STUDIES ON THE IMMUNOLOGICAL SIGNIFICANCE OF THE ACUTE PHASE PROTEINS OF MAN AND EXPERIMENTAL ANIMALS

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ABSTRACT:

Studies on the Immunological Significance of the Acute Phase Proteins of Man and Experimental Animals.

Evidence is presented indicating that:

Rabbit acute phase protein (Cx-reactive protein) is analogous, for experimental purposes, to human acute phase protein (C-reactive protein).

Injections of Freund's-type incomplete adjuvant regularly produces a detectable level of Cx-reactive protein in rabbits.

Serum containing adjuvant-produced Cx-reactive protein possesses the ability to enhance a specific immune response to a simultaneously injected antigen. Such ability is similar to that possessed by adjuvant itself.

Human C-reactive protein and rabbit Cx-reactive protein share a common antigenic constituent.

Cx-reactive protein contains an antigenic constituent identical with one present in normal (negative for Cx-reactive protein) rabbit serum.

Samples of Cx-reactive protein contained in acute phase sera of different rabbits appear to be antigenically identical.

Cx-reactive protein has an electrophoretic mobility, in agar, similar to that of a Beta-globulin.

Chickens and guinea pigs do not produce proteins directly analogous to Cx-reactive and C-reactive proteins.

I. INTRODUCTION

Any damage to the body tissues of a warm blooded animal is invariably followed by some change in the constituents of the circulating blood (1). Such change may include variations in the quantity of constituents normally present in the blood and/or the appearance of substances considered to be not normally present. One such substance is C-reactive protein (CRP). Since its discovery in 1930 CRP has been shown to occur in a wide variety of diseases. Extensive studies on the physical, chemical and biological properties of CRP have provided much useful information in respect to this interesting protein. Despite this, the site of origin, distribution in tissues, possible function and immunological significance of CRP, are all still obscure. The studies outlined, herein, represent an attempt to determine the immunological significance of CRP. Their design is a consequence of an extensive review of the pertinent literature. The essence of this literature is cited at length. This is purposeful and is considered a necessary prerequisite to the proper understanding of the subject and to the adequate design of the experimental procedures used.

The thesis, itself, represents the outcome of part of an overall research plan, the aim of which is to determine the biological significance of C-reactive protein. As the results of one phase of the study became apparent, each of the subsequent phases presented themselves in a natural fashion and together they formed a logical sequence of five separate, but integrated, experiments.

For the sake of clarity, the aim, materials and methods, results, discussion and summary of each of these experimental phases will be presented separately.

A summary and discussion of the overall study is included finally.

II. Definition of Terms

The following definitions are made to facilitate the reading of the review of the literature which is to follow.

1. C-reactive protein (CRP).

(a) It is an abnormal protein which occurs in human plasma, serous exudates and damaged body tissues following any type of noxious stimulus to the body tissues. It possesses the unique property of forming a precipitate with the somatic polysaccharide of Diplococcus pneumoniae (D. pneumoniae) in the presence of calcium ions and is antigenically distinct from all other serum proteins.

(b) It is not usually detectable in "normal" serum (Normal serum in this sense is considered to be obtained from an individual exhibiting no clinical or laboratory evidence of disease).

(c) It appears in the blood plasma and pathological transudates during the active phase of the body's reaction, after the onset of any tissue insult. It falls to undetectable levels in the convalescent phase serum soon after the removal of the inciting cause.

2. Acute phase serum. Serum obtained from warm blooded animals in the acute stages of body response to any non-physiological stimulus, as compared to the convalescent phase serum which is obtained during the recovery stage of the body reactivity.

3. Agar gel double diffusion test. Ouchterlony double diffusion method (2). This is a method for visualizing precipitation reactions in a semi-solid medium such as an agar gel. Antigen and antibody are put in respective wells at a distance from each other calculated to permit optimal interaction of the diffusing ingredients

in the gel medium.

4. Immunoelectrophoresis. This is a method by which mixtures of antigens and antibodies may be investigated by combining the Ouchterlony double-diffusion method with prior electrophoretic seperation of the antigen on the same agar plate. Increased resolving power over either method alone is obtained in this way. This technic was first described by Grabar and Williams (3).

5. Capillary precipitation test. A procedure for visualising precipitation reaction in a glass capillary tube in which antigen and antibody are allowed to rise and mix through capillary action.

III. REVIEW OF THE PERTINENT LITERATURE.

As defined earlier CRP reacts in a unique manner with the somatic polysaccharide of D. pneumoniae, but does not react with the other antigens present in the organism. Due to the fact that the Forssman antigen and the somatic polysaccharide of D. pneumoniae possess some similar properties, it is felt appropriate to discuss the different antigens of D. pneumoniae.

1. Antigens of Diplococcus pneumoniae.

Diplococcus pneumoniae (D. pneumoniae) were found independently in human saliva by Stenberg and Pasteur in 1881 (4). In 1909 - 1910 Neufeld and Handel discovered that the organisms could be divided into specific groups by immunological methods (4). This discovery was soon confirmed by Dochez and Gillespie who differentiated types I, II, III and a heterologous group IV (4). Subsequently Cooper et al separated group IV into some 30-odd types (5). At present there is a total of 77 recognised types (6).

Antigenic Structure of D. pneumoniae.

To date the following antigens have been isolated and characterised from D. pneumoniae.

(a) Soluble specific substance.

(b) Nucleoproteins.

(c) Somatic polysaccharide.

(d) Forssman antigen.

(e) "M" protein.

(a) Soluble specific substance, also termed as capsular polysaccharide, is responsible for immunological specificity. This substance was first discovered in 1917 by Dochez and Avery who found that it was present not only in the intact bacterial cell, but also in filtrates of D. pneumoniae (7). In 1923 Heidelberger and Avery described a method for the concentration and purification of the soluble specific substance of type II (8). At that time there was doubt as to whether the specific substance itself consisted of polysaccharide. However, in 1924 it was shown that the specific soluble substance was actually polysaccharide in nature and a cell constituent, not a contaminant (9). It was also demonstrated that there existed marked chemical differences between type II and type III specific soluble substances. In 1925 Heidelberger, Goebel and Avery described refinements in the method for the isolation of the specific soluble substance from types II and III (10). The immunological significance of the soluble specific substance was described in a subsequent paper (11). It is now known that each pneumococcus type is characterised by a specific capsular polysaccharide which determines its antigenicity and also its potential virulence.

(b) Nucleoproteins. In 1923 the chemical and immunological properties of the nucleoproteins of D. pneumoniae were described (12). In the following years, numerous other studies were carried out on the chemical and immunological properties of cell constituents of D. pneumoniae. A review and summary of these observations was presented in 1925 by Avery and Heidelberger (11). It is now known that pneumococcal nucleoproteins are comprised of a mixture of proteins. Unlike the capsular polysaccharides, the nucleoproteins do not exhibit type specificity. They however exhibit species specificity and are closely related to the nucleoproteins of the streptococci.

(c) Somatic C-polysaccharide (C-polysaccharide) In 1930 Tillet, Goebel and Avery presented the results of an investigation of a polysaccharide substance derived from a rough strain of pneumococcus type II (13). The substance was chemically and immunologically distinct both from type specific capsular polysaccharide and species specific nucleoprotein. These workers called this polysaccharide substance, Fraction C. The following properties of the somatic C-polysaccharide of D. pneumoniae have been recorded in the literature:-

i. It is an amorphous product, soluble in water and insoluble in organic solvents.

ii. It is a nitrogenous polysaccharide yielding about 6.2% nitrogen, but contains no amino-nitrogen.

iii. It yields about 3% reducing sugars.

iv. It contains phosphoric acid firmly bound in organic combination which is slowly liberated at 100° C by normal solutions of hydrochloric acid or sodium hydroxide.

v. Its immunological specificity is not impaired by the prolonged action of pepsin at pH 2.0 or trypsin at pH 8.1.

vi. A solution of this substance shows no reaction when tested for protein by the standard color tests (i.e. Millon's test, biuret test, Xanthoproteic test and the Hopkins-Cole test). It does not give any precipitate with trichloracetic acid, with picric acid nor with sulphosalicylic acid.

vii. After treatment with nitrous acid, like type specific polysaccharide, it is slowly hydrolysed with corresponding loss in its immunological activity, and appearance of reducing sugars, in a quantity 30% of the polysaccharide.

viii. It gives a strong positive Molisch test.

ix. C-polysaccharide obtained from smooth (S) and rough (R) forms of all D. pneumoniae is identical and is bound to the pneumococcal body itself, and like the nucleoproteins is species specific.

x. Injection of C-polysaccharide in mice and rabbits elicits no toxic manifestations.

xi. It is antigenically inert in purified form.

xii. It is present in very small amounts in the intact D. pneumoniae.

xiii. Its molecule is small enough to pass through parchment membrane.

xiv. Its chemical composition and its serological reactivity separate it from both type specific capsular polysaccharide and species specific nucleoprotein.

In 1946, McCarty and Avery succeeded in obtaining a special

form of somatic polysaccharide from D. pneumoniae which they called Cx-polysaccharide (14). This substance probably represents a more highly polymerised form of C-polysaccharide. Subsequently, in 1951 Anderson and McCarty described a simple procedure for the preparation of Cx-polysaccharide (15). The precise difference between the two polysaccharides has not been determined to date. The two substances appear to be identical in their reactivity with CRP and either substance is capable of removing all CRP from solution.

(d) Forssman antigen (F-polysaccharide). In 1931, Howard and Shorb first discovered the presence of an heterophile antigen in D. pneumoniae (16). In a series of subsequent publications they have described the chemical and immunological properties of this substance as it occurs in the intact cell (17-19). Goebel et al in 1943 isolated it in pure form and called it F-polysaccharide (20). They also did a comparative study on the physical and chemical properties of C and F-polysaccharides. In 1943, the immunological properties of C and F-polysaccharides were studied by Goebel and Adams (21).

F-polysaccharide was found to have the following properties.

i. In the intact cell it is thermostable.

ii. Boiling with acid or alkali or digestion with proteolytic enzymes destroys the antigen.

iii. Unlike C-polysaccharide which passes into solution with the autolysis of D. pneumoniae, F-polysaccharide is firmly bound to the detritus of the autolysed organism.

iv. In the purified dried form it appears as a water

soluble, amorphous powder, free from protein and nucleic acid.

v. It is non-dialysable and gives a faintly opalescent solution which foams on shaking, differing in this respect from C-polysaccharide.

vi. When boiled with mineral acids or when treated with nitric acid it deposits a "lipid-like" precipitate which can be extracted with chloroform. Solution of C-polysaccharide remains clear.

vii. Electrophoretic studies have shown that like C-polysaccharide, purified preparations of F-polysaccharide appear as a single homogenous substance. However, these two substances exhibit a decided difference in mobility.

viii. F-polysaccharide is a lipocarbohydrate. The carbohydrate moiety is bound in firm chemical union to a fatty acid of high molecular weight. The lipid comprises some 6% by weight and can be split off by vigorous chemical treatment. The carbohydrate moiety is either similar to, or identical with, C-polysaccharide on the basis of chemical and immunological studies.

ix. On chemical analysis F and C-polysaccharides are indistinguishable, both liberating the same amount of reducing sugars, nitrogen (6%), amino-nitrogen, phosphorous and amino-sugars on acid hydrolysis. Neither contains protein, purine, nucleotides, nucleic acid or polypeptides.

x. Purified preparations of F-polysaccharide are fully antigenic, stimulating in the rabbits the sheep red cell hemolysin and precipitins for F-polysaccharide but little or no precipitins for C-polysaccharide. C-polysaccharide on the other hand is completely inert

antigenically.

xi. F-polysaccharide differs from other lipocarbohydrate preparations obtained from gram negative bacilli in that it is non-toxic to rabbits and mice.

xii. Both F and C-polysaccharides give a precipitin reaction against a rough pneumococcal strain antiserum. Absorption of such antiserum with F-polysaccharide removes completely precipitins both for the homologous substance and for C-polysaccharide. However absorption with C-polysaccharide removes all the homologous precipitin, but does not completely remove precipitins for the F-polysaccharide. Whether the C-polysaccharide occurs in the intact organism as an individual substance, in chemical combination with some cellular constituents which render it antigenic, or whether it is an artifact derived from F-polysaccharide during the autolytic process, are questions not clearly answered to date. Considering the experimental data which show that rabbits immunized with F-polysaccharide give rise to antibodies which are weak precipitins for C-polysaccharide but precipitate the homologous antigen (21), it is possible that C-polysaccharide exists in the organism independently of F-polysaccharide and that the heterogenic reaction is due to the presence of a common constituent carbohydrate fraction.

(c) The other known antigens of D. pneumoniae are "M" protein (22) and an inactive polysaccharide substance closely related to chitin (23). Whether the inactive polysaccharide substance is a bacterial substance or a contaminant from the culture medium, is not known. The "M" protein of D. pneumoniae is similar to the "M" protein of the Streptococcus and is found in both smooth and rough strains of D.

pneumoniae is type specific but shows dissimilar reactivity between different strains.

2. Discovery of C-reactive Protein.

C-reactive protein (sometimes referred to as acute phase protein) was first detected in 1930 by Tillet and Francis. These workers observed that the somatic C-polysaccharide of Diplococcus pneumoniae gave a precipitin reaction with acute phase sera from patients with pneumococcal infection, but not with sera obtained after the crisis (24). They further observed this reaction in a number of other infectious diseases such as acute rheumatic fever, bacterial endocarditis and staphylococcal osteomyelitis. Ash in 1933 confirmed and extended these findings in sera obtained from children during the acute stage of infections caused by gram negative bacilli of the colon-typhoid group (25). This reactive substance, found only in the acute phase serum, was termed C-reactive substance, due to the fact that it reacted with fraction C, the then newly discovered pneumococcal somatic polysaccharide. Later as the protein nature of the C-reactive substance was revealed by Abernathy and Avery (26), it was renamed C-reactive protein (CRP), the name itself being coined by MacLeod and Avery in 1941 (27).

In 1939 Lofstrom described a substance which occurred in the blood serum in the acute stage of diseases of varying origin and which caused non-specific capsular swelling of D. pneumoniae types 27 and 28 and at times of other types as well (28). Lofstrom designated this substance "non-specific capsular swelling substance" (NCS). In 1944 he proved that NCS and CRP were one and the same (29). This was further

confirmed by Hedlund in 1959 (30).

CRP was at first thought to be a pneumococcal antibody. Even though it was found in other conditions with an etiology unrelated to D. pneumoniae, its presence was regarded as an anamnestic reaction (25). However, later work revealed several properties whereby CRP differed from "classical" antibody. Unlike classical antibody CRP is usually demonstrable predominantly in the acute phase serum, is non-specific in respect to the inciting agent, precipitates along with the albumin fraction following "salt" fractionation of serum, is antigenically distinguishable from any other serum protein normally present, requires calcium ions to enable it to precipitate with C-polysaccharide, and such precipitation can be prevented by the use of calcium binding agents such as oxalate or citrate. In contrast C-polysaccharide antibody reacts with C-polysaccharide even in the absence of calcium ions, is found in the globulin fraction after salt fractionation, and can be detected in highest titre in serum samples obtained after the acute phase of the illness is over. The detection of CRP in the serum was shown to be a valuable clinical measure of the activity of various disease processes. Little was known about the origin, function and fate of this protein, due mainly to the fact that a suitable laboratory experimental system was not available.

Abernathy in 1937 demonstrated that, like that of humans, the sera of monkeys infected with D. pneumoniae also contained a material which gave a precipitin reaction with C-polysaccharide (31). Simultaneous experiments on rabbits showed that the acute phase serum of the rabbit did not react with C-polysaccharide. However, subsequent work

by Lofstrom showed that the acute phase serum of rabbits caused non-specific capsular swelling of certain pneumococci, similar to that occuring with human acute phase serum (32,33). His studies provided presumptive evidence for the presence of a protein in rabbit acute phase serum similar to CRP. In 1951 Anderson and McCarty succeeded in isolating in crystalline form, from rabbit acute phase serum, a protein analogous to CRP (15). This acute phase protein of the rabbit reacts with a closely related form of C-polysaccharide designated as Cx-polysaccharide. It reacts with Cx-polysaccharide in the same manner as CRP reacts with classical C-polysaccharide. The rabbit acute phase protein has been termed as Cx-reactive protein (CxRP), and has been shown to be remarkably similar to CRP in its general properties and the conditions which govern its appearance in the blood. In this respect, CxRP and its production in rabbits is considered to be an adequate experimental analogy to the human system. Attempts have been made to see whether other animal species also produce acute phase protein. Nedlund using Lofstrom's capsular swelling reaction (28), has attempted to demonstrate the capability of mice, hens and guinea pigs to produce NCS, in acute phase sera, with some claims to success (34).

3. The Appearance of C-reactive Protein in Various Diseases.

Numerous papers have been published describing various pathological and physiological states in which CRP has been detected in the serum (25,33-38). These are listed below and their significance will be discussed.

(a) Diseases of the cardiovascular system.

It has been well documented that the appearance of CRP is of

value in the diagnosis and management of cardiovascular diseases (25,33-58). CRP has been shown to be invariably present in cases of myocardial infarction (25,33-46). Its presence appears to be very helpful in the differential diagnosis of coronary insufficiency particularly in determining the presence or absence of myocardial necrosis (38,39,44,46). In cases of old transmural infarction, left bundle branch block and the Wolf-Parkinson syndrome electrocardiogram changes may be inconclusive, yet CRP is invariably detected if myocardial necrosis is present. Following a single episode of myocardial necrosis, CRP appears in the blood 10-24 hours later and disappears usually in one to two weeks. Boltax and Fischel recorded a case showing the presence of CRP within 8 hours after an attack of confirmed myocardial infarction (42). Many cases have been recorded in which CRP appeared in the blood of the patient prior to the appearance of changes in the electrocardiogram (43). Thus the early appearance of CRP may aid in the diagnosis of myocardial infarction. However, the absence of CRP in a single serum specimen cannot be relied upon to reject the diagnosis of myocardial infarction, due to the fact that CRP may not appear until 48 hours after an acute myocardial infarction. A series of CRP tests is useful in evaluating the onset as well as the subsidence of inflammatory and necrotic processes in the heart following myocardial infarction.

In cases of rheumatic heart disease numerous reports have appeared on the detection of CRP and its value as an index of disease activity (25,36-38,47-57). CRP is usually present in rheumatic cases and persists as long as the rheumatic process is active, a few instances

have been recorded where CRP did not accurately mirror the rheumatic activity (53,54). However, in cases where the rheumatic process is inactive, CRP is always absent. Based on these observations the presence of CRP may be considered as the most reliable index of the presence of rheumatic activity.

(b) Diseases of the Lung and Upper Respiratory Tract (25,33,37,38).

CRP invariably appears in the blood in the acute stage of pneumonia and purulent sinusitis. In acute pneumonia the CRP titre reaches its maximum level in the early stage of the disease, then gradually decreases and disappears after 6 to 10 days. Relapses and complications can be revealed by its appearance in the blood (38).

(c) Diseases of the Gastro-Intestinal System.

CRP is usually present in cases of ulcerative colitis (37), and ischio-rectal abscess (35,37). In cases of acute appendicitis CRP is present irregularly, hence its determination is of little value in the differential diagnosis of appendicitis (38). Other gastro-intestinal diseases such as peptic and duodenal ulcer, unless complicated by neoplastic growth or superimposed bacterial infection do not show the presence of CRP (35,36).

(d) Diseasesof the Liver, Biliary Ducts and Pancreas (34,36,37).

The detection of CRP may be of diagnostic aid in the diseases of the liver and biliary ducts (34). CRP is usually present in diseases such as acute cirrhosis of the liver, acute cholycystitis, cholelithiasis (with inflammation), acute pancreatitis, and in carcinoma of the liver, biliary ducts or pancreas. In cases of infectious hepatitis CRP titres seldom reach high levels and usually fall to zero rapidly,

whereas in malignancies of the pancreas, biliary ducts and in acute cholecystitis the titres are often constant and high. In cases of cholelithiasis proper (non-inflammatory) CRP is absent. Presence of CRP in the blood of a patient with a typical attack of gall bladder colic is a good indication that the gall bladder is inflamed (34).

(e) Diseases of the Kidney and Urinary Tracts (35-38).

Pyogenic infections of the kidney and urinary tracts, such as pyelonephritis and acute cystopyelonephritis are invariably accompanied by the appearance of CRP in the blood. Functional disturbances, degenerative diseases and chronic infections seldom induce a CRP response.

(f) Diseases of Possible Immunologic Origin (35-38,51,59-61).

Observations on the appearance of CRP in rheumatoid arthritis have been widely recorded (35,38,51,59-61). CRP in rheumatoid arthritis is usually present in the active stage of the disease. In general, as the condition of the patient improves CRP disappears, however in some cases it may persist for a long period despite subjective and objective clinical improvement (51). A few cases of active clinical rheumatoid arthritis showing the absence of CRP have also been recorded (51,61).

In diseases such as dermatomyositis and osteoarthritis CRP is usually not present in the blood (59). However, in systemic lupus erythematosus (35,36), gout (during acute attacks) (59), rheumatoid spondylitis (61) and polyarteritis nodosa (59) CRP appears almost invariably in the blood.

(g) "Hypersensitivity" Diseases (62- 64).

Diseases in which hypersensitivity is considered to play a prominent etiological role fail to show the presence of CRP, unless

they are complicated by added bacterial infection.

(h) Metabolic Disease and Diseases of the Ductless Glands (35,36,38).

These diseases do not appear to induce a CRP response.

(i) Diseases of the Skin (35,36,65).

CRP appears only irregularly in skin diseases (non-inflammatory). However, bacterial infections of the skin are invariably accompanied by the appearance of CRP in the blood.

(j) Neoplastic Diseases (35,36,60,66,67).

Extensive surveys of the various neoplastic diseases with regard to the appearance of CRP have been presented (35,36,38). CRP is usually present in the blood in widespread malignant diseases. The malignant diseases most often showing the presence of CRP are hypernephroma, Hodgkin's disease, lymphosarcoma, carcinoma of the bronchus, liver, lung, bile ducts, stomach, colon, pancreas and prostate, especially when associated with widespread metastasis. Sprunt and Hale have recommended CRP determination as a valuable tool in the diagnosis of advanced cancer (66).

(k) Hematological Diseases (36,38).

Uncomplicated hematological diseases do not induce a CRP reponse.

(l) Tuberculosis (25,33,35,36,60,68,69).

There are contradictory reports as to the usefulness of the detection of CRP in cases of pulmonary tuberculosis. Lofstrom examined the sera of 54 cases of pulmonary tuberculosis. Using the non-specific capsular swelling reaction method (CSR) he showed that NCS was present in 23 of the cases (33). He also observed that the sera taken during the first week of illness or during an acute recurrence contained NCS,

while the sera taken during the second week of illness were more often negative (three out of eight being positive). This was despite the fact that the accompanying pulmonary changes, fever and the sedimentation rate showed that the disease had by no means entered an inactive stage. His observations are supported by other workers (35,37,68). Rountree and Rantz examined seven cases of early active pulmonary tuberculosis and found that CRP was present in only three of the sera even though acid-fast bacilli were cultured from each case, very close to the time the serum was tested for the presence of CRP (35). Contrary to these findings Hedlund observed the presence of CRP in the serum of all thirteen cases suffering from active pulmonary tuberculosis while six convalescent cases did not show the presence of CRP (38). His results have been confirmed by others (60,69). Walsh et al have recommended that CRP determination is more of a diagnostic aid in inactive rather than active cases of pulmonary tuberculosis (69).

(m) Leprosy (70,71).

A large proportion of active cases of Lepromatous leprosy show the presence of CRP. Active cases of tuberculoid leprosy also show the presence of CRP but in a lesser proportion. Lepromatous leprosy when complicated with secondary amyloidosis generally shows the presence of CRP. At present no information is available on the relationship of secondary amyloidosis and CRP response.

(n) Pyogenic Bacterial Infections (35,36).

CRP invariably appears in the blood in cases suffering from a pyogenic infection.

(o) Infectious Hepatitis (34,36-38,72).

Hedlund in 1947 examined the sera of 26 cases of acute infecti-

ous hepatitis and found NCB in 3 cases (38). However, in his later studies in 1961 on 400 cases of infectious hepatitis he observed the presence of CRP in more than 50% of the cases (34). He observed that CRP appeared mainly during the prelcteric stages of the disease. His results have been confirmed by Selman and Halpern (36). Contrary to these observations Havens, Eichman and Knowlton (72), and Yocum and Doerner (37) failed to observe the presence of CRP in all their cases of infectious hepatitis. Havens, Eichman and Knowlton however used C-polysaccharide as the means of detecting CRP. The reasons why the reports in the literature often seem at variance, may be twofold. First serum samples may be taken at different stages of a single disease in different studies. Furthermore, the use of C-polysaccharide as a means of detecting CRP appears to be less sensitive when compared to the use of specific antiserum to CRP. This latter point will be discussed more fully under "Methods of Detection of CRP" (p. 20).

(p) Mumps (37,68,73).

Cases of mumps both with and without complications have been studied. Stein and Smith studied 51 cases of uncomplicated and 25 cases of complicated mumps (mumps with orchitis) (68). The percentage of cases showing CRP was 72.7% and 84% respectively. Their findings are confirmed by Parker, Stackiw and Wilt who observed the presence of CRP in 21 out of 38 uncomplicated cases studied (73).

(q) Poliomyelitis (33,68,73).

In 1943 Lofstrom examined 18 cases of poliomyelitis and demonstrated the presence of NCB in 17 cases (33). Presence of CRP in poliomyelitis has since been confirmed by others (68,73).

A study of the literature appertaining to the appearance of

CRP in virus diseases suggests that CRP appears in the early phase of the disease. As the inflammatory process in most virus diseases is not very severe and lasts only for a short period, the CRP titre does not reach a high level and disappears from the blood early in the disease (35,37).

(r) Helminthic, Protozoal and Fungal Infections.

These infections are usually chronic in nature and rarely induce CRP response. The available data shows that systemic coccidioidomycosis and amebic hepatitis (complicated with abscess) induce CRP response.

(s) Traumatic States (35,36,74,76).

It appears that any traumatic episode, of sufficient magnitude, invariably is accompanied by the appearance of CRP in the blood.

(t) Physiological States (36,38,56,60 76-84).

Reports on the appearance of CRP in the normal pregnancy are contradictory. Medlund failed to detect the presence of CRP in the blood of 25 pregnant women examined in all trimesters of pregnancy (38). Similar results have been reported by others (36,56,77). However other reports showing the presence of CRP in the blood in pregnancy have also appeared (60,78-84). The appearance of CRP in pregnancy, a normal physiological phenomenon, is hard to explain. It seems that during pregnancy minor injuries to the placenta which are not revealed by other objective or subjective signs may be responsible for a CRP response. An alternative explanation may be that the leakage of incompatible fetal red blood cells into the maternal circulation sets up an immune reaction in the mother with a resultant CRP response. No work has been done in either regard.

CRP is usually present in the peripheral blood during and after labour (77,79-84). However in menstruation CRP does not appear in the peripheral blood (36).

4. Methods for Detecting C-reactive Protein.

CRP in human serum can be demonstrated by the following methods:

(a) Precipitation Tests -

i. In serological tubes.

ii. In capillary tubes.

iii. In Agar Gel by double diffusion technics.

iv. In Agar Gel by Immunoelectrophoretic technics.

(b) Non-specific Capsular Swelling Reactions.

(c) Complement Fixation Tests.

(d) Latex Fixation Tests

(e) Heamagglutination Tests.

(f) Fluorescent Antibody Technics.

(g) Intracutaneous Tests.

(a) Precipitation Tests.

Four methods have been used generally:

i. Tube method.

This was the method first used by Tillet and Francis (24). Since then it has been used with some modifications by various other workers (85,86). The test is performed as follows:

Equal quantities of serum and C-polysaccharide solution are mixed. The mixture is incubated at 37°C for 24 hours and kept at 4°C overnight. The test is read the next day. In positive tests a

precipitate is formed and settles down, and on shaking can be readily dispersed. In this method instead of C-polysaccharide, Cx-polysaccharide or CRP antiserum (ACRP) can also be used. Calcium ions are necessary when testing the serum with C-polysaccharide or Cx-polysaccharide.

ii. Capillary Precipitation Test (Microprecipitin Test).

This test is based on the principles outlined by Lancefield in 1938 (87) and modified by Swift, Wilson and Lancefield (88). Selman and Halpern have described the common pitfalls in the conduct of the test, together with the measures required to correct them (36). The test is performed as follows:

A capillary tube 0.4 to 0.6mm x 75 mm is one third filled with specific CRP antiserum (ACRP) and subsequently to 2/3 with the sera. The tube is then allowed to stand straight in a plasticine rack and incubated at 37°C for two hours and subsequently at 4°C overnight. The tube is read by oblique light. If no precipitate is seen the test is read as zero. A maximum precipitation which almost fills the tube is read as ++++ all gradations between the extremes are encountered. In this test instead of ACRP, C-polysaccharide or Cx-polysaccharide can also be used but the presence of Ca ions is then obligatory.

The precipitation tests (i and ii) employing ACRP are very sensitive and can detect CRP in quantities up to 3 micrograms per ml of serum (89). An accurate quantitation of CRP in undiluted serum is not possible when undiluted C- or Cx-polysaccharide is used. However, with the use of ACRP a semiquantative estimation of CRP can be made from the amount of precipitate formed. When using C- or Cx-polysaccharide the semiquantitative estimation of CRP in the serum is made

by the highest dilution of C- or Cx-polysaccharide precipitated by the serum under test.

iii. Agar Gel Diffusion Technics.

In the study of CRP an agar gel diffusion technic was first used by Libretti, Kaplan and Goldin (90). They used a modification of Oudin's technic as described by Munoz and Becker (91), and studied the penetration of precipitating bands formed due to CRP and ACRP reaction. They further used a modified Ouchterlony technic as described by Nalbert, Swick and Sonn (92). The antigenic constituents of CRP were shown to consist of, at least, 3 distinct components.

A micro-method of agar gel diffusion technic has been described by Parker, Stackiw and Hill (73). Detection and quantitation of CxCP by this technic is described in detail under "Material and Methods" (page 54).

iv. Immunoelectrophoresis (73,93,94).

This technic has also been used to study the biological properties (mobility) of CRP.

(b) Non Specific Capsular Swelling Reaction.

Lofstrom in 1939 described a serological reaction which he found to be due to a substance which occurred in acute phase sera and which caused swelling of the capsules of D. pneumoniae types 27, 28 and sometimes also of types 9,10,16,17,18, 21 and 23. The best results were obtained by using about twenty times greater dilutions of the bacterial bodies than in the regular slide agglutination method. Agglutination followed when the swelling was marked. This reaction, which occurred only with acute phase sera from a variety of diseases

was termed the non specific Capsular Swelling Reaction (CSR) (28). The presence of Calcium ions is obligatory in this reaction. Lofstrom's original description of the test is as follows (33):

"A normal loopful of bacterial suspension is placed on a slide and mixed with a loopful of serum, after which a loopful of Loeffler's alkaline methylene diluted 1:2 is added for staining. A cover glass is put on and a half hour later the preparation is ready for observation under the microscope. An immersion lens and magnification of at least one thousand times is used. Two phenomena are looked for under the microscope. Firstly capsular swelling, which generally occurs instantaneously, and secondly, agglutination which often requires a certain length of time to become stabilized".

A suitable basic density for the suspension corresponds to a twenty fold dilution of Burrough's and Wellcome's opacity tube no.3, which gives a concentration of one to five bacteria per field of vision. This concentration is called density 1, and non specific capsular swelling substance titre 1. The titration of NCS in the serum is done using a constant amount of serum against increasing bacterial concentrations (29). The test is little used in clinical practice.

(c) Complement Fixation Test (C. F. Test).

MacLeod and Avery in 1941 described the specificity of the CRP and ACRP-rabbit reaction by the means of the C. F. Test (27). Muschell and Weatherwax in 1954 presented a modification of this method applicable to the detection of CRP in human serum (78). Later, in 1956, Graf and Rappert presented a quantitative immunochemical method based on complement-fixation for the determination of CRP (95).

The test is performed as follows:

0.1 ml each of complement, buffer and ACRP-rabbit are added to 0.1 ml of varying dilutions of human test serum and the mixture incubated at 4°C overnight. The next day 0.1 ml of sensitised sheep red blood cells are added. After incubation in a water bath at 37°C for 30 mins. the test is read. Controls include normal human serum, normal rabbit serum, complement, ACRP and red blood cells and are put up in the same manner as the test itself. ACRP prepared in animals other than rabbit can also be used. The test has been used for the quantitation of CRP.

(d) Latex Fixation Test.

The demonstration of antibodies by means of precipitin reactions may be rendered more sensitive, and quantitative, by means of agglutinating particles of polysterene latex. The behaviour of such particles in electrolytes of various ionic strenghts and with varying levels of pH has been studied and a laboratory test for rheumatoid arthritis has been developed, based on the use of these particles (96). A similar type of latex fixation test used for the determination of CRP in serum has been described by Singer et al (97).

The test is performed as follows:

Equal volumes of serial two fold dilutions of serum and ACRP coated polysterene latex particles. (0.1 ml of stock suspension of latex and 0.05 to 0.1 ml of ACRP - 5000 to 1000 micrograms per ml - in 10 ml of physiological saline solution) are mixed. The mixture is incubated in a water bath at 56°C for 90 mins., centrifuged at 2300 RPM for 3 mins. and then read microscopically for the agglutination of latex particles, grading the quantity from zero to 4-plus.

Agglutination in a dilution 1:20 (or greater) is regarded as a positive test. The test seems to be quite promising and it is said that it can be used to quantitate CRP.

(e) Hemagglutination Test.

An hemagglutination test for the demonstration of CRP was first described by Gal and Miltenyi in 1955 (98). The test is based on the observation that C-polysaccharide will passively absorb onto the surface of various erythrocytes. It has been found that red blood cells sensitized in this manner are agglutinated to high titre by sera containing CRP.

The test is performed as follows:

A 5% suspension of erythrocytes is suspended in a 0.5% solution of C-polysaccharide. After incubation in a water bath at 37°C for 2 hours the sensitised erythrocytes are washed twice with physiological saline. Equal volumes of a 1% suspension of sensitized erythrocytes in physiological saline and varying dilutions of inactivated serum (inactivated at 56°C for 30 mins.) are mixed and incubated in a water bath at 37°C (repeatedly shaken during incubation). After a further incubation at 4°C overnight the test is read for agglutination of the red cells. Calcium ions are not obligatory for this test.

When applying the hemagglutination method in clinical practice, titres of less than 1: 150 are not considered as positive, as it has been shown that many normal sera agglutinate sensitized sheep red blood cells to a titre upto 1:75.

(f) Fluorescent Antibody Technics.

Methods have been described which employ fluorescent antibodies either by direct or indirect methods, as described by Coons et al (99,100). In essence these methods consist of labelling antibodies with a fluorescent dye, allowing the labelled antibodies to react with their specific antigen, and observing the reaction product under a fluorescent microscope. The principle can be applied to a variety of methods (101). Such fluorescent antibody technics have been made in the study of CRP. The technic has a limited application to the detection of CRP in the blood (102). However, it offers a good opportunity for the immunological study of CRP (103).

(g) Intracutaneous Test.

Francis and Abernathy in 1934 reported a delayed cutaneous reaction to the somatic C-polysaccharide (104). The reaction was positive only in the acute phase and became negative in convalescence. The reaction was nonspecific as it could be elicited not only in cases of pneumonia, but in other infections as well. Normal human subjects gave no response. Finland and Dowling in 1935 confirmed these results (105). In 1937 Francis and Abernathy published a detailed report of studies on C-polysaccharide and noted that the cutaneous reaction to C-polysaccharide could not be elicited in fatal cases during the acute stage of the disease (85). The reason for failure to elicit the skin reaction in fatal cases is not clearly understood. It is supposed

that the tissue reactivity to C-polysaccharide is depressed by toxic products of the disease in fatal cases.

The test is performed as follows:

0.1 ml of a solution containing 0.1 mg of C-polysaccharide is injected intra-dermally. Physiological saline is used as a control. The inoculated sites are observed frequently during the following 24 hours. The character of the positive cutaneous reaction is distinctive and has been described as follows (65):

"Within 15 to 30 mins. following the intra-dermal inoculation of 0.1 mg of C-substance there appears at the site of injection a wheal surrounded by a zone of erythema. This reaction in its early development resembles the capsular-polysaccharide skin test but the zone of erythema is less intense and pseudopods extending out from the central wheal so frequently seen in the latter are rarely encountered. Within an hour this acute reaction has usually faded and is then followed by a delayed reaction, an oedematous erythema. The centre of the area of delayed erythema is frequently dark reddish brown, sometimes haemorrhagic, around which there may be a pale halo and beyond this a bright red erythema. The delayed reaction begins to appear in 2 to 3 hours, is well marked in 6 to 10 hours, persists for 18 to 24 hours and fades leaving a residual brown stain. The size of the reacting area may reach 5 cm. in diameter but is usually 2 to 3 cm. in diameter when maximum. There is frequently tenderness at the site of the injection. The skin test is considered

positive if delayed reaction occure with an area of erythema larger than 1 cm. in diameter. The reaction is called doubtful if the erythema is between 5 and 10 mm. in diameter and negative if the erythema is less than 5mm. by 5mm."

The intracutaneous test for detection of CRP in human sera is of academic interest only as the much more simple, sensitive and faster tests are routinely used.

Sensitivity of the Various Tests Used for the Determination of CRP in Serum.

Various reports have appeared in the literature as to the sensitivity of the various tests used in the detection of CRP in serum:

(a) MacLeod and Avery compared the sensitivity of the C-polysaccharide-CRP precipitin (C-polysaccharide precipitin) test with the ACRP - CRP precipitin (ACRP precipitin) test and found the ACRP precipitin test more sensitive (27). McCarty compared the Cx-polysaccharide - CRP precipitin test with the ACRP microprecipitin test and found the latter more sensitive (89).

(b) Hedlund compared CSR with the ACRP microprecipitin test and found the two equal in sensitivity (106). However McCarty states that CSR has a sensitivity of the same order of magnitude as the C-polysaccharide precipitin test (107).

(c) Cel and Miltenyi compared the hemagglutination (HA) test with the C-polysaccharide precipitin and the ACRP microprecipitin test and found the HA test equal in sensitivity to the C-polysaccharide

precipitin test (98).

(d) Muschel and Weatherwax compared sensitivity of the C.F. test with the ACHP microprecipitin test and found that the C.F. test was found more sensitive (73). Contrary to these observations Rapport and Graf found the latter two tests equal in sensitivity (95).

(e) Singer et al compared the latex fixation test and ACHP microprecipitin test and observed that both tests correlated with each other, in sensitivity (97).

(f) Libretti, Kaplan and Goldin have mentioned that the agar gel diffusion test can detect the presence of small amounts of CRP in the serum (90).

As the various tests for the detection of CRP in the serum are based on different principles, it is hard to make a definite statement as to which test is most sensitive. However, considering the various reports mentioned above it seems that the ACHP microprecipitin test is sensitive enough to detect the presence of quite small amounts of CRP in serum.

5. Mode of Production of Acute Phase Protein.

Acute phase protein can be produced both in man and animals by a variety of stimuli.

In 1943 Lofstrom used sulphur (33), and in 1944 Medlund used several other agents (106), all parenterally, and found them to be effective in producing acute phase protein. In 1953 Wood used certain adjuvants for the stimulation of CRP production (109,110). Experiments with substances that do not produce an inflammatory reaction but exert a direct cytotoxic effect on bone marrow and the

lymphoid tissue have also been conducted (111-113).

As a result of these and other experiments it has been shown that acute phase protein appears in the blood following most types of inflammatory stimuli. These include the following:-

Artificially induced skin lesions using solid CO_2, heating in an incubator at 37°C, exposure to irradiation, injection of radio-mimetic drugs, manganese, gold and copper salts, sulphur, sterile milk, typhoid vaccine, living or dead micro-organisms, pepsin digested suspensions of a variety of rabbit tissues such as heart, lung, liver, kidney and muscle, amino-acids, peptone ordinary saline, purified preparations of CRP, seromucoid from rabbit acute phase serum, heterologous rabbit blood and a variety of adjuvants have all been found to induce CRP response (15,34,108-116). Trauma induced as a result of cardiac or intra-peritoneal puncture does not result in a CRP response.

Various agents differ from each other in their stimulatory activity. A greater acute phase protein response is achieved after stimulation with a relatively small dose of typhoid vaccine than with sulphur (34). Parallel to the stimulatory activity of various substances varying reactibility on the part of individual rabbits has also been observed.

The individual acute phase protein response has been of practical use in the preparation of CRP antiserum. Wood administered crystalline CRP to rabbits showing good CRP response and observed that these animals produced a high titre CRP antiserum (109).

Hedlund has shown that after repeated injection of manganese salts in sick individuals CRP response gradually decreased in strenght

sometimes disappearing completely and remaining negative despite renewed injections of manganese, but on the administration of sulphur the usual acute phase response was again produced (108). In rabbits a series of repeated injections with a constant dose of manganese and sulphur yielded higher CSR titres at the beginning of the series than at the end (108). Montella and Wood found that after repeated injections of thorotrast (colloidal thorium dioxide) the CRP response to thorotrast diminished and ultimately disappeared (115). The authors believed that the reticulo-endothelial system (RES) was effectively blocked by the thorotrast and thus the production of CRP by the R.E.S. was impeded. They also stated that another explanation might be direct toxic effects of thorotrast on the cells (other than R.E.S.) which produce CRP. Hedlund further observed a reduced CSR effect in rabbits after a second injection of typhoid vaccine and attributed the reduction in CSR titre to the antibodies produced in response to typhoid vaccine (34).

Experiments to induce CRP response in humans have been limited. Injections of manganese salts, sulphur, typhoid vaccine, and sterile operations have been shown to induce CRP response (15,74,75,108, 117). CRP response after the artificial induction of fever is irregular. Experimentally induced fever of 10 to 20 hours duration as a result of the continuous intravenous administration of saline containing formalin-killed aerobacter-aerogenes has been shown to induce CRP production (117). However elevation of body temperature by heating human subjects in an ultrapandorous apparatus did not induce CRP response (34).

Attempts have been made to evaluate the CRP detection as a

screening procedure for blood donors and a bases for predicting minor non-hemolytic transfusion reactions of the recipients of blood. Knights, Hutchins and Morgan observed that all their patients who experienced chills after transfusion, showed CRP in the serum (36). They considered that this was probably a type of immune reaction.

6. The Appearance Time of Acute Phase Protein.

Many statements have been made about the appearance of acute phase protein in man and animal. Gartens using CSR demonstrated NCS in the blood 24 hours after sterile operations on the knee (74). Bjornesjo, Werner and Odin, demonstrated CRP 12 to 18 hours after operations (116). The shortest time noted for the appearance of CRP is 8 hours after the clinical onset of myocardial infarction (42). Anderson and McCarty in experiments on human volunteers showed that CRP appeared in the blood 18 hours after a single subcutaneous injection of triple typhoid vaccine (16). Hedlund observed the appearance of CRP 12 hours after the onset of the inciting stimulus (119). The shortest appearance time of CRP under experimental conditions after a single intravenous injection of typhoid vaccine has been shown to be 6 to 12 hours (120).

The appearance time of CxRP has been studied most extensively. Hedlund noted the appearance of NCS in serum 20 hours after a single intramuscular injection of 1.0 ml of typhoid vaccine (34). Anderson and McCarty observed CxRP in the serum 12 to 18 hours after stimulation. Artificial heating of rabbits in an incubator at 37°C has been shown to induce CxRP response in 6 hours time.

7. The Occurrence of Acute Phase Protein in Animals.

The acute phase protein has been found in man (24), monkey (31), and rabbits (15). Earlier efforts by Lofstrom to induce an acute phase response in horses and guinea pigs failed (33). However Hedlund in 1961 using the CSR technic reported the occurrence of MCS in acute phase serum sampled of mice, hens and guinea pigs (34).

8. Physical, Chemical and Biological Properties of C-reactive Protein.

CRP is reported to possess the following properties:

(a) When present in human acute phase serum or as a crystaline, preparation, it gives a precipitin reaction with C- and Cx-polysaccharide. For this reaction traces of ionized calcium are obligatory even though sodium chloride may be present in optimal concentration. The precipitability of calcium free acute phase serum is completely restored upon the additions of calcium chloride to a concentration much lower than that normally present in the blood (26).

(b) It is protein in nature. The chemical analysis shows the nitrogen content to be 14.66% with no detectable level of phosphorus (86).

(c) When heated to a temperature above 65°C, acute phase serum loses its property of precipitation with C-polysaccharide. At these temperatures many other serum proteins are also denatured (26).

(d) Salt fractionation of acute phase serum or pathological transudates precipitate a CRP-lipid complex along with plasma albumin between 50 and 75% salt (ammonium sulphate) saturation (26,121).

(e) CRP in serum, as well as in pathological transudates, is associated with lipids. In this form CRP is sensitive to calcium ions, forming a precipitate (122). Dialysis of the whole acute phase serum or pathological transudate against a 0.01% calcium chloride so-

lution fails to precipitate CRP. However, if the precipitate obtained by 50 to 75% salt saturation is itself dissolved in normal saline or distilled water, and then dialysed against 0.1% calcium chloride solution, the CRP lipid complex is precipitated out. After removal of the lipids CRP is still precipitated between 50 to 75% salt saturation but is no longer sensitive to calcium ions shown by its solubility upon dialysis against calcium chloride solution (27).

It seems that the solubility of CRP is conditioned by the interaction of two factors:

i. The presence of certain lipids with which, it is intimately associated.

ii. The marked sensitivity of this CRP-lipid complex to calcium ions.

Although important in determining the solubility of the C-reactive protein, lipids are not essential in the C-polysaccharide-CRP reaction since they can be extracted, without impairing the reaction. However for the precipitation reaction with C-polysaccharide, traces of calcium ions are necessary irrespective of the presence or absence of lipids (26,121,122).

(f) Addition of an excess of ionised calcium to acute phase serum precipitates CRP along with other normal serum proteins but from this state separation of CRP in a purified form is difficult (122).

(g) Crystalline preparations of CRP have been obtained from pathological transudates (86,121), but not from acute phase sera.

(h) Crystalline C-reactive protein has a very low solubility in distilled water in the absence of other serum proteins.

Solutions of crystalline CRP on dialysis against distilled water precipitate out. However, the addition of other normal serum proteins such as albumin exert a stabilising effect on CRP as shown by its easy solubility and absence of precipitation upon dialysis against distilled water. Temperature also has an effect on the solubility of CRP. Solutions of relatively low concentrations (0.1 to 0.5%) prepared at 37°C tend to precipitate on cooling (86).

(i) CRP has its isoelectric point at pH 4.82 as determined by free electrophoresis (121). Electrophoresis studies on CRP have shown its mobility to be in line with the fast moving components of gamma globulin. Detailed discussions on the electrophoretic studies of CRP will be described elsewhere.

(j) The ultracentrifugal studies of crystalline preparations of CRP in a centrifugal field of 240,000 g. show that the protein moves as a single, sharp and homogenous peak. Throughout the run the S_{20}^{w} of CRP has been shown to be 7.5 (121).

(k) Immunological studies on CRP have been shown it to be antigenically identical in all human sera (123), but distinct from all normal serum proteins. CRP antiserum prepared against purified preparations of CRP from human acute phase serum show a trace reaction with normal human serum (27). However, CRP antiserum prepared by immunising rabbits with crystalline CRP obtained from human pathological transudates does not show any cross-reaction with normal human serum (86). The CRP-ACRP (CRP-antiserum) reaction is independent of calcium ion requirements. CRP antiserum has been prepared successfully in a variety of animals such as the horse, goat, sheep, cat, guinea pig

and rabbit.

(l) CRP is thought to consist of more than one component when studied in gel diffusion and cross-absorbtion experiments (30, 90,93,124). Libretti, Kaplan and Goldin using gel diffusion technics have shown that CRP consists of three antigenic components (90). Their results have been confirmed by others (93,124). The cross absorption experiments on human acute phase sera have all shown that CRP consists of at least two components (30,124). Contrary to these observations Kapport and Graf have reported that CRP consists of one antigenic component only (95).

(m) Studies on CxRP have shown it to be remarkably similar to CRP in regard to its general properties and the conditions which govern its appearance (15). In the presence of calcium ions rabbit acute phase serum and crystalline preparations of CxRP give a precipitin reaction with Cx-polysaccharide but not with C-polysaccharide. However CxRP-Cx-polysaccharide precipitin reaction is completely inhibited by Cx-polysaccharide at a concentration of 0.25 mg/ml (15). CxRP-Cx-polysaccharide precipitates can be redissolved in sodium citrate solution. CxRP in rabbit acute phase serum is also associated with lipide. Salt fractionation of rabbit acute phase serum between 50-75% salt (ammonium sulphate) saturation precipitates a CxRP-lipid complex along with the albumin fraction. Dialysis of this fraction against 0.01% calcium chloride solution results in precipitation of the CxRP-lipid complex. Delipidisation of rabbit acute phase serum does not effect the precipitability of CxRP in salt fractionation, but renders it insensitive to calcium ions as shown by the absence of any precipitation

on dialysis against 0.01% calcium chloride solution. Crystalline preparations of CxRP, free from any contaminant normal rabbit serum proteins, have been successfully prepared. CxRP, although antigenically identical in all rabbits, is distinct from other normal rabbit serum proteins. Specific antiserum to CxRP has been prepared in a variety of animal species.

(n) It appears that both CRP (25,79,80,82) and CxRP (34) can be produced at any time in extra-uterine life. It has also been shown that CRP does not pass through the placenta (79,80,82,84) is not present in colostrum and is not present in human milk (125,126).

(o) The first electrophoretic studies on CRP were done by Perlman, Bullowa and Goodkind in 1943 (127). By moving boundary electrophoresis they studied acute phase sera before and after the removal of CRP by C-polysaccharide and also determined the electrophoretic components of serum obtained by the fractionation method of McLeod and Avery (122). They were unable to determine conclusively the fractions of serum with which CRP migrated, but suggested on the basis of indirect evidence that it was an alpha-globulin. In 1954 Wood, McCarty and Slater by means of free electrophoresis found that crystalline or lipid-bound CRP migrated as a beta-globulin (121). Addition of purified lipid-bound CRP to a normal serum gave an identical electrophoretic pattern, under similar conditions. When zone electrophoresis of an acute phase serum in a starch medium was conducted, CRP was located in the fast moving gamma-globulin region. Addition of purified lipid-bound CRP to a normal serum gave identical results under similar conditions. It was further shown that the retarded mobility of CRP on starch medium was

independent of any interaction of other serum proteins. Subsequently to this work other electrophoretic studies on CRP have confirmed these results (128,131).

Recently immunoelectrophoretic studies on CRP have shown its mobility to be that of a fast gamma-globulin ($gamma_1$ or $beta_2$) (73).

Some indication of an antigenic similarity between the acute phase proteins of different species have been found by McLeod and Avery in 1941, who showed that CRP antiserum prepared in rabbits gave a precipitin reaction with monkey acute phase serum. In 1960 Gotschlich and Stetson in a study on human, monkey and rabbit acute phase proteins demonstrated cross reactivity by capillary precipitation, double diffusion in agar, passive cutaneous anaphylaxis and delayed skin reactions (132). The fact that the acute phase proteins of man, monkey and rabbit gave a precipitin reaction with Cx-polysaccharide indicates that these acute phase proteins are probably related antigenically. This interpretation is further supported by the results of double diffusion studies which show partial crossing between the acute phase proteins from man, monkey and rabbit (103,132).

9. Site of Origin, Function and Fate of C-reactive Protein.

The site of origin of CRP is still unknown. In this current literature, however there are certain reports of studies which suggest many possible sites of origin and tend to rule out others. The following points have arisen from this literature:

(a) Attempts have been made to detect NCS in lymphocytes, fluid lymph, lung, liver, kidney, spleen tissue taken from rabbits with a high CSR titre. Similarly, attempts have been made to detect CRP in human leukocytes taken and tested during the acute phase of pneumonia. All such attempts have yielded negative results (34).

(b) In 1961 Kushner and Kaplan put forward a hypothesis that CxRP is produced locally in the tissues, at or adjacent to the site of inflammation (103). Using a fluorescent antibody technic they showed the presence of CxRP within the necrotic muscle fibres at an inflammatory site produced, after a latent period of 8 to 10 hours, by the intramuscular injection of typhoid vaccine. However CxRP could not be detected in the necrotic tissue before its apperance in the blood, thus the secondary deposition of CxRP from the blood at the site of inflammation cannot be excluded.

(c) CRP response to inflammatory stimuli is retained following induction of granulocytopenia by nitrogen mustard (133-a). This suggests that circulating granulocytes play only a minor part in the mechanism of CRP production, if at all.

(d) Reduction of the acute phase response and the attendant disappearance of CRP has been observed many times clinically in the treatment of various diseases with ACTH, cortisone, salicylates and amidopyrine. However cortisone and salicylates have been shown to be ineffective in suppressing the experimental induction of CxRP in rabbits (120,134).

(e) Administration of the inflammatory steroids (methyl prednisolone) and fluorometholone prevent the appearance of CxRP following the subcutaneous injection of an adjuvant (mineral oil - Aquaphor emulsion). Fluorometholone has also been shown to block the CxRP response following the injection of acute phase seromucoid, tobacco mosaic virus (TMV), and to the intravascular formation of soluble antigen-antibody complexes. However, it does not block the CxRP response induced by total body irradiation (250 r) or by injection of thorotrast (thorium dioxide) 2.0 ml/kg, and it is only partially effective in blocking the response due to intra-

venous injections of varidase (5000 units) (135).

The inability of fluorometholone to block CxBP responses to X-irradiation and thorotrast is in distinction to the activity displayed against CxBP response induced by other stimuli. This difference may be attributed to the presence of alternate pathways via which production of CxBP may be stimulated. There may even be more than one mechanism for the actual production of CxBP. Another explanation may be related to differences in the intensity of the inflammatory processes, elicited by the various stimuli. The data available at present is insufficient to explain these apparently contradictory findings.

(f) It has been contended that CxBP response is elaborated by some cellular component of the reticuloendothelial system (RES), in response to appropriate stimuli (109,113,115,136). This contention is supported by the experimental evidence that extensive blockade of the RES with thorotrast markedly reduced the capacity of rabbits to elaborate CxBP (115). Although suggestive this evidence is far from unequivocal. It may well be that thorotrast is able to block equally well both the RES and some other quite unrelated system which controls CxBP production. This is supported by the evidence that the CxBP response can be prevented by fluorometholone without modifying the specific antibody response to occur after the injection of a suitable dose of BSV (135).

(g) "In vitro" studies on the physiological activities of CRP have been done and the following observations recorded:

i. Acute phase human sera exert no leukocidal effect, yet purified preparations of CRP, used in the same concentrations which exist in acute phase sera, have been shown to be toxic towards normal human leukocytes. It appears that the normal serum proteins present in serum

may act as stabilizing agents against the toxic effects of CRP (137).

ii. Acute phase human serum stimulates normal human leukocytes to migrate twice as rapidly as does the normal human serum. Although purified preparations of CRP, when used in concentrations of 0.004 mg/ml, appear to stimulate a similar increase in the rate of leukocyte migration, the observation may not be valid as the negative control used was saline and not a normal serum fractionated in a similar manner as that used to provide the purified CRP. Concentrations higher than 0.005 mg/ml were shown to rapidly destroy the leukocytes (137).

iii. Pretreatment of bacteria with purified preparations of CRP enhances the phagocytic activity of leukocytes in heparinized normal whole blood. Yet the parallel addition of similar bacteria to the same batch of purified CRP and a further aliquot of the same heparinized whole blood, previously mixed together, showed no increase in phagocytic activity of leukocytes, after the subsequent addition of the bacteria. It has also been observed that addition of heparin to CRP reduces its reactivity with CRP antiserum when used in the agar gel double diffusion technic (138). It has been postulated from this observation that the bacteria and the heparin have a competitive action for the surface of the CRP molecule. Addition of heparin to the mixture of CRP and whole blood appears to bind the CRP molecule and render it unavailable for use in coating the bacteria, and therefore there is not the expected increased phagocytic activity on the subsequent addition of the bacteria. However pretreatment of bacteria with CRP alters the bacterial surface in such a manner that their affinity for the leukocytes in the heparinized normal

whole blood is enhanced.

iv. It has been suggested that a possible function for CRP is to combine with antigen early in a pathological tissue reaction, in this way rendering the antigen harmless, until such time as the specific antibodies are developed (74). This view is upheld by the experimental evidence that partially purified CRP shows neutralizing and complement-fixing activity in low titre by cytopathogenic inhibition tests on monkey kidney cells and by complement-fixation tests, respectively (139).

(b) The significance of CRP in the antibody production mechanism is still unknown. A correlation between the early production of CRP and the subsequent production of a significant titre of precipitating antibodies to an introduced antigen has been shown (109, 140). Further it has been shown that substances e.g. adjuvent, which induce CxRP response in rabbits, also enhance the production of antibody to antigens introduced at a separate site (110). These results have lead to speculation that the acute phase protein is directly concerned in the chain of events that lead to antibody production. On the other hand suggestions have been made that the degree of inflammatory reaction in the body influences the quantity of antibody production (141,142), and if so the appearance of CxRP may be correlated with the amount of antibody merely because it is related to the amount of inflammation produced by the injection of antigen. Good has noted that individuals with either congenital or acquired hypogammaglobulinemia, including the most severe cases, are capable of giving a number of normal acute phase responses, including CRP (133-b). He has suggested that the formation of CRP is not directly linked with the production of antibodies. In the same vein Riley, Coleman and Nokama have noted that doses of X-irradiation sufficient to inhibit antibody synthesis do not alter the ability of the rabbit to synthesise CxRP (113,135).

IV. MATERIALS AND METHODS.

Materials:

The sources of all commercial materials and origins of all stock antigens described hereunder, are listed, for the sake of clarity, in appendix form at the end of this thesis.

1. Antigens:

(a) Echovirus antigens:

Stock Echovirus types 22,24,25 and 28 were grown in roller tube cell cultures of trypsinized second generation human amnion cells of blood group type "O". The titres (TCD_{50}) of the viruses used to infect the cells were $10^{5.5}$, $10^{5.3}$, $10^{6.1}$ and $10^{6.5}$ per 1.0 ml respectively. A 0.1 ml aliquot of the appropriate virus type was used to inoculate each tube of cell culture. The tubes were incubated at 37°C in stationary racks and read every day. When the cytopathogenic effect (CPE) was complete in each tube, the infected tubes were stored at -20°C. All the stored tubes were subsequently thawed and frozen alternatively for a total of 6 cycles. The virus was then inactivated at 56°C for 30 minutes in a water bath. Pools of each virus type was prepared separately. Each pool was centrifuged at 4°C in a refrigerated centrifuge at 3000 RPM for 1/2 hour, the clear supernatent fluid was removed and stored at -20°C.

(b) Adenovirus antigen:

Stock Adenovirus type 5 was grown in trysinized second generation human amnion cells of blood group type "O". 0.2 ml of Adenovirus type 5 superinfected tissue culture fluid was used as the inoculum for each tissue bottle. The subsequent procedure for the preparation of the antigen was

The following antisera used were obtained from commercial sources:

(a) Rabbit - produced antiserum to CRP ..... (ACRP-rabbit)

(b) Horse - produced antiserum to CRP ..... (ACRP-horse)

(c) Goat - produced antiserum to CRP ..... (ACRP-goat)

(d) Horse - produced antiserum to CxRP ..... (ACxRP-horse)

(e) Goat - produced antiserum to CxRP ..... (ACxRP-goat)

(f) Rabbit - produced antiserum to human crystalline serum albumin.

(g) Rabbit - produced antiserum to normal horse serum (AHS-rabbit)

(h) Sheep - produced antiserum to rabbit serum.

3. Test Sera:

(a) CRP Positive Serum:

A pool of acute phase human sera (10 sera, 1.0 ml of each) was prepared. These sera were taken from patients suffering from a variety of illness and were kindly supplied by the Department of Bacteriology, Winnipeg General Hospital, and were known to give a + + or more reaction in the capillary precipitation test, using commercial rabbit-A.C.R.P.

(b) CRP Negative Serum:

A pool of normal human sera (10 sera of 1.0 ml of each) was prepared. These sera were taken from clinically healthy adult individuals and showed no precipitation reaction in the capillary precipitation test using rabbit-ACRP.

(c) CxRP Positive Serum:

An acute phase response was produced in rabbits by inoculating 5.0 ml adjuvant subcutaneously at separate sites in two equally divided doses of 2.5 ml. The animals were bled 24 hours after the injection and the serum separated. The serum was tested for the presence of CxRP by the means of

double diffusion in agar gel using horse-ACxEP. Only sera showing the presence of a strong CxEP-specific precipitation line by the above method, were used.

(d) CxEP Negative Serum:

Normal rabbit serum was tested for the presence of CxEP diffusion in agar gel using ACxEP-horse. The serum showed no CxEP.

For the detection of CxEP in agar gel double diffusion studies, positive and negative controls (known CxEP positive and negative sera) were obtained from specimens previously tested in the Virus Laboratory, Department of Bacteriology and Immunology, University of Manitoba, Winnipeg.

(e) Normal Horse Serum:

Supplied from a commercial source.

4. Agar Gel Slides.

A hot solution of Agar (2% w/v in barbital buffer) was poured onto standard microscope slides in a volume of 2.5 ml per slide and then allowed to gel. These agar covered slides were stored at 4°C in a humid atmosphere in petri dishes, until used.

5. Adjuvant.

A Freund's - type incomplete adjuvant was prepared by mixing 9 parts of Bayol F and 1 part Arlacel A (Mannide Mono-oleate). After mixing the preparation was stored at room temperature until used.

6. Buffer Solutions.

(a) Veronal Buffered Saline:

85.0 gm of sodium chloride, 5.75 gm of diethylbarbituric acid and 3.75 gm of sodium barbitone were dissolved in 2,000 ml of distilled water in the order described. This was used as stock solution and diluted 1:5 for use. 0.1 ml of a 10% solution of magnesium sulphate $\cdot 7H_2O$

(c) Decolorizing Solution:

A solution of 5% glacial acetic acid in 100.0 ml of distilled water was prepared.

8. Animals:

All animals used in the experiments were healthy and young. The weight of rabbits, guinea pigs and chickens was 2.5 to 4.5 Kg, 275 to 300 gm, 2 to 2.5 Kg, respectively. The guinea pigs and hens were bred in the animal house of the Department of Bacteriology and Immunology, and Animal Science Department University of Manitoba, Winnipeg respectively. The rabbits were a mongrel breed but secured from a reliable local source. After arrival in the laboratory animal house the rabbits and chickens were kept under quarantine and observed for any evidence of disease for a period of two weeks. All animals were found to be healthy and removed to three separate rooms according to the animal species. All animals were kept in individual cages.

9. Bleeding Procedures:

Care was taken to conduct all bleeding operations and blood collection under aseptic conditions. The blood was collected in sterile one ounce universal containers and allowed to clot overnight at room temperature. The serum was removed after centrifugation at 2000 RPM for 15 minutes, and stored separately in the deep freeze at -20^{0}C.

Rabbits were bled through a small incision made in the marginal ear vein with a Bard-Parker scalpel blade. After bleeding the cut was sealed with Collodion. Each subsequent bleeding was done at a new site.

Chickens were bled from a vein under the wing, while the guinea pigs were bled by cardiac puncture after the animals were under ether

anesthesia. The needles used in these bleeding operations were 20G x 1 1/2" B.D.

10. Inoculation Procedures.

(a) Rabbits: Subcutaneous inoculations were made in the thigh region of the hind legs, intravenous inoculations in the marginal ear veins, while the intraperitoneal inoculations were made through the central abdominal wall in one of the lower quadrants.

(b) Guinea pigs and Chickens: Subcutaneous inoculations were made in the inguineal region while the intramuscular inoculation were done in the thigh muscles.

For the various inoculations the following types of needles were used.

Needles:

Subcutaneous injection:	19 G	1-1/2"	B-D needle
Intramuscular injection:	21 G	1"	B-D needle
Intraperitoneal injection:	20 G	1-1/2"	B-D needle
Intravenous injection:	26 G	1/2"	B-D needle

11. Capillary Precipitation Test:

The capillary microprecipitation method of Swift, Wilson and Lancefield was used to determine the presence of CHP in human sera, or Cx-polysaccharide reacting substances in human and animal sera (88). Glass capillary tubes 1.3 - 1.5 mm x 75 mm were used in the test. A column of 2.0 cm of CHP antiserum was drawn in the tube. The tube was wiped clean on the outside with tissue and then placed in contact with human test serum of which 2.0 cm was drawn up in the tube. Care was taken not to allow any air bubbles to form between the two reacting sera. The tube was then in-

verted four times in order to insure an adequate mixing of the two fluids. The tube was then wiped clean with tissue, and the fluid in the tube allowed to rise 10mm from the lower end in order that the bottom miniscus was 5mm above the plasticine level in the rack. The tube was then placed vertically in the plasticine rack and incubated at 37°C for 2 hours and then refrigerated at 4°C overnight. CRP negative and positive sera were also tested in the manner described above and served as negative and positive controls respectively.

For the detection of any Cx-polysaccharide-reacting substance present in human and animal sera a solution of Cx-polysaccharide (kindly supplied by Dr. Gotschlich of Rockefeller Centre, New York) was used in a concentration of 1.25 mg. per ml in phosphate buffered saline containing 0.001 M calcium chloride, in a manner similar to that described above.

Reading and Interpreting of the Test:

Reading was made by holding the rack to a light source with the capillary tubes in an oblique position. The rack was held above eye level and aimed toward a dark background with the light directed at the capillary tubes from above.

A negative reaction was one in which no precipitate appeared.

A positive reaction was one in which a precipitate appeared. The results were recorded by defining a very slight precipitate as a trace, a definite, but a slight reaction (1.0 mm of precipitate) as one plus; a moderately strong reaction (2.0 mm in height) as two plus; and so on. The height of the packed precipitate in the capillary tube was measured with a small millimeter ruler.

12. Cytopathogenic - Inhibition Test (Virus Neutralization Test):

Virus neutralizing antibody titre was determined both in pre- and post-virus inoculation sera. The test is described in detail in the Virus Laboratory Manual, Department of Bacteriology and Immunology, University of Manitoba, Winnipeg.

Briefly the test was as follows:

All sera were inactivated at 56°C for 30 minutes.

Doubling dilutions (1:4 to 1:1024) of each serum were prepared. To each serum dilution an equal volume of virus suspension (100 TCD_{50} as determined previously on MKTC was added. The serum virus mixture was then inoculated into each of a set of four MKTC tubes.

The following controls were set up:

(a) Virus control: 0.2 ml of 100 TCD_{50}, and 1 TCD_{50} of virus suspension inoculated into each of a set of four MKTC tubes.

(b) Serum control: 0.2 ml of serum (1:4 dilution) inoculated into each of a set of four MKTC tubes.

(c) Cell controls: 0.2 ml of diluent inoculated into each of a set of four MKTC tubes.

All dilutions were prepared in Hank's balanced salt solution.

All tubes were incubated at 37°C in stationary racks. Readings were made on the fourth day for echoviruses and on the seventh day for adenoviruses. All the controls were correct. The end point of virus neutralizing antibody was taken as the highest serum dilution that completely neutralized 100 TCD_{50} of the virus.

13. Complement Fixation Test:

The technic was based on the principles described by Fulton

and Dumbell and modified by Delaat as outlined in the Virus Laboratory Manual, Department of Bacteriology and Immunology, University of Manitoba.

Briefly the technic was as follows:

The unit of virus antigen was determined by titration against two units of known human adenovirus antiserum. Subsequently two units of antigen, contained in a 0.1 ml volume were mixed with an equal volume of doubling dilutions of inactivated test serum (inactivation by heat at 56°C for 30 minutes).

The serum dilutions ranged from 1:4 to 1:1024. To each mixture of antigen and antiserum, 0.1 ml of pooled guinea pig complement (2.5 HD_{50} per 0.1 ml) was added. After overnight incubation at 4°C, 0.1 ml of 1% sensitized sheep red blood cells (sensitized cells) were added. Readings were taken after incubating the test plates, at 37°C for 30 minutes in a water bath. The titre to which complement was fixed was taken at the point where 50% or less of the cells were hemolysed.

Controls:

The following controls were set up in a manner similar to the technic described above.

(a) Complement Control:

i. 0.2 ml of saline, 0.1 ml complement (2.5 HD_{50}) and 0.1 ml sensitized cells, showed 100% lysis.

ii. 0.2 ml saline, 0.1 ml complement (1 HD_{50}) and 0.1 ml sensitized cells, showed 50% lysis.

iii. 0.2 ml saline, 0.1 ml complement, (0.5 HD_{50}) and 0.1 ml sensitized cells, showed 25% or less lysis.

(b) Antigen (Virus) Hemolytic Control:

0.1 ml antigen (2 units of antigen (0.1 ml), 0.2 ml saline and 0.1 ml of sensitised cells, showed no lysis.

(c) Antigen (Virus) Anticomplementary Control:

0.1 ml antigen, 0.1 ml saline, 0.1 ml complement (2.5 HD_{50}) and 0.1 ml sensitised cells showed 100% lysis.

(d) Cell Control: (also served as saline control)

0.3 ml saline and 0.1 sensitised cells showed no lysis.

(e) Test Serum Anticomplementary Control:

0.1 ml serum, 0.1 ml saline, 0.1 ml complement (2.5 HD_{50}) and 0.1 sensitised cells showed 100% lysis.

(f) Normal Antigen Control:

0.1 ml normal antigen (same dilution as for 2 antigen units of virus antigen), 0.1 ml serum, 0.1 ml complement (2.5 HD_{50}) per 0.1 ml sensitised cells showed 100 % lysis.

(g) Positive Serum Control:

0.1 ml antigen, 0.1 ml positive serum (2 units of known adenovirus antiserum per 0.1 ml), 0.1 ml complement (2.5 HD_{50}) and 0.1 ml sensitised cells, showed no lysis.

(h) Negative Serum Control:

0.1 ml antigen, 0.1 ml negative serum (serum known to have no adenovirus antibody), 0.1 ml complement (2.5 HD_{50}) and 0.1 ml sensitised cells, showed 100% lysis.

The diluent and saline used in the above test was veronal buffered saline pH 7.4.

14. Double Diffusion Studies in Agar Gel:

The micro-method as described by Parker, Stackiw and Wilt was used (73). Briefly the technic was as follows:

With the aid of a plastic mold prepared in the Virus Laboratory, Department of Bacteriology and Immunology, University of Manitoba, Winnipeg, a pattern of eight peripheral wells, having a diameter of 2.0 mm, were made in the agar layer. The central well was made with the aid of the tip of a Pasteur pipette to give a diameter of 3.0 mm. The reacting reagents along with the control reagents were placed in individual wells by means of capillary tube using a separate tube for each well. The prepared slides were placed in a petri dish and incubated at room temperature in a draught-free humid atmosphere for a period of 24 hours. The slides were then washed, stained and read.

The following will illustrate the procedure described above.

(a) Detection and Quantitation of CxBP in Rabbit Serum by Means of Double Diffusion in Agar Gel:

A micro-method was used for this purpose (73). In an agar covered slide, one central and eight peripheral wells were arranged in a manner already described. Doubling dilutions of test serum, ranging from 1:1 to 1:32 were prepared in sterile physiological saline. With the aid of capillary tubes, one for each well, the various reagents were placed into the appropriate wells in the following pattern:

ACxBP - horse	Central Well
CxBP negative serum (negative control)	Well 1
CxBP positive serum (positive control)	Well 2

Test serum,			dilution 1:1	Well 3
"	"	"	1:2	Well 4
"	"	"	1:4	Well 5
"	"	"	1:8	Well 6
"	"	"	1:16	Well 7
"	"	"	1:32	Well 8

The prepared slide was incubated at room temperature for 24 hours in a humid chamber, washed in physiological saline, dried at room temperature, and then fixed and stained. A typical result on such a stained slide is shown in figure 1.

There is a reaction between CxHP negative serum (well 1) and ACxHP-horse (central well) as shown by the set of lines formed between the central well and well #1. These lines indicate that ACxHP-horse contains antibodies to contaminant normal rabbit serum proteins present in the CxHP preparation used to produce ACxHP. There is also a reaction between CxHP positive serum (well #2) and ACxHP-horse (central well) as shown by the set of lines between the central well and well #2. However, comparison of the two sets of lines shows the presence of an extra line in the space between the central well and well #2 (shown by one arrow). This line is indicative of the presence of an extra substance (CxHP) in the CxHP positive serum and the result of a precipitin reaction between the CxHP and its homologous antiserum (ACxHP-horse). The line is thus a CxHP-ACxHP precipitation reaction line. The CxHP-ACxHP reaction line merges with a line in front of well 3 as shown by two arrows. This is a reaction of complete identity and indicates the presence of CxHP in the test serum.

The presence of CxFP in the test serum can be traced to a serum dilution 1:4 as shown by the presence of the CxFP-ACxFP reaction line.

(b) Detection and Quantitation of a Human Serum Albumin in Antibodies in Rabbit Serum by Double Diffusion in Agar Gel:

The same method as described for the detection and quantitation of CxFP in rabbit serum was used. The pattern of the various reacting substances in the various wells was as follows:

Human serum albumin solution (2% w/v)	Central Well
Rabbit normal serum (negative control)	Well 1
Rabbit produced antiserum to human serum albumin (positive control)	Well 2
Test serum, dilution 1:1	Well 3
" " " 1:2	Well 4
" " " 1:4	Well 5
" " " 1:8	Well 6
" " " 1:16	Well 7
" " " 1:32	Well 8

The results are shown in figure 2.

There is no reaction between rabbit normal serum (negative control) and human serum albumin solution (HSA). However the positive control shows a broad precipitation line adjacent to well #2 (shown by an arrow). The broad precipitation line is characteristic of serum albumin in precipitation in a gel medium. The precipitation band is continued around well #3,4,5,6, and 7 and shows a reaction of identity

throughout.

The titre of antibodies to human serum albumin in the test serum is 1:16, when tested by this method.

15. Immunoelectrophoresis.

Rabbit CxSP positive and CxSP negative sera were studied simultaneously by a modified micro-method.

Briefly the technic was as follows:

In an agar covered slide (as for double diffusion) two wells, each 2.0 mm in diameter were made. The wells were placed 3.0 mm apart from each other and placed equidistant from either end of the slide. With the aid of glass capillary tubes, CxSP positive and negative sera were placed in the separate wells, and electrophoresed by means of a current of 25-30 milliamps and a potential difference of 75-85 volts across 4 slides. Following electrophoresis two troughs were cut in the agar parallel to each other in the long axis of the slide, each trough being situated 2.0 mm lateral to each well. Each trough was 6.5 cm in length and 0.1 cm in width and was so placed as to leave 0.5 cm of the uncut agar at each end. The troughs were cut with the aid of 2 Bard-Parker scalpel blades by binding tape 0.1 cm apart. With the aid of a glass capillary tube each trough was filled with ACxSP-horse. The agar slide was then placed in a petri dish and incubated at room temperature in a drought-free area for a period of 24 hours. The slides were then washed, stained and read.

16. Washing and Staining Methods:

(a) Washing and Drying:

Each agar slide was washed separately by submerging it in

liberal amounts of physiological saline (300 ml for each slide) for 24 hours. Saline was changed three times. The agar gel slide was then taken out of the saline and covered with a filter paper and allowed to dry at room temperature for 24 hours. A clear agar film adherent to the slide surface was formed which was easily stained as follows:

(b) Staining:

The washed slide was fixed by submerging it in fixative solution for a period of five minutes. The fixed slide was then covered with stain for ten minutes and then submerged in decolorising solution to remove the excess stain.

The stained slides were allowed to dry at room temperature before reading.

17. Serum Protein Fractionation.

A modification of the serum protein fractionation technic as described by Anderson and McCarty was used (15). The method of serum protein fractionation was as follows:

The serum diluted with an equal volume of physiological salt solution and brought to 0.5% saturation with ammonium sulphate by the addition of 32 g. of the solid salt for each 100 ml of the diluted serum (0.5 saturation causes precipitation of most of the serum globulins). The mixture was then allowed to stand at room temperature for two hours and then passed through a filter paper (no. 40, 15.0 cms. W & R Balston Ltd., England). The clear filtrate was brought to 0.75% saturation by the additon of 17.5 g. of solid ammonium sulphate per 100 ml of the filtrate. (0.5 - 0.75% saturation causes precipitation of most of the serum albumin, and most of the lipid bound acute phase pro-

tein, if present in the serum). The mixture was allowed to stand at room temperature overnight and filtered. The precipitate was dissolved in distilled water in a volume half of the original serum volume. The solution was transferred to a cellophane bag and dialysed first against 3 changes of liberal amounts, in excess of 500.0 ml, of distilled water for 24 hours, and then against three changes of liberal amounts of 0.1% Calcium chloride solution for a further period of 24 hours (dialysis against 0.01% Calcium chloride solution causes precipitation of lipid bound acute phase protein).

Experiment I

This experiment was a pilot study designed only to determine the effects on rabbits of certain procedures and manipulations, which were to be utilised in later experiments.

Its purpose was to study the efficacy of the proposed procedures in producing a CxBP response in rabbits following the administration of:

1. Adjuvant (Freund's incomplete adjuvant) along with an inactivated virus antigen (infected human amion cells).

2. Adjuvant along with sterile physiological saline.

3. Sterile physiological saline.

4. The trauma associated with the type of bleeding procedure (marginal ear vein bleeding) to be considered the most efficacious.

Further it was aimed to observe:

1. At what time CxBP appeared in the peripheral blood after administration of the stimulating agents.

2. Any quantitative difference in CxBP response between the various stimulating agents.

3. Any dimunition in CxBP response after repeated stimulations.

The detection and quantitation of CxBP in the serum was performed by double diffusion studies in agar gel.

Experimental

The experiment was performed on twelve healthy adult rabbits. The rabbits were grouped as follows:

Group I 4 Rabbits (#1,2,3, and 4)

Group II 3 Rabbits (#5,6, and 7)

Group III 3 Rabbits (#8,9 and 10)

Group IV 2 Rabbits (#11 and 12)

Stimulating agent used:

1. Virus and adjuvant, Echovirus types 24, 25, 28 and 22 were used as virus antigen for rabbits 1,2,3, and 4 of group I respectively.

Day 1 and 22 of the experiment: A homogenous mixture of 5.0 ml of adjuvant and 5.0 ml virus antigen was injected subcutaneously in 4 equally divided doses in each rabbit. Simultaneously 1.0 ml of virus antigen was injected in the marginal ear vein of each rabbit.

On days 8, 15 and 31 of the experiment: 0.1 ml of virus antigen was injected into the marginal vein of each rabbit.

2. Adjuvant and sterile physiological saline, for all rabbits of group II.

On days 1 and 22 of the experiment: An homogenous mixture of 5.0 ml of adjuvant and 5.0 ml of saline was injected subcutaneously in 4 equally divided doses in each rabbit. Simultaneously 1.0 ml of saline was injected in the marginal ear vein of each rabbit.

3. Sterile and physiological saline, for all rabbits of group III.

Day 1 and 22 of the experiment: 10 ml of saline in 4 equally divided doses was injected subcutaneously in each rabbit. Simultaneously 1.0 ml of saline was injected in the marginal ear vein of each rabbit.

On days 8, 15 and 31 of the experiment: 1.0 ml of saline was injected in the marginal ear vein of each rabbit.

4. Untreated controls:

No stimulating agent, group IV.

Results:

The results are shown in Tables I and II. Table I shows the CxEP response in each rabbit. Injection of virus plus adjuvant and saline plus adjuvant both induced a CxEP response in the blood when tested 24 hours after injection. The injection of saline only, in group III induced an irregular and poor CxEP response. There seemed to be no difference in CxEP response between Groups I and II following injections on day 1 and 22 of the experiment.

In rabbit #2 CxEP response after intravenous (IV) injection of virus antigen alone was irregular and less marked when compared to the CxEP response obtained following combined IV and subcutaneous injections of the antigen. CxEP response after IV injection of saline on day 8, 15 and 31 is irregular both in Group II and III. Group IV did not show any CxEP in the blood throughout the experiment. Hence the CxEP response in groups I, II and III can be attributed to the stimulatory agents used rather than the bleeding procedure. There seemed to be no decrease in CxEP response after repeated stimulations under identical conditions.

Table II shows the virus neutralising antibody titres in each rabbit of group I. As each rabbit was injected with a different echovirus antigen no conclusion can be drawn in regard to a correlation between CxEP response and the final virus neutralising antibody titre.

Discussion:

Medlund showed that acute phase protein can be produced by a variety of agents (34). He also showed that minor injuries caused during

bleeding and inoculation procedures did not induce CxBP response. Our observations in this respect are in agreement with Hedlund's finding. The injection of various echovirus antigens or saline by the intravenous route produced an irregular CxBP reponse. Administration of saline by combined subcutaneous and intravenous injections also produced an irregular CxBP response. However incorportion of adjuvant along with viral antigen or saline produced a regular and better CxBP response. Adjuvant because of its oily nature is not easily eliminated from the body, and hence may have prolonged stimulatory effect on the CxBP producing mechanism. In accordance to Hedlund's observations we have also observed that the bleeding, through a small nick in the marginal ear vein, did not produce a detectable level of CxBP. It seems that a certain threshold of injury must be reached to induce CxBP response, and the usual inoculation and bleeding procedures appear not to exceed that threshold.

CxBP response following repeated stimulation under similar conditions was also studied. The results obtained are not in agreement with those of other workers. Hedlund used manganese and sulphur (106), while Montella and Wood used thorotrast to stimulate CxBP production (115). It was observed that CxBP response to any of these agents after repeated injections of the agent gradually diminished and ultimately disappeared. The difference may be attributed to the diverse nature of the agents, used to stimulate CxBP production. Thorotrast, sulphur and manganese salts are inorganic metallic salts and may block CxBP producing cells, hence decrease in CxBP response after repeated injections.

However this does not explain Hedlund's observation that the usual acute phase protein response could be obtained by changing the type of stimulating agent. In individuals who gradually became refractory to the repeated injections of manganese salts to a point where the production of CRP could no longer be stimulated at all, injections of sulphur would still produce an apparently normal CxRP response (108). If repeated injections of inorganic metallic salts block all CRP producing cells the usual CRP response with a different stimulating agent have a predelition for different groups of cells each capable of producing CRP. A further explanation may lie in the timing and intervals between injections being different for each substance capable of producing a refractory response.

Summary:

The CxRP response in rabbits after the administration of a variety of different stimulating agents was studied.

The CxRP response produced following the use of different stimulating agents varied, depending upon the type of stimulating agent used.

Administration of physiological sterile saline or a virus antigen induced an irregular CxRP response. The incorporation of an adjuvant with either saline or a virus agent induced a good CxRP response.

Daily bleeding through a small nick in the marginal ear vein of the rabbits did not induce CxRP response.

Repeated stimulation under similar conditions did not cause a diminished CxRP response .

CxRP titre usually reached its peak within 24 hours after the administration of the stimulating agent. The persistence of the CxRP titre depended upon the type of stimulating agent used.

Experiment II

This experiment was designed to determine the effect on the production by rabbits of precipitating antibodies following the administration of human serum albumin together with the following:

1. The individual rabbit's own acute phase serum (known to be CxRP positive).

2. The individual rabbits own normal serum (known to be CxRP negative).

3. Sterile physiological saline.

In addition it was hoped to determine:-

1. The effect on the CxRP response following repeated stimulations under similar conditions.

2. Any possible correlation between the CxRP response and the precipitating antibody titres to human serum albumin produced in each rabbit.

The CxRP and the precipitating anti-human serum albumin antibody (A-HSA) titres were estimated by double diffusion studies in agar gel.

Experimental

Fourteen rabbits were used. Rabbits were numbered from 21 to 34. 5.0 ml of blood was taken from each rabbit prior to the commencement of the experiment proper. To evaluate the CxRP response in these rabbits each rabbit was then injected subcutaneously with 5.0 ml of Freund's incomplete adjuvant in two equally divided doses at two sites. A further 5.0 ml of blood was then taken from each rabbit 24 hours after the adjuvant injection. The pre- and post-adjuvant inoculation serum samples (serum #1 and #2 respectively) of each rabbit were tested for the presence of, and titre of, CxRP and A-HSA. It was felt that only those rabbits able to produce a good CxRP response should be utilized for subsequent comparative studies.

Hence, this screening procedure.

The results of this procedure are seen in Table III.

As seen in Table III rabbit #26 showed the presence of CxMP both in sera #1 and #2. Rabbit #27 showed no CxMP in either sera, while in rabbits #23 and 28 CxMP could only be detected in undiluted serum, specimen #2. None of the rabbits showed the presence of A-MSA in either sera.

Based on the CxMP titre of serum #2 of each individual rabbit, ten rabbits numbering 21,22,24,25,29,30,31,32,33 and 34 were selected for further studies in this experiment. The rabbits were considered good producers of CxMP.

The animals were grouped as follows:

Group I Rabbit #21,24,30 and 32

Group II Rabbit #22,33

Group III Rabbit #25,29,31, and 34

The CxMP titres of serum #2 of each individual rabbit in each group are shown in table IV.

On day 19 of the experiment the animals of group I were inoculated with 10.0 ml of adjuvant subcutaneously in 4 equally divided doses. On day 20 of the experiment 50.0 ml of blood was taken from each animal in groups I and III. The serum (#3) of each rabbit was separated, tested for the presence and titre of CxMP and then stored individually. The CxMP response of these rabbits is shown in Table V.

On day 50 of the experiment, animals of all three groups were examined to see if they were still healthy. Rabbit #32 was found to be suffering from an ear infection, and was therefore not included in the experiment.

On day 51 of the experiment animals of group I and III were in-

oculated intraperitoneally with 20.0 ml each of their own stored serum (#3), while the animals of group II were inoculated intraperitoneally with 20.0 ml of sterile physiological saline. The temperature of the inoculated sera and saline was allowed to reach that of room temperature before inoculation. Immediately after the intraperitoneal injection all animals of group I, II, and III were inoculated intravenously with sterile 2% w/v solution of human serum albumin (HSA) in a dose of 15mg/kg body weight. The body weight of each rabbit is shown in table VI.

On days 79,86,93,100 and 107 of the experiment each rabbit was given further injections, the same dose of HSA subcutaneously.

Results:

The CxBP and A-HSA titres of individual rabbits are shown in tables VII and VIII respectively. They are further shown graphically in figure 3. The geometric means of CxBP and A-HSA titre of each group are shown in table no. IX and X respectively and further shown graphically in figure 4. In calculating the geometric means, the CxBP or A-HSA titres of less than 1:1 were not recorded. Although the mathematical geometric mean of a group of figures which includes a zero figure is not absolutely correct, the presentation of the data by calculating the geometric mean and ignoring zero values, allows a clearer picture of the CxBP and A-HSA titres of the three groups to be seen. The A-HSA titres of individual rabbits of the three groups are shown in figure 5.

All animals except rabbits #24 (of group I) and 31 and 34 (both of group III) produced A-HSA. All animals except rabbits #22,24 and 29 showed regular CxBP response to subcutaneous injections of HSA. Rabbits #21 and 30 (of group I) were the first to show the appearance of A-HSA, on day 86 of the experiment. Sera of rabbits #22 and 33 (group II) showed the

appearance of A-HSA, on days 93 and 100 respectively, of the experiment. Sera of rabbits #25 and 29 (of group III) showed the appearance of A-HSA on day 100 of the experiment. The maximum A-HSA titre in rabbits #21 and 30 (of group I) was reached before that of rabbits #22 and 33 (group II), and 25 and 29 (of group III). Rabbits #21,22,25,30, and 33 showed a greater CxIP response to subcutaneous injections of HSA at the time of the first appearance of A-HSA. Sera of rabbit #21 and 30, on day 80 of the experiment showed, 24 hours after the injection of HSA, a CxIP titre of 1:4 and no detectable A-HSA. However, on day 87 of the experiment, 24 hours following another injection of HSA, the sera of both rabbits showed the presence of A-HSA and a CxIP titre of 1:8 (rabbit #21) and 1:16 (rabbit #30). Until the termination of the experiment, these rabbits showed a CxIP response to each injection of HSA equal or greater than the CxIP response shown on day 80 of the experiment, when no A-HSA antibodies were detected. Sera of rabbits #25 and 33 on day 94 of the experiment showed a CxIP titre of 1:4 and no detectable A-HSA 24 hours after the injection of HSA. However, on day 101 of the experiment, 24 hours following another injection of HSA, the sera of both rabbits showed the presence of A-HSA and a CxIP titre of 1:16 (rabbit #25) and 1:8 (rabbit #33). Similarly rabbit #22 showed an increase in CxIP titre from 1:2 (on day 87) to 1:4 (onday 94) associated with the first appearence of A-HSA. Following the appearance of A-HSA, CxIP response to each subcutaneous injection of HSA in rabbits #22,25 and 33 was shown to be higher than the CxIP response obtained by similar injections given before A-HSA appeared. In rabbit #29 CxIP response to subcutaneous injection of HSA before and after the appearance of A-HSA showed little difference.

Discussion:

Usually, following a suitable antigenic stimulus, warm blooded animals produce specific humoral globulin substances termed antibodies. It is difficult to define precisely those properties which determine the ability of a molecule to act as an antigen. The molecular weight of a substance has been considered one of the factors necessary before that substance will act as an antigen (144-a). Antigenicity is generally possible over a molecular weight of 10,000. Although smaller molecules may act as haptens, they are incapable of stimulating antibody production on their own. Antigen of small molecular weight are quickly absorbed into the general circulation and are apparently eliminated within a short period.

To stimulate antibody production it appears that an antigen undergoes some process of modification, the nature of which is, as yet, unknown. However, there is a general agreement that the antigen is carried in some way to antibody producing cells, and that it then resides in an intra-cellular location. Phagocytic cells attempt to actively engulf any "foreign" agent either particulate or soluble, living or dead. This mechanism of active phagocytosis can be either helped or hindered by numerous factors. The acquired antibody, through its agglutinating, opsonising and other activities helps in the engulfment of antigens by phagocytes. Knisely, Bloch and Warner have observed that a fibrinogen-like, blood protein coating is formed around inert particles such as India ink or kaolin before they are ingested by the macrophages of the liver (145). Two protein factors which increase phagocytosis of human polymorphonuclear cells have been characterized in normal plasma (146,147). Acute phase protein

has also been shown to enhance the phagocytic activity (138) and mobility of normal human leukocytes (137).

Numerous methods have been used to intensify and prolong antibody production to an injected antigen. One such method is the use of adjuvants . Adjuvants are usually non-antigenic in themselves, but if mixed with antigens cause an enhanced antibody response (144-a). It is said that of the variety of adjuvants used, preparations of mineral oil are most effective (148). The first observations with mineral oil were made in France by Le Moignic and Pinoy (149). Freund modified the original procedure by emulsifying water-in-oil with the aid of lanolin (150, 151).

The mechanism of adjuvant action is not understood. However, it has been shown that: 1. Administration of an adjuvant alone induces the production of acute phase substances which include acute phase proteins.

2. Administration of an adjuvant enhances the production of antibody to many types of antigen when simultaneously administered at a separate site (110).

3. Incorporation of an adjuvant with an antigen moves back in time the appearance of antibodies in new-born rabbits (152, 153).

4. Incorporation of adjuvant with antigen retards both the distribution of the antigen (148,154) and its rapid elimination from the body (155).

Based on these observations the following hypotheses may be postulated as to the mechanism by which adjuvants augment antibody production, when mixed with the antigen and administered parenterally:-

1. The adjuvant forms a depot of persisting antigen.

2. It causes a more efficient dissemination of the antigen.

3. It non-specifically stimulates the antibody forming mechanism.

4. It enhances the production of acute phase substances which may modify the antigen in some manner which renders it more readily phagocytosed and thus is carried to antibody-producing sites and provides a greater stimulus to the antibody producing mechanism.

In this study an attempt was made to show that the action of adjuvant in the augmentation of antibody production probably mediated through its enhancing effect on the production of acute phase substances which include acute phase proteins. Acute phase response in rabbits was produced by injecting Freund's incomplete adjuvant. The acute phase sera was individually stored and injected back into the same rabbits, after a resting period of 32 days, along with a soluble protein antigen (HSA) both by different parenteral routes and at quite separate sites. The lapse of a period of 32 days between the injection of adjuvant and the injection of the rabbits own acute phase sera was on the presumption that any non-specific stimulating effect of adjuvant on the antibody producing mechanism would not be still active after this time. However, no studies were performed to confirm this presumption.

The results of the experiment show the following:-

a) That the rabbit's own acute phase serum (CxRP positive) when administered just prior to, but separately from soluble protein antigen (HSA) induces an early antibody response. The precipitating antibodies to HSA (A-HSA) in rabbit #21 and 30, given their own acute phase serum, showed a more rapid appearance.

b) That the A-HSA titres produced are greater and are also attained

earlier in these animals. It is possible that the simultaneous administration of the antigen and acute phase serum provides an opportunity for the necessary acute phase substance which includes CxRP to act upon the injected antigen (HSA) and modify it in some manner whereby it is rendered more accessible to the antibody producing mechanism.

The stored acute phase serum samples (CxRP positive) of the animals of group I were each insufficient in quantity to allow further amounts to be administered with each subsequent injection of HSA. Therefore subsequent injections were of HSA alone. After repeated injections of HSA the animals in the various groups showed no significant differences in the final A-HSA titre obtained. This observation was not expected as only one injection of acute phase serum was given (day 51). Hence only the time of the initial appearance and the highest titre obtained of A-HSA, in the various groups of treated animals, is of significance in drawing any conclusion.

Repeated stimulation of rabbits under similar conditions did not show any appreciable difference in the CxRP response of the rabbits. This supports the observations made in experiment #1. Furthermore the appearance of A-HSA did not seem to reduce the CxRP response in individual rabbits. This observation is in disagreement with that of Hedlund, who when using a second injection of the vaccine 62 days after the first (34). He was of the opinion that the reduction in CSR titre might be due to the production of antibodies to the vaccine. However, it is known that if an antigen is reintroduced into the body while antibody resulting from a previous injection of the same antigen is still circulating, an antigen-antibody complex is formed (144-b). Further is has been shown that passive

administration of soluble antigen-antibody complexes in rabbits induces a CxRP response (136). In view of these observations we feel that in Hedlund's experiment (34) reduction in CSR titres following the second injection of typhoid vaccine is unlikely to be due to the presence of circulating antibodies to the vaccine. The results of our experiment show that the CxRP response to subcutaneous injections of HSA was either the same or greater after antibodies to HSA had appeared (see table VI).

The increase CxRP response might well be due to a more effective combined stimulus of the injected antigen and circulating soluble antigen-antibody complexes formed as a result of the injected antigen.

The total absence of antibody response HSA in rabbits 24,31 and 34 cannot be explained. No inference can be drawn as to whether or not there is any correlation between the CxRP and A-HSA titres.

Summary:

The effect of the administration of acute phase serum (known to contain CxRP) on the production of precipitating antibodies to human serum albumin (HSA) was studied.

Rabbits receiving a single injection of their own acute phase serum (CxRP positive) showed an early antibody response to the simultaneously injected antigen (HSA). The precipitating anti-human serum antibody titre in this group of animals reached a maximum earlier than it did in either group of animals given saline along with HSA, or the group given their own normal (CxRP negative serum).

The appearance of precipitating antibodies to HSA did not result in a reduction in the subsequent CxRP response of the same rabbits.

The available data allowed no correlation to be made between the

CxP titres and the A-BSA titres. It does appear that acute phase serum contains a substance or substances which are capable of enhancing antibody response. It is possible that this is CxP, as the only quantitive differences know between resting normal rabbit serum and acute phase rabbit serum is the presence of CxP only in the latter. The other changes appear to be due to a quantitive change in serum proteins already present. (See Immunoelectrophoretic studies.)

Experiment III

This experiment was designed to determine the effect on the production of CxFP, complement fixing (CF) and virus neutralizing antibody titres following the administration of:

1. Inactivated virus antigen (adenovirus type 5 grown in human amnion cells group "O") plus an adjuvant (Freund's incomplete adjuvant).

2. The same inactivated virus antigen plus sterile physiological saline.

Further it was aimed to observe:

1. Whether any correlation existed between CxFP response, complement-fixing antibody levels and the final virus-neutralising antibody levels obtained.

2. Whether any diminution of CxFP titres occurred following repeated stimulation under similar conditions. The estimation of CxFP in rabbit sera was made by means of diffusion studies in agar gel.

Experimental

The experiment was performed on eight healthy adult rabbits. The rabbits were grouped as follows:

Group I - Rabbit #50,51,52, and 53

Group II - Rabbit #54,55,56, and 57

Stimulating Agent:

Group I: Virus and adjuvant

On days 2 and 23 of the experiment: An homologous mixture of 5.0 ml inactivated virus antigen and 5.0 ml adjuvant was injected subcutaneously in 4 equally divided doses into each rabbit. Simultaneously 1.0 ml of the virus antigen was injected intravenously into

each rabbit.

Group II: Virus and sterile physiological saline.

On days 2 and 23 of the experiment: An homologous mixture of 5.0 ml of the inactivated antigen in 4 equally divided doses was injected subcutaneously into each rabbit. Simultaneously 1.0 ml of the virus antigen was injected intravenously into each rabbit.

On days 9, 16 and 32 of the experiment 1.0 ml of the virus antigen was injected intravenously into every rabbit of both groups, I and II.

Results:

The CxRP, CF and virus neutralizing antibody titres obtained are shown in tables XI, XIII respectively. The CF antibody titre obtained in each individual rabbit of group I and II is shown in figures 6 and 7 respectively. The mean log CxRP and CF antibody titres of the two groups of rabbits are shown in tables XIV and XV respectively, and further shown graphically in figure 8. In calculating the geometric means, CxRP titres of less than 1:1 and CF antibody titres of less than 1:4 were not counted. The mathematical mean calculated from a group of figures which includes one or more zero figures is not absolutely correct. However, the presentation of the data of this experiment by calculating the geometric means and ignoring the zero values allows a clearer picture of the CxRP and CF titres of the two groups to be presented. The defect inherent in such calculations is felt not to unduly interfere with the accuracy of this method of presenting the result.

All animals, except rabbits #56 and #57 showed no CxRP response on day 1 of the experiment (before the administration of any stimulatory agent). The presence of CxRP in rabbits #56 and #57 on this day may be the

result of a minor injury or subclinical infection. All animals except #54 and 55 showed a CxFP response on day 3 and 24 of the experiment, following the administration of the stimulatory agents by combined intravenous and subcutaneous injection. Rabbits #54 and 55 showed no CxFP response on day 3 and only a poor response on day 24. The CxFP response following the administration of the virus antigen by the intravenous route alone, on days 10, 17 and 33 was irregular in all rabbits except rabbits #52 and 53. There is no decline in subsequent CxFP response following the second combined subcutaneous injections in the rabbits except for rabbits #50 and 51.

All animals showed the production of CF antibodies. However animals of group I (virus plus adjuvant) showed a consistently higher CF antibody titre than those of group II (virus plus saline). In group II rabbits #56 and 57 showed the presence of CxFP on day 1 (before the administration of any stimulatory agent), and showed a good CxFP response throughout the experiment. Rabbits (#56 and 57), however, showed no better CF antibody response than rabbits #53 and #54 who showed a poor irregular CxFP response throughout.

The virus neutralising antibody titre in all animals on day 1 was less than 1:4 except in rabbit #56 who showed a titre greater than 1:16. The end point of the titre in this rabbit was not determined. After the administration of the varying stimulatory agents both groups of animals showed the production of virus neutralising antibodies. Rabbit #50 was sacrificed on day 3 of the experiment, hence the virus neutralizing antibody of this rabbit was determined on the serum specimen collection of this day. The two groups of rabbits showed no sig-

nificant difference in the final virus neutralising antibody titres.

Discussion:

In this experiment an attempt is made to show that the effect of adjuvant, in augmenting the production of antibody to an antigen, might be due to its effect on enhancing production of acute phase substances in the serum. The results showthat the administration of the adjuvant induced a greater CxHP and CF antibody response to adenovirus type 5. However, it exerted no enhancing effect on the final virus neutralizing antibody titres. No explanation can be given as to the failure of adjuvant in augmenting final virus neutralizing antibody titres. Further no definite conclusion can be made whether the higher CxHP response in animals of Group I was responsible for the consistently higher CF antibody titres in that group of animals.

In experiments #I and II it was observed that repeated stimulation under identical conditions does not lead to any diminution in the CxHP response. This observation is again supported by this experiment. In experiment I it was also observed that the intravenous administration of virus antigen induces irregular CxHP response. Again this observation is supported by the result of this experiment. With the available data no definite conclusion can be drawn as to any difference seen to exist between the CxHP, CF and final virus neutralizing antibody titres produced by the individual rabbits in the two variously treated groups of animals.

Summary:

The effect of the administration of adjuvant on the production of CxHP, CF and the final virus neutralising antibody titres was studied

in two groups of rabbits.

The group given virus plus adjuvant showed a better CxHP and CF response than the group given virus mixed with saline. However, the final virus neutralising antibody titres showed no significant difference between the two groups of animals.

There was no decline in CxHP response after repeated stimulation under similar conditions.

Experiment IV

This experiment was designed to demonstrate any immunological cross-reaction which may occur between rabbit and human acute phase serum samples. The experiment was divided into four parts, designed to:

Part 1. Demonstrate any cross-reaction which may occur between rabbit acute phase serum (CxRP positive) and both ACRP-goat and ACRP-horse.

Part 2. Demonstrate the presence or absence of any cross-reaction between CxRP positive serum and ACRP-rabbit.

Part 3. Demonstrate any cross-reaction which may occur between pooled human acute phase serum (CRP positive) and both ACxRP-goat and ACxRP-horse.

Part 4. Demonstrate the presence or absence of any cross-reaction between normal horse serum and both rabbit and human acute and normal phase sera.

The method utilised throughout all four parts of this experiment was the agar gel double diffusion test, as outlined on page 54.

Results:

Part 1. The demonstration of cross-reaction between CxRP positive serum and ACRP-goat and ACRP-horse sera.

Figure 9. Shows the results of interactions in agar gel between:

(1) ACRP-goat (central well) and

(2) (a) CxRP negative serum (well #1)

(b) CRP negative serum (well #2)

(c) CRP positive serum (well #3)

(d) CxRP positive sera from different rabbits (well #4,5,6,7 and 8).

Both CxRP negative and CRP negative sera show no reaction. However, there is a reaction between CxRP positive sera and ACRP-goat (CxRP-ACRP reaction) as shown by the line between the central well and wells #4,5,6,7 and 8. The various CxRP sera show a reaction of complete identity against ACRP-goat as shown by the continuity of the CxRP-ACRP reaction line. There is a reaction between the CRP fusiform band between the central well and well #3. The posterior wall of this fusiform band stretches between well #2 and well #4. At its midpoint it is merged by the CxRP-ACRP reaction line (line situated between the central well and well #4), as shown by an arrow. This indicates a reaction of partial identity between CRP positive serum and CxRP positive serum.

Figure 10. Shows the results of the reactions between:

(1) CxRP positive serum (well #2), and ACRP-horse (well #3) and ACxRP-horse (well #1).

(2) CRP positive serum (well #4), and ACxRP-horse (well #1) and ACRP-horse (well #3).

CxRP positive serum shows the reaction both with ACRP-horse and ACxRP-horse as indicated by the line present between well #2 and #3 (CxRP-ACRP reaction) and well #1 and #2 (CxRP-ACxRP reaction) respectively. The CxRP-ACRP reaction line merges into the posterior wall of one of the CxRP-ACxRP reaction lines and is shown by a single

arrow. This indicates the presence of an antigenic substance in CxHP positive serum reacting both with ACHP-horse and ACxHP-horse.

CHP positive serum shows a reaction with ACHP-horse and ACxHP-horse as indicated by the lines between wells #3 and #4 (CHP-ACHP reaction) and wells #1 and #4 (CHP-ACxHP reaction). The CHP-ACxHP reaction line shows a splitting as indicated by a double arrow and the CHP-ACHP reaction line merges into the posterior portion of this line.

Part 2. Demonstration of the absence of cross reaction between CxHP positive serum and ACHP-rabbit.

Figure 11. Shows the results of reaction between:

1. ACHP-rabbit (central well) and:
2. (a) CxHP positive serum (well #1)
   (b) CxHP negative serum (well #4)
   (c) CHP positive serum (well #2 and #5)
   (d) CHP negative serum (well #3 and #6)

There is a reaction line between CHP positive serum and ACHP-rabbit (CHP-ACHP reaction) as indicated by the arcs. There is no reaction between any other sera and ACHP-rabbit.

Part 3. Demonstration of the cross-reaction between CHP positive serum, and ACxHP-goat and ACHP-horse.

Figure 12. Shows the results of reactions between:

(1) ACxHP-goat (central well) and
(2) (a) CHP negative serum (well #1)
   (b) CHP positive serum (well #2)
   (c) CxHP positive serum of various rabbits wells #3, #4, #5, #6, and #7.

(d) CxRP negative serum (well #8)

There is no reaction between CRP negative serum and ACxRP-goat. There is a reaction between CxRP positive sera and ACxRP-goat (CxRP-ACxRP). The CxRP-ACxRP reaction line (between the central well and well #7) is shown by a double arrow. The various CxRP positive sera show complete identity in their reaction against ACxRP-goat. There is a reaction between CxRP negative serum and ACxRP-goat. This reaction is probably due to the presence of antibodies to normal rabbit serum proteins found in ACxRP-goat. (This phenomenon has been explained under Materials and Methods page 55). There is a reaction between CRP positive serum and ACxRP-goat (CRP-ACxRP reaction) as shown by the line between the central well and well #2. The CRP-ACxRP reaction line merges with a bend into the CxRP-ACxRP reaction line formed between the central well and well #3 and is shown by a single arrow.

Figure 13. Shows the results of reactions between:

(1) ACxRP-horse (central well) and

(2) (a) CRP negative serum (well #1).

(b) CRP positive serum (well #2).

(c) CxRP positive sera of various rabbits (well #3, #4, #5, #6, and #7).

(d) CxRP negative serum (well #8).

There is no reaction between the CRP negative serum and ACxRP-horse (CxRP-ACxRP reaction). The CxRP-ACxRP reaction line between the central well and well #7 is shown by a double arrow. The various CxRP positive sera show complete identity in their reaction against ACxRP. The reaction between CxRP negative serum and ACxRP-horse is due to antibodies to normal rabbit serum proteins in ACxRP-horse. There is a reaction between CRP positive serum and ACxRP-horse

(CRP-ACxRP reaction) as indicated by the line between the central well and well #2. This is shown by a single arrow.

Part 4. Demonstration of the absence of cross-reaction between normal horse serum, and CRP and CxRP negative and positive sera.

Figure 14. Shows the results of reactions between:

(1) Normal horse serum (central well) and

(2) (a) Anti-horse serum (rabbit) (well #1)

(b) CxRP negative serum (well #2)

(c) CxRP positive serum (well #3,#6,#7 and #8)

(d) CRP positive serum (well #4)

(e) CRP negative serum (well #5)

There is a reaction between normal horse serum and anti-horse serum (rabbit) as shown by a broad line between the central well and well #1. There is no reaction between normal horse serum and any other sera in well #2 to #8.

Discussion:

Previous studies on CRP have shown that they are remarkably similar to one another in regards to their general properties and the conditions which govern their appearance (15). A further similarity between the two proteins was revealed when Gotschlich and Stetson in 1960 demonstrated the immunological cross-reactivity of the two proteins by means of a variety of tests (132). The results of this study confirm and extend these findings.

As shown in figure 14, human and rabbit normal and acute phase

sera did not cross-react with normal horse serum. Hence the results of cross-reaction between ACRP-horse and ACxRP-horse were due to the reaction between common human and rabbit serum antigens and horse serum. As normal goat serum was not available, tests for reactivity between human and rabbit normal and acute phase sera, with normal goat serum, could not be performed. However, no such cross-reaction has ever been reported. Hence it was assumed that the cross-reaction resulting from the use of ACRP-goat and ACxRP-goat were due to a reaction between human and rabbit acute phase sera, and the goat prepared antisera.

The CRP negative serum showed no cross-reaction with any of the antisera (ACRP or ACxRP).

The CxRP negative serum showed no cross reaction with ACRP-goat (figure 9). However, it did show a cross-reaction with ACxRP-goat and ACxRP-horse (figures 12 and 13 respectively), but, this cross-reactivity was due to non-specificity of the two ACxRP antisera used, as indicated by the absence of CxRP-ACxRP reaction line shown by the double arrows in figures 12 and 13.

The positive serum showed a cross-reaction against ACRP-goat, ACRP-horse, ACRP-rabbit, ACxRP goat and ACxRP-horse (figures 9,10,11,12,13 respectively). The results of figures 10, 12 and 13 suggest the presence of an antigenic substance in the CRP positive serum, which:

(a) Reacts with ACxRP

(b) Is either identical or similar to a substance present in CxRP positive serum (figure 12) and

(c) Is not present in the CRP negative serum. The splitting of the CRP-ACxRP reaction line shown in figure 10 is not understood.

The above results extend the findings of Gotschlich and Stetson, who in their double diffusion studies demonstrated cross-reaction between CRP positive serum and ACxP-sheep (132).

The CxP positive serum shows a cross-reaction with ACP-goat, ACP-horse, and ACxP-goat and ACxP-horse (figures 9,10,12, and 13). In figure 9 the CxP-ACP reaction line merges with a spur into the CRP-ACP reaction line which extends beyond the merging point. This phenomenon is a reaction of partial identity between CRP and CxP positive sera. The results of figure 10 support and enlarge these findings. Thus the observations to be made from figures 9 and 10 suggest that there is an antigen for CxP positive serum which:

(a) Reacts with ACP,

(b) Is either identical or similar to a substance present in CRP positive serum and,

(c) Is not present in CxP negative serum,

(d) Is identical in all CxP positive sera.

If CxP and CRP share a certain antigenic component, rabbits on immunization with CRP should not produce antibodies to that component of CRP which is shared by CxP and is therefore not foreign to rabbit. This hypothesis is confirmed by the results of figure 11 which show no cross-reaction between CxP positive sera and ACP-rabbit.

Summary:

The immunologic cross-reactivity between human and rabbit, normal and acute phase sera, was studied by agar gel double diffusion studies.

It has been shown that CxP positive serum cross reacts with ACP-goat and ACP-horse but not with ACP-rabbit.

The CRP positive serum cross reacts with ACxP-goat and ACxP-

horse.

It is suggested that the acute phase proteins of man and rabbit share a common antigen which is responsible for the cross-reaction.

ACRP-rabbit does not show the presence of antibodies to that component of CRP which is shared by CxRP.

CxRP in various rabbit acute phase sera is antigenically identical.

Experiment V

In experiment no. IV observations were made on the immunological cross-reactions which occur between human acute phase serum and rabbit acute phase serum, by using antisera to CRP and CxRP. This experiment was designed to determine the following:

1. Whether the production of an acute phase protein analogous to CRP and CxRP, occurred in chickens and guinea pigs. If such a protein was present, and was similar to CRP and CxRP, it should be precipitated by salt fractionation using ammonium sulphate (75% saturation) and following redissolving should be reprecipitated by dialysis against 0.01% w/v calcium chloride ($CaCl_2$) solution.

2. Whether there was any cross-reaction between chicken sera (normal and acute phase) and antisera to CRP and CxRP.

3. Whether there was any cross-reaction between guinea pig sera (normal and acute phase) and antisera to CRP and CxRP.

Experimental:

Seven chickens and twelve guinea pigs were used for this experiment. They were grouped as follows:

Group I - Chickens #1,2,3, and 4

Group II - Chickens #5,6, and 7

Group III - Guinea pigs #1,2,3,4,5, and 6

Group IV - Guinea pigs #7,8,9,10,11, and 12

All animals were bled (serum #1) before inoculation.

Stimulating Agent:

Groups I and III: An acute phase response was stimulated by subcutaneous injection of 2.0 ml of adjuvant in two equally divided doses in each animal.

Groups II and IV: An acute phase response was stimulated by intramuscular injection of 1.0 ml of triple typhoid vaccine in each animal.

Bleeding:

5.0 ml of blood was taken from all animals, on three consecutive days following the inoculation of the stimulatory agents. (Sera #2 and #3 and #4 respectively).

Capillary Tube Precipitation Test:

Cx-polysaccharide solution was used to detect the presence of Cx-polysaccharide reacting substances in serum samples (#1,#2,#3, and #4) from each animal. CRP positive and negative human sera served as positive and negative controls respectively.

Salt Fractionation Study:

Two pools of twenty-five ml of each normal and acute phase serum were prepared from guinea pigs of group III and were used for fractionation studies. Likewise two pools of normal and acute phase serum from six rabbits were prepared and used for fractionation studies for negative and positive control purposes respectively.

Double Diffusion Study in Agar Gel:

Normal and acute phase sera (sera #1,#2,#3, and #4) of each animal of all four groups were tested against ACRP-horse and ACxRP-horse for the demonstration of any cross-reaction. CRP negative and CRP positive serum were used as negative and positive controls for ACxRP-horse.

Results:

Capillary precipitation test:

No Cx-polysaccharide reacting substances were present in nor-

mal or acute phase sera of the chickens and the guinea pigs.

Salt Fractionation Study:

Rabbit acute phase pooled serum: The precipitate formed on the addition of 75% ammonium sulphate saturation was easily dissolved in distilled water. On dialysis against 0.01% calcium chloride solution a precipitate was formed. This precipitate was dissolved in normal saline and when tested by agar gel double diffusion it was shown to contain CxPP.

Rabbit pooled normal serum, guinea pig pooled normal serum and guinea pig pooled acute phase serum when individually treated under similar conditions showed no precipitate on dialysis against 0.01% calcium chloride solution.

Double Diffusion Study in Agar Gel:

None of the sera (#1,#2,#3, and #4) from the chickens and guinea pigs showed any cross-reaction against ACPP-horse or ACxPP-horse.

Discussion:

Studies on acute phase proteins of man, monkey and rabbit have shown them to be remarkably similar in most of their properties. They are all present only in acute phase serum, give a precipitin reaction Cx-polysaccharide and exhibit an immunological cross-reactivity (132). Salt fractionation studies on human and rabbit acute phase sera have shown that if the precipitate obtained at 50-75% salt saturation is dissolved in distilled water or normal saline and dialysed against 0.01% calcium chloride solution, the acute phase protein-lipid complex is precipitated out.

Attempts to demonstrate the production of acute phase proteins

by animal species other than man, monkey and rabbit have been made. Lofstrom in 1943 attempted to demonstrate the production of NCS in horses and guinea pigs (33). His attempts did not succeed. However, Hedlund in 1961 using Lofstrom's CSR technic demonstrated the presence of NCS in the acute phase sera of guinea pigs, hens and mice and his opinion was that these animals were also capable of producing acute phase protein (34). Based on the evidence that NCS of rabbits behaves like NCS of man in respect to Cx-polysaccharide precipitation and salt fractionation studies, attempts were made in this experiment, to demonstrate the presence of an analogous substance in the acute phase sera of guinea pigs and hens.

The results of this study have shown that normal and acute phase sera of both chickens and guinea pigs did not exhibit the presence of any Cx-polysaccharide reacting substance. Furthermore, salt fractionation studies on guinea pig pooled sera (normal and acute) showed that guinea pig sera did not possess a substance which behaves like CxCP in forming a precipitate on dialysis against 0.01% calcium chloride solution. Immunological studies in agar gel double diffusion tests, further showed the absence of any substance giving a cross-reaction with ACRP-horse or ACxCP-horse.

On the basis of the results of this study it is felt that chickens and guinea pigs do not produce an acute phase protein analogous to CRP and CxCP. In expressing this view the criterion used was that any acute phase protein analogous to CRP or CxCP would demonstrate a similar precipitation reaction with Cx-polysaccharide. As regards the salt fractionation study we feel that it is possible that any acute phase protein of guinea pig, if present in acute phase serum, may be precipitable

at a salt saturation below 50% or greater than 75%. Further it is also possible that the acute phase protein of guinea pig unlike CRP and CxRP may not be associated with lipids, hence no precipitation on dialysis against calcium chloride solution.

An answer to the question whether animal species other than man, monkey and rabbit produce any acute phase protein may possibly be given after immunising heterologous animals with pooled normal and pooled acute phase serum of the animal under study and compare any immunological cross-reactivity which may be shown by agar gel double diffusion studies.

Summary:

Chickens and guinea pigs do not produce acute phase proteins analogous to CRP and CxRP.

Normal and acute phase sera of chickens and guinea pigs do not show any cross-reaction with ACRP-horse and ACxRP-horse in agar gel double diffusion tests.

Experiment VI

This experiment was designed to observe the electrophoretic mobility of CxHP in rabbit acute phase sera by immunoelectrophoresis.

Experimental

Paired CxHP negative and positive sera of four rabbits were simultaneously subjected to immunoelectrophoresis. The CxHP titre in each of the CxHP positive sera was previously determined by double diffusion in agar gel and was known to be 1:8. The immunoelectrophoretic study was performed in the manner described under materials and methods. (p. 57)

Results:

Figure 15 shows the results of immunoelectrophoresis of CxHP negative and positive sera obtained from the same rabbit. Wells #1 and #2 contain CxHP negative and positive sera respectively. The trough on the lateral side of each well contains ACxHP-horse. It can be seen from the figure that in the region of CxHP positive serum an extra line is present (shown by an arrow, labelled CxHP). All four paired sera from the different rabbits used showed the same picture. If this additional precipitation line is due to CxHP then this suggests that its mobility is similar to that of a gamma-1 globulin (or beta-2 globulin). The absolute identity of the extra line in the acute phase sample has yet to be determined. It is most likely CxHP, but absorption studies will be required to confirm its identity. It is interesting to note that before there is a faint line of identity formed by the CxHP negative serum, which merges completely with the apparently extra line formed by its CxHP counterpart alongside.

Discussion:

The agar gel double diffusion studies used for the detection and quantitation of CxRP previously showed that the ACxRP-horse contains both CxRP and contaminant normal rabbit serum protein. This impurity in ACxRP-horse is also evidenced in this study as shown by lines other than the CxRP line between the CxRP positive serum and ACxRP-horse, and duplicated in the interaction lines between CxRP negative serum and ACxRP-horse. However the simultaneous study of a paired CxRP negative and CxRP positive serum in the same agar slide presents no difficulty in locating any additional line which may be seen only in the gamma-1 or beta-2 region of the CxRP positive serum (as a result of interaction of CxRP and its homologous antibodies in ACxRP-horse). The present study suggests that the additional line seen in positive serum and which is most likely due to CxRP has the mobility of a gamma-1 globulin (or beta-2 globulin), and that possibly CxRP is present in normal rabbit sera but that tests other than immunoelectrophoresis are too insensitive to detect its presence.

Summary:

The electrophoretic mobility of CxRP rabbit acute phase serum was studied. The mobility of an additional acute phase protein which is probably CxRP has been shown to be in the region of the gamma-1 globulins (or beta-2 globulins). This "additional" protein shows a strong precipitation line by the immunoelectrophoresis technique. A faint line of identity, produced by the normal (CxRP negative) serum and which merges completely with the "additional" line seen in the CxRP positive serum, suggests that CxRP may be present in normal rabbit serum in an amount which is detectable by less sensitive techniques.

Summary of Overall Findings.

The following points have arisen from the five studies. They are listed together with the experiment(s) from which they originated.

(a) The procedure utilised to bleed the rabbits, throughout the entire study, itself did not produce CxRP (Pilot Exper. #I).

(b) Antigen plus saline injections only occasionally produced CxRP (Pilot Exper. #I).

(c) Antigen plus incomplete Freund's type adjuvant injections regularly produced CxRP (Pilot Exper. #I and III).

(d) The CxRP response does not diminish following repeated stimulating injections (Pilot Exper. #I, Expers. #II and III).

(e) A single injection of a rabbit's own acute phase (CxRP- positive) serum given with the first dose of an immunising course of human serum albumin (HSA), resulted in an earlier appearance and earlier maximum titre of HSA antibody, when compared with the two groups given either saline or a rabbit's own normal serum with the first dose. (Exper. #II)

(f) The appearance of HSA antibodies did not result in a diminution of the CxRP response following subsequent stimulation in the same rabbit (Pilot Exper. # II).

(g) The CF antibody response, and CxRP response, is greater when adjuvant is used in conjunction with Adenovirus (APC) type 5 as antigen, than when saline is used. The titre of neutralising antibody to the same virus, however, shows very little difference between the saline and adjuvant groups (Exper. #III).

(h) Rabbit acute phase serum (CxRP-positive) cross-reacts with ACRP-horse and ACRP-goat, but not with ACRP-rabbit (Exper. #IV).

(i) Human acute phase serum (CRP-positive) cross-reacts with ACxCP-goat and ACxCP-horse (Exper. #IV).

(j) h/ and i/ suggest that CxCP and CRP share a common antigen.

(k) ACRP has no antibodies to that component of CRP which is shared by CxCP (Exper. #IV).

(l) The CxCP produced by different rabbits is antigenically identical (Exper. #IV).

(m) Proteins, similar to CxCP and CRP, do not seem to appear in chicken and guinea pig acute phase sera (Exper. #V).

Discussion of Overall Findings.

It has been adequatly demonstrated that adjuvant enhances the specific immune response of warm blooded animals to the concommitant parenteral administration of an antigen. The phenomenon is constantly utilised for the everyday production of diagnostic antisera in the laboratory.

This study has shown that the use of adjuvant regularly produces detectable levels of CxRP in the serum of the recipient rabbit. Such serum is considered as acute phase serum.

The mechanism(s) whereby adjuvant promotes such activities are not understood.

Possible ways in which adjuvant may work are as follows:-

(a) Adjuvant forms a depot of persisting antigen. This may allow the slow release of small amounts of antigen, from the site of injection, to antibody forming cells. Thus, behaving as if multiple small doses of antigen were injected daily over a period of time; a process known to produce a good antibody response.

(b) Adjuvant may stimulate the antibody forming system in a non-specific manner.

(c) Adjuvant may, by causing local tissue damage and inflammation, cause an increase in the systemic mobilization of phagocytic cells which engulf antigen particles and allow a more efficient and widespread dissemination of the antigen to sites of antibody production.

(d) Adjuvant may stimulate the production of an intermediate humoral substance which, itself alone, is the agent responsible for the subsequent enhancement of the specific immune response. Such a substance might be involved in the hypothetical explanations (b) and (c).

The first theoretical explanation, (a), is unlikely to be correct,

as it has been adequately recorded that the immune response may be enhanced when the antigen and adjuvant are injected at sites far removed from one another (110). Furthermore, this study has shown that acute phase serum (CxCP-positive) produced by adjuvant, one month previously, will itself act as an adjuvant. A single injection only, of such serum, if given at the commencement of an immunization course, is capable of accellerating and enhancing a specific immune response. Resting serum from this same rabbit, taken prior to the use of any experimental procedure, had no such action.

This would support the concept that the heightened immune response resulting from the use of adjuvant is mediated by the activity of some endogenous factor which appears in the serum following the administration of adjuvant. The continued presence of adjuvant, acting in some other manner over a period of one month after its administration, is a possibility which has not been excluded by this study. The use of acute phase serum produced following mechanical trauma, may well clarify this point.

Adjuvant has been shown to enhance the C.F. antibody response to Adenovirus type 5 but not the final neutralising antibody response.

This cannot adequately be explained. It is possible that the size of the antigen molecule is in some way involved, as outlined in the discussion following Experiment #II.

The antigenic studies on human acute phase serum (CRP-positive) and rabbit acute phase serum(CxCP-positive) have shown that CRP and CxCP share a common antigenic constituent. This common antigenic constituent behaves as an antigen present in normal rabbit serum which becomes incorporated as part of the molecule which reacts as CxCP, in rabbit acute phase serum. This is supported by the results of parallel immunoelectrophoretic analysis of normal and acute phase serum obtained from the same

rabbit. The acute phase serum sample reveals a prominent precipitation arc in the beta-globulin region which at first glance appears identical to those found in the counterpart normal serum. However, the normal serum shows the presence of a faint line which merges with that in the acute phase serum, as a reaction of complete identity. This further suggests that, at least a portion of the CxRP molecule, is present in normal rabbit serum.

Thus, CxRP appears to be an unusual protein which becomes detectable in rabbit serum following adjuvant stimulation. Such serum (acute phase), in which CxRP is found, is itself capable of reacting as an adjuvant. CxRP possibly contains a constituent antigenically identical to a constituent present in normal (CxRP-negative) rabbit serum.

Samples of CxRP present in acute phase serum samples obtained from different rabbits appear to be antigenically identical.

Guinea pigs and chickens do not appear to produce proteins directly analogous to CxRP.

Further Work Recommended:

(a) Attempts should be made to crystalise CxRP.

(b) The local and systemic inflammatory reaction should be studied in rabbits following the parenteral administration of varying amounts of crystaline CxRP.

(c) Indirect evidence suggests that CxRP (and CRP) may be one of the agents responsible for the endogenous production of fever. This should be studied in rabbits using CxRP.

(d) The possibility that CxRP is nothing more than a repolymerization or a degradation product of serum proteins normally present, should be investigated. This is primarily a biochemical problem, however.

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SOURCE OF MATERIALS USED.

NAME OF THE MATERIAL	SOURCE OBTAINED
1. Antigen	
(a) Echovirus types 22,24,25 and 28..... grown in trypsinized second generation Human Amnion Tissue Culture Cells.	Federal Laboratory of Hygiene, Ottawa
(b) Adenovirus type 5 grown in..... trypsinized second generation Human Amnion Tissue Culture Cells group "O".	Stock Culture of the Virus Lab. Department of Bacteriology and Immunology, University of Manitoba, Winnipeg.
(c) Human Crystallized Serum Albumin.....	Connaught Medical Research Lab., University of Toronto, Toronto, Ontario.
(d) Triple Typhoid Vaccine.....	
2. Antisera	
(a) ACRP-rabbit.....	Difco Laboratories, Detroit 1. Michigan, U.S.A.
(b) ACRP-horse.....	" " "
(c) ACRP-goat.....	" " "
(d) ACxRP-horse.....	Hyland Laboratories, Los Angeles, California, U.S.A.
(e) ACxRP-goat.....	" " " "
(f) Rabbit -produced Antiserum to..... Normal Horse Serum.	" " " "
(g) Rabbit produced Antiserum to Human....	" " " "
Crystallized Serum Albumin.....	" " " "
3. Sera	
Normal Horse Serum.....	Connaught Medical Research Lab., University of Toronto, Toronto
4. Adjuvant	
Bayol F.....	Imperial Oil Ltd., Winnipeg, Man.
Arlacel A.....	Atlas Chemical Industries, Inc., Chemical Division, Welmington, D.E.L.

NAME OF THE MATERIAL	SOURCE OBTAINED
5. Agar	
Special Nobel Agar.....	Difco Laboratories, Detroit 1, Michigan, U.S.A.
6. Capillary Tubes	Fischer Scientific, Toronto
7. Centrifuge	
International Portable Refrige-..rated Centrifuge Model PR -2 Serial No 150-E	International Equipment Co. Boston, Mass., U.S.A.
8. Dialysing Tube	
Dialysing Tube - Visking Dia- .. meter 19 mm	Canadian Laboratory Supplies, Winnipeg
9. Electrophoretic Power Pack	
Reco Electrophoresis Migration.. Chamber Model E-800-2	Research Equipment Corporation, Oakland California, U.S.A.
10. PH - Meter	Beckman Zeromatic pH Meter Palo Alto, California.
11. Tissue Culture Cells	
(a) Monkey Kidney Cells.....	Connaught Medical Research Laboratories Toronto. Sent at a 1:1200 dilution of packed cells in propagating media (0.5% lactalbumin hydrolysate, 2.0% horse serum in Hank's balanced salt solution).
(b) Human Amnion Cells.....	Virus Laboratory, Department of Bacteriology and Immunology, University of Manitoba, Winnipeg, Manitoba
12. Filter and Pads	
(a) Filter Paper.....	W & R Balston Ltd., England.
(b) 15.0 cm Seitz Filters.....	" " "

TABLE NO. I

THE PRODUCTION OF CxHP AND ITS TITER* IN RABBITS GIVEN VARIOUS STIMULATING AGENTS.

STIMULATING AGENT	GROUP NO.	RABBIT NO.	DAY OF EXPERIMENT																	
			2	3	4	5	6	7	8	9	10	15	16	17	22	23	24	31	32	33
VIRUS AND ADJUVANT	I	1	8	8	8	4	4	4	4	4	0	0	0	0	0	8	8	4	2	0
		2	16	16	8	2	1	0	0	0	0	0	1	0	0	8	4	0	4	0
		3	4	4	2	0	0	0	0	0	0	0	0	0	0	2	1	0	0	0
		4	4	4	4	2	1	1	0	4	0	0	0	0	0	4	4	0	0	0
SALINE AND ADJUVANT	II	5	4	16	4	4	4	4	1	2	2	0	0	0	0	2	1	0	0	0
		6	4	4	4	4	4	2	1	0	0	0	0	0	0	8	4	0	1	1
		7	8	8	8	4	4	4	4	2	0	0	0	0	0	4	4	0	0	0
SALINE	III	8	2	2	1	1	0	0	0	0	0	0	0	0	0	0	0	4	1	2
		9	2	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
		10	1	1	0	0	0	0	0	0	0	0	1	1	0	1	1	0	0	0
NIL	IV	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* RECIPROCAL OF SERUM DILUTION.
0 - NO CxHP DETECTED IN UNDILUTED SERUM.

TABLE NO. II

Virus Neutralising

Antibody Titres* in 4 rabbits, immunised by echovirus types 24,25,28 and 22 respectively plus incomplete Freund's type adjuvant.

ECHOVIRUS TYPE	RABBITS NO.	DAY OF EXPERIMENT	
		2	41
24	1	< 4	512
25	2	< 4	512
28	3	< 4	1028
22	4	< 4	128

* RECIPROCAL OF SERUM DILUTION

TABLE NO. III

CxRP AND A-HSA TITRE* IN RABBITS BEFORE AND AFTER INOCULATION WITH ADJUVANT.

RABBIT NO.	CxRP TITRE		A-HSA TITRE	
	SERUM #1	SERUM #2	SERUM #1	SERUM #2
21	0	8	0	0
22	0	8	0	0
23	0	1	0	0
24	0	4	0	0
25	0	4	0	0
26	1	2	0	0
27	0	0	0	0
28	0	1	0	0
29	0	2	0	0
30	0	4	0	0
31	0	4	0	0
32	0	2	0	0
33	0	4	0	0
34	0	8	0	0

A-HSA = HUMAN SERUM ALBUMIN ANTIBODY.

#1 = PRE-ADJUVANT INOCULATION SERUM.

#2 = POST-ADJUVANT INOCULATION SERUM.

* = RECIPROCAL OF SERUM DILUTION.

0 = NO CxRP OR A-HSA DETECTED IN UNDILUTED SERUM.

TABLE NO. V

CxRP TITRE IN RABBIT SERA FOLLOWING INOCULATION WITH ADJUVANT (GROUP I) AND IN NORMAL RABBIT SERA (GROUP III).

GROUP NO.	RABBIT NO.	CxRP TITRE* IN SERUM #3+
I	21	4
	24	4
	30	8
	34	2
III	25	0
	29	0
	31	0
	34	0

* RECIPROCAL OF SERUM DILUTION

\+ GROUP I = SERUM AFTER INOCULATION WITH 10.0ml OF ADJUVANT
GROUP III = NORMAL SERUM - NO INOCULATION.

TABLE NO. VI

WEIGHT OF RABBITS AND DOSE OF HUMAN SERUM ALBUMIN SOLUTION (HSA) EACH RECEIVED.

GROUP NO.	RABBIT NO.	WEIGHT OF THE RABBIT IN KG.	DOSE OF HSA SOLUTION (2% W/V) INJECTED
I	21	2.961	2.2 cc
	24	2.843	2.15 cc
	30	3.180	2.5 cc
II	22	4.0	3.0 cc
	33	4.065	3.0 cc
III	25	4.420	3.3 cc
	29	3.236	2.5 cc
	31	2.90	2.2 cc
	34	4.	3.0 cc

* - RECIPROCAL OF SERUM DILUTION

\+ - GROUP I - INOCULATED I.P WITH OWN ACUTE PHASE SERUM (CxRP POSITIVE) ALONG WITH HSA, I.V ON DAY 51

GROUP II - INOCULATED I.P WITH STERILE PHYSIOLOGICAL SALINE ALONG WITH HSA, I.V ON DAY 51

GROUP III - INOCULATED I.P WITH OWN ACUTE PHASE SERUM (CxRP NEGATIVE) ALONG WITH HSA, I.V ON DAY 51

ALL RABBITS IN EACH GROUP SUBSEQUENTLY INOCULATED WITH HSA, S.C ON DAYS 79, 86 93, 100 AND 107.

HSA - HUMAN SERUM ALBUMIN SOLUTION 25% W/V

I.P - INTRAPERITONEAL

I.V - INTRAVENOUS

S.C - SUBCUTANEOUS

TABLE NO. VII

CLOT TIME IN RABBITS FOLLOWING THE ADMINISTRATION OF VARYING STIMULATING AGENTS.

GROUP NO.	RABBIT NO.	DAY OF EXPERIMENT																
		52	55	58	61	65	73	76	80	86	87	93	94	100	101	107	108	112
I	21	4	1	2	2	2	2	1	4	2	8	1	16	0	16	1	4	1
	24	2	0	0	2	0	0	0	0	0	0	0	0	0	2	0	4	0
	30	2	0	0	0	0	0	0	4	0	16	0	8	0	16	0	16	0
II	22	1	1	0	1	2	0	0	0	0	2	0	4	0	4	0	4	0
	33	4	4	1	2	2	0	2	4	0	2	0	4	0	8	0	8	1
III	25	2	0	0	16	0	0	0	4	0	4	0	4	1	16	1	8	0
	29	2	2	0	0	0	0	0	1	0	0	0	4	0	4	0	4	0
	31	4	1	1	0	0	0	4	4	0	2	0	4	0	4	0	4	4
	34	2	0	0	1	0	0	0	4	0	8	0	8	0	2	0	4	0

TABLE NO. VIII

A-HSA TITRE* IN RABBITS FOLLOWING THE ADMINISTRATION OF VARYING STIMULATING AGENTS.‡

GROUP NO.	RABBIT NO.	DAY OF EXPERIMENT															
		52	55	58	61	71	76	80	86	87	93	94	100	101	107	108	112
I	21	0	0	0	0	0	0	0	16	8	32	16	2	2	32	8	16
	24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	30	0	0	0	0	0	0	0	2	2	16	2	2	2	8	4	1
II	22	0	0	0	0	0	0	0	0	0	2	2	2	1	4	8	2
	33	0	0	0	0	0	0	0	0	0	0	0	4	4	8	4	4
III	25	0	0	0	0	0	0	0	0	0	0	0	4	4	4	4	2
	29	0	0	0	0	0	0	0	0	0	0	0	4	4	4	4	4
	31	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	34	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

A-HSA - HUMAN SERUM ALBUMIN ANTIBODY.
* - RECIPROCAL OF SERUM DILUTION.
‡ - SEE TABLE VII

GEOMETRIC MEAN OF C-NP TITRES SHOWN IN TABLE NO. VIX.

GROUP NO.	DAY OF EXPERIMENT																
	52	55	58	61	65	71	76	80	86	87	93	94	100	101	107	108	112
I	2.5	1	1.26	1.59	1.26	1.26	1	2.5	1.26	5	1	5	X	8	1	6.35	1
II	2	2	1	1.41	2	X	1.41	2	X	2	X	4	X	5.65	X	5.65	1
III	2.37	1.19	1	2	X	X	1.41	2.81	X	2.81	X	4.75	1	4.75	1	4.75	1.4

X - NO GEOMETRIC MEAN TAKEN AS ALL FIGURES WERE ZERO.

TABLE NO. X

GEOMETRIC MEAN OF A-HSA TITRES SHOWN IN TABLE VIII.

GROUP NO.	DAY OF EXPERIMENT																
	52	55	58	61	65	71	76	80	86	87	93	94	100	101	107	108	112
I	X	X	X	X	X	X	X	X	3.17	2.5	8	3.17	1.58	1.58	6.35	3.17	2.5
II	X	X	X	X	X	X	X	X	X	X	1.41	1.41	2.81	2	5.65	5.65	2.81
III	X	X	X	X	X	X	X	X	X	X	X	X	2	2	2	2	1.68

X - NO GEOMETRIC MEAN TAKEN AS ALL FIGURES WERE ZERO.

TABLE NO. XI.

C.F.P TITRES* IN RABBITS GIVEN VARYING STIMULATING AGENTS.

STIMULATING AGENT	GROUP NO.	RABBIT NO.	DAY OF EXPERIMENT																						
			1	3	4	5	6	7	8	9	10	11	16	17	18	23	24	25	26	27	28	29	32	33	34
		50	0	4	4	1	1	1	1	0	0	0	0	0	0	0	2	2	2	1	1	1	RABBIT DIED		
VIRUS AND ADJUVANT	I	51	0	16	2	4	1	1	0	0	4	2	1	1	1	0	4	2	1	0	0	0	0	0	0
		52	0	4	4	2	2	2	2	2	4	2	0	2	1	0	4	4	2	2	1	1	0	1	1
		53	0	4	4	2	2	1	1	1	1	0	4	2	1	0	4	4	4	1	0	0	0	1	0
		54	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	1
VIRUS AND SALINE	II	55	0	0	0	0	0	0	0	1	1	0	0	0	1	0	1	0	0	0	0	0	0	1	0
		56	1	2	4	4	4	1	1	0	0	0	0	1	1	1	4	2	1	1	1	1	0	2	1
		57	2	1	0	1	1	2	1	1	0	0	0	1	0	0	4	1	1	0	0	0	0	2	0

* = RECIPROCAL OF SERUM DILUTION
0 = NO C.F.P DETECTED IN UNDILUTED SERUM

TABLE NO. XII

COMPLEMENT FIXING ANTIBODY TITRES* IN RABBITS FOLLOWING THE ADMINISTRATION OF TWO DIFFERENT STIMULATING AGENTS. (INCOMPLETE FREUND'S-TYPE ADJUVANT AND SALINE)

STIMULATING AGENT	GROUP NO.	RABBIT NO.	DAY OF EXPERIMENT						
			1	5	9	17	23	28	37
VIRUS AND ADJUVANT	I	50	0	0	64	32	64	256	RABBIT DEAD
		51	0	4	32	64	64	128	128
		52	0	0	64	64	64	256	128
		53	0	0	64	64	128	512	256
		54	0	0	32	32	32	128	64
VIRUS AND SALINE	II	55	0	0	32	16	32	128	64
		56	0	0	32	16	32	128	128
		57	0	0	32	64	64	64	128

* RECIPROCAL OF SERUM DILUTION

0 TITRES LESS THAN 1:4

TABLE NO. XIII

VIRUS NEUTRALIZED ANTIBODY TITRES* IN RABBITS FOLLOWING THE ADMINISTRATION OF TWO DIFFERENT STIMULATING AGENTS. (FREUND'S-TYPE INCOMPLETE ADJUVANT AND SALINE)

STIMULATING AGENT	GROUP NO.	RABBIT NO.	DAY OF EXPERIMENT	
			1	37
VIRUS AND ADJUVANT	I	50	< 4	512**
		51	< 4	256
		52	< 4	128
		53	< 4	128
VIRUS AND SALINE	II	54	< 4	256
		55	< 4	128
		56	> 16	128
		57	< 4	256

* RECIPROCAL OF SERUM DILUTION

** SERUM TESTED ON DAY 30.

TABLE NO. XV

MEAN LOG* OF THE COMPLEMENT FIXING ANTIBODY TITRES SHOWN IN TABLE XVI.

STIMULATING AGENT	GROUP NO.	DAY OF EXPERIMENT						
		1	5	9	17	23	28	37
VIRUS AND ADJUVANT	I	X	.15	1.73	1.73	1.88	2.4	2.2
VIRUS AND ADJUVANT	II	X	X	1.5	1.4	1.58	2.03	1.95

X - NO MEAN LOG TAKEN AS ALL FIGURES WERE ZERO

* - LOG TO THE BASE 10.

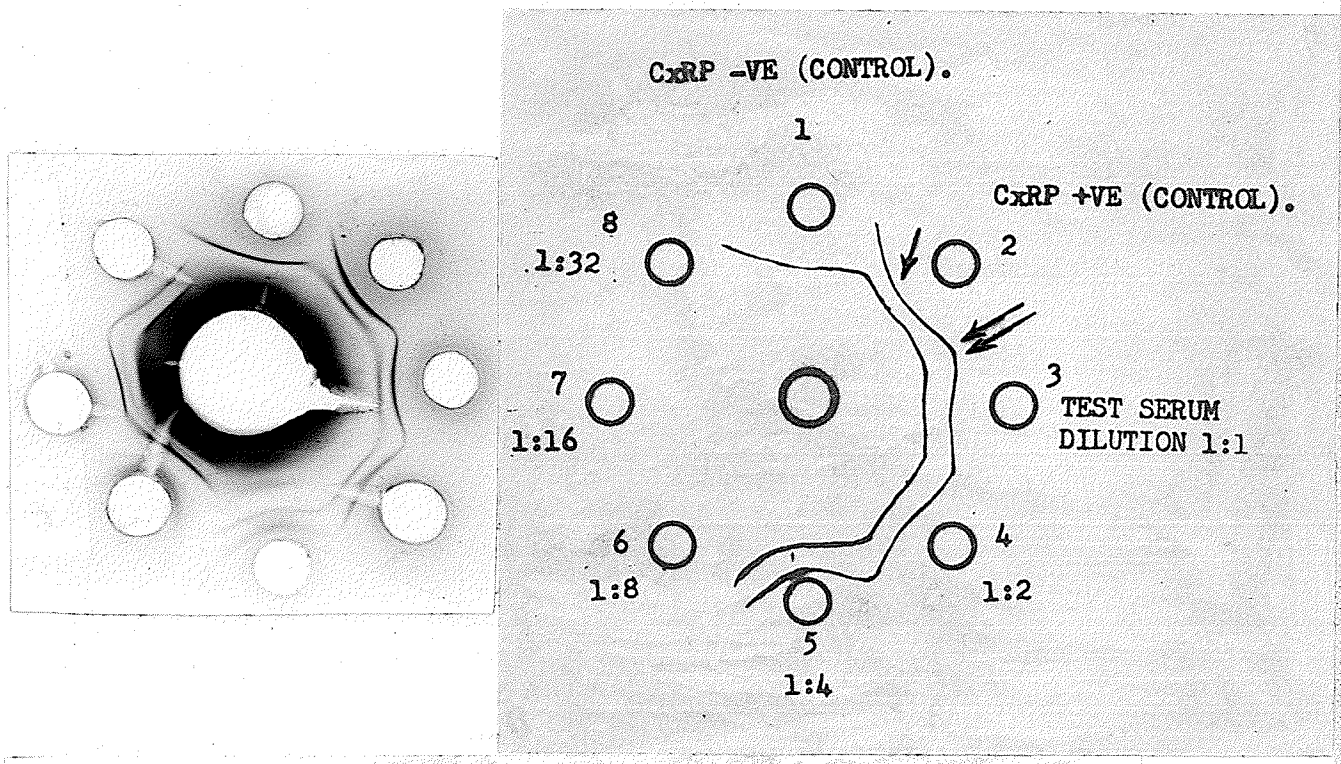


Figure 1. Detection and quantitation of CxRP in rabbit serum by double diffusion in agar, the central well contains ACxRP-horse.

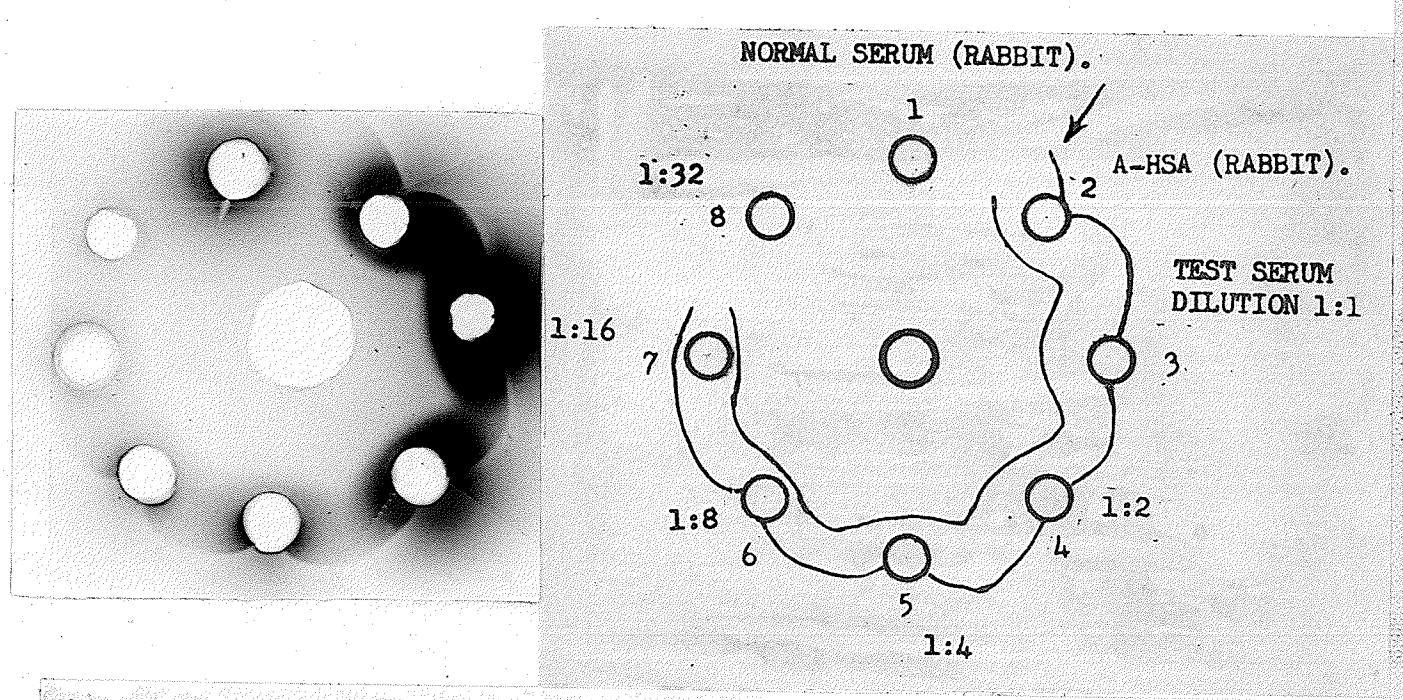


Figure 2. Detection and quantitation of precipitating antibodies to human serum albumin (AHSA) in rabbit serum by double diffusion in agar, the central well contains human serum albumin 2% W/v.

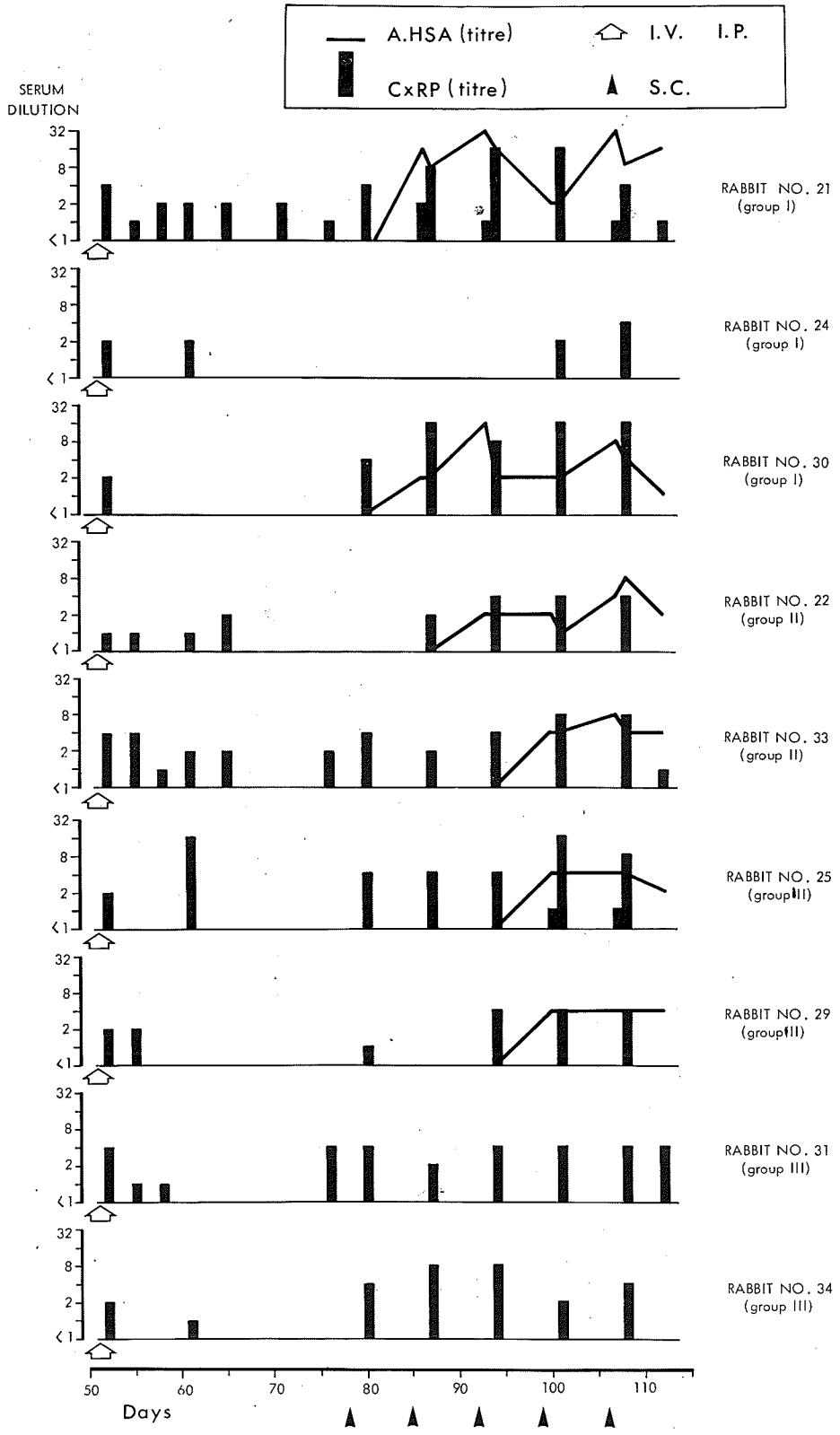


Figure 3. CxRP and human-serum-albumin antibody (AHSA) titres after initial intraperitoneal (I.P.) administration of rabbit's own acute phase serum (Group 1), sterile physiological saline (Group 2), and rabbit's own serum (Group 3), along with intravenous (I.V.) injection of human-serum-albumin (HSA) followed by subcutaneous injections of HSA to all groups at the indicated intervals.

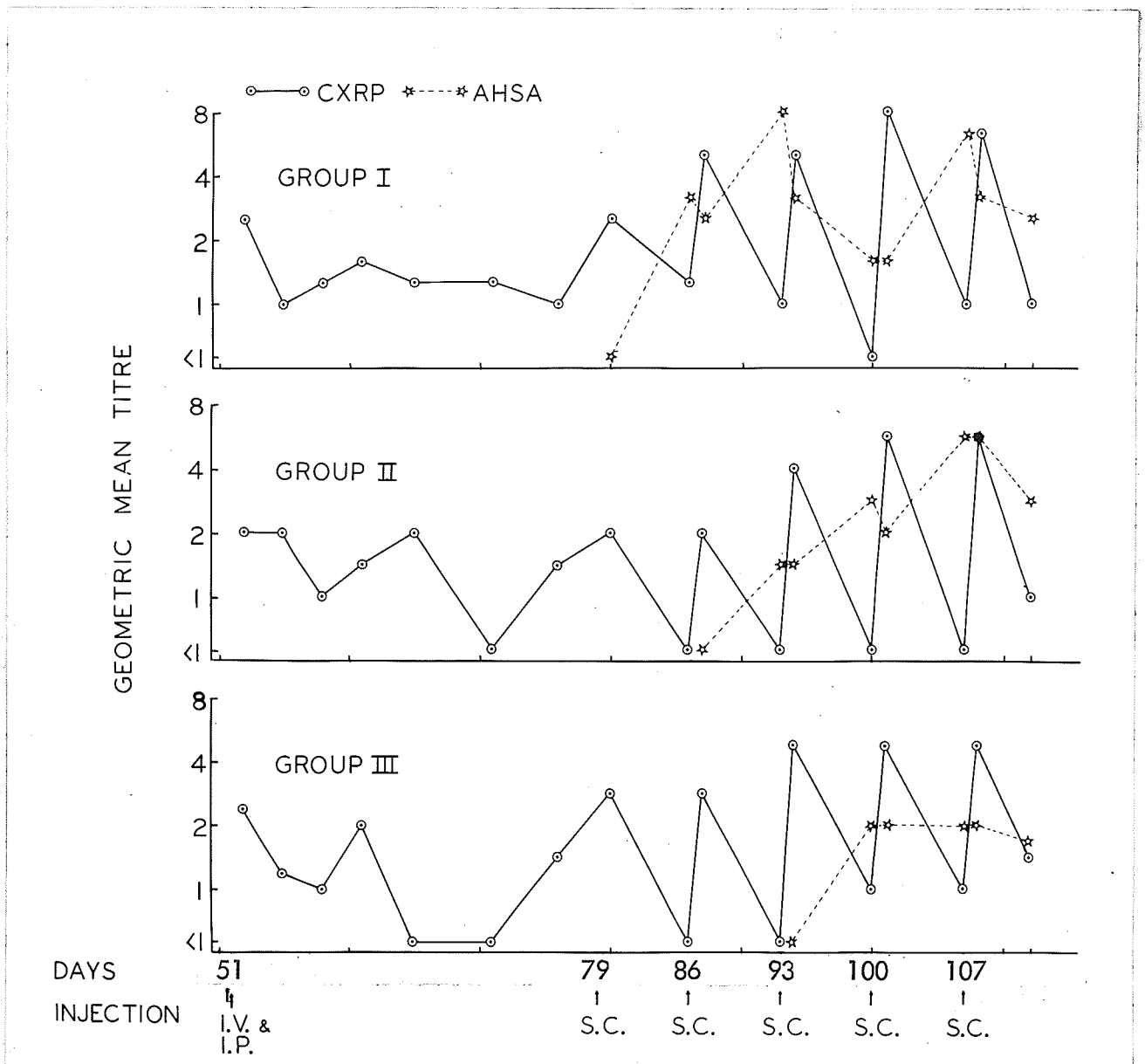


Figure 4. Geometric mean titres of CxRP and human-serum-albumin antibody (AHSA) of groups 1,2,3. See fig.3 for explanation.

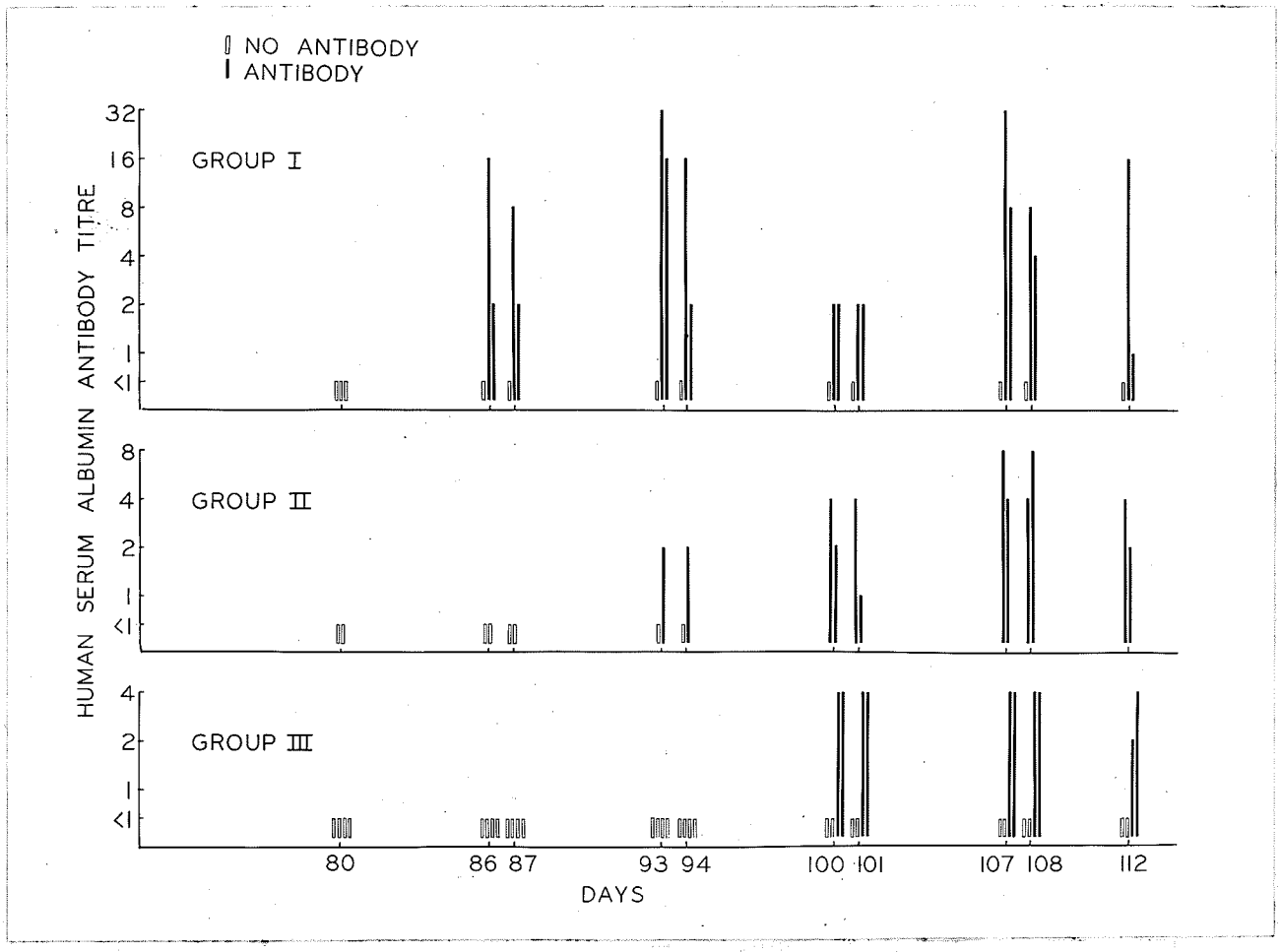


Figure 5. Human serum albumin antibody titre of individual rabbits.
Reading from left to right:
Group I rabbit # 24, 21 and 30.
Group II rabbit # 33 and 22.
Group III rabbit # 31, 34, 25 and 28.
See fig. 3 for explanation.

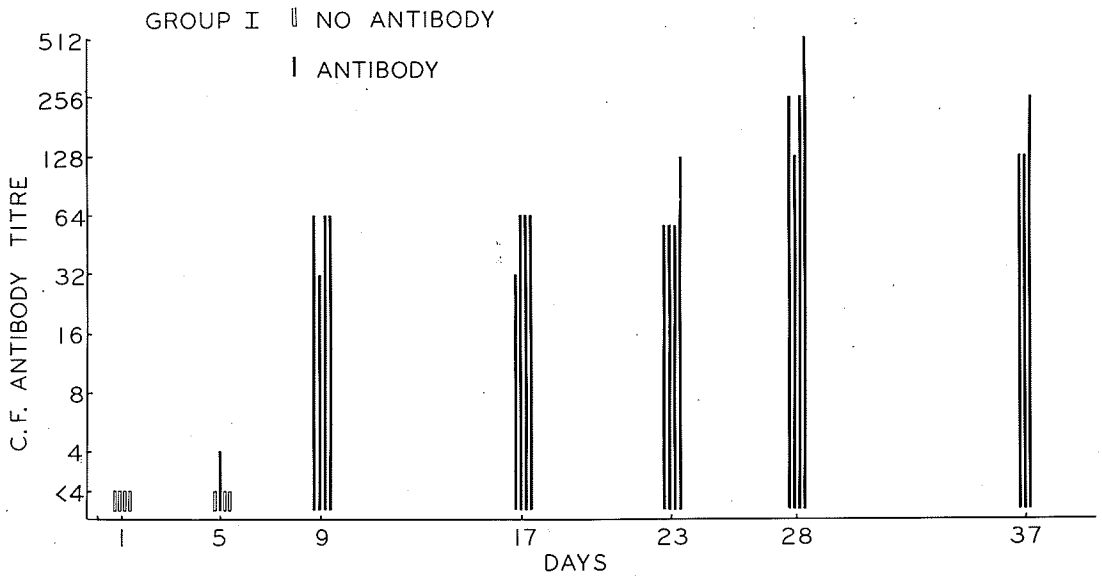


Figure 6. Complement fixing (CF) antibody titres to Adenovirus type 5 of individual rabbits in group I at the indicated intervals after the following immunization schedule: days 2 and 23 virus-adjuvant mixture subcutaneously (S.C.) along with virus intravenously (I.V.),days 9,16 and 32 virus alone I.V. Rabbit # from left to right at each indicated interval 50,51,52 and 53.

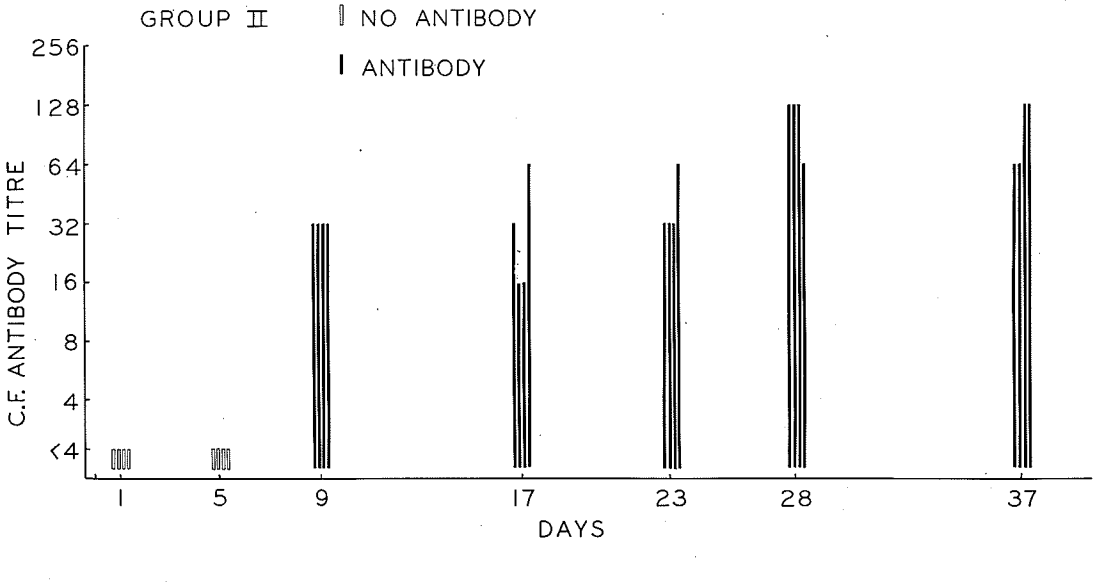


Figure 7. As fig.6 except that for these rabbits (group II) the virus was mixed with sterile physiological saline instead of adjuvant in the first injections (days 2 and 23).Rabbit # 54,55,56 and 57.

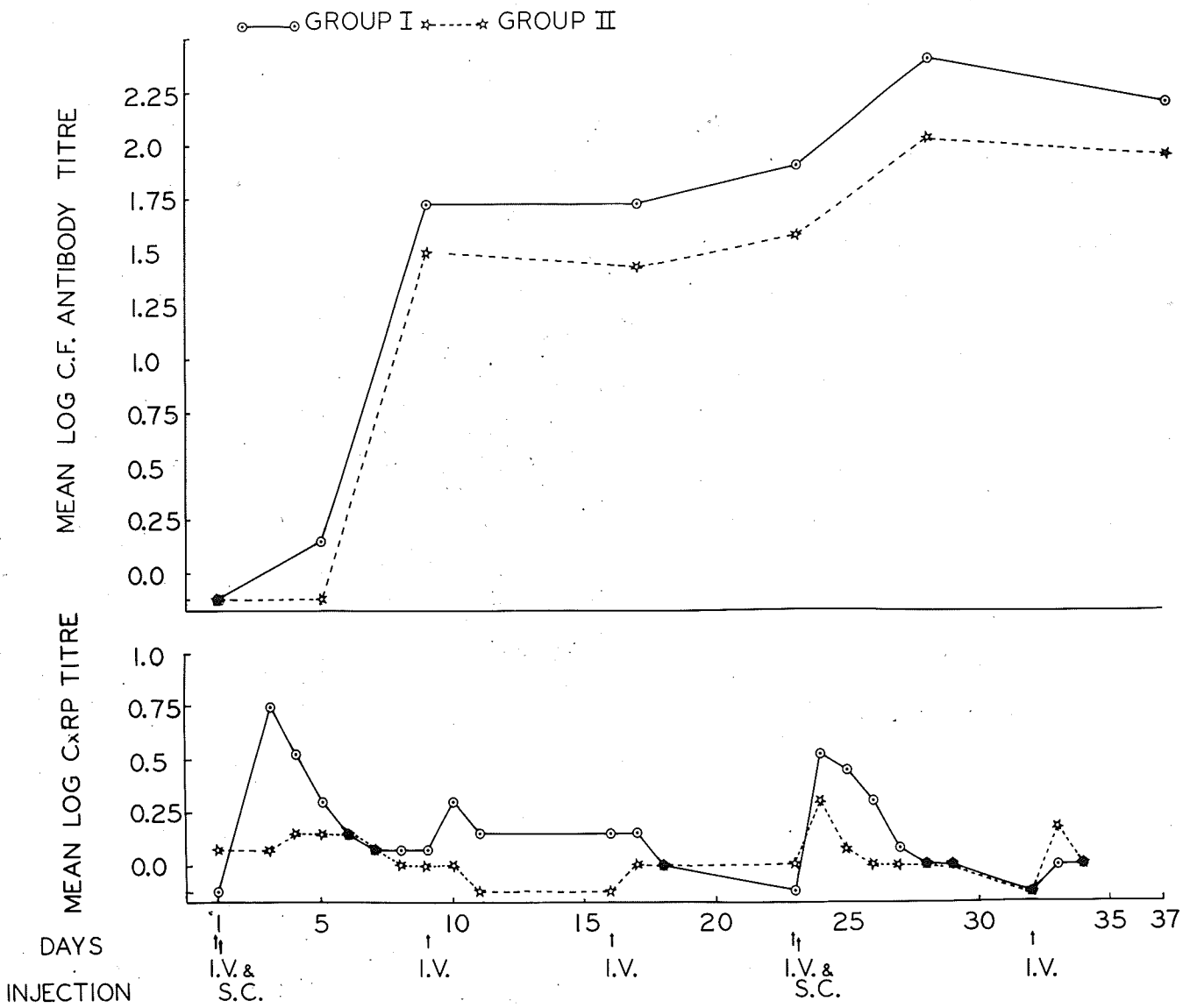
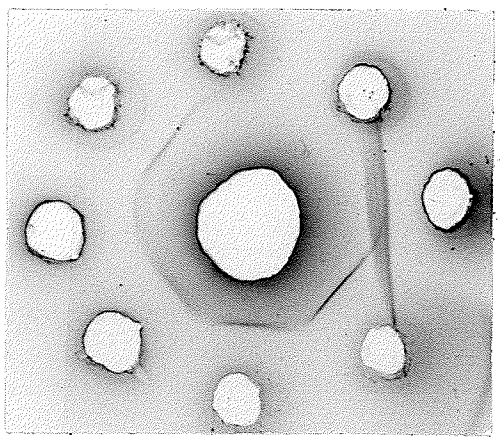


Figure 8. Mean log. C.F. antibody titre to Adenovirus type 5 and mean log. CxRP titre of groups I and II shown in figures 6 and 7.
Group I Virus and adjuvant treated rabbits.
Group II Virus and saline treated rabbits.



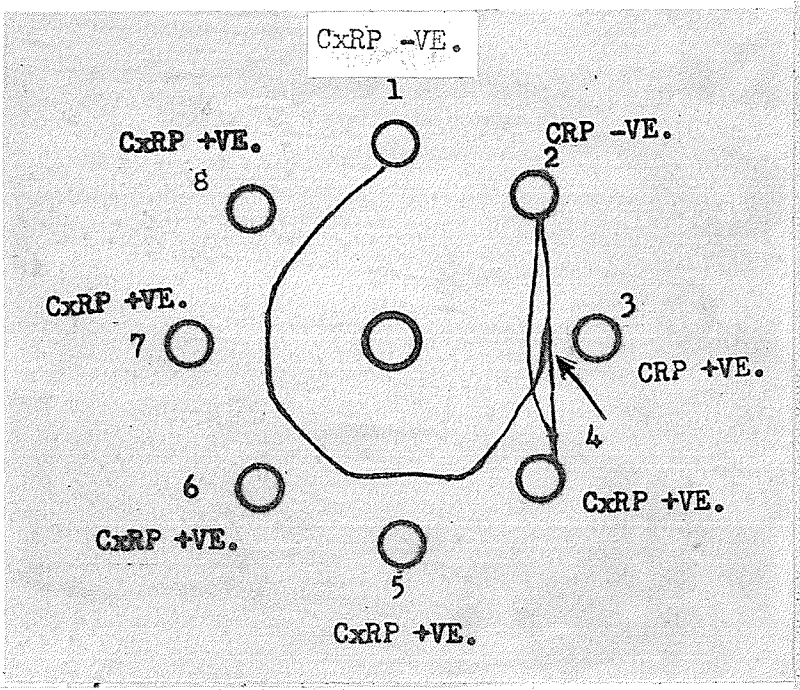
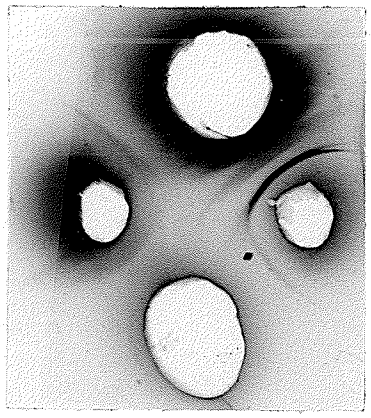


Figure 9. Demonstration of a cross-reaction between CxRP-positive sera and ACRP-goat (central well), as shown by double diffusion in agar.



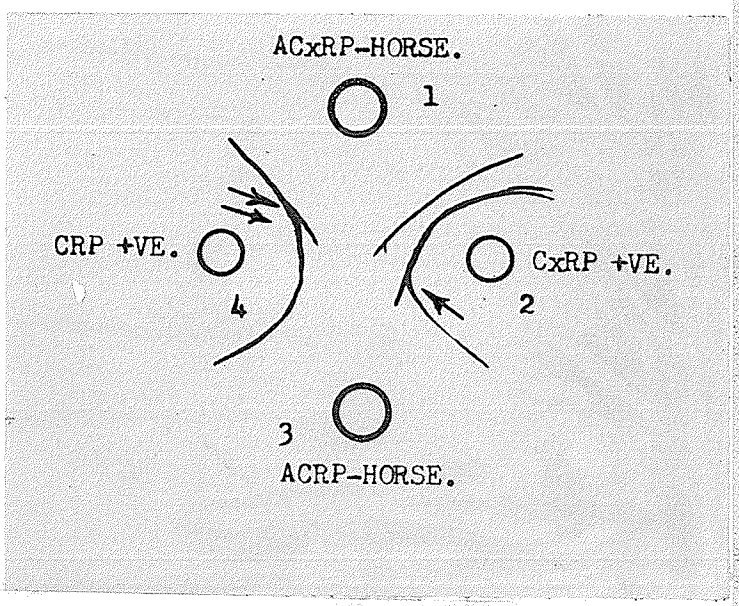
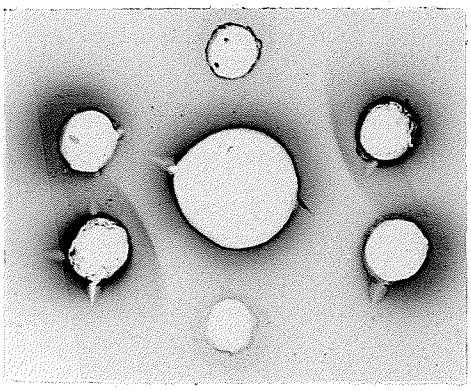


Figure 10. Demonstration of a cross-reaction of CRP-positive and CxRP-positive sera, with ACRP-horse and ACxRP-horse, as shown by double diffusion in agar.



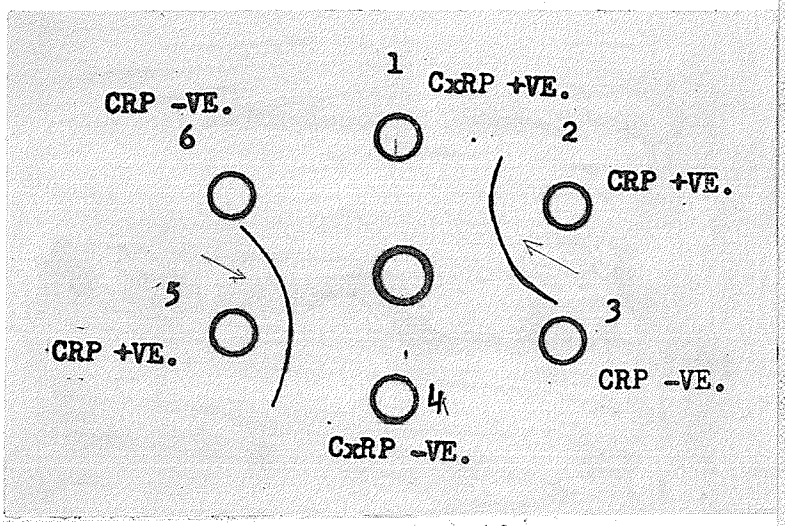
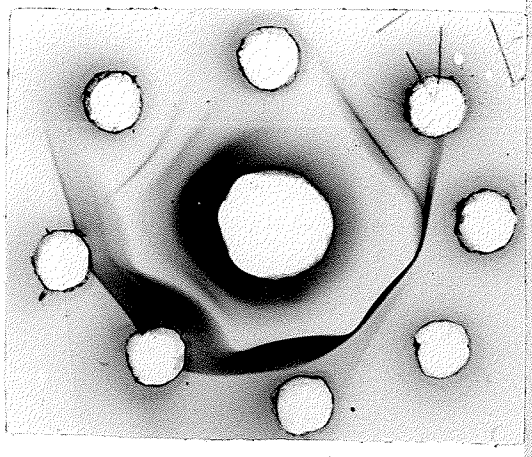


Figure 11. Absence of cross-reaction of CxRP-positive, CxRP-negative and CRP-negative sera, with A-CRP-rabbit, as shown by double diffusion in agar.



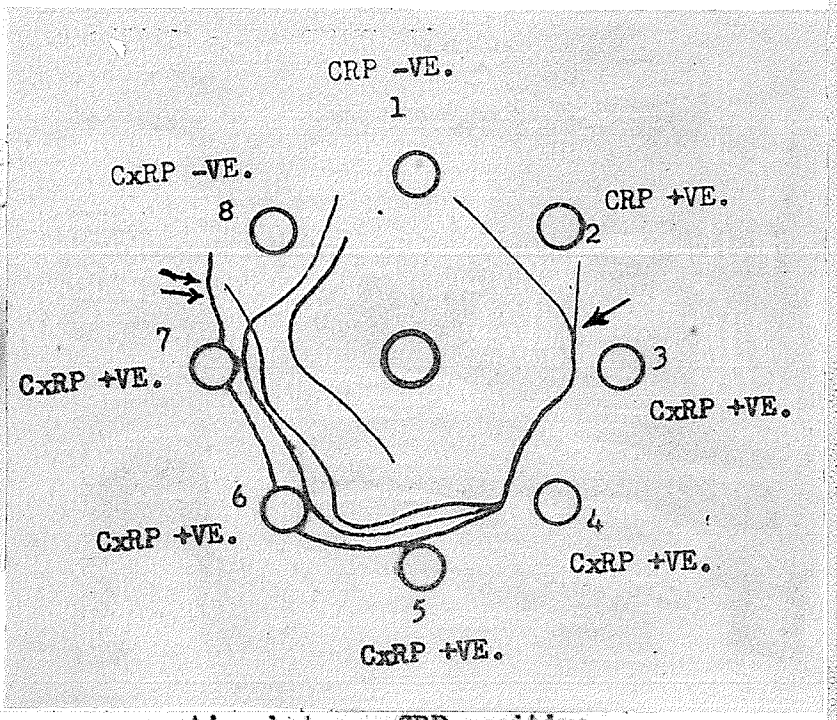
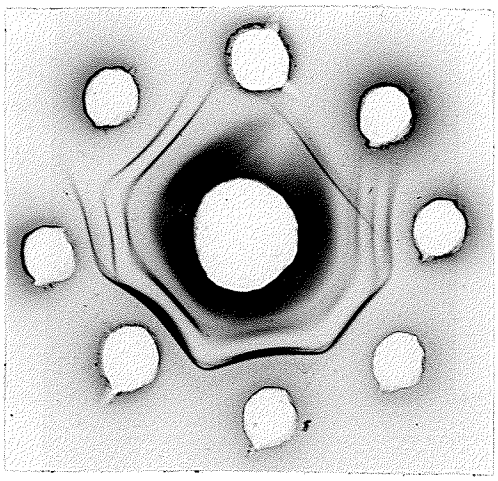


Figure 12. Demonstration of a cross-reaction between CRP-positive serum and A-CxRP-goat (central well), as shown by double diffusion in agar.



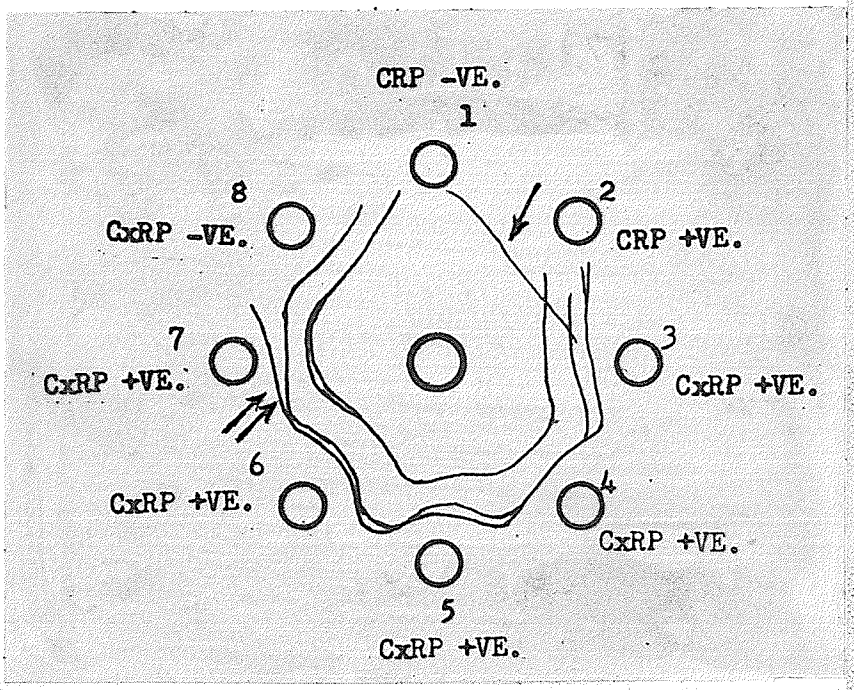
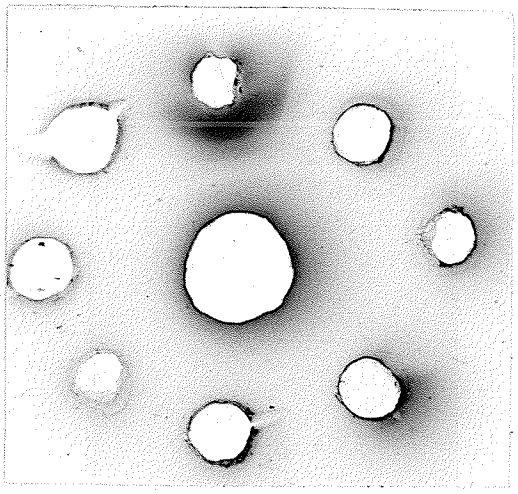


Figure 13. Demonstration of a cross-reaction between CRP-positive serum and ACxRP-horse (central well), as shown by double diffusion in agar.



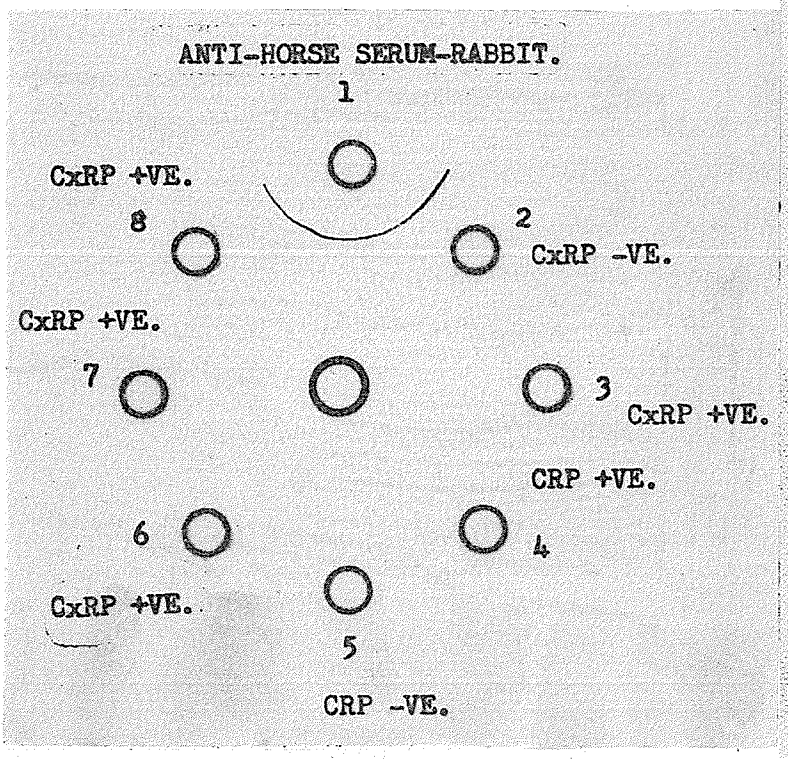
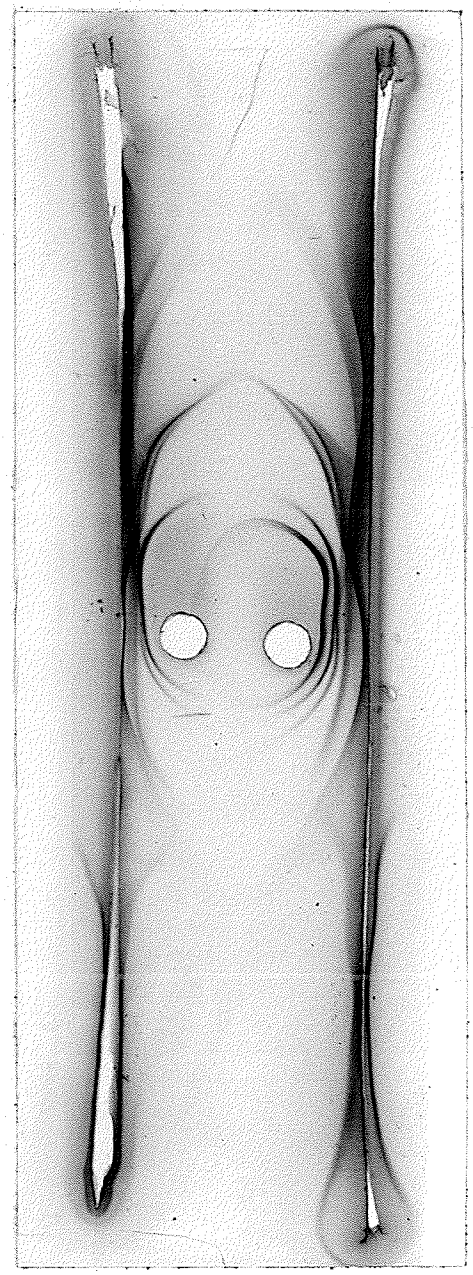


Figure 14. Absence of cross-reaction of CxRP-negative, CxRP-positive, CRP-negative and CRP-positive sera, with normal horse serum (central well), as shown by double diffusion in agar.



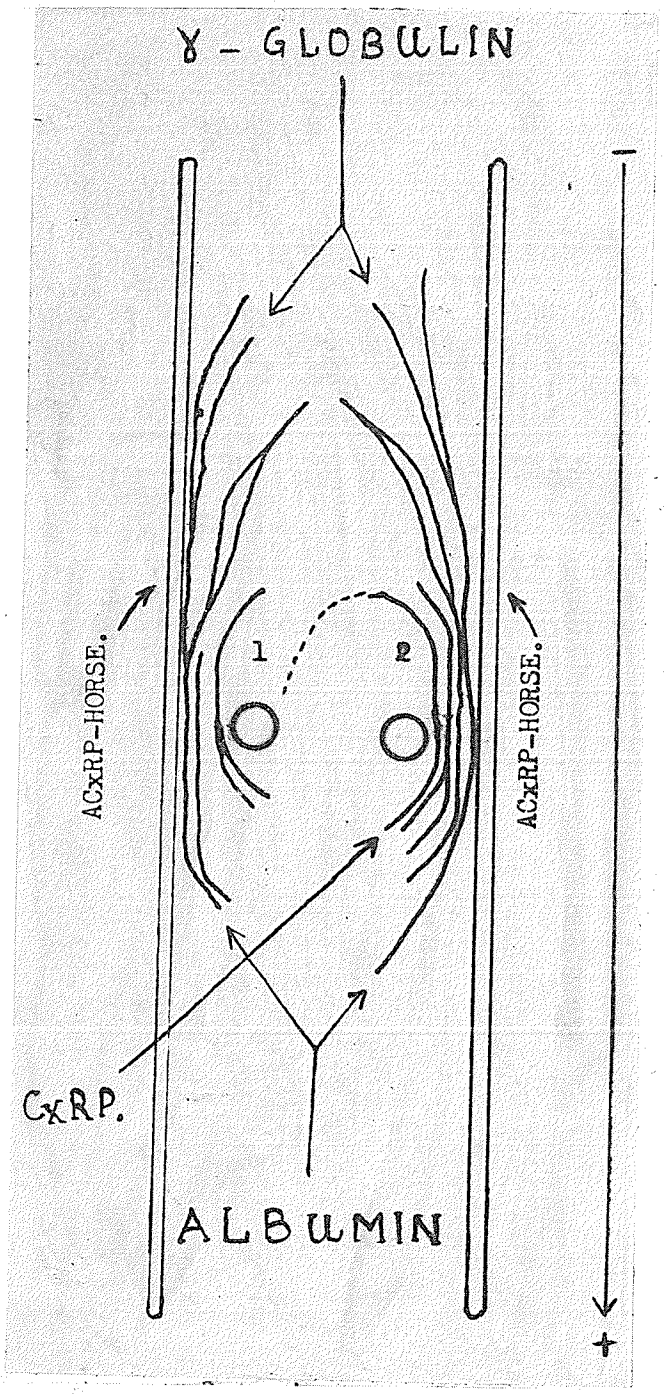


Figure 15. Immune electrophoretic comparison of normal CxRP-negative serum (well 1) and post-adjuvant stimulated CxRP-positive serum (well 2).
The troughs contain CxRP-antiserum produced in horse.