissue culture fluid utilizing 7% polyethylene glycol (71) and stirring at 4° C for one to two hours. The precipitate was collected by centrifugation at 10,000 rpm, resuspended in a small amount of 0.05M Tris buffer containing 0.155M NaCl and layered on a 4 ml gradient cushion of sucrose (30-50% wt/wt). The suspension was centrifuged in a SW 41

rotor at 35,000 rpm for three hours and the visible virion band was removed through the side of the tube with a syringe and needle and stored at -70° C.

### Radioactive Labelling of Rubella Virion RNA

This procedure has been previously described (2) and is based on the addition of 5-$^{3}$H uridine (50 uCi/ml, New England Nuclear Corp.) to 72 hour viral cultures thirty minutes after treatment with actinomycin D (5 µg/ml, Merck, Sharpe, and Dohme) and harvesting virus 18-24 hours later.

### Influenza Virus

The influenza virus used for electron microscopy was the Influenza A/New Jersey 1976 strain fixed in formaldehyde and suspended in isotonic PBS as a vaccine (Merrell-National Laboratories).

### Neutralization Test (NT)

The NT was performed with Vero monkey kidney cells grown in cluster plates (Costar) of twenty-four 16 mm wells per plate. C-reactive protein was added to an equal amount of heated (56°, 30 minutes) CRP-negative, NT antibody-negative serum and two-fold dilutions of this were made in PBS containing 2% reconstituted lyophilized guinea pig serum (BBL, Division of Becton, Dickinson, and Co.). Each serum dilution was mixed with an equal volume of rubella virus suspension containing 30 $TCID_{50}$ per 0.1 ml. The virus-serum mixtures were

incubated overnight at 4° C and for ten minutes at 37° C just prior to inoculating 0.1 ml of each serum dilution per well in quadruplicate. The cultures were put at 37° C, refed with fresh media one hour after inoculation and on the fifth day after infection, and were read on the eleventh day by checking for hemagglutination in a 1:2 dilution of the culture fluid (72). The neutralization titer was expressed as the reciprocal of the highest serum dilution which blocked viral growth as measured by hemagglutinin production.

## Complement Fixation Test

The standard microtitration technique employing guinea pig complement and 4 units of antigen was used (73). In addition to C-polysaccharide, whole rubella virion antigen and soluble rubella antigen were employed in tests with CRP. Whole rubella antigen was prepared by ammonium sulfate precipitation of several liters of Vero monkey kidney cell grown virus. The resulting precipitate was resuspended in 0.05M Tris 0.10M NaCl, pH 7.2 and layered on a 15-65% (wt/wt) sucrose gradient and centrifuged at 22,500 rpm for 18 hours at 4° C. Fractions were collected from the bottom of the tube and those fractions comprising the HA peak were pooled and frozen at -70° C. Soluble rubella antigen was prepared from the supernatant of this centrifugation which was dialyzed against 0.05M Tris 0.10M NaCl pH 7.2 for 24-48 hours at 4° and then concentrated three to six times with aquacide (Calbiochem). Tween-80 (L. Light and Co., Ltd.) was added to a final concentration of 0.125% along with ether to a

final concentration of 50% and this was continuously stirred for 30 minutes at 4° C. The mixture was then centrifuged at 3,000 rpm for 30 minutes at 4° C and the aqueous phase removed and stirred under a stream of nitrogen to remove trace ether. This material was also stored at -70° C.

Pneumoccal C-polysaccharide (CPS)

This procedure was modified from that of Schiffman et al. (74). A Cs-capsulated strain of pneumococcus (kindly provided by Dr. Gerald Schiffman, Department of Microbiology and Immunology, State University of New York, Downstate Medical Center, Brooklyn, New York) was grown overnight in fresh beef heart infusion broth supplemented with 1% neopeptone (Difco Laboratories). Glucose was then added to a final concentration of 1% and incubation of the culture was continued with periodic neutralization with 3N NaOH of the lactic acid formed. At the end of the exponential phase of growth, the bacterial cells were harvested by centrifugation and allowed to autolyze in 30 ml of 5% sodium acetate at 37° C for 18 hours. The insoluble cellular debris was removed by centrifugation and the protein was removed by shaking with chloroform-butanol (Sevag method), after which procedure the aqueous phase was fractionated with ethanol. The fraction precipitating between 55 and 75% ethanol was mainly CPS. This was further purified by gel filtration on Sephadex G-200 (Pharmacia) in 0.2N ammonium acetate. Effluent fractions were assayed by determining

their density after lyophilization. Fractions from the resulting peak were resuspended, pooled and relyophilized.

## C-reactive protein (CRP)

This method of isolation is slightly modified from that described by Osmand et al. (53). Ascites fluid (collected with the kind assistance of Dr. Ernest Kohorn, Department of Obstetrics and Gynecology, Yale University School of Medicine, New Haven, Connecticut) from patients with gynecological malignancies was clarified by low speed centrifugation and stored at 4° C after the addition of 0.01M sodium azide. The affinity chromatography procedure was based on the calcium dependent binding of CRP to pneumococcal C-polysaccharide (CPS). CPS was coupled to cyanogen bromide activated Sepharose 4B (Pharmacia) as described by Wofsy and Burr (75), and 30 ml of gel was packed into a small column. Clarified ascites fluid (60 ml) was washed through the column with 0.05M Tris pH 8.0 with 0.155 M NaCl and 2.0 mM $CaCl_2$ until the $A_{280}$ of the effluent was less than 0.020 and then the CRP was eluted with Tris saline containing 2.0 mM citrate. The resulting fractions were assayed for CRP by immunodiffusion against anti CRP (Behring Diagnostics) and the most strongly positive were pooled and stored at 4° C until used.

## Quantitation of CRP by Immunodiffusion

Tests were done on 75 x 25 mm glass slides covered by 2.5 ml of gel consisting of 0.4% agarose in 0.01M Tris buffer containing 0.1%

sodium azide. Two-fold dilutions of CRP containing material were made in Tris saline and 20-μl aliquots of these were placed in wells 5 mm in diameter and 3 mm apart. Results were read after 18 hours of incubation in a moist chamber at room temperature. The titer of CRP was expressed as the reciprocal of the highest dilution giving a visible precipitate. The concentration was expressed in units/μl with one unit/μl defined as that dilution giving a sharp precipitate at the mid point between the antigen and antibody well.

## Immunoelectrophoresis

The micro method described by Scheidegger (76) was used.

## Standard Test for Virolysis

The procedure used was described by Schluederberg et al. (2) but was modified by substituting purified CRP for the serum which was mixed with equal aliquots of guinea pid complement and 5-$^3$H uridine-labelled rubella virus. After overnight incubation at 4° C, an aliquot of RNase A (60 μg/ml, Sigma Chemical Co.) was added to the test sample and its accompanying controls and these tubes were incubated for two hours at 37° C. After this incubation, the contents of each tube were layered on a 2 ml claifying cushion of 20% (wt/wt) sucrose. Virions were sedimented through the sucrose layer in an sw 51.1 rotor at 40,000 rpm for two hours at 4° C and the radioactivity of the pellet was measured.

### Measurement of Radioactivity

Virion pellets were suspended in 400 µl of solubilizer (NCS) (Amersham/Searle) and incubated at room temperature for 30 minutes. This was then added to 5 ml of Spectrofluor (Amersham/Searle) and counted in a Beckman model LS-150 scintillation counter to an accuracy level of at least 5% after non-radioactive stimulation of fluors had fully subsided (1-2 days). When acid-soluble radioactivity was measured, aliquots of the supernatant from acid precipitation were mixed with three volumes of NCS and incubated at room temperature for 30 minutes before mixing with the scintillation fluid.

### Estimation of Lytic Activity

CRP and control associated lysis was compared to that effected by a standard rubella convalescent serum (MG) which was tested after inactivation at 56° C both with heated (negative control) and unheated (positive control) guinea pig complement. The difference in cpm between these two controls was considered to represent 100% efficient viroloysis. Thus the difference in cpm between a given sample's pelletable RNA and the negative control's was expressed as per cent maximal lysis.

### Preparation of Rubella Virus for Electron Microscopy

Rubella virus in sucrose stored at -70° C was rapidly thawed and mixed with equal aliquots of CRP, CRP and guinea pig complement, complement or buffer as noted with the experimental results. After varying incubation periods at 37° C, a drop of the sample was placed

on a 300-mesh parlodian and carbon coated grid and left for five minutes. The excess fluid was withdrawn with filter paper and the grid was turned down on a drop of 2% PTA for 30 minutes. The excess stain was then removed and the specimen was ready for examination.

### Preparation of Influenza Virus for Electron Microscopy

A slight modification of the procedure reported by Edwards et al. (77) was utilized. The vaccine virus was diluted 1:4 with 0.05M Tris buffer containing 0.155M NaCl, 2.3 mM $CaCl_2$ and 1.0 mM $MgCl_2$ or with the reagents to be tested. After incubation for one hour at 37° C and overnight at 4° C, the samples were pelleted at 15,000 rpm for 75 minutes, resuspended in distilled water and negatively stained.

## IV. Results

### Purification of Pneumococcocal E-polysaccharide (CPS)

Fifteen liters of beef-heart infusion media were seeded with 75 ml per liter of one day old cultures of Streptococcus pneumoniae $A_{66}R_2$TRIDRS $C_{55}$ and grown for 24 hours yielding approximately 3.0 grams of CPS after autolysis and ethanol fractionation. This was placed on a sephadex G-200 column, 3.5 x 42 cm, and eluted with 0.2N ammonium acetate. The resulting fractions were concentrated by lyophilization before testing. The active fractions, comprising a single peak, were pooled and relyophilized before use.

## Preparation of CPS - Sepharose for Affinity Chromatography

Approximately 2 grams of CPS was disolved in 8 ml of 0.1M $NaHCO_3$ and this was added to 300 ml of the activated sepharose 4B CNBr treated (76) intermediate. This material was stirred overnight at 4° C and then the buffer with uncombined CPS ligand was filtered off. Analysis of this wash for the presence of CPS was done by measuring its ability to block a CRP immunoprecipitin test. Two-fold dilutions were made and each was mixed with an equal amount of C-reactive protein positive ascitic fluid.In double diffusion assays, the CPS solution employed as a ligand inhibited a CRP-anti CRP precipitate to a dilution of 1:16,000; the wash had no demonstrable CPS activity indicating all of the CPS had been bound to the sepharose.

## Isolation of C-reactive Protein (CRP)

Affinity chromatography as described in materials and methods and employing small amounts of ascites fluid as a CRP source was highly successful. The ascites pool employed had a CRP concentration of 4 units/μl and passage through the column removed all demonstrable CRP. Elution of the CRP with Tris saline containing citrate eluted all CRP within one column bed volume and the resulting CRP peak typically had concentrations of 4-8 units/μl. Immunoelectrophoresis of the CRP against goat anti-human serum (Hyland Div., Travenol Laboratories, Inc.) failed to show any precipitate while a single line was seen against anti CRP. This evidence indicated the CRP isolated in this fashion was immunologically pure. SDS-acrylamide gel electro-

phoresis demonstrated a major band at MW 23,000, the same result obtained by Osmand et al. (53). It should be noted that the CRP thus isolated was in citrate buffer and before use, it was necessary to add additional calcium to the buffer to a final concentration of 2.3 mM. Serial determinations of CRP concentration by immunodiffusion showed the protein to be antigenically stable for greater than two weeks at 4° C.

### Quantitation of C-reactive Protein by Immunodiffusion and Protein Determination

The concentration of one unit of CRP per microliter, as defined by immunodiffusion against our standard anti-CRP serum diluted 1:8 with Tris saline, was found to correspond to a protein concentration of 120 μg/ml as measured by the method of Lowry et al. (78).

### Non-Immune Virolysis Mediated by C-reactive Protein

Because experiments quantitating immune rubella virolysis provided the genesis for these investigations and because the technique as reported by Schluederberg et al. (2) could be modified so that purified CRP could be used in place of serum, a number of these experiments were performed. However, for unknown reasons there was poor reproducibility among experiments as well as a number of experiments in which the virus controls exhibited marked lysis probably indicating that the virus employed was damaged. There was only a single experiment where the duplicates of each sample matched each other and the controls did not invalidate the results. The results

are displayed in Table 1. Each sample was approximately equivalent in protein concentration, discrepancies being equalized with BSA present at a final concentration of 5%. Each sample had an aliquot of either CRP or the buffer in which the CRP was eluted. The BSA was dissolved in pH 7.3 veronal buffer containing 0.145M NaCl 0.25mM $CaCl_2$ and 8mM $MgCl_2$, so there was sufficient cation concentration to permit complement activation even though the concentrations of these two ions varied slightly among samples. It should be noted that the virus in the negative buffer had 25% lysis as compared to the positive control. This was not noted when whole serum (e.g. guinea pig complement) was present. C-reactive protein induced lysis almost one-half as efficiently as antibody in the presence of both active and heated complement. It induced no lysis in the absence of guinea pig serum. The degree of lysis was significantly more than with the corresponding controls (samples 7 and 8). The addition of CPS inhibited CRP-effected virolysis.

It is not likely that this single experiment amidst numerous unsuccessful ones is highly significant. It perhaps suggests that CRP can mediate virolysis in the presence of one or all of the complement components. It is indeed unfortunate that this sensitive technique for quantitating virolysis could not be more successfully utilized.

Table 1. Lysis of Rubella Virus Following Incubation in CRP-Containing or Control Reaction Mixtures[1]

| | Sample | Content | Arc CPM Virus Pellet | Lytic Efficiency[2] (%) |
|---|---|---|---|---|
| Controls: | 1 | Buffer[3] | 935 | 25 |
| | 2 | Buffer + C' | 1058 | 12 |
| | 3 | Buffer + ΔC' | 1254 | 0 |
| | 4 | Buffer + C' + CPS | 1125 | 0 |
| | 5 | Buffer + C' + immune serum | 354 | 100 |
| | 6 | Buffer + ΔC' + immune serum | 1130 | 0 |
| | 7 | CRP + C' + immune serum | 354 | 100 |
| Test: | 8 | CRP | 1157 | 0 |
| | 9 | CRP + C' | 796 | 43 |
| | 10 | CRP + ΔC' | 750 | 49 |
| | 11 | CRP + C' + CPS | 1048 | 11 |

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[1] Incubation overnight at 4° and 2 hours at 37° in the presence of RNase. See materials and methods for details.

[2] Samples 5 and 6 defined as 100% and 0° efficiency.

[3] Buffer used was a pool of CRP-negative fractions collected in the column eluate. Bovine-serum albumin was added to both CRP and buffer control to stabilize the virions.

## Complement Fixation by C-reactive Protein Using Whole Rubella Virions and Pneumococcal C-polysaccharide as Antigens

Experiments were set up to see if complement fixation by rubella virus and CRP could be demonstrated. To standardize conditions, initial tests were conducted with CRP and CPS, a combination previously shown to fix complement (49, 53). The diluent used was VBD and two units of hemolysin (BBL, Division of Becton, Dickenson, & Co.) were used. The potency of the CRP preparation was 6 units/μl and two-fold dilutions were carried to 1:64. The CRP was found to be consistently anticomplementary, indicating that it was binding or inhibiting complement in the absence of CPS. Further tests were done using a mixture of equal aliquots of guinea pig complement and human C as well as CRP carried to higher dilutions in an attempt to diminish its anticomplementariness. The mixture of g.p.C' and human $C_1$ was found to have minimal hemolytic activity and this approach was abandoned.

The anticomplementary activity of the CRP preparation was eliminated in subsequent tests in which CRP was diluted in a pool of rubella CF-negative serum. Two-fold dilutions of CRP 1:2 in antibody-negative serum were run against two-fold dilutions of a stock solution of CPS in distilled water in a checkerboard type pattern to determine optimal dilutions of antigen and CRP. For increased sensitivity, one unit of guinea pig complement was used in these tests. All dilutions of CRP fixed complement using the CPS antigen. One hundred per cent or 4+ fixation was observed for CRP dilutions 1:8 through

1:32. This represented approximate CRP concentrations of 600 μg/ml through 150 μg/ml.

Following these positive results, CRP (1:2 in antibody-negative serum) diluted 1:4 and 1:16 was tested for complement fixation with whole rubella virion antigen as well as with soluble rubella antigen and CPS was utilized as a positive control antigen. The results of this experiment as well as the previous one are displayed in Table 2. The dilutions of the virion and soluble antigens represent four units as defined by titration against a standard CF-positive immune serum. One unit and 0.5 units of guinea pig complement were used. The results here are disappointing. CRP did not fix one unit of complement with the CPS antigen as it had previously. Why this failed to occur is not understood. Additionally, the complement fixation with CPS and virion antigen was also seen with an identical dilution (1:4, 1:8) of negative serum only.

The conclusions to be drawn are threefold. 1) C-reactive protein isolated using the described technique has its anticomplementariness reduced by dilution in human serum. 2) CRP has been found to fix complement in the presence of CPS as previously reported (49, 53). 3) No evidence was obtained that CRP has CF activity in the presence of soluble rubella antigens or whole rubella virion antigen.

## C-reactive Protein Mediated Neutralization of Rubella Virus

The ability of CRP to neutralize rubella virus was examined using the standard neutralization test performed in this laboratory. CRP

Table 2a. Complement Fixation By C-Reactive Protein And C-polysaccharide Antigen

| | CRP | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1:2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 |
| CPS 1:30 | 2[1] | 3 | 4 | 4 | 4 | | | | |
| CPS 1:60 | 2 | 3 | 4 | 4 | 4 | | | | |
| CPS 1:120 | 2 | 3 | 4 | 4 | 4 | | | | |
| CPS 1:240 | 2 | 3 | 4 | 4 | 4 | 3 | 3 | 3 | 3 |
| CPS 1:480 | | | | | 2 | 2 | 2 | 2 | 2 |
| CPS 1:960 | | | | | 1 | 1 | 1 | 1 | 1 |
| CPS 1:1920 | | | | | ± | 1 | 1 | 1 | 1 |
| vBD | - | - | - | - | - | - | - | - | - |

| | Neg Serum | | | | |
|---|---|---|---|---|---|
| | 1:2 | 4 | 8 | 16 | 32 |
| CPS 1:30 | 2 | - | - | - | - |
| CPS 1:240 | | ± | - | - | - |

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[1] Numbers recorded represent degree of fixation on a scale from 0 (-) to 4, where - represents complete hemolysis and 4 represents complete fixation (no hemolysis). 3 represents nearly complete fixation, with a button of RBCs only very slightly smaller than in a 4, and only minimal hemolysis.

Table 2b. Complement Fixation By C-reactive Protein Using C-polysaccharide, Whole Rubella Virion Antigen and Rubella Soluble Antigen

| | 1 Unit C' | | | 0.5 Units C' | | |
|---|---|---|---|---|---|---|
| | CRP | 1:4 | 1:16 | CRP | 1:4 | 1:16 |
| CPS 1:120 | | –[1] | ± | | 2 | 3 |
| Vag 1:4 | | – | – | | 2 | 3 |
| Sag 1:8 | | – | – | | ± | ± |
| VBD | | – | – | | – | – |

| | 1 Unit C' | | | 0.5 Units C' | | |
|---|---|---|---|---|---|---|
| | Neg. Serum | 1:4 | 1:16 | Neg. Serum | 1:4 | 1:16 |
| CPS 1:120 | | ± | – | | 4 | ± |
| Vag 1:4 | | – | ± | | 3 | 3 |
| Sag 1:8 | | – | – | | ± | |
| VBD | | – | – | | – | |

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[1] Numbers reccrded represent degree of fixation as explained in Table 2a.

was diluted 1:2 in a pool of antibody negative sera from individuals who later went on to sero convert with positive rubella HAI titers. This resulted in a CRP serum concentration of 240 µg/ml. The five samples tested and their neutralization titers are listed in Table 3. Sample 3 had sufficient CPS added to it to bind all CRP present. Sample 4 had no CRP present, only the buffer in which the CRP was eluted. Sample 5 was CRP diluted 1:2 in 0.155M NaCl with 2.3 mM CaCl and 1.0mM $MgCl_2$ instead of the negative serum. All neutralization titers were two or less than two; the slight variation among samples is not significant.

There is no evidence that CRP can neutralize rubella virus _in vitro_ under the conditions employed.

## _Electron Microscopy of Rubella Virus Incubated with C-reactive Protein_

Rubella virus purified by PEG precipitation followed by sucrose gradient centrifugation was suspended in pH 7.4 0.05M Tris with 0.155M NaCl. The buffer was also adjusted to have a final bovine serum albumin (Sigma Chemical Co.) concentration of 5% as well as being 2.3 mM $CaCl_2$ and 1.0mM $MgCl_2$. This control sample of virus plus CRP-negative column elutant was compared to a test sample, virus incubated with CRP having a concentration of 4 units/µl. The incubation period was one hour at 37° C. Because the samples were not pelleted, the number of virions seen was small.

The sample of virus control, picture 1, yielded sparse but intact virions with discreet borders and regular surface projections, whereas those virions exposed to CRP, picture 2, had less distinct borders with a halo surrounding the individual particles. Such a finding is

Table 3. C-Reactive Protein Mediated Neutralization of Rubella Virus

| Sample Number | | Neutralization Titer |
|---|---|---|
| 1 | Negative serum | 2 |
| 2 | Negative serum & CRP | < 2 |
| 3 | Negative serum & CRP & CPS | < 2 |
| 4 | Negative serum & CRP negative buffer | 2 |
| 5 | CRP & Tris saline with $CaCl_2$ & $MgCl_2$ | < 2 |

These samples were tested with a number of post rubella vaccination sera which served as positive controls and exhibited positive NT titers.

typical of virus coated with antibody (79) and suggests that CRP, the same size as IgG and similar to immunoglobulin in many ways, may have coated the virion envelope.

This experiment was repeated but without diluting the virus in Tris saline, so that virus in "negative buffer" was compared to virus in CRP-containing buffer. In this case, neither sample had calcium ions present thus preculding CRP binding to its recognized substrate. Both samples appeared to be identical with mildly hazy and irregular borders with definite surface projections. No halos were seen. Though both samples indicated that the virus was less well preserved than in the initial control preparations examined, the lack of difference between samples in the absence of calcium-dependent CRP virion binding could be interpreted as giving support to the hypothesis that the differences seen in the initial experiment were due to CRP-virion binding and not due to effects of the CRP buffer.

## Electron Microscopy of Influenza A Virus Incubated with C-reactive Protein

The formaldehyde-fixed influenza A virus was selected for incubation with CRP because previous microscopy yielded micrographs which demonstrated the viral envelope and envelope glycoproteins in good detail. It was thought that this virus might provide better insight into a possible CRP-envelope interaction. Picture 3 shows the appearance of the vaccine virus diluted with Tris saline. Note its relatively

intact appearance with regular surface projections. Picture 4 is of the virus incubated with only the buffer in which the CRP was suspended. It too is relatively intact and appears similar to picture 3, the virus control. Picture 5 is of the virus incubated with CRP at an approximate concentration of 90 µg/ml. The virus is considerably disrupted with negative stain penetrating many more particles than in the CRP-negative buffer or the Tris saline controls. The envelope and surface projections appear modified and are certainly less distinct. Pictures 6 and 7 are of the virus incubated with an equal amount of CRP in the presence of guinea pig complement. In scanning the grid,single virions were quite sparse and several large aggregates of virus were noted. These were interpreted as aggregates formed by complement fixed to the virion surface. In addition, cellular debris seen in the virus suspension was noted to have membrane holes typical of those caused by activated complement. Pictures 8 and 9 are of the virus incubated with CRP and heated complement and the virus plus complement alone. No aggregation or lysis was noted in these samples; picture 8 appears quite similar to pictures 3 and 4, the virus control in Tris saline and the CRP-negative buffer control. This suggests that heated guinea pig serum confers protection on the viral envelope exposed to CRP.

These photographs suggest that CRP can bind to the influenza viral envelope and that once bound it can fix complement and aggregate virion particles.

## V. Discussion

This study was undertaken to determine the virolytic potential of C-reactive protein, an acute phase reactant discovered more than forty-five years ago. Though it has been extensively studied, CRP's fundamental role as a component of the acute reaction to injury is not well understood. There is an intriguing similarity between CRP and the immunoglobulins in that they share an ability to mediate many of the same *in vitro* reactions. This as well as the accumulated evidence suggesting that CRP is an important part of the acute antibacterial defense provides a creditable foundation for the CRP mediated virolysis hypothesis. The finding of Schluederberg et al. (2) that five of nine sera from serologically confirmed rubella susceptibles effected rubella virolysis in the presence of guinea pig complement almost as completely as a rubella convalescent serum also suggests that factors other than antibody can sensitize enveloped virus for complement lysis.

The possibility exists that there are numerous serum factors which participate in non-specific neutralization and lysis of virus. Because of this, the experiments described were conducted whenever possible with purified CRP in a simple Tris saline buffer. The affinity chromatography method of CRP purification yielded a pure protein as demonstrated by immunoelectrophoresis and SDS acrylamide-

gel electrophoresis. That the CRP was active was verified by demonstrating its ability to bind to CPS as well as to fix guinea pig complement in the presence of CPS.

C-reactive protein was initially studied using a system to quantitate virolysis. $^3$H-uridine was used to label the rubella virus genome and after incubation with CRP and guinea pig complement, ribonuclease was added in sufficient quantity to degrade the genomes of those virions lacking an intact viral envelope. Pelletable radioactivity represented unlysed virus and thus CRP-complement mediated virolysis could be compared to antibody (standard convalescent serum) complement induced virolysis. Certain technical considerations were found to be important in these experiments. The rubella virus appeared to be exquisitely sensitive to varying ionic and protein environments. It was necessary to add BSA to the reaction system to stabilize the virus in the Tris or veronal buffer. Even this was not entirely effective in reproducing the stability achieved with virus incubated in human serum, and further attempts to match the pH and cation concentration of human serum in the experimental buffer system were not sufficient to eliminate the lysis seen in samples of virus and buffer alone. Presumably the 25% lysis seen in the virus plus negative buffer control reflects the damage to the virion secondary to prolonged exposure to temperatures of 4° C or greater.

The experiment described did not clearly demonstrate that C-reactive protein could sensitize the rubella virus for complement

lysis. It did indicate that the viral envelope in the presence of CRP and a heat stable constituent(s) of lyophilized guinea pig had its integrity compromised, providing ribonuclease with access to the virion core. Neither heated or unheated complement alone brought about such a change. The addition of CPS prevented this envelope alteration, presumably by binding CRP, indicating that CRP was an essential reactant. Yet virus incubated with CRP alone manifested no envelope permeability change either. Whether the surface effects of CRP and complement piling up on the envelope borders changed its permeability is not known. Clearly however, the combination of CRP and guinea pig serum effected a change in virion structure in this particular experiment.

Because the non-immune virolysis experiments failed to establish that C-reactive protein-activated guinea pig complement was responsible for the observed virolysis, the ability of the preparation of CRP to fix complement was examined. Preliminary to tests employing viral substrates, tests were run with the C-polysaccharide as "antigen". Repeated trials found CRP diluted in VBD to be anticomplementary, binding or inhibiting complement in the absence of CPS. Further tests were then carried out using CRP diluted in a pool of CF negative serum from rubella susceptibles. This scheme removed all trace anti-complementariness. Subsequently, the dilutions of stock CRP and CPS necessary for maximal complement fixation were defined. Using the

resulting dilution of CPS as a positive control antigen, and bracketing the ideal CRP dilution, CF tests were done using whole rubella virion antigen as well as soluble antigen. The results did not indicate that complement fixation occurred. It is not clear whether a more sensitive assay of complement consumption would have changed the findings.

In another attempt to determine the outcome of CRP-viral envelope interaction, the neutralization test was done. CRP was suspended in the negative serum pool used previously for CF work and the tests were then performed with only slight modification of technique from that used routinely to test serum for neutralizing antibodies in which complement is present as 2% guinea pig serum. The unequivocally negative result with this method seems to indicate that CRP in low levels ($\leq$ 60 μg/ml) in concert with the guinea pig complement concentration employed in the test is not capable of reducing rubella infectivity.

None of the above methods directly measured envelope-CRP interaction _per se_ and electron microscopy was chosen as one remaining tool available with this potential. This technique is a sensitive one in studying virolysis (80) and it requires only minimal handling of virus.

The results of microscopy work done with rubella seem clear in two aspects. First, it is apparent that CRP has some disruptive

effect on the virion envelope. Secondly, this effect seems to occur as a result of actual CRP-envelope binding as indicated by the halos seen around CRP incubated virions. Future work was done with formaldehyde fixed influenza A virus because of increasing problems of instability of purified rubella virus preparations.

The results of work done with influenza virus also suggest that CRP has a direct effect on the virion envelope leading to disruption or a change in permeability that permits the entry of negative stain to the virion interior. The addition of unheated guinea pig serum to virus incubated with CRP led to agglutination of virions. In addition, typical C'-induced membrane holes were seen in virions and in cell debris coincidently suspended in the influenza vaccine. Presumably, these cell membranes as well as the viral envelopes had been modified by the incorporation of virus-specified proteins. Complement alone did not induce these changes. Activation of complement with subsequent virolytic activity appeared to be mediated by CRP. A puzzling observation was the apparent protection conferred on influenza virus exposed to CRP by heated guinea pig serum. Virus incubated with CRP alone appeared to have envelope damage. Where virus was incubated with CRP in the presence of heated guinea pig serum, this was not seen. The mechanism or significance of this phenomenon is not known nor was this noted in the rubella virolysis experiments.

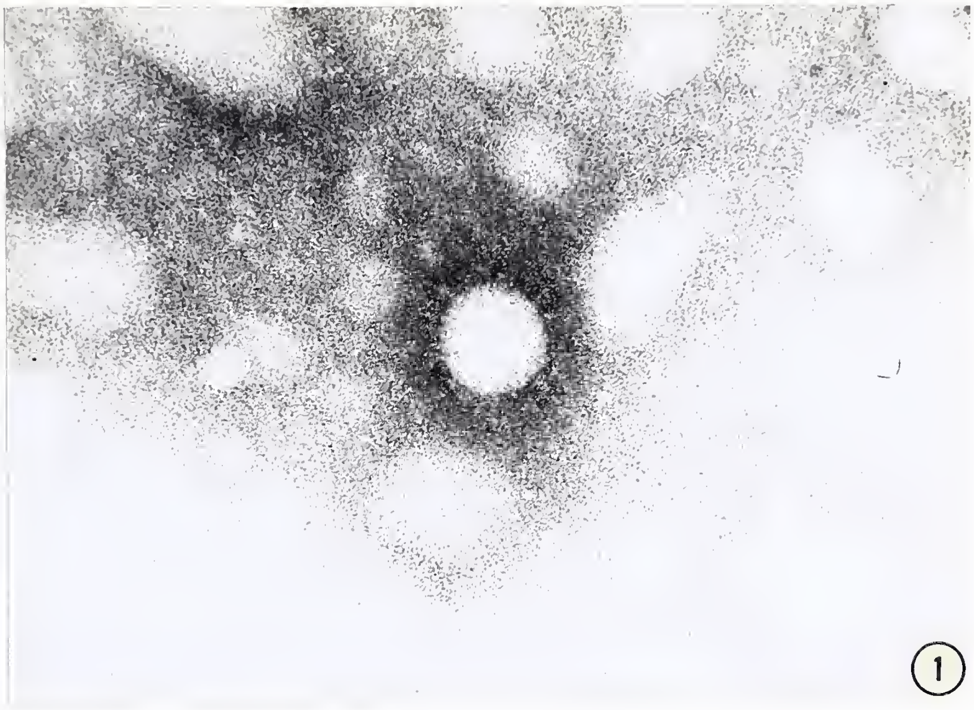
The dramatic agglutination of influenza virions by CRP and guinea pig serum was unexpected, as agglutination is not a usual consequence

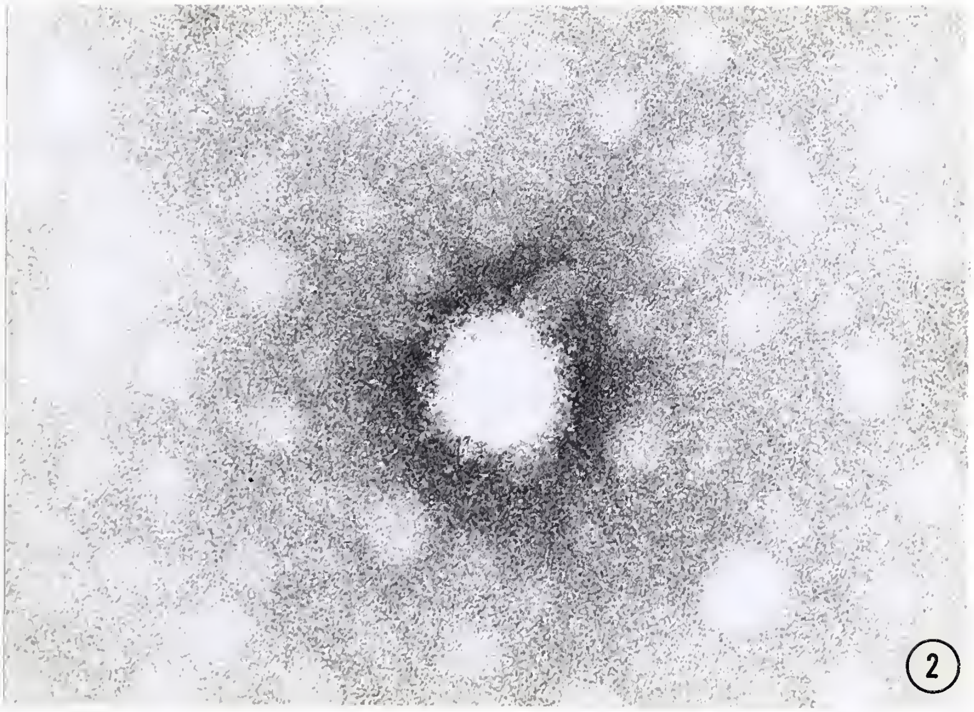
of guinea pig complement fixation. It is unfortunate that there was insufficient time to confirm or extend these preliminary findings. It would be desirable, for example, to attempt to repeat these observations using viable unfixed influenza virions.

This work provided preliminary evidence that C-reactive protein at concentrations seen in acute-phase human serum can interact with the envelope of rubella and formalin-fixed influenza virus in vitro to change membrane meability or configuration. Interaction with serum proteins may ensue which can lead to agglutination or to virolysis. The significance of these findings is unclear, although there is reason to suspect that CRP instigated phenomena may apply to all enveloped viruses. It should be noted that CRP and complement are present in extracellular fluid, where they presumably are able to interact with virus released from host cells or budding through host cell membranes. They are also able to interact with the virus during viremia. In addition, the possibility of CRP-complement interaction with cells bearing viral antigens has been raised. Perhaps CRP plays a very direct role in host defense against viral disease in the days following infection but before an immune response has been mounted.

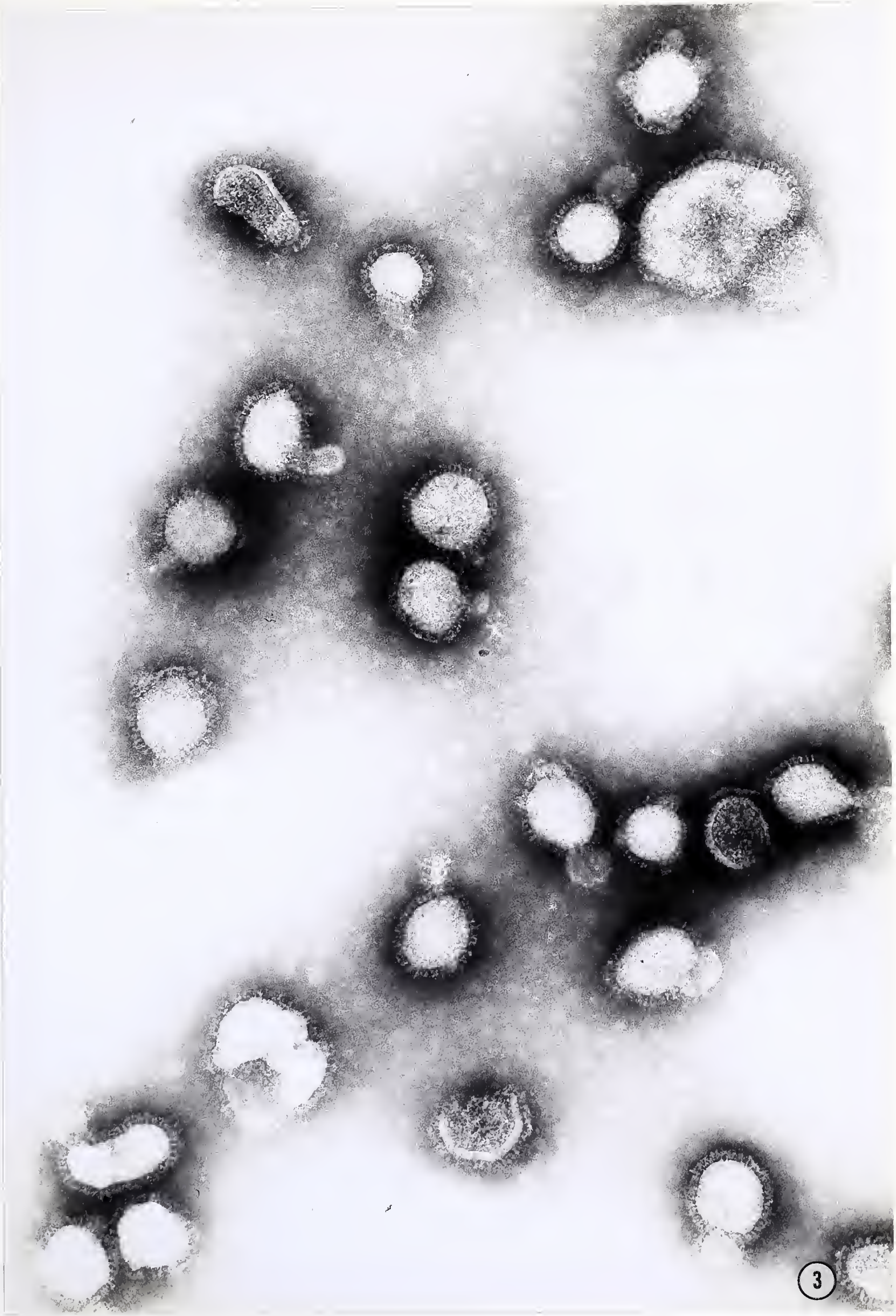
Picture 1 (above) is a rubella virion negatively stained after incubation in CRP-negative column elutant. Its well demarcated border and discreet surface projections are easily seen.

Picture 2 (below) is a negatively stained rubella virion from the sample incubated in CRP. Though the virion core seems intact, the envelope border is not discreet and there appears to be a halo of material fixed to the particle. Both particles are magnified 220,000 x.


①


②

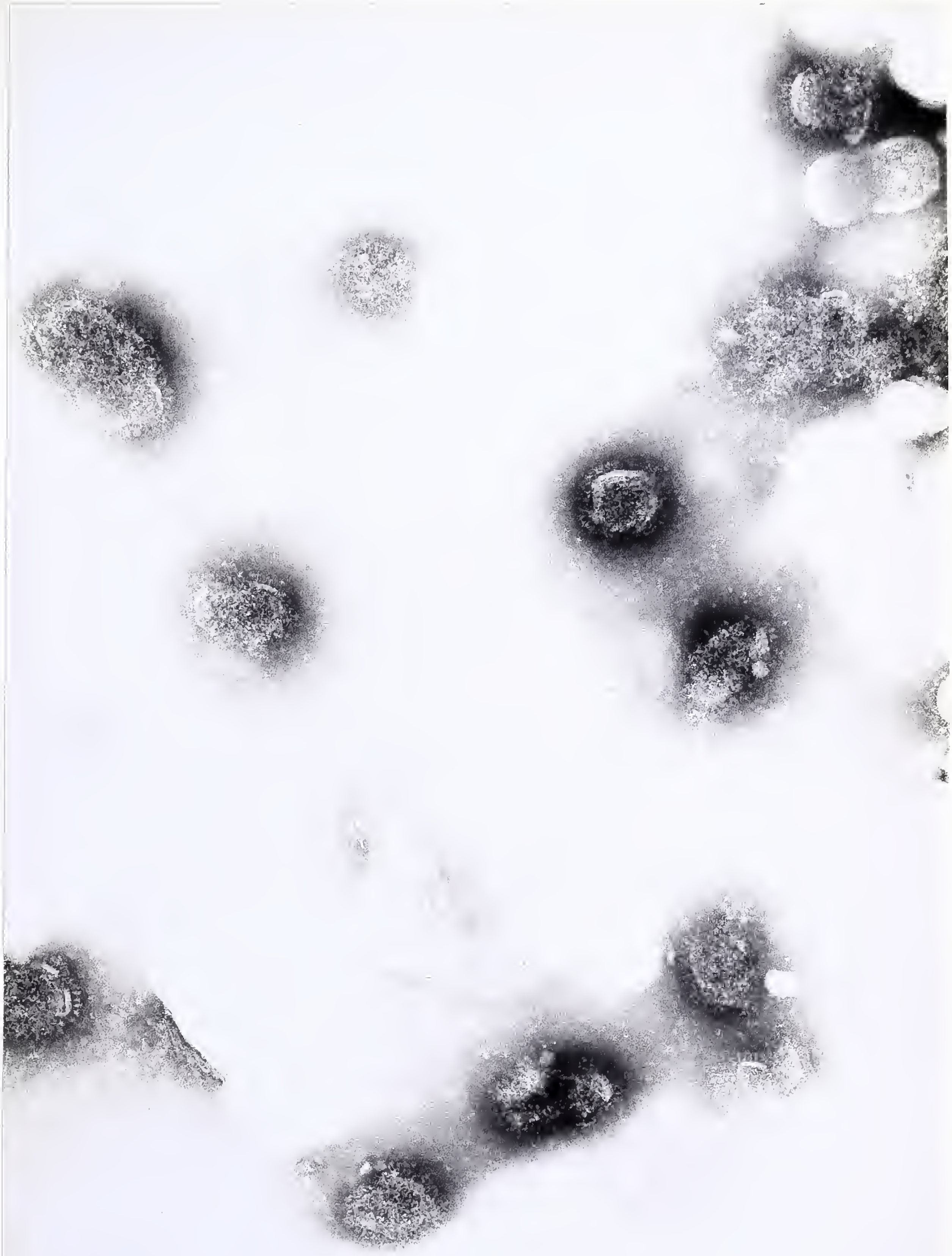
Picture 3 is a negatively stained preparation of formalin-fixed influenza A virus suspended with Tris saline. These virions are intact with the regular envelope glycoprotein projections characteristic of influenza. Magnified 130,000 x.


3

Picture 4 is a negatively stained preparation of the formalin fixed influenza A virus incubated with only the buffer in which the CRP was suspended. These particles are relatively intact and appear similar to those in picture 3, the virus control group. Magnified 130,000 x.

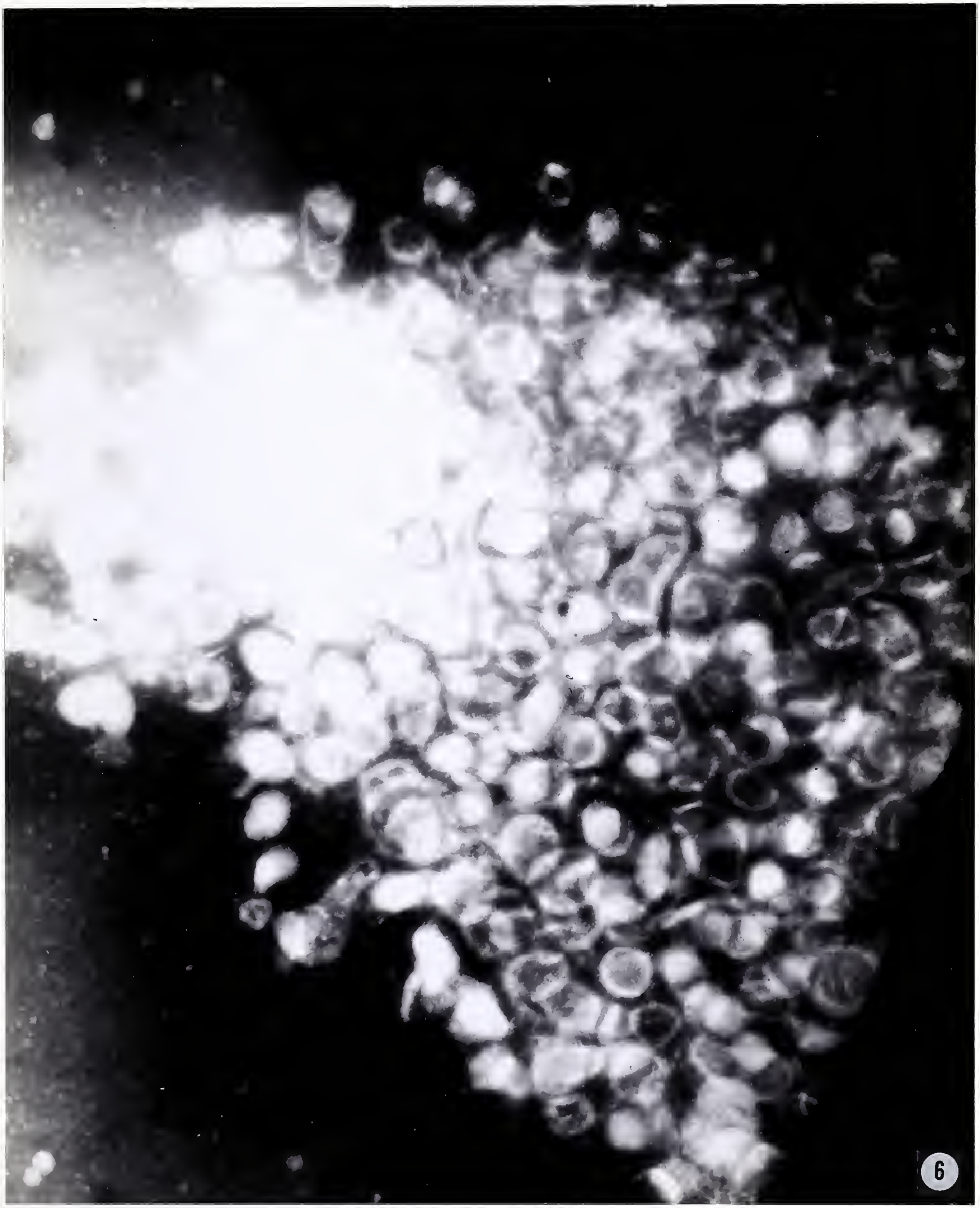
④

Picture 5 is a negatively stained preparation of the influenza A virus which has been incubated with CRP. The virions appear considerably disrupted with negative stain penetrating to the core of many of the particles. The envelope and surface projections appear modified and are less distinct than those seen in the virus control. Magnified 130,000 x.



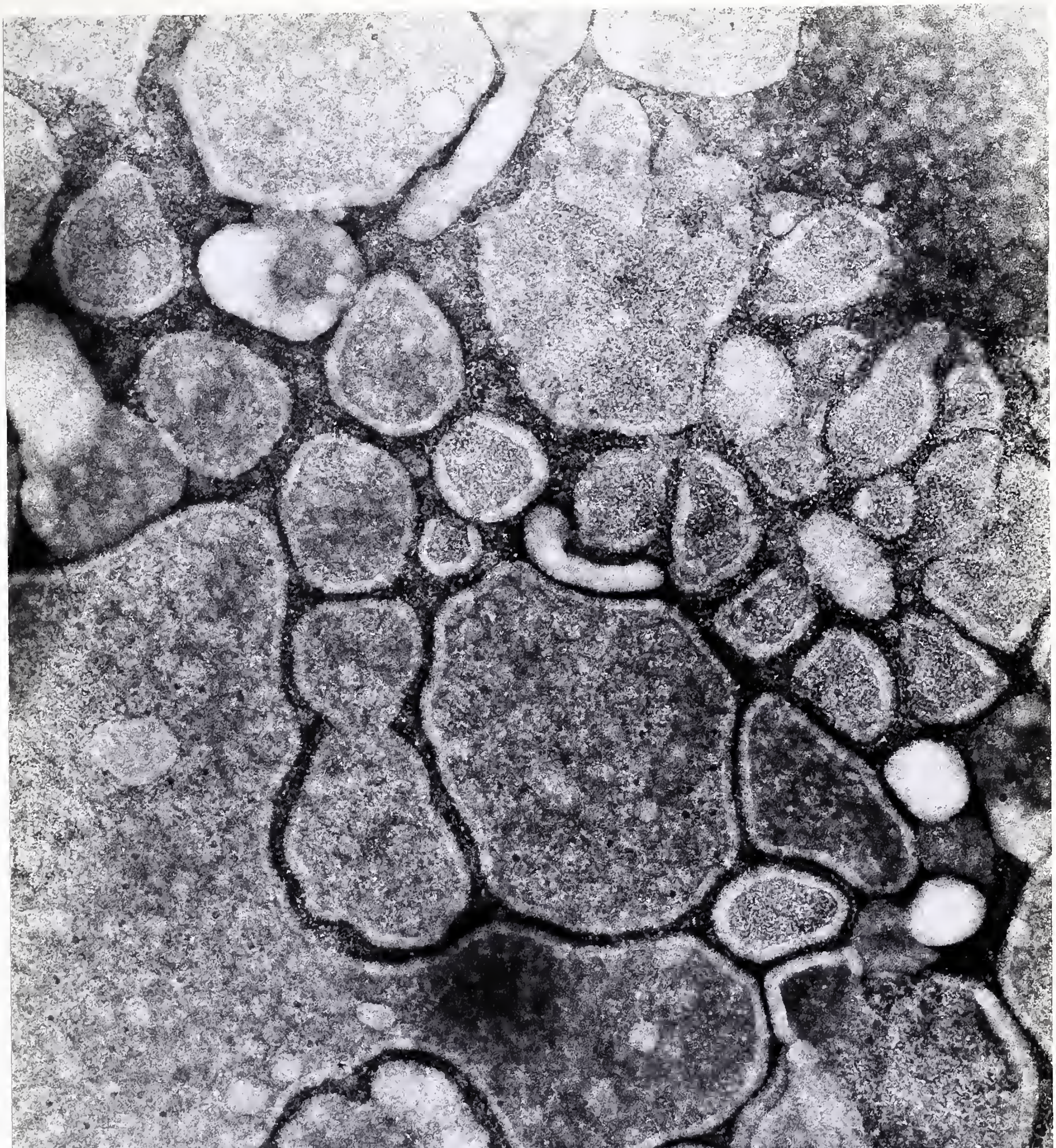
5

Picture 6 is a negatively stained preparation of the influenza A virus incubated with both CRP and guinea pig complement. Large aggregates of virions such as the one above were seen; single virions were quite sparse. Many of the particles in this aggregate have stain which has completely penetrated the interior indicating the presence of envelope defects. Magnified 130,000 x.



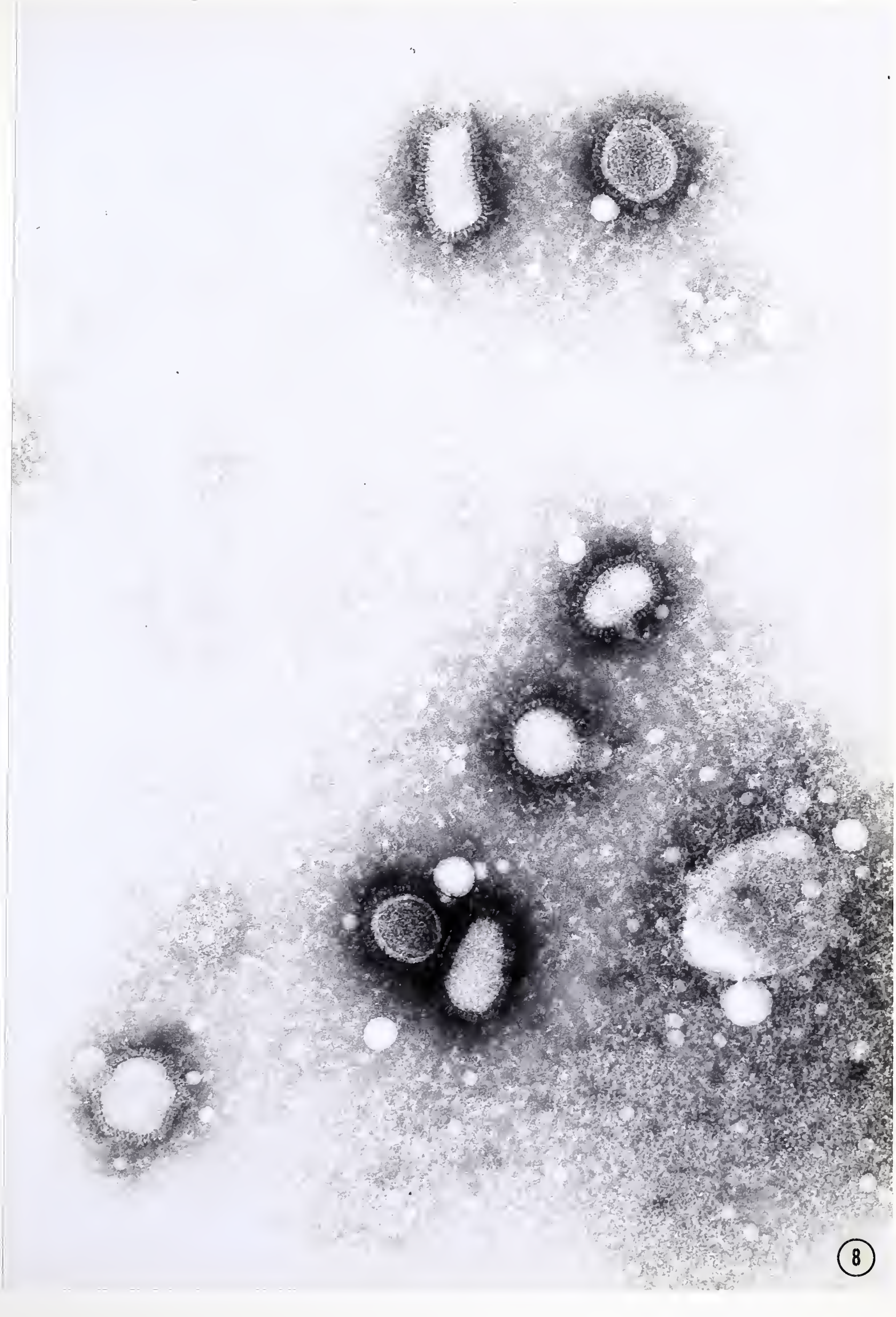
6

Picture 7 is a negatively stained preparation of the influenza A virus incubated with CRP and guinea pig complement. Most of the particles in this micrograph are pieces of cell debris coincidently suspended along with the virus in the vaccine preparation. Note the many complement mediated membrane lesions seen as small dark holes. Magnified 130,000 x.



⑦

Picture 8 is a negatively stained preparation of the influenza A virus incubated with CRP and guinea pig complement heated at 56° C for 30 minutes. These particles are intact and strongly resemble those in picture 3, the virus control. Magnified 130,000 x.



⑧

Picture 9 is a negatively stained preparation of the influenza virus incubated in guinea pig complement (guinea pig serum) alone. The particles also appear intact and resemble the virus control particles. Magnified 130,000 x.

⑨

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