Isolation of Reagins with a Cellulose Immunosorbent

by

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CHAPTER I
GENERAL INTRODUCTION

The existence of immunity to disease can be recognized in animals, including man, which have recovered from a bacterial or viral infection. These animals are found to be resistant to a subsequent infection of the same type. The serum of such animals is capable of causing agglutination, opsonization or lysis of the organism whose previous attack has been overcome. The factor in the serum responsible for this reaction is referred to as an antibody and the substance which induced its formation, in this case the virus or bacteria, is called an antigen.

The nature of antigens

Antibodies are formed not only as a result of infection but also in response to the administration of certain substances of large molecular size derived from bacterial, plant and animal cells. Antibody formation can also be elicited by the injection into animals of innocuous materials such as cells or proteins of a foreign species. Lipids (1), polysaccharides (2) and conjugated proteins (3) were also shown to be antigenic.

Several prerequisites are necessary for a substance to be antigenic in experimental animals.

(a) The administered compound must be foreign to the circulation of the animal, must have large molecular weight and a certain degree of complexity. Small molecules are not antigenic by themselves. However, when they are linked covalently to a large carrier molecule, such as a protein, they may
acquire antigenicity (4). Also, weakly antigenic substances become more so when adsorbed onto some particulate matter.

(b) Antigens must have chemical groups of sufficiently rigid structure in their molecules to serve as antigenic determinants (5). Groups, such as paraffin chains, the configuration of which are easily changed, cannot act as antigenic determinants. This fact explains the inability of long-chain fatty acids to induce antibody formation. Therefore, the atoms of the antigenically determinant regions must be in fixed spatial relationships to be efficient.

(c) According to Burnet (6), the response of an organism towards a foreign substance consists in the recognition of its "self" or "not-self" character. Normally, the organism becomes tolerant to most of its constituents in the course of its development. However, in some clinical conditions, such as autoimmune haemolytic anemia, antibodies may be produced also to the host's own tissues (auto-antigens). Nevertheless, generally, these auto-antibodies are formed against organs (such as the lens of the eye, thyroid gland, kidney, testis and brain tissue) which are anatomically isolated from the antibody forming cells ( reticulo-endothelial system) and which are, therefore, regarded as foreign or "not-self" by the immune system. Auto-antibodies may also be formed against organs other than the ones mentioned above if these organs suffer damage, which changes their "self" character (7).

In an attempt to elucidate the main characteristics of antibody-antigen reactions, Landsteiner (4) in his classical
work with azoproteins showed that the specificity of an antigen is not due to the molecule as a whole but only to certain atomic groupings, "active patches", on the surface of the macromolecule. A large protein molecule, such as serum albumin or γ-globulin, will have a complex configuration and may possess a variety of such determinant groups. Therefore, Landsteiner (4), Haurowitz (8), Pressman (9), Campbell (10), Karush (11) et al used simple, well-defined chemical compounds coupled to proteins by covalent bonds. These conjugated proteins induced the formation of antibodies specific to the artificial determinant groups, which were named haptens by Landsteiner (4), as well as to the antigenic determinants of the protein carrier, and antibodies were also directed against the region of the conjugate in which linkage between the hapten and the protein is located. The study of these hapten-protein conjugates gave tremendous impetus to the investigations into the problems of antigenicity. The classical work of Landsteiner clearly showed that antibodies could be formed against simple compounds of known chemical constitution and that these antibodies exhibited a high degree of specificity with respect to structurally related compounds.

Another powerful tool in studies of antigenicity is the use of synthetic polypeptides and polypeptidyl-proteins of known amino-acid composition for studying the effects of variations in the degree of crosslinking, molecular size and percentage helical structure on antigenic behaviour. It has been found that, in general, homopolymers are not antigenic (12, 13, 14).
Various co-polymers of glutamic acid and lysine, however, induce the formation of antibodies (15) and multilinear co-polymers of tyrosine, glutamic acid, alanine and lysine are even more antigenic. It would also appear that in order to be immunogenic, an antigen must be susceptible to in vivo modification or degradation and that the antigenic fragments must be retained by the host's antibody forming cells (16). Non-digestible artificial polymers and native non-digestible substances, such as gum acacia, are deposited in tissues without apparent degradation and do not induce antibody formation.

These studies greatly furthered the knowledge concerning the nature of antigenic determinant groups of synthetic polypeptides. However, the results cast no light on the structure of the determinant groups in natural protein antigens. For the elucidation of this problem another approach was suggested, namely the degradation of the protein antigen by chemical or enzymatic processes into small fragments. However, it was essential that these fragments still retained their antigenic groups as demonstrated by their ability to inhibit specifically the reaction between the native antigen and the homologous antibodies (17, 18). This method was particularly successful in studies concerning the antigenic constitution of human serum albumin (19) and fibroin (20).

Incubation of antigens with their respective antisera results in a specific combination of the antigen and antibody as was first shown by Ehrlich (21). This interaction between antibodies and their appropriate antigens may result in different
in vitro and in vivo manifestations, i.e. the neutralization of toxins, agglutination or lysis of foreign cells, the formation of a precipitate.

IN VITRO MANIFESTATIONS OF ANTIBODY-ANTIGEN REACTIONS

The precipitin reaction

The appearance of a flocculant precipitate on addition of soluble antigen to serum containing the homologous antibody, is the most frequently studied reaction in immunology. In general, the amount of precipitate formed on addition of increasing amounts of antigen to a constant amount of antiserum increases steadily, reaches a maximum and finally decreases until precipitation is completely inhibited. This behaviour is represented quantitatively by a typical curve, known as the precipitin curve (Fig. 1). It is convenient to divide the whole range of the reaction into the following three zones: (i) antibody excess zone, where insufficient antigen has been added, (ii) equivalence zone (Heidelberger) where both reactants are precipitated quantitatively and (iii) antigen excess zone, where precipitation is progressively decreased.

To elucidate the mechanism of the precipitin reaction Heidelberger and Kendall studied a convenient model system using the Type III pneumococcus polysaccharide as antigen and a homologous horse antiserum (22). On the basis of their experiments they developed the quantitative precipitin test for the determination of the amount of antibody present in various biological fluids.

Several hypotheses have been proposed to explain the
Increasing amounts of antigen added to constant amount of antiserum

Fig. 1. Precipitin curve.
mechanism of the precipitin reaction. The theory which at present is most generally accepted was put forward independently by Marrack (22) and Pauling (24). This "framework" (or "lattice") theory is based on the postulate that both the antigen and antibody molecules are polyvalent, i.e. that each of these molecules possesses more than one combining site. Accordingly, in the region of antibody excess the precipitate consists of small aggregates composed primarily of antibody molecules, which are crosslinked by a small number of antigen molecules. In the equivalence zone, where maximum precipitation occurs, the antigen-antibody complexes are crosslinked into larger and more compact aggregates, consisting of an alternating and recurring antigen-antibody pattern. Addition of excess antigen over the amount required to combine with all antibody sites causes disruption of this compact framework and results in the formation of smaller complexes, until these become too small to form a precipitate and remain in solution. This theory has been fully confirmed by recent experimental data. Most antigens were shown to be polyvalent (ovalbumin, thyroglobulin and viviparous hemocyanin were proven to have 4,40 and 231 antigenically determinant groups per molecule, respectively) and precipitating antibodies were shown to possess two combining sites (24, 25, 26, 27, 28).

**Agglutination**

The serum of an animal which has been immunized with cellular antigens, such as red blood cells of another species, will often cause clumping of the cells when added to the cell
suspension. This phenomenon called agglutination is another in vitro manifestation of antibody-antigen reactions. The mechanism of agglutination is considered to be similar to that of the precipitin reaction; the cells coated with the antigen molecules are crosslinked by divalent antibody molecules into a three-dimensional network. Soluble protein antigens may be adsorbed onto, or linked by covalent bonds to, erythrocytes, thus rendering the cells susceptible to agglutination by the appropriate antisera. These agglutination techniques will be discussed in more detail in a later chapter.

Complement fixation and lysis

When the combination of antigen and antibody takes place in the presence of fresh serum, certain serum factors (C'1, C'2, C'3, C'4) are incorporated into the immune complexes. These serum constituents are collectively named complement; their uptake is called complement fixation. If the antigen is part of some cellular entity (erythrocytes, microorganisms, etc.) the antigen-antibody interaction will be accompanied by the lysis of the cell.

IN VIVO REACTIONS OF ANTIGENS AND ANTIBODIES

In vivo neutralization of toxins and viruses

Antibodies produced in animals against toxins or viruses are well adapted to the neutralization of the biological activities of the offending organisms. The action of these antibodies seems likely to consist of a simple blocking of the active groups responsible for the toxic or viral activity.
Hypersensitive reactions

When an individual forms antibodies against a foreign, pathogenic substance he becomes resistant to further infection. Due to an aberration of the immunological mechanism, however, an individual may become highly sensitive to certain antigens and subsequent exposure to these antigens may produce harmful, often fatal, reactions. These states of hypersensitivity are of two types, i.e. immediate and delayed. Conditions of immediate hypersensitivity such as anaphylaxis and atopic allergy can be passively transferred into a non-sensitive individual with serum and, therefore, this type of hypersensitivity is obviously associated with circulating antibody. The characteristic manifestations of immediate hypersensitivity are accompanied by the liberation of pharmacologically active substances such as histamine, serotonin and bradykinin which are produced as a result of antibody-antigen combination. On the other hand, no circulating antibodies have been demonstrated in the delayed type of hypersensitivity and passive transfer requires the transfer of cells (leukocytes) or cell extracts (32). This type of sensitivity includes tuberculin sensitivity (33), contact allergy (34), experimental "auto-allergies" (35) and possibly homograft rejection (36). The inflammatory reaction characteristic of delayed hypersensitivity is not mediated by histamine.

Anaphylactic shock

This reaction was first described by Portier and Richet (37), who observed that an initial injection of an antigen into dogs followed 10-21 days later by a second, so-
called "shocking dose" of the same antigen caused violent illness, and often, death. This condition, which is called anaphylaxis, is generally accepted to be due to an antibody-antigen reaction. The first, sensitizing injection of the antigen initiates the production of antibodies, some or all of which remain fixed to tissue cells. The time interval which must elapse before anaphylactic shock can be provoked coincides with the period necessary for antibody formation. These antibodies then combine with the antigen upon a subsequent injection and induce the anaphylactic response. By the administration of small sublethal doses of antigen repeatedly, however, sensitized animals may be rendered refractory to shock indefinitely. Anaphylactic reactions exhibit a high degree of specificity, i.e. shock can be provoked only by the antigen used for sensitization. Whilst shock organs and symptoms vary from species to species, it has been shown that the reaction is a result of the contraction of smooth muscle (38). Excised smooth muscle tissue, such as uterine or intestinal strips from sensitized guinea pigs, can be used for in vitro demonstration of anaphylactic reactions (Schultz-Dale technique) (39, 40). In many cases sensitivity to an antigen can be transferred from an actively sensitized animal to a normal animal by injecting the serum of the first into the latter. Passive transfer methods, such as passive systemic and passive cutaneous anaphylaxis techniques, have been used to gain quantitative information on antibody-antigen reactions.
Arthus phenomenon (41)

Previously sensitized animals show intense local inflammatory reactions consisting of edema, infiltration of polymorphonuclear leukocytes and hemorrhage followed by secondary necrosis when reinjected subcutaneously with the sensitizing antigen. This type of hypersensitivity is associated with large amounts of precipitating antibody (42) which is not fixed to the tissues.

Atopic sensitivity

Perhaps the most common manifestation of an aberrant and harmful immunological mechanism is encountered in conditions of atopic sensitivity (43). Relatively innocuous substances which are usually harmless to normal subjects, render other individuals sensitive to them and cause asthma, hay fever, urticaria, dermatitis, etc. Substances which have been found to induce this type of hypersensitivity include pollens, danders, dust, foods, molds, bacteria, fungi, drugs and even simple chemicals. Atopic hypersensitivity develops spontaneously as the result of exposure to such substances (referred to as allergens) either by inhalation, ingestion or occasionally through epidermal contact. Von Pirquet (44) devised the term "allergy" for such hypersensitive states.

The participation of antibodies in the various atopic syndromes has been shown repeatedly. When the specific allergen is injected intradermally into a sensitive individual, a characteristic wheal and erythema reaction is obtained at the site of the injection, due to the antibody-antigen reaction taking place.
This reaction may also be elicited by passive transfer in normal, non-allergic individuals by injecting a small amount of serum obtained from an allergic individual and subsequently challenging the same site with the allergen (44).

The involvement of antibodies in immunity, autoimmune diseases, anaphylaxis and allergy has been established beyond any doubt. However, there is no general agreement as to what mechanisms underlie the different manifestations of the reactions between antigens and the homologous humoral and/or cellular antibodies. It is not clear whether the same molecular species is involved in more than one antibody manifestation as postulated by the "unitarian" hypothesis or whether several molecular species are involved. A simple solution to this question is not possible until pure preparations of antibodies formed against a single, well-defined antigen are available for the studying of immune phenomena. In an attempt to elucidate the nature of antibodies in conditions of hypersensitivity, the present study has been directed towards the evaluation and development of specific methods for the isolation of different types of antibodies present in sera of ragweed-sensitive individuals.

The nature of antibodies

Many attempts have been made to establish chemical or physicochemical differences between normal and antibody globulins. From earlier electrophoretic studies, it was concluded (46) that most antibodies are associated with the \( \gamma \)-globulin fraction of serum having electrophoretic mobilities of the order of \(-1 \times 10^{-5} \, \text{cm}^2/\text{volt/sec}\) at pH 8.6 and ionic strength 0.1. More recently, however, with the use of better
techniques, such as immuno-electrophoresis, it has been amply demonstrated that various factors, including the length and route of immunization, the nature of the antigen, and the species used (47) all have an influence on the physicochemical properties of the antibody molecules produced. In fact, the immunoglobulins are an electrophoretically heterogeneous population of molecules extending from the region of the slowest migrating γ-globulins into the region of α-globulins (48, 49). The sedimentation coefficient of γ-globulins is $7 \times 10^{-13}$ sec (7S) corresponding to a molecular weight of about 160,000, but an increasing number of studies (50, 51) show that antibodies may have sedimentation coefficients as high as 19S and several intermediate values have also been reported. The three major types of immune globulins are the following: (i) 7S γ-globulins which comprise more than 85% of the total immune globulins, and have a molecular weight of the order of 160,000.

(ii) γ1A- (or β2A-) globulins, which represent about 10% of the total, and have a slightly higher electrophoretic mobility than that of the 7S γ-globulins. The main ultracentrifugal component has a sedimentation coefficient of about 7S, but these globulins may also contain material having sedimentation coefficients of 10S, 13S, 15S (52). These substances may be aggregates since they are dissociated into 7S units by mercaptan treatment.

(iii) γ1M- (or β2M-) globulins which make up about 5% of immune globulins, and have a sedimentation coefficient of 19S. (M.wt.$\approx 10^6$). Reductive cleavage of disulfide bonds of these molecules results in the formation of inactivated 7S fragments.
The amino acid residues present in the combining region(s) are oriented so as to provide a complementary fit with the antigenically determinant group. As a result of this stereochemical complementarity, weak forces, such as van der Waals, hydrogen bonds and hydrophobic as well as ionic interactions come into play and hold the antigen and antibody molecules together. According to a postulate advanced by Marrack (53), Heidelberger (54), and Pauling (55), this close and complementary fit explains the high specificity observed.

Antibody molecules have been recently shown to possess two combining sites, both of which seem to be directed against the same antigenic group (56). Porter was able to split the rabbit antibody molecule of the 7S γ-globulin type into three fragments by digestion with papain (57, 58, 59). Two of these fragments contain one antibody site each, which are still capable of combining with the homologous antigen. The third fragment is devoid of antibody activity. Similar results were obtained by digestion of antibody molecules with insoluble papain followed by cysteine reduction (60). On the other hand, Nisonoff et al (61) showed that pepsin digestion of precipitating antibodies yields a bivalent fragment, which can be further split into two univalent fragments upon reduction of a single disulfide bond (62). Although the detailed mechanism of antibody biosynthesis is far from having been established, all these findings may suggest that the univalent portions are first formed, possibly by a mechanism such as proposed originally by Pauling (24), and then combine with the third fragment to form the complete anti-
Heterogeneity of antibodies

The basic postulate of the unitarian hypothesis (64) is that the administration of a single pure antigen to an animal elicits the formation of only a single kind of antibody. However, several investigators have found that antisera produced in response to immunization with a "pure" antigen contained several different antibodies. In retrospect, this could have been expected on theoretical grounds, since an antigen molecule by virtue of its macromolecular nature has a great many regions, which may act independently as templates for the formation of various antibodies (65, 66). Moreover, one could expect that even a single antigenic determinant group may induce the formation of a variety of antibodies differing in the configuration of their combining sites, which would result in different strength of the bonds between interacting antibody and antigen molecules. Studies of the dissociation of specific antibody-antigen precipitates show that the ease of dissociation of the precipitates differs for the different complexes formed (67).

Reference has already been made to the different electrophoretic and sedimentation properties shown by antibodies. Grossberg (68) separated electrophoretically 7S antihapten antibodies into components of different mobilities. Palmer et al (69) showed differences in their chromatographic behaviour. In the case of anti-insulin antibodies formed in the guinea pig Yagi et al (70) found the activity located in the \(~\beta\) and \(~\gamma\)-globulin regions, the two fractions having different mobilities and antigenic specificities. Further evidence for the hetero-
geneity of antibodies of the same molecular weight came from studies of antibody fragments obtained by papain digestion (71, 72). Thus, when digests were chromatographed on CM-cellulose, fractions I and II contained unequal amounts of protein. This finding was explained by the supposition that one part of the antibody population contained two identical fragments both of which fell into fraction I, while other antibody molecules yielded two active fragments which fell into fraction II. In some cases further subfractions were obtained containing active fragments from a whole host of different types of antibodies in the same serum.

ANTIBODIES IN SERA OF ALLERGIC INDIVIDUALS

Differences in physicochemical and biological properties among antibodies elicited by the same antigen are well illustrated by the complex antibody systems produced by allergic individuals. Because these antibodies are usually present in concentrations lower than detectable by standard physicochemical procedures (73, 74, 75), the elucidation of their nature has been seriously hindered. The skin-sensitizing antibodies produced "spontaneously" by allergic individuals are associated with the electrophoretically faster $\gamma_1$-globulins (76, 77), while blocking antibodies present in the sera of individuals after hyposensitization treatment are localized in the slower $\gamma_2$-globulins (78, 79, 80).

Skin-sensitizing antibody or reagin

Intradermal injection of the specific allergenic extract into allergic individuals leads to the formation of a
characteristic reaction which consists of a wheal surrounded by erythema. Prausnitz and Küstner (45) showed that a similar reaction can be obtained by injecting the allergic patient's serum into a non-allergic individual and by challenging the sensitized site, usually 24-48 hours later, with the allergen. This procedure is known as the passive transfer test of Prausnitz and Küstner or, for short, as the P-K test. The skin-sensitizing antibody has been shown to remain attached to the skin for periods of months (81). The P-K test has been used routinely for the demonstration and titration of skin-sensitizing antibodies in sera of allergic individuals. It was also shown (82) that when blood from an allergic donor is transfused into a non-allergic recipient, skin-sensitizing antibodies disappear rapidly from the circulation to become fixed in the skin, conjunctiva and mucosae. Exposure of the recipient to the specific allergens results in clinical symptoms similar to those of the donor. It may also be mentioned that accumulation of eosinophils has been observed at the site of the allergic reaction (83). Recently, using Rebuck's skin-window technique, it was shown that the eosinophilotactic activity arising from the combination of reagin with homologous allergen could be induced at the site of the skin reaction, both in allergic individuals and in non-allergic volunteers sensitized by passive transfer (84). Moreover, the reaction was shown to be specific and could not be induced by local application of histamine, serotonin and bradykinin in the absence of the specific allergen.
Skin-sensitizing antibodies differ from ordinary (immune) antibodies in many respects. They do not precipitate with the homologous allergen in vitro, do not readily cross the placental barrier from mother to foetus and lose their ability to sensitize normal skin after heating at 56°C for 1-10 hours. Furthermore, mixing the allergen with allergic serum does not modify the subsequent reaction of the allergen, when injected into sensitized normal skin. This latter observation together with the non-precipitability of skin sensitizing antibodies has been attributed (85, 86) to the possibility that reagins are either incomplete or univalent antibodies, and that, consequently, sufficiently large insoluble aggregates would not be produced even if combination of these antibodies with the appropriate allergens did occur in vitro. The alternative explanations are (i) that reagins are present in concentrations below the limits of detection by conventional precipitin methods and other common immunological techniques, and (ii) their affinity in vitro for the allergen is small.

Our knowledge concerning the chemical nature of skin sensitizing antibodies is still very fragmentary. Nevertheless, all observations made to date indicate that skin sensitizing antibody is much more labile than "conventional" antibodies produced on immunization. Storage of allergic sera at 4°C under sterile conditions for periods longer than 6 months causes considerable loss of activity. On the other hand, reagins present in serum fractions of relatively low total protein content lost their activity on storage at 4°C even for short periods.
Freezing and thawing of whole sera or of serum fractions was also found to result in serious losses of their skin sensitizing ability (76). Subjection of allergic sera to mild techniques, such as dialysis against 0.01 M phosphate buffer at pH 7.5 in the cold was associated with losses of reaginic activity of the order of 50% (87), while recovery of serum proteins was almost quantitative. The chemical heterogeneity of skin-sensitizing antibodies is well illustrated by their behaviour on exposure to acids. Thus, skin-sensitizing activity of certain allergic sera was partially or completely destroyed on incubation at pH 3 for 2 hours, whereas other sera were not affected by this treatment (74). Like immune antibodies, skin-sensitizing antibodies are degraded with papain. However, the rate of degradation of skin-sensitizing antibodies was found to be much slower and complete inactivation required 24 hours of digestion. However, unlike immune antibodies, skin-sensitizing antibodies are inactivated by treatment with mercaptoethylamine at a concentration of 0.1 M, in the absence of papain (88, 89, 52). Under these conditions antibodies of the 19S and 81A types are degraded. This inactivation may be the result of the reductive cleavage of the disulfide bonds of skin-sensitizing antibodies. The results obtained in the studies on the enzymatic and chemical degradation of skin-sensitizing antibodies indicate, that during these reactions the portion of the molecule responsible for its attachment to the skin becomes destroyed or dissociated from the rest of the molecule.

The weight of all this evidence supports the view that
skin-sensitizing antibodies differ radically from immune antibodies in their chemical nature and, as will be shown later, also in their physico- and immunochemical behaviour.

**Blocking antibody**

The usual hyposensitization treatment of allergic individuals consists of a series of intracutaneous injections of the specific allergen and induces the formation of a heat stable humoral antibody, which is called the blocking antibody. This antibody is capable of inactivating the allergen in vitro. Blocking antibodies, in contrast to reagins, are thermostable, they neither sensitize the skin, nor become fixed to it, and they pass the placenta from maternal to foetal circulation. Generally, it is believed that a significant clinical improvement is achieved by such hyposensitization treatment, which is manifested by the patient's ability to tolerate exposure to higher concentrations of the allergen. This effect is considered to be, at least in part, due to the protective capacity of blocking antibodies, which seems a reasonable inference, since the development of blocking antibodies appears to be the major immunologic response during immunization.

**Demonstration of antibodies in sera of allergic individuals**

As mentioned earlier, both reaginic and blocking antibodies fail to give visible precipitates, agglutinates or other readily measurable products of reaction when incubated with the homologous allergen(s) in vitro. That the combination of reagin and blocking antibody with the appropriate allergen does take place in vitro, has been demonstrated by many recent
investigations using special techniques. Incubation of sera from ragweed- and grass-sensitive individuals with homologous immunosorbents prepared by coupling the constituents of ragweed or grass pollen to polystyrene \((90, 74)\) erythrocytes \((90, 30, 91, 92)\) or cellulose \((93)\) resulted in the complete and specific removal of skin-sensitizing and/or blocking antibodies from the appropriate sera. It is important to point out that the removal of these antibodies did not result in a measurable decrease in the protein concentration of the serum. These and other results demonstrated that firm combination occurred between the allergens and skin-sensitizing and/or blocking antibodies in vitro, and that the concentration of allergic antibodies may be as low as \(0.01 - 1 \mu g/ml\) \((94)\).

Another approach for the detection of allergic antibodies involves the use of cellular elements of the blood, primarily the white cells, and the appropriate allergen-antibody systems. Thus, the amount of histamine liberated in vitro in bloods of allergic individuals in the presence of ragweed extract was found to be significantly higher than that in normal bloods \((95, 96)\), indicating that a specific interaction took place.

Coupling or adsorbing the antigen onto some suitable particulate matter results in an increase in the effective mass of antigen-antibody complexes, enhancing thus the sensitivity of the agglutination methods for the demonstration of minute amounts of antibodies. Using collodion particles "sensitized" with ragweed allergens, Cohen and Weller \((97)\) were able to
demonstrate clumping regularly with sera of treated allergic individuals, but the sensitivity of the method was low. Higher sensitivity was achieved with the use of red blood cells pre-treated with tannic acid according to Boyden's method (33). Accordingly, Feinberg et al (98) found that 21% and 67% of the sera of non-treated and treated allergic patients, respectively, exhibited positive hemagglutination tests. Subsequently, however, it was found that ragweed allergens were desorbed from the sensitized cells, leading to the inhibition of the hemagglutination reaction and to false negative results (99). To overcome this limitation, Sehon et al (90, 30, 100) attached ragweed and grass allergens to rabbit erythrocytes via bis-diazotized benzidine, thus forming stable covalent bonds between the red blood cells and the antigen molecules. Using this technique, all sera of both treated and non-treated ragweed- and grass-sensitive patients gave positive results. The test was shown to be specific and highly sensitive and has been adopted for the detection of antibodies in sera of individuals allergic to coffee bean (101), penicillin (102, 103), horse serum (104), milk (105), and egg-white proteins (106). However, no simple relationship could be found between the hemagglutinating and skin-sensitizing (P-K) titers of these sera. In earlier studies conducted in this laboratory some parallelism seemed to exist between these two manifestations, but higher values of hemagglutinating titers were observed with sera of treated individuals who had produced blocking antibodies. Recently, sera of non-treated allergic patients were also shown
to contain very low amounts of blocking type of antibody, the hemagglutination titers of such sera being primarily due to these blocking antibodies (87). Furthermore, on concentration of the chromatographic fractions of sera containing only blocking antibodies, these fractions were shown by the micro-Ouchterlony technique to possess anti-ragweed precipitins. Tentatively, these precipitating antibodies were considered to be blocking antibodies (107).

To elucidate the relations amongst these various antibody factors, a number of studies have been undertaken in an attempt to separate the different types of antibodies by the application of physicochemical and immunochemical methods. Chromatography of sera on DEAE-cellulose yielded fractions which contained hemagglutinating antibodies devoid of skin-sensitizing activity (87); these antibodies had the properties of normal, divalent, immune antibodies. The fractions containing skin-sensitizing activity, on the other hand, were never devoid of hemagglutinating activity, so the possibility that reagins also possessed hemagglutinating ability could not be ruled out. Serum fractions, separated by zone electrophoresis (108), precipitation with ammonium sulphate (108) or ultracentrifugation (50), which contained skin-sensitizing or blocking antibodies had also invariably hemagglutinating ability. The inability of eliminating completely hemagglutinating activity from fractions possessing skin-sensitizing activity by physicochemical procedures might be construed as indicating that hemagglutinating and skin-sensitizing functions are different manifestation of a complex molecule, or that they are
associated with different molecules having similar physico-
chemical properties (75).

**Antigenic relations between skin-sensitizing antibodies
and other globulins**

The relationship of reagins to other serum proteins
has been the subject of much study and controversy. From
electrophoretic, ultracentrifugal and chromatographic pro-
properties of skin sensitizing antibodies it would appear that
these antibodies may belong to the immunoglobulins of the
\( \gamma_1 \) type. This possibility received support from a recent
study (77) in which complete removal of reaginic activity from
three sera of treated ragweed-sensitive individuals was achieved
by absorption of the sera with an antiserum specific for \( \gamma_1 \-
globulins. These sera were also fractionated on Sephadex G-200,
the eluates possessing reaginic activity were shown to be com-
posed of \( \gamma_{1A} \)-globulins, 7S \( \gamma \)-globulins and \( \gamma_{1M} \)-globulins, but
the distribution of skin-sensitizing activity seemed to parallel
best that of the \( \gamma_{1A} \)-globulins (109). It should be mentioned
here that \( \gamma_{1A} \)-globulins have a tendency to complex with other
serum proteins (110). Therefore, it is also conceivable that
skin-sensitizing antibodies, identified as \( \gamma_{1A} \)-globulins, are
complex molecular species (75), one of their building blocks
being \( \gamma_{1A} \)-globulin, and that this latter component might be
responsible for the fixation of skin-sensitizing antibodies to
tissues (111). In this connection it is noteworthy that passive
sensitization of normal human skin with reagins was blocked with
normal human \( \gamma_{1A} \)-globulins (112). Using radio-immunoelectro-
phoresis, it was demonstrated that the ragweed-binding capacity
of allergic sera was associated with 7S $\gamma$-globulins and also with the $\gamma_{1A}$- and/or $\gamma_{1M}$-globulins (113). From these results, however, one cannot infer as to whether or not any of these ragweed binding antibodies can be identified with reagins, since even sera of non-treated allergic individuals possess antibodies in addition to reagins, which are capable of combining with the allergen (107). The results of preliminary studies by Heremans and Vaerman on the other hand, suggest that $\gamma_{1A}$-globulins may carry the skin-sensitizing activity of allergic sera (114). Thus, on the basis of all the results of radio-immunoelectrophoretic experiments and in the light of the findings derived from electrophoretic, ultracentrifugal, chemical, chromatographic and immunologic studies of antibodies in sera of allergic individuals, it may be suggested that blocking antibodies are 7S $\gamma$-globulins and that skin-sensitizing antibodies may belong to the class of $\gamma_{1A}$- and/or $\gamma_{1M}$-globulins.
CHAPTER II

THE ISOLATION AND PURIFICATION OF ANTIBODIES

Ever since antibodies have been identified as the factors responsible for the various immune manifestations, a great number of studies have been made to isolate antibodies in a "pure" state. Availability of "pure" antibody preparations, i.e. free of "normal" globulins and other serum components, is of prime importance in many studies. Pure preparations are particularly essential in any attempt aiming at the elucidation of the factors underlying the mechanisms of antibody-antigen interactions and for investigations of the structure and formation of antibodies (115, 62, 59). Purified, radioactive labelled antibodies may also be used as specific reagents for the identification and localization of tissue components, and in an attempt to destroy foci of cancerous cells (116).

Isolation procedures generally used for the preparation of pure antibodies may be divided into two groups according to the principles on which the experimental methods are based:

(a) non-specific methods, which involve the fractionation of antisera on the basis of physicochemical properties common to both antibody and non-antibody globulins, and

(b) specific methods, in which advantage is taken of the specific combination of antibodies with the appropriate antigens.

Non-specific methods

Since non-specific methods of separation depend on
physicochemical procedures, which cannot distinguish between the closely similar properties of immune and other globulins, these methods lead at best to an increase in the ratio of antibody globulin to the total amount of proteins in the mixture. Purification in this sense would include all procedures which result in the elimination of albumin from immune sera, e.g. by the precipitation of globulin fractions containing antibodies. The lower solubility of globulins allows their preferential precipitation by salts (ammonium sulphate, sodium sulphate, sodium phosphate) (117, 118, 110), or by organic solvents (119) (methanol, ethanol, acetone), which lead to the dehydration of serum proteins and thus to precipitation. In the majority of fractionation methods using salts, only heterogeneous mixtures of serum proteins were obtained. By precipitation at the isoelectric point at low ionic strength, Felton (120) obtained two fractions from anti-pneumococcus horse serum and found that the euglobulins which precipitated at pH 6.8 contained all the antibody activity, while the pH 5 fraction was inactive. However, most preparations obtained by this method proved to be mixtures of proteins, because protein-protein interactions are maximal at low salt concentrations.

Fractionation with cold ethanol is the basis of the method developed by Cohn and his co-workers (121), widely used for the large-scale preparation of human γ-globulins for therapeutic or prophylactic purposes. The use of organic solvents has several advantages over salting out methods: marked effects may be achieved by minor variations in pH, ionic
strength, temperature, protein and solvent concentration; time consuming dialysis to remove salts is avoided and purer, more homogeneous preparations can be obtained. With respect to antibody content, purification by a factor of 5 to 7 can generally be achieved.

Other methods take advantage of the ability of certain cations and anions to form complexes with proteins, which possess distinct solubility properties. Thus, Isliker and Antoniades (122) succeeded in purifying antibodies by a factor of two by extracting the gamma-globulin fraction precipitated in the presence of 5mM zinc lactate with different concentrations of glycine and tartrate. Fractionation of human serum using zinc salts was thoroughly investigated by a number of workers (121, 123). On the other hand, addition of aluminum chloride (0.05M, pH 4.7) resulted in the precipitation of all serum proteins, leaving 80% to 90% of the γ-globulins, with a purity of 95%, in solution (124). Between pH 5 and 6 γ-globulins bear a net positive charge in contrast to most other proteins. This leads to interactions between γ-globulins and anions such as the salicylate, oxalate, tartrate ions and certain negatively charged polymers such as polyacrylate (125), polymethacrylate (126) and polystyrene sulphonates (127), which results in the precipitation of γ-globulins. In one instance, the diphtheria antitoxin content of a purified fraction prepared by precipitation with polyacrylate exhibited a forty-fold increase over that of the original serum (125).

Ion-exchange chromatography has proved a most promising
technique for the separation of proteins (128, 129). However, the possibility of denaturation during adsorption and desorption should be borne in mind. Various cellulose derivatives, such as di- and triethylaminoethyl-(DEAE, TEAE) and carboxymethyl-(CM) cellulose (130, 129) have proved to be some of the most useful ion-exchangers.

Exploiting the differing electrophoretic mobilities of serum proteins, Kunkel et al fractionated larger volumes of sera by zone electrophoresis (131, 132). This method permits the utilization of various supporting materials, e.g. cellulose, starch- and other gels, glass beads, polyvinyl chloride particles. It is relatively simple and mild, and leads, in general, to good antibody recoveries.

Recently ultracentrifugation, with and without the use of density gradient, has been used for fractionation of serum proteins. Antibody activity was demonstrable in the 7S and 19S globulins, and Rockey and Kunkel (52) reported a new class of antibodies separated by density gradient ultracentrifugation, with sedimentation coefficient intermediate between these two values. Furthermore, these workers showed that like certain isohemagglutinins, the glucagon-reactive skin-sensitizing antibody from an allergic diabetic patient sedimented at a rate of 8-11S. Fractionation of allergic sera by gel filtration on Sephadex G-200 columns (109) yielded three protein fractions corresponding to sedimentation constants of 19S, 7S and 4S. Skin-sensitizing antibodies were found in a fraction between the 19S and 7S peaks. Another, less frequently used method is
ultracentrifugation in partition cells, designed for standard rotors in analytical ultracentrifuges (133). This method lends itself to the calculation of the sedimentation coefficient of antibodies, based on the determination of the antibody concentration or activity in the top or bottom compartment before and after centrifugation, even if their concentration is below the sensitivity of the Schlieren optical system (134).

It is apparent from this survey, that although non-specific methods of purification lead to the isolation of antibodies in good yields, the purity of the preparations is quite low. Nevertheless, fractions enriched in antibody by a factor of 10-20 can be readily isolated and fractionation of hyperimmune sera may lead to highly purified antibody preparations.

**Specific methods**

These methods exploit the specific combination between antibodies and the homologous antigens and involve the following steps: (i) formation of insoluble antibody-antigen complexes, (ii) isolation of these complexes from all other serum proteins, (iii) dissociation of the complexes under mild conditions, and (iv) separation of the released antibodies from the antigens. Since reactions of antibodies with antigens are associated with considerable decrease in free energy, drastic conditions are often required to liberate the antibody from the complex, which might lead to some denaturation of the antibody molecules. Also, separation of antibody and antigen molecules after dissociation may involve considerable difficulty, which can be circumvented, however, by rendering the antigens
insoluble during the dissociation process or by coupling the antigens to inert and insoluble polymers.

Since some antigen-antibody reaction were shown to be exothermic (135), raising the temperature caused at least partial dissociation of the antigen-antibody complexes. Thus, cold agglutinins adsorbed onto red cell stroma at 4°C were eluted from the washed complexes at 37°C to yield preparations of high purity (136). Moreover, antibodies differing in their affinity for the red-cell antigens have been successfully subfractionated by elution at increasing temperatures (137).

The use of concentrated salt solutions for the elution of antibodies has been limited to systems where the antigen is a polysaccharide. Heidelberger and Kabat, using 15% NaCl solutions, succeeded in eluting antibodies to pneumococcus polysaccharide which were 60-100% precipitable with the antigen, the yield of purified antibodies, however, was only 0.5-24% of the antibody present in original serum (138).

Selective enzymatic degradation of the antigen offers another approach to the isolation of antibodies from specific complexes. Pope and Healey (139) subjected dyphteria toxin-antitoxin floccules to the action of pepsin at pH 3.0 and recovered 70% of the original antitoxin activity. Tetanus antitoxins were prepared by a similar procedure. Recently, the isolation of pure antibodies to gelatin was achieved (140) by the selective digestion of the antigen in the washed specific precipitates with collagenase, which degrades collagen and gelatin into small molecular weight products.
The presence of opposite charges on the complementary combining regions of the antibody and the antigen molecules has been shown by many investigations, (141, 142) and, hence, the participation of electrostatic forces in the antibody-antigen interactions appears certain (143). One should, therefore, expect that at a pH at which strong positive or negative charges are conferred on both molecules would result in the dissociation of the complexes. The effect of acids and bases on the dissociation of antigen-antibody complexes was studied systematically by Kleinschmidt and Boyer (144) and by Singer and Campbell (145), who found that the complexes are stable between pH 4.5 and 11; dissociation gradually increases outside this range, and is complete at a pH of about 2.4 or 12.3.

Acids and bases have been widely used for the elution of antibodies from antigen-antibody complexes. Sternberger and Pressman (146) developed a method for the purification of antibodies to protein antigens. The latter were coupled to diazotized arsanilic acid and the modified antigen was still capable of combining with the antibody produced to its original configuration. The specific precipitate was dissociated with calcium hydroxide, and the insoluble antigen was removed with calcium-aluminate at pH 12. By this method, antibodies could be recovered with a 30% yield. A general method based on the same principle has been recently developed for the isolation of pure antibodies (147). The protein antigen was first modified by reaction with N-acetylamidocysteine-thiolactone, which leads to the coupling of a number of sulphhydryl groups onto the molecule. The specific
precipitate was then prepared, freed from non-specific proteins and dissociated at pH 2.4. The thiolated antigen was precipitated by crosslinking with the 3,6-bis(acetoxymercurymethyl)dioxane, leaving the antibody in solution. Rabbit antibodies to ovalbumin and bovine serum albumin were obtained with 90% purity. A lower degree of purity (69%) was recorded for antiribonuclease preparations. The yield of antibodies varied between 22% and 78%.

The common feature of the specific purification methods discussed above is that they are applicable only to systems in which the antigen is insoluble or is rendered insoluble during the process of purification, depending on some special property of the particular antibody-antigen system. In such systems the antibody, after dissociation from the antigen, is readily separated from the latter by centrifugation. A more general method for the insolubilization of soluble protein antigens involves the adsorption or attachment of the antigen through stable, covalent bonds to some solid and inert supporting medium, thus forming an immunologically specific adsorbent, (immunosorbent). This principle has been employed by several investigators using a variety of insoluble matrices. The feasibility of such a technique was first demonstrated by Landsteiner and Van der Scheer (148) who coupled diazotized hapten to red-blood-cell stroma and used the conjugates for the absorption of antihapten antibodies. The antibodies were recovered by dissociation by dilute acetic acid in 50% purity.

Antigens were adsorbed on kaolin (149), charcoal (150)
or glass beads (151), and these antigen-coated particles were capable of removing antibodies specifically. However, to eliminate the risk of the antigen becoming desorbed under certain conditions the antigens have been covalently coupled to the supporting polymers in all the later studies. Froese and Sehon (152) used RBC-stroma to purify anti-arsanilate antibodies, which were eluted with solutions of the hapten. From precipitin curves, the purity and yield of these antibodies were calculated as approximately 60%. A number of variations of this technique and principles have been used by many workers (153, 154). In the procedure of Farah et al (154), the free amino groups of the protein-hapten conjugate were blocked with 2,4-dinitrophenyl groups, and as a consequence the conjugate acquired a strong negative charge. Specific precipitates were prepared and the antibodies were eluted with hapten in the presence of streptomycin, which precipitated the dinitrophenylated protein-hapten conjugate. Isliker (155) converted carboxylated or sulphonated resins to the corresponding acyl- or sulphonylchlorides which were then reacted with protein antigens. The recovery of antibodies was about 55% and their purity 75-90%. In recent years protein antigens have been coupled to diazotized polyamino-polystyrene (156, 157, 158). The resulting immunosorbent was capable of removing antibodies from the appropriate antiserum specifically, and when ragweed or grass pollen constituents were coupled to the polystyrene, it removed skin-sensitizing, blocking and hemagglutinating antibodies from sera of allergic individuals (74). Precipitating antibodies could
be readily eluted at pH 3, but by contrast, antibodies of allergic sera were recovered only in small amounts. Recently it was reported, that the original polyamino-polystyrene (not diazotized) had also the ability of binding protein antigens, and that the antigen coated polyamino-polystyrene adsorbed antibodies specifically (158, 159).

The use of cellulose as supporting medium was originated by Campbell et al (160), who designed a general method for the isolation of anti-protein antibodies. Cellulose was combined with p-nitro-benzylchloride, the nitro groups were reduced, diazotized and the polydiazonium compound was coupled to the protein antigens. Antibodies were specifically absorbed and subsequently eluted at pH 3.2; the yield and purity were about 90%. However, other workers using similar adsorbants (161) found the yield and purity of the purified antibody to be significantly lower, probably caused by leaching of the antigen during elution of antibodies. More recently, Gurvitch, Kapner and Nezlin have modified this procedure and extended it for the quantitative determination of antibodies (162). The cellulose powder was combined with N-(m-nitro-benzylloxymethyl)pyridinium chloride to give the corresponding N-(m-nitro-benzylloxymethyl)ether of cellulose. The nitro groups were reduced and the aminocellulose was diazotized and coupled to protein antigens. This immunosorbent was claimed to have a high capacity of the order of 300 mg for antibodies per gram of immunosorbent and the non-specific adsorption was negligible. From the results discussed in this Chapter it may be concluded that specific purification methods are generally superior to non-specific procedures, and that immunological
adsorbents prepared by coupling the antigen chemically to an insoluble framework, have proved highly efficient. In most cases, antibodies can be eluted only under conditions of extreme acidity or alkalinity, which may lead to some structural modifications of the antibody molecules. However, the retention of their combining capacity both in vitro and in vivo seems to indicate that no irreversible, drastic changes are imparted to the antibody using this technique.
CHAPTER III
PREPARATION AND EVALUATION OF CELLULOSE-ANTIGEN CONJUGATES FOR THE PURIFICATION OF PRECIPITATING ANTIBODIES

INTRODUCTION

A large number of studies have been made to develop general methods for the isolation of antibodies in sufficient quantity for chemical and physical characterization. In most attempts to insolubilize the antigen for use as a specific adsorbent several difficulties were encountered such as the non-specific adsorption of serum proteins, the low recovery of active antibodies after desorption and a low capacity of the adsorbent. Recently, these difficulties have been overcome to a considerable extent by a modification (162) of the method of preparation of cellulose immunosorbents originally developed by Luescher et al (160).

For a critical evaluation of this type of immunosorbent, the amino derivative of cellulose was synthesized according to the procedure of Gurvitch and various protein antigens were coupled to it through stable azo bonds.

EXPERIMENTAL

Preparation of amino-cellulose-suspensions

For the synthesis of the immunosorbent cellulose preparations, designated as Solka-Floc BW-40 and BW-200, which were obtained from Brown Co., Berlin, N.H., were used as starting materials. For the preparation of aminocellulose, the introduction of amino-groups was achieved by the spontaneous reaction between cellulose and N-(m-nitrobenzyloxy) methylpyridinium chloride (163), as shown in the flowsheet presented in Fig. 2.
To portions of the cellulose (30g) suspended in absolute ethanol (550 ml), acetylchloride was added (38.5 ml) and the mixture was gently refluxed for 1 hr. (164). After cooling, the dark brown supernatant liquid was filtered on a Buchner funnel and the purified cellulose was washed with absolute ethanol until it appeared white. The cellulose was then air-dried on the Buchner funnel.

N-(m-nitrobenzyloxy)methyl pyridinium chloride was synthesized according to the method of Kursanov and Solodkov (163). For this purpose m-nitrobenzylalcohol (6g, practical grade) and polyoxymethylene (paraformaldehyde) (4.8g) were dissolved in benzene (35 ml). Dry hydrochloric acid was then bubbled through the mixture for 2 hrs, the solution being magnetically stirred. After standing for 1 hr the reaction mixture started to separate into two phases their separation being complete after about 10 hours. The upper phase was removed in a separatory funnel, dried over sodium sulphate and the benzene was distilled off. The product was then subjected to vacuum distillation (4.25-4.75 mm Hg pressure) in a micro-Quickfit apparatus, the m-nitrobenzylchloromethylether, a pale yellow liquid, being collected in the fraction boiling at about 158°C. (The yield was 4g, 51%). The product was identified by its nuclear magnetic resonance spectrum (Fig. 3). The m-nitrobenzyl-chloromethylether (4g) was then added to pyridine (12g). The condensation product appeared as a white precipitate. After 24 hours it was filtered off, washed with petroleum ether and
Fig. 2

Flowsheet for the preparation of aminocellulose

\[
\begin{align*}
\text{m-nitrobenzyl chloromethyl ether} & \quad \text{N-(m-nitrobenzyl oxymethyl) pyridinium chloride} \\
\text{aminocellulose}
\end{align*}
\]
Nuclear magnetic resonance spectrum of m-nitrobenzylaxychloromethyl ether.

The high resolution NMR spectrophotometer (Varian H.R.60) was used, the sample was dissolved in a dioxane and carbontetrachloride solvent.
air-dried. The pyridinium salt was identified by its infrared spectrum (Fig. 4).

The coupling of N-(m-nitrobenzyloxy)methyl-pyridinium chloride to cellulose powder was performed according to the method of Gurvitch et al (165), with only minor modifications. Three preparations, differing in the degree of substitution were synthesized, using 2%, 5% and 7% solutions of the pyridinium-salt in 90% ethanol (25 ml), (90% ethanol was used in place of water to facilitate subsequent drying) containing 0.7%, 2.5% and 2.5% (W/V) sodium acetate, respectively. The solutions were well mixed with the pre-treated cellulose powder (12.5g) and the mixtures were dried in an incubator between 60-80°C for about 2 hours. For the reaction leading to the formation of a stable bond, the mixture was heated in an oven at 125°C. Heating time was 40 min. for preparations No. 1 and 2, 1 hour for preparation No. 3. The reaction mixture was thoroughly washed on a Buchner funnel with benzene and then with water to remove any unreacted pyridinium chloride. The polynitrobenzene derivative of cellulose was then dried at 65°C and was again washed with three 200 ml volumes of benzene, for the purpose of extracting any m-nitrobenzylalcohol which may have formed due to hydrolysis. Following this washing the cellulose was air dried on the Buchner funnel.

The reduction of the nitro groups was achieved with a 15% solution of sodium-hydrosulphite (75 ml) at 50-60°C. The suspension was stirred for 30 min, filtered and washed thoroughly with water, 30% acetic acid and again with water.
Infrared spectrum of N-(m-nitrobenzyloxymethyl)pyridinium chloride.
The product, referred to as aminocellulose, could be stored dry at room temperature over a period of months. The degree of substitution achieved was determined by direct nitrogen analysis by the Kjeldahl method performed on both the aminocellulose and the original unsubstituted cellulose powder.

Thoroughly dispersed suspension of aminocellulose was prepared by dissolution in an ammoniacal copper solution and subsequent regeneration of the cellulose. Dry aminocellulose powder (lg) was dissolved in ammoniacal copper solution (33 ml of solution consisting of 1.5g cupric hydroxide, 0.33g sucrose, 20 ml of ammonium hydroxide (sp. gr. 0.90) and 13 ml water). A further portion of ammonium hydroxide (40 ml) and, for the reprecipitation of the aminocellulose, warm water (70°C) were added. The suspension was centrifuged, the supernatant decanted, and the cellulose neutralized with 10% sulphuric acid and washed several times with water. It was stored as a well-dispersed aqueous suspension at room temperature, without the addition of bacteriostatic agents.

**Preparation of cellulose-antigen conjugates**

Prior to coupling of protein antigens to aminocellulose the latter was diazotized. 1 ml of aminocellulose suspension was filtered, dried in the oven and weighed to determine the volume of suspension containing lg of aminocellulose. This volume of suspension was centrifuged and the supernatant liquid discarded. The cellulose was resuspended in chilled 5% hydrochloric acid (250 ml), the mixture was placed in an ice-bath, and NaNO₂ (5g) was added. Diazotization was continued for
30 min, with careful cooling and continuous stirring. The suspension was then centrifuged in a pre-cooled centrifuge tube at 0°C, washed twice with cold water and once with cold borate buffer at pH 8.6*. (The whole washing procedure was performed as quickly as possible to prevent any decomposition of the diazocellulose, within about 30-45 min). The diazocellulose was resuspended in the antigen solution (76 ml) containing 2% protein in pH 8.6 borate buffer. The mixture was stirred overnight in the cold. The supernatant solution containing any excess protein was then separated by centrifugation, and the cellulose-antigen complex was washed extensively with 0.9% sodium chloride at pH 7 to remove any free antigen. The protein content of the supernatants was estimated spectrophotometrically at 280 mµ (using a Beckman D-K recording spectrophotometer) and the difference between the total amount of protein contained in all the supernatants and the amount of protein added before coupling was taken as a measure of the antigen bound to the cellulose. To block any free, unreacted diazonium groups in the conjugates, a saturated solution of β-naphthol in borate buffer at pH 8.6 was added. In the presence of free diazonium groups a distinctly orange coloured derivative of diazocellulose is obtained. No colour change was ever observed in these experiments upon the addition of β-naphthol, indicating thus that all reactive diazonium groups had been blocked. The

* In view of the known lability of diazonium compounds in alkaline medium, i.e. of diazonium hydroxides, in later experiments the diazotized aminocellulose was washed with iced water only.
cellulose-antigen conjugate was again washed with physiological saline solution until the supernatant was free of material absorbing at 280 m\(\mu\) then washed 3-5 times with glycine-HCl buffer at pH 3 and finally twice with phosphate buffer at pH 7.3.

**Isolation of antibody**

For the isolation of antibody the immunosorbent was suspended in the appropriate antiserum for 1-2 hrs with stirring. Generally, antisera were diluted two-fold before absorption to reduce the viscosity of the mixture. The maximum antibody binding capacity of the immunosorbent was determined by absorbing repeatedly 3-5 ml portions of the antiserum with the same immunosorbent and following the extent of antibody removal with the bis-diazotized benzidine hemagglutination test. When the hemagglutination titer of the supernatants was identical to the original serum, the immunosorbent was considered to be saturated with respect to antibody. The amount of antibody bound to the adsorbent was then calculated from the sensitivity of the hemagglutination method previously determined on the basis of a quantitative precipitin test done on the antiserum. The antibody binding capacity of the immunosorbent was found to vary with the antigen-antibody system used.

The cellulose-antigen-antibody conjugates were then washed free of any non-specific serum proteins with saline at pH 7, by repeatedly suspending it in saline and centrifuging, until the washings contained no protein as judged by absorption at 280 m\(\mu\) or less than 10\(\%\) /ml nitrogen as determined by the
micro-Kjeldahl procedure. This limit for the nitrogen content of washings was set arbitrarily, since an excessive number of washings would have been necessary to lower it further.

Elution of the antibodies was carried out batchwise, since the small particle size of the cellulose-suspension prevented the achievement of a reasonable flow rate in columnar operations. Eluting agents were either 1% NaCl adjusted to pH 3.2 with 0.1N HCl or a glycine-HCl buffer at pH 3, ionic strength 0.1. The conjugates were suspended in the acidic agents for a few minutes, and the dissociated antibody was separated from the cellulose-antigen conjugates by centrifugation. The pH of the supernatant, referred to hereafter as eluate, was adjusted to 7. Occasionally, at this stage a small amount of flocculant precipitate formed, this was centrifuged off before protein and antibody estimations were made.

**Determination of antibody content**

The antibody content of the whole antisera and of the eluates was determined by quantitative precipitin test performed by the adaptation of the method of Heidelberger and Kendall (22). One ml portions of the antiserum (or eluate) were added to a series of tubes each containing 1 ml volumes of the homologous antigen in two-fold decreasing concentrations. To begin with, the antiserum was diluted to give a maximum precipitate of about 100 µg/ml, as determined from a preliminary experiment. After mixing the two reactants, the solutions were incubated at 37°C for 2 hours, and then at 4°C for 48 hours. The precipitates
were separated by centrifugation and resuspended three times in 2 ml volumes of chilled saline. The washed precipitates were digested with sulphuric acid and were analyzed for nitrogen by the micro-Kjeldahl procedure (166). The precipitin curves were obtained by plotting the amount of specific precipitate against the amount of antigen added.

When glycine-HCl buffer was used for the dissociation of the antibody, the eluates were dialyzed against saline to remove the glycine before the precipitin test was set up. This was necessary because of the inhibitory effect of glycine and other amino acids on the formation of precipitates (167). Along with the quantitative precipitin test, the BDB-hemagglutination technique (90, 30, 100) was used extensively for the estimation of antibody content of sera and of eluates.

**Hemagglutination test (micro)**

Rabbit erythrocytes were used in the hemagglutination test. For this purpose blood was collected from the marginal ear vein from "normal", non-immunized rabbits into a flask containing an equal volume of Alsever's solution. For storage (up to three days) of erythrocytes the blood in Alsever's solution was kept at 4°C. The serum used for the preparation of the diluent was obtained from the blood of the same rabbit which supplied the erythrocytes. It was decomplemented by heating at 56°C for 30 minutes and diluted 100-fold with phosphate buffer at pH 7.3.

The bis-diazotized-benzidine (BDB) used for attaching the antigens to the red blood cells was prepared according to the method used previously in this laboratory (30) and was kept
in small vials at -20°C until required. Before coupling the antigen to erythrocytes, the frozen BDB was melted by rotating the vial in the palm and BDB solution (1 ml) was quickly added to phosphate buffer (14 ml) at pH 7.3, which had been pre-cooled to 4°C. This solution, referred to hereafter as BDB-phosphate solution, must be used as soon as possible because it deteriorates as evidenced by the formation of a brown colour.

Sensitization of the erythrocytes

The packed rabbit red blood cells were washed three times by resuspending in cold physiological saline and recentrifuging. The washed packed cells were then resuspended in an equal volume of saline. The optimal quantity of antigen was placed into a 15 ml centrifuge tube and mixed with 0.1 ml of 50% red cell suspension. To this mixture was added the optimal amount of BDB-phosphate (freshly prepared). The reaction was allowed to proceed at room temperature for fifteen minutes with occasional stirring. "Sensitized" cells were separated by centrifugation and the brownish supernatant was discarded. The cells were washed with 3.5 ml of diluent, recentrifuged and resuspended in diluent to a final volume of 5 ml to give a 1% (v/v) red blood cell suspension.

Standardization of the method

To establish the optimal relative amount of each batch of BDB for each of the antigens, the following procedure was used: with a constant amount of erythrocytes (i) the BDB-phosphate volume was varied keeping the antigen concentration constant, and (ii) the antigen concentration was varied and the
BDB-phosphate volume was kept constant. Each batch of sensitized cells was then tested with normal rabbit serum and with the appropriate antiserum. The proportions of BDB-phosphate solution to antigen were considered optimal when the highest titers were obtained with antisera and no reaction ensued with normal sera. For optimal sensitization the following amounts of materials were used: 0.1 ml of 50% red cell suspension, 0.5 ml BDB-phosphate solution and 3 ml of antigen solution in saline, containing 1 mg water-soluble ragweed extract (WSR), or 0.5 mg dialyzed residue of ragweed, or 0.6 mg horse serum albumin, or 6 mg bovine serum albumin (for the latter antigen, the amount of BDB-phosphate solution necessary was 2 ml). To remove non-specific agglutinins, all sera were treated with an equal volume of packed, non-sensitized rabbit erythrocytes (washed three times with cold saline) for 1-2 hours at room temperature or overnight in the cold. The cells used for this absorption, as well as for sensitization with the antigen were always from the same rabbit.

Performance of the microhemagglutination test (168)

For the hemagglutination experiments plexiglass plates, (purchased from Metrimpex, Budapest, Hungary), having 6 rows of 12 wells each were used. The wells have hemispherical bottoms and have 0.125 ml working capacity. (Plates with conical-bottom wells were also found to be convenient). A volume of 0.05 ml of diluent was placed into each well by means of a specially calibrated dropper, which delivers drops of uniform size (0.025 ml). The absorbed sera were serially diluted in halving dilutions
using stainless-steel loops of known (0.05 ml) volumes, and one drop (0.025 ml) of the sensitized cells' suspension was added to each well. The plates were gently shaken to ensure even dispersion of the cells, and covered by other plates to minimize evaporation. The reaction proceeded at room temperature and could be read after a minimum of 2 hours. Usually they were read after not more than twelve hours, although occasionally even after 30 hours at room temperature the patterns were found unchanged. The titer of the antiserum was expressed as the reciprocal of the highest dilution of antiserum which gave a positive pattern. The hemagglutination patterns were graded according to the recommendations of Stavitsky (169). Because the end-point of the hemagglutination test is somewhat subjective, each reading was compared against a control row of sensitized cells in diluent. In addition, for day-to-day comparison of the method, a serum of known antibody content and hemagglutination titer was employed. The sensitivity of the hemagglutination test was calculated for each antigen-antibody system. For the estimation of antibody content, the sensitivity of the test was expressed as \( \mu g \) antibody per ml per hemagglutinating unit; it represented the ratio of the number of \( \mu g \)-s of antibody contained in one ml of antiserum, as determined by the quantitative precipitin test, to the hemagglutinating titer. The sensitivity values ranged from 0.22 \( \mu g/ml/HA \) unit for rabbit anti-HSA to 0.0008 \( \mu g/ml/HA \) for the rabbit anti-ragweed (residue) system (107, 108).
the micro hemagglutination test is included in the Microtit kit, (Fig. 5)*, assembled by Takatsy, the originator of the micro-method (168). The stainless steel loops are attached to tapered metal handles and are supplied in three sizes (0.1, 0.05 and 0.025 ml) calibrated at the factory. For the purposes of this study they were recalibrated. For this purpose, a concentrated solution of bromphenol-blue dye was serially diluted with water with each of the loops. A volume of 20 μl of the resulting solutions were withdrawn from each well and diluted sufficiently to bring their optical densities into the linear range for this dye, which was determined by a preliminary calibration curve. Optical densities were read at 540 μm. The volumes of the loops were then calculated from the concentrations of the dye in subsequent wells. The results were found to agree with the values given by the manufacturer. - The droppers supplied are all the same size. They consist of a small length of stainless steel tubing, (internal diameter about 1/4 mm) encased in a specially shaped plastic tip that fits onto ordinary pipettes to form the complete dropper. The drop size was re-determined by weighing 50 drops of distilled water and was found to be 0.025 ml for each tip in the Microtit.

Antigen-antibody systems used

Antisera were produced in the rabbit against different protein antigens, such as bovine serum albumin (BSA), horse serum albumin (HoSA), water-soluble ragweed extract (WSR) and dialyzed residue of ragweed (DWSR). Albino rabbits of 3 to 4 kilograms were injected intravenously (into the marginal ear

* Obtained from Metrimpex, Budapest, Hungary.
The use of stainless steel loops (1) and the calibrated dropper (2) in the microhemagglutination technique.
veins) with 1 ml of a 1% solution of the antigen (BSA and HoSA) three times per week for four weeks. Blood samples were collected periodically starting six days after the last injection and were tested by ring test for antibody content. When a good titer had been reached, 50 to 70 ml of blood were taken from each rabbit from the marginal ear vein. The blood samples were allowed to clot at room temperature and then placed in the cold to allow the clot to retract. The clotted bloods were centrifuged, the sera were decanted and sterilized by filtration through Swinney-type Seitz filters. All sera were stored at 4°C until used.

In view of the poor antigenicity of ragweed, the dialyzed residue of water soluble ragweed extract was administered in Freund's adjuvant. Equal volumes of Freund's complete adjuvant (purchased from Difco Laboratories, Detroit, Mich., U.S.A.) and of a solution, containing 10 mg of antigen and 1.5 mg of stearylhydroxamic acid β-(diethylmethylammonium)ethyl ester iodide* per ml, were mixed and homogenized with the VirTis 45 homogenizer, purchased from the VirTis Co. Inc., Gardiner, N.Y. The following immunization schedule was used: each rabbit received one injection of the emulsion weekly over a period of one month. During the first two weeks, 0.06 ml of the emulsion was injected into each of the front footpads and in the following two weeks, 1 ml of the emulsion was given

* This compound was kindly supplied by Dr. W.C. Murphy of CIBA Ltd. and was found to stabilize the emulsion. This compound is identified as Preparation No. 20,150 of the CIBA Co.
intramuscularly. Subsequently, portions of 1 ml of the aqueous extracts of ragweed pollen in the presence of stearylhydroxamic acid β-(diethylmethylammonium)-ethyl ester iodide, were injected intraperitoneally at weekly intervals until the antibody concentration rose to a satisfactory titer. The animals were then bled from the marginal ear vein. About 120 ml of blood was obtained from each animal over a period of 3 days. The animals were re-immunized after a rest period of 3 months and received at this stage two intramuscular injections of the aqueous antigen solution over a period of 2 weeks and one intraperitoneal injection in the third week.

RESULTS

The aminocellulose preparations made with 2%, 5% and 7% concentrations of the N-(m-nitrobenzyloxymethyl)-pyridinium chloride differed significantly in appearance as well as in their nitrogen content (determined by the micro Kjeldahl procedure) and their antigen and antibody combining capacities (Table 1). Preparation No. 3, containing the highest amount of amino groups coupled to cellulose was used in all subsequent work for the preparation of this immunosorbent. Although preparation of the aminocellulose proved to be straightforward and simple, reprecipitation from ammoniacal copper solution was associated with some difficulties. The yellow amorphous product obtained at the end of the synthesis was difficult to dissolve and occasionally twice the usual amount of ammoniacal copper solution was necessary for complete dissolution. Also, addition of excessive volumes of lukewarm water was sometimes
### Table 1
Characteristics of amino-cellulose preparations

<table>
<thead>
<tr>
<th>Preparation</th>
<th>$N_2$ content gmN/100g cell</th>
<th>Antigen bound mg/g cell</th>
<th>Antibody bound mg/g cell</th>
<th>Concentration of N-(m-nitro*)</th>
<th>Colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.02</td>
<td>N.D.**</td>
<td>N.D.</td>
<td>2%</td>
<td>white</td>
</tr>
<tr>
<td>2</td>
<td>0.06</td>
<td>120</td>
<td>N.D.</td>
<td>5%</td>
<td>cream</td>
</tr>
<tr>
<td>3</td>
<td>0.22</td>
<td>188</td>
<td>294</td>
<td>7%</td>
<td>yellow</td>
</tr>
</tbody>
</table>

* N-(m-nitrobenzyl)oxymethyl)pyridinium chloride.
** Not determined
necessary before precipitation would commence. This latter
difficulty was obviated to a large extent by warming the water
to 70°C prior to the addition to the cellulose-solution. The
amino-cellulose suspension obtained after reprecipitation
represented 70 to 90% of the material dissolved and contained
a flocculent, evenly and finely dispersed cellulose derivative
with an enormous surface. This suspension could be kept at
room temperature for months, without any deleterious effects.

Several cellulose-antigen conjugates prepared with
different antigens such as BSA, HoSA, WSR and DWSR, were
capable of completely removing precipitating antibodies from
the homologous rabbit antisera. The specificity of this reaction
was demonstrated by the fact that these antisera were not deple-
ted of their antibodies by immunosorbents prepared with hetero-
logous antigens. For example, the incubation of a cellulose-BSA
conjugate with a rabbit anti-WSR serum or the serum of an
individual allergic to ragweed did not deplete the anti-ragweed
antibody content of these sera. Hemagglutination and P-K tests
done on these sera before and after exposure to the cellulose-
BSA conjugate showed no decrease. On the other hand, when the
cellulose BSA conjugate was incubated with the homolous rabbit
antiserum, i.e. with anti-BSA serum, all antibodies were removed
specifically and the hemagglutination titer was reduced to prac-
tically nil.

Cellulose-protein conjugates could be kept at 4°C in
aqueous suspensions, without loss of activity, for months.
Recovery of precipitating antibodies from cellulose-antigen-
antibody complexes was achieved by dissociation at pH 3 with physiological saline solution acidified with 0.1 N HCl or with a glycine-HCl buffer at pH 3. Elution was done batchwise, since attempts aiming at columnar operation invariably failed because of the blocking of flow by the small particles of the immunosorbent in suspension. Separating the cellulose from the supporting sintered glass disc or glass wool in the column by a plug of Sephadex G-25 did not increase flow rates. Mixing the immunosorbent with an equal volume of Sephadex G-25 was similarly unsuccessful*. In the batchwise method of elution, fractions of 5 to 10 ml volumes were collected. Complete elution generally required 8 to 20 such fractions depending on the amount of immunosorbent used and its degree of saturation with antibodies. It was found that the length of time the antibodies were allowed to stay on the immunosorbent had a direct adverse effect on the ease of elution. This is in agreement with the findings of Campbell et al (160).

The yield and purity of the antibody preparations were estimated by quantitative precipitin tests. The behaviour of the eluted antibodies was similar to that of the original antiserum. The precipitin curves for the BSA-anti BSA system are illustrated in Fig. 6. As can be seen, the characteristics of the precipitin curve obtained with purified anti-BSA antibodies correspond closely to those of the whole antiserum. In this instance, 84.4% of the antibody adsorbed was recovered and

* In a very recent publication Porter, R.R. et al (170) reported successful adaptation of this method to chromatographic procedures by mixing the immunosorbent with 19 volumes of Solka-Floc cellulose.
Precipitin curves obtained with whole antiserum and with antibodies eluted from the immunosorbent (BSA-anti-BSA system).
69% of the eluted protein was specifically precipitated with the antigen. It should be pointed out that the precipitin test on which the above results were based, was done after extensive dialysis and freeze-drying of the purified antibody. During dialysis against water some precipitate formed in the dialysis bags, which did not completely dissolve in saline. This precipitate was removed by centrifugation before freeze-drying. When the dry antibody preparation was redissolved in saline, some additional cloudiness appeared. All these findings point to some denaturation of antibody protein during the purification process and during dialysis and freeze-drying.

Special problems were encountered in working with ragweed-cellulose conjugates. In contrast to pure protein antigens, such as BSA for example, ragweed extracts are chemically and immunologically distinctly heterogeneous. Due to the low protein contents of WSR and its high degree of heterogeneity very large amounts had to be used for coupling; even so the attachment of the main antigenic component(s) was badly hindered by the many other substances present. This necessarily resulted in an "immunosorbent" of poor capacity for anti-ragweed antibodies. Better results were obtained with the dialyzed residue of WSR, which is significantly richer in proteins, containing 65% protein compared to 5% in WSR. As previously shown DWSR is antigenically more potent (171). Immunosorbents were prepared with dialyzed residue by coupling it 1.5-2 times higher concentrations than with "pure" protein antigens. This immunosorbent was capable of removing specifically precipitating rabbit antibodies directed against WSR or DWSR. Before exposing the
immunosorbent to the antisera, it was extensively washed with the pH 3 glycine-HCl buffer to eliminate the possibility of the antigen becoming desorbed during the elution of antibodies. This step was considered essential because of the lability of ragweed constituents even at neutral pH-s when in solution. However, the amount of material removed by the washing procedure was very little, and could be considered negligible. Nevertheless, this procedure was adhered to in all preparations of immunosorbents. Estimating the yield and purity of anti-ragweed antibodies was also made difficult by the heterogeneity of ragweed antigens. Precipitin tests at best give only approximate results as to the quantity of antibody directed against ragweed contained in antisera and in eluates. It has been shown in this laboratory, that for this system supernatant solutions in the equivalence zone still contained considerable amounts of free antibodies. Thus, the calculated value for the antibody content as determined by the precipitin test is a minimum value. Apparent recoveries of antibody ranged from 86 to 109% and at least 41% of the recovered protein was precipitable with ragweed antigens. The antibody-binding capacity of the immunosorbent was of the order of 250-300 mg protein per gram of sorbent.

The homogeneity of the purified antibody preparation was shown by ultracentrifugal analysis (Fig. 7), and moving boundary electrophoresis (Fig. 8). All ultracentrifugal experiments were carried out in the Spinco Model E optical ultracentrifuge at 55,000 r.p.m. and 0°C. The synthetic boundary cell was used preferentially, because of the relatively low protein
Fig. 7
Schlieren patterns obtained in the analytical ultracentrifuge for a purified anti-WSR antibody preparation

Photographs were taken at 8 min. intervals in the synthetic boundary cell.
Moving boundary electrophoretic patterns obtained with a purified anti-WSR antibody preparation

The electrophoretic analysis was performed in a Spinco Model II Tiselius Electrophoresis apparatus in veronal buffer pH 8.6, ionic strength 0.1. The schlieren patterns above were taken at 90 min. and 150 min. in the descending boundary.

The \(\xi\)-anomaly is stationary, the mobility of the protein peak \(-1.5 \times 10^{-5} \text{ cm}^2/\text{volt/sec}\).
concentration of the samples. Sedimentation coefficients were calculated by the method of Yphantis and Waugh (134). Two purified antibody preparations, specific for BSA and DWSR, were shown to contain one component only, with sedimentation coefficients of 6.3S and 7.2S, respectively (not corrected to standard conditions because of limited amounts). The results are in good agreement with the general observation that antibodies produced on active immunization for prolonged periods in rabbits or in man are globulins of the 7S type.

Electrophoretic analyses were carried out in the Spinco Model H Tiselius apparatus at 0.8°C, in the micro electrophoresis cell. Prior to electrophoresis the eluates were concentrated by pervaporation to a protein concentration of about 0.5g per cent and dialyzed against veronal buffer of pH 8.6, ionic strength 0.1, for 24 hrs. The Schlieren patterns obtained showed a single symmetrical peak with a mobility of \(-1.5 \times 10^{-5} \text{ cm}^2/\text{volt/sec}\), this value is of the same order as the mobility of \(\gamma\)-globulins.

Essentially the same results were obtained using an aminocellulose preparation kindly provided by Dr. A.E. Gurvitch of the Institute of Epidemiology and Microbiology of the Academy of Medical Sciences, Moscow, U.S.S.R. The cellulose-antigen conjugate was washed with pH 8.6 borate buffer after the elution of antibodies, and was stored in phosphate buffer at pH 7.3 at 4°C for a further experiment. This immunosorbent, however, possessed only about half of its original capacity for antibodies when re-used in the subsequent experiment.
An ideal antigenically specific adsorbent should contain active antigen, should be insoluble under the experimental conditions used, should have sufficiently high capacity for the antibody and should not adsorb heterologous serum proteins. These requirements were fulfilled by the cellulose-antigen conjugates prepared from N-(m-amino benzyloxy)cellulose ether ("amino-cellulose") according to the procedure of Gurvitch. These immunosorbents showed an unimpaired antigenic activity with respect to the homologous antibodies and had very little or no affinity for non-antibody proteins.

The number of amino groups in the cellulose could not be estimated from titration curves, since there was no clear cut inflection point in these curves, suggesting that some of the amino groups were buried in the matrix of the cellulose and became only gradually accessible for reaction with hydrogen ions in the titration process. Direct nitrogen analysis on the other hand gave an upper limit for the number of amino groups since conceivably some nitro groups had not been reduced. As mentioned previously, those aminocellulose preparations containing the highest number of diazotizable amino groups proved to possess the highest antibody-binding capacity. Elution of the antibodies (absorbed out specifically from antisera) was achieved by lowering the pH to 3. This method was shown to be successful, although the antibodies could not be recovered quantitatively. This is in accordance with the concept of the heterogeneity of antibodies inasmuch as some of the antibodies
presumably remained strongly bound to the immunosorbent, while those with lower affinities for the antigen were easily eluted at pH 3. According to this view the eluted purified antibodies do not represent the whole spectrum of antibodies originally present in the antisera but only the ones with lower affinity for the antigen and this has to be borne in mind in any further studies of antibodies prepared by this method. However, this technique offers a practicable method for the preparation of antibodies in reasonable yields, on a 100-200 mg scale. It is felt that the development of an immunologic adsorbent ideal in every respect for the isolation of antibodies in pure form will be possible only when the precise structures of antigenically determinant groups and of the antibody combining sites are established, and when the factors responsible for antigen-antibody combinations are fully elucidated.
CHAPTER IV
THE USE OF CELLULOSE-ALLERGEN CONJUGATES FOR THE ISOLATION OF ANTIBODIES FROM SERA OF ALLERGIC INDIVIDUALS

INTRODUCTION

It has been repeatedly shown that skin-sensitizing antibodies present in sera of allergic individuals do not yield detectable precipitates on incubation with the homologous allergens. This failure was attributed, as discussed in Chapter I of this thesis, to different causes such as their inability to combine with the homologous antigen in vitro, the univalency of these antibodies or their being present in concentrations below the threshold of detectability. However, in spite of the absence of a visible reaction, combination between skin-sensitizing antibodies (reagins) and the corresponding allergens does occur in vitro as was shown in earlier studies done in this laboratory (100, 30). It was found that all sera of non-treated* or treated* ragweed sensitive individuals invariably agglutinated red blood cells to which ragweed pollen constituents were coupled via bis-diazotized benzidine. However, it was established that there was no clear cut relationship between skin-sensitizing and hemagglutinating titers. Also, polystyrene-allergen (74) and red blood cell-allergen (92) conjugates were capable of removing specifically antibodies from allergic sera. However, these antibodies could not be eluted in significant quantities from these immunosorbents.

* For the sake of brevity, allergic individuals who have received hyposensitization treatment are referred to as "treated" individuals, and those who have received no such treatment, as "non-treated" individuals.
and further characterization of the antibodies could not be carried out. Recently, Augustin (172) claimed to have isolated skin-sensitizing antibodies; however, no details of her procedure were published. In the present study an attempt was made to isolate skin-sensitizing antibody from sera of allergic individuals by means of cellulose-ragweed conjugates.

**EXPERIMENTAL**

**METHODS AND MATERIALS**

**Preparation of cellulose-allergen conjugates**

The experimental procedures and the materials were similar to those described in the previous Chapter for the preparation of cellulose-antigen conjugates. The aminocellulose preparation containing the largest number of aminogroups was used. For the preparation of the immunosorbent 2.7g of the dialyzed residue of water-soluble ragweed extract (DWSR) was coupled to one gram of aminocellulose.

**Allergic human sera**

Allergic sera containing skin-sensitizing antibodies were obtained from treated and non-treated ragweed-sensitive individuals through the courtesy of Dr. S.O. Freedman of the Allergy Division, McGill University Medical Clinic at the Montreal General Hospital, Montreal, Canada. All sera were sterilized by Seitz filtration and kept at 4°C.

**Absorption of sera with cellulose-ragweed conjugates**

The incubation of allergic sera with the immunosorbent and the recovery of the sera after absorption were performed as described in Chapter III. Elution of antibodies from the
cellulose-ragweed-antibody complexes was attempted by dissociation with physiological saline solution acidified to pH 3 with dilute hydrochloric acid, or with diluted normal human serum acidified to pH 3, or with 6M urea solution, or with normal human serum diluted ten-fold with saline and containing urea in 6M concentration.

The antibody content of the sera (and of the corresponding filtrates and eluates) of the treated and non-treated allergic individuals was determined both by the in vitro BDB-hemagglutination technique (30), as described in Chapter III of this thesis, and by the in vivo passive transfer test of Prausnitz and Küstner (45), referred to hereafter as the P-K test.

Passive transfer (P-K) test

The titers of skin-sensitizing antibodies were obtained by the Prausnitz-Küstner (P-K) reaction using 0.05 ml portions of the test solutions. These solutions, prepared in two-fold serial dilutions, were injected subcutaneously into the back of volunteers who gave no skin-reaction to ragweed. The sites were challenged 24 hours later with 0.025 ml of a water soluble extract of ragweed pollen and after 20 minutes the reactions were graded from 0 to 4+ depending on the size of the wheal and erythema. The site which still gave a minimal positive reaction was taken as the endpoint of the titration for skin-sensitizing antibodies and the titer was expressed as the reciprocal of the dilution of the serum (or eluate) injected. The challenging antigen was the dialyzed residue of water-soluble ragweed extract.
At a concentration of 50μg/ml or, in some experiments, the water-soluble ragweed extract (WSR) at a concentration of 1000 PNU*. Antigen solutions were sterilized by filtration through Oxoid membranes, since in an earlier study done in this laboratory (173) Seitz filtration was shown to remove some of the allergens as compared with membrane filtration.

**RESULTS**

On incubation of sera of ragweed-sensitive individuals with cellulose-ragweed conjugates, skin-sensitizing antibodies were removed from these sera, as demonstrated by the in vivo P-K test. The removal of these antibodies was always accompanied by a parallel disappearance of the hemagglutinating factor(s). The specificity of these reactions was demonstrated by experiments similar to those performed with precipitating antigen-antibody systems (Chapter III). When sera containing skin-sensitizing antibodies were incubated with cellulose-antigen conjugates prepared with unrelated antigens, such as BSA or HoSA, the skin-sensitizing titer of the recovered sera did not show any significant decrease. Similar results were obtained using the in vitro hemagglutination technique.

The capacity of the immunosorbent for skin-sensitizing antibodies could not be determined quantitatively. However, it was established that 25 mg of the cellulose-ragweed conjugate was capable of removing at least 90% of the antibodies present in 27 ml of a sera from a ragweed-sensitive individual, as determined by the P-K passive transfer test, with reference to the reactions obtained with serially diluted unadsorbed serum.

* 1 protein nitrogen unit (PNU) = 10^-5 mg of phosphotungstic acid precipitable nitrogen.
Attempts to elute allergic antibodies from the cellulose-allergen-antibody conjugate with glycine-hydrochloric acid buffer at pH 3 did not lead to their recovery in measurable amounts. On a few occasions, it was found that the eluates gave threshold P-K reactions in very low dilutions. However, these results could not be reproduced. Similarly, the hemagglutination titers of the eluates were found to be negligible, within the accuracy of the method.

In order to investigate the reasons for the failure to recover skin-sensitizing and hemagglutinating antibodies by dissociation at pH 3 the following control experiments were performed. (i) Four sera of allergic individuals were treated with hydrochloric acid at pH 3 for 30 minutes. Following this treatment it was found that skin-sensitizing activities of two sera were unchanged. However, acid treatment caused considerable reduction of the P-K titers of the other two sera. There was no obvious difference between these sera since both groups of sera included one treated and one non-treated serum. Only those two sera which retained their skin-sensitizing activities on exposure to acidic medium were used in all subsequent experiments. (ii) Purified rabbit anti-ragweed antibodies were coupled to aminocellulose and the cellulose-antibody conjugate was used to pick up ragweed antigens from a solution of DWSR. The resulting cellulose-antibody-antigen complex was then incubated with a reaginic serum. Skin-sensitizing antibodies were removed by the conjugate as was shown by both the P-K and the hemagglutination tests performed on the supernatants. (The
decrease of P-K and hemagglutination titers was not due to the inhibition of the reactions by free antigen in the conjugate, as demonstrated by the inability of the conjugate to inhibit the hemagglutination reaction between a rabbit anti-WSR serum and WSR). Since pH 3 was shown to be effective in breaking the bond between rabbit antibody and the antigen, it was expected that acid treatment of the complex would result in the release into solution of antigen-reagin units. However, direct skin tests failed to show the presence of the antigen-reagin complexes.

From the results of these experiments it may be concluded that the failure to recover skin-sensitizing and hemagglutinating antibodies from the immunosorbent may be attributed to the inadequacy of the elution method used or to possible denaturation of these moieties on the surface of the immunosorbent.

Since specific antibody-antigen precipitates are dissociated partially by concentrated urea solutions (67), an attempt was made to use 6M aqueous urea to elute reagins from the immunosorbent. Because the effect of urea on skin-sensitizing antibodies was not known, the following control experiment was done first. Sera of allergic individuals were diluted ten-fold with 6M urea and incubated at room temperature for 4 hours. Aliquots of the same sera were "treated" with a saline solution in the same way to provide a blank experiment. All samples were dialyzed against many changes of saline overnight and diluted the next day for a P-K test to determine skin-
sensitizing activity. The results of this test showed that exposure to 6M urea for 4 hours partially destroyed skin-sensitizing activities of all sera*, although dilution and dialysis in absence of urea did not impair the activities. Nevertheless, since in some cases about 50% of skin-sensitizing activity was retained after urea treatment, elution by means of 6M urea was attempted. For this purpose 20 mg immunosorbent containing reagins from 3 ml serum were stirred with 2 ml 6M urea for 1 hour, the supernatant was centrifuged off, dialyzed against saline and skin-tested. Results were uniformly negative, no skin-sensitizing activity could be found in the eluates, demonstrating thus that this means of elution is also inadequate.

The failure of pH 3 and 6M urea to elute reagins could be attributed to the denaturation of the antibody factors by the eluting agents or to the inability of these agents to dissociate the allergen-reagin complexes. Since it is known that reagins are very labile when exposed to conditions different from those in whole serum, the following experiment was performed to examine the above possibilities. Two eluting agents were prepared. Normal human serum (NHS)** was diluted ten-fold and (i) its pH was lowered to 3 by the addition of 0.1 hydrochloric acid (ii) another aliquot was dissolved in 6M aqueous

* It is worthwhile to mention that resistance of skin-sensitizing antibodies to urea in the various sera paralleled closely their ability to withstand exposure to pH 3.

** "Normal" human serum designates serum obtained from an individual who is free of all common allergies.
urea. Both were stirred with two 20 mg portions of the immuno-
sorbent containing antibodies from 4 ml of the serum of a
treated and of a non-treated ragweed sensitive individual.
Elution was continued for 30 minutes and the supernatants
separated by centrifugation. The eluates were then neutralized
with a dilute sodium bicarbonate solution to pH 7.3, Seitz
filtered and diluted for skin tests. A control experiment was
done using NHS diluted ten-fold with saline for the elution of
antibodies from 20 mg immunosorbent in the same manner. All
eluates had a volume of 2 ml, which represented a two-fold
increase in concentration compared to the original serum used
to saturate the cellulose. Results are shown in Table 2.
As can be seen, both NHS at pH 3 and NHS in 6M urea led to the
elution of significant amounts of skin-sensitizing antibodies.
The highest skin-sensitizing titer was obtained when NHS at
pH 3 was used to elute antibodies from an immunosorbent, which
had been exposed to a "treated" serum*. On the other hand,
NHS + 6M urea proved less efficient, although the skin-
sensitizing activities of these eluates were still considerable.
NHS treatment of the immunosorbent resulted in eluates in which
no skin-sensitizing activity could be detected. All eluates
having skin-sensitizing activity also showed ability to agglu-
tinate red blood cells sensitized with ragweed, although the
titers obtained were generally low. The control experiments
in which NHS at pH 7 or saline at pH 3 were used as eluting
agents yielded eluates of no demonstrable hemagglutinating
activity.

* The sera from the treated and non-treated ragweed-allergic
individuals had identical P-K titers.
Table 2
Skin-sensitizing antibody (SSA) and hemagglutinating antibody (HA) titers of eluates recovered from immunosorbents

<table>
<thead>
<tr>
<th>Serum used for saturation of immunosorbent</th>
<th>Eluting agent</th>
<th>SSA (P-K)* titers of eluates</th>
<th>HA* titers of eluates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treated**</td>
<td>Saline at pH 3</td>
<td>(&lt;12.5)</td>
<td>0</td>
</tr>
<tr>
<td>&quot;</td>
<td>NHS at pH 7</td>
<td>(&lt;12.5)</td>
<td>0</td>
</tr>
<tr>
<td>&quot;</td>
<td>NHS at pH 3</td>
<td>400</td>
<td>32</td>
</tr>
<tr>
<td>&quot;</td>
<td>NHS + 6M urea</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>Non-treated**</td>
<td>Saline at pH 3</td>
<td>(&lt;12.5)</td>
<td>0</td>
</tr>
<tr>
<td>&quot;</td>
<td>NHS at pH 7</td>
<td>(&lt;12.5)</td>
<td>0</td>
</tr>
<tr>
<td>&quot;</td>
<td>NHS at pH 3</td>
<td>50</td>
<td>16</td>
</tr>
<tr>
<td>&quot;</td>
<td>NHS + 6M urea</td>
<td>50</td>
<td>8</td>
</tr>
</tbody>
</table>

* P-K and HA titers are expressed in terms of dilutions of the original serum.

** Before adsorption the P-K titers of both treated and non-treated sera were 1600; hemagglutination titers were 256 and 128, respectively.
DISCUSSION

Although cellulose-allergen conjugates were capable of removing specifically skin-sensitizing and hemagglutinating antibodies from allergic sera, these antibodies could not be isolated in a pure form. Nevertheless, on the basis of the results obtained some conclusions can be drawn concerning the nature of skin-sensitizing antibodies present in allergic sera.

The results of hemagglutination and P-K tests of the allergic sera before and after adsorption with the immunosorbent indicated that both of these activities were removed by the immunosorbent. The specific removal of hemagglutinating and skin-sensitizing antibodies by this immunosorbent may be considered as further evidence for the in vitro combination of all the antibodies in allergic sera with the allergen. The capacity of the immunosorbent for skin-sensitizing antibodies could not be determined absolutely. However, small amounts (25 mg) of the cellulose-allergen conjugate were shown to be able to adsorb all antibodies from large volumes (20-30 ml) of allergic sera. By comparison, the same quantity of immunosorbent bound about 8 mg of antibodies from rabbit sera. The removal of the antibodies was shown to be specific and was not merely caused by inactivation of these antibodies on the surface of the immunosorbent.

The method of elution with glycine-HCl buffer which had been successfully used in the isolation of rabbit precipitating antibodies (Chapter III) was not found satisfactory for the elution of active skin-sensitizing antibodies.
by analogy it was inferred that elution at pH 3 destroyed skin-sensitizing activity of the antibodies. Attempts to demonstrate the presence of $\gamma_1A$- or $\gamma_{1M}$-globulins in the eluates by precipitation in agar on micro Ouchterlony plates by means of antisera specific for these proteins were unsuccessful, probably because of the very low concentration of the denatured antibody factors. On the other hand, the possibility existed that the bonds between allergen and skin-sensitizing antibodies had actually not been dissociated, and that the skin-sensitizing antibodies had remained attached to the immunosorbent.

This consideration was discounted by experiments in which rabbit antibodies were coupled to diazotized cellulose, the conjugate exposed in turn to ragweed antigens and to allergic sera. As was shown in this study, the bond between rabbit antibodies and ragweed was susceptible to dissociation under acidic conditions. Yet, when the cellulose-antibody-WSR-reagin conjugate was treated with acid, no skin-activity could be demonstrated in the eluates. Nevertheless, the lack of any skin reactions with these eluates cannot be considered to be adequate proof for the absence of skin-sensitizing activity, since the corresponding control experiment, namely dilution of the original allergic sera and addition of the neutralizing amount of allergen (WSR) before injection, had not been performed.

Since the action of acidic medium was deemed too drastic, milder methods of elution were investigated. As shown in a concurrent study in this laboratory (67), concentrated urea solutions were capable of dissociating specific antibody-antigen
precipitates to a considerable extent. An attempt was made therefore to elute skin-sensitizing antibodies from the immuno-
sorbent with 6M urea. However, these experiments also proved
fruitless; no activity could be found in the eluates.

Both unsuccessful attempts, aimed at the elution of
reagins, were performed using media free of proteins, namely
pH 3 saline and aqueous 6M urea. Since the effect of these
agents on the whole allergic sera was much less pronounced, it
seemed possible that both the acidic medium and 6M urea would
prove to be harmless to skin-sensitizing antibodies in the
presence of serum proteins, these proteins acting as stabilizing
agents. This hypothesis was indeed shown to be at least in
part valid. After the isolation of all antibodies from allergic
sera with the immunosorbent, the resulting conjugate was treated
with the pH 3 and 6M urea eluting agents to which normal human
serum had been added. In both cases active skin-sensitizing
antibodies were shown to be present in the eluates. Recovery
of these antibodies was not nearly as high as in the case of
precipitating antibodies, but significant amounts (25%) were
nevertheless recovered. The elution of active skin-sensitizing
antibodies in this fashion conclusively showed that the process
of adsorption did not have an adverse effect on reaginic acti-
vity. Consequently, one may infer that the loss of activity
caused by acidic saline and 6M urea was probably due to con-
formational changes of the skin-sensitizing antibody molecule
brought about by these agents in the absence of stabilizing
serum proteins. The changes suffered by the skin-sensitizing
antibody molecule in the absence of proteins may affect both
the portion of the molecule responsible for its fixation to human skin and the antibody combining site, since both skin-sensitizing and hemagglutinating activities were lost during elution.

At the present time it is not clear which fraction of the serum proteins is responsible for their protective action. It would seem possible that higher recoveries of skin-sensitizing antibodies could be obtained by incorporating into the eluting agents \( \gamma \)-globulins, \( \gamma_1 \) -globulins or \( \alpha_2 \)-macroglobulins i.e. those serum proteins which have been implicated as possible carriers of reaginic activity or which may form a part of the complex reagin molecule. The possibility exists that a well chosen single protein could be used to replace whole serum in the elution of antibodies. If the electrophoretic mobility of this protein was sufficiently different from the eluted antibodies either by its own nature or by the introduction of charged groups, then electrophoresis of the mixture would be expected to yield "pure" skin-sensitizing antibodies.
GENERAL DISCUSSION

The purpose of the present investigation was the evaluation of an aminocellulose derivative (162) as the insoluble matrix for the preparation of immunosorbents and the use of such specific immunosorbents for the isolation of skin-sensitizing antibodies from sera of ragweed sensitive individuals. It was anticipated that these factors, once isolated, could be characterized with respect to their physicochemical and immunochemical properties. Previous attempts to isolate skin-sensitizing antibodies in pure form were unsuccessful (175)*, primarily because of the extraordinary lability of these factors when separated from other serum proteins.

Cellulose was selected as the backbone for the preparation of the immunosorbent because of its hydrophilic nature (49, 174) and because of the reported efficiency of this particular amino derivative of cellulose for the purification of precipitating rabbit antibodies (162). The preparation of the N-(m-nitrobenzyloxymethyl) pyridinium chloride and its coupling to cellulose were described in the first part of this thesis. The amino-cellulose thus prepared was used in a preliminary series of experiments for the purification of rabbit antibodies directed against BSA and ragweed antigens with a view to evaluating its performance. It was found that the immunosorbents prepared with these antigens had indeed the capacity to remove from homologous antisera about 300 mg of the corresponding antibodies per gram of sorbent.

The extent of the recovery of the antibodies and the

* As mentioned before, Augustin, R. has claimed to have isolated reagins (172).
purity of the eluates, however, fell somewhat short of expectations. The total protein recovered in the eluates was about 80-90% of the antibody protein picked up by the immunosorbent and about 70% of the recovered protein was precipitable specifically with the antigen. When the ragweed-anti-ragweed system was used in the experiments, the amount of precipitable antibody was even less, of the order of 40%. The results of electrophoretic and ultracentrifugal analyses performed on the purified preparations showed that these antibodies were globulins and were "homogeneous" with respect to their electrophoretic and ultracentrifugal properties. This finding may suggest that, although not all of the proteins present in the eluates are precipitable with the antigen, the non-precipitable portion consists likely of denatured antibody-protein and not of heterologous serum proteins, since it would seem very improbable, that only the $\gamma$-globulin fraction of serum proteins would be adsorbed non-specifically by the immunosorbent. Additional argument supporting this interpretation is the fact that very little, if any, non-specific "uptake" was observed with heterologous adsorbents. Thus, one would conclude that elution at pH 3 may have had a detrimental effect on some of the antibody molecules resulting in loss of antibody activity. An alternate possibility is that during elution of antibodies small amounts of antigen were washed off the immunosorbent, and this resulted in partial inhibition of the precipitin reaction. Since for the batchwise elution of antibodies the total time of exposure of the cellulose-antigen-antibody con-
jugate to low pH may be as long as 6-8 hours, it is also possible that some antigenic determinants may have been hydrolyzed off during this length of time. The lower purity of anti-ragweed antibodies prepared by this method would support this criticism, since ragweed antigens are not stable. Therefore, in future work this aspect of the problem needs further scrutiny; any antigen present in the eluates could be detected after concentration by pervaporation-dialysis of the eluates or by labelling the antigen with radio-isotopes.

The immunosorbent prepared with ragweed antigens was shown to remove specifically antibodies from sera of ragweed sensitive individuals. Adsorption with the immunosorbent resulted in a concomitant disappearance of both hemagglutinating and skin-sensitizing factors from these sera. From the results of experiments using acidic medium and 6M urea for the elution of skin-sensitizing antibodies from the immunosorbents it was concluded that these agents have a damaging effect on the skin-sensitizing activity of allergic antibodies. However, it was noted that when whole allergic sera were treated with these agents only partial inactivation took place and skin-sensitizing and hemagglutinating activities of some sera were totally unaffected. It seemed, therefore, that the serum proteins exerted a beneficial protective effect on the antibody constituents. This hypothesis is actually supported by the results of the experiments in which normal human serum along with pH 3 and 6M urea was used for the elution of antibodies. These experiments also clarified the problem, whether the skin-
sensitizing antibodies lost their activity when first isolated from their normal environment by adsorption onto the immuno­
sorbent or inactivation was caused by the eluting media in the absence of stabilizing serum proteins. The results show that the latter happened, since in the presence of serum proteins active skin-sensitizing antibodies were washed off the immuno­
sorbent. Elution of skin-sensitizing antibodies with normal serum and a dissociating agent such as pH 3 or urea has only theoretical significance, since by this means one merely transfers the antibody factors from an allergic serum into a normal one. However, the fact that this can be done, points the way toward developing methods for the isolation of pure reagins by incorporating various single proteins into the eluting media. At the present time, however, there is no evidence for the existence of pure reagin as such, since it seems likely that to remain active, reagin molecules must remain attached to some serum protein, a possible candidate being the $\alpha_2$-macroglobulin (176). Hence, by incorporating $\alpha_2$-macroglobulins into the eluting agents one would expect to recover most of the skin-sensitizing antibodies from the immunosorbent.

In spite of the unavailability of pure skin-sensitizing antibodies, a number of recent studies have sug­
gested that these antibodies might be $\gamma_{1A}$-globulins (112, 114). However, most of the evidence to date is only circumstantial and convincing as it may be, direct experimental confirmation
must wait until preparations of pure reagins can be obtained. Thus, the problem of isolating these factors from allergic sera in a pure form remains as challenging as ever.
BIBLIOGRAPHY


