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FROM THE INSTITUTE OF MEDICAL MICROBIOLOGY, DEPARTMENT OF BACTERIOLOGY,
UNIVERSITY OF GÖTEBORG, SWEDEN
HEAD: PROFESSOR Ö. OUCHTERLONY

STUDIES ON C-REACTIVE PROTEIN BY MEANS OF IMMUNOPRECIPITATION METHODS

BY

LARS-ÅKE NILSSON

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Akademisk avhandling

SOM MED VEDERBÖRLIGT TILLSTÅND AV MEDICINSKA FAKULTETEN VID GÖTEBORGS UNIVERSITET FÖR VINNANDE AV MEDICINE DOKTORSGRAD OFFENTLIGEN FÖRSVARAS I FÖRELÄSNINGSSALEN, INSTITUTIONERNA FÖR MEDICINSK MIKROBIOLOGI, GÖTEBORGS UNIVERSITET, LÖRDAGEN DEN 11 MAJ 1968 KL. 9 F.M.

AV

LARS-ÅKE NILSSON

Med. lic.

GÖTEBORG 1968
ORSTADIUS BOKTRYCKERI AKTIEBOLAG

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Contents

This survey is based on the following publications, which are referred to in the text by Roman numerals as follows:

I. Immunodiffusion analysis of human, monkey and rabbit acute phase proteins.
Int. Arch. Allergy *32*:545—562, 1967.

II. Qualitative analysis of acute phase protein antisera with the comparative interference diffusion-in-gel technique.
Int. Arch. Allergy *33*:16—28, 1968.

III. Comparative testing of precipitation methods for quantitation of C-reactive protein in blood serum.
Acta path. microbiol. scand. In press.

IV. C-reactive protein in a random sample of Swedish men aged fifty. Distribution and relation to clinical manifestations.
Co-author: G. Tibblin.
Acta med. scand. In press.

V. C-reactive protein in apparently healthy individuals (blood donors) related to age.
Acta path. microbiol. scand. In press.

Nomenclature

Acute phase protein without indication of species is used as the designation of the C-reactive protein equivalents in man, monkey and rabbit. Acute phase serum is used as a designation of blood serum containing acute phase protein. C-reactive protein (CRP) and Cx-reactive protein (CxRP) are used as designations of acute phase protein from man and rabbit respectively. Unless otherwise indicated, the designation CRP refers to the human acute phase protein.

Introduction

C-reactive protein (CRP) is one of a number of plasma constituents which have been designated as acute phase reactants (91). CRP seems to be unique among these in that it has generally been reported not to be demonstrable in healthy subjects (15, 27). CRP was detected in 1930 by *Tillett & Francis* (100) as a serum "precipitin" which reacted with "fraction C", a polysaccharide which could be extracted from R as well as S strains of pneumococci (101). Tillett and Francis found the "precipitins" in the sera from patients acutely ill with lobar pneumonia but not 1—3 days after the crisis. Furthermore, sera from patients suffering from diseases other than pneumonia, such as streptococcal and staphylococcal infections and rheumatic pericarditis, showed this special type of reactivity. Some years later *Ash* (9) in an extended study demonstrated "precipitins" against "fraction C" in serum during the acute stages of additional infectious diseases caused by gram-positive as well as gram-negative bacteria. Furthermore, she pointed out the relationship between fever and increased sedimentation rate on the one hand and the presence of C-precipitating substance on the other.

In a series of papers from the Rockefeller Institute (3, 62, 63, 66, 111) some of the physico-chemical and immunological properties of the substance were elucidated. Its protein nature was suggested by *Abernethy & Avery* (3) in 1941 and the designation, C-reactive protein, was applied (63). Due to its presence during the active stage of a disease it has also been called acute phase protein (37, 82). The C-polysaccharide — CRP reaction is dependent on the presence of calcium ions (3). Recent investigations by *Gotschlich & Liu* (30) have indicated that the major CRP-reacting part of the C-polysaccharide is *N*-acetylgalactosamine phosphate. The C-polysaccharide — CRP reaction was shown to be specifically inhibited by various phosphate monoesters of low molecular weight (29, 44). CRP occurs in serum associated with a lipid (62), which reacts with anti-β-lipoprotein (108).

In 1937 *Abernethy* (2) showed that acute phase sera from monkeys, but not from rabbits, infected with pneumococci were precipitated by the C-polysaccharide. Monkey acute phase protein was shown to cross-react with rabbit anti-human CRP (63). The occurrence in the rabbit of an analogous substance was described by *Anderson & McCarty* (7) in 1951.

The rabbit protein reacted only with a specially prepared form of the pneumococcal C-polysaccharide, the so-called Cx-polysaccharide, and not with the C-polysaccharide prepared as originally described. On the other hand human CRP reacts with both C- and Cx-polysaccharide. According to the latter authors the C-polysaccharide should be a somewhat more degraded form of Cx-polysaccharide (7). Due to its reactivity the protein found in rabbits was labelled CxRP. An immunological relationship between man, monkey and rabbit acute phase proteins was demonstrated in 1960 by *Gotschlich & Stetson* (31) employing the comparative double diffusion-in-gel technique.

Independently, *Löfström* (58, 59) in 1939 noted that under certain conditions a substance occurred in blood serum which caused swelling of the capsules of some pneumococci, among others, of types 27 and 28. The substance was called "nonspecific capsular swelling substance", and, by cross-absorption studies, was shown to be identical with CRP (60).

In 1941 *MacLeod & Avery* (62) isolated CRP from human serum and showed its antigenicity in rabbits (63). The protein was crystallized in 1947 by *McCarty* (66). In the capillary tube precipitation test devised by *Anderson & McCarty* (6) antiserum against human CRP (CRPA) was employed for the demonstration of CRP. This technique was shown to be at least ten times as sensitive as the precipitation reaction with pneumococcal C-polysaccharide (109), and it is still the most commonly employed routine technique for demonstration of CRP.

The clinical significance of the presence of CRP in blood serum was further elucidated by a large number of studies during the following years. The comprehensive studies of *Hedlund* (37) and *Keitel, Diesner & Bannert* (48) should be specially mentioned. Reviews on the subject have been given by *Good* (27) and *Schwarz* (93) among others. From these works it appears that the presence of CRP in blood serum can be regarded as an indication of an inflammatory or necrotic process in the organism. Especially the diagnostic value of CRP in acute rheumatic fever (6, 37, 110) and myocardial infarction (37, 53, 54) has been pointed out. It has been assumed that CRP vanishes during recovery from the disease and is generally not demonstrable in healthy subjects. In this connection it is interesting to note that *Vedros & Fishel* (103), separating normal sera by starch block electrophoresis, obtained fractions in the albumin and the γ-globulin regions which reacted with Cx-polysaccharide but not with CRPA. Their findings were thought to indicate that CRP was constantly present in serum but that its detection in normal serum might be masked through the presence of a substance in serum migrating with the α_2-globulins in starch electrophoresis.

CRP has been shown to be present during pregnancy (10, 34, 68, 70, 71,

84, 86, 94) but does not seem to pass the placental barrier (10, 34, 70, 71, 84, 86) or to be present in the milk (34, 59, 76). Since CRP has been demonstrated in very young infants it seems that the capacity to form CRP is present early in life (10, 34, 70, 71, 84, 86).

The antigenic nature of CRP has been studied by *Libretti, Kaplan & Goldin* (56, 57) who employed diffusion-in-gel analysis. These authors demonstrated 1—3 precipitation lines when CRP-containing blood serum or pleural fluid was analysed by means of CRPA, whereas crystalline CRP always gave three lines. These results were interpreted to indicate the presence of three antigens in CRP. It was concluded that the concentrations of these different components varied in different sera. In the later report (56), however, a denaturation of CRP was also considered as a cause of the multiple precipitation lines. *Nishimura* (75), as well as *Nilsson & Hanson* (34, 74) in diffusion-in-gel analyses obtained results which could be interpreted as confirmation of a serological heterogeneity of CRP. Other authors have been unable to show more than one precipitation line when analysing CRP—CRPA systems with this technique (e.g. 23, 67, 81, 85, 97). Diffusion-in-gel analyses have not revealed any antigenic differences in CRP from different individuals with various diseases (56, 61, 81, 97). Findings indicative of a heterogeneity of CRP have however been reported by *Hedlund* (36) and *Fishel, Vedros & Rothlauf* (20) in absorption experiments with pneumococci and/or pneumococcal C-polysaccharide.

The electrophoretic behaviour of CRP has been the subject of many controversial reports. *Perlman, Bullowa & Goodkind* (82) employing free electrophoresis, as well as *Hornung & Morris* (45) using paper electrophoresis, by indirect means concluded that CRP probably migrated with the α-globulin fraction of serum. On the other hand the results of *Wood, McCarty & Slater* (111) as well as *Hansen, Marner & Ejby-Poulsen* (32) indicated that CRP moved with the β-globulins in free electrophoresis. The former authors (111) stated that it moved with the fast γ-globulins at zone electrophoresis on starch. *Hedlund & Brattsten* (38) employing continuous zone electrophoresis on starch localized the CRP in the γ-globulin fraction whereas *Philipson & Tveterås* (84) and *Gedin & Porath* (24) using zone electrophoresis in vertical columns with a cellulose medium found CRP to move intermediate between β- and γ-globulins. Immunoelectrophoretic studies have also given somewhat conflicting results, most authors, however, refer the mobility of CRP in agar gel or on paper to the β- (11, 23, 25, 40, 67, 83) or γ-region (12, 13, 61, 112, 113) or between these regions (1, 81, 98). *Fishel* (19) observed that the position of CRP in immunoelectrophoretic patterns produced at different pH values ranging from 6.9 to 8.6 did not change relative to the origin whereas *Davila* (16) found

the localization of CRP to vary between the albumin and the γ-globulin regions in conjunction with pH changes between 7.0 and 8.3. The discrepant electrophoretic mobilities reported, have been explained by differences in experimental conditions and the varying adsorption of CRP to the supporting media (16, 23, 25, 32, 38, 67, 84). Differences in the state of aggregation of CRP have also been considered (41). Indications of an electrophoretic heterogeneity of CRP have been reported by several authors (14, 39, 75, 81, 92, 96). *Anzai, Sato, Fukuda & Carpenter* (8) demonstrated three immunoelectrophoretic types of CRP, designated *β*, *γ* and 2H, and their findings were interpreted to indicate that CRP consisted of two proteins differing in electrophoretic mobility but with identical antigenicity. *Hokama, Coleman & Riley* (42) ascribe these findings to an interaction between CRP and low levels of Lewis and/or H blood group substances in serum.

According to *Gotschlich & Edelman* (28) the CRP molecule probably consists of subunits with a molecular weight of 21,500. These authors proposed two possible models for the CRP, each consisting of six subunits. The six-subunit model should probably represent only one of several forms since the molecular weight of CRP was found to range between 110,000 and 144,000 by utracentrifugal analysis.

The aim of the author's investigation

The capillary tube precipitation test for demonstration of CRP has been considered satisfactory for clinical routine work. However, for more detailed analysis of the properties and occurrence of CRP, a more sensitive and accurate test for determination of CRP was considered necessary. Since diffusion-in-gel techniques have proved to be of value for qualitative and quantitative immunological analyses, some of these were applied to study of CRP with the following main purposes.

1. To study the immunodiffusion characteristics including the antigen mosaics of acute phase proteins from man, monkey and rabbit.
2. To evaluate some immunological techniques for determination of CRP.
3. To examine quantitatively the CRP in some populations and to analyse these results in relation to certain clinical and laboratory variables, as well as the age, of the individuals.

Materials

Native acute phase protein antigens (I, II)

In order to minimize denaturation of the antigens, studies of the antigen mosaics of acute phase proteins were performed with freshly drawn samples which were used either within 24 h after sampling or after storage at —20° C. Ascites from a woman suffering from a metastasizing cancer of the caecum was used as the human CRP reference antigen. Acute phase sera were obtained from monkeys and rabbits injected 24 h earlier with typhoid-paratyphoid vaccine or live virulent pneumococci, type 27.

Purified acute phase protein antigens (I, III)

Purified human CRP was used for production of antisera in animals (I) and for standardization of the quantitative determination technique (III). The method of *Wood et al.* (111) employing precipitation with C-polysaccharide was used for preparation of CRP (I). A modification of the chromatographic technique elaborated by *Hokama & Riley* (43) was also employed (I, III). After preparation of "crude CRP" according to the latter authors, purification was pursued by subsequent gel filtration on Sephadex G-200. This procedure gave a material which presumably was immunologically pure, since immunization with a total amount of 1.4 mg CRP emulsified in Freund's adjuvant for a period of 4 months did not stimulate rabbits to formation of antibodies other than those to CRP as tested by the diffusion-in-gel technique (III). The diffusion coefficient in agar gel of this purified CRP was measured with the angle plate technique of *Allison & Humphrey* (5) based on a principle given by *Ouchterlony* (77). It was not found to deviate significantly from that of native human CRP (III).

Acute phase protein from monkey was also purified with the chromatographic method of *Hokama & Riley* (43) (I). Since the monkey acute phase protein was used only for immunization purposes a purity as high as that of the human CRP was considered unnecessary, and further purification by gel filtration was therefore not performed. In contrast to the human and monkey proteins, rabbit acute phase protein was not retained by the DEAE-cellulose with the buffer system used (0.05 M sodium citrate buffer, pH 7.0, containing 0.1 M NaCl) (73).

Human blood sera

For the methodological and population studies blood sera from the following populations were used:

50-year-old men (III, group A; IV). These men were randomly selected

from the register of the Revenue Office of Göteborg and comprised all the men living in Göteborg in 1963, born in 1913 on dates which are multiples of three. Nine hundred and seventy-three men were originally selected, 855 (88 %) of whom were clinically examined in the hospital. The clinical part of the study as well as an analysis of the nonparticipating group has been described by *Tibblin* (99). At the time when the CRP analyses were performed, 835 of the 855 sera were available for study. Quantitative immunodiffusion analyses for CRP were performed on 826 of these sera. All sera were stored at —20° C until used for analysis.

Blood donors (V). Blood sera were obtained from the Blood Bank of the Sahlgren Hospital, Göteborg, from 405 blood donors of different ages. Physical examination of the donors was not performed, but anamnestic inquiries were made regarding certain diseases such as syphilis, malaria, tuberculosis, hepatitis, asthma or other allergic disorders. Donors who have had these diseases are not admitted. The donors are also requested to state diseases occurring since the preceding donation. The sedimentation rate exceeded 16 mm/h in only 7 of the 395 donors in whom it was determined. These 7 cases were not confined to any special age group.

Patients (III, group B). One hundred and thirty-five samples were selected from the sera sent to the Bacteriological Laboratory of the Sahlgren Hospital, Göteborg, for CRP determination. Only sera found CRP positive with the capillary tube precipitation technique (6) were included in this series.

Antisera to acute phase proteins

Antisera against human CRP (CRPA) were produced in a goat (I), a monkey (I) and rabbits (III) by injection with purified CRP (I, III).

The antisera obtained from goat were absorbed with a CRP negative serum to remove precipitating antibodies other than those against CRP. Commercial CRPA antisera*), from rabbit as well as pooled from sheep and goat, were also employed. Antiserum against monkey CRP was produced in a hen by injection of purified monkey CRP. Antibodies against monkey serum proteins other than acute phase protein were removed by absorption with a CRP negative monkey serum. An antiserum against rabbit CxRP produced in goat*) was employed after absorption with normal rabbit serum. Portions of each of the three acute phase protein antisera were absorbed with the two heterologous native acute phase protein antigens. The adequacy of the absorption of the antisera was tested by means of the diffusion-in-gel technique with the absorbing antigen.

*) Hyland Laboratories, Los Angeles, Calif., USA

Immunological methods

The comparative double diffusion-in-gel method of *Ouchterlony* (77) was used in the modifications of *Hanson* (33) and *Wadsworth* (105). The elaborated interference diffusion-in-gel technique was used as described in II. Regular and comparative immunoelectrophoretic analyses were performed as described by *Wadsworth & Hanson* (106). The capillary tube precipitation technique for demonstration of CRP was that described by *Anderson & McCarty* (6) (III). The single radial immunodiffusion (halo) technique according to *Mancini, Carbonara & Heremans* (64) was performed as described in III.

The antigen mosaics of acute phase proteins (I, II)

The antigen mosaics were studied with the comparative double diffusion-in-gel technique (77). Interpretation of precipitation patterns obtained with this technique has been discussed by several authors (e.g. 51, 79, 80) and has withstood experimental trial at least in regard to comparative analysis of antigens. The patterns obtained in the present study (I) were type reactions I (reaction of fusion), III (reaction of partial fusion) and IV (reaction of inhibition), and they were interpreted according to *Ouchterlony* (79).

When each of the acute phase proteins from the three species was analysed with its corresponding homologous or heterologous antisera with the double diffusion-in-gel method one precipitation line was obtained. Results obtained when human CRP was analysed with the monkey anti-human CRP were considered especially indicative of an immunological homogeneity of the human acute phase protein. This was not in accord with the results of *Libretti et al.* (57) as well as others (20, 75, 92). Some of the author's previous studies were also thought to indicate an immunological heterogeneity of CRP since some CRP—CRPA systems gave two precipitation lines (34, 74). A crossing of these lines was obtained in some instances. These findings were later re-evaluated (I) and it was suggested that these early results were probably due to denaturation of CRP in the analysed sera or possibly to imbalance of the systems tested. By means of the comparative diffusion-in-gel technique it was shown that splitting of native CRP into serologically reactive fragments might occur after some months of storage at $+4^{\circ}$ C (I).

Neither did immunoelectrophoretic analyses give evidence of an immu-

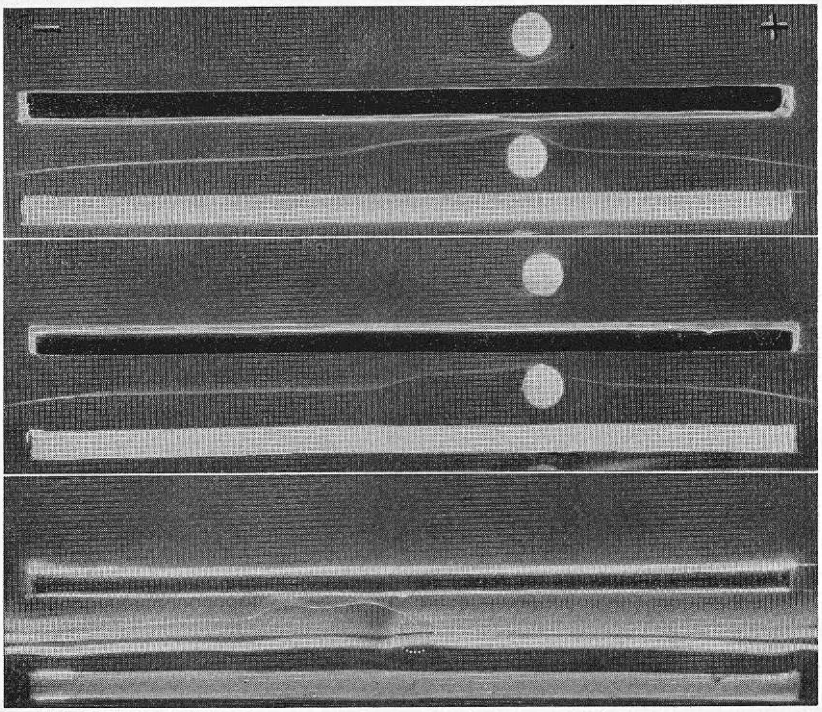

Fig. 1 a and b. Regular and comparative immunoelectrophoretic analyses of human CRP-containing sera (circular start basins) by means of CRPA (clear longitudinal basins) and the human CRP reference antigen (opaque longitudinal basins). a) Serum with a high CRPconcentration. b) Serum with a low CRP concentration.
Fig. 1 c. Comparative immunoelectrophoretic analysis of a purified human CRP preparation before gel filtration (circular start basin) by means of sheep anti-human serum (see I) (upper longitudinal basin) and a CRP negative human serum (lower longitudinal basin).

nological heterogeneity of acute phase protein. However, an electrophoretic heterogeneity of acute phase protein was demonstrated in each of the three species analysed; this was especially evident when the comparative immunoelectrophoretic technique was employed. The results obtained with a partly purified human CRP preparation indicated at least four groups of particles with different electrophoretic mobilities but with indistinguishable antigen composition (I) (Fig. 1). The patterns obtained in analyses of the purified human CRP preparation as well as the native acute phase protein antigens were not in accord with a continuous spectrum of differently charged particles as suggested by *Schultze & Heremans* (90).

Comparative testing was performed on acute phase proteins from man, monkey and rabbit by means of unabsorbed and absorbed homologous antisera in order to integrate the antigenic factors revealed. The precipitation

TABLE 1

Antigen mosaics of acute phase proteins from man, monkey and rabbit

Species	Antigenic determinants				
	a	b	c	d	e
Man	+	+	+	−	−
Monkey	−	+	+	−	+
Rabbit	−	−	+	+	−

patterns obtained were in accord with findings published by other authors (31, 55). The present results indicated at least five different antigenic determinants labelled *a*, *b*, *c*, *d* and *e* on the three acute phase proteins (I). These occurred in varying combinations on the proteins studied. One of the determinants (*c*) was common to all three acute phase proteins, one (*b*) was common to the human and monkey acute phase proteins whereas the other three were found one in each species, the human (*a*), the monkey (*e*) and the rabbit (*d*) proteins, but could not be demonstrated in the other two (Table 1). Thus differences in antigen mosaic were indicated for the three acute phase proteins, and on the basis of the patterns of reaction obtained in the comparative analyses it was concluded that the antigenic determinants of each of the three acute phase protein antigens were situated on the same molecule and unable to diffuse independently. These conclusions were confirmed by analysis of the corresponding antisera with the comparative interference diffusion-in-gel technique (II).

Methodological aspects

Comparative interference diffusion-in-gel analysis (II)

The comparative interference diffusion-in-gel technique was elaborated in order to facilitate comparison of different antisera by means of a multideterminant antigen *). When such an antigen is analysed in the regular diffusion-in-gel technique with an antiserum containing antibodies against

*) antigen particles carrying determinants of different specificity

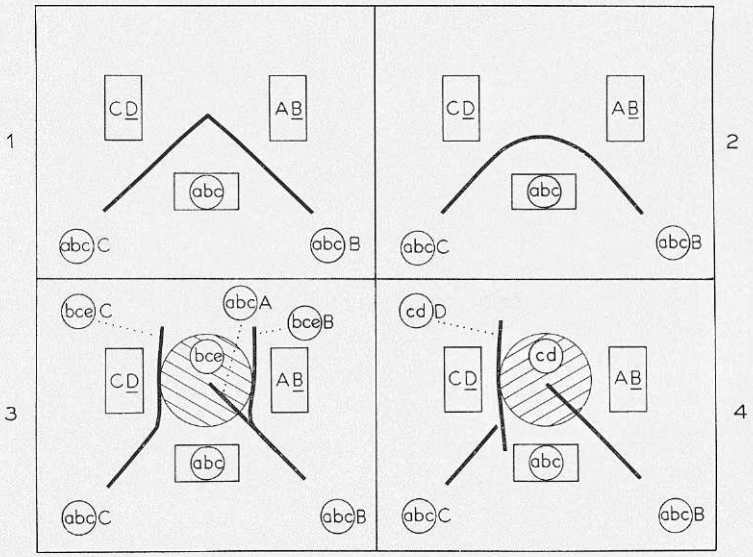

Fig. 2. Postulated (1) and obtained (2) reactions when goat anti-rabbit CxRP (*CD*) and goat anti-human CRP absorbed with CxRP (*AB*) were compared by means of human CRP (*abc*). The different patterns obtained with the comparative interference diffusion--in-gel technique with monkey CRP (*bce*) (3) and CxRP (*cd*) (4) in the circular basins demonstrate the different antibody contents of the compared antisera. Underlined antibodies are leading in the diffusion.

two or more of these determinants, the initial composition of the precipitation line depends on which of the antibodies is leading at the diffusion. This depends mainly on the concentration of the different antibodies but might also be due to differences of e.g. molecular weight. *Ouchterlony* (78) in 1960 postulated that a reaction of mutual inhibition (one of his type IV reactions) should occur when two antisera with different leading antibodies are compared by means of a multideterminant antigen with determinants specific for each of the antibodies (Fig. 2:1). This reaction pattern could not be experimentally verified with the various homologous and heterologous acute phase protein — anti-acute phase protein systems studied, instead a reaction of fusion (reaction type I) was always obtained (Fig. 2:2). These results were in accord with those obtained by *Korngold & van Leeuwen* (52) and *Jennings* (46, 47). The results obtained in I and II, contradictory to Ouchterlony's postulation, initiated further work to confirm the presumed character of the antibodies contained in the antisera employed for the analyses. To elucidate some of the factors involved, the comparative interference diffusion-in-gel technique was elaborated. In this technique an additional circular basin is established in the expected area of interference of the regular comparative technique (Fig. 2:3 and 2:4). The reagents in the regular basins are allowed to diffuse for 5—7 h when

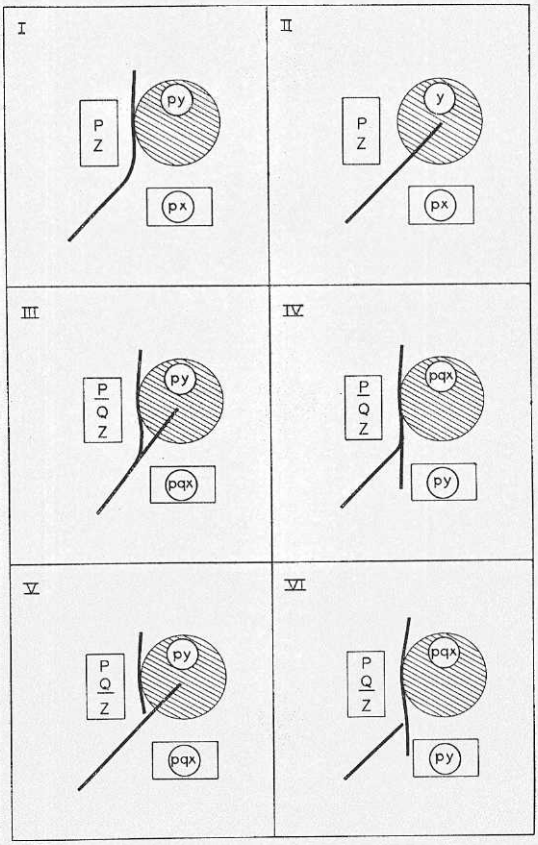

Fig. 3. The six characteristic interference reaction patterns obtained with the comparative interference diffusion-in-gel technique. Antigen determinants are designated with lower-case letters, antibodies with upper-case letters. *p*, *P* and *q*, Q are hypothesized constituents revealed by the test system, *x*, *y* and *Z* are hypothesized constituents not revealed by the test system.

the precipitates begin to become visible. Then a suitable antigen mixed with agar is poured into the circular additional basin and the influence of this diffusing antigen on the developing precipitation lines can be studied. Thus the antigen-agar mix in this additional basin functions as a reactant reservoir as well as an area of reaction. Incorporation in the circular basin of an antigen with an antigen determinant reacting with the leading reacting antibody in the test antiserum, would interfere with the regular extension of the precipitation line causing a deviation or inhibition (Fig. 2:3 and 2:4, left). On the other hand an antigen without determinants reacting with the pertinent antibody would not influence the formation of the precipitation line (Fig. 2:4, right).

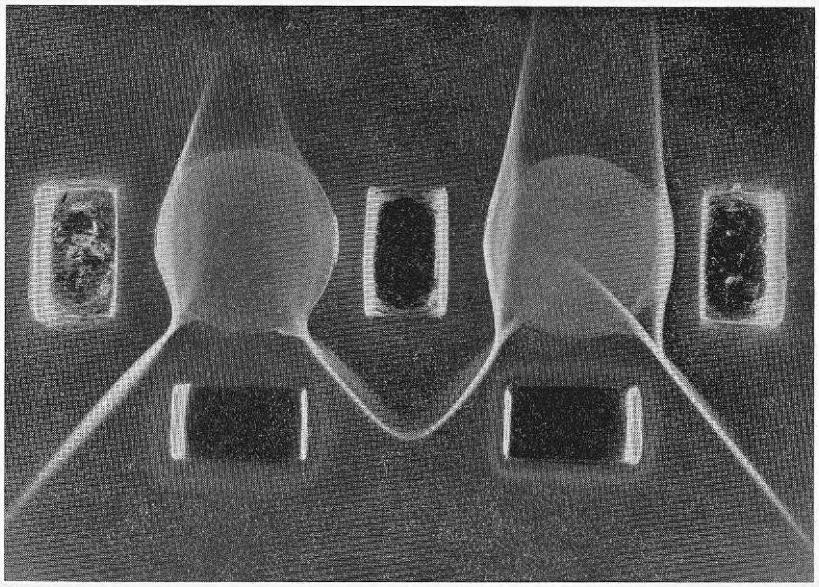

Fig. 4. Comparative interference analyses of CRPA absorbed with CxRP (upper outer basins) and anti-CxRP (central basin) by means of human CRP (lower basins and left circular basin) and monkey CRP (right circular basin).

As test reactants were used the native acute phase protein antigens from man, monkey and rabbit and homologous and heterologous antisera to these. Both unabsorbed antisera and antisera absorbed with the two heterologous acute phase protein antigens were used. In total nine antisera with different contents of antibodies to various acute phase protein determinants were employed. A careful check of the appropriate absorption of these antisera was performed with the diffusion-in-gel technique. By means of these tentatively designated reactants precipitation lines with an assumed initial composition could be established. (II, Table II). When the influence of the three acute phase protein antigens (in the circular basins) on precipitation lines which had been formed by different antibodies was studied, six characteristic interference reaction patterns were obtained (Fig. 3). These patterns were in complete agreement with those postulated on the basis of the presumed antigen mosaics and antibody contents of the reactants used. A photograph of a representative comparative interference diffusion-in-gel plate comprises Fig. 4.

Thus it was concluded that the regular comparative technique is of limited differentiating value when different antisera are compared by means of a multideterminant antigen. With the comparative interference diffusion-in-gel technique, it is possible to differentiate antisera with regard to the specificities of contained antibodies.

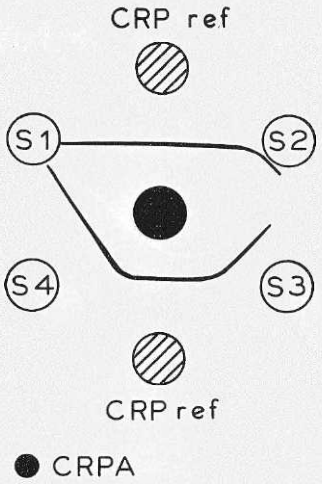

Fig. 5. Comparative double diffusion-in-gel analysis (7-basin arrangement) for demonstration of CRP in four blood sera (S 1 — S 4) by means of CRPA (center basin) and a CRP reference antigen (upper and lower hatched basins).

The type patterns described by Ouchterlony in 1960 for comparative analysis of antisera have since been accordingly revised (80, Fig. 19.16) on the basis of the present results (II).

Comparative double diffusion-in-gel analysis (III)

For demonstration of CRP in blood sera the microplate technique of *Wadsworth* (105) was used. A seven-basin arrangement was employed with the central basin filled with antiserum (CRPA), two opposed basins with a CRP-containing reference antigen and the four remaining basins with the samples to be analysed (Fig. 5). With this arrangement two parallel reference CRP—CRPA precipitation lines are obtained. A very low CRP concentration in the sample analysed is sufficient to cause a discernible deviation of the reference precipitation line. By testing dilutions of a serum sample with known CRP concentration the sensitivity of the technique was estimated at about 0.5 µg/ml. This arrangement of basins and reactants is more easily interpreted than the five-basin arrangement used earlier (34, 74) (Fig. 6). A definite advantage of the diffusion-in-gel technique is the control of the immunological specificity of the precipitates obtained and furthermore zonal effects occurring in some techniques (68, 69, 95) are eliminated. In addition, any adverse effects of hemolysis in the serum sample are minimized by diffusion-in-gel techniques.

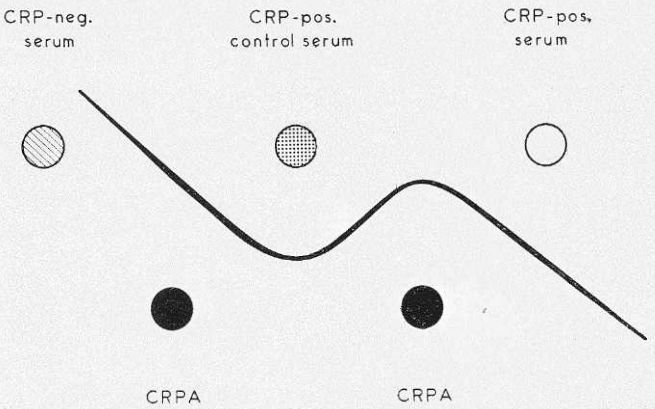

Fig. 6. Comparative double diffusion-in-gel analysis (5-basin arrangement) for demonstration of CRP in two blood sera (upper outer basins) by means of CRPA (lower basins) and a CRP reference antigen (upper central basin).

Capillary tube precipitation technique (III)

The technique was performed as described by *Anderson & McCarty* (6) with the following modification. In one of the series analysed (III, group A) the incubation period at 4°C was extended from 24 h to 48 h. This increased the sensitivity of the technique mainly by converting some of the negative results to trace reactions.

Since preliminary investigations had shown that most of the sera contained only low concentrations of CRP an attempt to further increase the sensitivity of the technique was made by using the employed commercial antiserum diluted 1:4. In total, CRP was demonstrated in 25% of the sera from 50-year-old men when the employed antiserum was used undiluted. When the same antiserum was used diluted 1:4, CRP was shown in 42% of the sera. The dilution of the antiserum provides more optimal conditions for the precipitation reaction to occur at low CRP concentrations (III). This is in accord with the considerations of *Nakamura, Magsaysay, Ford & Kunitake* (69).

Halo technique (single radial diffusion) (III)

The halo technique as described by *Mancini et al.* (64) is a simple, accurate and sensitive technique for immunochemical quantification of e.g. serum proteins. In this technique the antigen is allowed to diffuse from a circular basin punched out in an antiserum-containing agar gel. During diffusion circular antigen-antibody precipitates are formed in the immune serum-agar mix. These continue to grow until balance between the reacting components is attained. The area of the precipitate is pro-

portional to the antigen concentration which can be determined from comparison with simultaneously analysed reference antigens of known antigen concentrations.

The use of the halo technique for quantification requires a certain knowledge of the antigen to be tested. Thus the antigenic properties must have been established especially in regard to its possible occurrence as several antigenic components. The immunological homogeneity of CRP as revealed by diffusion-in-gel analysis was shown in I and II.

Quantitative immunochemical techniques require either highly purified antigens or antisera which are specific against the antigen to be measured. The last alternative is the most practical and usually employed. However, for standardization of the technique, at least if absolute and not only relative values of the antigen concentrations are desired, a highly purified antigen preparation is also required. A simple procedure for preparation of immunologically pure CRP has been described (III). It is important that the purified reference antigen has the same diffusion properties as the corresponding "native" antigens and that these do not vary among themselves when present in different sera. Thus, *Fahey and McKelvey* (18) found that about twice as much of a 9S IgA preparation as compared with a 7S IgA preparation was necessary to give the same area when tested with the halo technique. The studies of *Hokama, Coleman & Riley* (41) indicated that the CRP present in sera from different individuals interacted with the agar to various degrees. Although the findings obtained in the present studies do not contradict an interaction with the agar the variability of this interaction of CRP from different individuals was not confirmed. On the contrary the diffusion constants for CRP in different sera as well as in the reference antigen preparation varied only within the limits of experimental error when carefully balanced systems were analysed (III). Nor did the results of gel filtration of ten CRP-containing sera indicate any differences in molecular weight since the CRP always appeared in the effluent at the same elution volume. It was concluded that from this point of view, under the experimental conditions used, the halo technique would be suitable for quantification of CRP.

In the original report of *Mancini, Vaerman, Carbonara & Heremans* (65), the halo plates were incubated for 14 days and this time was also used in the present investigation to ascertain that balance was attained. For routine work this is a very long time and subsequent experience showed it to be unnecessarily long. With adjustment of the antiserum concentration the diffusion time can be as short as 24 h even at relatively high CRP concentrations (73), and this time is usually sufficiently short for clinical demands. It should be mentioned that an increase of the antiserum concentration leads to a decreased sensitivity of the technique (64).

The calculated error of the halo technique expressed as the coefficient of variation ranged between 3.4 and 5.1% for the CRP concentrations tested and is thus slightly larger than the corresponding figure, 2%, obtained by *Mancini et al.* (64). This error is however still acceptable from a practical point of view.

The sensitivity of the halo technique was estimated at approximately 1 μg CRP/ml by testing dilutions of a CRP-containing serum with known CRP concentrations. This is in accord with the corresponding figures for the albumin — anti-albumin system studied by *Mancini et al.* (64).

Comparison of immunoprecipitation methods for demonstration of CRP (III)

In the literature the following methods have been employed for demonstration of CRP:

1. Reaction with pneumococcal C-polysaccharide
   a) Precipitation (100)
   b) Skin test (4, 21)
   c) Hemagglutination (22)
2. Nonspecific capsular swelling (59)
3. Reaction with specific antiserum (CRPA)
   a) Precipitation in capillary tubes (6)
   b) Complement fixation (68, 85)
   c) Agglutination of antiserum-coated latex particles (95)
   d) Immunodiffusion methods (57, 92)
   e) Fluorescent antibody technique (26)
4. Reaction with certain surface active substances (APC-test) (102)

This study concerns only precipitation in capillary tubes and some immunodiffusion methods.

The main drawback of precipitation with pneumococcal C-polysaccharide is the laborious preparation of the substance. The introduction by *Anderson & McCarty* (6) of the CRPA precipitation test performed in capillary tubes was an important step forward and furthermore this method was shown to be at least ten times as sensitive as precipitation with C-polysaccharide (109). The precipitation reaction with C-polysaccharide was found not to be consistently positive at CRP concentrations below 0.1 mg/ml whereas the precipitation reaction with CRPA would detect the presence of less than 0.01 mg/ml (109). The nonspecific capsular swelling method of *Löfström* (59) was shown by *Hedlund* (35) to have approximately the same sensitivity as the capillary tube precipita-

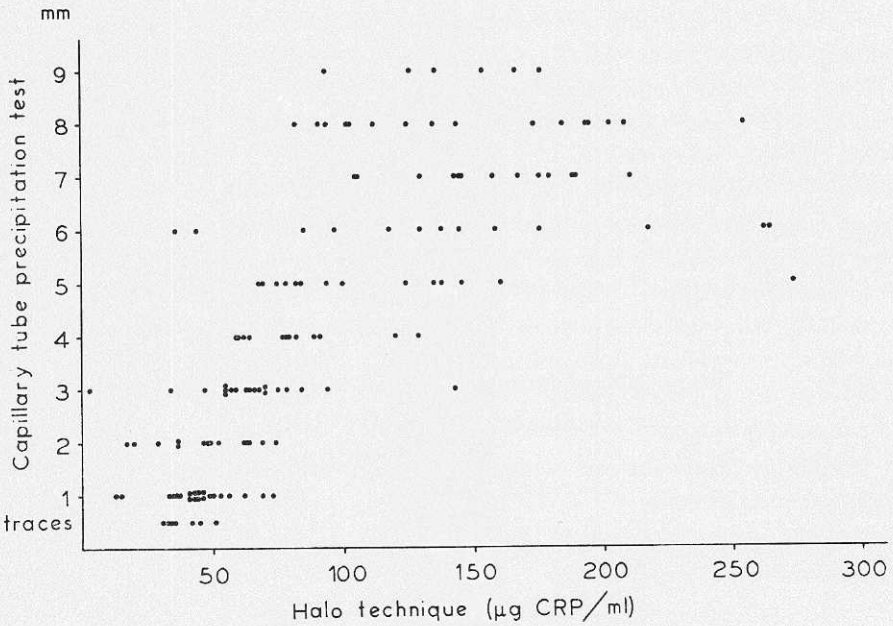

Fig. 7. Relation between CRP concentration determined by the halo technique and by the capillary tube precipitation test in 135 CRP-containing sera.

tion technique. Löfström's technique, however, although having interesting theoretical implications, has not been widely used mainly because of difficulties in standardization of the readings.

Nilsson & Hanson (74) pointed out the greater sensitivity of the CRPA precipitation technique when performed in a micromodification (105) of Ouchterlony's diffusion-in-gel technique (77). In these studies about 50% of sera obtained from blood donors and hospitalized patients were shown to contain CRP, although no CRP was demonstrated with the capillary tube technique. These analyses were extended and the greater sensitivity of the comparative double diffusion-in-gel technique was confirmed in the study on sera obtained from randomly selected 50-year-old men (III, group A). In this study a different basin arrangement was used (Fig. 5) by means of which CRP could be demonstrated in about 80% of the sera reacting negatively in the capillary tube precipitation technique.

Besides its higher sensitivity, the comparative double diffusion-in-gel technique provides a control of the specificity of the reaction. According to the results obtained with this technique the capillary tube technique gave false positive reactions in about 1% of the analysed sera.

A comparison of the capillary tube precipitation technique and the

immunodiffusion techniques (comparative double diffusion and halo techniques) for quantification of CRP in serum was also made. CRP was estimated in 135 serum samples from patients with these techniques (III, group B). It was shown that the distribution of CRP values obtained with the halo technique was very great within each CRP level as determined with the capillary tube technique (Fig. 7). Only at traces and 9 mm of precipitate was the maximum CRP concentration (halo technique) less than twice the minimum concentration, and at some levels (1 and 6 mm) the maximum CRP concentration exceeded the minimum concentration by more than five times. It was concluded that the halo technique is better suited than the capillary tube technique for quantification of CRP due to its greater specificity, sensitivity and accuracy. Furthermore the amount of antiserum required is very low.

The limit of sensitivity of the capillary tube precipitation technique, reported to be about 10 μg CRP/ml (109) is confirmed by the findings obtained in the present study (III, Table 5).

Population studies (IV,V)

According to the literature CRP is a protein not normally demonstrable in serum. Its appearance is elicited by various stimuli, the common denominator of which seems to be the induction of an inflammatory reaction or tissue necrosis. Available evidence points to CRP as a very sensitive indication of inflammatory disease or tissue necrosis and that changes of the CRP level reflect the course of the disease (15, 22, 48, 50, 53, 59, 109, 110).

Knights, Hutchins, Morgan & Ploompuu (49) demonstrated the presence of CRP in 12 % of a large series of blood donors, but, on the basis of available data assumed that a positive reaction was indicative of a pathological state existing in the donor. Preliminary studies performed by *Nilsson* (72) revealed an increasing prevalence rate of CRP-containing sera with increasing age.

In the present analyses sera obtained from a series of randomly selected 50-year-old men (IV) and a series of blood donors of different ages (V) were tested for the presence of CRP with qualitative and quantitative immunodiffusion techniques.

In the analysis of sera from 50-year-old men the presence of CRP was demonstrated in 84% with the comparative double diffusion-in-gel technique. This prevalence rate was considered high since there was a

rather low incidence of clinically apparent disease in the series investigated and the majority of the men were at active work (99). With the capillary tube precipitation test CRP could be demonstrated in only 25% of these men. Most of the positive reactions (21% of the total series) were trace reactions (precipitate < 1 mm). The discrepancy between the results obtained by the capillary tube precipitation technique and the diffusion-in-gel technique was ascribed to the higher sensitivity of the latter (III). The difference in sensitivity level, between the immunodiffusion techniques used in the present study and the techniques used for demonstration of CRP by other authors, would explain why they generally have not demonstrated CRP in subjects without apparent disease. For this reason it was considered worthwhile to ascertain at which CRP concentration a relationship to pathological conditions could be noted.

Elevation of the erythrocyte sedimentation rate is another nonspecific indication of a pathological process in the organism. Since there is a well-documented parallelism between prevalence of CRP and sedimentation rate (6, 48, 59, 87, 88, 104, 110), the latter variable was chosen to be compared with the CRP concentration. In addition, symptoms from the respiratory tract indicative of bronchitis at different stages as well as smoking habits were compared with the CRP concentration; because of the covariation of these variables (17, 107) it was expected that they would show similar relations to CRP concentration. It was found that the prevalence rates of sera with CRP concentrations equal to or higher than 1—4.2 μg/ml increased with increasing sedimentation rate, increasing cigarette consumption, as well as increasing severity of respiratory symptoms. On the other hand the prevalence rates of sera containing trace amounts of CRP as well as the negatively reacting sera decreased when these variables increased. On the basis of these findings it was therefore suggested that the presence of trace amounts (< 1 μg/ml) of CRP is of minor clinical significance if incipient or declining disease can be excluded.

Studies performed on sera from 98 healthy children (89) revealed a much lower incidence of CRP-containing sera (17%) than found in the present study on sera from 50-year-old men (84%) (IV). For this reason it was thought worthwhile to examine the prevalence rates of sera with different CRP concentrations and their relation to the age of apparently healthy individuals (V). A series of blood donors was considered suitable since there are uniform health demands for the admittance to donation of blood. The similarity in state of health within the different age groups was indicated by the even distribution of the very few high sedimentation rates (> 15 mm/h) recorded, as well as similar prevalence rates for CRP concentrations exceeding 4.2 μg/ml. Values exceeding this level could

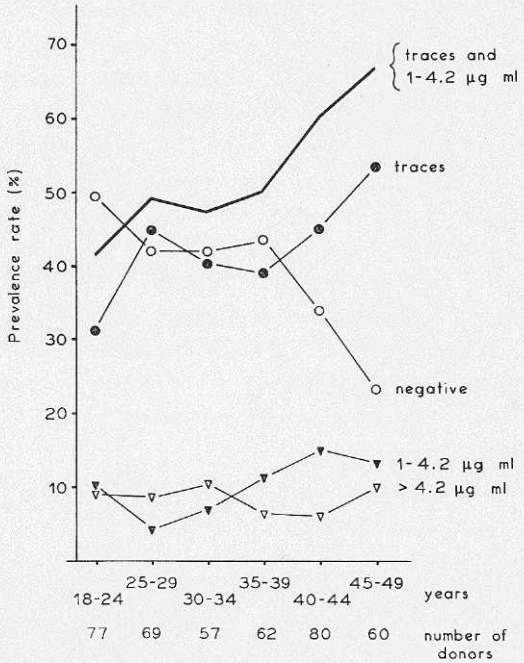

Fig. 8. Prevalence rates of sera with different CRP concentrations related to age in a series of 405 blood donors.

probably be considered as an expression of morbidity (IV). On the other hand the prevalence rates of sera without demonstrable CRP decreased significantly from 49 % in the age group 18—24 years to 23 % in the age group 45—49 years (Fig. 8). There was an increase in prevalence rates of sera with trace amounts of CRP and, although to a lesser degree, in prevalence rates of sera with CRP concentrations between 1 and 4.2 µg/ml with increasing age. Since the presence of trace amounts ($<$ 1 µg CRP/ml) could not be shown to be related to clinically demonstrable diseases (IV) it was suggested that the presence of trace amounts and possibly in some instances concentrations up to about 4 µg/ml is indicative of changes associated with aging.

Consideration of the implications of these results raised the question of the normal limit for blood serum CRP. It was proposed that CRP might occur in low concentrations in normal subjects, that is, in subjects without clinically demonstrable disease. To evaluate a positive finding within the low range in a single specimen as an indication of incipient or declining disease or "normal", a follow-up testing of the individual is necessary. Further studies on clinical series and long-term follow-up studies of healthy subjects should elucidate this question.

Summary

1. The antigen mosaics of acute phase proteins from man, monkey and rabbit were studied by means of the comparative double diffusion-in-gel technique. The results obtained indicated the presence of at least five antigenic determinants in the three different acute phase proteins. One factor (*c*) is common to all three species, another (*b*) is found in man and monkey but not in rabbit, while three others are probably specific for man (*a*), monkey (*e*) and rabbit (*d*). In each type of acute phase protein the pertinent antigenic determinants are situated on the same molecule and unable to diffuse independently. No indications of an immunological heterogeneity of the proteins were obtained, whereas immunoelectrophoretic studies indicated an electrophoretic heterogeneity. In order to confirm the multispecificity of the acute phase proteins an immunotechnique, a comparative interference diffusion-in-gel technique, was elaborated. This method is particularly useful for characterization of the antibody content of antisera to multispecific antigens.
2. The capillary tube precipitation technique, the double diffusion-in-gel technique and the halo (single radial diffusion) technique were employed for estimation of C-reactive protein and the results obtained with these techniques were compared. The greater sensitivity, specificity and accuracy of the immunodiffusion techniques as compared to the capillary tube precipitation technique was demonstrated. It was concluded that the halo technique is well suited for routine quantification of C-reactive protein.
3. Sera obtained from a series of 50-year-old men and a series of blood donors of different ages were tested for the presence of CRP by means of the aforementioned immunodiffusion techniques. It was found that C-reactive protein may be present in low concentrations (trace amounts and possibly up to about 4 μg/ml) in sera from apparently healthy individuals. The prevalence rate of sera with low CRP concentrations increased with increasing age.

Acknowledgements

It is a pleasure to express my indebtedness for support and assistance to my colleagues at the Institute of Medical Microbiology where this work was carried out.

My sincere thanks are due to Professor Örjan Ouchterlony for his encouraging guidance and constructive criticism throughout the work. I am also indebted to Docent Lars Åke Hanson for valuable advice and criticism as well as to Laborator Gösta Vahlne for support and providing me with some of the materials.

To my collaborator Dr Gösta Tibblin I wish to express my thanks for helpful suggestions on the population studies. I should like to thank Dr Lars Ryttinger and Docent Per Hedlund for their support which facilitated the progress of this work. I am also obliged to Lektor Sven Eriksson for help with the statistical aspects.

Mrs Lisbeth Björk has shown an unstinting loyalty throughout the work and I am most grateful for her skilful and reliable technical assistance. Mr Charles Wadsworth revised the English text and I appreciate his criticism during this phase of the work. I am also greatly indebted to Miss Sally Bodin for excellent typing of the manuscripts.

This work was made possible by grants from the faculty of Medicine, University of Göteborg, The Swedish Society for Medical Research, The Ellen, Walter and Lennart Hesselman Foundation for Scientific Research and AB Immuno Research.

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