STUDIES ON HUMAN REAGIN F.T.S. KISIL 1970

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ABSTRACT

Evidence to suggest that reagins were not associated with IgG, IgA or IgM was obtained from observations of the different distributions of the highest concentration of these immunoglobulins and reaginic activity following fractionation of allergic sera. For their isolation and characterization, reagins were adsorbed specifically onto specially synthesized ragweed immunosorbents and eluted with glycine-HCl at pH 2.5, or 2M NaI at pH 9.1, or 6M urea at pH 7.4. The presence of human or rabbit serum protein was required for maximal recovery of reaginic activity. The specific elution of a portion of reagins was accomplished with hapten-like constituents of ragweed pollen. An identification of the antigenic determinants characteristic of reagins as those of the IgE class of immunoglobulins was made from antisera produced by the immunization of animals with the reaginic eluates.

The heterogeneity and diversity of the allergen-reagin system was demonstrated with respect to (i) the antigenic and allergenic composition of ragweed pollen extracts, (ii) the specificities of reagins, and (iii) the skin-fixation property of reagins. A relationship of the reactivity of allergens with reagins was established on the basis of their electrical charges.

Attempts to develop a method for the treatment of allergic individuals by inactivation of reagins *in vivo* with mercaptan were shown to be partially successful in a model system using monkeys sensitized intradermally with human reaginic serum.

STUDIES ON HUMAN REAGIN

by

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TO

MY WIFE, MOTHER AND LATE FATHER

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LIST OF ABBREVIATIONS

A-R	allergen-reagin
AHS	allergic human serum
BDB	bis-diazotized benzidine
BSA	bovine serum albumin
EMA	ethylene-maleic anhydride
D.C.	direct current
DWSG	dialysed water soluble extract of Timothy grass pollen
DWSR	dialysed water soluble extract of ragweed pollen
g	gram
G	gravitational unit
$\Gamma/2$	ionic strength
HSA	human serum albumin
Kg	kilogram
M	molar (solution)
mc	millicurie
2-ME	2-mercaptoethanol
mg	milligram
N	normal (solution)
NCS	normal cord serum
NHS	normal human serum
O.D.	optical density
%	percent
PBS	phosphate buffered saline, pH 7.4
PNU	protein nitrogen unit
RBC	red blood cell
RSA	rabbit serum albumin
r.p.m.	revolutions per minute
S	sedimentation coefficient
SAS	saturated ammonium sulfate (solution)
U-F	ultra-filtrate
U.V.	ultraviolet
V	volt
v	volume
w	weight
ω	angular velocity (radians)
WSR	water soluble extract of ragweed pollen

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CHAPTER I

SOME CONCEPTS OF IMMUNOLOGY AND IMMUNOCHEMISTRY

Introduction

Since ancient times it has been known that animals and humans after recovery from a contagious disease acquired immunity to the disease, since on subsequent contact with the same pathogenic organism the infection did not reappear or was of a less severe nature. It is known today that the serum obtained from such individuals contains a factor which is capable of neutralizing the disease producing organism. This factor is referred to as antibody, and the material which induced its formation is referred to as antigen. The formation of antibodies can also be induced by naturally occurring biological macromolecules in addition to bacterial or viral antigens.

In some cases, however, immunity may not be of a protective type and may induce an untoward or even fatal reaction following the re-exposure of the animal to certain antigens. This state of altered reactivity as opposed to immunity, is referred to as hypersensitivity and may be attributed to an aberration of the immunological mechanism.

The earliest documented observations of hypersensitivity reactions in man were recorded by Hippocrates (1) and Lucretius (2) who noted that 'What food is to one may be fierce poison to others'. In 1565, Botallus observed that certain individuals began to sneeze when exposed to certain flowers (3). The symptoms of this condition, referred to today as hayfever, were described by Bostock in 1819 (4). Evidence that the condition was due to pollen was presented by Elliotson who observed that the period of worst

symptoms coincided with the pollination of grasses in the haying season (5). The works of Gordon (6), Phobus (7), and Wyman (8) all implicated pollen as the causative agent. Because of the apparent relationship of this condition with the season of the year, the disease became known as hayfever.

The first diagnostic skin test for hayfever was performed in 1865 by Blackley who also showed that the pollen of grasses was capable of inducing hayfever symptoms in allergic persons (9, 10); Dunbar clearly established that pollens were the causative agents (11).

A similarity between hayfever and other forms of hypersensitivity reactions such as serum sickness, urticaria and anaphylaxis, was observed by Weichardt (12), Wolf-Eisner (13) and Meltzer (14). Anaphylaxis, which is an acute form of immediate hypersensitivity, was first observed by Magendie in 1839 (15). Flexner, in 1894, found that rabbits tolerated the first injection of dog serum, but often died of shock on reinjection (16). This phenomenon was further studied by Richet (17) and Portier (18) and to contrast it with active immunity or prophylaxis they designated this condition anaphylaxis. Friedberger postulated in his humoral theory that, following the reaction of circulating antibody with injected antigen, a substance called anaphylatoxin was released into the serum and caused a shock-like state (19). The severity of the anaphylactic response could be diminished in sensitized animals by multiple injections of low doses of antigen given at closely spaced intervals. A similar procedure was employed by Noon for the desensitization treatment of hayfever in man (20).

In 1906, Von Pirquet coined the terms "allergy" for hypersensitivity and "allergen" for the antigen causing the hypersensitivity (21). The first experimental studies in allergy were performed by Koch in 1890 (22).

He reported that guinea pigs did not show any response when first injected with tubercle bacilli; however, subsequent injections of the same antigen into the sensitized animals caused inflammatory responses. Similarly, Behring observed that guinea pigs could be sensitized with diphtheria toxin (23).

By 1910, Dale (24) and Abel and Kubota (25) had recognized that histamine was one of the pharmacological mediators implicated in the in vivo antigen-antibody reactions leading to anaphylaxis. Dale had also proposed that the reaction of tissue-bound antibodies with the antigen initiated the anaphylactic response (26). Previously, Otto (27) and Dale (26) had demonstrated that hypersensitivity could be transferred to a normal animal with the serum of a sensitive animal. These experiments provided conclusive evidence that (i) humoral antibodies could fix to or sensitize normal tissue, and (ii) pharmacological mediators, such as histamine were responsible for the anaphylactic response. The Schultz-Dale test, using smooth muscle tissue sensitized actively or passively with antibodies is still employed as a sensitive in vitro test for the detection of antigen-antibody interactions (26, 28).

Antigens

Substances such as proteins, polysaccharides, polypeptides, or polynucleotides which possess their own individual characteristic structure(s) and steric configuration(s), may induce antibody formation when administered to animals. This property of provoking the synthesis of antibodies is called immunogenicity (29). In contrast, the term antigenicity refers to the capacity of substances to react with homologous antibodies. Although immunogenicity is normally associated with macromolecules foreign to the host, under special circumstances antibody production may be elicited by

the host's own proteins; such as those which (i) have undergone slight physical and/or chemical modification so that they were regarded as being foreign, or (ii) proteins such as thyroglobulin, lens protein or spermatozoa, which are normally stored and secreted away from immunologically competent cells but which may reach these cells following trauma or disease of the corresponding organs (thyroid, ocular lens, testis). Similarly, substances which are not normally immunogenic can be rendered so through chemical modification of their structure. Thus, gelatin can be made immunogenic by the introduction of tyrosine residues onto the molecule (30).

The reactive portions of the antigen molecule inducing antibody formation are referred to as antigenic determinants or antigenic sites. Complex immunogenic substances, such as proteins, may possess several antigenic determinants. In studies with enzymatically degraded albumin it was revealed that at least three antigenically distinct fragments reacted with antibodies produced against the native molecule and that each fragment reacted only with its specific antibody (31).

Simple substances of low molecular weight, cannot by themselves induce antibody formation, but may become immunogenic when attached to a suitable carrier molecule. To distinguish these compounds from true immunogens, Landsteiner coined the term hapten for these substances (32). Haptens have proven to be an invaluable tool in the study of immunogenicity and in establishing the quantitative and molecular aspects of antigen-antibody interactions.

Antibodies

Following stimulation with an immunogen, animals will synthesize globular serum proteins, called antibodies, which possess the unique property of combining specifically with homologous antigens or related materials

with similar stereochemical properties. This specificity is a reflection of steric complementarity between the antigenic determinant groups and the antibody combining sites.

The introduction of electrophoretic techniques (33) led to a partial resolution of the complex spectrum of serum proteins, and to the demonstration that antibody activity was associated with a wide spectrum of globulins (34). By immunoelectrophoresis (35) the globulins have been further resolved into antigenically distinct classes. These antigenic differences of immunoglobulins are also referred to as isotypic specificities i.e., they are common to all individuals of the same species and differentiate classes and types of immunoglobulins (36, 37). The immunoglobulins have been designated as IgG, IgA, IgM, IgD and IgE (38, 39, 40, 41, 42, 43, 44). All of these immunoglobulins coexist in human sera but occur in different concentrations, differ in antibody content, rates of synthesis and catabolism, placental transfer and in their physicochemical and immunochemical properties (Table I).

(i) <u>IgG or γG-globulins</u> (γ_2; γ_{ss}; 6.6Sγ; 7Sγ)

The majority (80%) of the immunoglobulins belong to this group. IgG is characterized by a molecular weight of about 150,000 and a sedimentation coefficient of approximately 7S (45). IgG has a relatively low carbohydrate content of 2% (46). At pH 8.6 it possesses the lowest anodic mobility. This property of low net negative charge is exploited for its isolation and purification employing ion-exchange chromatography on DEAE-cellulose (47, 48, 49). Antibodies against many gram positive bacteria, viruses, and toxins are associated with IgG.

The abundance of IgG in serum has greatly facilitated structural studies of the immunoglobulin molecule. First evidence that the IgG

TABLE I

Summary of Some Properties of Human Immunoglobins

	IgG	IgA	IgM	IgD	IgE
Synonyms	γG, 7Sγ, γ_2, γ_{ss}	γA, β_2A, γ_1A	γM, 19Sγ, β_2M, γ_1M	γD	γE, IgND
Physicochemical					
Sedimentation constant	6.5-7.0	7, 10, 13, 15, 17	18-20, 30	6.2-6.8	8
Molecular weight	150,000	180,000; 385,000*	900,000	183,000	196,000
Total carbohydrate (%)	2.9	7.5	11		10
Immunochemical					
Heavy-chain classes	γ	α	μ	δ	
Light-chain types	κ , λ	κ , λ	κ , λ	κ , λ	κ , λ
Molecular formula	$\gamma_2\kappa_2$, $\gamma_2\lambda_2$	$\alpha_2\kappa_2$, $\alpha_2\lambda_2$ ($_2$ $_2$)T*, ($_2\lambda_2$)T*	$(\mu_2\kappa_2)_5$; $(\mu_2\lambda_2)_5$	$\delta_2\kappa_2$, $\delta_2\lambda_2$	$\varepsilon_2\kappa_2$, $\varepsilon_2\lambda_2$
Allotypes					
Gm specificity (γ chain)	yes	no	no	no	
Inv specificity (κ chain)	yes	yes	yes	?	
Number subclasses known	4	2	2		
Biological					
Serum Con (mg%)	800-1680	140-420	50-190	0.3-10	0.0017-0.006
Synthesis rate (mg/kg/d)	20-40	2.7-5.5	3.2-16.9	0.03-14.9	
Catabolic rate (% I.V. pool/d)	4-7	14-34	14-25	18-60	
Distribution (% in I.V. pool)	48-62	40	65-100	63-86	
Antibody Acitivty	yes	yes	yes	yes	yes
Placental passage	yes	no	no	no	no
Presence in cerebrospinal fluid	yes	yes	no		
Skin sensitization					
heterologous species	yes	no	no		yes
homologous species	no	?	no	no	yes
Complement fixation	yes	no	yes		no?

* In exocrine secretions, IgA has a dimeric structure and an attached T chain

molecule was constructed of several polypeptide chains was provided by starch gel electrophoresis in an acid-urea medium of the reaction products obtained by reduction and alkylation of IgG (50). As a result of these experiments, two types of polypeptide chains, termed heavy (H) and light (L) chains were eventually identified (51). Each of the two H chains had a molecular weight of the order of 55,000 and possessed 95% of the total carbohydrate content of intact IgG, and each of the two L chains had a molecular weight of about 20,000 (50). The heavy and light chains are separable from each other by gel filtration on the basis of their size (52).

On the basis of results obtained from enzymatic, chemical and immunochemical studies the structural relationships illustrated in Figure 1 have been established (53). Thus, characteristic antigenic determinants of IgG are located in the Fc portion of the H chains, while the remaining portion of the H chain, known as Fd piece (54, 55), is located in the Fab fragments contiguous with the L chain. By reduction of Fab, Fd is dissociated from the L chain.

Enzymatic digestion with papain cleaves the 7S IgG molecule into three 3.5S fragments which can be separated by ion-exchange chromatography on CM-cellulose (56). By this method, two Fab fragments and one Fc fragment were obtained from each IgG molecule. Each of the Fab fragments has a molecular weight of 52,000 and is univalent since it contains only one antibody combining site. On the other hand, the Fc fragment is devoid of antibody activity, but carries most of the antigenic determinants unique to IgG and can be crystallized under appropriate conditions (57). Furthermore, its unique structural features play a dominant role in (i) the binding to microsomes during IgG synthesis (58), (ii) the placental transfer of IgG (59), (iii) its fixation to guinea pig skin in the passive cutaneous anaphylaxis (PCA)

reaction (60), (iv) the interaction of IgG with rheumatoid factor (61) and (v) complement fixation (62).

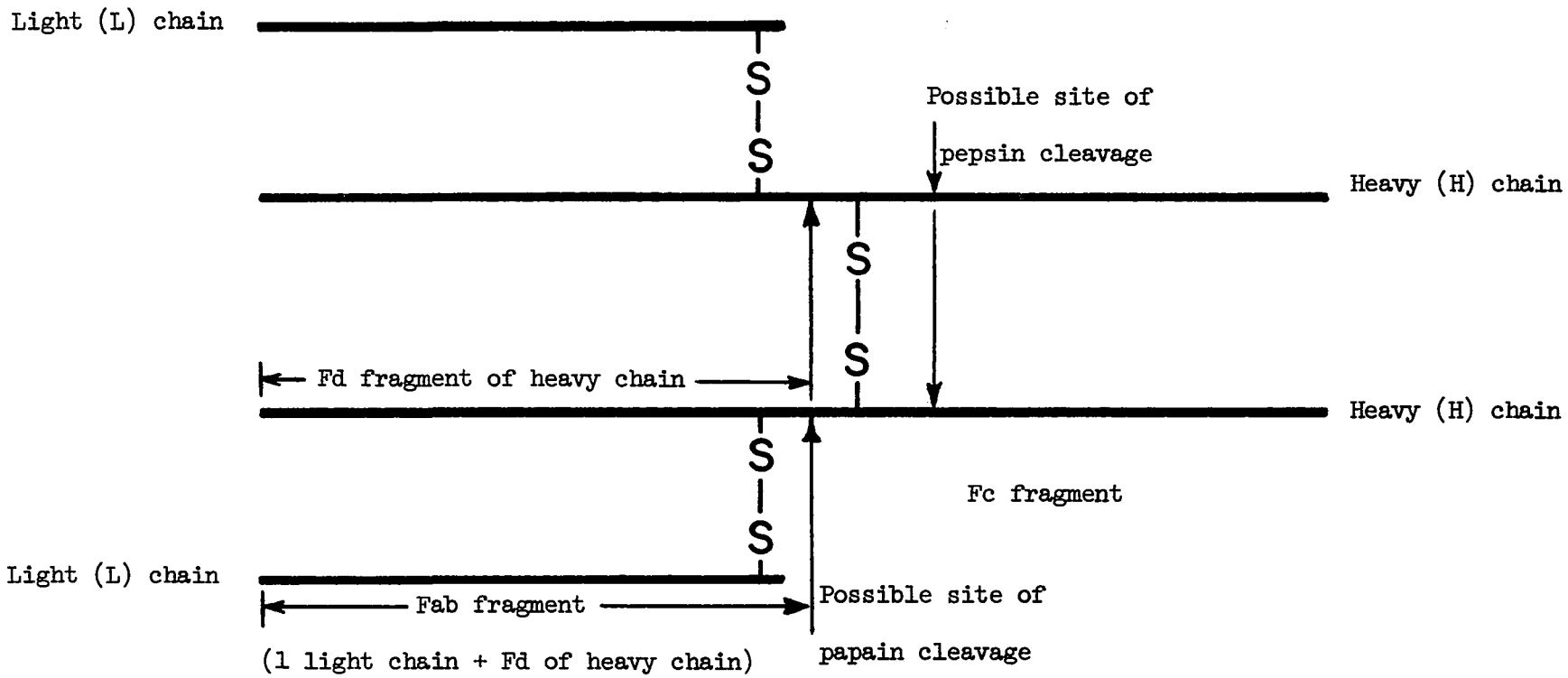
All immunoglobulins within a species share common antigenic determinants which are located on the L chains. Two antigenically distinguishable types of light chains, κ and λ have been recognized, each being found only on separate immunoglobulin molecules (63, 64, 65). About 70% of the light chains of immunoglobulins of normal sera are of type κ and 30% of type λ. Antibody activity has been demonstrated in immunoglobulins possessing either the κ or λ L chains (66, 67). A small amount of light chains possess neither antigenic determinant.

Four subgroups of IgG have been so far detected by means of rabbit antisera prepared against individual myeloma IgG proteins. These antisera were rendered specific to the antigenic determinants of the individual myeloma IgG (subgroup determinants) by absorption with both types of light chains (or Bence-Jones proteins), and by absorption with the γ-chain (heavy chain of IgG) from another myeloma IgG in order to remove antibodies against common antigenic determinants found on all IgG immunoglobulins (68). Similarly, specific antisera against the subgroups have been prepared in monkeys immunized with normal IgG proteins isolated from a pool of normal human sera (NHS) (69). These antisera on immunoelectrophoretic analysis of human sera revealed four subclasses of IgG which have been designated as γG_1, γG_2, γG_3, and γG_4 (70).

Antigenic specificities which distinguish polymorphic forms of immunoglobulins not present in all individuals of the same species are known as allotypes. IgG of man possesses two groups of allotypic specificities, represented by the Gm groups associated with the Fc region of γ-chains (71) and the Inv groups which are located on the κ type light chains (72,73).

FIGURE 1

Schematic structure of IgG


Light (L) chain
S
S
Possible site of
pepsin cleavage
Heavy (H) chain
S
S
Fd fragment of heavy chain
Heavy (H) chain
S
S
Fc fragment
Light (L) chain
Fab fragment
(1 light chain + Fd of heavy chain)
Possible site of
papain cleavage

This specificity is designated as Inv [1, 2] or Inv [3] when leucine or valine respectively occupy amino acid position 191 in the light polypeptide chain. For λ type light chains the allotypic differences are designated as Oz+ or Oz- when lysine or arginine is respectively present at position 190 (74).

(ii) IgA or γA-globulins (β_2A, γ_1A)

This class represents about 10% of the immunoglobulins and has the electrophoretic mobility of fast γ-globulins (75). The IgA molecule is composed of two H chains, known as α-chains, and either two κ or two λ chains, has a relatively high carbohydrate content of about 10% and a molecular weight of approximately 170,000. The IgA globulins tend to aggregate into complexes of higher molecular weight: the main component (monomer) has a sedimentation coefficient of approximately 7S and the minor components (about 15% of IgA globulins) have sedimentation coefficients ranging from 9S to 15S (76).

Studies to determine antibody activities of IgA have proven to be difficult due to their similarities with IgG in their sedimentation and electrophoretic properties. Nevertheless, highly enriched IgA preparations have been obtained by a combination of salt precipitation, preparative electrophoresis, and ion-exchange chromatography (75, 77, 78, 79). (Studies to develop procedures for the isolation of IgA are described in Chapter III). Certain rheumatoid factors, isoagglutinins and antibodies to Brucella and Diphtheria have been demonstrated to be associated with IgA globulins (80, 81, 82).

In addition to their presence in serum, γA-globulins are detected in colostrum, nasal and bronchial secretions, saliva, tears and in urine (83, 84, 85). The concentration of IgA in these secretions is higher than that

of the other immunoglobulins. The secretory γA-globulins, however, differ antigenically from the serum γA-globulins. This difference is due to the presence of a glycoprotein termed secretory or transport piece (T piece) (86). Secretory 11S γA-globulins with a molecular weight of 385,000 are composed of a dimer of 7S IgA linked by disulfide and non-covalent bonds to one 4.2S T piece with a molecular weight of 58,000 (87). The site of attachment of the T piece is uncertain. T piece by itself can be demonstrated in the saliva of agammaglobulinemic individuals lacking detectable IgA as well as in the saliva of neonates whose sera are also deficient in IgA (88). In these cases, the T piece has a more variable electrophoretic mobility in comparison with T piece from IgA (89). Although its function is unknown, T piece may facilitate the transport of the locally synthesized IgA molecules into secretions and may also make the secretory γA-globulin more resistant to proteolysis. Secretory γA-globulins appear to be more resistant to reduction since no changes in their ultracentrifugal properties can be detected following treatment with 0.5M mercaptan. In order to dissociate the reduced and alkylated γA-globulin into individual H and L chains, 2M urea must be employed. This indicates that the components of the immunoglobulin molecule which are split by the reduction are held together in a stable configuration by non-covalent forces (86). γA-globulins are not transported across the placenta.

Two antigenically distinguishable types of γA-globulins have been demonstrated with antisera prepared against IgA myeloma proteins (90, 91, 92). The subgroup determinants were found to be located on the α-chains. A minor group (10% of the γA myelomas) was shown to be antigenically deficient when compared to the major group.

(iii) IgM or γM-globulins (19Sγ, β_2M, γ_1M)

These globulins represent 5 to 10% of the total immunoglobulins. In (almost) all species γM antibodies are the first type to be detected following immunization with antigens or with hapten-protein conjugates (93, 94, 95). γM-globulins can be isolated by preparative ultracentrifugation (96) or by a combination of chromatography and gel filtration (55). Its molecular weight is approximately 900,000 and the molecule appears to be composed of five 7S subunits linked by inter-μ-chain disulfide bonds (97).

The 7S subunits obtained from IgM by reduction with mercaptoethanol were found to be univalent and, therefore, did not precipitate on combination with the antigen (98). Although the 7S subunits still contain two heavy and two light chains, their univalency is at variance with the divalency of the 7S γG-globulins (99). More recent studies with rabbit γM-globulins have shown that the whole molecule contains five strong and five weak antibody combining sites (100). Each 7S unit of the IgM globulin molecule consists of two μ-chains and two κ or λlight chains. The γM-globulins contain about 10% carbohydrate. Since the 7S subunits of IgM differ in (i) their electrophoretic mobility, (ii) carbohydrate content, and (iii) antigenic properties with respect to the characteristics of 7S γA- or γG-globulins, the γM-globulins do not represent polymers of either (101). In addition to the 19S components, two minor components with sedimentation coefficients of the order of 29S and 35S also exist (102).

The γM-globulins do not cross through the placenta. Isohemagglutinins, cold agglutinins, rheumatoid factors and antibodies to gram negative bacteria are associated with γM-globulins (103). In neoplastic diseases involving lymphoid cells, abnormally high quantities of γM-globulins

may be found in the serum. This disease is referred to as Waldenström's macroglobulinemia.

(iv) IgD or γD-globulins

This immunoglobulin represents about 1% of the total immunoglobulins and is present in normal adult sera in a concentration of 8 mg%. The IgD molecule is composed of two heavy chains (δ) and two κ or λ light chains. The molecular weight of the γD-globulin is 183,000, its sedimentation coefficient being 6.1S (41, 104, 105). The γD-globulins are labile to extremes in pH or ionic strength forming Fc and Fab like fragments and they lose their antigenicity following incubation at 60°C for 4 hours. IgD has been separated from other immunoglobulins in a myeloma serum by ion-exchange chromatography on DEAE-cellulose and by gel filtration on Sephadex G-200 (41, 104).

Although the function of γD-globulins is at present unknown, the serum levels of IgD were reported to be considerably elevated in patients with cystic fibrosis, visceral larva migrans syndrome or cirrhosis (104). By an indirect immunofluorescent technique, Heiner demonstrated IgD antibodies in the sera of patients with disseminated lupus erythematosus (104).

In addition to their presence in serum, γD-globulins have been demonstrated in other fluids such as colostrum, nasal secretions, adult saliva, gastric juices and also in the urine of patients with IgD myeloma (104, 106).

(v) IgE or γE-globulins (myeloma ND)

γE-globulins were discovered by radio-immunodiffusion techniques in studies to determine the nature of the immunoglobulin(s) associated with skin-sensitizing activity (42, 43, 107). However, the basic physico-chemical properties of this immunoglobulin have been obtained from studies

of a myeloma protein ND (44, 108, 109, 110). The structure of the ND -globulins (assumed to be identical to those of IgE) was found to be similar to that of the other immunoglobulins: i.e. two heavy ε-chains of molecular weight of 75,500 each and two light λ-chains of molecular weight of 22,500 each, are linked to form the immunoglobulin which has a molecular weight of the order of 196,000. The sedimentation coefficient is about 8S. In addition to 8 methionine residues on each ε-chain, 24 cysteine residues have been found on the Fc portion of the ND-globulin molecule (110). The carbohydrate content of about 10% is similar to that of IgA and IgM. The electrophoretic mobility of IgE in agarose at pH 8.6 is that of γ_1-globulins and extends anodally from the point of application (43). IgE and myeloma ND possess identical antigenic properties (111, 112). IgE is capable of fixing to or sensitizing human or monkey skin (113, 114, 115). However, this skin-fixation property as well as its ability to react with homologous antisera, is destroyed by heating at 56°C for four hours (116). A direct correlation between skin-sensitizing antibody activity and IgE was shown. These findings will be elaborated in Chapter II.

A sensitive inhibition technique, the radio-immunosorbent assay (RISA) was used to determine γE-globulin concentrations in serum. In this technique (117) antibodies, prepared against the Fc portion of the myeloma ND, were coupled to dextran. The resulting "reversed immunosorbent" was rendered specific to the Fc portion of myeloma ND (γE-globulins) by saturation with appropriate amounts of normal human serum,* γG-globulins, Bence-Jones proteins, and the Fab portion of the myeloma ND. The principle of the inhibition test is based on the fact that normal γE-globulins from the sample being analysed are bound to the immunosorbent and partially inhibit

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* It must be presumed that the normal sera, in fact, lacked IgE.

the subsequent absorption of radioactively labelled myeloma ND. The degree of inhibition is directly proportional to the amount of IgE in the sample. Therefore, to determine the concentration of γE-globulins, the degree of inhibition of uptake of labelled IgND is compared to standard curves obtained with known amounts of IgND. It was claimed that this technique could detect γE-globulins at concentrations of the order of 0.005 to 0.01 µg/ml.

Using the RISA method it was determined that normal sera contain γE-globulins in concentrations of the order of 330 ng/ml. In individuals with proven cases of allergy the serum levels of IgE are about 3.5 times higher. The concentration in cord sera is of the order of 30 ng/ml. γE-globulins were also demonstrated in the saliva of the myeloma-ND patient and its concentration using the standard single radial immuno-diffusion method was found to be 0.4 mg/ml (44).

Bence-Jones Proteins and γ_T Globulins

Certain patients with multiple myeloma may excrete in their urine Bence-Jones protein, which is characterized by its precipitation on heating to 45-56°C and its redissolution on boiling (118). Bence-Jones proteins may be found in the form of the monomer, with a molecular weight of about 22,000 and a sedimentation coefficient of about 2.5S, and in the form of a dimer with a molecular weight of about 45,000 and a sedimentation coefficient of 3.4S (119). The Bence-Jones proteins represent the light chains of the immunoglobulins, and two antigenically distinguishable types, κ and λ, exist (120, 121). Dimers of the Bence-Jones proteins, formed by

covalent disulfide bonds, are mainly of the λ type, whereas non-covalently linked dimers and free monomers appear to be of the κ type (122).

Low molecular weight immunoglobulins or γ_L-globulins are antigenically related to the light chains and probably represent incomplete light chains. Their presence has been demonstrated in the serum (123) and in the urine (124) of normal individuals. There are two types of serum γ_L-globulins, differing in both electrophoretic mobility (cathodic γ_2 and anodic γ_1) and sedimentation coefficients (2S and 3S respectively) (125). The urinary γ_L-globulins have a molecular weight of about 12,900 and sedimentation coefficient of about 1.7S. Like the Bence-Jones proteins, the γ_L-globulins exhibit unusual thermosolubility properties (126). However, the size and immunochemical properties of the γ_L-globulins indicate that they are not identical to the light chains (127). The significance of the γ_L-globulins is not clear, but it has been suggested that they may be break-down products of serum immunoglobulins (128).

Antibody Combining Site(s)

Structural analysis of the immunoglobulins had revealed that antibody activity was restricted to the Fab portions of the molecule (56). Reduction of the Fab fragments and exposure to 6M guanidine hydrochloride resulted in unfolding of the chains and destruction of their antibody activity. Subsequent reoxidation and removal of the denaturing agent permitted the molecule to restore its initial configuration, along with its binding properties (129). Thus, the specificity of antibodies must be related to the unique chemical and configurational properties located in these regions of the molecule. The regions which can interact with the antigenic determinants or haptens are referred to as the antibody combining sites. Characteristic differences in the amino acid composition of specific

antibodies have been found in the N-terminal portions of both heavy and light chains (130). By means of the technique of affinity labelling it was demonstrated that both H and L chains were involved in the antibody combining site (131). Isolated H chains (132, 133) and L chains (134) have been demonstrated to possess some combining affinity. The antibody combining site is primarily located in the Fd portion of the heavy chain (137), with the homologous L chains serving to enhance binding activity (135, 136).

Most of the knowledge concerning antibody combining sites has been obtained with immunoglobulins from species other than man. However, the formation of interspecies 7S molecular hybrids of chains from diverse mammalian species indicates that close similarities must exist between the immunoglobulins of most, if not all, species (138). Thus, it appears that the basic immunoglobulin structures and antibody combining properties so far observed may also apply to human immunoglobulins.

The Precipitin Reaction

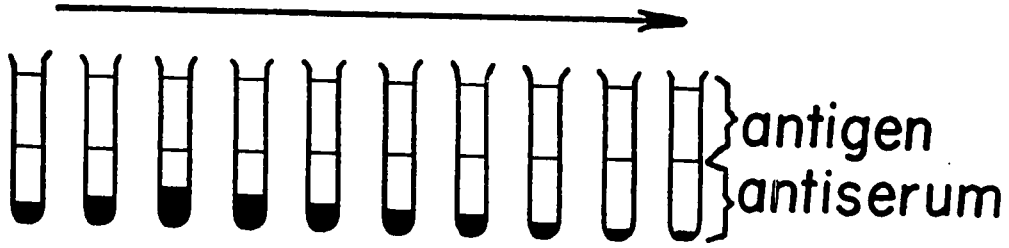
Among the most common *in vitro* reactions of homologous antigen-antibody systems one may cite the readily visible precipitation of antigen-antibody complexes when the two are mixed in appropriate ratios. In general, the amount of precipitate formed on the addition of increasing amounts of antigen to a constant amount of antiserum is represented quantitatively by the precipitin curve, (Figure 2). It is evident from this curve that the amount of precipitate consisting of both antigen and antibody increases at first in the antibody excess zone, reaches a maximum in the equivalence zone where both reactants are precipitated quantitatively, and then decreases in the antigen excess zone where precipitation is quantitatively inhibited. This variation in precipitation was explained by the lattice framework

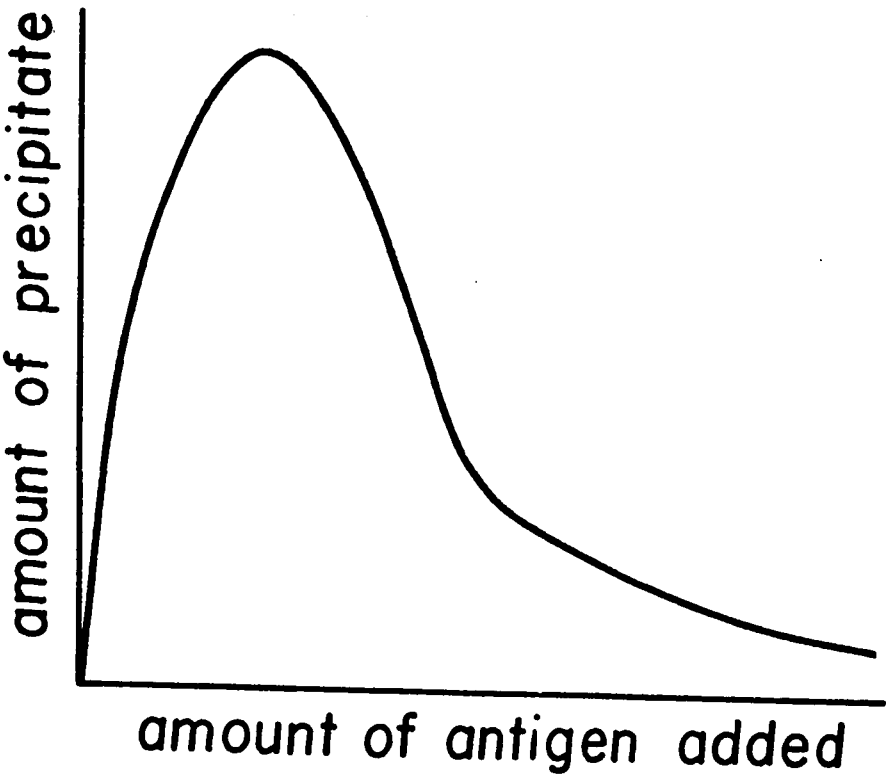
FIGURE 2

Typical precipitin curve

TYPICAL PRECIPITIN CURVE

Increasing amounts of antigen added to constant amount of antiserum





theory proposed by Marrack (139) and Pauling (140). Accordingly, the precipitate formed with low amounts of antigen, i.e. in antibody excess, is composed primarily of antibody, cross-linked by antigen. In the region of maximum precipitation, the antigen-antibody complexes are cross-linked into larger and more compact three-dimensional aggregates, consisting of an alternating and recurring antibody-antigen pattern. With concentration of antigen greater than that required to combine with all antibody sites, the compact regular antigen-antibody framework is loosened, resulting in the formation of smaller aggregates. In extreme antigen excess, each antibody molecule is combined with the number of antigen molecules equivalent to the valency of the former. In this state, since no cross-linking occurs, the complexes remain in solution. Thus, if antibody molecules were univalent, such as the Fab fragments, or if the antigens were similarly univalent (e.g. haptens), polymeric aggregates could not be formed and the corresponding antigen-antibody complexes would be soluble. Consequently, the addition of hapten to a multivalent hapten-protein conjugate and the homologous antibody will result in inhibition of precipitation.

CHAPTER II

HYPERSENSITIVITY AND ANTIBODIES IN ALLERGIC INDIVIDUALS

Introduction

Allergy or hypersensitivity is defined as the altered and increased immunological response of an individual towards an innocuous substance and usually results from prior exposure to the same or to a chemically related substance. Hypersensitivity states are divided into two main classes: (i) of the immediate type, and (ii) of the delayed type. This classification is based in part, on the speed of onset of the reaction in the sensitive individual, and in part, on the underlying mechanism of these reactions. The immediate forms of hypersensitivity are characterized by (i) the rapid onset of the symptoms or reactions leading to pathological changes, usually within a few minutes following exposure to the allergen; (ii) the presence of circulating antibodies in the sensitized individual (thus immediate hypersensitivity can be transferred with serum); and (iii) the reactions, due to the allergen-antibody combination, which are mediated through histamine and other pharmacologically active agents acting on the blood vessels and smooth muscle tissues. Anaphylaxis and atopic sensitivity are two examples of the immediate type hypersensitivity. By contrast, delayed type hypersensitivity is characterized by (i) the late onset of detectable reactions; these may require a few hours or even days after exposure to the allergen for the maximum reaction to occur; (ii) the reaction is due to direct cellular interactions and may be transferred with sensitized cells; (iii) with few exceptions (141) circulating antibodies have not been demonstrated to be involved in these reactions; consequently

the sensitivity is not transferable with serum from the sensitive individual; rather, the transfer can be effected by either white cells or extracts of these cells. Examples of delayed hypersensitivity include tuberculin hypersensitivity, contact allergy, autoimmune allergies, and phenomena related to transplantation immunity.

Immediate type hypersensitivity manifests itself in a variety of local or systemic reactions. These will be briefly described.

(i) Cutaneous Reactions

Wheal and flare skin reactions may be elicited in sensitive individuals by the local intradermal injection of the sensitizing antigen. The reactions are similar to the skin reactions to histamine described by Lewis (142). The maximal reactions appear within a few minutes and do not lead to necrotic lesions. A variation of the cutaneous reaction is the passive cutaneous anaphylaxis reaction or PCA, which was introduced by Ramsdell (143) and thoroughly investigated by Ovary (144). The reaction is employed as a sensitive test for the detection of small quantities of antibodies and is performed as follows: antibody is injected intradermally into a local site of a normal recipient animal, and after a suitable time interval during which antibody fixes to the skin and non-specific irritation due to the injection subsides, antigen and an indicator dye, such as Evans blue, are injected intravenously. The ensuing flare reactions due to capillary dilation are made visible by the extravasation of the dye. Reversed passive cutaneous anaphylaxis can be performed when the antigen is an immunoglobulin capable of fixing to the skin. In the reversed PCA test, antibody and indicator dye are injected intravenously to elicit the reaction with the tissue-bound antigen.

(ii) Anaphylaxis

Anaphylactic shock is one of the most dramatic manifestations of the in vivo combination of antigen and antibody. The rapid onset of the reaction which is mediated through pharmacologically active substances (histamine, serotonine, acetylcholine and kinins) is regarded as a prototype of hypersensitivity reactions. It is similar to the cutaneous reaction but is on a larger systemic scale. The pattern of the reaction differs somewhat in different species and depends upon the sensitized tissues (shock organs) which are involved in the reaction with the antigen.

(iii) Serum Sickness

This condition has sometimes been referred to as a protracted anaphylaxis since symptoms appear only after 8 - 12 days following sensitization and may last for weeks or months (145). This behavior is attributed to the persistence of the antigen in the sensitized individual. The presence of circulating antigen leads to the formation of harmful antigen-antibody complexes. Sensitizing antigens include not only foreign sera but also such substances as penicillin which may become coupled covalently to the host's own proteins (146).

(iv) Atopic Sensitivity

Certain forms of hypersensitivity were observed to differ from the three classes discussed above. They were therefore, termed atopy (meaning strangeness) by Coca and Cooke (147). The principle manifestations of atopic hypersensitivity include such diseases as asthma, urticaria (hives), angio-oedema (general swelling) and hayfever; and the atopic individuals may show typical wheal and flare reactions after skin testing with the allergen.

Hayfever is one of the most common atopic conditions on the American continent. This hypersensitivity state is induced in susceptible individuals by pollens from trees, grasses, and other weeds such as short (dwarf) or giant ragweed. The unique seasonal appearance of the pollens of different plants accounts for some of the variations in severity of symptoms in allergic individuals. The atopic condition is characterized by watery exudation from the mucous membranes of the upper respiratory tract and conjunctiva, which results in violent and often protracted sneezing, nasal discharge and lacrimation.

Spontaneously occurring atopic hypersensitivity has been primarily a disease of man. However, dogs were shown to be capable of developing "spontaneous" pollen sensitivity, and their cutaneous, respiratory and anaphylactic sensitivity could be transferred to normal dogs (148, 149). Also, asthma-like symptoms were displayed in horses "spontaneously" sensitized to mouldy hay (150).

Inhalant allergenic substances, such as ragweed pollen and house dust, have been analyzed by various methods in an attempt to identify and to characterize the determinant group(s) responsible for the allergenic activity. Berrens (151) postulated that the allergenic activity of diverse substances may be attributed to specific chemical structures such as N-glycosidic linkages, the sugars being linked to proteins by the ε-amino groups of lysine. In another study it was demonstrated that the introduction of N-glycosidically bound sugars onto β-lactoglobulin resulted in increased skin reactivity as revealed by direct skin tests in milk sensitive patients (152). From the results of these and of several other studies, Stanworth concluded that the skin activity of five different allergens (ragweed, rye grass, horse dander, ipecacuanha, and house dust) was

invariably associated with a protein-carbohydrate conjugate with a sedimentation coefficient in the range of 2.2 to 3.8S (153). It was also noted that human epithelial glycoproteins had similar structures. Thus, on the basis of these observations, it was suggested that reaginic antibodies could be produced as a result of an autoimmune mechanism which regarded the epithelial tissues as being altered and, thus immunogenic.

Early investigations of the prevalence of hypersensitivity revealed that "spontaneous" sensitization of about 10 to 15% of the total population was determined by a genetic predisposition (154, 155). This observation, together with the poor immunogenic properties of the allergens, would appear to indicate that a unique responsiveness of the immune mechanism might result in the atopic condition. However, attempts to demonstrate abnormalities of the immune response in atopic individuals lead to conflicting results. Thus, vaccination with typhoid bacilli (156) suggested that the atopic individuals produced larger quantities of antibodies. On the other hand, antibodies produced against diphtheria toxoid exhibited poorer precipitable properties but had better skin-sensitizing abilities (157). In contrast to these observations, no differences in the levels of precipitating antibody or skin reactivity to dextran or pneumococcal polysaccharide antigens could be detected following immunization of both atopic and normal individuals with these substances (158). Since the experimental approaches might not reproduce the conditions prevailing during spontaneous sensitization, the results of these studies must be considered with certain reservations since they may not be directly related to the reactions actually occurring in atopic states.

It also appears from some studies that atopic individuals may exhibit an increased permeability of their gastric mucosa (a genetic trait) which

leads to an increased immune response (159, 160). Similarly, it was also suggested that atopic individuals with respiratory hypersensitivities had a respiratory mucosa with an increased permeability to the inhaled allergens (161). About 65-75% of ragweed allergic individuals were shown to become readily sensitized to unrelated allergens following experimental exposure to them. In addition, an inherited capacity of allergic individuals to become sensitized to inhaled allergens was demonstrated by epidemic outbreaks of asthma following exposure to castor bean dust (162). In these cases, about 20% of the allergic individuals showed positive skin reactions on direct skin tests with castor bean extracts.

Allergic individuals may suffer from more than one of these manifestations simultaneously or at different times, e.g. asthma and hayfever. Furthermore, their sensitivities may be directed against several non-related allergens, e.g. ragweed and grasses. Thus, what appears to be inherited by an allergic individual is not a sensitivity to any specific group of allergens, but rather a tendency to form a unique form of antibody capable of triggering off the inflammatory reaction on combination with the specific allergen (154).

ANTIBODIES IN ATOPIC INDIVIDUALS

Biological Properties

The first recorded observation of passively transferred immediate hypersensitivity was made by Ramirez, who transfused a non-allergic recipient with the blood from a horse sensitive individual (163). He observed that immediate sensitivity ensued on challenging the recipient with horse serum. He suggested that this effect was due to a humoral factor present in the serum of the atopic individual and that on transfusion, this factor

became fixed to the skin, conjunctivae and mucosae and remained fixed to these tissues for several weeks. Since this skin-sensitizing factor did not form a precipitate with the corresponding allergen(s), nor fixed complement, the term reagin* was used to designate this unusual antibody (164). The persistence of the atopic symptoms following exposure of the passively sensitized recipient to the homologous allergen was shown to be due to those tissue-bound reagins (165).

Atopic hypersensitivity can also be transferred locally with the serum from a sensitive individual to the skin of a normal individual (166). Typical wheal and flare reactions are obtained when the sensitized skin is injected (challenged) usually 24 or 48 hours later, with the specific allergen. The procedure is known as the Prausnitz-Küstner passive transfer or P-K test, and has been widely used to demonstrate skin-sensitizing antibodies. The P-K test has also been used routinely for the titration of skin-sensitizing antibodies, the P-K titer being equivalent to the reciprocal of the highest dilution of the allergic serum which is still capable of causing a detectable reaction.

In view of the complexity of the mechanism of the skin reaction, it would be expected that the intensity of the allergic skin reaction would depend not only on the concentration of skin-sensitizing antibodies and allergen, but also on the concentration of the biochemical mediators in the reactive site which may differ from one allergic individual to another and which determines the overall response. The P-K titer obtained with a given allergic serum is not a constant value, since different end points are obtained in different non-allergic recipients and also in different

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* The terms reagin and skin-sensitizing antibody are used interchangeably in this thesis.

regions of the skin of a given individual (167).

Among the other unusual biological properties of skin-sensitizing antibodies is their failure to pass through the placenta from maternal to foetal circulation and their retention by the choroid plexus (168, 169). As a result, no skin-sensitizing antibodies are detected in either cord serum or cerebro-spinal fluid.

Physicochemical Properties

All observations made to date indicate that skin-sensitizing antibodies are much more labile than the usual types of immune antibodies. Moreover, like other antibodies they are heterogeneous with respect to their chemical properties. Thus, skin-sensitizing activity is lost on heating at 56°C, and the minimal time required for their heat inactivation varies from serum to serum, extending up to periods of ten hours (170). The chemical heterogeneity is further illustrated by their behavior on exposure to acids. For example, the skin-sensitizing activity of certain allergic sera is partially or completely destroyed on incubation at pH 3 for two hours, whereas other sera are not affected by this treatment (171).

All of the procedures mentioned so far have no detectable effect on 'conventional' antibodies produced on immunization. Therefore, the foregoing observations may suggest that reagins may readily undergo conformational changes resulting in the inactivation or masking of the groups responsible for skin-fixation. It was claimed that the presence of an α_2-macroglobulin exerted a protective effect on skin-sensitizing activity (172). However, it was not established whether the α_2-macroglobulin complexed with skin-sensitizing antibodies, or whether the protective effect was due to the stabilizing properties of proteins in general.

Unlike immune antibodies, skin-sensitizing antibodies can be inactivated with 0.1M mercaptoethylamine (173, 174). Further proof of their chemical heterogeneity was provided by the demonstration of two types of reagins, one sensitive and the other resistant to treatment with mercaptoethanol; some sera contained both types of reagins (175).

In view of the evidence for the extreme lability of skin-sensitizing activity it may be somewhat surprising to find that reagins are more resistant than immune antibodies to enzymatic digestion with papain. Immune antibodies were degraded within 30 minutes of digestion, whereas complete inactivation of skin-sensitizing antibodies required about 24 hours of digestion (174).

The weight of all this evidence supports the view that skin-sensitizing antibodies differ radically from immune antibodies. This conclusion is further substantiated by an examination and comparison of their physicochemical properties.

By a variety of techniques, skin-sensitizing antibodies have been demonstrated to possess properties which are different from those of immune antibodies of the IgG type*. Thus, during fractional precipitation of allergic sera with either ammonium sulfate (176) or sodium sulfate (177) at concentrations which precipitated immune antibodies it was demonstrated that the bulk of skin-sensitizing antibodies remained in the supernatants.

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* The positive demonstration that reaginic activity is commonly associated with IgE represents the culmination of painstaking efforts in many laboratories over a period of time extending more than 20 years. Nevertheless, the inclusion in this thesis of a historical survey of the investigations leading to the final identification of this immunoglobulin class has been deemed appropriate to illustrate the complexity of the problem and the difficulties which had to be overcome.

The fractionation of allergic sera by various electrophoretic methods, such as Tiselius electrophoresis (178), electrophoresis convection (179), zone electrophoresis (180) and continuous paper electrophoresis (176) clearly demonstrated that the electrophoretic mobility of serum fractions rich in skin-sensitizing activity was higher than that of immune antibodies. The activity was associated mainly with γ_1- (or β_2-) globulins. By immunoelectrophoresis, the fractions were shown to contain mixtures of γA- and γM-globulins, including also small amounts of γG-globulins. Furthermore, the electrophoretic studies revealed that there was no apparent correlation between the protein concentration of the isolated serum fractions and the skin-sensitizing activity of these fractions (180). In retrospect, this observation may have been the first to suggest that skin-sensitizing antibodies were not associated with the then known immunoglobulins. Relatively high skin-sensitizing activity was also demonstrated in fractions with very low protein concentrations, indicating that skin-sensitizing antibodies were present in the sera of allergic individuals in exceedingly low concentration and possessed a high specific biological activity.

During early attempts to determine the sedimentation properties of reagins it was inferred that skin-sensitizing activity was associated with the heavier serum components (181). In another study, skin-sensitizing antibodies were found to be associated with 12.4, 14.1 and 22.5S components (182). However, these observations were challenged by other investigators who found that skin-sensitizing activity could be found in fractions devoid of 19S components (183).

An alternative explanation for the diverging conclusions of these results might be that skin-sensitizing activity is associated with neither the 7S nor 19S components, but with components having sedimentation

coefficients intermediate between these two values (184). In one study it was shown that the average sedimentation coefficient of reagins was 7.7S (185) while in another study the sedimentation coefficients were of the order of 8 to 11S (186).

In view of the different methods used for the preparation and the analysis of serum fractions, the results of many studies can only be interpreted to indicate that skin-sensitizing activity was not associated with the 7S globulins but with heavier serum components. Moreover, the evaluation of the skin-sensitizing activity of the serum fractions was performed in some studies in terms of the size of the P-K reactions, rather than in terms of an absolute quantitative method, the dilution technique.

The results obtained in studies using allergic sera fractionated by ion-exchange chromatography on DEAE-cellulose suggested that skin-sensitizing antibodies were physicochemically heterogeneous (187). The bulk of immune antibodies were eluted first with buffers of low ionic strength, whereas skin-sensitizing activity was eluted in several later fractions with buffers of increasing concentrations.

Chromatography on molecular sieves, such as Sephadex G-200, supported the claims that skin-sensitizing activity was associated with serum components with sedimentation coefficients greater than 7S but less than 19S (188, 189). The eluates containing skin-sensitizing activity were found to be composed mainly of γA-, γG- and small amounts of γM-globulins.

Antigenic Relationship of Reagins to Other Globulins

From a consideration of the physicochemical and immunochemical properties of the proteins which were associated with skin-sensitizing activity, around 1964 it appeared that skin-sensitizing activity parallelled best the distribution of γA-globulins. This conclusion was based not on the

direct identification of skin-sensitizing antibodies as γA-globulins, but on circumstantial evidence. Thus, immunoelectrophoretic analysis of serum fractions, isolated by various procedures and containing skin-sensitizing activity, revealed γA-globulins in addition to one or more of other serum proteins, such as γG- and γM-globulins, siderophilin, transferrin, haptoglobin, and α_1-glycoprotein (177, 183, 188, 189, 190). By means of radio-immunoelectrophoresis, it was demonstrated that IgG, IgA and IgM all possessed antibody activity against ragweed pollen antigens (191, 192). Since this procedure could not assess the main biological property of reagins, namely their skin-fixation (or skin-sensitizing) capacity, no correlation between skin-sensitizing activity and antigen binding activity could be made. Nevertheless, these results demonstrated that a multiplicity of antibodies directed against the antigenic components of ragweed coexisted in allergic serum.

In various studies the bulk of skin-sensitizing activity against such diverse allergens as ragweed, moulds and snake venom, was found in preparations which were enriched in γA-globulins (193, 194, 195). It was also observed that secretions such as saliva, sputum (84, 196) and tears (197), which contained reaginic activity, contained appreciable amounts of γA-globulins. On the other hand, both γA-globulins and skin-sensitizing antibodies were absent from cord serum of atopic mothers (198).

The observation that α-chains (199) as well as intact IgA (200) were able to block passive sensitization of normal skin with skin-sensitizing antibodies was taken as further proof that α-chains possessed skin-fixing properties and that reaginic activity was associated with γA-globulins.

On the basis of the ultracentrifugal heterogeneity of γA-globulins (76) and of skin-sensitizing antibodies (186), it was not inconceivable

at the time to conclude that reagins might have belonged to the group of γA-globulins with higher sedimentation coefficients.

The evidence presented so far has demonstrated that γA-globulins and fractions rich in skin-sensitizing activity share similar physicochemical properties. To investigate this relationship more closely, the technique of specific precipitation (immune absorption) was employed. This procedure makes use of the characteristic and unique antigenic property of each immunoglobulin. However, owing to the two distinct polypeptide chains of the immunoglobulin molecule, antisera prepared against any one type of immunoglobulin will contain antibodies directed against the antigenic determinants characteristic of both H and L chains. Only after absorption of an antiserum with either the light chains or with heterologous immunoglobulin(s), will antibodies against the common determinant be removed, and the absorbed serum will then react only with the specific antigenic determinants of the H chain. Using an antiserum rendered specific in this manner, it was shown that the specific precipitation of IgG did not alter the titer of skin-sensitizing activity of an allergic serum fraction. On the other hand, complete removal of skin-sensitizing activity was accomplished by immune adsorption with a specific antiserum of γA-globulin and it was concluded that some antigenic determinants associated with skin-sensitizing antibodies were identical to those of γA-globulins* (188, 194).

Although the results from many investigations supported the view that skin-sensitizing activity was associated with γA-globulins, other observations contradicted this conclusion. Thus, some allergic sera, which

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* Recently, the experiments by Ishizaka et al. (204) revealed that the anti-γA antiserum used originally by Fireman et al. (188), actually contained anti-γE antibodies which were responsible for the precipitation of reaginic activity.

possessed high skin-sensitizing activity, did not contain any detectable γA-globulins (201, 202, 203). Also, on closer inspection of allergic serum fractions obtained by ion-exchange chromatography it was found that the bulk of reaginic activity was not associated with γA-globulins (205, 206). The demonstration that γG- and γM-globulins could bind labelled antigens, in addition to γA-globulins (175, 191), further illustrated the multiplicity of immunoglobulins which were capable of binding labelled antigen. In other experiments, a heterogeneity of reagins was demonstrated by the existence of two types of reagins, which were separated by ion-exchange chromatography and molecular sieving; one group was associated with γM-globulins and was inactivated by mercaptoethanol and by heat, the other was associated with γG-globulins and was resistant to mercaptan treatment and to heating (207). From the results of another study, it was suggested that skin sensitizing-antibodies may be associated with a unique subgroup of γG-globulins (175). However, in a third study, the absorption of a serum fraction containing the highest skin-sensitizing activity with specific antisera to γG- or to γA-globulins did not alter the reaginic activity (205, 208). These observations threw doubt on the previous conclusions that reaginic activity was associated with γA-globulins.

It has already been mentioned that passive sensitization of normal skin with reagins could be blocked by prior intradermal injection of either intact γA-globulins or their α-chains (199, 200). In later investigations, however, it was found that purified myeloma γA-globulins were not effective in this reaction and that only heterologous reaginic fractions could successfully block passive sensitization (209). Thus, on the basis of these results, it was concluded that both skin-fixing and skin-sensitizing properties were associated with the same molecule, which was not a γA-globulin.

To investigate the possibility that reagins may be associated with an as yet unidentified immunoglobulin, a reagin rich fraction containing apparently γA- and γG-globulins only, was used to immunize rabbits. The resulting antiserum was absorbed with appropriate immunoglobulins or their constituent chains until no antibody activity could be demonstrated against the α, μ and γ heavy chains (including the subgroups γ_1, γ_2, γ_3, γ_4) and the κ and λ light chains. This absorbed antiserum nevertheless, was still capable of completely removing all reaginic activity from an allergic serum fraction which had initially contained only γA-globulins in detectable amounts (42). On the other hand, the immune absorption of this reaginic serum fraction with a specific antiserum against γA-globulins did not alter its skin-sensitizing activity but completely removed the ragweed binding γA-globulins, as revealed by radio-immunoelectrophoresis.

Reagins did not appear to be associated with γD-globulins since on ion-exchange chromatography the elution patterns of this globulin did not parallel the distribution of skin-sensitizing activity, nor was the activity removed by the immune absorption with a specific anti-γD antiserum (210, 211). Also, γD-globulins did not bind radioactively labelled ragweed (107). Skin-sensitizing activity could not be co-precipitated with γM-anti-γM complexes (113). However, co-precipitation of skin-sensitizing activity could be achieved with γG-globulins and an antiserum against the light polypeptide chains (42).

All these observations indicated that reagins were not associated with either γA-globulins or with the other known immunoglobulins, but with a new class of immunoglobulin which possessed unique antigenic determinants and was designated as γE-globulin.

From the analysis of the antigenic properties of reaginic antibodies it was evident that γE-globulins contained unique antigenic determinants which presumably were located on the heavy polypeptide chains. Since the anti-γE antisera had been rendered specific by absorption with Fab fragments, the specific antigenic determinants of γE-globulins must be localized on the Fc portion of the molecule. Moreover, since both γE-globulins and skin-sensitizing activity were co-precipitated by antisera to light chains it was concluded that γE-globulins and reagins must contain light chains. Furthermore, it was shown by radio-immunoelectrophoresis, that radioactively labelled anti-Fab antibodies combined with the γE-anti-γE complexes (107) and that both κ and λ types of light chains were present in γE-globulins (113).

The results of a series of experiments provided a direct demonstration of the association of reaginic activity with γE-globulins (107). Fractions of an allergic serum containing the highest concentration of skin-sensitizing activity were obtained by successive purification procedures which included precipitation with ammonium sulphate, ion-exchange chromatography on DEAE-cellulose, recycling molecular sieving on Sephadex G-200 and ion-exchange chromatography on DEAE-Sephadex. After these procedures the reaginic preparation contained only trace amounts of γD-, γG- and γA-globulins and was, on a weight basis, about 200 times more active than the original allergic serum. Using specific antisera against γG- and γA-globulins, the contaminants of γG- and γA-globulins were removed. The skin-sensitizing activity of this final preparation, which contained only γE- and γD-globulins was about 1000 times higher than the original allergic serum. Furthermore, the removal of γE-globulins from this preparation by precipitation with a specific anti-γE antiserum was accompanied by the complete loss of reaginic

activity. From a consideration of all of these observations it was concluded that γE-globulins and reagins are identical.

By gel filtration of allergic sera on Sephadex G-200, skin-sensitizing antibodies are characteristically eluted in fractions extending from the descending portion of the first peak to the ascending portion of the second peak, the majority being eluted in the latter (189, 212). This finding indicates that there is a size distribution for skin-sensitizing antibodies. In another study, rechromatography of two Sephadex G-200 fractions, led to the separation of two reaginic γE-globulin components, both of which were shown to bind allergens (204). Following sucrose-density gradient ultracentrifugation, one fraction of γE-globulins was shown to have a sedimentation coefficient of 8S, and the other 11S. On the basis of these results it was suggested that the 11S component might represent either a polymer of γE-globulins, or an aggregate of γE-globulins with some other protein.

Recently, a comparative study of the relationships between skin-sensitizing activity and immunoglobulins from three different species has been reported (213). Reaginic sera from man, dogs and monkeys were fractionated by gel filtration on Sephadex G-200. Although some of the human and canine reaginic sera contained skin-sensitizing antibodies directed against two different allergens while the monkey reaginic serum was against a single allergen, the results indicated that the distribution of skin-sensitizing activity was similar in all three species. Moreover, no obvious correlation between skin-sensitizing activity and the major immunoglobulins IgG, IgA or IgM could be demonstrated. Similar conclusions were made from immune absorption studies with specific antisera against the γG-, γA- and γM-globulins of all three species: specific precipitation of these

immunoglobulins did not affect the skin-sensitizing activity of the fractions. On the other hand, specific antisera, which had been prepared against the reagin rich serum fractions and which were rendered specific to reagins by absorption with selected reagin-free allergic serum fractions, were able to remove the skin-sensitizing activity from each allergic serum. Since no antibody activity against the corresponding γG-, γA-, or γM-globulins could be demonstrated in the specific antisera, it was suggested that an immunoglobulin analogous to human IgE may be a carrier of reaginic activity in the dog and monkey. The same conclusions were made in another study which demonstrated that rabbit reaginic antibody was not of the γG-, γA-, or γM-globulin classes (214).

Immunological Properties

One characteristic feature of conventional antibodies of the IgG type is their capacity to form precipitates on combination with the homologous antigen. Therefore, the consistent failure of reagins to give a visible precipitate with allergens prompted early investigators to classify reagins into a distinct group of antibodies. To account for an apparent lack of precipitating antibody activity of reagins, three possibilities were proposed: (i) skin-sensitizing antibodies are univalent, (ii) the affinity of skin-sensitizing antibodies for the allergens is very small, or (iii) skin-sensitizing antibodies are divalent and have a high affinity for the allergen, but their concentration is too low to be detected by the standard precipitin techniques (181, 215). In light of present knowledge of the properties of skin-sensitizing antibodies, sufficient evidence has been now accumulated to indicate that the third possibility is the correct one.

The *in vitro* combination of reagins with allergen has been demonstrated by the use of immunologically specific adsorbents (immunosorbents).

Ragweed was either attached to insoluble supports (171, 216, 217, 218) or insolubilized (219) to form allergenically active immunosorbents. These immunosorbents were shown to remove skin-sensitizing antibodies specifically and completely from reaginic sera. However, no demonstrable decrease in the protein concentration or a change in the distribution of proteins of allergic sera, as determined from comparative electrophoretic and ultracentrifugal analysis, could be shown (171). These results indicated that skin-sensitizing antibodies must be associated with serum components which are present in concentrations lower than 100 μg/ml which is the limit of detectability by the Schlieren optical systems used.

Because of the low serum concentration of γE-globulins, the allergen-binding activity of γE-antibody (i.e. reagin) was demonstrated by an indirect method (220). In this technique allergen-antibody complexes were first formed by incubating the allergic serum with radioactively labelled allergen. These complexes were then selectively precipitated with antisera specific for each of the immunoglobulins. To prevent the possible loss of trace amounts of the three component allergen-γE-anti-γE complexes, the precipitation was performed in 0.5% agarose. Finally, the radioactivity of the washed precipitates was measured to determine the amount of allergen bound by each immunoglobulin. The results from these studies demonstrated that only the binding of allergens by the γE antibodies could be correlated with skin-sensitizing activity. Furthermore, from a comparison of the allergen-binding values for γE-globulins and the amounts of allergens precipitated with a rabbit anti-ragweed antiserum, it was inferred that the minimum dose of γE antibody required to elicit positive P-K reactions was of the order of 10^{-6} to 10^{-5} μg N*. Since the specificity of the rabbit

* N = protein nitrogen

antibodies directed against ragweed components may differ from those of the human antibodies, the minimal dose required to elicit positive cutaneous reactions can obviously be only a gross approximation.

Rabbit anti-γE antibodies, at about the same concentration were observed to elicit reversed PCA reactions in both the human and monkey (110). Since reversed PCA reactions could not be obtained with rabbit antisera against γA- or γG-globulins, it was concluded that only γE-globulins had the ability to become fixed to certain target cells in man and monkey. These cells have been identified as mast cells in the latter species (221).

The presence of γG-, γA- and γM-globulins in the skin of atopic and non-atopic individuals has been demonstrated by the fluorescent antibody technique (222). However, since rabbit antibodies to γG-, γA- or γM-globulins do not elicit cutaneous reactions, it could be concluded that these immunoglobulins are fixed to cells which do not participate in immediate type hypersensitivity reactions, or that the γG-, γA- and γM-globulins are not capable of forming the proper aggregates required for inducing skin reactions.

Allergen-antibody complexes, prepared with γE-globulins and ragweed allergens were shown to induce immediate-type skin reactions (223). The intensity of the skin reactions varied with the composition of the complexes and depended on the amount of allergen added. Maximum reactions were obtained by mixtures of reaginic γE-globulins with slight excess of the ragweed allergen preparations. Fractionation of these reagin-allergen complexes by sucrose density gradient ultracentrifugation revealed the presence of two major components: a 9.7S component which was presumably composed of one IgE antibody molecule and two molecules of the allergen,

and another component with a sedimentation coefficient of 13.1S, believed to be composed of two IgE antibody molecules and three allergen molecules. The 13.1S component elicited greater skin reactions than the 9.7S component. Moreover, the skin reactivities were more intense with preformed complexes than with complexes formed in vivo by the separate injections of the identical doses of IgE antibody and ragweed allergen into the same skin site.

Antibodies which bind to target cells, sensitizing them for anaphylaxis have been referred to as cytotropic antibodies (224). Homocytotropic antibodies sensitize the cells of the same species and include human IgE reagins which can sensitize human tissues (skin, appendix (225), ileum and uterus (226)) belong to this class. On the other hand, antibodies, such as human γG-globulins, which do not sensitize human tissues, but are capable of sensitizing guinea pig tissues (60) are referred to as heterocytotropic antibodies.

Human reaginic antibodies have been demonstrated to be capable of eliciting PCA reactions in the monkey (115) and of passively sensitizing monkey ileum (227) and lung (228, 229). The ability of the γE-globulins to sensitize these tissues was lost following the removal of IgE by precipitation with a specific anti-γE antiserum. Since no localization of the human reagins in passively sensitized monkey skin could be demonstrated with antisera specific for human γG-, γA-, γM-, or γD-globulins, it was concluded that only the γE immunoglobulins fixed to the monkey skin. In addition, the reversed PCA reactions elicited in monkeys by anti-human IgE antisera demonstrated that γE-like proteins are present in monkey skin (112). These results also indicated that human and monkey γE-globulins share common antigenic determinants and that the human and monkey target

cells which can fix human reagins must have some structural similarities. Recently, γE-globulins have been isolated from monkey serum and demonstrated to be similar to human IgE (230). Moreover, rabbit antisera to monkey IgE were capable of absorbing human reaginic antibodies from the sera of ragweed allergic individuals.

From the observations that leukocytes from atopic patients released histamine following exposure to the homologous allergen, it was concluded that reagins (γE antibodies) sensitized these cells or became fixed to them (231, 232). This conclusion is further supported by the finding that normal leukocytes can be passively sensitized with reaginic serum for this reaction.

Various forms of agglutination techniques have been applied to the detection of antibodies in the sera of allergic individuals. The hemagglutination procedure in which antigens are linked to erythrocytes by stable covalent bonds with bifunctional reagents, such as bisdiazotized benzidine, has been widely used (233, 234). It appeared at the time that skin-sensitizing antibodies contributed only a minor portion of the total hemagglutinating activity of allergic sera. More recently, however, ragweed reaginic serum fractions which contained only γE-globulins have been demonstrated to agglutinate sensitized red cells. Therefore, these results indicated that reaginic γE-antibodies must be at least divalent (204). The hemagglutination titers further increased on the additions of an anti-γE antiserum, indicating that the reaginic γE antibodies had combined with the ragweed allergens on the erythrocytes.

Blocking Antibodies

The presence of blocking antibodies has been demonstrated in the sera of both treated and non-treated atopic patients, as well as in the sera

of normal individuals immunized with ragweed antigens (187, 235). It is capable of reacting with the allergen (236).

In contrast to reagins, blocking antibodies appear to have no affinity for human skin or mucous membranes (165), and pass through the placenta (237). Also, unlike reagins, blocking antibodies are not inactivated by heat at 56°C (238), are stable under various conditions of storage and handling (174, 187, 235), and are not degraded by reduction with 0.1M mercaptoethanol (174). Human blocking antibodies can elicit PCA reactions in the guinea pig and are, therefore referred to as heterocytotropic antibodies (174).

Antibodies which have the ability of blocking the P-K reaction in man are also formed by other species on immunization with the appropriate allergen (174, 176). On chromatography, the majority of blocking antibodies is eluted in fractions from DEAE-cellulose with buffers of low ionic strength (187). These fractions are composed mostly of γG-globulins and possess hemagglutinating activity. Moreover, at sufficiently high concentrations human blocking antibodies readily formed precipitin lines in agar with the homologous allergens. The precipitin bands showed reactions of identity between blocking antibodies from both rabbit and human antisera and ragweed extracts. These results indicate that all sera examined contained precipitating antibodies against one or more antigenic determinants on the same ragweed constituent.

PURPOSE AND SCOPE OF THE PRESENT INVESTIGATION

The main interest in human reaginic antibodies arises from the fact that they represent an unusual immunoglobulin with the unique property of fixing to or sensitizing certain tissues, in addition to their ability of combining specifically with the homologous allergen. The characterization of reagins which are responsible for the manifestations of hypersensitivity reactions is of fundamental importance for (i) the understanding of the mechanism of this altered immune response in allergic individuals, and (ii) a possible rational approach to the management of this condition. The purpose of the work reported in this thesis was to develop methods for the isolation, inactivation and characterization of the immunoglobulin class(es) possessing properties of reagin(s). As it will become evident, the investigation proved difficult because of the intrinsic characteristics of the system studied, i.e. (i) reagins are labile, (ii) they are present in extremely low concentrations in the sera of allergic individuals and, (iii) they share similar physicochemical properties with some other immunoglobulins.

In Chapter III are described attempts to develop methods for the isolation of IgA for the purpose of elucidating its previously reported association with reagin. The procedures were subsequently modified and used for the rapid fractionation of allergic sera to obtain highly enriched reaginic preparations.

To exploit the specific antibody combining properties of reagins, immunochemical methods employing immunosorbents were used. In the experiments described in the first section of Chapter IV, a chemically simple immunosorbent was prepared by the polymerization of the aqueous

extract of ragweed pollen with ethylene-maleic anhydride. A model system using rabbit antiserum to ragweed was first used to establish suitable conditions for the recovery of antibodies adsorbed to immunosorbent and to investigate the possibility of using these menthods for isolating reagins from human ragweed allergic sera. The second section of Chapter IV deals with the use of amino-cellulose for the same purposes. In addition, the elution of reagins from immunosorbent by their displacement with haptens is described. The final outcome of these experiments was to reveal the unique antigenic identity of reagins, identifiable with IgE, by the use of antisera prepared against reagins eluted from immunosorbent.

The elucidation of the nature of reagins has been hindered, partly because of previous failures to isolate the antibodies from the sera of allergic individuals in a pure state, and partly because of the unavailability of pure allergens. Both situations originate from the complexity and heterogeneity of mixtures of proteins in general, containing these two components of the antibody-antigen system involved in common allergy. The experiments reported in Chapter V were designed to investigate the nature of the allergen-reagin system in ragweed allergy in man.

Despite the hyposensitization treatment introduced by Noon in 1919 (20) for allergic individuals, a large number of allergic persons do not respond well to this procedure and an urgent need for satisfactory remedy still exists. In Chapter VI the results of an investigation into the possibility of employing mercaptans for the <u>in vivo</u> inactivation of reagins are reported.

In view of the broad scope covered in this thesis, the various aspects of the study have been presented in what seemed to be a logical order, rather than in the chronological order in which they were investigated.

CHAPTER III

DEVELOPMENT OF METHODS FOR THE ISOLATION OF IgA FROM NORMAL HUMAN SERUM AND FOR THE FRACTIONATION OF SERA FROM RAGWEED ALLERGIC INDIVIDUALS

Introduction

At the beginning of this project, the accumulated evidence presented in Chapter II from various laboratories implicated γA-globulins as the carrier of reaginic activity. Thus, the bulk of reaginic activity appeared usually to be associated with the γA-globulins isolated by various procedures from allergic sera (84, 177, 183, 188, 189, 190, 193, 194, 195, 196, 197). Moreover, the selective removal of γA-globulins from allergic sera by precipitation with antisera, apparently rendered specific to γA-globulins, resulted in the depletion of these sera of their skin-sensitizing activity (188, 194). Consequently at the time it was deemed appropriate to embark on an investigation designed to develop more efficient methods for the isolation of IgA in the expectation of clarifying the nature of skin-sensitizing antibodies.

METHODS AND MATERIALS

Immunochemical Methods

(a) Immunoelectrophoresis

Immunoelectrophoresis was performed by the micro-method of Sheidegger (239) using the LKB apparatus* . Precleaned microscope slides were coated with a 0.1% agar** solution in water. Agar gel for the electrophoresis

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* LKB Producter. Sweden, Model 6800A.

** Special Agar Noble, Difco Laboratories, Michigan.

was prepared by dissolving 1 g of agar in 100 ml of veronal buffer,* pH 8.6, ionic strength 0.033, heated at 80°C and poured onto the pre-coated slides in a frame placed on a horizontal leveling table. The agar was permitted to gel for 15 minutes after which the slide and tray assembly was placed in a moist chamber for at least 2 hours before use. A pattern consisting of longitudinal troughs and wells in the center of each slide was cut in the gel with a punch. The wells were opened by removal of the gel plug with a needle and gentle aspiration. Samples were inserted into the wells by capillary tubes. The slide-tray assembly was placed onto the buffer filled electrode chambers and electrical contact was established by rayon wicks soaked in veronal buffer. For electrophoresis, a D.C. voltage of 250 V (9.25 V/cm) was applied for 40 minutes.

After the electrophoretic run, the gel in each pre-cut longitudinal trough was removed with a gel knife. The trough(s) were filled with the appropriate antiserum(a), and the precipitin arcs were allowed to develop by incubating the slides for 16-24 hours in a moist chamber.

Before staining the slides, the excess and unreacted proteins were removed by washing the slides in saline for at least 16 hours with one change of the saline bath. The final wash was with distilled water for 2 hours. The slides were covered with 'lintless' filter paper strips and dried at room temperature. The filter paper strips were peeled off

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* The veronal buffer used in the electrode chambers was prepared by dissolving 3.684 g diethyl barbituric acid, 20.618 g sodium barbiturate and 8.204 g sodium acetate.$3H_2O$ in 2 liters of distilled water. For use in the preparation of agar gel, 1 volume of this veronal buffer was diluted with 3 volumes of distilled water.

and the slides were stained with a solution* of either azo-carmine-B, or light green. Excess stain was removed by washing the slides with repeated changes of a 5% solution of acetic acid followed by distilled water. The slides were dried with a hot air blower and stored for future reference.

(b) Preparation of antiserum

An antiserum to NHS was prepared in rabbits by repeated intradermal injections with an emulsion prepared by mixing 1 volume of the protein (5 mg) in saline with an equal volume of complete Freund's adjuvant. The animals were reinjected at intervals of 1, 3, and 6 weeks after the initial immunization. Blood was collected from the marginal ear veins, ten days following the last immunizations, allowed to clot at room temperature for two hours and then overnight at 4°C. The serum was separated from the clot by centrifugation at 5000 X G for 10 minutes, decanted and sterilized by filtration through millipore** membranes (0.45 μ pore size). Rabbit antiserum to human IgA was obtained from Behring-Werke.***

Human Sera

Whole blood was obtained from a myeloma patient with elevated serum levels of IgA. The blood was allowed to clot for several hours at room temperature and overnight at 4°C. The serum was separated from the clot by centrifugation at 5000 X G for 10 minutes.

Ten normal non-allergic individuals each donated a volume of 50 ml of whole blood. The sera were obtained as previously described and pooled.

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* Dye solutions at a concentration of 0.5% (w/v) were prepared in 5% acetic acid.

** Millipore Filter Corporation, Bedford, Massachusetts.

*** Behringe-Werke, Marburg, Germany.

Whole blood was obtained from non-treated ragweed allergic individuals (Gre and Wh) and from patients (Gr, Kh and We) receiving hyposensitization treatment. The sera were sterilized by filtration through millipore membrane (0.45 μ pore size) and stored at -15°C.

Vertical starch gel electrophoresis

Vertical starch gel electrophoresis with a discontinuous buffer system* was performed according to the method of Smithies (240). The hydrolysed starch** (61 g) was dissolved in 500 ml of Tris-citrate buffer, pH 8.65, heated to 78°C. To reduce the possibility of the formation of air bubbles in the gel, the molten starch gel solution was briefly boiled under reduced pressure. The molten starch was then quickly poured into a horizontally levelled perspex mould and covered with a perspex sheet containing moulds for the sample wells. To improve the uniformity and the physical stability of the starch gel, the entire assembly was cooled at room temperature over a period at least 6 hours before use. The top mould was then removed and 30-50 μl of each sample to be analysed was inserted with a micro-pipette into one of the pre-formed sample wells. Molten petrolatum was poured over the sample wells to seal them and the gel surface was covered with Saran-Wrap.***

The mould containing the starch gel was clamped in a vertical position with the bottom of the gel in the borate buffer. Filter paper (Whatman 3MM) was used to effect an electrical connection between the top of the gel and the top buffer compartment. Silver-silver chloride electrodes were used; the positive electrode was immersed in 5% solution

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* Discontinuous buffer system:
Starch gel buffer: 0.076M tris, 0.005M citric acid, pH 8.65.
Electrode buffer: 0.3M boric acid, 0.005M NaOH, pH 8.65.

** Connaught Laboratories, Toronto, Canada.

*** Saran-Wrap, The Dow Chemical Co., Midland, Michigan.

(w/v) of sodium chloride. Electrophoresis was performed at room temperature for 15 hours at a potential of 7 V/cm D.C. The polarity of the electrodes was chosen so that albumin migrated downwards. At the completion of an electrophoresis run, the gel was removed from its mould and sliced into two halves. The gel was stained for 10 minutes with 0.5% amido black (w/v) in a solution composed of 9 parts of methanol and 1 part of acetic acid. To remove the excess stain the gel was washed with a methanol-water-acetic acid solution (5:5:1).

For the specific identification of IgA and other serum proteins, the vertical starch gel electrophoresis technique was employed in conjunction with immunodiffusion analysis in agar. For this purpose, at the completion of electrophoresis, the gel was sliced into two equal portions with each containing one-half of the fractionated sample. For localization of the zones one part was stained with amido black in a 5% acetic acid solution, and to minimize shrinkage of the starch gel methanol was omitted from both the stain and wash solutions. A 2 mm wide longitudinal strip of starch gel along the course of the electrophoresed sample(s) was cut out from the unstained half of the gel using an apparatus devised from two parallel spaced dermatome blades.

For the immunodiffusion analysis a glass plate horizontally levelled which had been pre-coated with a 0.1% agar solution in water and dried at 80°C was overlaid with 1% agar in borate buffered saline, pH 8.65 at 80°C, and allowed to cool to room temperature. The solidified agar formed a layer approximately 3 mm deep. Parallel troughs 2 mm wide and 10 mm apart were cut into the agar; the agar plugs from alternate pre-cut troughs were removed and the 2 mm wide segments of starch gel containing

the electrophoresed sample were transferred into the open troughs. The electrophoresed proteins were permitted initially to diffuse from the starch gel into the surrounding agar gel for a period of 16-24 hours after which the agar plugs from the adjoining pre-cut troughs were removed and filled with appropriate antisera (anti-IgA or anti-NHS). The precipitin arcs were permitted to develop for 24-48 hours in a moist chamber.

Preparation of DEAE-cellulose* for ion-exchange chromatography

DEAE-cellulose was suspended in distilled water and washed on a Buchner funnel with 0.5 N NaOH and then with distilled water to approximately neutral pH. The DEAE-cellulose was resuspended in a large volume of 0.01M phosphate buffer, pH 8.0, stirred for several hours after which it was permitted to settle. The supernatant containing the 'fines' was decanted and replaced with fresh buffer. The procedure of stirring, settling, decanting and washing with fresh buffer was repeated until the pH of the washing fluid was constant at pH 8.0, and the supernatant was cleared of the fines. A slurry of the DEAE-cellulose was poured into columns and packed under nitrogen pressure (20 lbs/in^2) until the bed volume was constant. The initial eluting buffer was percolated through the packed DEAE-cellulose for several column volumes before its use.

Preparation of DEAE-Sephadex A 50 for ion-exchange chromatography

DEAE-Sephadex A 50 was allowed to swell in distilled water for 20 hours and was regenerated by washing successively with 0.5 N NaOH, distilled water, 0.5 N HCl and distilled water. The pH of the slurry was adjusted to 8.0 with NaOH and the DEAE-Sephadex was then equilibrated

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* DEAE-cellulose, through 325 mesh screen. Brown Co., Berlin, New Hampshire.

with 0.1M Tris-HCl, pH 8.0, containing 0.5M sucrose. Excess buffer was filtered off and the gel was used as a moist cake.

Preparation of Sephadex G-25 for desalting by gel filtration

Dry Sephadex G-25 (coarse) was allowed to swell in distilled water for at least 3 hours. The Sephadex was resuspended by stirring and, after settling, the supernatant fluid containing the 'fines' was removed by decantation. The gel was washed in 0.1M Tris-HCl buffer, pH 8.0 and poured as a slurry into glass chromatography columns. To ensure complete equilibration of the gel with buffer, several column volumes of the Tris-HCl buffer were percolated through the Sephadex filled columns.

Fractionation of γA-myeloma serum with ammonium sulfate at increasing concentrations

A preliminary experiment was carried out to determine the concentration of ammonium sulfate at which γA-globulins were preciptated from human serum. To a volume of 10 ml of a γA-myeloma serum, 0.5 ml portions of a saturated solution of ammonium sulfate (SAS) pH 7, 22°C, were added dropwise and with constant stirring. The precipitate which formed after the addition of each portion of SAS was recovered by centrifugation at 6000 X G for 10 minutes at 22°C, dissolved in a volume of 4 ml of distilled water and dialysed against PBS.

Immunoelectrophoretic analysis employing a polyvalent antiserum to normal human serum was used to identify the proteins in these fractions.

Fractionation of normal human serum (NHS pooled) by a single step salting-out procedure

One volume of SAS pH 7, 22°C, was added to 1.22 volumes of pooled NHS; the final concentration of ammonium sulfate corresponding to 45% saturation. The preciptate was isolated by centrifugation at 6000 X G

For 10 minutes at 22°C, and washed by resuspension in a small volume of 45% SAS solution and recentrifuged. This washed precipitate was dissolved in distilled water in a volume of about one-half the original serum volume, and then dialysed at 4°C against 0.01M phosphate buffer, pH 8.0. The preciptate which formed after dilaysis was removed by centrifugation at 10,000 X G for 10 minutes.

Ion-exchange chromatography on DEAE-cellulose

The fraction of normal human serum, precipitated at 45% SAS and dialysed against the initial 0.01M phosphate buffer pH 8.0, was applied to the DEAE-cellulose column and elution was continued with the same buffer at 4°C. The effluent was monitored at 253 mμ using the LKB Uvicord (Model 4701A) recording apparatus. Base-line conditions for the recorder were established with the 0.01M phosphate buffer. Following the elution and recovery of the protein components the washing of the column was continued with the same buffer for an additional 24-48 hours. Then the buffer on top of the DEAE-cellulose bed was withdrawn and replaced with the second buffer of the same pH and increased molarity. The 5 phosphate buffers* used for the step-wise elutions from DEAE-cellulose were:

(I)	0.01M	phosphate	buffer, pH 8.0
(II)	0.02M	"	"
(III)	0.035M	"	"
(IV)	0.04M	"	"
(V)	0.05M	"	"

The eluates containing protein were pooled and concentrated to small volumes by pervaporation with simultaneous dialysis against saline, and

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* Buffers appropriately diluted from stock 0.05M phosphate buffer, pH 8.0, solution prepared by dissolving 0.3284 g NaH_2PO_4 and 6.7590 g $NaHPO_4$ per liter distilled water.

analysed by immunoelectrophoresis. These eluates were coded by two numbers, the Arabic number indicating the concentration of ammonium sulfate (percent saturation) used for preliminary precipitation and the Roman numeral indicating the eluting buffer employed, (e.g. fraction 45). Fraction 45[III] was further analysed by vertical starch gel electrophoresis used in conjunction with immunodiffusion analysis as described in Methods.

Hyperimmunization as a biological test for protein purity

A 0.5 ml portion containing 2.5 mg of the γA-globulin preparation (45[III]) was emulsified with an equal volume of complete Freund's adjuvant and injected both intradermally and intramuscularly into a rabbit. The immunization was similarly repeated 1 week and three weeks after the first injection. Blood was withdrawn 10 days following the last immunization and the antiserum was separated from the clotted blood by centrifugation at 3000 X G.

This rabbit antiserum was employed in an experiment designed to reveal the identity of the antigenic component(s) in the purified IgA preparation. For this purpose, the original unfractionated human serum was electrophoresed in agar. Then the antibody troughs were filled with the rabbit antiserum and the precipitin arcs were allowed to develop.

Ultracentrifugation

The Spinco model E analytical ultracentrifuge was used to determine the sedimentation constant of th IgA preparation 45[III]. The protein was dissolved in saline to a concentration of 1 g%. The average rotor temperature was 25°C and the rotor* speed was 59,780 r.p.m. The sedimentation coefficient was calculated using the standard formula

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*Analytical Rotor An-D, Spinco Model E., Palo Alto, California.

$$s = \frac{dx/dt}{\omega^2 x}$$

where x is the distance of the peak from the center of rotation at time t. The distances were determined from the schlieren patterns, photographed at different times of centrifugation; ω is the angular velocity. Since this protein preparation was shown to be a heterogeneous mixture of globulins, the recalculation of the sedimentation coefficients for "standard" conditions was not deemed necessary.

Human allergic sera

Single salting-out procedure

The identical procedure employing SAS, (pH 7.0, 22°C) at a final saturation of 45%, as described for fractionation of NHS, was used with allergic sera Gr, Wh and We. These globulin fractions will be referred to in general as AS[45]. In this case, desalting of the ammonium sulfate fractions was accomplished by gel-filtration on Sephadex G-25 instead of dialysis against buffer.

Desalting by gel filtration on Sephadex G-25

The dissolved fractions were applied to the top of the bed of Sephadex (previously equilibrated with 0.1M Tris-HCl, pH 8.0) and allowed to flow into the gel. At the moment when it disappeared through the surface, a small amount of buffer was used to wash the sample completely into the gel. The column was then connected to a reservoir of 0.1M Tris-HCl buffer, pH 8.0 and gel filtration was continued. The effluent was monitored for protein at 280 mμ. Eluates containing protein, as evidenced by an increase in the optical density were pooled.

Sequential salting-out procedures

Allergic sera, Gre and Kh, were fractionated sequentially at three different levels of saturation with ammonium sulfate. The first fraction was obtained by salting-out at 30% final saturation at 22°C; the precipitate, (herein referred to as fraction 30) was recovered by centrifugation at 6000 X G for 10 minutes at 22°C; to the clear supernatant a sufficient amount of SAS was added to raise the level of saturation to 35%. This second precipitate which will be referred to as fraction 30-35 was isolated by centrifugation as described previously. The quantity of SAS which was required to raise the saturation of the remaining supernatant to 45% was added and the precipitate which formed was recovered by centrifugation. This last fraction is designated as 35-45. All three precipitates were each washed once by resuspension in a small volume of 30%, 35% or 45% SAS solution respectively and then centrifuged. The wash fluids were discarded. Each precipitate was dissolved in distilled water and the fractions were immediately desalted by gel filtration on Sephadex G-25.

DEAE-Sephadex A 50 batch chromatography

The globulin fraction of allergic serum Gr obtained at 45% saturation with ammonium sulfate, and those of allergic sera Gre and Kh obtained at saturations of 30% and 35-45% were individually stirred with the moist DEAE-Sephadex for 1 hour at 4°C. A ratio of 500 mg of protein (globulin fraction) to 1 g dry weight of DEAE-Sephadex A 50 was used. The resulting gel-protein slurry was filtered through a Buchner funnel and washed with the eluting buffer, 0.1M Tris-HCl, pH 8, containing 0.5M sucrose. For elution of the next protein fraction the washed gel was then resuspended in the next buffer of increasing molarity and the

procedure for stirring, filtering and washing of the gel was repeated. The buffers contained 0.5M sucrose and increasing concentrations of Tris-HCl. Fractions eluted with 0.1M, 0.2M, 0.3M, 0.4M and 0.6M Tris-HCl buffers, pH 8.0 will be referred to by Roman numerals as I, II, III, IV and VI respectively. The filtrates, together with the corresponding washings were concentrated by "dialysis" against dry sucrose, dialysed against PBS, and reconstituted to the original serum volume. A flow sheet illustrating the entire fractionation procedure is shown in Figure 3. Skin-sensitizing activity of the allergic serum fractions was determined in duplicate by the P-K test.

Prausnitz-Kustner passive transfer (P-K) tests

Skin sites on the backs of normal non-allergic human volunteers were sensitized by intradermal injections with 0.05 ml of the allergic serum samples. Twenty-four hours later, these sites were challenged by injecting 0.025 ml of WSR (1000 protein nitrogen units/ml). Twenty minutes after challenge the positive reactions were graded from 1+ to 4+, according to the size of the wheal and the surrounding flare.

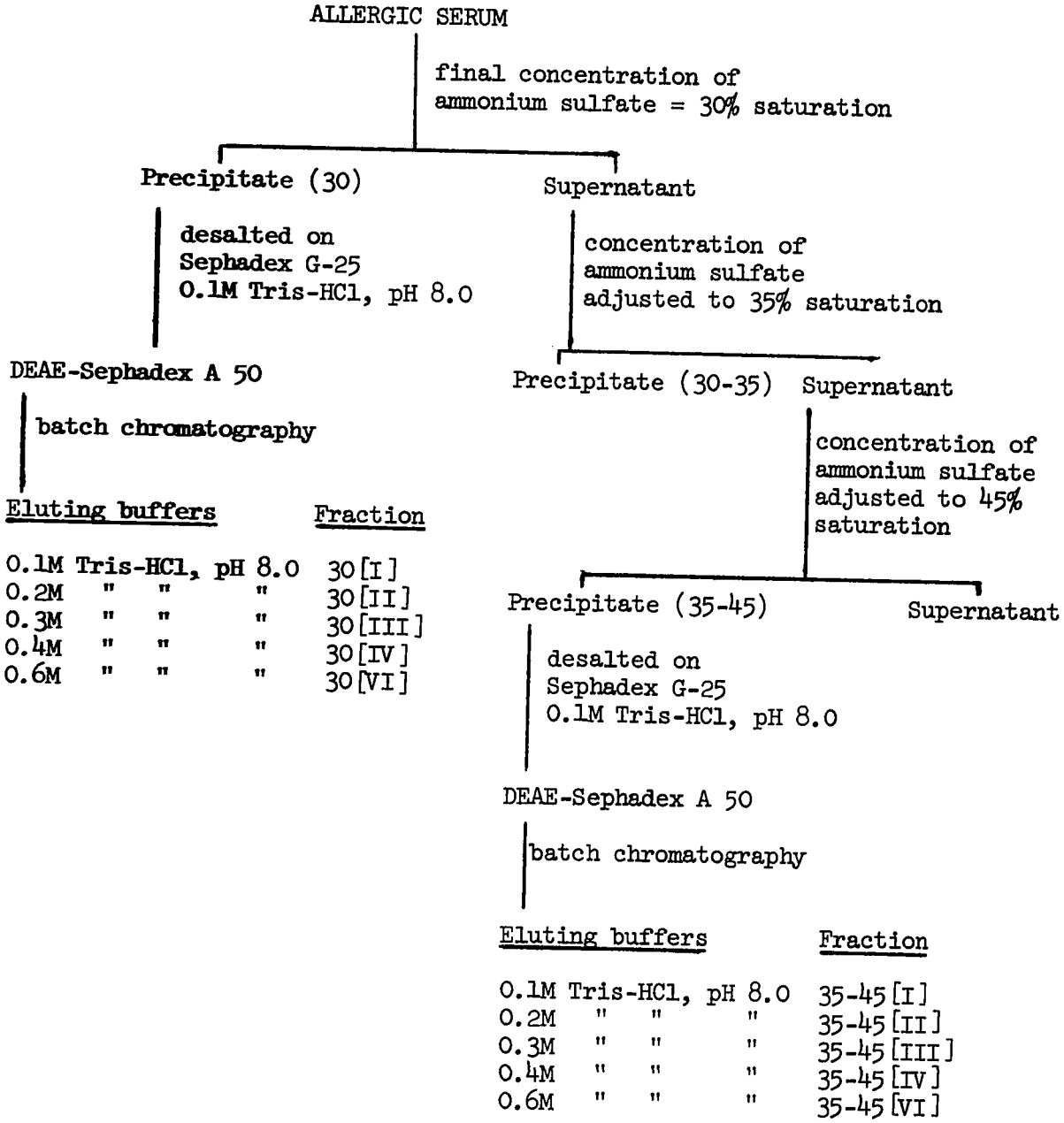
RESULTS

Fractionation of γA myeloma serum with ammonium sulfate at increasing concentrations

The salting-out of proteins from the myeloma serum was first observed when the saturation with ammonium sulfate reached a minimum level of 28.5% (Table II). IgG was the first immunoglobulin to be salted-out and

FIGURE 3

Flowsheet for the fractionation of allergic sera by salting-out with ammonium sulfate at increasing concentrations, followed by ion-exchange chromatography on DEAE-Sephadex A 50. Elution performed with Tris-HCl, pH 8.0 buffers containing sucrose, 0.5M.



Eluting buffers	Fraction
0.1M Tris-HCl, pH 8.0	30 [I]
0.2M " " "	30 [II]
0.3M " " "	30 [III]
0.4M " " "	30 [IV]
0.6M " " "	30 [VI]

Eluting buffers	Fraction
0.1M Tris-HCl, pH 8.0	35-45 [I]
0.2M " " "	35-45 [II]
0.3M " " "	35-45 [III]
0.4M " " "	35-45 [IV]
0.6M " " "	35-45 [VI]

TABLE II

Fractionation of a human γA myeloma serum with ammonium sulfate at increasing concentrations

Total Volume (ml) SAS* added to 10 ml serum**	Final % Saturation with ammonium sulfate	Amount*** of Globulin	
		IgA	IgG
3.5	25.9	-	-
4.0	28.5	-	+++
4.5	31.0	-	++++
5.0	33.3	+	+++
5.5	36.7	++	++
6.0	37.5	++++	+
6.5	39.4	+++	-
7.0	41.4	+++	-
7.5	42.8	++	-
8.0	44.4	+	-
8.5	45.9	-	-
9.5	48.7	-	-
11.5	53.4	-	-
13.5	56.4	-	-

* SAS = saturated ammonium sulfate solution, pH adjusted to 7 with NaOH.

** Serum from a patient with myeloma with elevated levels of serum IgA.

*** Relative amount of immunoglobulin in arbitrary units estimated from immunoelectrophoretic analysis.

was found in all fractions obtained in the range of 28.5 to 37.5% saturation with ammonium sulfate. IgA was detected in fractions salted-out between 33.3 and 44.4% saturation. Thus, a small portion of IgG was separated from IgA by this procedure. The results indicated that IgA could be salted-out completely at 45% saturation with ammonium sulfate.

Fractionation of the NHS pool by a single salting-out procedure

At 45% saturation with ammonium sulfate, all the γA-globulins were precipitated from the serum. By immunoelectrophoresis, it was confirmed that the remaining proteins in the supernatant were devoid of IgA.

Column chromatography of the NHS globulin fraction (NHS [45]) on DEAE-cellulose

Typical optical density profiles of the step-wise elution with the 5 phosphate buffers at constant pH are illustrated in Figure 4. A major portion of IgG was eluted with the initial buffer I . IgG and IgA were recovered in eluate II. IgA was the only immunoglobulin detected by immunoelectrophoresis in eluate III (Figure 5, part a); approximately 10 mg of IgA, which represented 10-15% of the total serum concentration, was isolated from an original volume of 50 ml of NHS. Although IgA was also recovered in eluate IV, this chromatographic fraction was found to contain proteins with the electrophoretic mobility of β-globulins. Transferrin and α_2M-globulins were eluted with buffer V.

Ultracentrifugal analysis of the isolated IgA preparation

A single peak only was observed during the entire ultracentrifugation period of 80 minutes at maximum rotor speed (Figure 6). The sedimentation

FIGURE 4

Chromatography on DEAE-cellulose (2.5 x 30 cm column) of normal human serum fraction NHS [45] using step-wise elution with phosphate buffer at constant pH and increasing ionic strength.

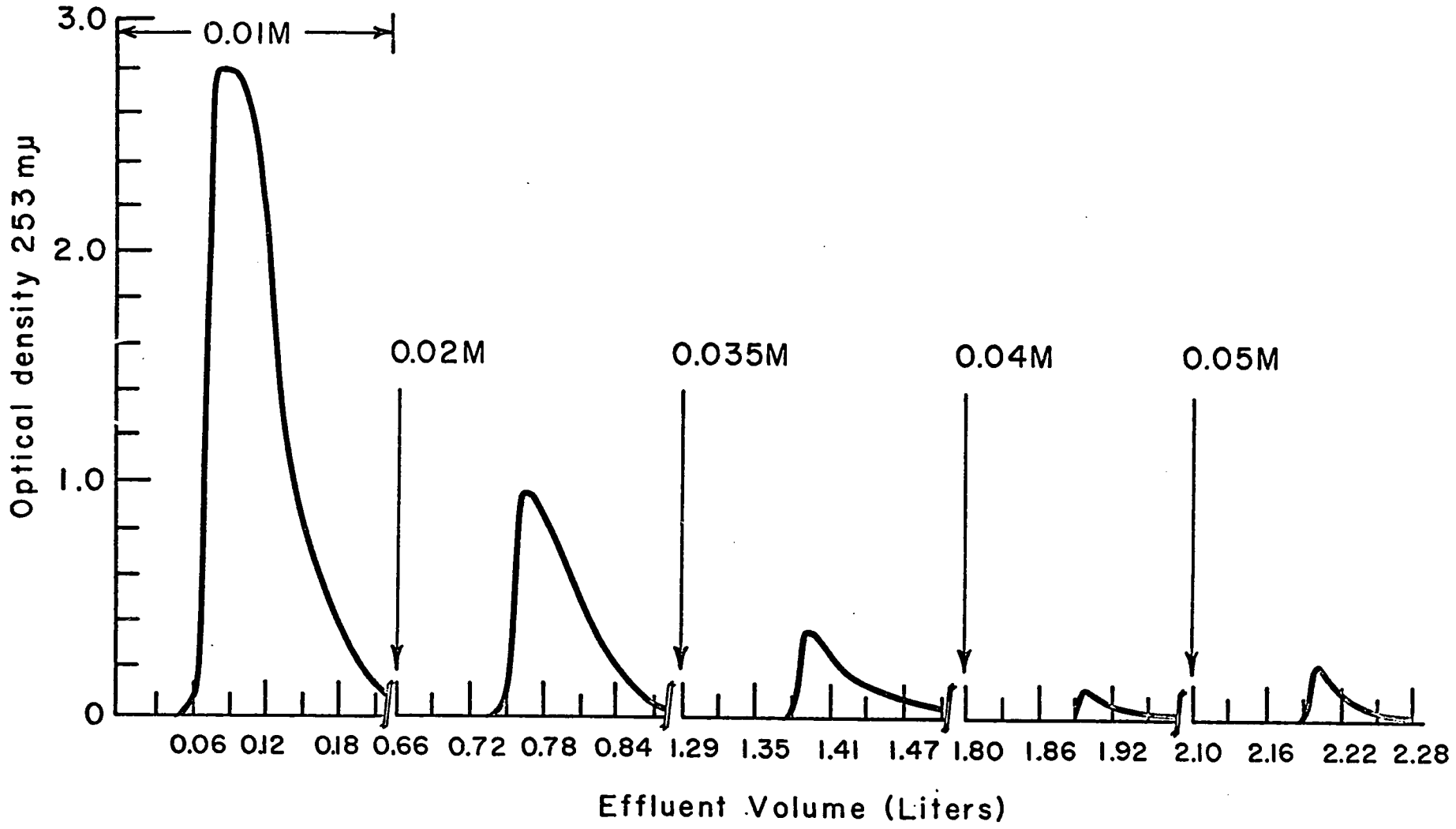

3.0
2.0
1.0
0
Optical density 253 mμ
0.01M
0.02M
0.035M
0.04M
0.05M
0.06 0.12 0.18 0.66 0.72 0.78 0.84 1.29 1.35 1.41 1.47 1.80 1.86 1.92 2.10 2.16 2.22 2.28
Effluent Volume (Liters)

FIGURE 5

Immunoelectrophoretic analysis in agar with veronal buffer $\Gamma/2 = 0.033$, pH 8.6; electrophoresis at 9.25 V/cm for 50 minutes.

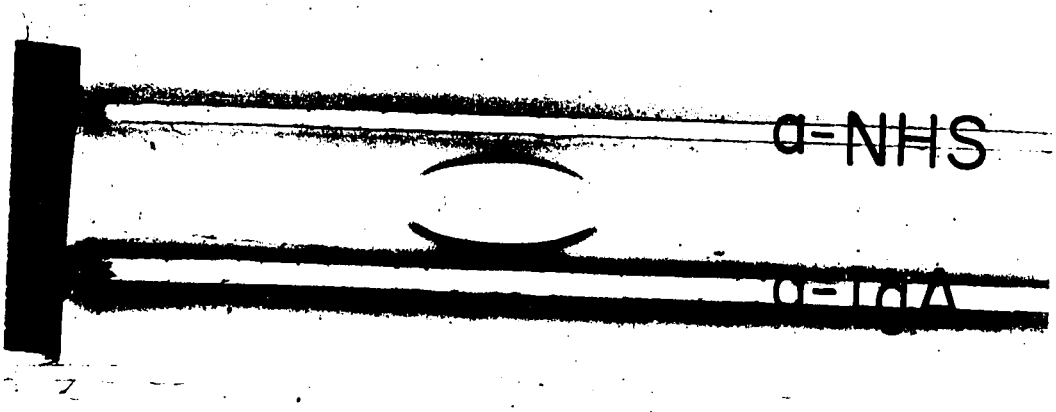
(a) Immunoelectrophoretic pattern of serum fraction NHS 45 [III]; antibody troughs were filled with a horse antiserum to whole human serum (a-NHS) or, rabbit antiserum to human IgA (a-IgA).
(b) Immunoelectrophoretic analysis of a rabbit antiserum (a-NHS 45[III]) produced by hyperimmunization with serum fraction NHS 45 [III]. Center well was filled with NHS prior to electrophoresis.

(a)

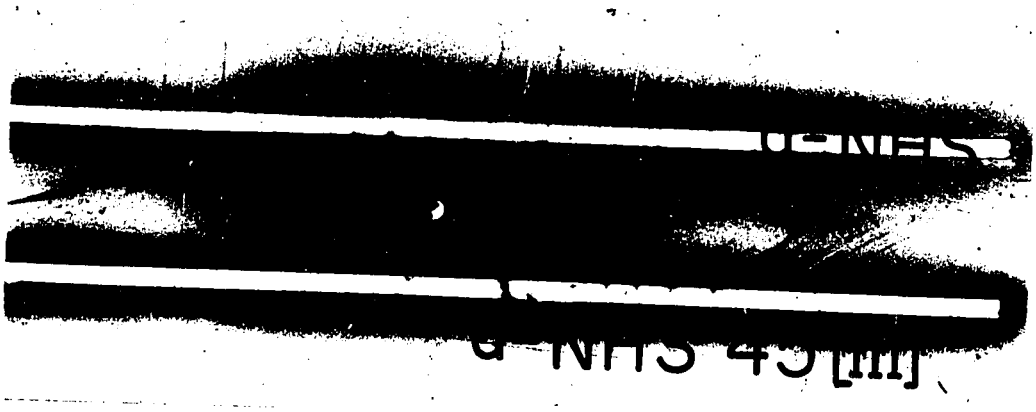
(b)

⊕ ⊖

(a)



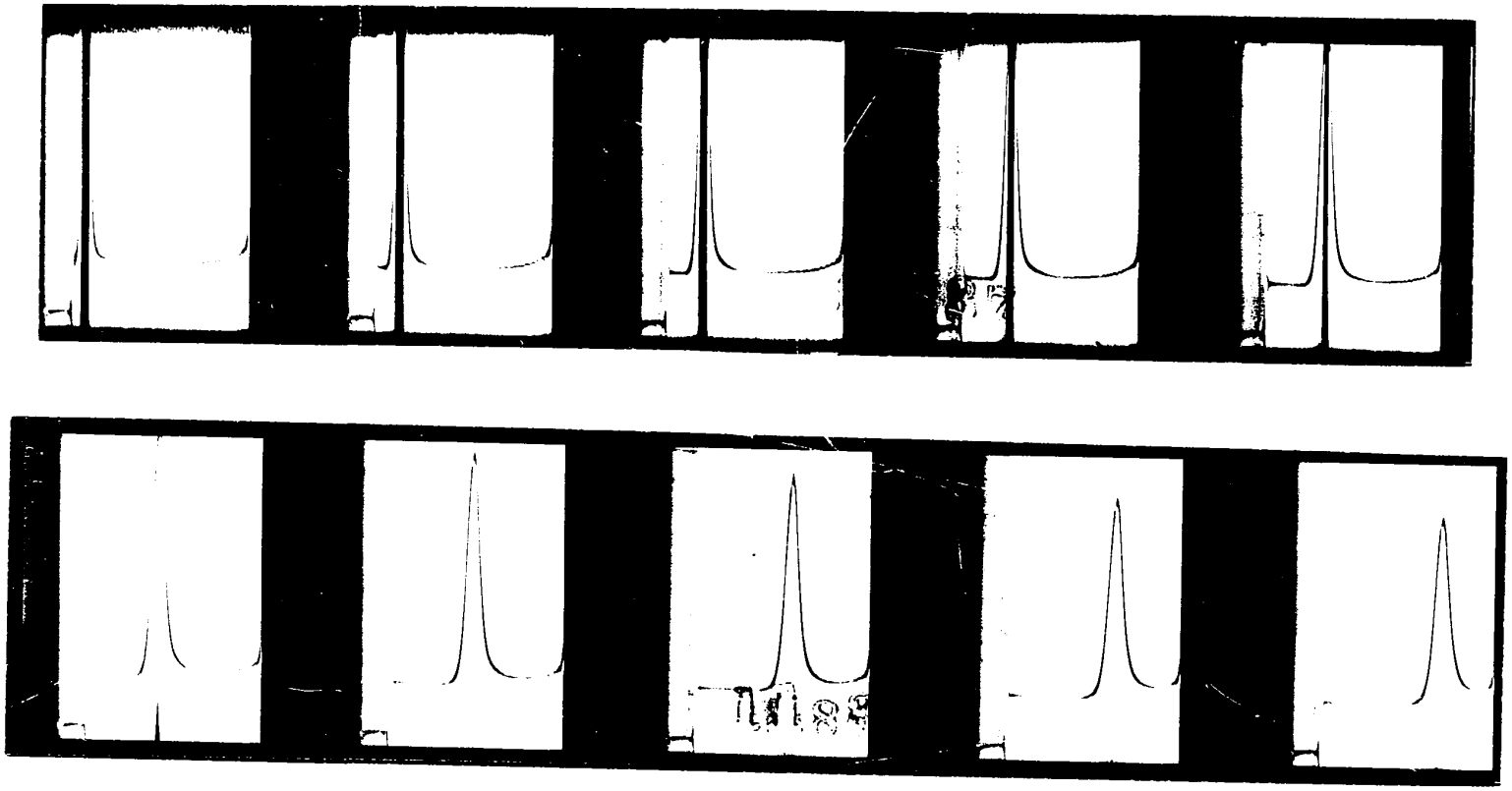
(b)



⊕ ⊖

FIGURE 6

Ultracentrifugal sedimentation pattern for serum fraction NHS 45 [III] in saline; sedimentation from left to right; concentration of protein = 1 g%; centrifugation at 59,780 r.p.m.; pictures taken at intervals of 8 minutes.



coefficient of the IgA preparation was calculated to be 7.1S.

Vertical starch gel electrophoresis combined with immunodiffusion analysis in agar

At least 12 differentially stained areas could be distinguished for the electrophoresed NHS (Figure 7). A single diffusely stained area, anodal to the point of application, was observed for the electrophoresed IgA preparation. The identification of IgA from NHS in the stained gel was not possible because of the presence of other globulins with similar electrophoretic mobilities which contributed to the staining of the same general area. A positive correlation between IgA and this stained area was made with immunodiffusion analysis in agar by comparing the positions of the precipitin arcs which formed between IgA and a rabbit antiserum specific to IgA. Moreover, with a polyvalent antiserum to NHS, IgA was the only protein detected in the purified IgA preparation. The only discernible difference between the purified IgA and the IgA present in NHS was that the precipitin arc formed with the latter was longer by approximately 2 mm in the anodal portion. This observation suggested that the isolated IgA was less heterogeneous with respect to size and/or charge distribution than the native population of serum proteins with antigenic determinants of IgA.

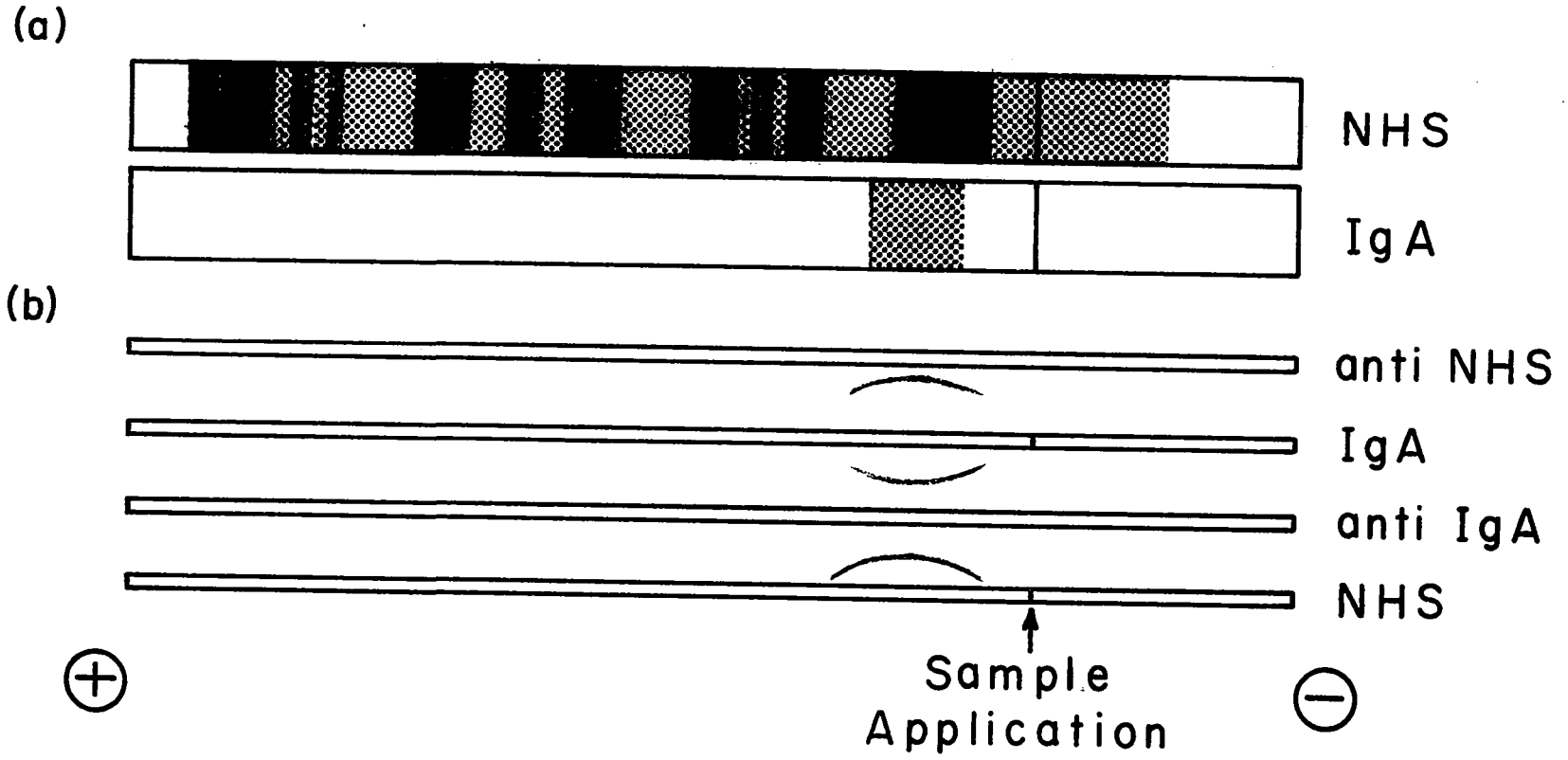
Hyperimmunization with purified IgA

By means of the immunoelectrophoretic technique the antiserum produced by repeated immunization with the purified IgA preparation was demonstrated to contain antibodies to IgA, IgG, transferrin and α_2-macroglobulins. Therefore, it must be concluded that the other proteins to which antibodies had been produced must have been present in the

FIGURE 7

Diagramatic representation of vertical starch gel electrophoresis and of immunodiffusion analysis in agar.

(a) immunodiffusion analysis
(b) starch gel stained with amido black to reveal the protein zones


(a)
NHS
IgA
(b)
anti NHS
IgA
anti IgA
NHS
Sample
Application

original IgA preparation, but in concentrations too low to be detected directly by immunoelectrophoretic analysis.

Desalting on Sephadex G-25

It was found that samples with volumes not exceeding one-third of the bed volume of the packed Sephadex G-25 could be rapidly desalted and re-equilibrated with other buffers. Substantially lower amounts of precipitate (if any) formed following desalting by gel-filtration on Sephadex G-25 as compared with the amount of precipitate formed in an identical ammonium sulfate fractionated serum preparation which was desalted by dialysis against PBS. From these observations it was inferred that desalting by gel filtration had reduced denaturation to a minimum and hence this technique was employed in all subsequent experiments requiring desalting and reequilibration with other buffers.

Batch chromatography of the globulins from allergic sera (AS[45]) on DEAE-Sephadex A 50

The order of elution of immunoglobulins IgG, IgA and IgM from DEAE-Sephadex A 50 employing the batch elution procedure, was the same as from DEAE-cellulose. IgG was the only immunoglobulin separated from the other two (IgA and IgM); its presence was detected in all fractions. The recoveries* of IgG were of the order of 50 to 62% (Table III).

The elution profiles of IgA varied with the three allergic sera chromatographed. Thus, with serum We, IgA was first detected in eluate 45-II, whereas with Gr and Wh, IgA was eluted later in eluates 45 [III]. The recovery* of IgA from sera Gr and We was of the order of 56 to 67%. For ion-exchange chromatography on DEAE-cellulose, the lowest concentration of phosphate employed was 0.01M; for DEAE-Sephadex this value was higher

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*Since elution with the 0.6M Tris-HCl buffer, pH 8.0 was not performed in the chromatography of allergic serum Wh, the yields of reagin and of the three immunoglobulins have not been incorporated into these values.

TABLE III

Distribution of P-K activity and immunoglobulin concentrations in eluates following batch chromatography of the globulins of allergic sera (AS [45]) on DEAE-Sephadex A 50

Serum Gr, DEAE Sephadex eluate	P-K Titer	Immunoglobulin concentration* (mg%) γG	γA	γM
I	0	400	0	0
II	160	375	0	0
III	320	140	25	10
IV	80	80	60	42
VI	0	12	10	28
Original serum	800	1500	155	210
reconstituted**	500			
Recovery (%)	62	67	61	38

Serum We, DEAE-Sephadex eluate	P-K Titer	Immunoglobulin concentration* (mg%) γG	γA	γM
I	0	310	0	0
II	100	270	6	4
III	200	110	30	12
IV	100	56	25	36
VI	0	6	12	20
Original serum	800	1350	170	100
reconstituted**	400			
Recovery (%)	50	56	43	72

Serum Wh, DEAE-Sephadex eluate	P-K Titer	Immunoglobulin concentration* (mg%) γG	γA	γM
I	100	290	0	0
II	800	200	0	0
III	400	140	17	0
IV	200	40	17	0
Original serum	2400	1200	180	56
reconstituted**	1200			
Recovery (%)	50	59	19	0

* Immunoglobulin concentrations determined by means of single radial immunodiffusion technique with specific antibody incorporated into agar (Immuno-Plate).

** P-K titers determined from a pool of aliquots from each fraction, with the final volume adjusted to equal the original serum volume.

by a factor of 10. Thus, with higher salt concentration, euglobulins including IgM remained in solution and thereby contributed to their greater recovery following ion-exchange chromatography. IgM was eluted simultaneously with IgA for allergic sera Gr and We; however, in marked contrast to the high recovery of IgM (72%) from serum We, IgM was not recovered from serum Wh.

Reaginic activity was detected in all fractions of allergic serum Wh but was absent from eluates 45[I]and[IV]for allergic sera Gr and We. No obvious correlation between the distribution of reaginic activity and of immunoglobulins IgG, IgA, or IgM could be made. Approximately 50 to 62% of the original reaginic activity was recovered following the fractionation of allergic sera.

Fractionation of allergic sera with ammonium sulfate at increasing concentrations of ammonium sulfate followed by ion-exchange chromatography on DEAE-Sephadex A 50

The distribution of reaginic activity and of immunoglobulins IgG, IgA and IgM following batch chromatography of allergic serum Kh; fractions Kh 30 and Kh 35-45 are shown in Table IV part (a) and (b) respectively; and similar distributions for allergic serum Gre, fractions Gre 30 and Gre 35-45 are given in Table V part (a) and (b) respectively.

A common observation with both allergic sera was that higher amounts of IgG were eluted on ion-exchange chromatography of fractions 30 than of fractions 35-45. On the other hand, for the latter fraction, higher recoveries of IgA, IgM and of reaginic activity were obtained. However, the elution profiles of reaginic activity were not similar for the two allergic sera; the highest P-K titer was found in eluate 35-45 [II] for serum Kh and in eluate 35-45 [III] for allergic serum Gre. Moreover,

TABLE IV

(a) Distribution of reagins and immunoglobulins following batch chromatography of allergic serum fraction Kh 30 on DEAE-Sephadex A 50

DEAE-Sephadex eluate	P-K Titer	Immunoglobulin concentration (mg%) γG	γA	γM
I	0	287	0	0
II	0	173	2	0
III	0	40	12	1
IV	0	2	4	5
VI	0	0	0	0

(b) Distribution of reagins and immunoglobulins following batch chromatography of allergic serum fraction Kh 35-45 on DEAE-Sephadex

DEAE-Sephadex eluate	P-K Titer	Immunoglobulin concentration (mg%) γG	γA	γM
I	10	56	0	0
II	200	25	5	0
III	100	10	47	11
IV	50	0	13	15
VI	0	0	2	10

The P-K titer given by allergic serum Kh before fractionation was 6400. The original concentration of immunoglobulins was as follows: IgG, 1500 mg%; IgA, 215 mg%; IgM 110 mg%.

TABLE V

(a) Distribution of reagins and immunoglobulins following batch chromatography of allergic serum fraction Gre 30 on DEAE-Sephadex A 50

DEAE-Sephadex eluate	P-K Titer	Immunoglobulin concentration (mg%) γG	γA	γM
I	10	380	0	0
II	20	212	2	0
III	20	33	4	0
IV	10	16	3	12
VI	0	2	5	8

(b) Distribution of reagins and immunoglobulins following batch chromatography of allergic serum fraction Gre 35-45

DEAE-Sephadex eluate	P-K Titer	Immunoglobulin concentration (mg%) γG	γA	γM
I	10	18	0	0
II	100	11	1	0
III	300	9	12	13
IV	80	4	32	27
VI	0	2	7	13

The P-K titer given by allergic serum Gre before fractionation was 3200. The original concentration of immunoglobulins was as follows: IgG, 1110 mg%; IgA, 250 mg%; IgM, 115 mg%.

while low but definite reaginic activity was detected in eluates Gre-30 [I]to [IV] inclusive, the corresponding eluates of serum Kh were devoid of reagins.

The chromatography of serum fraction 30 revealed that higher recoveries of IgG in eluate I were obtained from serum Gre than for serum Kh; the amounts were 380 mg% and 287 mg% respectively. The higher yields of IgG could not be attributed simply to higher serum levels of IgG since serum Gre actually contained lower amounts of IgG than serum Kh. Conversely, with serum Gre, which possessed a higher concentration of IgA, the total amount of IgA recovered from fraction Gre 35-45 was 52 mg as compared to 67 mg of IgA recovered from fraction Kh 35-45. Thus, the variation in profiles of elution and recovery of the immunoglobulins depended on their individual physicochemical nature and was independent of the serum immunoglobulin concentrations.

DISCUSSION

At the beginning of this project the method employed for the isolation of IgA was the precipitation procedure with zinc sulfate developed by Heremans et al (75). Briefly, it consisted of multiple steps involving the removal of euglobulins by dialysis against low ionic strength buffer followed by: (i) precipitation with zinc sulfate and removal of the salted-out proteins by filtration through filter paper, (ii) 'spontaneous' precipitation induced by raising the temperature and removal of the precipitated proteins by centrifugation, (iii) preparative electrophoresis of the supernatant proteins and (iv) precipitation with ammonium sulfate to recover the IgA. However, in the present investigation

consistent failures to obtain the 'spontaneously' forming precipitate were experienced. Moreover, the steps involving filtration and centrifugation, as described by Heremans did not prove to be satisfactory for the complete removal of the precipitates which did form.

Another method for the isolation of IgA had been developed by Havez et al (241), who employed salting-out at neutral and acid pH. IgA was separated from other proteins by ion-exchange chromatography on DEAE-cellulose and elution with phosphate buffers at constant pH and stepwise increasing molarity. This procedure proved to be reproducible, but the authors cautioned that the IgA isolated represented only a small fraction of the total heterogenous population of γA-globulins. Their low yields of IgA could be attributed, in part, to losses which occurred at each fractionation step. Thus, from a consideration of the methods available for the isolation of IgA at the time this study was initiated, it was clear that an ideal procedure should preferably consist of only a minimum number of fractionation steps and it was visualized that to preserve the activity of reagins one would have to avoid (i) elevated temperatures, (ii) low pH and/or (iii) prolonged contact with solutions of high salt concentration.

To develop simple and efficient methods for the isolation of IgA, a myeloma serum with elevated levels of γA-globulins (and normal levels of IgG) was selected for preliminary experiments designed to determine the concentration of ammonium sulfate at which salting-out of the γA-globulins from solution occurred. It was observed from immunoelectrophoretic analysis that the myeloma γA-globulins possessed electrophoretic mobility (and therefore electrical charge) similar to the IgA from normal

human sera. The high concentration of γA-globulins facilitated their detection in the serum fraction obtained by sequentially increasing the concentration of ammonium sulfate. Since only a small amount of protein, consisting of IgG was precipitated in the range of 28.5 to 31% saturation with SAS, an initial salting-out step to effect a partial separation between γG- and γA-globulins was not performed at this stage. Instead, a single salting-out procedure employing ammonium sulfate at a final concentration corresponding to 45% saturation was used to precipitate essentially all of the IgA and to provide an enriched preparation for subsequent purification procedures. Ion-exchange chromatography on DEAE-cellulose was successful for the isolation of only a small portion of the total IgA from normal human sera. Even though extensive washing of the DEAE-cellulose followed the elution of the major portion of IgG, this procedure was still inadequate for the complete removal of IgG from subsequent chromatographic fractions. Owing to the similarity in their net electrical charge some of the IgA and IgG were eluted simultaneously. Similarly, IgA was eluted with globulins possessing a higher net negative electrical charge such as haptoglobulins and transferrin. Since IgA could not be recovered from such mixtures, it is obvious that separation of IgA from globulin mixtures on the basis of differences in their charge is not an efficient method. Moreover, from an identification of the antigenic components which reacted with antibodies produced by hyperimmunization with apparently "pure" IgA, it was evident that the combination of procedures employing salting-out and ion-exchange chromatography were not sufficient to resolve the globulin mixtures into immunologically pure IgA preparations.

Theoretically, immunization with a pure immunoglobulin preparation, e.g. IgA, would elicit the formation of antibodies directed to the characterisitic antigenic determinants of the corresponding immunoglobulin class. (In addition, antibodies would also be produced to the antigenic determinants located on the light polypeptide chains which are common to all immunoglobulin molecules). However, in spite of elaborate isolation and purification techniques employed for the isolation of IgA, the antisera produced always required absorption with other globulins to render the antisera specific for IgA. These problems were compounded by the fact that the globulin preparation used for the absorptions were themselves immunologically impure (with the possible exception of IgG which is the first immunoglobulin to be eluted in ion-exchange chromatography and is therefore not contaminated with other proteins).

Due to the low concentration of skin-sensitizing antibodies, they had escaped detection by standard physicochemical techniques which similarly were grossly inadequate to detect trace protein contaminants in the "purified" IgA preparations. Thus, it is not surprising that divergent results were obtained in different investigation, in which apparently specific antisera had been used to remove selectively IgA from fractions of sera enriched with reagins. In some studies, skin-sensitizing activity was co-precipitated with IgA (188, 194), whereas in another study similar absorptions of IgA did not alter the reaginic activity (205, 208). From a consideration of the results of the present study it may be postulated that low concentrations of unidentified protein(s) present in "purified" immunoglobulin preparations, elicited the production of the homologous antibodies, which were in fact responsible for precipitating reagins.

Although polymeric forms of IgA exist in normal sera (76) the bulk is comprized of 7S γA-globulins. Thus, it was expected that the isolated IgA would possess a sedimentation coefficient value of approximately 7S. Attempts to demonstrate polymeric forms of IgA by starch gel electrophoresis were not successful. The smooth continuity of the IgA-anti-IgA precipitin arcs observed by immunodiffusion analysis of the electrophoresed proteins, indicated that there were no sharp zones of concentrations due to polymers of IgA, and it was concluded that the IgA which was isolated from NHS was composed of the 7S form only. On the other hand, a diffusely stained area of starch gel, obtained with the purified IgA, suggested that these globulins were electrophoretically heterogeneous. By comparison with γA-globulins of unfractionated NHS, the mobility of the purified IgA preparation was somewhat restricted to the more cathodic globulins. This result was consistent with the observations that more IgA could be eluted with buffers of increased ionic strength, i.e. the more negatively charged IgA with greater anodic mobility.

A simple and rapid method employing (i) salting-out with ammonium sulfate, (ii) desalting and re-equilibration with other buffers by gel filtration and (iii) ion-exchange batch chromatography on DEAE-Sephadex A 50 with elution at constant pH and step-wise increasing molarity, was developed for the fractionation of allergic sera. Using such procedures a maximum recovery of reaginic activity of the order of 62% was achieved. This value is twice as high as the recovery reported for experiments using chromatography on DEAE-cellulose (187). A subsequent modification to this fractionation procedure was attempted by using three different concentrations of ammonium sulfate to separate reagins from IgG blocking

antibodies. The fractionation was continued as before by ion-exchange chromatography on DEAE-Sephadex and resulted in a 20 fold enrichment in the specific reaginic activity.

Although allergic sera Kh and Gre were fractionated simultaneously under identical conditions, the elution profiles of reaginic activity and of the immunoglobulins IgG, IgA and IgM were different for each serum. These observations illustrated the heterogeneity of both reagins and immunoglobulins. Fractions of allergic sera possessing the highest skin-sensitizing activity always contained IgG, and 4 out of 5 allergic sera also contained IgA. However, since the high reaginic activity did not parallel the distribution of either immunoglobulin it was concluded that reagins were not associated with either IgG or IgA. This conclusion was also supported by the observations that the values for the recovery of reaginic activity did not parallel those of IgG, IgA or IgM but were intermediate to the recoveries of IgG and IgA. Moreover, these results suggested that immunoglobulins with ion-exchange elution properties intermediate to those of IgG and IgA were the actual carriers of reaginic activity. In more recent studies allergic sera were fractionated by column chromatography on DEAE-Sephadex A 50 using gradient elution (206, 242). The bulk of reaginic activity was found in fractions containing little or no IgA and the rest was found in separate fractions containing both IgG and IgA (208). The results of fractionations of allergic sera with rivanol (243) or sodium sulfate (244) also suggested that the majority of reagins were not associated with the bulk of IgA. The mounting evidence to support the notion that reaginic activity was not associated with IgA (201, 202, 203) strongly suggested that the experimental

approach in the investigations designed to establish the nature of reagins be modified. In particular, since the techniques employed were obviously incapable of separating proteins with similar physicochemical properties, the isolation of proteins employing specific immunochemical methods seemed more promising. These attempts are described in the next chapter.

CHAPTER IV

ISOLATION OF REAGINS WITH SPECIFIC IMMUNOSORBENTS

SECTION A IMMUNOSORBENTS PREPARED WITH ETHYLENE-MALEIC ANHYDRIDE COPOLYMER

Introduction

Skin-sensitizing antibodies in common with other antibodies, can be specifically and completely removed from the sera of ragweed allergic individuals with appropriate immunosorbents (171, 216, 217, 218, 233, 245). In this procedure advantage is taken of the unique feature of antibodies to combine with their homologous antigens which are insolubilized either by being linked through stable covalent bonds to insoluble (and inert) supporting matrices through cross-linking with polyfunctional reagents. Since the coupling of antigens to the supporting matrix may involve the antigenic determinants themselves, or other groups closely situated to these determinants, and thus lead to immunochemically inactive products, the choice of the supporting medium with regard to the composition and structure depends primarily on the physicochemical properties of the antigen. In general, a good immunosorbent should satisfy the following criteria (i) the specificity of the insolubilized antigen as regards the structure of its determinant groups, should not be altered during the preparation of the immunosorbent, (ii) the immunosorbent should have a high capacity to absorb homologous antibodies without binding non-specifically other serum proteins and (iii) the adsorbed antibodies must be elutable without denaturation from the immunosorbent.

Adsorbed antibodies can be recovered by dissociation and/or elution from the immunosorbent by non-specific means, e.g. (i) by the addition of hydrogen bond breaking agents such as urea, (ii) by lowering the pH to 2-3, or (iii) by alteration of the ionic strength and dielectric constant of the medium. In addition, specific means involving the displacement of antibodies by the addition of univalent haptens have been recently employed, e.g. a hapten bearing one of the antigenic determinants of myoglobin was used to displace the homologous antibodies from an immunosorbent (246). Moreover, in an earlier study in this laboratory (245), skin-sensitizing antibodies were desorbed from erythrocyte-allergen conjugates by antibodies possessing a greater affinity for the antigenic determinants. Recently, a chemically simple and rapid procedure using ethylene-maleic anhydride copolymer (EMA) to insolubilize protein antigens was adapted for the preparation of high capacity immunosorbents (219, 247). In experiments to be described in this section, the effectiveness of the EMA immunosorbents was first evaluated with a model system using rabbit antibodies to ragweed pollen constituents, and the knowledge thus gained was applied to the isolation of reaginic antibodies from the sera of ragweed allergic individuals

MATERIALS AND METHODS

Preparation of water soluble ragweed extract

Approximately 50 g of short ragweed pollen (ambrosia artemisiaefolia) was defatted with diethyl ether at room temperature in a Soxhlet extraction apparatus for 24-48 hours until all of the ether soluble pigmented material was removed. The ether was then evaporated off at reduced

pressure in a desiccator. The dry ether extracted pollen was stirred with 500 ml of distilled water for two days at 4°C. The insoluble material was removed by filtration on a Buchner funnel, and the water extract (WSR) was clarified by centrifugation at 15,000 X G for 20 minutes at 4°C, and then frozen and lyophilized.

To prepare the dialysed water soluble extract of ragweed pollen (DWSR), lyophylized WSR was dissolved in distilled water, insoluble material removed by centrifugation at 15,000 X G for 20 minutes, the supernatant dialysed exhaustively against distilled water and finally lyophilized.

Preparation of low molecular weight dialysable components from ragweed

(A) Two methods were employed for the isolation of low molecular weight components from aqueous extracts of ragweed pollen :

(i) WSR was dialysed against distilled water. The dialysate containing the ragweed components with molecular weight extending to approximately 6000 was concentrated by lyophilization, dissolved in a small volume of distilled water, applied to a column of Bio-Gel P-2 and eluted with water. The first half of the total ragweed constituents eluted were pooled and lyophilized. This preparation was designated Fraction A*.

(ii) Diaflo** apparatus and the UM2 Diaflo membranes with a 'cut off' range for substances with molecular weight above 1,000 was employed for the ultrafiltration of WSR. The ultrafiltrate with molecular weight below 1,000 is referred to as U-F*.

(B) Casein* (100 ml of 1% solution in 0.1M ammonium bicarbonate) was digested with α-chymotrypsin (10 mg in 0.001N HCl) for 6 hours at 37°C.

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*The author is grateful to Dr. N.A. Attallah for the preparation of ragweed Fraction A and casein digest, and to Dr. E.R. Centeno for preparation of U-F.

**Diaflo, Amicon Corporation, Mass. U.S.A.

The mixture was lyophilized, dissolved in a small volume of distilled water and dialysed against distilled water. The dialyste was concentrated by lyophilization.

Preparation of rabbit antiserum to DWSR

Antiserum to DWSR was prepared in rabbits by repeated intradermal injections with an emulsion of DWSR (10 mg) in complete Freund's adjuvant.

Hemagglutination test (micro)*

The bis-diazotized benzidine (BDB)-hemagglutination micro technique was employed to assess the titers of ragweed antisera (216, 218, 233). For the sensitization of the erythrocytes, i.e. for the attachment of the antigen to rabbit red blood cells (RBC), a volume of 0.1 ml of a 1 : 1 suspension of rabbit RBC in PBS was dispensed in 3 ml of the appropriate antigen solution to which the optimum amount of BDB** was added and the mixture was incubated at room temperature for 15 minutes. The sensitized cells were washed twice with diluent***, resuspended in 5 ml of diluent, and volumes of 0.05 ml were added to antisera (0.05 ml) in two-fold serial dilutions. Sensitized cells added to diluent served as controls.

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* Microtit Kit-Metrimpex, Budapest, Hungary.

** BDB = 0.3214 g benzidine dihydrochloride dissolved in 45 ml HCl (0.11M) at 0°C, and 0.175 g $NaNO_2$ in 5 ml water at 0°C added dropwise. After 30 minutes, 1.3 ml portions were dispensed into vials and quick frozen in dry ice acetone mixture. For use, the BDB was quickly thawed and 1 ml was added to 14 mls PBS and was used immediately.

*** Diluent was prepared by diluting 1 ml of the decomplemented serum (from the same donor which supplied the erythrocytes)to 100 ml with PBS.

To remove non-specific agglutinins, all antisera were incubated with an equal volume of packed, non-sensitized rabbit erythrocytes (washed three times with cold PBS) for 1 - 2 hours at room temperature. The cells for this absorption, as well as for the sensitization with the antigen were always from the same rabbit donor. The optimum amounts of antigen and BDB solution were established with a constant amount of erythrocytes in the following way, (i) the BDB solution volume was varied keeping the antigen concentration constant, and (ii) the antigen concentration was varied and the BDB solution volume was kept constant. Each batch of sensitized cells was then tested with the appropriate antisera and with diluent. The proportions of BDB to antigen were considered optimal when the highest titers were obtained with antisera and no reaction (agglutination) ensued with the diluent.

For the optimal sensitization the following amounts of materials were used: 0.1 ml of 1 : 1 suspension of red blood cells, 1.5 ml BDB solution and 3 ml of antigen solution in PBS, containing 2 mg/ml WSR, or 0.5 ml BDB solution and 0.5 mg/ml of DWSR.

Preparation of ethylene-maleic anhydride allergen conjugates (EMA immunosorbents)

Ethylene-maleic anhydride copolymer* was used as the backbone crosslinking material for the preparation of immunosorbents by coupling DWSR by the "graft" polymerization technique. Rabbit or human serum albumin (RSA or HSA) was used as part of the insoluble matrix, onto which DWSR was grafted for the preparation of immunosorbents to be

* EMA DX-840-31 was kindly supplied by Monsanto Limited (Canada).

used for the isolation of the antibodies from rabbit antisera and human allergic sera, respectively.

The immunosorbent was prepared* by copolymerizing RSA or HSA (50 mg dissolved in 50 ml of saline) with a small amount (1 ml) of EMA 1% in acetone solution at 0°C with constant stirring. After 30 minutes a DWSR solution (10 ml containing 500 mg) was added drop-wise followed by additional EMA solution (12 ml). The mixture was stirred for 1 hour at 0°C, and then extensively washed with PBS saline until no more protein was detected spectrophotometrically at 280 mμ in the wash liquids. To reduce the possibility of desorption of antigenic material during subsequent elution procedures, the immunosorbents were pretreated by washing extensively with 0.1M glycine-HCl buffer, pH 3 and with saline, until the eluates had no U.V. absorbing material.

Absorption of Antisera with EMA Immunosorbents

The washed pretreated immunosorbent was dispersed in Sephadex G-25 (coarse) and the slurry was packed in a column (2.5cm x 30cm). Several column volumes of PBS were passed through before use. The immunosorbent was saturated with antibodies by slowly passing an excess of antiserum through the column; an immunosorbent was judged to be saturated when the effluent (i.e. the serum passing through) had antibody activity as measured by either the ring test or hemagglutination for the rabbit antisera to DWSR, and P-K test for the human allergic sera.

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* The author is grateful to Dr. E.R. Centeno for the preparation of the EMA immunosorbents.

Absorption of normal human serum with EMA Immunosorbent

Normal human serum obtained from a non-allergic individual Ra, was also used to "saturate" the immunosorbents to minimize if not to eliminate any non-specific uptake of protein in the subsequent steps involving the specific antiserum. The immunosorbents were washed extensively with PBS until the optical density (O.D.) at 280 mμ of the eluate did not exceed a value of 0.020.

Elution of antibodies from EMA Immunosorbents

(i) Rabbit antibodies to DWSR:

Attempts were made to elute adsorbed antibodies from immunosorbents by displacement with the low molecular weight dialysable components of ragweed, Fraction A or U-F. Since there was a distinct possibility that the antibodies could be desorbed non-specifically due to changes in the dielectric constant and salt concentration of the medium on addition of Fraction A, the following experimental procedure was employed. An EMA ragweed immunosorbent which was saturated with rabbit antibodies was eluted sequentially with (i) a chymotryptic digest of casein (400 mg in a volume of 1.75 ml), (ii) low molecular weight ragweed components i.e. Fraction A (400 mg in a volume of 1.75 ml) and (iii) 2M NaCl to remove residual antibodies. The eluates were separately pooled, concentrated by "dialysis" against dry sucrose and then exhaustively dialysed against PBS. Protein concentrations were determined spectrophotometrically at 280 mμ. Antibody activity was assayed by the BDB micro-hemagglutination test.

(ii) Human antibodies to DWSR:

A sequential elution procedure was employed using first 2M NaCl solutions followed by re-equilibration with saline and second Fraction A in attempts to displace human antibodies from EMA immunosorbents saturated with antibodies to DWSR by prior absorption of allergic sera. These eluates were exhaustively dialysed against PBS.

In one experiment following elution with 2M NaCl and Fraction A or U-F a 0.1M glycine-HCl buffer, pH 2.5 was employed in a final attempt to displace skin-sensitizing antibodies from the immunosorbent. This eluate was adjusted to pH 7.4 with sodium bicarbonate and then concentrated to a small volume. All eluates were analysed by the P-K and micro-hemagglutination tests.

RESULTS

Elution of rabbit antibodies to DWSR from EMA immunosorbents*

Antibody activity was recovered in each case by elution with the casein digest, ragweed Fraction A, or 2M NaCl (Table VI). Thus, some antibodies could be eluted non-specifically with the casein digest and with 2M NaCl. However, the specific elution with low molecular weight ragweed components, i.e. Fraction A, was at least four times more active for displacing rabbit antibodies from the immunosorbent than either casein digest or 2M NaCl. Moreover, the specific hemagglutinating activity of the eluate, which was expressed as the hemagglutinating titer per mg of

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* The results of this investigation were presented at the 6th International Congress of Allergology in Montreal, November 1967 (248).

TABLE VI

Hemagglutinating activity (HA) of rabbit anti-ragweed antibodies eluted from immunosorbent.

	Protein (mg)	HA titer*
Amount applied	600	81,000
" not retained	460	40,960
" eluted (i) casein digest	2	40,960 (0.04)
(ii) Fraction A or U-F	6	327,680 (1.00)
(iii) 2M NaCl	18	40,960 (0.37)

* HA titer normalized to a protein concentration of 0.150 g%. Figures in parenthesis refer to HA activity relative to that obtained on elution with Fraction A or U-F, taken as unity.

protein eluted, was larger than that of the original serum. By immunoelectrophoretic analysis (Figure 8), it was demonstrated that IgG was the main protein recovered on elution with (i) casein digest, or (ii) ragweed Fraction A or (iii) 2M NaCl.

Elution of human antibodies from EMA immunosorbent

The O.D. profiles obtained by the sequential elution with 2M NaCl, Fraction A and 0.1M glycine-HCl buffer at pH 2.5 are illustrated in Figure 9. An increase in the O.D. of the second peak can be attributed to the U.V. absorbing ragweed polypeptides used for the elution, (a similar O.D. profile was observed with fraction U-F). The amounts of protein eluted are shown in Table VII. Since 210 mg of protein had been retained by the immunosorbent and a total of approximately 22 mg had been eluted, these yields indicated that approximately 188 mg of protein were still bound to the immunosorbent. None of the eluates contained skin-sensitizing or hemagglutinating activity. However, during the sensitization of the volunteers' skin for P-K testing, immediate wheal and flare reactions were observed on the intradermal injections of the eluates obtained with ragweed fraction A or U-F. These reactions were considered to be due to the presence of allergen-reagin complexes (A-R). To investigate this possibility, the following experiments were performed. Skin sites on a normal volunteer were sensitized with allergic serum and challenged 48 hours later with several ten-fold dilutions of these A-R eluates, (Table VIII, part a). In this case, positive wheal and flare reactions were elicited with the A-R eluate in dilutions as high as 1:200. No such reactions were observed when the eluates A-R (1:200 dilution) were used to challenge control skin sites "sensitized" with a normal human serum.

FIGURE 8

Immunoelectrophoretic pattern of eluates obtained from EMA immunosorbent saturated with rabbit antiserum to DWSR. Sample wells #1 were filled with whole rabbit antiserum to DWSR. Fractions obtained on elution with:

well (2) dialysable constituents of casein chymotryptic digest
well (3) ragweed Fraction A
well (4) 2M NaCl

Electrophoresis was performed in agar with veronal buffer, $\Gamma/2 = 0.033$, pH 8.6, at 9.25 V/cm for 40 minutes. Antibody troughs were filled with a sheep antiserum to rabbit serum proteins.

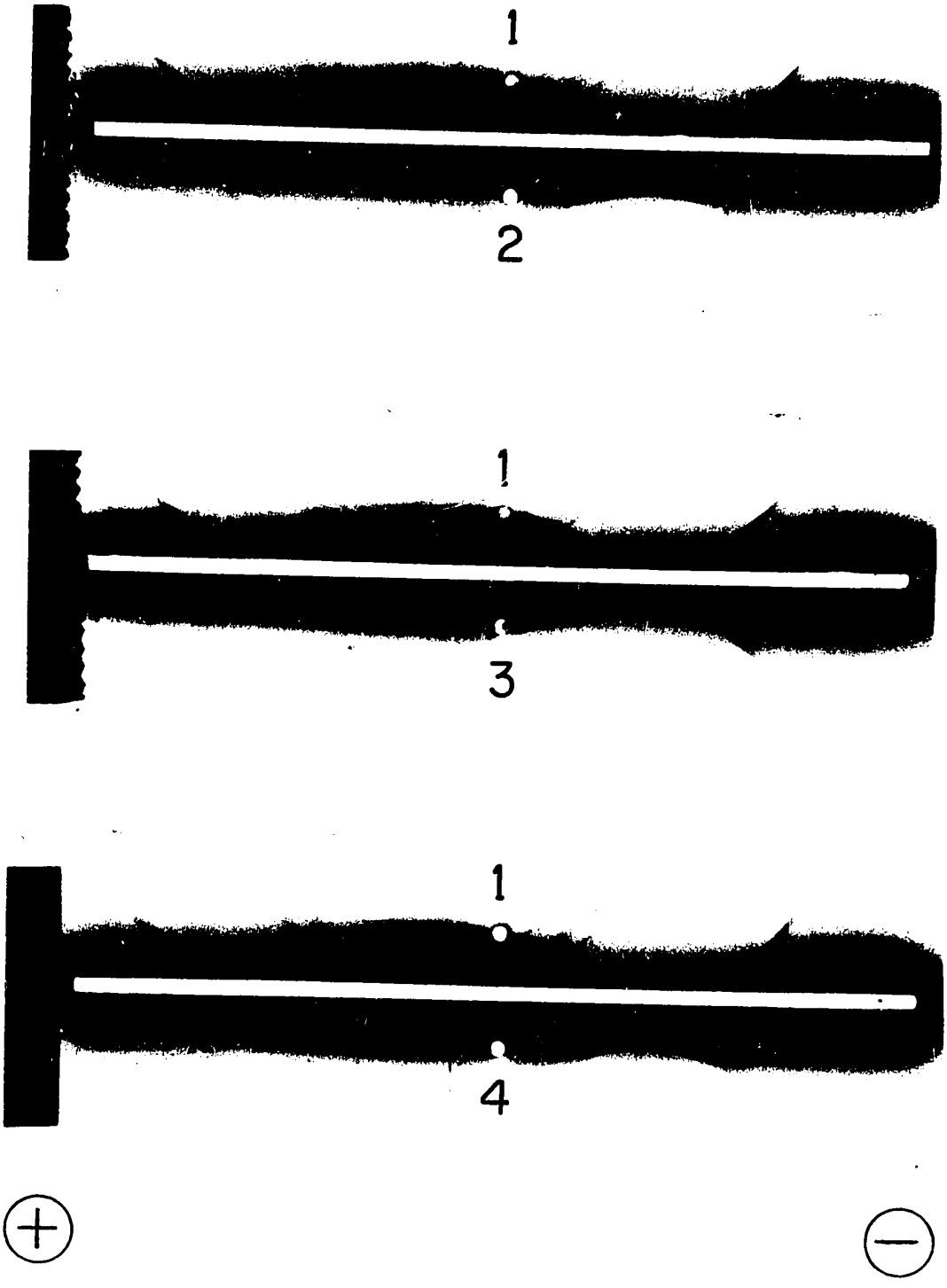

1
2
1
3
1
4
+
−

FIGURE 9

Protein profiles on elution from EMA immunosorbent. The globulin fraction of allergic serum Kh (1300 mg) was applied. Elutions were accomplished with (i) 2M NaCl; (ii) Fraction A (400 mg); and (iii) 0.1M glycine-HCl, pH 2.5.

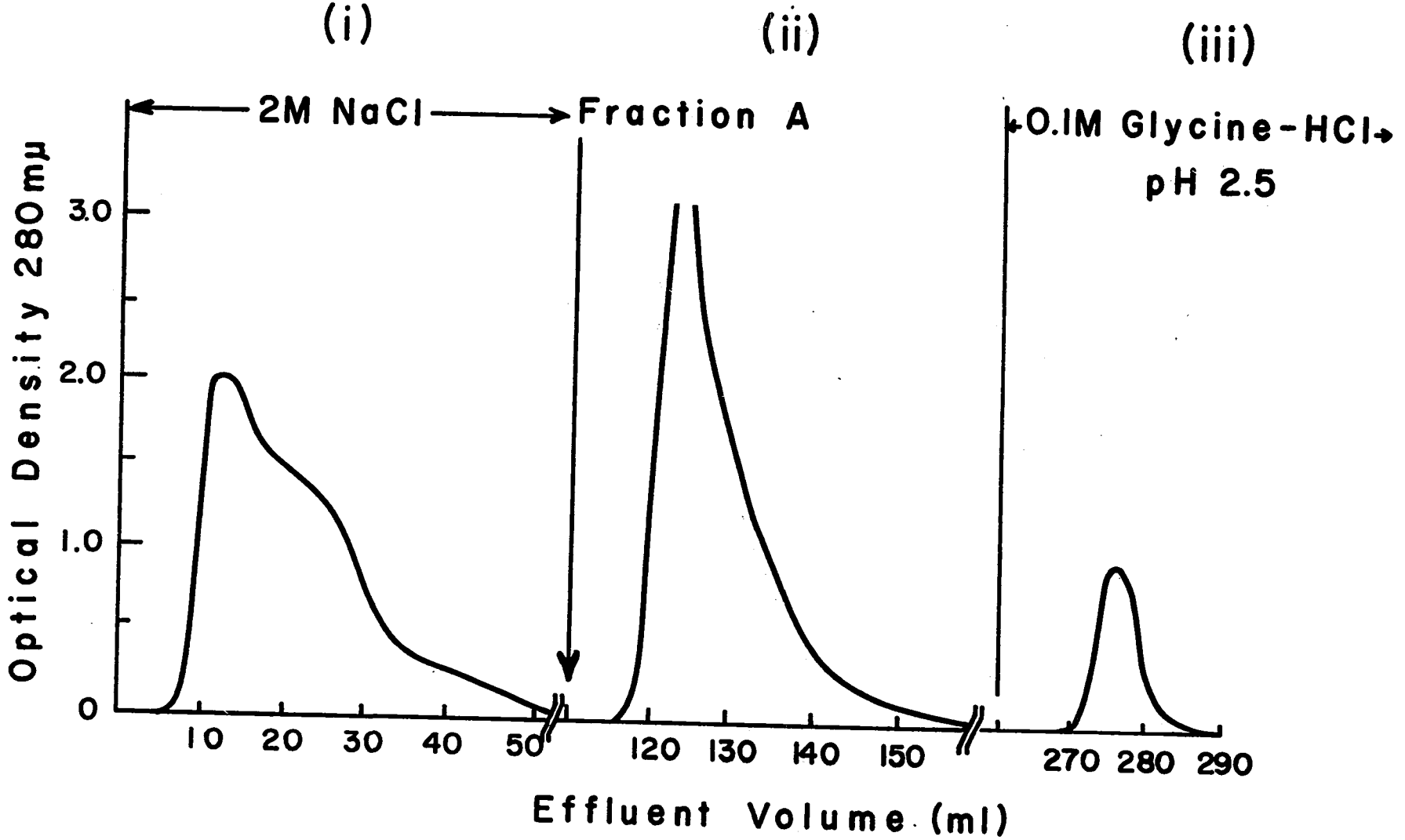

(i)
(ii)
(iii)
2M NaCl
Fraction A
0.1M Glycine-HCl
pH 2.5
Optical Density 280mμ
3.0
2.0
1.0
0
10 20 30 40 50
120 130 140 150
270 280 290
Effluent Volume (ml)

TABLE VII

Chromatography of allergic serum Kh on immunosorbent.

	Amount (mg)	HA titer	P-K titer
Protein applied	1330	32,800	640
" not retained	1120	1600	640
" eluted* (i) 2M NaCl	21	0	0
(ii) Fraction A	<1	0	0
(iii) Glycine-HCl, pH 2.5	<1	0	0

* HA and P-K assays performed with ten-fold concentrated eluates.

TABLE VIII, part a

Experiment 1: Demonstration of skin activity in eluates recovered from immunosorbents by displacement with ragweed Fraction A.

Skin sites sensitized* with	Titers of challenging material	
	A-R	2M NaCl
allergic serum	200	0
NHS (control)	1	0

*Volumes of 0.05 ml of allergic serum Kh (1:50) and NHS Ra (1:50) injected intradermally into normal non-allergic volunteers.

Eluates A-R and those obtained with 2M NaCl were used to challenge skin sites sensitized with allergic serum and NHS. Titers represent the highest dilution giving positive reactions. A zero titer (0) indicates an absence of reaction.

TABLE VIII, part b

Experiment 2: Demonstration of blocking of passive sensitization with A-R.

Day 1	Day 2	Day 3
Sensitization with	Sensitization** with	Reactions observed on challenge with WSR
2M NaCl eluate	allergic serum*	3+
A-R eluate	allergic serum*	-
allergic serum*	saline	3+

*Allergic serum Kh (1:50).

**Reinjection of sites sensitized on day 1.

In the second experiment performed in the same normal volunteer, the A-R eluate was injected intradermally into different skin sites and, 24 hours later, the same sites were injected with reaginic serum at a dilution of 1:200 (Table VIII, part b). On this occasion no reactions were observed. An additional 24 hours later, on intradermal injection of WSR into these sites, suprisingly no reactions were elicited. On the other hand, control skin sites sensitized with the same dilutions of the allergic sera gave 3+ reaction when challenged with WSR in identical concentration. Taking an overall view of these data, it may be suggested that these results indicate that allergen-reagin complexes were present in the A-R eluates.

Immunoelectrophoretic analysis of the eluates revealed that 2M NaCl had displaced albumin, haptoglobin and β-globulins in addition to IgG, (Figure 10, part a). Similar proteins were eluted with ragweed Fractions A (or U-F) although the IgG was reduced in concentration. Albumin was the major serum protein constituent detected in the eluate obtained at pH 2.5.

Elution of normal human serum proteins from EMA immunosorbent

The amounts of protein eluted with 2M NaCl from an EMA immunosorbent saturated with 20 ml of a normal human serum, Ra, was 38 mg. When this immunosorbent was again saturated with the same serum the amount of protein eluted with 2M NaCl was 36 mg. In a similar experiment with NHS, Go, the amounts of protein eluted with 2M NaCl were 13 and 12 mg. An immunoelectrophoretic analysis and comparison of 2M NaCl eluates from EMA immunosorbents previously saturated with allergic serum Kh, or with NHS Ra, is shown in Figure 10, part (b). These eluates were composed of albumin, α- and β-globulins and only small amounts of γG-globulins were detected.

FIGURE 10

Immunoelectrophoretic analysis of eluates obtained from EMA immunosorbents.

part a Immunosorbent saturated with allergic serum Kh; fractions obtained on elution with:

well (1) 2M NaCl
well (2) ragweed Fraction A
well (3) gly-HCl, at pH 2.5

Antibody troughs were filled with sheep antiserum to rabbit serum proteins.

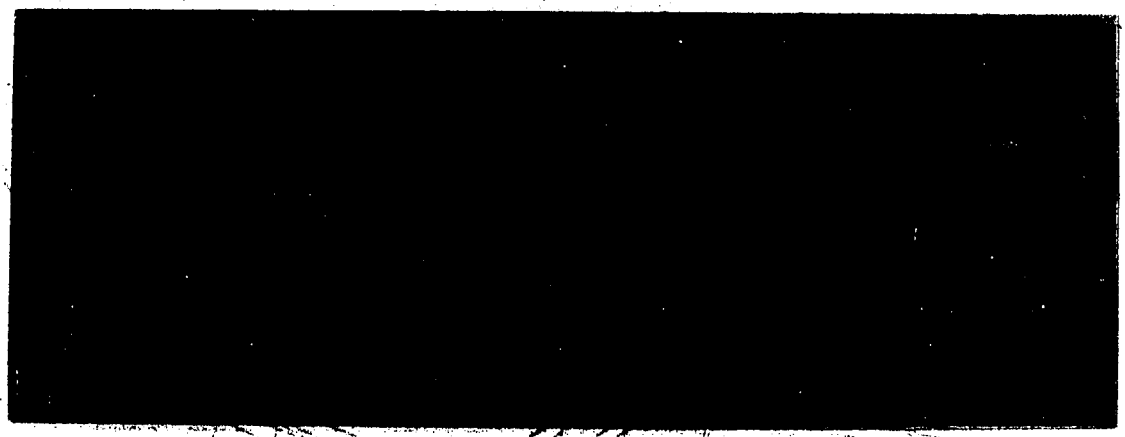
part b Immunoelectrophoretic comparison of eluates obtained with 2M NaCl from immunosorbents saturated with allergic serum Kh or normal human serum Ra.

well (1) original allergic serum, Kh
well (2) 2M NaCl eluate, allergic serum, Kh
well (3) 2M NaCl eluate, normal serum, Ra

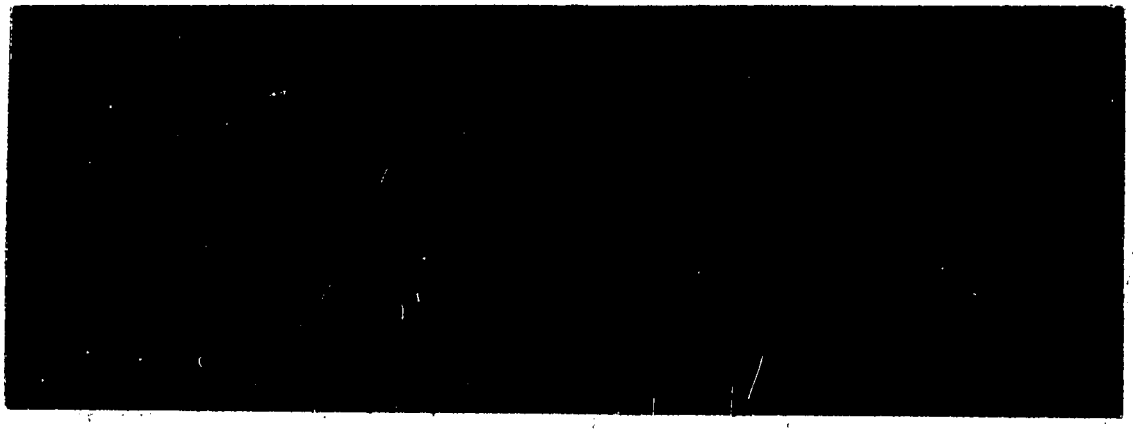
Antibody troughs were filled with rabbit antiserum to human serum proteins.

Electrophoresis was performed in agar with veronal buffer $\Gamma/2 = 0.033$, pH 8.6 at 9.25 V/cm for 40 minutes.

(a)



(b)



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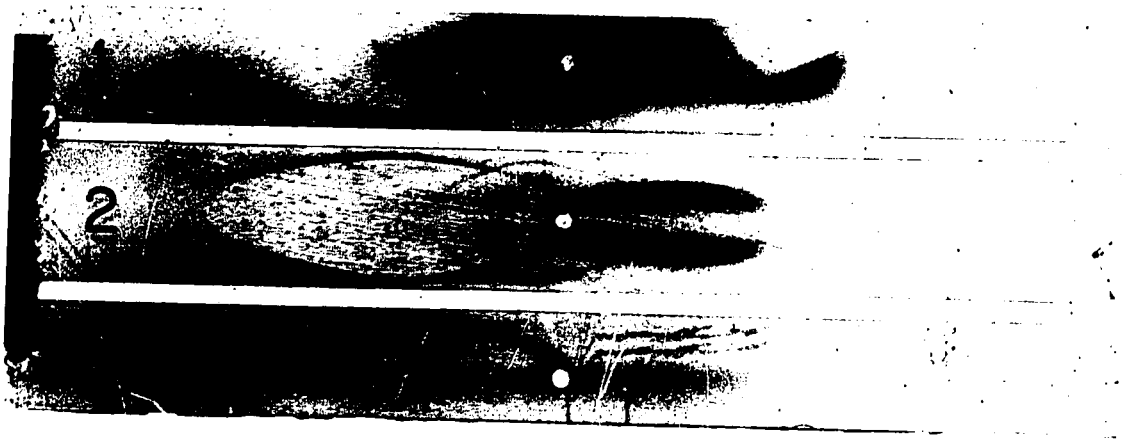
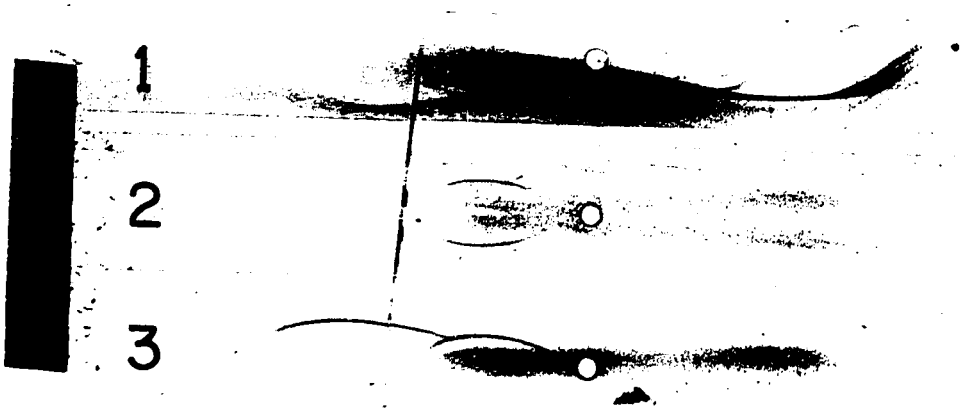
(a)



(b)



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DISCUSSION

A simple technique was employed for the preparation of ragweed immunosorbents. A graft polymerization procedure was used to overcome difficulties in the insolubilization of small peptides such as those found in aqueous ragweed extracts. This method consisted in preparing first a tightly cross-linked insoluble EMA-protein matrix, by the reaction of EMA with a large molecular weight protein (e.g. RSA or HSA) without blocking all of its amino groups; onto this framework was then "grafted" the low molecular weight antigens by subsequent reaction with a second portion of EMA.

Immunosorbents prepared by such procedures were capable of removing antibodies from immune sera. Their capacity to adsorb ragweed antibodies could not be determined owing to the heterogeneity of ragweed components. However, in another study in this laboratory (247), employing immunosorbents prepared by the insolubilization of bovine serum albumin (BSA) with EMA, it was found that 400 to 700 mg of rabbit anti-BSA antibody could be eluted from 1 g of immunosorbent with 8M urea at neutral pH or with glycine-HCl buffers at acid pH.

In the present study it was demonstrated that displacement of antibodies from the immunosorbent using low molecular weight ragweed components resulted in enrichment of antibody activity. In this case the recovery of the antibodies may be compared to elution with homologous hapten as has been successfully developed with EMA immunosorbents for the isolation of antibodies to the C-terminal heptapeptide of sperm-whale myoglobin (246).

The elution of rabbit anti-ragweed antibodies with 2M NaCl is reminiscent of the classical findings of Heidelberger et al (249) which revealed that concentrated solutions (15%) of sodium chloride were capable of dissociating polysaccharide antigens from their specific immune precipitates and therefore suggests that antigenic determinants of ragweed pollen constituents may involve polysaccharide groups.

In the present study it would probably have been more appropriate to use in these experiments as a control eluting agent, low molecular weight components of another pollen extract with no cross reacting allergens, rather than the casein chrymotryptic digest. However, such components were not readily available at the time of the study.

In view of the usefulness of the mildness of the procedure employing Fraction A for the elution of rabbit anti-ragweed antibodies it was considered that a similar technique could be employed for the elution and isolation of human skin-sensitizing antibodies without the accompanying hazard of denaturing these antibodies at low pH.

However, the displacement of antibodies with low molecular weight components of ragweed pollen was found to be unsatisfactory for the recovery of skin-sensitizing activity since these were obtained in the form of allergen-reagin complexes. This conclusion was based on the following three observations, (a) intradermal injection of the A-R eluates (which had been extensively dialysed for the elimination of the eluting, low molecular weight constituents) produced immediate wheal and flare reactions on injection into the skin of normal volunteers, (b) skin sites sensitized with allergic sera reacted when challenged with the A-R eluates in dilutions which did not elicit immediate skin reactions and (c) sensitization of normal skin sites with the A-R eluates apparently blocked further fixation

of skin-sensitizing antibodies present in the whole allergic serum. In this last case, skin sites sensitized with the A-R eluates may have contained sufficient ragweed allergens to neutralize skin-sensitizing antibodies passively transferred to the same skin sites. Ishizaka et al (209) reported that sensitization with enriched reaginic preparation of one specificity (e.g. ragweed) blocked subsequent passive sensitization with reaginic antibody of a heterologous system. Thus, to demonstrate conclusively that skin sites were indeed blocked by the A-R eluates to further passive sensitization, and not rendered merely inert to ragweed, it would have been more appropriate to use an allergic serum of a different specificity (e.g. ovalbumin) rather than a ragweed allergic serum for the second step of the sensitization of the skin. However, such allergic sera were not available at the time of these experiments. The skin activity of Fractions A and U-F revealed the fact that these low molecular weight extracts of ragweed pollen were allergenically multivalent. Furthermore, the apparent failure to remove the allergenic fractions by dialysis, indicated that the reagins in the A-R complex possessed a high affinity for the skin-active components. The use of 2M NaCl as an eluting agent resulted in the displacement of the same types of proteins regardless of the nature of the sera used to saturate the EMA immunosorbents. Therefore, it may be concluded that some absorption processes involved in the saturation of the immunosorbents are independent of the presence of serum antibodies. On the other hand, if the immunosorbent functioned as an ion-exchange resin it would be expected that comparable rabbit and human proteins would have been displaced by 2M NaCl, whereas in actual fact in the former case only rabbit γG-globulins were displaced from the immunosorbents in contrast to the latter in which human albumin, α- and β-globulins were eluted.

The binding of human serum globulins to ragweed pollen had been demonstrated by an indirect antiglobulin test (250). Agglutination of ragweed pollen previously incubated with human serum occurred only after the addition of antiserum to γG-globulin. Both normal and ragweed allergic serum globulins were capable of becoming adsorbed onto pollen. Moreover, this binding property of human serum globulins was specific for ragweed pollen as it could be neutralized only by ragweed extracts and not by grass pollen or its extracts.

In another study using immunosorbents prepared by the insolubilization of wheat gliadin with EMA in conjunction with the serum of a wheat allergic individual, the authors reported that some reaginic activity was recovered by elution with 2M NaCl (251). Although IgA, IgM or IgD could not be detected in these eluates, IgG was demonstrated by an indirect method by an examination of the antibody specificities produced after immunization of animals with the immunosorbent-antibody complexes. This procedure elicited the formation of antibodies to IgG in addition to β_{1c} and two other unidentified proteins.

These results appear to be at variance with those of the present investigation. However, it should be stressed that even though identically prepared immunosorbents were employed with similar antigen-antibody systems in the present study, comparable results were not obtained with the ragweed antisera from two different species (man and rabbit). Thus, fallacious conclusions may be arrived at from a comparison of results obtained with different antigen-antibody systems.

From an overall analysis of the results, it can be concluded that adsorption of a restricted range of other serum proteins can occur in addition to immuno-specific absorption of homologous antibodies, and that

the nature of these adsorbed proteins depends in part on the type of immunosorbent used and in part on the source of the antisera.

Although the procedures described in this chapter did not lead to the direct isolation of reagins, it is believed that the results obtained pointed to the potential usefulness of immunosorbents for the isolation of reagins or reagin-allergen complexes; the salient features of the method being the mild and immunochemically specific methods of elution using low molecular weight constituents of allergenic extracts.

SECTION B IMMUNOSORBENTS PREPARED WITH AMINO-CELLULOSE AS SUPPORTING MEDIUM

Introduction

A simple and rapid procedure for the preparation of a ragweed immunosorbent was described in the preceding section. Although the successful recovery of rabbit antibodies, in a model system, by elution with low molecular weight components of ragweed pollen was demonstrated, this procedure when applied to the recovery of reagins from human sera led to the isolation of reagin-allergen complexes rather than the reaginic antibody alone.

In previous studies in this laboratory it had been demonstrated that reagins could be removed completely from the sera of ragweed allergic individuals with immunosorbents consisting of the aqueous extract of ragweed pollen coupled to diazotized polyamino styrene (171) or to cellulose (218). The elution of the adsorbed reagins was accomplished with acidic buffers at pH 3 or urea at neutral pH but only in the presence of NHS. Although the nature of the serum protein(s) responsible for the protecting or stabilizing effect was not determined, the possibility arose that a single protein could be used to replace whole serum in this procedure. Thus, if the physicochemical properties of this protein were sufficiently different from the eluted antibodies, it can be visualized that reagins could be subsequently recovered by the use of mild physicochemical methods. In the experiments to be described, an investigation was undertaken to identify the nature of the protective protein(s) and to use them in the isolation of reagins with cellulose immunosorbents.

MATERIALS AND METHODS

Preparation of a globulin fraction of human sera

A pool of NHS and a pool of normal cord serum (NCS) were fractionated by salting-out with ammonium sulfate at a final concentration corresponding to 45% saturation, according to the procedure described in Methods, Chapter III. The globulins were desalted by gel filtration on Sephadex G-25 previously equilibrated with PBS.

Isolation of Bence-Jones Proteins

Bence-Jones proteins* types κ and λ were obtained from the urine of two multiple myeloma patients, Gr and Cu respectively. The urinary proteins were precipitated at room temperature by the addition of solid ammonium sulfate to a concentration of 3 molar, and were recovered by centrifugation at 8000 x G for 10 minutes at 22°C. The precipitates were washed once by resuspension in 3M ammonium sulfate solution and recentrifuged. The washed precipitates were dissolved in distilled water and the proteins were precipitated with ammonium sulfate at 2 molar concentration. These precipitates were centrifuged and washed once with 2M ammonium sulfate, centrifuged, dissolved in water and dialysed against 0.005M phosphate buffer, pH 8.0. The small amounts of precipitate which formed after dialysis were removed by centrifugation and the proteins were separately chromatographed on DEAE-cellulose at constant pH and with stepwise increasing ionic strength. The λ type Bence-Jones protein was eluted with the 0.005M phosphate buffer, while the κ type was eluted with the 0.01M phosphate buffer. The eluates containing the Bence-Jones

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* Reference rabbit antisera to κ and λ type Bence-Jones proteins were kindly supplied by Dr. W.D. Terry, of the National Institutes of Health.

proteins were concentrated by "dialysis" against dry sucrose and finally dialysed against PBS.

Preparation of Amino-cellulose

An amino derivative of cellulose was synthesized according to the procedure described by Gurvitch (252). Briefly, cellulose powder pretreated with acetylchloride was reacted with N-(m-nitrobenzyloxymethyl) pyridinium chloride (253) to give the corresponding m-nitrobenzyloxymethyl cellulose ether. A solution of 7% of the pyridinium salt was used since at this concentration the highest number of amino groups were coupled to the cellulose (254). The nitro groups were reduced with a 15% solution of sodium hydrosulfite and the resulting amino-cellulose was washed thoroughly with water.

To improve the capacity of the amino-cellulose, the surface area was increased by dissolving it in an ammoniacal copper sulfate solution follwed by precipitation in the form of a fine suspension. In this procedure, 1 g of dry amino-cellulose was dissolved in a volume of 66 ml of ammoniacal copper sulfate solution consisting of 3 g cupric hydroxide, 0.66 g sucrose, 20 ml ammonium hydroxide and 40 ml water. After stirring for 0.5 hour at room temperature a further portion of ammonium hydroxide (80 ml) was added and any remaining insoluble material removed by filtration through glass wool. This filtrate was added to 4 liters of distilled water at 70°C. The amino-cellulose was precipitated by cooling the solution to room temperature and by adjusting the pH to 7 with 10% sulfuric acid (v/v). The precipitated amino-cellulose was recovered by centrifugation, washed with water and stored as a well dispersed aqueous suspension.

Preparation of amino-cellulose-allergen conjugates (immunosorbent)

Prior to coupling protein antigens to amino-cellulose, the latter was diazotized. First, to determine the volume of suspension containing 1 g of amino-cellulose, a volume of 1 ml of the suspension was filtered, dried at 100°C and weighed. According to this value the required volume of suspension was centrifuged and the supernatant liquid was discarded. The amino-cellulose was resuspended in 5% hydrochloric acid (250 ml), the mixture was placed in an ice-bath, and $NaNO_2$ (5 g) added. Diazotization was continued for 30 minutes at 0°C with continuous stirring. The diazotized cellulose was then centrifuged at 5000 x G in pre-cooled tubes and washed with distilled water at 0°C until the pH of the washing was approximately 6. The washings were performed as quickly as possible to prevent decomposition of the diazocellulose. To test for free diazonium groups a small portion of the diazocellulose was mixed with a few drops of a saturated solution of β-napthol in borate buffer at pH 8.6. In the presence of free diazonium groups a distinctly orange coloured derivative of diazocellulose was obtained.

The diazocellulose (1 g) was resuspended in DWSR (2.3 to 2.5 g) dissolved in a volume of 750 ml borate buffer pH 8.6 and the mixture stirred for 20 hours at 4°C. To remove uncoupled proteins the cellulose-allergen conjugate, i.e. immunosorbent, was centrifuged at 5000 x G for 10 minutes, the supernatant was decanted and the immunosorbent resuspended in saline. These procedures were repeated until the optical density at 280 mμ of the wash fluids was below 0.01. To reduce the possibility that some ragweed antigens become released during subsequent elution, the immunosorbent was pretreated by washing successively with saline,

0.01M Tris-HCl, pH 9.1; 2M NaI in the Tris-HCl buffer, pH 9.1; saline; 0.1M glycine-HCl, pH 2.5 and finally with saline at neutral pH.

Absorption of allergic sera with immunosorbent

In view of the known lability of skin-sensitizing antibodies at low pH, a preliminary experiment was performed to determine the effect of acid on the P-K activity. In these experiments 6 allergic sera were used: sera Ju and Gre from non-treated ragweed allergic individuals and Kh, Gr, We, and Za from ragweed allergic patients receiving hyposensitization treatment. A volume of 1 ml of each serum was diluted with an equal volume of saline and the pH adjusted to 2.5 - 3 with 0.1N HCl. After a period of 30 minutes the pH was raised to 6.5 - 7 by the addition of 0.1N $NaHCO_3$. All serum samples were sterilized by filtration through membranes (0.45 μ pore size) and analysed for skin-sensitizing activity by the P-K test.

Only those allergic sera which retained high skin-sensitizing activity were selected. The immunosorbent was suspended in allergic serum (in a ratio of 1 g to 125 ml serum containing 60 ml saline) and the mixture was stirred for two hours at room temperature. The immunosorbent-antibody complex was washed free of non-specifically adsorbed proteins by repeated centrifugations and resuspensions in fresh saline until the OD_{280} was below 0.01.

Elution of absorbed antibodies

Elution of the antibodies was carried out batchwise since the small particle size of the immunosorbent prevented a satisfactory flow rate in columnar operations. Four eluting agents were employed in

attempts to recover the antibodies from the immunosorbent. These were

*(i) 0.1M glycine-HCl at pH 2.5

(ii) 6M urea at pH 7.4

*(iii) 2M NaI in 0.1M Tris-HCl. at pH 9.1

(iv) a ragweed hapten preparation at pH 7.4

In each of these cases a stabilizing protein was incorporated into the eluting agents. To investigate the nature of the stabilizing protein(s), the effect of (i) NHS; (ii) globulins of NHS; (iii) globulins of normal cord serum (NCS); and (iv) HSA, all at the same protein concentration of 7 mg/ml were used during the elutions with acid or urea. In addition, rabbit serum albumin** (RSA) was also used with gly-HCl buffers at pH 2.5. For these experiments the immunosorbent-antibody complex was divided into portions of 20 mg each and centrifuged; the supernatants were discarded and replaced with a volume of 2 ml of the eluting agent (either 0.1M gly-HCl at pH 2.5 or 6M urea at neutral pH) containing one of the protein preparations described and the mixtures stirred. Elution with gly-HCl was carried out at 0°C and with urea at room temperature using an incubation period of 5 minutes. The immunosorbents were removed by centrifugation and the supernatnats recovered. The gly-HCl eluates were neutralized by adding sodium bicarbonate and then dialysed against PBS. Urea eluates were dialysed separately against PBS. All eluates were sterilized by membrane filtration. The recovery of skin-sensitizing activity was determined by P-K tests. When RSA was employed as the stabilizing protein,

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*For the sake of brevity, eluant (i) will be referred to as gly-HCl and eluant (iii) as NaI.

**Pentex. Kankakee, Illinois.

the recovery of reagins was determined by PCA tests in monkeys. To determine the minimum concentration of protein which would still exert a stabilizing effect, NHS and HSA in concentrations ranging from 0.07 to 70 mg/ml were incorporated into gly-HCl buffers; the reagins were recovered from 20 mg portions of immunosorbent-antibody complex and their activity evaluated by P-K tests.

The possibility that a milder procedure employing sodium iodide for the elution of reagins from immunosorbent was investigated. A 4M solution of NaI was prepared in degassed distilled water and a portion titrated for free iodine with 0.1N sodium thiosulfate using a starch indicator. Sodium thiosulfate in an amount equal to a 10% excess, in relation to that required to neutralize the iodine, was added to the sodium iodide solution. For use, 1 volume of the 4M NaI was mixed with 1 volume of 0.2M Tris-HCl buffer, pH 9.1 containing stabilizing protein. Prior to the elutions, the pH of the immunosorbent-antibody complex was raised to 9.1 by washing once with 0.1M Tris-HCl, pH 9.1 buffer containing HSA at a concentration of 7 mg/ml. The immunosorbent was centrifuged and the supernatant was recovered, dialysed against PBS, sterilized, and saved for examination by P-K. This procedure also served as a control to examine for the possible elution of reagins by the Tris-HCl buffer at pH 9.1. For elution of antibodies the immunosorbent-antibody complex was suspended in 2M NaI in the Tris-HCl containing HSA at a concentration of 7 mg/ml, stirred at room temperature for 15 minutes and then centrifuged. The supernatant was withdrawn and dialyzed first against the Tris-HCl buffer at 15°C* to remove NaI

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* To avoid precipitation of NaI which occured at 12°C, the dialysis was performed at 15°C.

and then against PBS at 4°C; it was sterilized by membrane filtration and analysed by P-K. For additional controls the allergic serum was diluted 1:200 with 2M NaI solution in the Tris-HCl and dialysed first against Tris-HCl buffer at 15°C and then against PBS at 4°C.

Sequential elutions from the immunosorbent

In view of the successful recovery of skin-sensitizing activity following elution with either gly-HCl or with NaI (see results, page 115) the possibility arose that two groups of reagins which differed in their elutability had been displaced from the immunosorbent. To explore this possibility, the elutions were performed sequentially from the same aliquot of immunosorbent.

(i) Multiple elution with 2M NaI followed by multiple elution with gly-HCl

In this experiment, elution from 20 mg of immunosorbent-antibody complex was made with a volume of 2.5 ml of NaI containing HSA (7 mg/ml). The mixture was stirred for 15 minutes, centrifuged, the supernatant was removed and dialysed against the Tris-HCl buffer and then against PBS. These procedures were repeated four more times.* The immunosorbent was then washed twice with saline and resuspended in a volume of 2.5 ml of the gly-HCl buffer containing HSA (7 mg/ml) at 0°C, stirred for 5 minutes and centrifuged at 0°C. The supernatant was withdrawn and immediately neutralized with sodium bicarbonate and dialysed against PBS.

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* In these multiple elution experiments, each eluate will be referred to by the number corresponding to the order of the elution, i.e. 1, 2, 3, 4 or 5.

These procedures were repeated four more times.

(ii) Multiple elution with gly-HCl followed by multiple elution with NaI

In these experiments, a fresh aliquot of the immunosorbent-antibody complex was used in conditions identical to those described in section (i) except that in this case the five elutions were first performed with the gly-HCl-HSA buffer. The immunosorbent was then washed once with saline and once with the Tris-HCl buffer and incubated with 2.5 ml portions of NaI buffer, containing HSA (7 mg/ml). The elution with NaI was repeated four more times.

The specific isolation of reagins using hapten

The specific displacement of reagins from immunosorbent was attempted with a ragweed haptenic preparation. In this experiment 40 mg of immunosorbent-antibody complex was suspended in a volume of 2 ml of a solution containing 200 mg of hapten and 10 mg of HSA. After stirring for a period 0.5 hour at room temperature the immunosorbent was removed by centrifugation, the supernatant recovered and dialysed extensively against 2M NaI in 0.1M Tris-HCl, pH 9.1, at 15°C to dissociate the reagin-hapten complexes and to facilitate the removal of the haptens, then against the Tris-HCl buffer alone and finally against PBS. Finally, to displace residual antibodies, the washed immunosorbent was stirred in a volume of 2 ml of a solution of the NaI and HSA (7 mg/ml). To test for the specificity of the elution with the ragweed haptens, an immunosorbent was prepared by coupling the non-dialysable constituents of an aqueous extract of Timothy grass pollen (DWSG) using the same conditions described for the preparation of DWSR immunosorbents. The DWSG immunosorbent (30 mg)

was saturated with an allergic serum from a grass sensitive individual, Na. In this experiment, the elution was performed with a solution (1.5 ml) containing 150 mg of the ragweed hapten and 7.5 mg HSA.

Identification of the antigenic determinants characteristic of reagin

The purpose of these experiments was to isolate reagins in amounts sufficient for the production of specific antisera, to be used for the subsequent characterization of reagins by immunochemical procedures. For this purpose, allergic sera Kh and Gre (original serum volumes of 400 and 340 ml respectively) were fractionated by salting-out with ammonium sulfate and by ion-exchange chromatography on DEAE-Sephadex A 50. These procedures and their results have been described in Chapter III. Allergic serum fractions Kh 35-45[II] and Gre 35-45[II] containing high skin-sensitizing activity, were used to saturate quantities of 670 and 630 mg of the immunosorbent, respectively. In addition, allergic serum fraction Kh 30[I] which was devoid of reaginic activity but which was presumed to contain the blocking antibodies was employed to saturate 500 mg of immunosorbent.

The elutions from the saturated immunosorbents were performed initially with 4 portions (80 ml each) of NaI containing HSA (5 mg/ml) as described previously. The immunosorbent saturated with allergic serum fraction Gre 35-45[II] was divided into two equal portions; one-half was eluted with four 40 ml portions of NaI containing HSA and the other half was similarly eluted with NaI containing RSA as the stabilizing protein. All of the eluates were separately dialysed against the Tris-HCl buffer and then against PBS, concentrated and finally dialysed against PBS. Meanwhile, the immunosorbents were washed with saline to remove the NaI

and the multiple elutions were continued at 0°C with 3 portions (80 ml each) of gly-HCl in the presence of HSA or RSA. These eluates were neutralized with $NaHCO_3$, dialysed against PBS, concentrated and finally dialysed against PBS.

The eluates recovered from immunosorbents were analysed for IgE allergen-binding antibodies by radio-immunodiffusion techniques employing specific antiserum to IgE*. In Ouchterlony analysis performed in agarose gel (1 g%) the antiserum to IgE was placed into the central well and aliquots of the concentrated eluates obtained with the NaI and gly-HCl buffers were placed into the outer wells. Also, the allergic serum fraction Kh 35-45[II] which had been used to saturate the immunosorbent was added to one of the outer wells. In immunoelectrophoretic analysis, also performed in agarose, an aliquot of a γE myeloma serum was placed into the sample wells and the first eluates obtained with NaI or with gly-HCl (i.e. eluate 1) were then added to these wells. Following electrophoresis the antiserum specific to IgE was placed into the antibody trough. In both Ouchterlony and electrophoretic analysis, immunodiffusion was carried out for 48 hours following which the agarose plates were washed for a period of 24 hours to remove the unreacted proteins.

DWSR was labelled with I^{131} according to the method used by Yagi (191). In this procedure, volumes of 0.2 ml aliquots each of DWSR (200 μg) in borate buffer pH 9, KI (2×10^{-4}M) and Chloramine-T (1.41 mg/ml in borate buffer) were added to 0.2 ml NaI^{131} (2 mc). The mixture was

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* Antiserum to IgE and a γE myeloma serum sample were kindly provided by Dr. K. Ishizaka of Children's Asthma Research Institute and Hospital, Denver, Colorado.

incubated at room temperature for a period of 5 minutes and a volume of 0.2 ml Na_2SO_3 (1.26 mg/ml in borate buffer) was added. The labelled DWSR was dialysed overnight. In the radio-Ouchterlony analysis the wash fluid was removed from the wells and the DWSR-I^{131} was added to the central antibody well. For radio-immunoelectrophoretic analysis, the agarose was removed from the antibody trough and DWSR-I^{131} added. Immunodiffusion was carried out for 36 hours after which the agarose plates were washed in saline for 48 hours with frequent changes of the saline bath. The plates were dried and placed in contact with Kodak Industrial X-Ray film type KK. After suitable exposures (6 - 24 hours) the films were developed with Diafine developer* and fixed in the usual manner.

Immunization of guinea pigs

To remove the bulk of the HSA or RSA, NaI eluates 1 to 4 and gly-HCl eluates 1 and 2 were first pooled and then fractionated by salting-out with ammonium sulfate at a concentration corresponding to a final saturation of 47%. The precipitate was dissolved in distilled water and desalted by gel filtration on Sephadex G-25 which had been previously equilibrated with PBS. The protein eluate was concentrated to a volume of 1 - 2 ml.

For the induction of immune paralysis to serum proteins other than to IgE, adult guinea pigs were injected intravenously (by the intracardiac route as recommended by Dr. Ishizaka) with an amount of 10 mg of a fraction of NCS obtained by salting-out with ammonium sulfate at

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* Acufine Inc., Chicago, Illinois.

incubated at room temperature for a period of 5 minutes and a volume of 0.2 ml Na_2SO_3 (1.26 mg/ml in borate buffer) was added. The labelled DWSR was dialysed overnight. In the radio-Ouchterlony analysis the wash fluid was removed from the wells and the DWSR-I^{131} was added to the central antibody well. For radio-immunoelectrophoretic analysis, the agarose was removed from the antibody trough and DWSR-I^{131} added. Immunodiffusion was carried out for 36 hours after which the agarose plates were washed in saline for 48 hours with frequent changes of the saline bath. The plates were dried and placed in contact with Kodak Industrial X-Ray film type KK. After suitable exposures (6 - 24 hours) the films were developed with Diafine developer* and fixed in the usual manner.

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* Acufine Inc., Chicago, Illinois.

a concentration corresponding to a saturation of 50%. The animals were then injected intra-muscularly with 0.2 ml of an emulsion made with equal volumes of the concentrated eluates and complete Freund's adjuvant. A separate group of animals was directly immunized with the eluates obtained from immunosorbent saturated with allergic serum fraction Kh 30[I], i.e. in this case the animals were not pretreated with NCS. All animals were reimmunized one and two months after the initial injection; blood was collected from them by intra-cardiac puncture. To remove antibodies which might have been formed against light chains, an amount of 1 mg each of Bence-Jones proteins κ and λ was added to 2 ml of the immune sera prior to their examination by immunoelectrophoresis and by radio-immunodiffusion Ouchterlony* techniques. For reference purposes in the latter method, a myeloma IgE was added to two of the allergic sera and the precipitin arcs were developed with a rabbit antiserum specific to IgE. Ragweed antigen E, labelled with I^{125}, was added to all of the wells in the Ouchterlony agarose plates.

The antisera produced by immunization with the eluates containing reagins were examined by the reversed PCA test in the monkey (112). In this procedure, 0.025 ml of the antiserum, in ten-fold serial dilutions, was injected intradermally and the reactions were made visible by an intravenous injection of a volume of 5 ml of a 1% solution of Evans blue in PBS. A normal guinea pig serum similarly injected in identical dilutions served as control.

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* Kindly performed by Dr. A. Yurchak, E.J. Meyer Memorial Hospital, State University of New York, Buffalo, New York.

RESULTS*

The effect of acid treatment on the skin-sensitizing activity of allergic sera

A comparison of skin-sensitizing activity of allergic sera before and after treatment with HCl is shown in Table IX. The reaginic titer of four allergic sera decreased by at least one-half of the original activity, and in two of the sera, Ju and We, the activity was reduced four-fold. Thus, only allergic sera Kh and Gre, which still possessed high titers after exposure to acid, were used for the saturation of immunosorbents with reagin. The recovery of reagins from immunosorbent-antibody complexes was equally efficient in the presence of all protein preparations (Table X, part a). However, the reaginic activity in eluates obtained with gly-HCl buffer at pH 2.5 was 4-8 times higher than that in eluates isolated with urea at neutral pH. It was also found that RSA had the same capacity as HSA to stabilize the reaginic activity on elution at low pH (Table X, part b). By varying the protein concentration of HSA or NHS it was found that the maximum reaginic activity was recovered at protein concentrations of the order of 7 mg/ml or greater (Table XI) and that about one-half of the reaginic antibody activity was inactivated on decreasing the protein concentration within the range of 0.7-1.4 mg/ml. Moreover, at the lower concentration of 0.07 mg/ml no reaginic activity was detected in the eluates. Therefore, for maximal recovery of reagin, the elutions were performed in the presence of HSA at concentrations from 3-7mg/ml.

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* The results given on pages 111-118 inclusive were presented at the the Canadian Federation of Biological Societies in Montreal 1970 (255).

TABLE IX

The effect of acid treatment on the skin-sensitizing activity of allergic sera.

	serum	P-K titer original	P-K titer after acid treatment*
(a)			
	Ju	400	100
	Gre	1600	800
(b)			
	Za	400	200
	Kh	3200	1600
	We	400	100
	Gr	400	200

*Allergic sera were initially diluted two-fold with saline, 0.1N HCl was used to adjust the pH to 2.5-3. After incubation for 30 minutes at room temperature, the pH was adjusted to 7, with sodium bicarbonate.

(a) Allergic sera obtained from non-treated ragweed allergic individuals.

(b) Allergic sera obtained from ragweed allergic patients undergoing hyposensitization treatment.

TABLE X

Effect of human serum proteins on the stabilization of reaginic activity during their elution from immunosorbent.*

(a)

Proteins**	P-K titer	
	gly-HCl elution	urea elution
NHS	200	50
Globulin fraction of NHS	200	25
Globulin fraction of NCS	200	25
HSA	200	25

(b)

Proteins**	PCA titer
	gly-HCl elution
RSA	32
HSA	32

* A quantity of 20 mg of immunosorbent was used in each elution experiment.

** All protein preparations were employed at a concentration of 7 mg/ml.

(a) The immunosorbents were saturated with ragweed allergic serum Kh. The P-K titer given by serum Kh was 1:3200.

(b) The immunosorbents were saturated with ragweed allergic serum Gre. The PCA titer given by allergic serum Gre in monkey was 1:512.

TABLE XI

Effect of varying the stabilizing protein concentration on the recovery of reagins from immunosorbent by elution at pH 2.5.

Protein concentration (mg/ml)	P-K titer of eluates in presence of	
	NHS	HSA
70	200	200
7	200	200
1.4	100	100
0.7	100	100
0.07	0	0

The P-K titer given by the allergic serum Kh was 1:3200.

Isolation of reagins

The experiments using 2M NaI at pH 9.o in the presence of HSA (7 mg/ml) proved to be successful for the isolation of reagin. A P-K titer of 1:200 was given by such eluates obtained from 20 mg of immunosorbent saturated with allergic serum Kh.

(i) The sequential elutions employing first 2M NaI and then gly-HCl buffers demonstrated the presence of reagins with two distinctly different affinities for the immunosorbent (Figure 11, part A). Thus, P-K activity was associated only with the first three NaI eluates (1, 2 and 3) and was absent from eluates 4 and 5 (Figure 11, part A(i)). However, the subsequent eluates 1 and 2 obtained with gly-HCl also possessed reaginic activity, (Figure 11, part A(ii)).

(ii) When the elution procedure was reversed (Figure 11, Part B), the bulk of the reaginic activity was recovered by elution first with gly-HCl in fractions 1,2 and 3 but not in eluates 4 and 5 (Figure 11, part B(i)); moreover, some reagins were subsequently eluted also with NaI (Figure 11, part B(ii)).

A similar elution pattern emerged for reagins of allergic serum Gre (Figure 12). However, in this latter case, the recoveries of reaginic activity were lower and no reaginic activity was observed in any of the NaI eluates (Figure 12, part B(ii)) following the multiple elution with the gly-HCl (Figure 12, part B(i)).

The results from the sequential elution experiments demonstrated the presence of (at least) two groups of reagins; one being eluted with NaI and the other with gly-HCl. Moreover, these experiments showed that the dissociation of reagins from immunosorbent was not complete with wither NaI or gly-HCl alone.

FIGURE 11

Recovery of reagins by sequential elutions from immunosorbent (25 mg) saturated with allergic serum Kh.

Part A Eluates obtained first with 2M NaI in 0.1M Tris-HCl, pH 9.1, (part A(i)) and secondly from the same aliquot of immunosorbent with 0.1M gly-HCl, pH 2.5, (part A (ii));

Part B Eluates obtained first with 0.1M gly-HCl pH 2.5, (part B(i)), and secondly from the same aliquot of immunosorbent with 2M NaI in 0.1M Tris-HCl, pH 9.1 (part B(ii)).

A volume of 2.5 ml of the eluting agent was used for each of the multiple elutions and the eluates are referred to by the numerals 1, 2, 3, 4 and 5 which correspond to the order of elution. The eluting agents contained HSA at a concentration of 7 mg/ml.

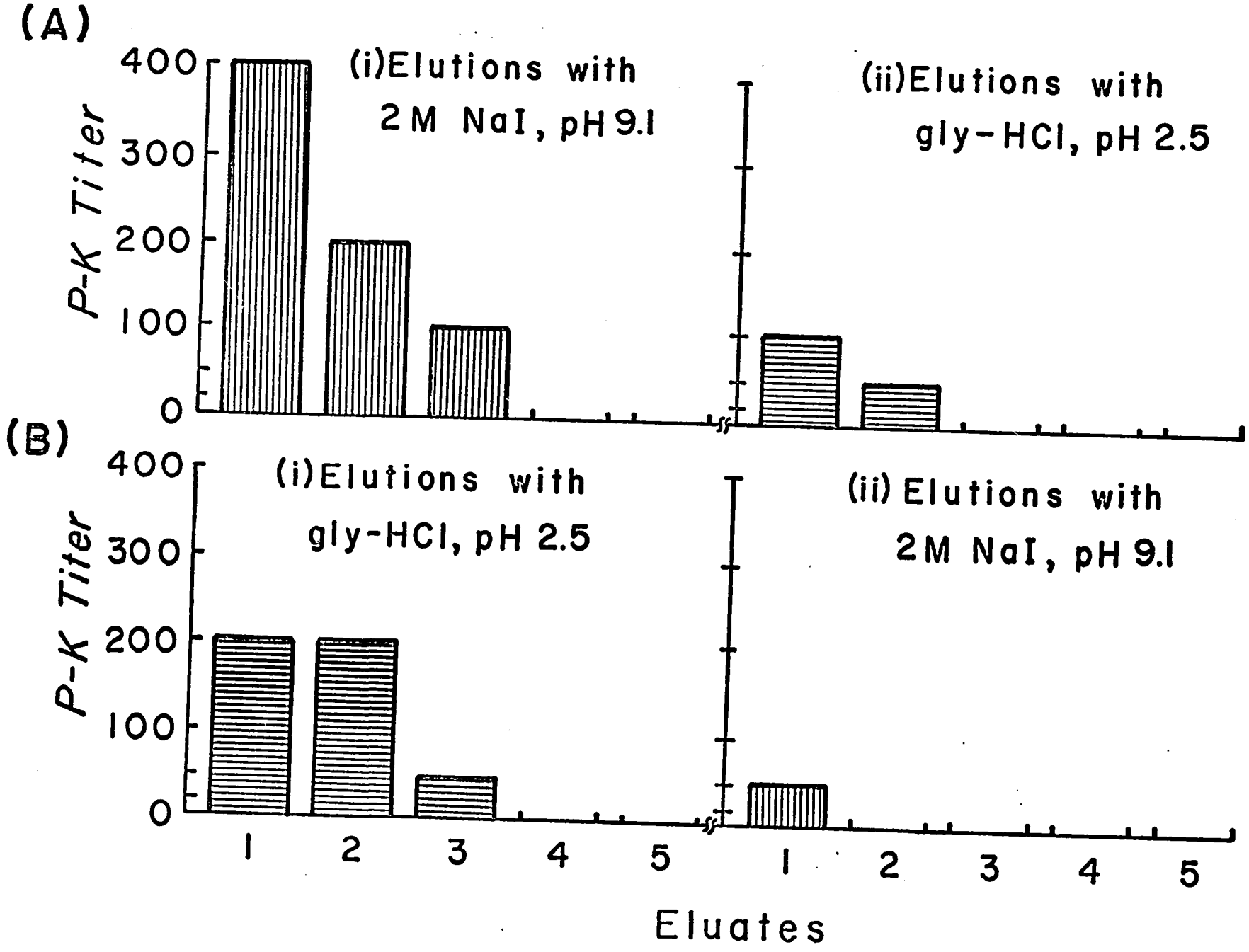

(A)
(i)Elutions with
2 M NaI, pH 9.1
(ii)Elutions with
gly-HCl, pH 2.5
P-K Titer
400
300
200
100
0
(B)
(i)Elutions with
gly-HCl, pH 2.5
(ii) Elutions with
2M NaI, pH 9.1
P-K Titer
400
300
200
100
0
1
2
3
4
5
1
2
3
4
5
Eluates

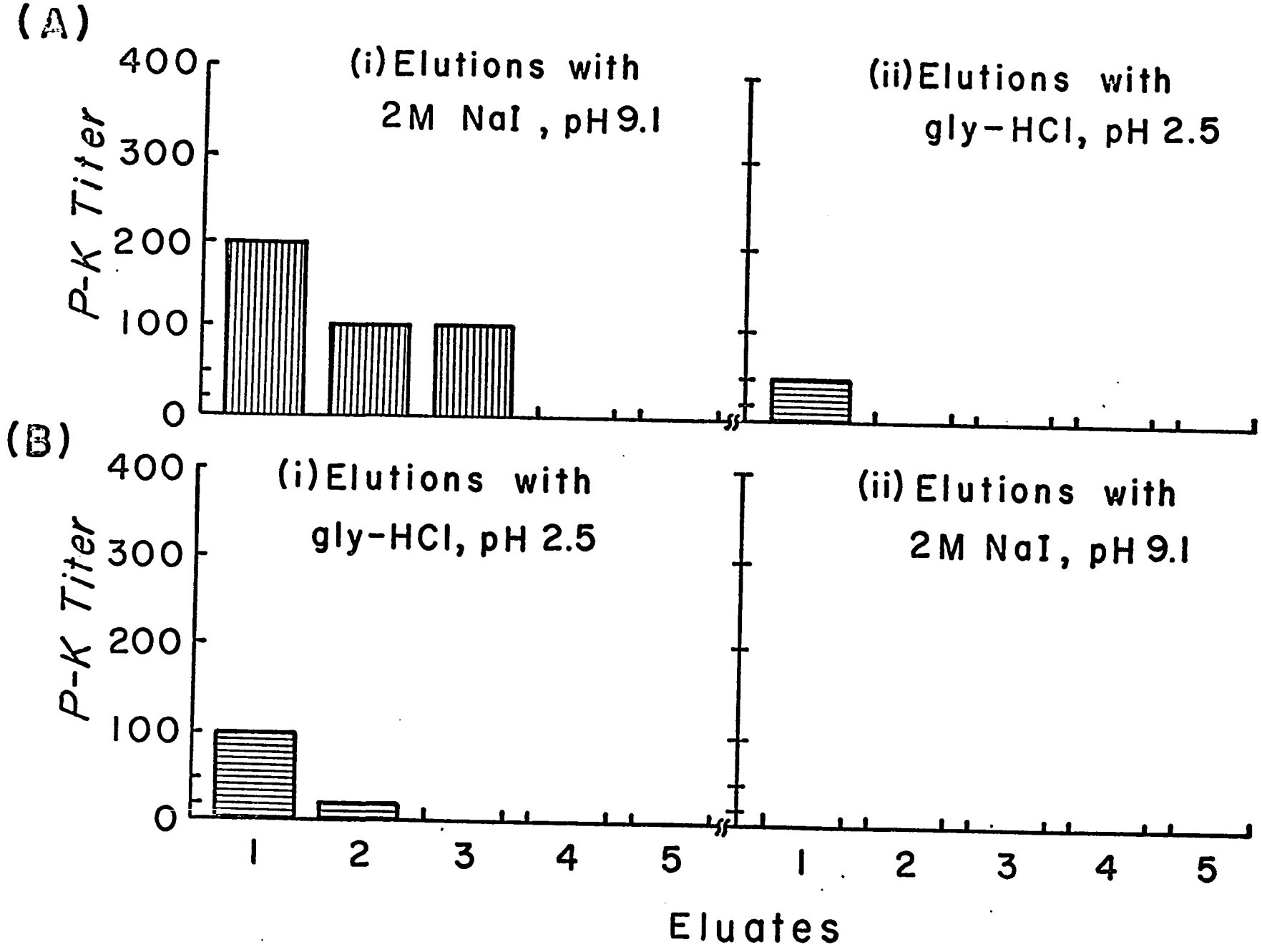
FIGURE 12

Recovery of reagins by sequential elutions from immunosorbent (25 mg) saturated with allergic serum Gre.

Part A Eluates obtained first with 2M NaI in 0.1M Tris-HCl, pH 9.1 (part A(i)) and secondly from the same aliquot of immunosorbent with 0.1M gly-HCl, pH 2.5, (part A(ii));

Part B Eluates obtained first with 0.1M gly-HCl pH 2.5, (part A(i)), and secondly from the same aliquot of immunosorbent with 2M NaI in 0.1M Tris-HCl, pH 9.1 (part B(ii)).

A volume of 2.5 ml of the eluting agent was used for each of the multiple elutions and the eluates are referred to by the numerals 1, 2, 3, 4 and 5 which correspond to the order of elution. The eluting agents contained HSA at a concentration of 7 mg/ml.


(A)
400
300
200
100
0
P-K Titer
(i) Elutions with
2M NaI , pH 9.1
(ii) Elutions with
gly-HCl, pH 2.5
(B)
400
300
200
100
0
P-K Titer
(i) Elutions with
gly-HCl, pH 2.5
(ii) Elutions with
2M NaI, pH 9.1
1
2
3
4
5
1
2
3
4
5
Eluates

Specific isolation of reagin from immunosorbent with hapten

The eluate obtained by displacement with the ragweed haptens (Figure 13, part a) had a P-K titer of 1:25. Following this specific elution, additional reaginic activity at a titer of 1:200 was recovered from the same immunosorbent by elution with 2M NaI. The specificity of the elution of reagins with haptens was demonstrated by the fact that reagins against the allergens of grass pollen were not displaced by the ragweed haptens from appropriately saturated grass allergen immunosorbent (Figure 13, part b). In this latter case, reagins were recovered from immunosorbent by elution with 2M NaI; the P-K titer of eluates with specificities to the allergens of grass pollen was 1:100.

(A) Characterization of antibodies recovered from immunosorbents

On immunoelectrophoresis the mobility of the myeloma IgE was anodal to the point of application (Figure 14, part a). The presence of radioactively labelled precipitin arcs was revealed by radio-autographic analysis (Figure 14, part b). Moreover, the mobility of the labelled precipitin arcs - obtained on reaction of the material, eluted with NaI and gly-HCl, with an antiserum specific to IgE* and I^{131}-DWSR - coincided with the mobility of the visible precipitin arcs. This procedure demonstrated that the eluted IgE antibodies were still capable of reacting with the I^{131}-DWSR. In these experiments, a γE myeloma serum* was added to the sample wells prior to the addition of the eluates and before electrophoresis. In a control experiment, it was determined that the I^{131}-DWSR did not react or bind with the myeloma IgE alone.

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* This antiserum and the γE myeloma serum were kindly provided by Dr. K. Ishizaka.

FIGURE 13

Demonstration of the specificity of the elution of reagins by displacement off immunosorbent with haptens.

(a) The immunosorbent, prepared by coupling DWSR to amino-cellulose, was saturated with serum from the ragweed allergic individual (Kh). The elutions were performed (1) with ragweed haptens, and (2) with 2M NaI in 0.1M Tris-HCl buffer at pH 9.1 from the same immunosorbent. The eluates were exhaustively dialysed prior to the P-K test. Skin sites sensitized with these eluates were challenged with DWSR (10 μg/ml).

(b) The immunosorbent prepared by coupling DWSG to amino-cellulose, was saturated with serum from the grass allergic individual (Na). The elutions were performed (1) with ragweed haptens, and (2) with 2M NaI in 0.1M Tris-HCl buffer at pH 9.1 from the same immuno-sorbent. The eluates were exhaustively dialysed prior to the P-K test. Skin-sites sensitized with these eluates were challenged with DWSG (10 μg/ml).

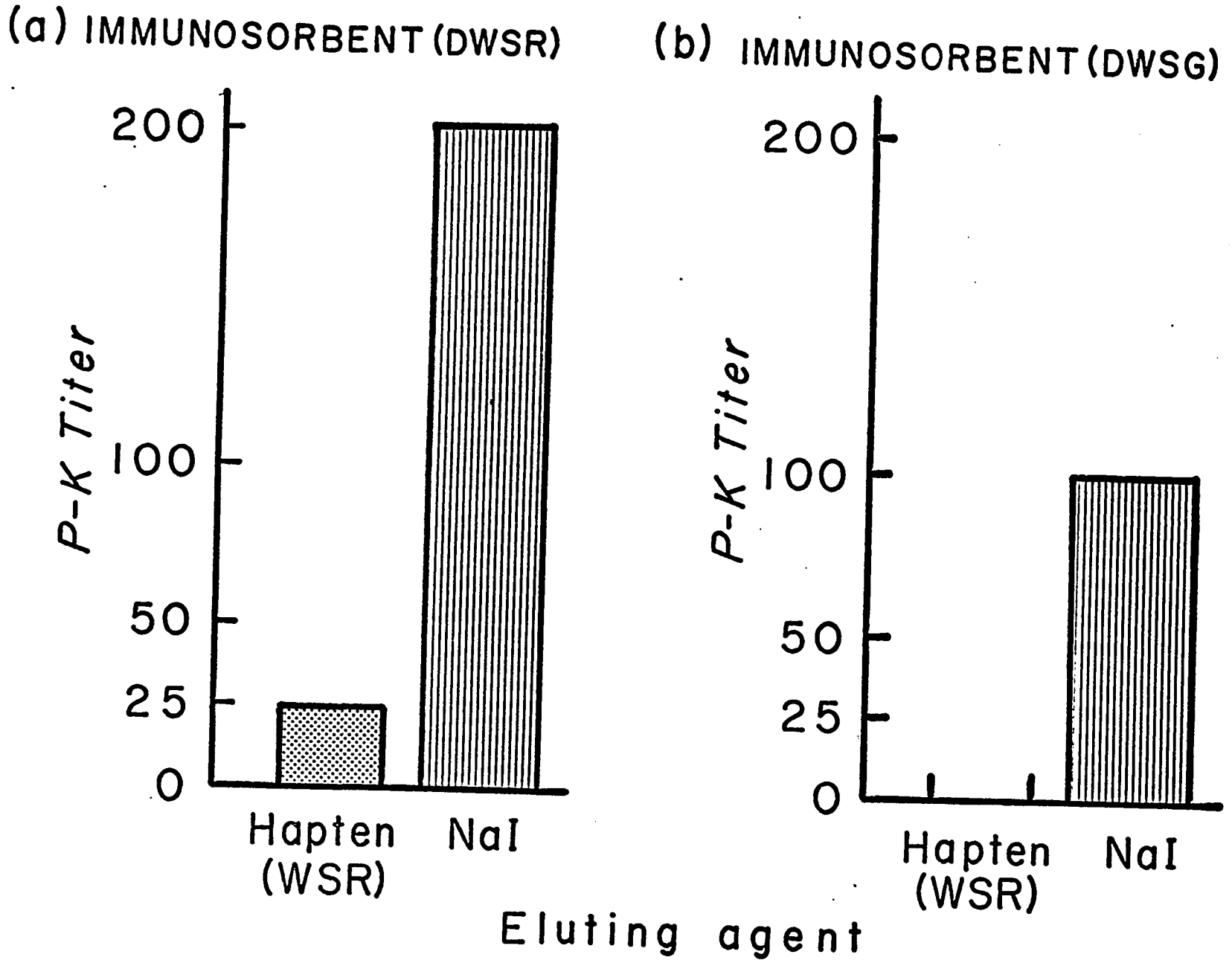

(a) IMMUNOSORBENT (DWSR)
(b) IMMUNOSORBENT (DWSG)
P-K Titer
200
100
50
25
0
Hapten (WSR)
NaI
Eluting agent

FIGURE 14

Immunoelectrophoretic pattern of eluates recovered from immunosorbent saturated with reaginic serum fraction Kh 35-45[II].

(a) Stained slide.

well (1) Eluate obtained with 2M NaI in 0.1M Tris-HCl buffer, pH 9.1.

well (2) Eluate obtained with 0.1M gly-HCl buffer at pH 2.5.

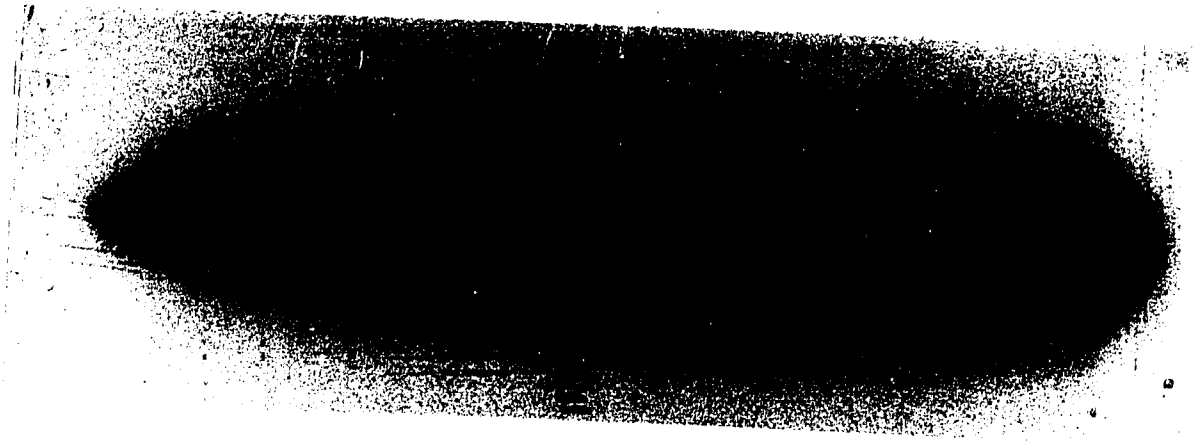
A γE myeloma serum was also added to both sample wells prior to electrophoresis.

The immunoelectrophoretic analysis was done in agarose with veronal buffer, pH 8.6 $\Gamma/2 = 0.033$, for 40 minutes at V/cm. An antiserum specific to IgE was added to the antibody trough after electrophoresis. I^{131}-DWSR was also added to the antibody trough after immunodiffusion had been carried out for 48 hours and the slides had been washed to remove unreacted proteins.

(b) Radio-autograph of the above slide.

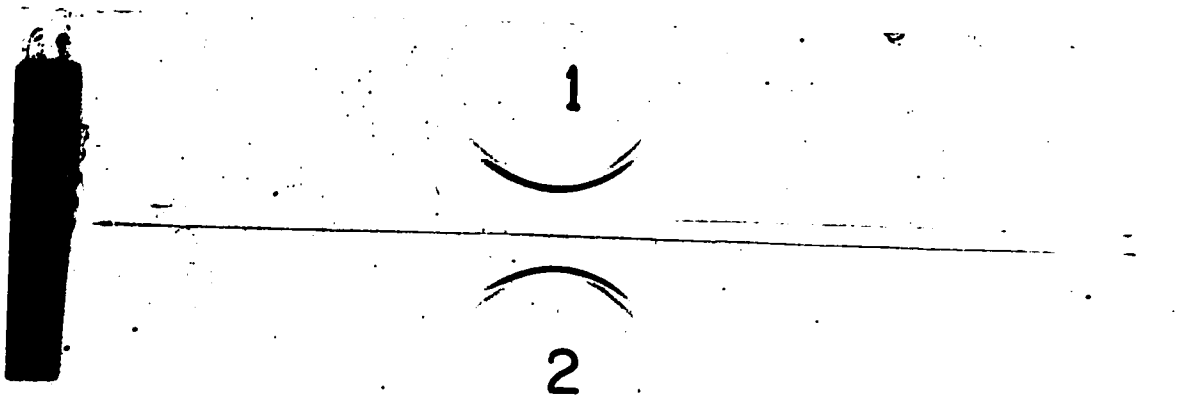
(a)

(b)

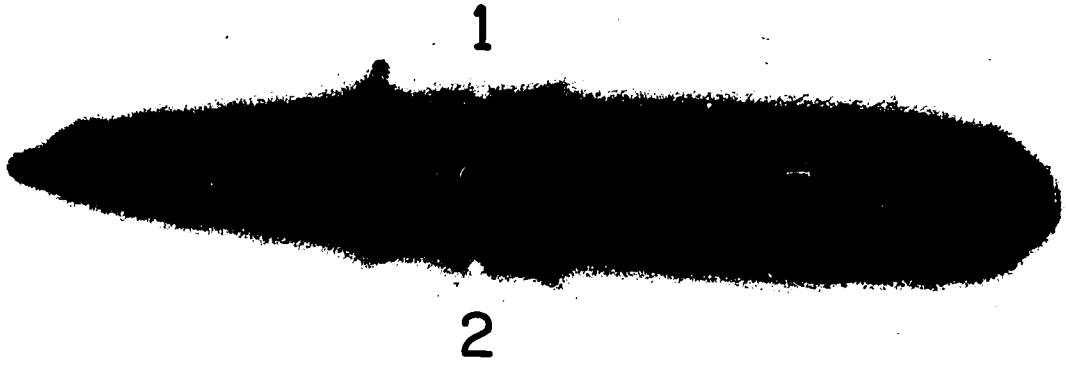


⊕ ⊖

(a)



(b)



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Whereas the analysis of eluates obtained with NaI and gly-HCl by the Ouchterlony procedure did not reveal any precipitin bands, evidence for the presence of IgE in these eluates was obtained by radio-autography (Figure 15); it was thus demonstrated that the NaI eluates 1, 2, 3 and 4 and gly-HCl eluates 1 and 2 contained DWSR binding antibodies of the IgE class.

The binding of labelled DWSR was not observed with either IgA or IgM during immunodiffusion analysis of eluates isolated from immunosorbents saturated either with the reaginic fraction Kh 35-45[II] or with the IgG fraction Kh 30[I] (which was devoid of reagins). For immunosorbents saturated with this latter serum fraction, the presence of IgG ragweed-binding antibodies was demonstrated by radio-immunoelectrophoresis in both NaI and gly-HCl eluates (Figure 16). The radioactive precipitin arcs coincided with the visible precipitin arcs obtained on reaction of the eluates with an antiserum specific to IgG. In these experiments a normal human serum was added to the sample wells prior to the addition of the eluates and before electrophoresis.

(B) Characterization of antibodies present in antisera produced in guinea pigs by immunization with eluates recovered from immunosorbents

(i) Antisera obtained from guinea pigs in which immune paralysis had been induced to serum proteins with NCS

In the analysis of 5 allergic sera by the Ouchterlony procedure employing antiserum to the reaginic eluates, a precipitin band was observed with two of the allergic sera (CB, GC.) to which IgE had been added (Figure 17, part a) thus, demonstrating again that the reaginic eluates used for immunization of guinea pigs contained IgE. Moreover,

FIGURE 15

Radio-autographic pattern of eluates recovered from immunosorbent saturated with allergic serum fraction Kh 35-45 [II]. The center well was filled with an antiserum specific to IgE (provided by Dr. K. Ishizaka). Wells 1, 2, 3, and 4 contained eluates 1, 2, 3, and 4 obtained with 2M NaI in Tris-HCl buffer, pH 9.1: wells 5, 6, and 7 contained eluates 1, 2 and 3 obtained with 0.1M gly-HCl buffer, pH 2.5. Well 8 contained allergic serum fraction Kh 35-45[II]; DWSR-I^{131} was added to the center well 48 hours after immunodiffusion had taken place and the Ouchterlony slides had been thoroughly washed.

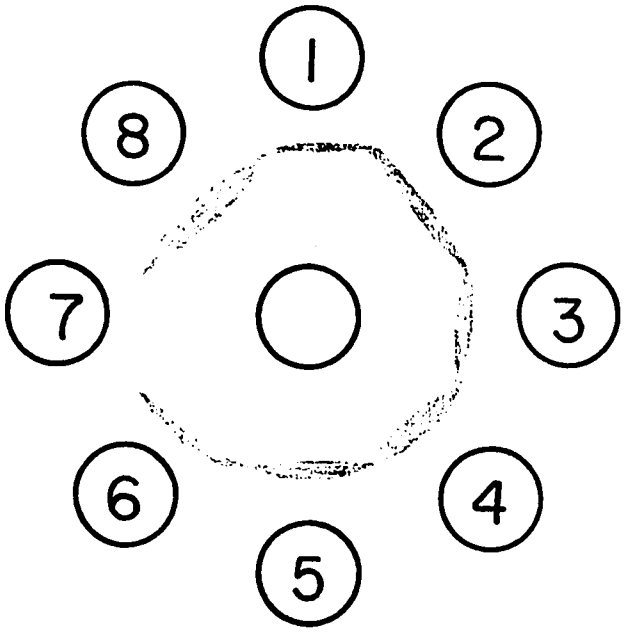

1
2
3
4
5
6
7
8

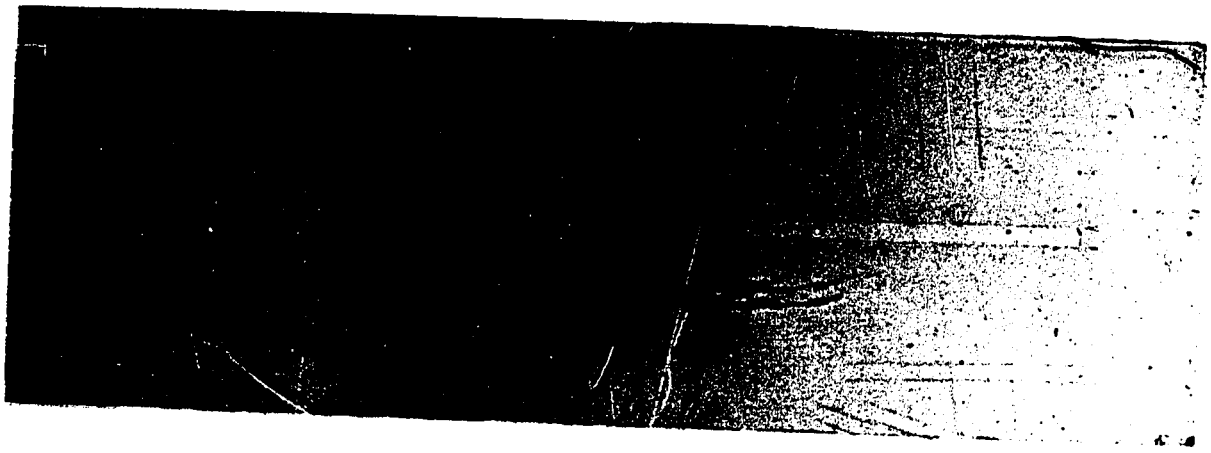
FIGURE 16

Immunoelectrophoretic pattern of eluates recovered from an immunosorbent which had been saturated with allergic serum fraction Kh 30[I] (devoid of reaginic activity).

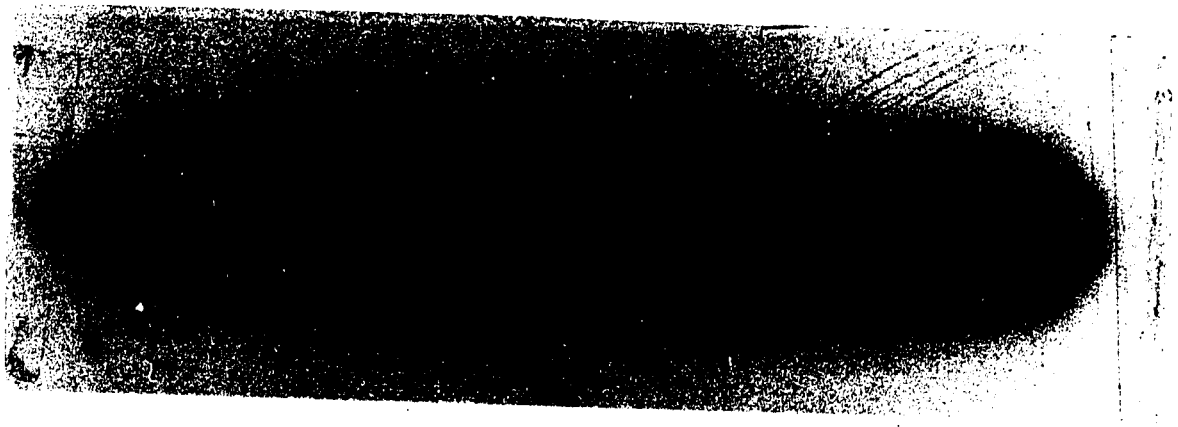
(a) **Stained slide.** (1) Eluate obtained with 2M NaI in 0.1M Tris-HCl buffer at pH 9.1; (2) Eluate obtained with 0.1M gly-HCl at pH 2.5. A normal human serum was added to both sample wells prior to electrophoresis which was performed in agarose with veronal buffer, $\Gamma/2 = 0.033$, pH 8.6, at 9.25 V/cm for 40 minutes. An antiserum specific to IgG was added to the antibody trough after electrophoresis. I^{131}-DWSR was also added to the antibody trough after immunodiffusion had been carried out for 48 hours and the slides had been washed to remove unreacted proteins.

(b) Radio-autograph of above slide.

(a)

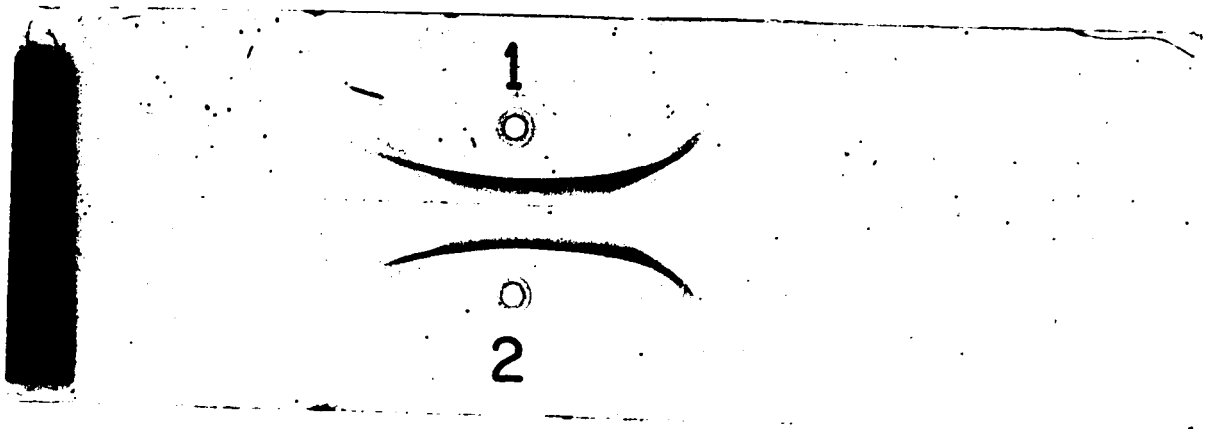


(b)



⊕ ⊖

(a)



(b)

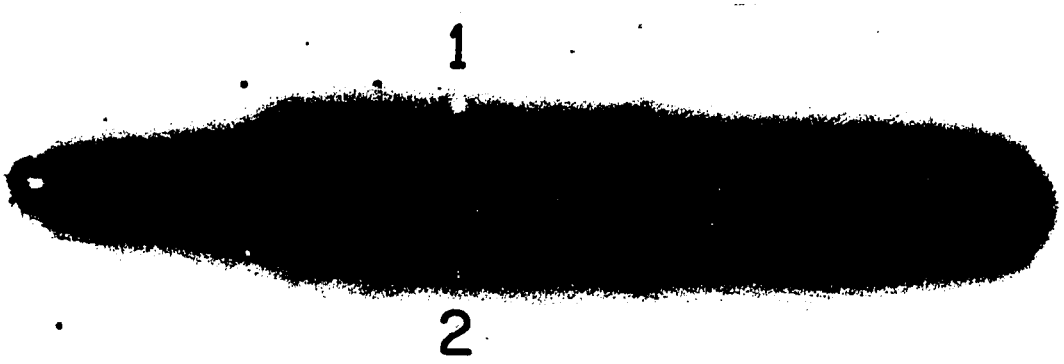


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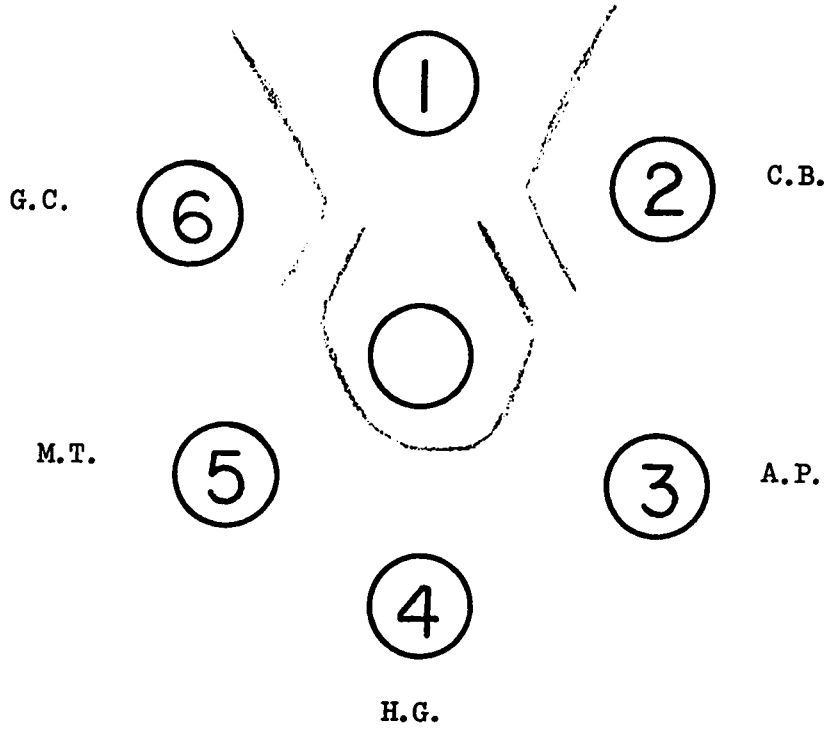
FIGURE 17

Ouchterlony patterns obtained with guinea pig antiserum to reagin.

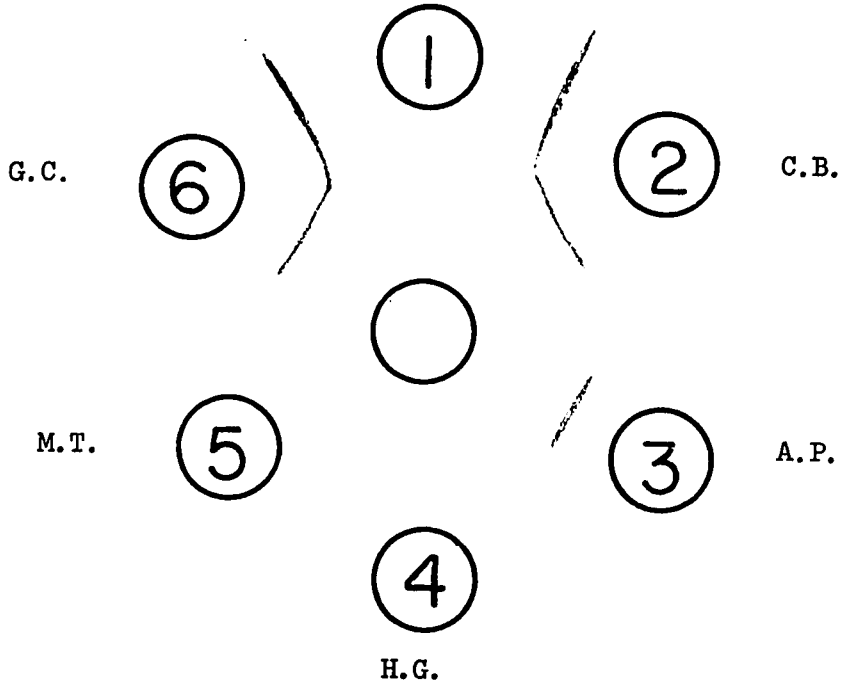
(a) Stained slide: The center well was filled with the guinea pig antiserum produced to the reaginic fractions eluted from immunosorbent, which had been saturated with allergic serum fraction Kh 35-45 [II]. (The guinea pig had been pre-treated with a preparation of normal cord serum injected intravenously before immunization, to induce immune paralysis to normal human serum proteins). Well 1 contained Dr. Ishizaka's antiserum specific to IgE. Allergic sera C.B., A.P., H.G., M.T., and G.C. were added to the outer wells as indicated. Myeloma IgE was also added to the outer wells 2 and 6 containing sera C.B. and G.C respectively. Ragweed antigen E labelled with I^{125} was added to all wells.

(b) Radio-autograph of the Ouchterlony plate.

(a)



(b)



these precipitin bands merged with those elicited on reaction with Ishizaka's antiserum to IgE. Furthermore, by radio-autography, it was revealed that these precipitin bands representing IgE-anti-IgE complexes incorporated I^{125}-antigen E (Figure 17, part b). A radioactive precipitin band was also formed between the allergic serum A.P. and the guinea pig antiserum, although in this case no corresponding visible precipitin band had been detected. It appears therefore, that IgE antibodies were present in serum A.P. in sufficient concentration to be detected without the addition of carrier IgE. An additional set of merging precipitin bands, closer to the central antibody well, were obtained with all 5 allergic sera on reaction with the guinea pig antiserum (Figure 17, part a). However, these precipitin bands did not bind the I^{125}-antigen E.

In the analysis of whole allergic sera Kh and Gre by immunoelectrophoresis (Figure 18, part b) with the guinea pig antiserum to the reaginic eluates it was found that antibodies had not been produced to the HSA present in the eluates for stabilizing reagins. Thus, it was concluded that an immune tolerance to this protein had been indeed induced by the intravenous pre-treatment with NCS. Antibodies to component(s) with the mobility of α-globulins were present in the guinea pig antiserum (Figure 18, part a).

Demonstration by the reversed PCA test in monkey of the presence of antibodies to IgE in the guinea pig antisera to reaginic eluates

The presence of antibodies to IgE in the guinea pig antiserum produced by immunization with the reaginic eluates was also demonstrated by the reversed PCA test in a monkey in which it was observed that the antiserum elicited positive cutaneous reaction, whereas normal guinea pig antiserum did not (Table XII).

FIGURE 18

Immunoelectrophoretic analysis of guinea pig antisera with allergic sera.

(a) Antiserum (1) from a guinea pig, which had been rendered tolerant to the proteins of normal cord serum, immunized with the combined eluates obtained with NaI at pH 9.1 and gly-HCl at pH 2.5, HSA having been added to the eluting buffers for stabilization of reagins. The immunosorbent was saturated with allergic serum fraction Kh 35-45[II].

(b) Antiserum (2) from a normal guinea pig immunized with the eluates obtained with NaI and gly-HCl from immunosorbent saturated with allergic serum fraction Gre 35-45[II]. Rabbit serum albumin had been added to the eluting buffers for stabilization of reagins.

(c) Antiserum (3) from a normal guinea pig immunized with the eluates obtained with NaI and gly-HCl from immunosorbent saturated with allergic serum fraction Kh 30[I] (devoid of reagins), HSA having been added to the eluting buffers.

The sample wells were filled with whole allergic serum Kh or Gre as indicated. Electrophoresis was performed in agarose with veronal buffer $\Gamma/2 = 0.033$, pH 8.6, at 9.25 V/cm for 40 minutes.

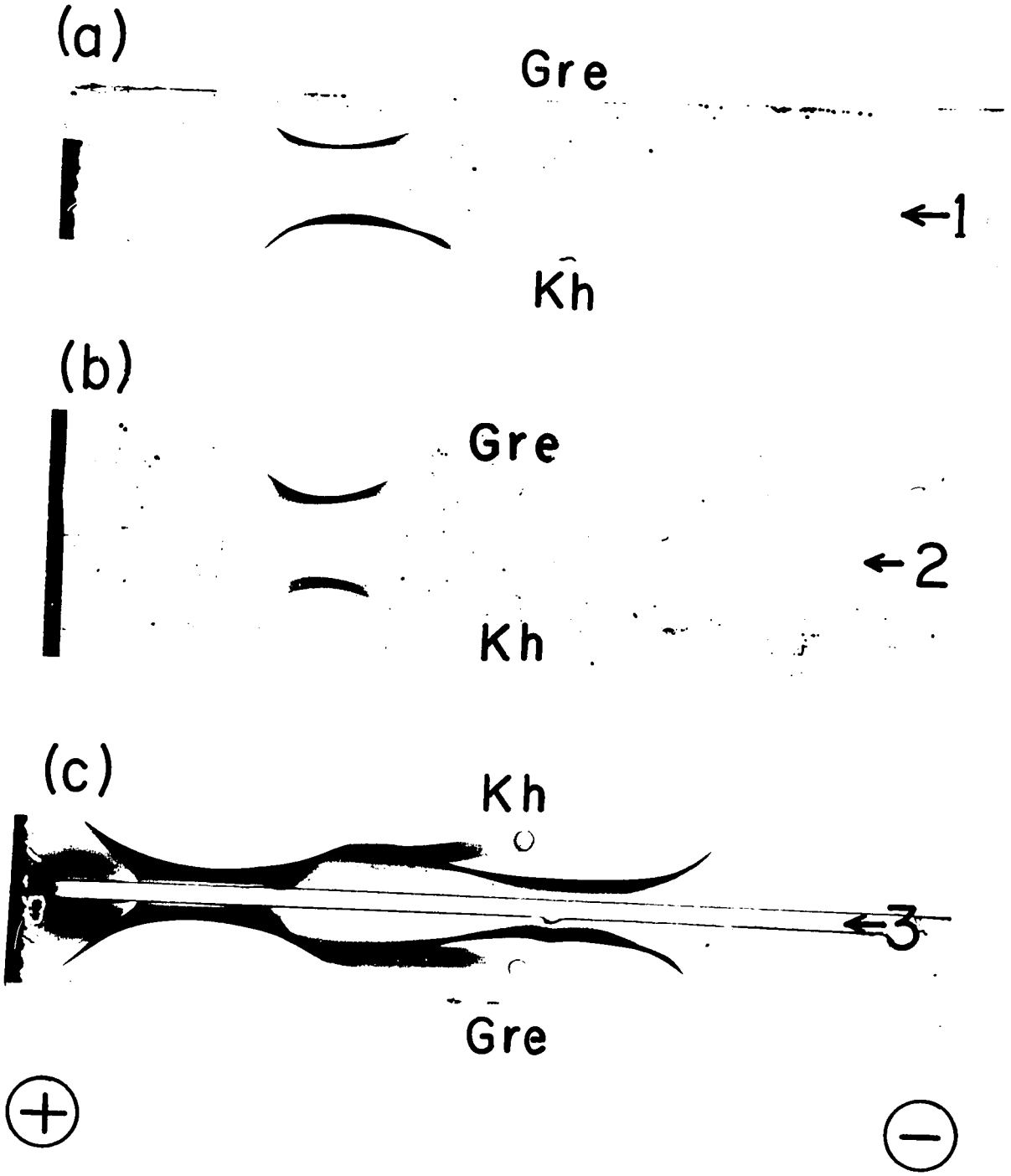

(a)
Gre
←1
Kh
(b)
Gre
←2
Kh
(c)
Kh
←3
Gre
⊕
⊖

TABLE XII

Demonstration by the reversed PCA test in monkey of the presence of antibodies to IgE in guinea pig antiserum to reaginic eluates.

Reciprocal of serum dilution	Cutaneous reactions* observed with immune serum**	normal serum
100	+	-
1000	+	-
10,000	-	-

* To render the cutaneous reactions easily visible, a volume of 5 ml of Evans blue (1%) was injected intravenously 10 minutes after the intradermal injections of the guinea pig antiserum. (A slight reaction (±) was also observed with both the immune and normal sera in a dilution of 1:10, which is probably attributable to non-specific irritation due to high serum protein concentrations).

** The guinea pigs had been rendered tolerant to the proteins of cord serum. The immune serum was obtained after the second course of immunizations with the combined NaI and gly-HCl eluates isolated from immunosorbent saturated with allergic serum fraction Kh 35-45[II]. A quantity of 1 mg each of Bence-Jones proteins, type κ and λ was added to a volume of 1 ml of the antiserum prior to its use for cutaneous injections.

(ii) Antisera obtained from animals which had not undergone any pre-treatment with serum

Antibodies to the human serum components with the mobility of an α-globulin were also detected in an antiserum produced by immunization with the reaginic eluates of serum Gre in which RSA was present as a stabilizing agent for reagins (Figure 18, part b). Thus, it must be concluded that a serum constituent with the mobility of an α-globulin had become adsorbed from the allergic serum and was subsequently displaced from immunosorbent by the elution with NaI and gly-HCl.

As can be seen from the immunoelectrophoretic analysis (Figure 18, part c) of whole allergic sera Kh and Gre with the guinea pig antiserum to the eluates from immunosorbent w ich had been saturated with allergic serum fraction Kh 30[I], this antiserum contained antibodies to IgG and also to HSA which had been present in the eluates for stabilizing reagins.

From analysis of 5 allergic sera by the Ouchterlony procedure (Figure 19, part a) with this guinea pig antiserum, two groups of precipitin bands were observed; one close to the central antibody well and the other approximately equi-distant from the central and outer wells. Neither of these bands corresponded to IgE-anti-IgE complexes since neither of them merged with the precipitin bands developed between well 1 (anti-IgE) and wells 2 (C.B. + IgE), and 6 (G.C. + IgE).

On radio-autographic analysis (Figure 19, part b) it was observed that I^{125}-ragweed antigen E was incorporated into these IgE-anti-IgE complexes. However, in contrast to the similar analysis of the guinea pig antiserum to the reaginic eluates (Figure 17), precipitin bands which formed between rabbit anti-IgE (well 1) and allergic sera C.B. and G.C. (wells 1 and 6 respectively), did not merge with those formed with this

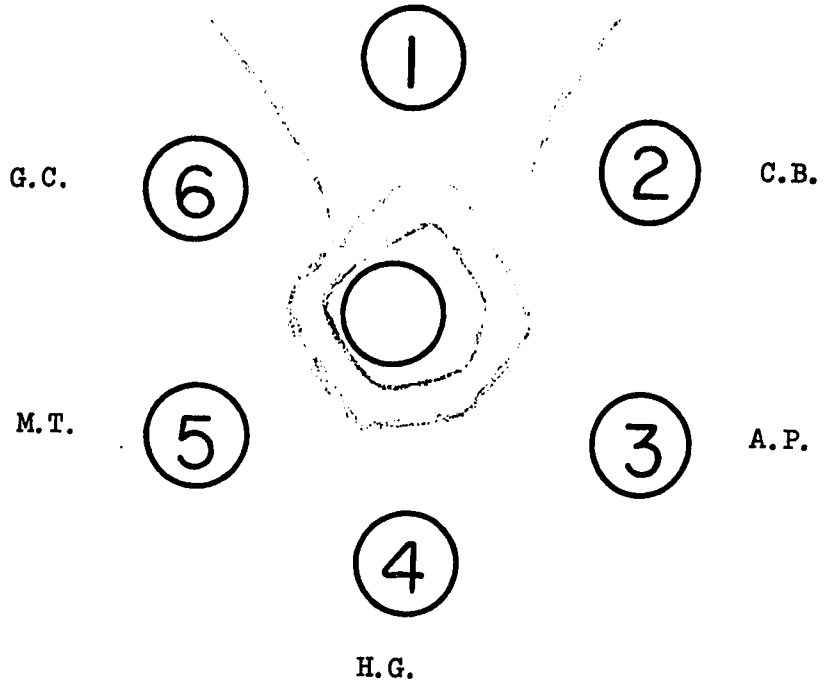
FIGURE 19

Ouchterlony pattern obtained with guinea pig antiserum produced by immunizations with eluates recovered from immunosorbents saturated with allergic serum fraction Kh 30[I].

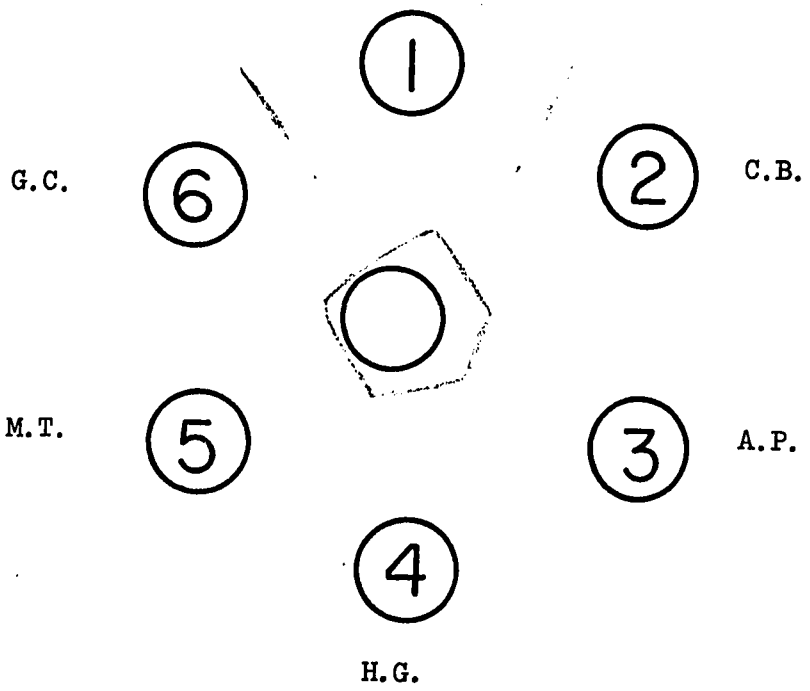
(a) Stained slide: The center well contained the guinea pig antiserum and well 1 contained antiserum specific to IgE. Allergic sera C.B., H.G., M.T., and G.C. were added to the outer wells as indicated. Myeloma IgE was also added to the outer wells 2 and 6 containing sera C.B. and G.C. respectively. Ragweed antigen E labelled with I^{125} was added to each well.

(b) Radio-autograph of the Ouchterlony plate.

(a)



(b)



guinea pig antiserum. Rather, the precipitin bands closest to the central antibody well obtained with all 5 allergic sera incorporated the I^{125}-antigen E (Figure 19, part b). Thus, on the basis of the observations from immunoelectrophoretic analysis which demonstrated the presence of antibodies to HSA and to IgG and from the Ouchterlony radio-autograph which revealed that the precipitin bands closer to the central antibody well contained I^{125}-antigen E, it was inferred* that the precipitin bands had indeed formed with the ragweed-binding IgG antibodies of the 5 allergic sera on reaction with antibodies to IgG present in the guinea pig antiserum.

DISCUSSION

In this phase of the investigation, it was demonstrated that reagins could be successfully eluted from cellulose-ragweed conjugates with 6M urea or at low pH in the presence of serum proteins, as long as these proteins were at a concentration higher than 0.7 mg/ml. Moreover, the addition of a single purified protein, such as HSA or RSA alone, resulted in the stabilization of reaginic activity during the elution process. Thus, the notion that stabilizing proteins were related to the globulins which were associated with reaginic activity does not appear to be valid (218). Therefore, HSA (or RSA) which possesses physico- and immuno-chemical properties distinctly different from those of reagin was employed as the stabilizing protein.

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* The continuation of these experiments employing appropriate reference human serum proteins and their specific homologous antisera had to be discontinued due to the closing down of the laboratory when Professor A.H. Sehon transferred from McGill University, Montreal, Quebec to the University of Manitoba, Winnipeg, Manitoba.

The purpose of this part of the study was to isolate reagins by means of specific immunochemical techniques using amino-cellulose for the preparation of the immunosorbent. Therefore, to maximize the amount of reagins absorbed onto the immunosorbent, the latter was saturated with allergic serum or its reaginic enriched fractions until the supernatants, after absorption, possessed reaginic activity approaching that of the original serum. For this reason, the yield of the eluted reagins cannot be referred to an original serum volume. Instead, arbitrarily, to evaluate the effectiveness of the dissociation of reagins a constant volume of the eluting agent for a given amount of immunosorbent was used in any one experiment, so that the activities of the eluates could be compared to each other. In an earlier investigation in this laboratory (218) using identically prepared immunosorbents, it had been found that approximately 8 mg of rabbit antibodies to DWSR was bound by 25 mg of the immunosorbent. These values are an indication of the capacity of the ragweed immunosorbents to adsorb homologous antibodies.

In addition to using urea or low pH for the dissociation of antibody-antigen complexes, it was found that sodium iodide at a concentration of 2M was a mild and effective agent for the elution of reagins from appropriately saturated immunosorbents. This concentration of NaI was chosen on the basis of the results of another study (256) in which it was demonstrated that about 82% of antibody-antigen complexes were dissociated by the chaotropic ions at this concentration. Furthermore, as shown by Avrameas (256), human isoagglutinins retained their antibody activity following exposure at this salt concentration for a period of 24 hours. By contrast, the use of higher salt concentrations, which were

capable of completely dissociating antibody-antigen complexes, or elution with acid, resulted in the denaturation of immunoglobulins as demonstrated by loss of antibody activity or changes in their electrophoretic mobility (256).

On the basis of the sequential displacement of reagins with two different eluting agents, it was concluded that the allergic sera used in this study contained two groups of reagins differing at least in their allergen binding affinity. Irrespective of the order of addition of the eluting agents to the immunosorbent, one group of reagins was eluted with NaI and the other with gly-HCl. In these experiments, the original, unfractionated allergic sera had been used to saturate the immunosrobents. It is important to note that in similar elution experiments from immunosorbents saturated with allergic serum fractions enriched with reagins, IgE was demonstrated in both NaI and gly-HCl eluates.

The specific isolation of reagins was accomplished by displacing them from saturated immunosorbents using low molecular weight haptens of ragweed pollen. These haptens have been shown to be capable of neutralizing completely and specifically the reaginic activity of ragweed allergic sera (257). The relatively low recovery of reagins by elution with hapten is not surprising since one would expect that only reagins with low affinity for the allergenic determinants of DWSR would be displaced by this method, particularly since the constituents of the low molecular weight fractions of WSR with haptenic properties may be present in very low concentrations.

To support this interpretation, it is to be noted that excessively large amounts of the haptenic preparation were required (by comparison with DWSR), for the complete neutralization of reaginic activity

of ragweed allergic sera (257). Moreover, it is to be pointed out that by zone electrophoresis on cellulose acetate it was shown that the haptenic preparation was composed of at least four components with distinct electrophoretic mobilities* and it still remains to be determined which of these components possesses neutralizing capacity. Thus to increase the yield of reagins by elution with hapten, either higher concentrations of the presently available haptenic preparations, or preferably more highly purified haptens should be employed.

Nevertheless, the immunochemical specificity of this elution procedure was demonstrated by the findings that reagins to ragweed allergens were displaced by ragweed haptens, while no reagins to the allergenic constituents of Timothy grass pollen could be displaced by these same haptens; however, the reagins to grass could be displaced by the subsequent elution with NaI.

The differential solubility of blocking antibodies and reagins in concentrated salt solutions was exploited for their separation from each other using a procedure established in an earlier study in this laboratory (176). Three fractions of allergic serum were obtained by salting-out with ammonium sulfate: the first contained the majority of the blocking antibodies but was devoid of reagins; the second fraction contained the remaining portion of blocking antibodies and some reagins; the third fraction contained reagins but was devoid of blocking antibodies (176). A further enrichment with respect to blocking

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* Personal communication (Drs. N.A. Attallah and A.H. Sehon).

antibodies or reagins was accomplished by ion-exchange chromatography on DEAE-Sephadex A 50 of the first and third allergic serum fractions described. Since it has been postulated that there is no essential difference between the sera of treated and non-treated allergic individuals, other than differences in the ratio of the amount of blocking antibodies to reagins (258) it is to be expected that the fractionation procedures employed led to the recovery of these antibodies in comparable ratios, irrespective of whether treated or non-treated allergic sera (Kh and Gre respectively) were used. Thus, for immunosorbents saturated with serum fractions enriched with reagins it is reasonable to assume that only reagins were adsorbed, in contrast to the adsorption of both blocking antibodies and reagins when unfractionated allergic sera were employed for the saturation of immunosorbents. This interpretation may account for the observation that eluate #4 (the fourth elution with NaI) from an immunosorbent which had been saturated with the reaginic serum fraction Kh 35-45[II], contained IgE ragweed-binding antibodies (Figure 17) whereas the corresponding fraction eluted from an immunosorbent saturated with the whole Kh serum was devoid of reaginic activity* (Figure 11, part A (i)).

In addition to the detection of IgE in the reaginic fractions eluted with 2M NaI and gly-HCl, pH 2.5, with the help of the antiserum obtained from Dr. Ishizaka, independent evidence for the presence of this immunoglobulin in these eluted fractions was adduced in this study by the production of specific anti-IgE antibodies in guinea pigs on

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* In these comparisons it is implied that IgE ragweed-binding antibodies are synonymous with reagins.

immunization with these fractions.

As mentioned earlier, the production of antibodies to proteins other than reagins was suppressed in these animals by inducing immune paralysis in them to most, if not to all, normal serum proteins. It was expected, in accord with the recent report of Henney and Ishizaka (259), that whole serum would induce immune paralysis in guinea pigs to the antigenic determinants of the light chains of immunoglobulins as well as to the characteristic antigenic determinants of the Fc portion of the heavy chains.*

For these reasons, a preparation of normal cord serum was used in the present study, since it is known that NCS is virtually free of reagins. As additional evidence for the effectiveness of this method to induce immune paralysis it is to be pointed out that neither antibodies to HSA nor to most of the normal human globulins were detected in the sera of these animals. However, immunization of these animals with the reaginic fractions eluted off specific immunosorbents led not only to the production of anti-IgE antibodies, but also surprizingly to antibodies reacting to a protein with the electrophoretic mobility of α-globulins. In view of the fact that cord sera lack IgA, these animals could not have been rendered tolerant to IgA. It is interesting to note that no anti-IgA antibodies were produced on immunization with the reaginic serum fractions. Therefore, this observation can be taken as further proof that reagins are not associated with IgA.

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* Nevertheless, as a precautionary measure in the present study, both κ and λ Bence-Jones proteins were used to "absorb" the guinea pig antisera to remove any antibodies directed against the antigenic determinants of κ and λ light chains.

Similar results were obtained with antisera prepared in normal guinea pigs (which had not been rendered tolerant to any serum proteins) on immunization with reaginic fractions eluted with NaI and gly-HCl in presence of rabbit serum albumin (and not HSA). Thus, as shown by immunoelectrophoresis (Figure 7, part c) this guinea pig antiserum contained antibodies to serum proteins with the mobility corresponding to α-globulins. Although the relationship of the ' α-globulin' to the reagin-ragweed allergen system was not elucidated, it is obvious that this protein had been bound to the immunosorbent. However, no attempt was made to establish if these ' α-globulins' possessed antibody-like sites capable of combining specifically with ragweed determinants and, at this time, it might be assumed that these globulins were adsorbed non-specifically to the immunosorbent. A similar complication had been encountered with the EMA-ragweed immunosorbent as described in Chapter IV, section A.

Further evidence for the production of anti-IgE antibodies in guinea pig was obtained in the present study by recourse to an indirect, biological procedure involving reversed PCA in monkey. Thus, the guinea pig antiserum had the ability of eliciting cutaneous reactions on intradermal injection into the monkey; this reverse PCA reaction had been previously shown by Ishizaka (112) to be due to anti-IgE antibodies and not to antibodies to human IgG, IgA, or IgM.

Since beside reaginic antibodies (IgE), all allergic sera contain blocking antibodies capable of combining specifically with allergenic constituents (171, 233, 245), proof for the presence of these different classes of antibodies in allergic sera was obtained by induction of antibodies in guinea pigs to fractions of allergic sera containing only one

distinct type of antibody. For this purpose, allergic serum fraction Kh 30[I] (devoid of reagins) was adsorbed onto the immunosorbent. The elutions were performed with NaI and gly-HCl and these fractions were used for the immunization of normal guinea pigs. As was demonstrated by the immunoelectrophoretic technique, antibodies to IgG were produced; in addition, antibodies to HSA which had been employed as the stabilizing protein, were also detected in the antisera. It is important to note that such antisera did not contain antibodies to proteins with the mobility of α-globulins; this observation is in agreement with the expectation that this serum fraction would not contain the ' α-globulin' and, consequently, these proteins were not available to become adsorbed onto immunosorbent.

The main potential of the methods developed in the experiments described in this chapter lies in the specific isolation of reagins from immunosorbent by displacement with homologous haptens. The application of this procedure is limited at the present time owing to the unavailability of haptens from the diverse allergenic substances capable of inducing the formation of reagins in susceptable individuals. Nevertheless, the immunospecific procedures developed for the isolation and identification of reagins with a unique immunoglobulin class have obvious application for the isolation and characterization of other antibodies.

CHAPTER V

THE HETEROGENEITY OF ALLERGEN-ANTIBODY SYSTEMS RELATED TO RAGWEED ALLERGY IN MAN

Introduction

In recent years evidence has accumulated to indicate the heterogeneity of antigen-antibody systems in ragweed allergy. The heterogeneity of skin-active components among the various constituents of ragweed pollen has been amply demonstrated (260). Moreover, in conformity with the evidence for the existence of multiple isotypic antibodies directed even against a single antigenic determinant group (261), skin-sensitizing antibodies have been shown to be heterogeneous with respect to their chemical properties. Thus, the skin-sensitizing activity of certain allergic sera is partially or completely destroyed on incubation at pH 3, whereas other sera are not affected by this treatment (171); the ability of skin-sensitizing antibody to sensitize human skin is lost on heating at 56°C, but the minimal time required for their heat-inactivation varies from serum to serum, ranging from one to ten hours, and it appears that reagins directed against some allergens are inactivated much faster than those reacting with other constituents of ragweed pollen extract (170). The existence of two types of reagins was also demonstrated on the basis of the difference in their resistance to inactivation by treatment with mercaptoethanol (175).

The purpose of this phase of the study was to investigate simultaneously the nature and degree of heterogeneity of the allergen-reagin systems in ragweed allergy.

MATERIALS AND METHODS

Sera were obtained from one non-treated ragweed allergic individual Ju, and from two patients receiving hyposensitization treatment Gr, Gu. The serawere sterilized by filtration through millipore membrane (0.45 μ pore size) and stored at -15°C.

Short ragweed pollen was obtained from Greer Drug and Chemical Corporation, Lenoir, N.C., and two batches from two different crops were used.

Preparation of Non-Dialysable Components from Aqueous Extract of Ragweed Pollen (DWSR)

Water soluble ragweed extract was prepared as described in Methods Chapter III, with some modifications. Defatted ragweed pollen (100 g) was stirred in distilled water (500 ml) for one day at 4°C, then homogenized with a Waring blendor for 5 minutes and stirred at 4°C for 48 hours. The insoluble material was filtered off and washed with distilled water in a Buchner funnel. The aqueous extract (WSR) was centrifuged for 20 minutes at 35,000 x G, 4°C, and the supernatant was dialysed against distilled water at 4°C for six days. This solution (DWSR) was then centrifuged as before and lyophilized.

Preparative Block Electrophoresis of DWSR on Sephadex G-25

Sephadex G-25, fine-beaded grade (Pharmacia) was allowed to swell in distilled water and was then washed to remove the "fines". It was then equilibrated by repeated washings with the buffer (Tris-maleate, 0.012M; NaOH to pH 8.6) and poured as a thick slurry into a levelled perspex tray (35.7 cm x 25 cm x 1.2 cm). Paper wicks (3MM Whatman chromatography paper)

extending 3.5 cm onto the gel surface and hanging over the ends of the tray served to drain off the excess of buffer, until a slot cut with a spatula did not collapse or fill with buffer. The entire assembly was then placed in a cold room. After 1 hour at 4°C the paper wicks were connected to the electrode vessels containing 0.072M Tris-maleate* buffer. One hour later a 1 mm wide transverse trough was cut out at approximately one-third of the distance from the cathodal end. The ends of this trough extended to within 2 cm from the sides of the tray. The sample of DWSR (300 mg in 2.5 ml buffer) was applied with a syringe and needle, and the trough was filled in with the gel slurry by gentle tapping and compression of the gel block. A marker dye (RBY**) was placed onto the gel adjacent to the trough in line with the sample application. The surface was covered with Saran Wrap***. Connection of the buffer chambers to the electrode chambers containing 20% KCl (w/v) was made with filter paper. Silver-silver chloride electrodes were employed. Electrophoresis was performed for 28.5 hours at 5.33 V/cm in the range of 35-45 milliamperes.

One half hour before the completion of the electrophoresis, a 2 cm wide strip of dry filter paper was placed on the surface of the Sephadex. It was removed at the completion of electrophoresis, dried at 110°C and stained for 5 minutes with Brilliant Blue (0.1% in 5% acetic acid). At the end of electrophoresis the 2 cm margins of Sephadex along the sides were removed

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* 0.072M Tris-maleate buffer, pH 8.6 was prepared by dissolving 53.28 g Tris-(hydroxymethyl)-amino-methane, 43.158 g maleic anhydride and 24.216 g NaOH to 6 liters.

** RBY reference dye, Gelman Instrument Co., Ann Arbor, Michigan.

*** Saran Wrap, The Dow Chemical Co., Midland, Michigan.

and discarded. Transverse segments, 2 cm wide, were cut out and the material eluted with saline on a glass sintered funnel. The optical density of the eluates was determined at 280 mμ and the values normalized to the original sample volume. All eluates, referred to as fractions, were dialysed extensively against distilled water at 4°C, centrifuged at 35,000 X G for 20 minutes and then lyophilized. To remove insoluble matter, the dry fractions were dissolved in a minimum volume of distilled water (3ml), membrane filtered (0.45 μ pore size), lyophilized again in weighed vials and stored in a desiccator at 4°C. Each fraction was eluted from a 2 cm segment of gel and was designated by a number representing the distance (in centimeters) from the line of application to the farthest line of migration of that fraction. Thus, for example, fraction 10 represents the eluate from the 2 cm segment of the gel confined in the region 8 to 10 cm anodally from the point of application; the fractions represented by a negative number were eluted from segments cathodal to the point of application.

Stock solutions containing 1 mg/ml were prepared by dissolving each fraction in phosphate-buffered saline (PBS), pH 7.4. Each solution was sterilized by filtration through membrane. From these stock solutions ten-fold serial dilutions in PBS were prepared.

For the determination of the extinction coefficient, $E^{1cm}_{1\%}$ at 280 mμ, lyophilized fractions were dried in vacuo over phosphorous pentoxide until constant in weight. Solutions at 1 g% were made in PBS and their optical densities determined at 280 mμ.

The electrophoretic fractions of DWSR (at concentration of 1 mg/ml) were analysed by the immunoelectrophoretic and Ouchterlony techniques with a rabbit antiserum to DWSR.

Evaluation of the relative skin activity of the electrophoretic fractions of DWSR by the P-K test

Skin sites of normal non-allergic volunteers were sensitized separately with 0.05 ml of the allergic sera, Gu and Ju (1:100 dilution). Twenty-four to forty-eight hours later the sites were challenged with 0.025 ml of ten-fold serially diluted fractions. Reactions were graded 20 minutes after challenge. Since in exploratory experiments no reactions were obtained on injection of any of the electrophoretic fractions into the skin of normal volunteers or into skin sites of volunteers "sensitized" with normal human serum (1:100 dilution), in subsequent experiments presensitization with normal human serum was dispensed with for control reactions. For controls, each fraction at the highest concentration (100 µg/ml) was injected into skin sites of the volunteers. To investigate further the variability of response due to different allergic sera, a third allergic serum, Gr, was used to sensitize skin sites in two other normal volunteers.

Comparison of two DWSR preparations

To explore the heterogeneity of ragweed allergens*, and the difference(s) from one batch of pollen to another, the electrophoretic fractionation of DWSR was repeated using a different preparation of DWSR isolated from the pollen of another crop. The skin activity of the electrophoretic fractions was determined by the same method as described previously.

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* An allergen is defined as the effective antigen which induces the formation of skin sensitizing antibodies in individuals predisposed to allergies. A skin reactive compound is clearly a substance which on injection into the skin of an allergic individual, or into the skin of a normal individual passively sensitized with allergic serum, induces a wheal and flare reaction. So far, no relation has been established between allergenicity and skin-activity of a given fraction of an allergen and tentatively these terms will be used interchangeably in this thesis.

Variability of skin activities of electrophoretic fractions of DWSR (preparation 2) as revealed with different allergic sera in different volunteers

The skin activities of the fractions isolated from the second preparation of DWSR were determined by the same method as described previously. Allergic sera Gu or Ju were used to sensitize skin sites in two different volunteers.

Variability of skin activities of electrophoretic fractions of DWSR (preparation 2) as revealed with the same allergic serum in different volunteers

The results (p. 146) obtained with the first electrophoretic preparations of DWSR already indicated that the difference in skin activity of the fractions depended on two variables, i.e. the allergic serum used for the passive sensitization and the volunteer's skin. To examine further these variables using the electrophoretic fractions of the second batch of DWSR, the same serum, Gr, was used to sensitize skin sites in two volunteers in order to eliminate, or bring out, the variability of the skin response with different allergic sera.

Variability of skin activities of electrophoretic fractions of DWSR (preparation 2) as revealed with different allergic sera in the same volunteer

To eliminate the variability of skin activities of electrophoretic fractions of DWSR, due to the volunteers, titrations with all three allergic sera (Gu, Gr and Ju) were simultaneously performed in one volunteer at a given time. This procedure was repeated in another volunteer.

Comparison of reactivity of two oppositely charged electrophoretic fractions of DWSR by challenging skin sites sensitized with allergic serum fractions with different net charge

Allergic serum Wh was fractionated by salting-out with ammonium sulfate (20°C, pH 7.5) at a final concentration corresponding to 45% saturation and by ion-exchange chromatography on DEAE-Sephadex A 50 as described in Methods, Chapter III. On the basis of their elution from DEAE-Sephadex A 50 it was expected that allergic serum fractions I and IV would differ most markedly in their electrical charge, fraction IV being the most negatively charged. For the passive sensitization of skin sites, serum fractions I and IV were used in different dilutions starting from 1:10. For the evaluation of the total reaginic content of these serum fractions the whole DWSR preparation containing all the skin active components was used.

The skin activities of the two oppositely charged electrophoretic fractions of DWSR, fraction -3 (cathodic mobility, i.e. positively charged) and fraction 24 (anodic mobility, i.e. negatively charged) at concentrations of 100 µg/ml, were compared by challenging skin sites sensitized separately with dilutions of the two allergic serum chromatographic fractions I and IV. The reactions which developed 20 minutes after challenge were measured in terms of the surface area of the flare and were recorded by tracing the boundary of the flare on Saran-Wrap; the surface area was determined by planimetry.

Since comparable skin reactions were obtained with the same dose of DWSR on challenge of skin sites sensitized with serum fractions I and IV in dilutions of 1:10 and 1:40 respectively (see results, Figure 30), these serum fractions were used in these dilutions for sensitization of another volunteer in a subsequent experiment.

Evaluation of the relative allergenic composition of the electrophoretic fractions of DWSR by the cross neutralization of P-K reactions

Cross neutralization tests were performed for the evaluation of the relative allergenic composition of the electrophoretic fractions. First, the neutralizing dose of two fractions (2 and 18) was determined with respect to each serum studied, (Gu, Gr, Ju,). For this purpose each fraction in various concentrations, was incubated with the allergic serum (1:100 final dilution) for 2 hours at room temperature and then overnight at 4°C. The mixtures were injected intradermally into normal volunteers and challenged 24 hours later with a 20-fold excess of the highest concentration of the fraction in question.

This large challenging dose of the fractions was used to exclude the possibility that the allergic serum and electrophoretic fraction constitued only a desensitizing mixture (170).

The minimum amount of the fraction required to completely neutralize the reaginic activity, as evidenced by the absence of a reaction on challenge, was taken as the neutralizing dose. Multiple skin sites were injected with the mixtures of the allergic sera containing the appropriate neutralizing dose of the fraction. These sites were challenged with other fractions of DWSR, each at a concentration of 50 µg/ml, to establish if they contained skin active components in addition to those found in the fractions used in the neutralization test. Mixtures of allergic serum and saline, instead of the electrophoretic fraction of DWSR, as well as of allergic serum and the respective neutralizing dose of the DWSR fractions, served as controls.

The allergenic relationships of four other electrophoretic fractions were also evaluated relative to those established for fractions 2 and 18

by the cross neutralization test. For this aspect of the study, fractions -3, 8, 14 and 24 were arbitrarily chosen as representative fractions on the basis of their different electrophoretic mobilities.

RESULTS*

Fractionation of DWSR by zone electrophoresis

The profiles of the optical densities and theyields of fractions of DWSR obtained by electrophoresis on Sephadex G-25 are shown in Figure 20. Although the bulk of the material remained at the zone of application, a relatively greater portion of the components of DWSR migrated towards the anode than to the cathode. The appearance of the lyophilized fractions varied for the mid-anodal components and brown and dense for the material migrating to the extreme anodal position. Yellow pigmented material which could not be completely eluted from the Sephadex was located in the region of fractions -1 and 2. The total recovery was of the order of 85%.

The maximum intensity of staining with Brilliant Blue of a filter paper imprint from the Sephadex block corresponded to the region of fractions 8 to 16, inclusive. These fractions also had the highest extinction coefficients $E^{1cm}_{1\%}$ at 280 mμ (Figure 21).

Skin activity of the electrophoretic fractions of DWSR

The skin activities of the fractions, evaluated in terms of P-K titers, are presented in Figures 22 and 23. When allergic serum Gu was used to sensitize the skin sites (upper portion of Figure 22), fractions 8 and 10 were found to have the highest skin activity. With another allergic serum (Ju) and in another volunteer (lower portion of Figure 22), fractions 6, 8,

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* The results given on pages 146-159 inclusive were presented at the VI International Congress of Allergology in Montreal, 1967 (262).

FIGURE 20

Electrophoretic fractionation of DWSR (preparation 1) on Sephadex G-25. The Sephadex gel was equilibrated with Tris-Maleate (0.012M) + NaOH to pH 8.6; applied 300 mg of DWSR in 2.5 ml of the buffer; electrophoresis was performed at a potential of 5.33 V/cm for 28.5 hours at 4°C.

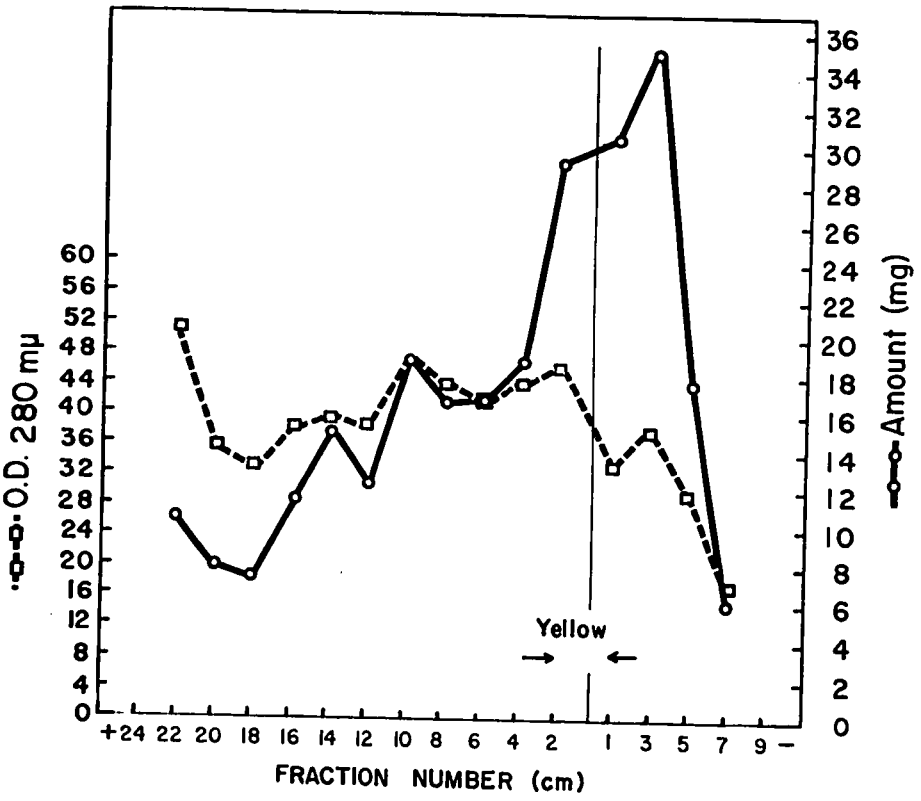

O.D. 280 mμ
Amount (mg)
Yellow
FRACTION NUMBER (cm)
+24 22 20 18 16 14 12 10 8 6 4 2 1 3 5 7 9 –

FIGURE 21

Extinction coefficients, $E^{1cm}_{1\%}$ at 280 mμ, of the electrophoretic fractions of DWSR. Solid line and circles, preparation 1; broken line and open circles, preparation 2.

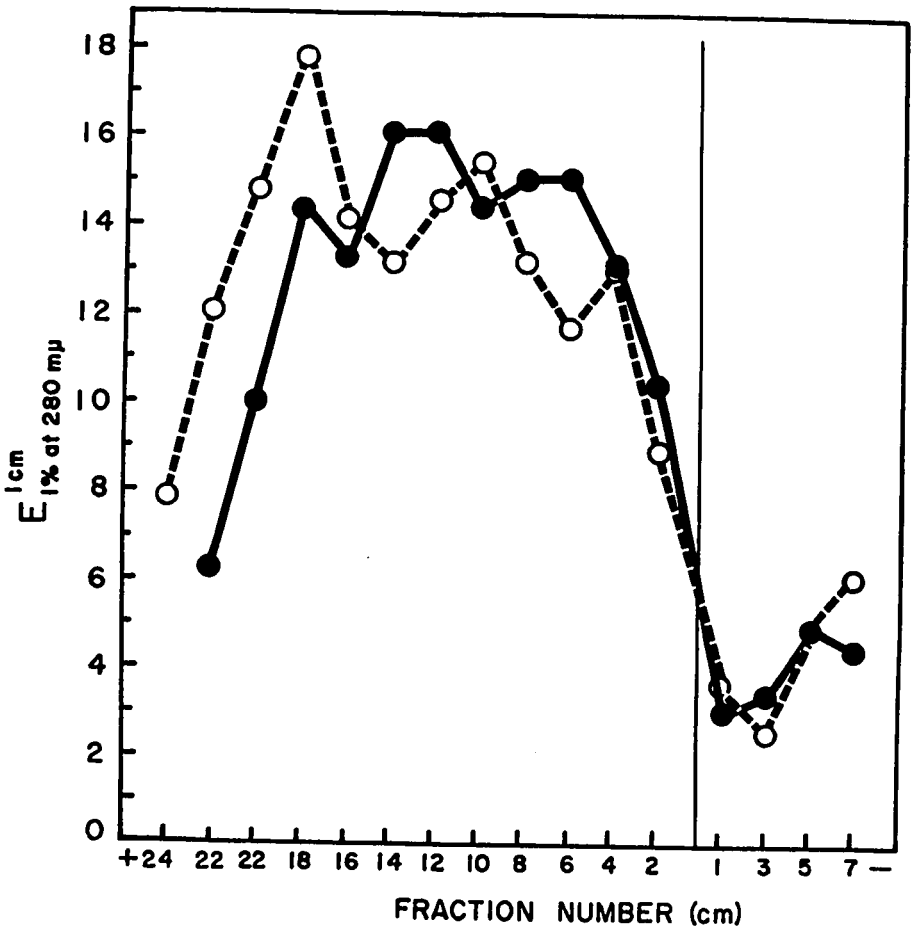

18
16
14
12
10
8
6
4
2
0
$E^{1cm}_{1\%\ at\ 280\ m\mu}$
+24 22 22 18 16 14 12 10 8 6 4 2 1 3 5 7 —
FRACTION NUMBER (cm)

FIGURE 22

Lowest concentrations of the electrophoretic fractions of DWSR (preparation 1) required to elicit positive skin reactions using two allergic sera in two volunteers.

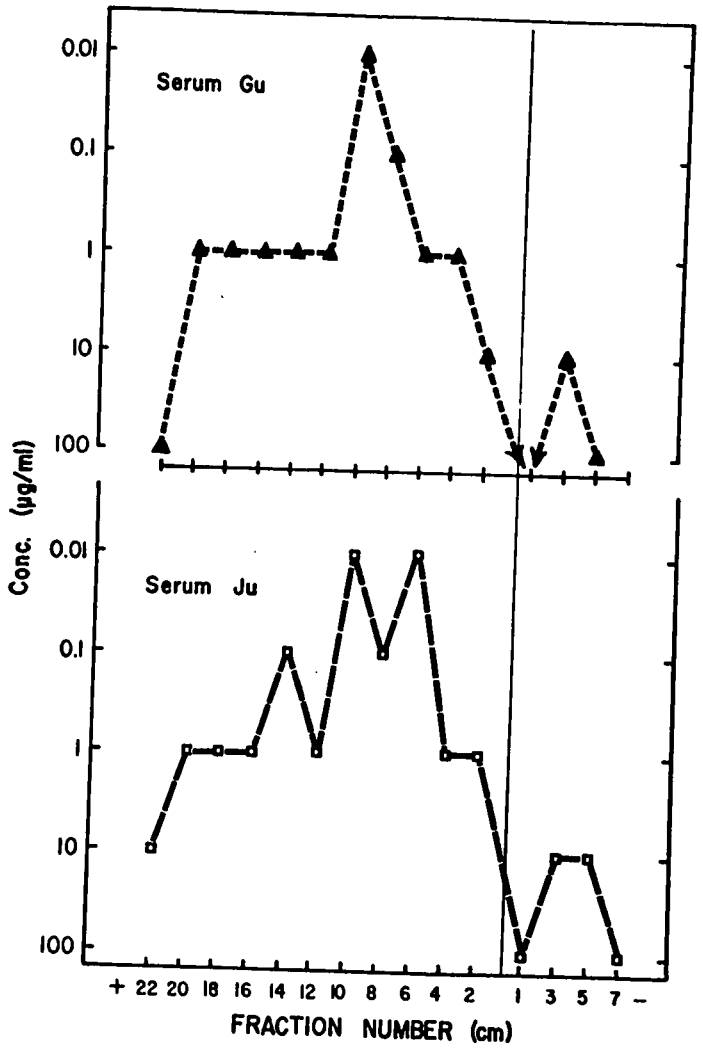
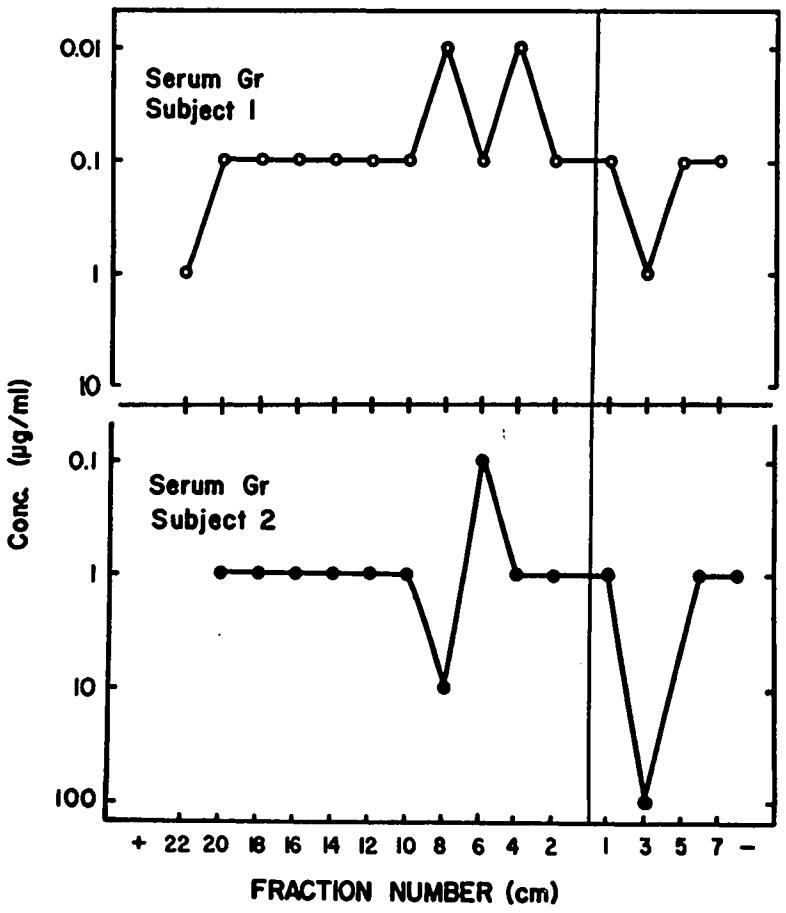

Serum Gu
Serum Ju
0.01
0.1
1
10
100
Conc. (µg/ml)
+ 22 20 18 16 14 12 10 8 6 4 2 1 3 5 7 –
FRACTION NUMBER (cm)

FIGURE 23

Lowest concentrations of the electrophoretic fractions of DWSR (preparation 1) required to elicit positive skin reactions using the same allergic serum in two volunteers.


0.01
0.1
1
10
Serum Gr
Subject 1
0.1
1
10
100
Serum Gr
Subject 2
Conc. (µg/ml)
+ 22 20 18 16 14 12 10 8 6 4 2 1 3 5 7 –
FRACTION NUMBER (cm)

10 and 14 all showed high skin activity. When a third allergic serum, Gr, was used to sensitize skin sites in two other normal volunteers (Figure 23), in one subject the highest skin activity was associated with fractions 4 and 8, whereas in the second volunteer fraction 6 was the most active and the skin activity of fraction 8 was lower by a factor of 100. In general, all fractions migrating to the cathode possessed lower skin activity, irrespective of the allergic serum used, and all anodal fractions possessed a higher, but more variable activity. These results demonstrated that the skin activity of the electrophoretic fractions of DWSR depends not only on the allergic serum but also on the volunteer used for the passive transfer tests.

Comparison of two DWSR preparations

A comparison of Figures 20 and 24 reveals that the profiles for the optical densities and yields were similar for these two DWSR preparations. Moreover, the general appearance (color, consistency) of the fractions was also similar in the two experiments; however, in addition to the yellow pigmented material in fractions -1 and 2, a violet-coloured material was found in fractions 18 to 22 of this second preparation. In general, the fractions isolated from the whole anodal portion of the Sephadex block had higher extinction coefficients than those from the cathode region; fractions near the zone of application had the lowest extinction coefficients (Figure 21).

By Ouchterlony analysis with a rabbit antiserum to DWSR, it was established that multiple antigenic components were present in most electrophoretic fractions (Figure 25). No precipitin arcs were obtained with the cathodic fractions -3 and -1 (at a concentration of 1 mg/ml), but all

FIGURE 24

Electrophoretic fractionation of DWSR (preparation 2) on Sephadex G-25. The Sephadex gel was equilibrated with Tris-Maleate (0.012M) + NaOH to pH 8.6; applied 300 mg of DWSR in 2.5 ml buffer; electrophoresis was performed at a potential of 5.33 V/cm for 28.5 hours at 4°C.

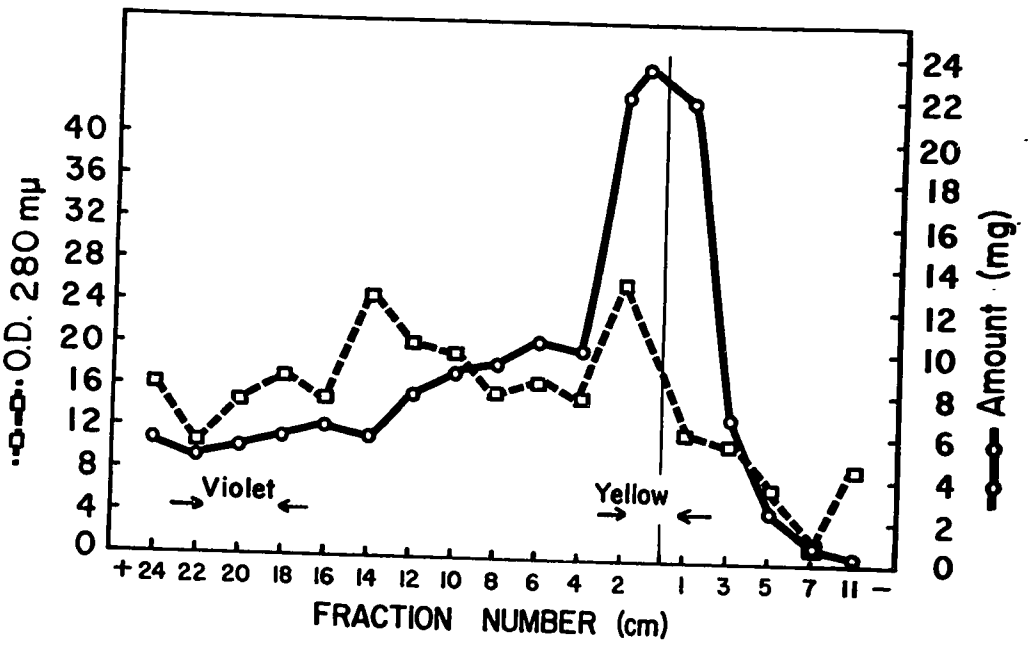
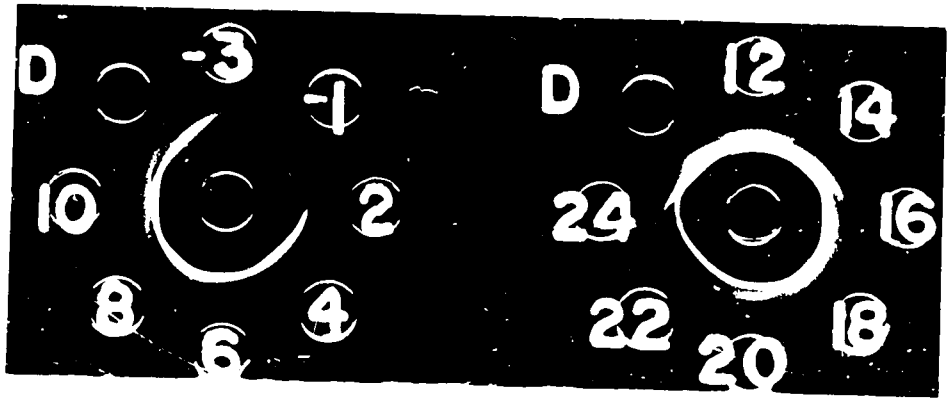

O.D. 280 mμ
Amount (mg)
Violet
Yellow
FRACTION NUMBER (cm)
+ 24 22 20 18 16 14 12 10 8 6 4 2 1 3 5 7 11 −

FIGURE 25

Ouchterlony immunodiffusion analysis of the electrophoretic fractions of DWSR. The central well contained a rabbit antiserum to DWSR and the peripheral wells contained the electrophoretic fractions as indicated and the unfractionated DWSR (D), at a concentration of 1 g% (w/v).


D
-3
-1
D
12
14
10
2
24
16
8
6
4
22
20
18

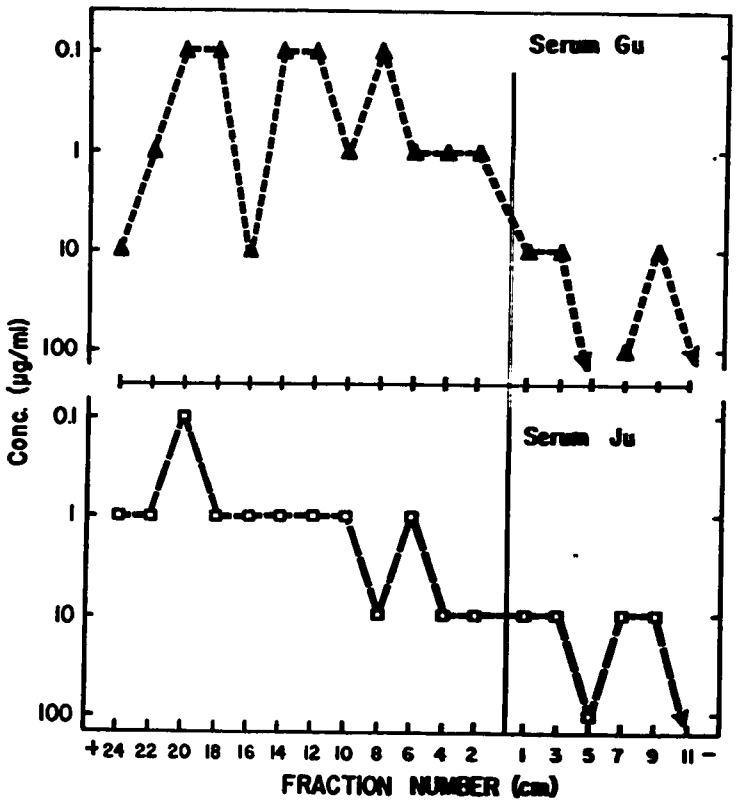
other fractions contained at least two common antigenic components. These components were revealed by the presence of a common faint precipitin band closer to the central antibody well, and another common heavy precipitin band nearer to the antigen wells. Fraction 12 had the greatest number of antigenic components. On immunoelectrophoresis, fractions -3 to 6 appeared to migrate towards the cathode; however, since fractions 2 to 6 migrated to the anode during electrophoresis in Sephadex, the cathodal mobility of these fractions in agar gel must be attributed to electro-osmotic effects. The other fractions, 8 to 24, moved to the anode and their electrophoretic mobility in agar could be correlated with that in Sephadex. None of the fractions appeared to be pure as judged by immunoelectrophoretic analysis, some fractions revealing at least three immunoelectrophoretically distinct components. Furthermore, by immunoelectrophoresis no difference could be detected between the electrophoretic fractions of the two DWSR preparations.

Variability of skin activities of electrophoretic fractions of DWSR as revealed with different allergic sera in different volunteers

Different profiles of skin activity were obtained for the two allergic sera Gu and Ju (Figure 26), which were, furthermore, different from those obtained with the first preparation. Thus, no obvious correlation in skin activity could be established between these two preparations of DWSR using two allergic sera. The only consistent feature observed in all skin tests with the electrophoretic fractions of both preparations of DWSR was that the skin activity was lower for the fractions migrating to the cathode, whereas fractions with anodal mobility possessed higher, but more variable, skin activities.

FIGURE 26

Lowest concentrations of the electrophoretic fractions of DWSR (preparation 2) required to elicit positive skin reactions using two allergic sera in two volunteers.


Serum Gu
Serum Ju
0.1
1
10
100
Conc. (µg/ml)
+24 22 20 18 16 14 12 10 8 6 4 2 1 3 5 7 9 11 −
FRACTION NUMBER (cm)

Variability of skin activity of electrophoretic fractions of DWSR as revealed with the same allergic serum in different volunteers

The results of using the same allergic serum in different volunteers (Figure 27) revealed identical profiles in skin activity for fractions -3 to 16, with the exception of fraction 2. However, fractions 18-22 which were highly active in the first volunteer showed a decreased activity in the second. It is also apparent from this Figure that the overall reactivity of the second subject was lower by a factor of ten, i.e. to elicit minimal skin reactions in this volunteer ten times higher concentrations of the fraction were needed. Identical profiles for the lowest concentrations of the electrophoretic fractions required to elicit positive skin reactions were obtained in duplicate skin tests performed in a volunteer sensitized with allergic serum Gr.

Variability of skin activities of electrophoretic fractions of DWSR as revealed with different allergic sera in the same volunteer

From the results of two experiments illustrated in Figure 28 it can be seen that the level of skin activity of the fractions with respect to the three allergic sera used depended on the allergic serum and decreased in the order of sera Ju, Gr and Gu. In the first subject, at least two groups of fractions, namely 2 to 4 near the zone of application and 16 to 20 in the anodal region, had higher skin activity. On the other hand, the skin activity of fractions 2 and 4 were lower in the second subject and higher skin activity was associated with fractions 10 to 12 (or 14) and 18 to 22.

FIGURE 27

Lowest concentrations of the electrophoretic fractions of DWSR (preparation 2) required to elicit positive skin reactions using the same allergic serum in two volunteers.

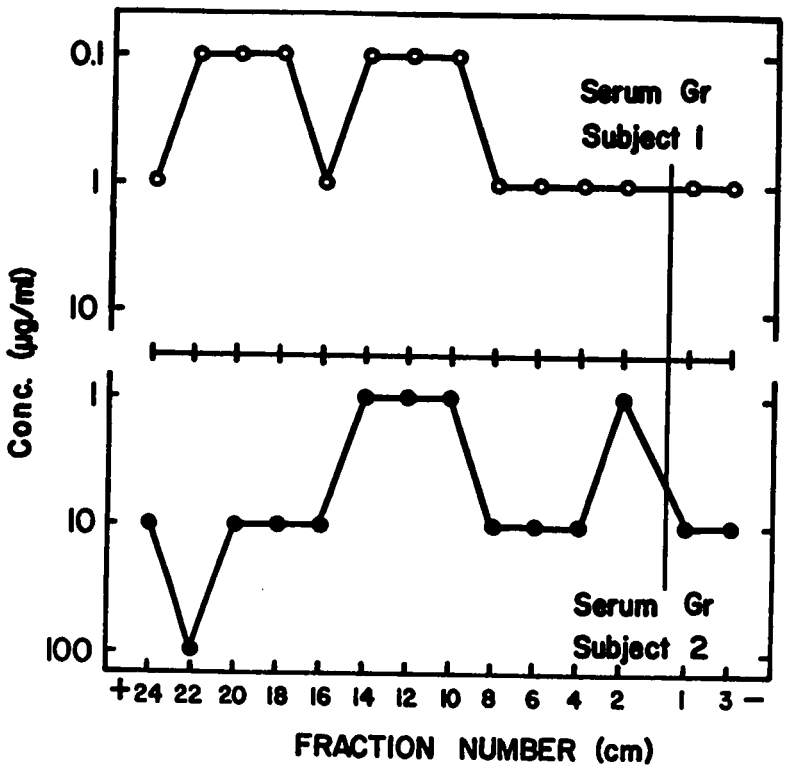
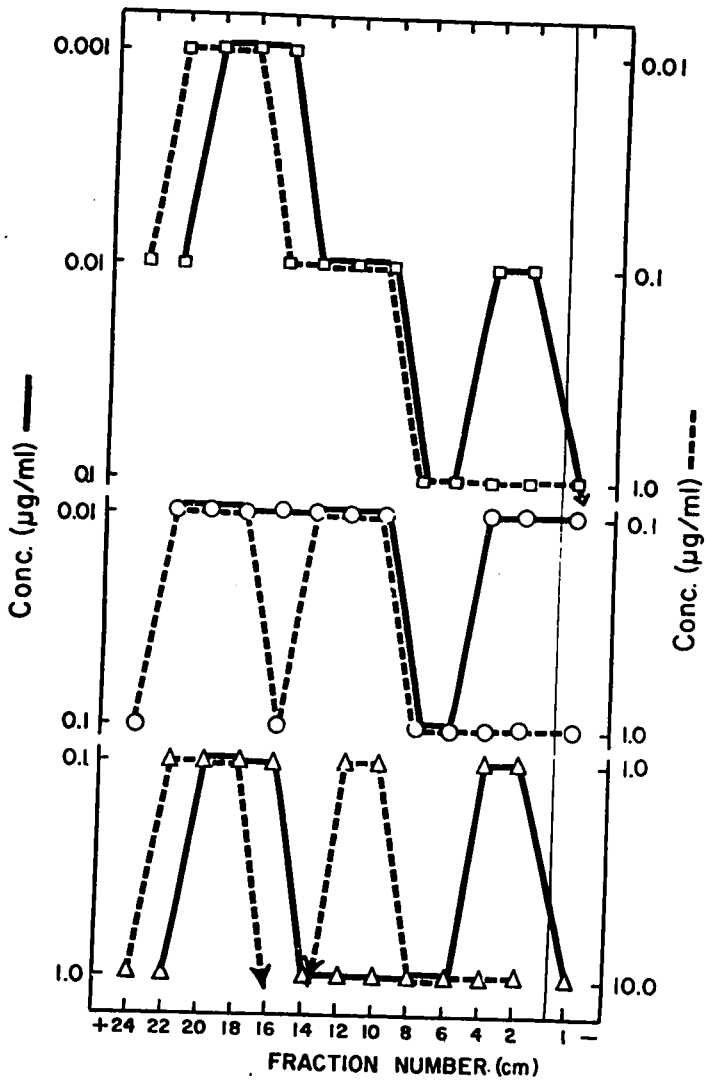

0.1
1
10
Conc. (µg/ml)
Serum Gr
Subject 1
1
10
100
Serum Gr
Subject 2
+24 22 20 18 16 14 12 10 8 6 4 2 1 3 –
FRACTION NUMBER (cm)

FIGURE 28

Comparison of the lowest concentrations of the electrophoretic fractions of DWSR (preparation 2) required to elicit positive skin reactions in two volunteers using three allergic sera; serum Ju (□), serum Gr (○), and serum Gu (△). The solid line represents reactivity in volunteer 1; the broken line represents reactivity in volunteer 2.


0.001
0.01
0.1
0.01
0.1
1.0
0.1
1.0
10.0
0.01
0.1
1.0
0.1
1.0
Conc. (µg/ml) ——
Conc. (µg/ml) ----
+24 22 20 18 16 14 12 10 8 6 4 2 1 —
FRACTION NUMBER (cm)

Comparison of reactivity of two oppositely charged electrophoretic fractions of DWSR by challenging skin sites sensitized with allergic serum fractions with different net charge*

By immunoelectrophoretic analysis (Figure 29), it was shown that the mobility of the globulins of serum fraction I were restricted to the extreme cathodic region, whereas the globulins of fraction IV possessed higher anodic mobility. Thus, these results confirmed that these serum fractions differed in their electrical charge.

The data given in Figure 30, part a and part b respectively demonstrated that (i) in sites sensitized with different dilutions of the less negatively charged serum fraction I the reactions obtained with negatively charged electrophoretic fraction 24 were consistently greater than those obtained with the positively charged fraction -3 (cathodic mobility) and (ii) in sites sensitized with different dilutions of the more negatively charged serum fraction IV the reactions obtained with the positively charged fraction -3 were consistently greater than those obtained with fraction 24 (negatively charged).

In a second volunteer sensitized with serum fraction I in dilutions of 1:10, the reaction was 10 cm^2 (averages of duplicates) with electrophoretic fraction 24, while no reactions could be elicited with fraction -3 (Figure 31, part a). Conversely, the reaction obtained with electrophoretic fraction -3 in skin sites sensitized with the more negatively charged fraction IV in dilutions of 1:40, was 12.5 cm^2 as compared to 8.4 cm^2 with fraction 24 (Figure 31, part b). Thus, in both volunteers the same relationship in reactivity between the electrophoretic fractions and allergic serum fractions was established.

As evidenced from the data in Figures 30 and 31, the reactions in

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*

These results were presented at the Canadian Federation of Biological Societies Meeting in Kingston, 1968 (263).

FIGURE 29

Immunoelectrophoresis of DEAE-Sephadex A 50 chromatographic fractions I and IV of allergic serum Wh, and of the preparation obtained by salting-out with ammonium sulfate at a concentration corresponding to a saturation of 45% (45% AHS). Electrophoresis was performed in agar with veronal buffer $\Gamma/2 = 0.033$, pH 8.6, at 9.25 V/cm for 40 minutes. The antibody troughs were filled with rabbit antiserum to normal human serum.

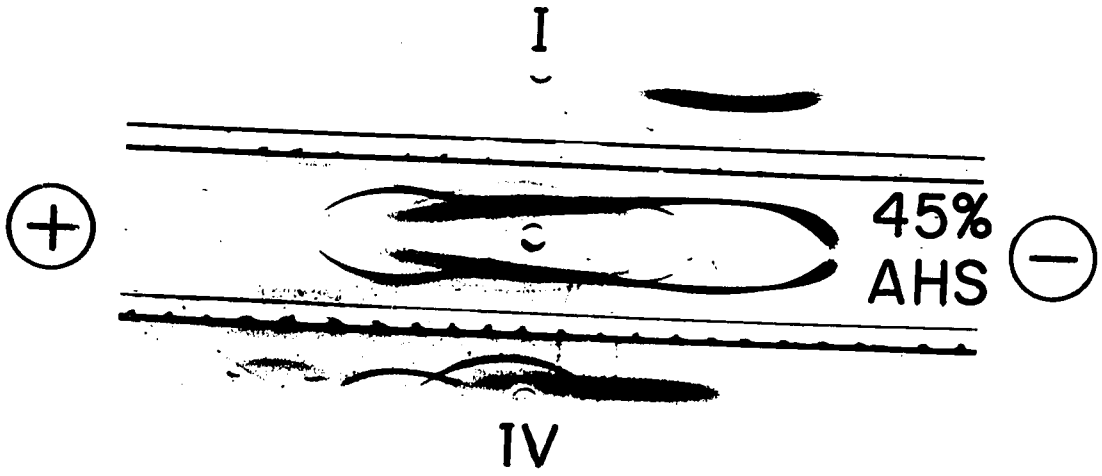

I
45%
AHS
IV

FIGURE 30

P-K reactions observed with chromatographic fraction 1 (part a) and fraction IV (part b) of allergic serum Wh, on challenge with DWSR (□), electrophoretic fractions -3 (○), and 24 (Δ). These three skin active preparations were tested at a concentration of 100 μg/ml.

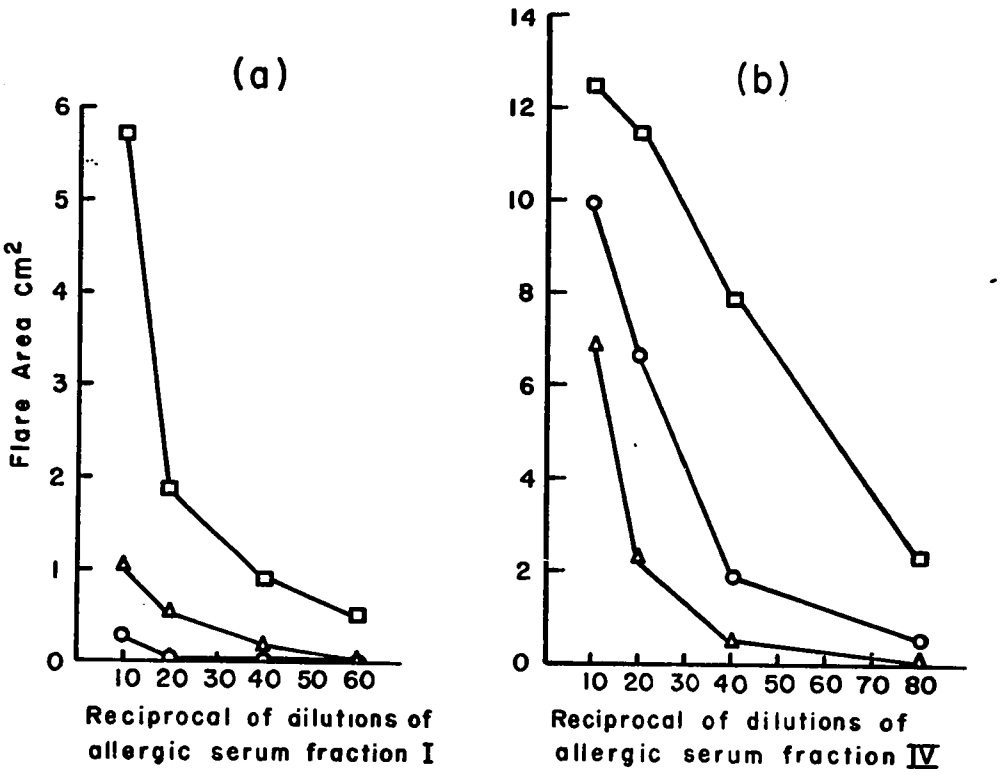
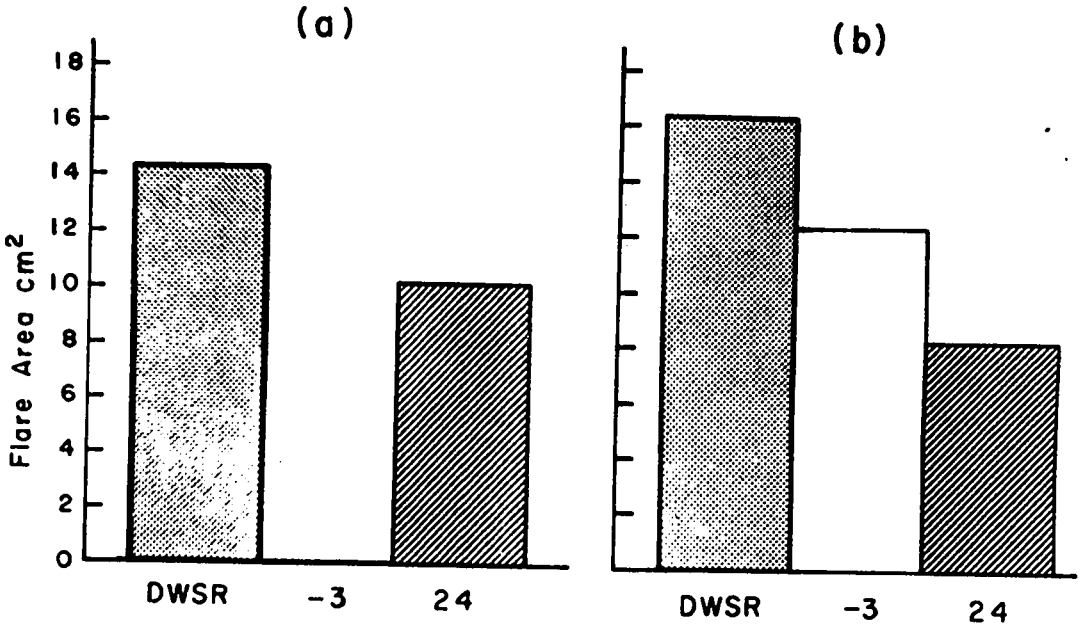

(a)
Flare Area cm²
0
1
2
3
4
5
6
10 20 30 40 50 60
Reciprocal of dilutions of allergic serum fraction I
(b)
0
2
4
6
8
10
12
14
10 20 30 40 50 60 70 80
Reciprocal of dilutions of allergic serum fraction IV

FIGURE 31

Variations in skin activity of DWSR and electrophoretic fractions -3 and 24, as tested with chromatographic fraction I (part a) and fraction IV (part b) of allergic serum Wh. These three skin active preparations were tested at a concentration of 100 µg/ml. The sizes of the reactions shown are the average of duplicate experiments in the same volunteer.


(a)
(b)
Flare Area cm²
0
2
4
6
8
10
12
14
16
18
DWSR
-3
24
DWSR
-3
24

the two volunteers given by DWSR with both serum fractions I and IV, were always larger than those given by the two electrophoretic allergen fractions. These maximal reactions may be attributed to the cumulative effects of all allergenic components present in DWSR.

Evaluation of the relative allergenic composition of the electrophoretic fractions of DWSR by the cross neutralization procedure*

The presence of several allergenic components in six of the electrophoretic fractions examined was demonstrated by the cross neutralization technique with three allergic sera (see Table XIII).

From the absence of skin reactions of serum Gu with the four other fractions -3, 8, 14, and 24, tested in relation to fractions 2 and 18, one would infer that this serum contains reagin(s) against one common group of allergens or against a single allergen present in all these six fractions. On the other hand, the positive reactions of serum Gr tested in an identical manner, indicated that at least two major allergenic groups were responsible for the induction of detectable reagins in this serum; one group was associated with fractions18 and 24 (Table XIII, part a), and the other group was associated with fractions -3, 2 and 8 (Table XIII, part b). In a similar fashion, two additional different allergenic groups were revealed by the cross neutralization experiments with serum Ju; one group was associated with fractions 8 and 18 (Table XIII, part a), and the other with fractions 2 and 14 (Table XIII, part b). Thus, on the basis of the overall results given in Table XIII it may be concluded that these six electrophoretic fractions contained a minimum of five different allergenic components and that each of the three allergic sera contained reagins only against a certain number of these allergens.

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*
These results were presented at theVI International Congress of Allergology in Montreal, 1967 (262).

TABLE XIII

Part (a)

Cross neutralization experiments between electrophoretic fraction 2 and five other fractions

Serum neutralized with fraction 2*	Reactions on challenge with fraction+					
	2**	-3	8	14	18	24
Gu	-	-	-	-	-	-
Gr	-	-	-	-	+	+
Ju	-	-	+	-	+	-

Part (b)

Cross neutralization experiments between electrophoretic fraction 18 and five other fractions

Serum neutralized with fraction 18*	Reactions on challenge with fraction+					
	18**	-3	2	8	14	24
Gu	-	-	-	-	-	-
Gr	-	+	+	+	-	-
Ju	-	-	+	-	+	-

* The neutralizing doses for these three allergic sera Gu, Gr and Ju were 0.5, 2.5 and 2.5 µg/ml respectively for electrophoretic fraction 2 and 0.1, 2.5 and 2.5 µg/ml respectively for electrophoretic fraction 18.

** The absence of reactions (-) given with the neutralizing fractions demonstrate that reagins in all sera had been indeed neutralized with respect to the allergen(s) in this fraction prior to sensitization of sites.

\+ All sites were challenged with fractions at a concentration of 50 µg/ml.

DISCUSSION

It was demonstrated previously (264, 265) in this laboratory that at least eleven components, separable by paper electrophoresis, were present in the water soluble extract of ragweed pollen (WSR). These components were detected by visual observation and by selective staining. Employing preparative electrophoresis on paper block (in borate buffer, pH 8.6), four distinct skin active components were isolated (170, 266). On the basis of cross neutralization tests, using the P-K reaction as the test system, it was inferred that the fractions near the zone of application and those migrating just anodally to it contained at least one additional allergen (or groups of allergens) not found in a fraction migrating to the extreme anodic position. Skin-sensitizing antibodies directed against these two groups of allergens were also heterogenous with respect to their physical properties, i.e. one class of reagins directed against the slow moving anodic allergens was resistant to heating at 56°C for 8 hours, whilst the other class, directed against the more anodic allergens was more heat labile and was destroyed on heating for 2 hours at 56°C (170).

In another study preparative paper block electrophoresis had been used for the isolation of a major protein component from DWSR which was denoted as fraction δ and was shown to possess all the major determinants responsible for the skin activity of DWSR (267, 268). Preparative starch block electrophoresis had also been used for the fractionation of ragweed pollen allergens (269, 270) and it was concluded from the results of direct skin testing in different dilutions that some fractions were more active in one group of 6 patients than in another group of

3 patients (270). Moreover, the finding that in some cases two different electrophoretic fractions had different skin activities in the same individual suggested the presence of multiple allergens with different allergenic determinants.

In the present study Sephadex G-25 was used as a supporting medium for preparative zone electrophoresis since it has several advantages over the supporting materials used in previous investigations. The preparation of the block is simple; the segments of the gel can be easily removed and the fractions can be eluted quickly and in high yields. This procedure thus minimizes contamination with neighbouring fractions by diffusion. In addition, Sephadex by virtue of its sieving properties may contribute to the separation of complex mixtures in terms of the difference in size of the corresponding components.

Although the overall physical appearance of the two DWSR preparations were similar, the optical densities and yields of the corresponding electrophoretic fractions (shown in Figure 20 and Figure 24) exhibited some differences, suggesting that the composition of various batches of pollen may not be identical. In another study (271) differences in chromatographic profiles, yields, chemical nature and immunodiffusion patterns of preparations of dwarf ragweed pollen had been observed. These differences were attributed to the variation in pollen crops, and not to the fractionation procedures.

If one assumes that carbohydrates are associated with fractions having low extinction coefficients and proteins with fractions of higher extinction coefficients at 280 mμ, the following conclusions may be drawn: (i) all fractions contain proteins and carbohydrates,

(ii) the bulk of material which remains at the zone of application is rich in carbohydrates, and (iii) the highest skin activity is associated mainly with fractions possessing higher amounts of proteins. In this connection it should be mentioned that skin activity of most allergens has been found to be associated with protein-carbohydrate complexes (153, 272).

The electrophoretic fractionation by itself was not sufficient to resolve the complex mixture of components of DWSR into immunochemically distinct single constituents, as revealed by immunodiffusion and immuno-electrophoresis with rabbit anti-DWSR serum. Moreover, these experiments clearly demonstrated the close similarity of the antigenic make-up of different DWSR preparations.

Although all electrophoretic fractions were skin active in P-K tests, the results of the cross-neutralization experiments indicated that these fractions contained a common allergenic component and at least four different skin active components possessing different electrophoretic mobilities. However, no two fractions were identical with respect to their allergenic composition.

An attempt was made to clarify the allergenic relationships among different electrophoretic fractions of DWSR. For a precise evaluation of the allergenic composition of the electrophoretic fractions, the cross-neutralization procedure would have had to be performed with each fraction and not only with fractions 2 and 18. However, the completion of this evaluation would have necessitated many more sites in the P-K tests which were not feasible. Therefore, the conclusions derived are based on the results obtained with neutralization with only fractions

2 and 18. Also, in view of the heterogeneity of skin-fixation properties of reagins (as determined by the divergence of the reactions obtained with the same allergenic fractions and a given reaginic serum in the skin of different volunteers) it would be difficult, if not meaningless, to establish any correlation of results from cross-neutralization studies using different subjects. Nevertheless, on the basis of the limited experimental data provided here, it may be predicted that an extremely complex pattern of allergenic composition would emerge, and from the reaction patterns given in Table XIII it is clear that (i) each fraction contained a different number of skin active components, and (ii) there was no simple relationship among the six electrophoretic fractions investigated.

It also became evident that each allergic serum contained a group of reagins which was different for every serum. Thus, allergic serum Gu possessed reagins directed only against a common allergenic component, while sera Gr and Ju each possessed two different groups of reagins against the corresponding allergenic components. It remains to be determined whether this uniqueness of reaginic antibody content of each allergic serum is the manifestation of the genetic factors determining the antibody synthesis of the allergic individual, or if it simply represents heterogeneity of the antibody response in different allergic individuals against a select group of allergenic components.

By comparing the lowest concentrations of any given electrophoretic fraction of DWSR required to elicit positive skin reactions obtained with the three allergic sera, it can be seen that these values varied from one allergic serum to another. Thus, with allergic serum Ju, fractions 6 and 10 were the most active whereas with serum Gu, only

fraction 10 showed a comparable activity (Figure 22). In addition, the concentration of any fraction required to elicit positive skin reactions with the same serum in the different volunteers varied by a factor of the order of 10 to 1000. Even when the same serum was used in the same volunteer on two separate occasions, the concentrations of the fractions required to elicit positive skin reactions varied by a factor of 10. However, the activity profiles in these experiments were similar. Differences in the P-K response by the same volunteer on different occasions were also observed by Stanworth and Kuhns (167). Therefore, the general level of skin reactivity of the volunteer may be attributed to changes in levels of pharmacological mediators in skin, which are involved in the wheal and erythema reaction. Since the activity profiles obtained with the same allergic sera in different volunteers were not identical it must be concluded that skin-sensitizing antibodies did not fix to or sensitize the skin of the different volunteers to the same extent. This is tantamount to saying that reagins are heterogeneous with respect to their skin-fixation properties.

In view of the fact that the skin reactivity of the electrophoretic fractions is a function of the amounts of tissue-bound reagins, all other factors being constant, lower skin reactivity of the fractions is a reflection of decreased amounts of the bound reagins. Thus, it is evident that the reagins of all three allergic sera directed against electrophoretic fractions 2 and 4, fixed to the skin to a lesser degree in one volunteer than in the other (Figure 28). In addition, with serum Gu, a different group of reagins with an apparent increased skin fixation capacity and directed against fractions 10 and 12 were demonstrated. Thus, the

same allergic serum possessed simultaneously two groups or reagins: one group with lower skin-fixation capacity and another group with higher skin-fixation capacity. Results from experiments designed to reveal differences in reactivity of the two oppositely charged electrophoretic fractions, which were used to challenge allergic serum fractions of different net charge, indicated that although the patterns of reactivity elicited were identical, the size of the reactions as compared with those obtained with DWSR were not similar in two volunteers (Figures 30 and 31). These observations were taken as additional evidence for the heterogeneity of the skin-fixation properties of reagins.

The study of Sela and Moses (273), using tailor-made synthetic antigens, demonstrated that the net charge of an antigen affects the charge on the antibody. Thus, using the same hapten coupled to synthetic polypeptides of different net charge they showed that the charge of the antibody was opposite to that of the carrier protein and was negligably affected by the nature of the hapten itself. Although it remains to be determined if the skin activity of the different ragweed pollen constituents is due to the same or different haptenic groups, which may be associated with the same or different carrier molecules, the results of this study indicate that positively charged ragweed pollen constituents gave a more intense skin reaction with negatively charged reaginic serum fractions, and, conversely, negatively charged ragweed pollen constituents were more reactive with the more positively charged allergic serum fractions. In accord with the findings of Sela and Moses, it is tempting to suggest that positively charged ragweed pollen constituents give rise to the formation of skin-sensitizing antibodies which have a

relatively larger number of anionic groups than those elicited by the more negatively charged constituents of ragweed pollen. A similar conclusion was arrived at in a contemporary study (274) in which two purified ragweed pollen constituents, antigen E (negatively charged) (275) and a new fraction Ra3 (positively charged) were used in conjunction with fractions of allergic sera differing in their charge and which had been isolated by ion-exchange chromatography.

CHAPTER VI

IN VITRO AND IN VIVO INACTIVATION OF REAGINS WITH MERCAPTANS

Introduction

The common hypo-sensitization treatment of allergic patients consists of parenteral administration of small doses of the allergen(s). The development of blocking antibody seems to be the major immunological response to this treatment (276). In contrast to reagins, the blocking antibodies have no affinity for human skin or mucosa (165) but are capable of reacting with and neutralizing the allergen (277).

Skin-sensitizing antibodies, unlike immune antibodies (IgG), are sensitive to reducing agents at low concentrations, and are inactivated by treatment with 0.1M mercaptoethanol (173, 174). If reagins could be inactivated *in vivo* by the administration of mercaptans to man, without concomitant inactivation of blocking antibody or without changing other vital functions, this procedure might have therapeutic potential for the treatment of allergic conditions mediated by skin-sensitizing antibodies. The possibility that reagins might be destroyed or inactivated *in vivo* by the administration of mercaptans, a procedure which had been previously shown to be effective *in vitro* with mercaptoethanol (173, 174), has been explored in this phase of the study.

MATERIALS AND METHODS

Reducing agents

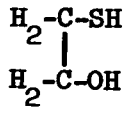
The six mercaptans, listed below (see also Figure 32), used to inactivate reagins were: 2-mercaptoethanol (2-ME), Mucomist (N-acetyl-cysteine), Acthiol (C-acetyl-cysteine. HCl), l-cysteine, d-penicillamine

FIGURE 32

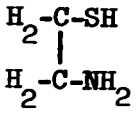
Structural formulae of the mercaptans used to inactivate reagins.

(1) Eastman Organic Chemical
(2) K & K Laboratories
(3) Sigma Chemical Co.
(4) Mead Johnson
(5) Laboratoires Joullie, (France)
(6) Merk, Sharp & Dohme

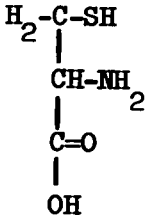
(1) 2-Mercaptoethanol



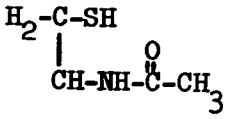
(2) Cysteamine (2-amino-ethanthiol)



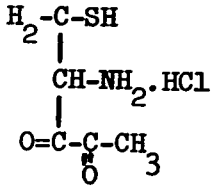
(3) l-Cysteine



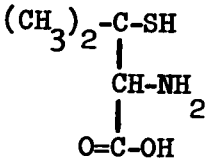
(4) Mucomist (N-acetyl-cysteine)



(5) Acthiol (C-acetyl-cysteine.HCl)



(6) d-Penicillamine (β-β-dimethylcysteine)



(β-β-dimethyl cysteine) and cysteamine (2-amino-ethanthiol). All mercaptans, with the exception of 2-ME and Mucomist*, were separately dried over phosphorous pentoxide. Stock solutions, containing 0.2M of the mercaptan were prepared in a phosphate-citrate buffer,** pH 7.1, which had been degassed and saturated with nitrogen gas immediately prior to use.

Standardization of WSR solution

Stock WSR solutions, 1 mg/ml, were prepared in distilled water. The nitrogen content of appropriately diluted samples of WSR was determined in duplicate by Kjeldahl analysis (278). Reagent grade chemicals were used throughout. The samples were digested by heating with 0.75 ml of concentrated sulfuric acid, 0.75 g (approximately) potassium sulfate (K_2SO_4) and 0.25 ml of a 10% solution of mercuric sulfate ($HgSO_4$),*** until clear. The mixtures were then boiled for an additional 15-20 minutes, and allowed to cool to room temperature. The digestion mixture was quantitatively transferred with a few milliliters of distilled water to the sample compartment of the micro-Kjeldahl apparatus. A volume of 5 ml of a sodium hydroxide-sodium thiosulfate ($NaOH-Na_2S_2O_3$)**** solution was added and steam distillation was carried out until at least 20 ml of condensate collected in a flask containing 5 ml of an indicator

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* Mucomist is supplied as a 20% (w/v) solution in phosphate-citrate buffer, pH 7.1.

** Disodium phosphate 0.04M, citric acid 0.005M, pH 7.1.

*** $HgSO_4$ was prepared by dissolving 10 g of HgO in 100 ml 4N H_2SO_4.

**** 200 g NaOH, 12.5 g $Na_2S_2O_3$ dissolved in and made up to 500 ml with distilled water.

solution.* The nitrogen was titrated with a solution of potassium bi-iodate $KH(IO_3)_2$, 0.003M, to violet-grey coloured end-point. The $KH(IO_3)_2$ was standardized by 'digesting' solutions of ammonium sulfate of known concentrations using the identical procedure outlined for the WSR samples. The 'digestion products' were similarly steam distilled and titrated (Figure 33).

The nitrogen content of WSR was expressed in terms of protein nitrogen units** (PNU). Solutions of WSR in PBS were made to contain 20,000 PNU/ml.

Passive cutaneous anaphylaxis (PCA)

Macaca Irus monkeys were used for the evaluation of the skin-sensitizing activity of human allergic sera by the PCA test. The chest and abdominal areas were closely shaved with electric clippers while the animals were restrained on their backs. Tranquilizers or sedatives were not used. To sensitize skin sites, a volume of 0.05 ml of the allergic serum samples was injected intradermally along the chest and abdominal regions. Occasionally, skin sites along the upper median area of the arms and thighs were used. Twenty-four hours following sensitization, the sites were challenged with an intravenous injection of 2.2 to 2.4 ml/kg body weight of a mixture containing equal volumes of WSR (20,000 PNU/ml) and Evans blue (2%). Positive reactions as evidenced by extravasation of dye were recorded 20 minutes after challenge and graded from 1+ to 4+. To detect and to rule out any non-specific skin reactions, control sites were 'sensitized' with the PBS which had been used in the preparation of serum dilutions.

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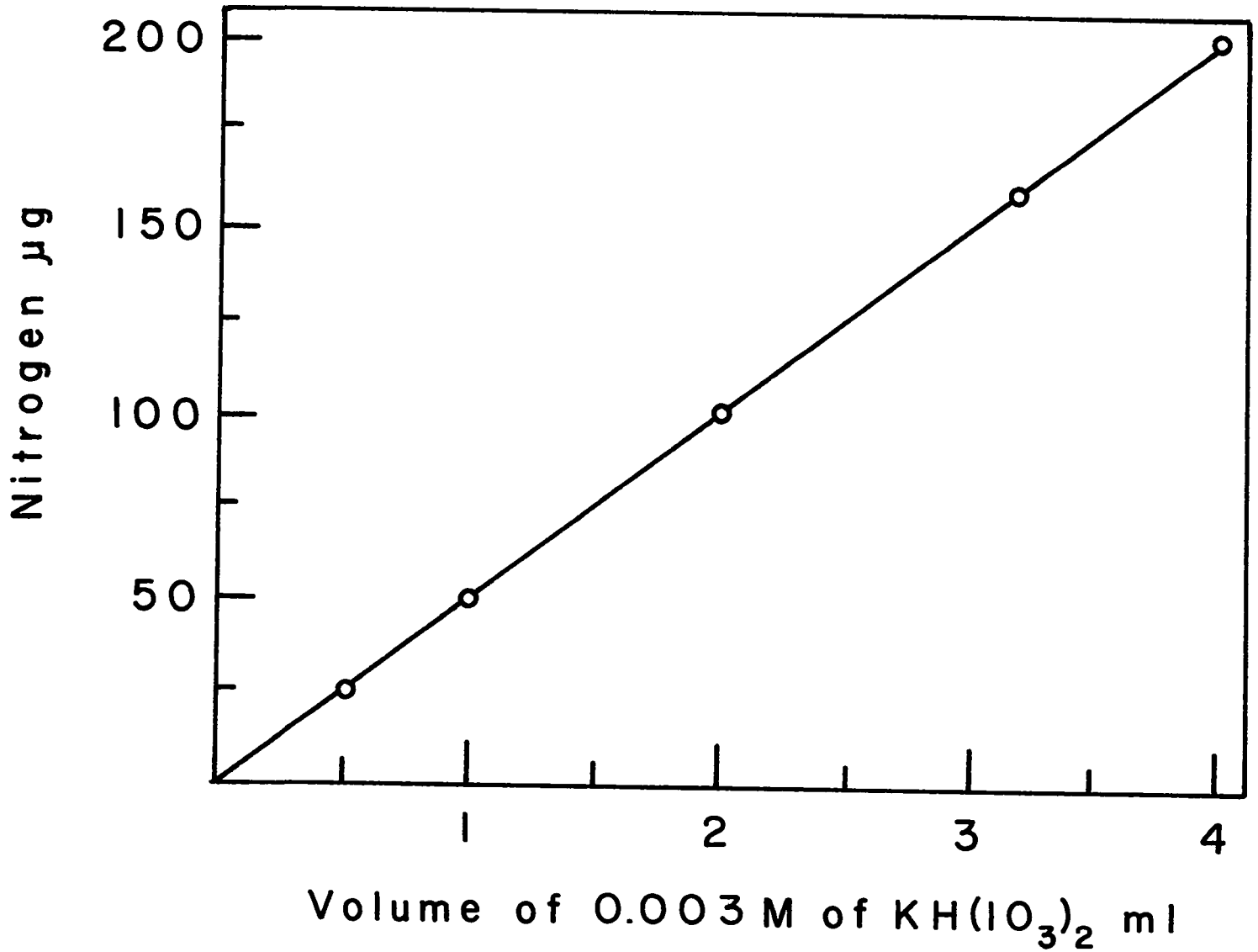
* Prepared fresh monthly, indicator in 0.6% boric acid containing 2 volumes of 0.2% methyl red in 95% ethanol and 1 volume of 0.2% methylene blue in 95% ethanol.

** 1 PNU = 10^{-5} mg phosphotungstic acid precipitable nitrogen

FIGURE 33

Standardization of potassium bi-iodate (0.003M) for the Kjeldahl analysis of nitrogen.


200
150
100
50
Nitrogen µg
1
2
3
4
Volume of 0.003 M of $KH(IO_3)_2$ ml

(A) In Vitro treatment of allergic serum with mercaptan

The *in vitro* inactivation of reagins by the various reducing agents was first investigated under carefully regulated conditions of temperature, concentrations, and time of incubation with mercaptan. The reduction reactions were quickly terminated by cooling and by the addition of iodoacetamide, (both procedures which were not possible in the *in vivo* experiments to be described later in this chapter).

To prevent the allergic serum from forming a gel during reduction with mercaptan it was initially diluted with an equal volume of the phosphate-citrate buffer. A volume of 0.5 ml of the diluted allergic serum was dispensed into test tubes, and a volume of 0.5 ml of the mercaptan, appropriately diluted from stock solutions was added to give a final concentration of 0.1M, 0.08M, 0.06M or 0.04M. The mercaptans l-cysteine, d-penicillamine and cysteamine were used only at final concentrations of 0.1M and 0.08M.

The rate of inactivation of skin-sensitizing activity was investigated by incubating mixtures of allergic serum and mercaptan at each concentration for varying lengths of time at 37°C. A "zero time control" was arbitrarily chosen as the serum sample to which 0.5 ml of iodoacetamide (in a concentration in excess of mercaptan) was added within 20 seconds following the addition of the mercaptan. The other mixtures of allergic serum and mercaptan were each incubated for 15, 30, 60, and 120 minutes, and the reactions were terminated by the addition of iodoacetamide in an amount which exceeded the original mercaptan concentration by 10%. The tubes containing allergic serum, mercaptan and iodoacetamide, were cooled in ice for 30 minutes after

which time the contents were individually transferred into 1/8 inch diameter dialysis tubings* and then dialysed against large volumes of phosphate buffered saline, pH 7.4 at 4°C. Serum samples treated with the six different mercaptans were dialysed separately.

To test the effect of all reagents (i.e. mercaptan, buffer and iodoacetamide) on the skin-sensitizing activity of allergic serum MacM, The controls illustrated in Table XIV, part a, were included. In control C-3, no iodoacetamide was used; instead the treated allergic serum sample was dialysed to remove 2-ME so as to permit reoxidation of the reduced material. All control samples were separately dialysed against PBS, sterilized by filtration through millipore membrane (0.45 μ pore size) and assayed for skin-sensitizing activity by the PCA test in monkeys and by P-K tests in human volunteers.

(B) In Vivo studies

(i) Control experiments

To investigate the possibility that reaginic activity might be restored in vivo following treatment with mercaptans, aliquots of the allergic serum in two-fold dilutions were incubated with 2-ME (0.1M final concentration) for 30 minutes at 37°C, and a volume of 0.05 ml of the mixtures was injected intradermally into a monkey. Allergic serum in two-fold serial dilutions similarly treated with saline, served as control.

To rule out the possibility that the mercaptans had either inactivated or altered the monkey skin for subsequent fixation of reagins, the following experiments were performed. One group of skin sites was injected intradermally with a volume of 0.05 ml of phosphate-

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* Union Carbide Corporation

TABLE XIV

Part a

Composition of control samples for the testing of the effect of buffer, mercaptan, and iodoacetamide on the skin-sensitizing activity of allergic serum, MacM.

Control	Allergic Serum (ml)	2-Mercapto-ethanol (ml)	Iodoacetamide (ml)	Buffer and/or Saline (ml)
C-1*	0.5	0	0	1.0
C-2**	0.5	0	0.5	0.5
C-3*	0.5	0.5	0	0.5

* Control samples C-1 and C-3 were incubated at 37°C for 30 minutes, and then dialysed against PBS.

** Control sample C-2 was incubated at 4°C for 30 minutes, and then dialysed against PBS.

Part b

PCA reactions observed in monkey and P-K reactions observed in human volunteers given by allergic serum control samples.

	Reactions* observed in	
Control	PCA	P-K
C-1	4	3
C-2	3	3
C-3	2	3
PBS	-	-

* Reactions were graded from negative (-) to 4+.

Note: These PCA and P-K passive transfer experiments were performed simultaneously in the same subjects used in another experiment, the results of which are given in Table XVI.

citrate buffer, pH 7.1; the second and third groups of skin sites received a volume of 0.05 ml of 2-ME, 0.1M, and Acthiol, 0.1M, respectively. Twenty-four hours later, allergic serum MacM, or Gr, in two-fold serial dilutions was injected into these skin sites. In addition, the allergic sera in two-fold serial dilutions were used to sensitize skin-sites which had not undergone any previous treatment. Forty-eight hours after the initial treatment of the skin sites (i.e. twenty-four hours following sensitization with the allergic sera) the sites were challenged with intravenous injection of a mixture of WSR and dye.

(ii) The inactivation of passively transferred human reagins in monkey by the in vivo administration of mercaptan

Four monkeys weighing from 2.2-2.5 kg were used for this phase of the study which was designed to determine the feasibility of using mercaptans in vivo to inactivate skin-sensitizing activity. Each of the experiments required four days for their completion and was performed as follows: On the first day, 0.05 ml of allergic serum MacM, in two-fold serial dilutions was injected in duplicate, intradermally along vertical rows on the right-hand side of the monkeys' chest. On day 2, a solution containing a dose of 0.25 g, 0.75 g, 1.0 g, or 1.25 g of Acthiol in 5 ml of phosphate-citrate buffer, was injected intravenously. On day 3, the procedure was the same as on day 1 except that the allergic serum in two-fold serial dilutions was injected intradermally along the left-hand side of the monkeys' chest. On the fourth day, the solution of allergen (WSR) with dye was injected for challenge intravenously into each animal and the PCA reactions were graded within twenty minutes. A flow sheet summarizing the procedures employed in these experiments is given in Figure 34.

FIGURE 34

Flow sheet for the experimental procedures designed to inactivate reagins by *in vivo* administration of the mercaptan Acthiol and to measure the degree of inactivation.

Day 1 Sensitization (Pretreatment)

Skin sites sensitized with 0.05 ml of allergic serum MacM, in two-fold serial dilutions.

Day 2 Treatment with mercaptan

Four monkeys received intravenous injection of 5 ml solutions of Acthiol in doses of 0.25 g, 0.75 g, 1.0 g and 1.25 g respectively.

Day 3 Sensitization (Post-treatment) Control

Skin sites sensitized with 0.05 ml of allergic serum MacM, in two-fold serial dilutions.

Day 4 Challenge

Skin sites challenged by intravenous injection of WSR and Evans blue dye solution; PCA reactions graded within twenty minutes.

RESULTS

(A) In vitro inactivation of skin-sensitizing activity by mercaptans

The effect of the exposure of reagins for varying times and different concentrations to 2-ME, Acthiol, and Mucomist as measured by the PCA test in monkey is shown in Table XV. It is evident that only 2-ME was able to inactivate completely skin-sensitizing activity when used in final concentrations of 0.1M, 0.08M and 0.06M for 15, 30 and 120 minutes, respectively. The size of the PCA reactions elicited with allergic serum MacM, treated for 0, 15 and 30 minutes with Mucomist, was about twice that obtained with allergic serum samples which had been treated with Acthiol for similar periods of time. Comparable PCA reactions were obtained with allergic serum treated with either Acthiol or with Mucomist for 60 and 120 minutes. Complete inactivation of skin-sensitizing activity was not obtained with either Acthiol or Mucomist, at the concentrations and incubation times employed in these experiments. The PCA titer determined from two-fold serial dilutions of untreated allergic serum McM was 1:256. By comparison, the PCA titer of allergic serum, McM, determined in another monkey was 1:64, i.e. the titer in this second animal was lower by a factor of 4. On the other hand, a P-K titer of 1:700 of the same allergic serum demonstrated that the test in human skin was about ten-times more sensitive than the monkey PCA for the detection of reagins.

The results from PCA tests in the second monkey (Table XVI) revealed that complete inactivation of skin-sensitizing activity was obtained with cysteine, d-penicillamine and cysteamine when used in concentrations of 0.1M and for periods of incubation ranging from 0 to 120 minutes.

TABLE XV

PCA reactions observed with mercaptan treated allergic serum MacM*

Mercaptan	Final Molarity	Time of Incubation (minutes) 0	15	30	60	120
2-Mercapto-ethanol	0.10	2	-	-	-	-
	0.08	2	1	-	-	-
	0.06	4	3	1	1	-
	0.04	2	2	1	1	1
Acthiol	0.10	2	1	1	1	1
	0.08	2	1	1	2	3
	0.06	1	1	1	2	2
	0.04	2	2	2	2	2
Mucomist	0.10	2	2	2	2	1
	0.08	2	2	2	2	2
	0.06	2	2	2	1	1
	0.04	4	4	2	2	2

* PCA titer of allergic serum MacM (not treated with mercaptan) was 1:256.

The figures in the time of incubation columns refer to the size of PCA reactions which have been graded from negative (-) to 4+.

TABLE XVI

PCA* and P-K** reactions observed with mercaptan treated allergic serum MacM

Mercaptan	Time of incubation (minutes)	Molarity 0.1M		Molarity 0.08M	
		PCA	P-K	PCA	P-K
2-Mercapto-ethanol	0	-	3	1	3
	15	-	-	-	1
	30	-	-	-	1
	60	-	-	-	-
		-	-	-	-
1-Cysteine	0	-	1	-	3
	15	-	2	-	3
	30	-	1	-	3
	60	-	1	-	3
	120	-	1	-	2
d-Penicillamine	0	-	3	2	3
	15	-	3	2	3
	30	-	3	-	3
	60	-	3	-	3
	120	-	3	-	3
Cysteamine	0	-	3	-	3
	15	-	2	-	3
	30	-	2	-	3
	60	-	2	-	3
	120	-	1	-	3

* PCA titer of allergic serum MacM (not treated with mercaptan) was 1:64.

** P-K titer of allergic serum MacM (not treated with mercaptan) was 1:700.

The PCA and P-K reactions were graded from negative (-) to 4+.

These mercaptans were equally effective at concentrations of 0.08M, with the exception of d-penicillamine which required incubation periods of 30 minutes or more to completely inactivate reagins. 2-ME which had been included for comparison in both experiments, completely inactivated reagins when used in concentrations of 0.1M for all incubation times and at 0.08M for periods of 15 minutes or longer.

Since low concentrations of skin-sensitizing antibody may have escaped detection by the PCA test in the second animal, it was deemed advisable to use a more sensitive assay for reagins. The same allergic serum samples treated with mercaptan were indeed found to possess skin-sensitizing activity when tested by passive transfer P-K tests in a human volunteer (Table XVI). Complete inactivation of reagins was obtained only with 2-ME when used at concentrations of 0.1M and 0.08M for periods of at least 15 and 60 minutes respectively. On the other hand, complete inactivation of reagins was not obtained with either cysteine, d-penicillamine or cysteamine at concentrations of 0.08M and 0.1M, even when incubated with the allergic serum for a period of 120 minutes.

From a consideration of all of these results and from a comparison of the PCA and P-K titers it must be concluded that the observation that an apparent inactivation of reagins had been achieved by three of the four mercaptans used in these experiments was in fact due to a decreased sensitivity of the PCA response in the second monkey, and that only 2-ME at a concentration of 0.1M was effective in inactivating skin-sensitizing activity when incubated with the allergic serum for at least 15 minutes.

An examination of the PCA and P-K reactions of the control samples C-1 and C-2 (Table XIV, part b) indicated that dilution of the allergic

serum with buffer or saline, or incubation with iodoacetamide in the absence of mercaptan, had no appreciable effect on reaginic activity. Treatment of the allergic serum with 2-ME alone (C-3) reduced the PCA reaction from 4+ to 2+. Although this difference in response may not be significant, the positive reactions nevertheless indicated that treatment of allergic serum MacM followed by dialysis, was not sufficient to completely inactivate reagins. Since the general purpose of these experiments was to explore the feasibility of inactivating reagins by the in vivo administration of mercaptans, these latter results with control C-3 suggested the possibility that similar in vivo conditions might be encountered in which reagins would not be completely inactivated by the administration of mercaptan without subsequent treatment with alkylating agents.

(B) In Vivo studies

(i) Control experiments

A PCA titer of 1:256 of allergic serum "treated" with saline indicated that a good response had been elicited (Table XVII). Since no PCA activity was detected in any of the allergic serum samples injected with 2-ME, it must be concluded that the in vivo conditions were not favourable for the restoration of reaginic activity. Also, as no differences in PCA reactions were observed between allergic serum injected in close proximity to and that injected remote from skin sites which had been sensitized with allergic serum containing 2-ME, it was concluded that the mercaptan had either lost its reducing capacity shortly after the intradermal injections, or did not diffuse away from the injection sites.

TABLE XVII

PCA titer given by allergic serum MacM treated* with 2-ME (0.1M) and not dialysed.

Reciprocal of allergic serum dilution	PCA reactions of allergic serum (MacM) treated with 2-ME(0.1M)	saline**	saline***
1	not done	4+	4+
2	not done	4+	4+
4	-	4+	3+
8	-	2+	2+
16	-	2+	3+
32	-	2+	2+
64	-	1+	1+
128	-	1+	1+
256	-	1+	1+
512	-	-	-

* Allergic serum MacM in two-fold serial dilutions starting at 1:4 was incubated with 0.1M 2-ME (final concentration) at 37°C for 30 minutes prior to sensitization of the monkey skin.

** PCA reactions of the allergic serum observed in skin sites sensitized adjacent to those sites which had been sensitized with the allergic serum and 2-ME mixture.

*** PCA reactions of the allergic serum observed in skin sites sensitized remote to those sites which had been sensitized with the allergic serum and 2-ME mixture.

The PCA titer (Table XVIII) of allergic serum MacM, determined in skin sites treated with Acthiol was 1:128 as compared to a titer of 1:64 determined in normal, non-treated skin sites. With another allergic serum Gr, a PCA titer of 1:256 was obtained in sites treated with buffer and 1:512 in sites which had not undergone any treatment or which had been treated with either Acthiol or with 2-ME.

In view of the inherent inaccuracies of the PCA test a difference in titer of a single two-fold serial dilution cannot be regarded as significant. Thus, it appears that the local *in vivo* administration of mercaptan to skin sites did not detectably alter the capacity of the skin to be sensitized by reagins, nor was the ability of tissues of the treated and sensitized skin sites affected to react on challenge with allergen.

(ii) The inactivation of passively transferred human reagins in monkey by the *in vivo* administration of mercaptan

Two sets of PCA titers were obtained in each monkey (Table XIX).

'Treated' titer:

The term 'treated' titer refers to the PCA titers determined from skin sites sensitized with allergic serum MacM, in two-fold serial dilutions. Twenty-four hours after these sensitizations Acthiol was injected intravenously. Thus, the mercaptan could conceivably react with and inactivate the skin-fixed reagins.

'Control' titer:

It was assumed that 24 hours after intravenous administration of Acthiol, its capacity to reduce proteins had been completely destroyed. Thus, the term 'control' titer refers to those PCA titers determined from skin sites which were sensitized on day 3 with the allergic serum in two-fold serial dilutions.

TABLE XVIII

Effect of local administration of mercaptan on PCA titer given by two allergic sera.

Allergic serum*	Pretreatment**	PCA titer***
MacM	none	64
MacM	Acthiol	128
Gr	none	512
Gr	Buffer	256
Gr	Acthiol	512
Gr	2-ME	512
Buffer	Acthiol	0
Buffer	2-ME	0

* Skin sites sensitized by intradermal injections of a volume of 0.05 ml of allergic serum in two-fold serial dilutions.

** 24 hours prior to sensitization with allergic serum a volume of 0.05 ml of either phosphate-citrate buffer, pH 7.1, or of mercaptan (0.1M) was injected into skin sites.

*** Reciprocal of highest dilution of allergic serum giving a positive reaction.

TABLE XIX

Effect of In Vivo administration of Acthiol on PCA titer observed with allergic serum MacM.

Dose of Acthiol (g)	PCA titer*		Decrease in titer by a factor of
	Control**	Treated***	
0.25	16	16	1
0.75	32	4	8
1.00	512	128	4
1.25	256	64	4

* Reciprocal of highest dilution of allergic serum giving a positive reaction.

** Control: (Post Acthiol administration) PCA titers determined from allergic serum in two-fold serial dilutions.

*** Treated: PCA titers determined from allergic serum in two-fold serial dilutions. Acthiol was injected intravenously, 24 hours after the sensitization of the monkey skin.

It is apparent from identical 'control' and 'treated' PCA titers that Acthiol in a dose of 0.25 g did not have any detectable effect on the skin-fixed reagins. The maximum decrease in PCA titer, by a factor of 8, was observed in the monkey which had received Acthiol in a dose of 0.75 g. Further increasing the Acthiol dose to 1.0 g or 1.25 g per animal, did not further decrease the PCA titers. However, several hours following the intravenous administration of Acthiol in a dose of 1.25 g, the particular animal exhibited signs of damage to its central nervous system and remained in a semi-conscious state for the rest of the experiment.

DISCUSSION

Various drugs such as anti-histamines which neutralize vasoactive agents liberated during the reaction of reagins and allergens, provide an immediate relief of the distresses experienced by an allergic individual, but this effect is temporary and no changes in the immunological parameters which lead to hypersensitivity reactions occur. On the other hand, long term treatments are based on a course of immunotherapy designed to alter the relative concentration of the two classes of antibodies (reagins and blocking antibodies) which participate in allergic reactions. While the mechanisms whereby improvement is brought about have not been elucidated, the relief experienced by allergic patients receiving hyposensitization treatment can be attributed, in part, to the formation of blocking antibodies which neutralize the allergens and thus prevent these from reaching and reacting with reaginic antibodies fixed to the cells of target organs (277) and, in part, to a decrease in the level of

reagins (279, 280). For the latter observation it has been suggested that reagins are either neutralized or that a partial tolerance to the specific allergen may be induced by hyposensitization treatment (281). However, hyposensitization treatments always carry the inherent danger of anaphylactic reactions. Moreover, immunotherapy with crude extract (282) or with low molecular weight preparations of allergens (281) have led to complications which arose from the formation of reaginic antibodies of different specificities from those before treatment. In the present phase of the investigation, the possibility that reagins might be inactivated in vivo was explored.

The selection of the mercaptans used in this study was based partly on the similarity of their chemical structure to 2-ME, which is known to inactivate reaginic activity, and partly on their low levels of toxicity. Cysteamine which forms a part of coenzyme A is most similar to 2-ME, the hydroxyl group being replaced by an amino group. Moreover, it was claimed to be capable of inhibiting both the tuberculin reaction in sensitized humans and the passive Arthus reaction in the skin of rabbits (283). The four remaining mercaptans are cysteine and its derivatives. Mucomist, or N-acetyl cysteine had been used in the treatment of chronic bronchitis in man (284). It is thought to exert its mucolytic action by breaking the disulfide bonds in mucus. Acthiol, as indicated in Figure 32, is the C-acetyl derivative of cysteine; d-penicillamine was chosen, because it had been shown to reduce the concentration of the serum macroglobulin paraprotein component (IgM), when administered to patients with Waldenström's macroglobulinemia (285).

The results of the in vitro control experiments clearly established that reagins were not demonstrably affected by such procedures as dilution

of allergic serum with the phosphate-citrate buffer, incubation with mercaptans at 37°C (for a minimum "zero" time), or dialysis against PBS. The importance of including the identical control (i.e. the same allergic serum in two-fold serial dilutions) throughout the PCA tests in the different monkeys was emphasized by a divergence of results of PCA from two experiments. In all in vitro experiments, the capacity of different mercaptans to inactivate reagins was always compared to that obtained with 2-ME. Therefore, the finding in one case (Table XVI) that reaginic activity appeared to have been completely inactivated when allergic serum was treated with 2-ME (0.1M) for a minimum 'zero time' was not expected, since complete inactivation of reagins had not occurred in previous experiments performed under identical conditions and similarly tested by PCA (Table XIV). A comparison of the control PCA titers obtained in two monkeys revealed that the second animal had either not been sensitized with the allergic serum to the same degree or had reacted on challenge with ragweed to a lesser extent than the first monkey. This indeed proved to be the case, as reaginic activity in the "zero time" mercaptan treated samples was detected by the more sensitive P-K test. A ten-fold difference between PCA and P-K titers of the same allergic sera have been reported (286, 287). In addition, two allergic sera have been found which consistently were unable to sensitize monkey skin for PCA reactions (288). Thus, the differences between PCA and P-K titers of allergic sera can be accounted for by the decrease in the degree of sensitization of monkey skin, and the variation of PCA reactions observed in monkeys appear to be similar to variations of P-K reactions observed in man.

It was found that 2-ME was unequivocally the most effective for the inactivation of reagins by the in vitro treatment of allergic serum. The other five mercaptans, in comparable concentrations, were capable of only partially reducing reaginic activity. Reaginic activity could be recovered when reduced allergic serum was allowed to reoxidize indicating that both skin-fixing and antibody combining capacity could be recovered. On the other hand, when the reduced preparation was treated with iodoacetamide (a procedure which by itself does not inactivate intact reagins) reaginic activity was not restored.

Since it was conclusively demonstrated that the mercaptans (2-ME and Acthiol) did not by themselves inactivate or alter the skin to fix passively transferred reagins, the failure to elicit positive PCA reactions in skin sites sensitized with a mixture of allergic serum and 2-ME, indicates that either the skin-fixing portion of reagin molecules or the antibody combining sites, or both, failed to reform in vivo to an active configuration following the elimination or inactivation of mercaptan.

The analysis of the amino acid composition of myeloma γE-globulins has revealed a relatively large number of methionine and cysteine residues (110). However, in the present study no changes were demonstrated in either PCA or P-K activity of allergic serum pre-treated with iodoacetamide. Since alkylation of sulfhydril groups with iodoacetamide proceeds faster than the reaction with amino or hydroxyl groups (289), it is unlikely that free sulfhydryl groups are necessary for either skin-sensitizing or antibody combining activity of reagins.

In another study (204) it had been revealed that following reduction and alkylation, the antibody activity was reduced by one-third and a five-fold increase in the concentration of the reduced and alkylated material was required to block passive sensitization in comparison with the untreated reaginic serum fraction. It must therefore be concluded that both the skin-fixing capacity and antibody combining sites of reagins are impaired by reduction and alkylation treatment.

In the in vivo studies, it was anticipated that inactivation and elimination of the injected mercaptan would occur in addition to its dilution and escape into extravascular spaces. Thus, only a small portion of the doses of Acthiol with reducing capability could conceivably reach the sensitized skin sites. The results of the in vitro experiments had already indicated that Acthiol at 0.1M was not sufficient to inactivate completely reagins of the six-fold diluted allergic serum. Nevertheless, an eight-fold reduction in PCA titer following in vivo treatment with mercaptan revealed that some skin-sensitizing antibodies had indeed been inactivated by the intravenously administered Acthiol.

The assay for reagins at one dilution was satisfactory for determining the presence or absence of reaginic activity in a large number of samples. However, to detect small differences in concentration of reagin, the allergic serum in two-fold serial dilutions was employed in the PCA tests. It is possible that the differences in PCA titers may be accounted for by the elimination of reagins from the passively sensitized monkey skin sites by processes of catabolism during the 48 hours between the two sensitizations. However, this seems unlikely in view of the fact that the animal treated with Acthiol in a dose of

0.25 g, identical PCA reactions were observed in skin sites sensitized two days apart with the allergic serum. It is worth noting that in another study designed to examine the effect of tranquilizers on the skin reactivity of monkeys sensitized locally with allergic sera, it was found that the PCA titer of a ragweed allergic serum was reduced from 125 to 25 following the intramuscular administration of acepromazine; a phenothiozine with a sulfhydryl group (290).

The cumulative results from the in vitro and in vivo experiments indicate that excessively large amounts of mercaptan, in doses toxic to the animals, would have to be administered to inactivate completely all reaginic activity. Thus, no satisfactory mercaptan was found which could be used safely to inactivate completely skin-fixed reagins, and which potentially could be administered to man for the same purpose.

GENERAL DISCUSSION

The most commonly used procedures for the isolation of a single immunoglobulin from a complex mixture of proteins, such as serum, employ techniques such as salting-out, ion-exchange chromatography and gel filtration. These methods are applicable for the isolation of proteins on the basis of their physicochemical properties. However, owing to the similarities of some properties of immunoglobulins belonging to the different classes and to the heterogeneity within any one immunoglobulin class, the techniques mentioned are only capable of providing highly enriched protein preparations. This limitation of the physicochemical isolation procedures was demonstrated by their failure to isolate immunochemically pure IgA from normal human serum. A more sensitive analytical technique of hyperimmunization with the IgA preparation in experimental animals was used to indirectly demonstrate the presence of other serum proteins contaminating the IgA. Therefore, this immunoglobulin preparation was not suitable for purposes of resolving unequivocally the possible association of IgA with reagins. Nevertheless, the observations that reaginic activity was separated from the bulk of IgG and IgA indicated that reagins were not intimately associated with either of these immunoglobulins.

Immunoglobulins with antibody activity are indistinguishable from other globulins by physicochemical criteria. The only distinguishable feature of antibody molecules is their ability to combine specifically with the homologous antigen molecule.

This property was exploited for the isolation of skin-sensitizing antibodies from the sera of ragweed allergic individuals. In a model system using a rabbit antiserum to the antigenic constituents of ragweed pollen, antibodies adsorbed onto immunosorbents prepared with EMA could be eluted with 2 molar solutions of NaCl. On the other hand, the highest specific antibody activity was obtained by dissociation of antibodies with dialysable fractions of aqueous extracts of ragweed pollen. However, only allergen-reagin complexes were isolated by identical procedures when human allergic serum was used. Although ragweed fractions A and U-F were both dialysable, it was apparent that on combination with reagins the resulting complexes were resistant to complete dissociation by extensive dialysis against saline.

Since antisera from two different species were used in the experiments, the different results obtained may be a reflection of differences in specificities and/or combining affinities of the rabbit and human antibodies to ragweed antigen and allergens respectively.

It was demonstrated in other experiments that the allergenic composition of DWSR was complex. In addition, reagins with specificities to one or more of the different allergenic groups were detected in allergic sera. Also, it is obvious that the insolubilization or coupling reactions of the constituents of DWSR during the preparation of the immunosorbents may have involved the allergenic determinants themselves, or groups closely situated to these determinants. This may have resulted in alterations in the antibody-binding capacity. The insolubilization of constituents of DWSR with EMA proceeds by

reaction with amino groups on the protein, whereas the coupling reaction to diazotized cellulose involves tyrosine residues of DWSR. Thus, from a consideration of these factors, it is conceivable that the specificities of the two immunosorbents may not have been identical.

In contrast to the recovery of serum proteins in eluates obtained at low pH from ragweed EMA immunosorbents, no proteins other than the added HSA could be detected in similar eluates recovered from amino-cellulose immunosorbents. These observations suggested that the degree of non-specific adsorption in the latter case was extremely low and that this condition could be attributed to low net electrical charge on the cellulose immunosorbent, by comparison with the EMA immunosorbent, which contains a large number of carboxyl groups.

To reduce the amount of these other proteins in relation to reagins, allergic sera were first fractionated by a combination of procedures employing salting-out with ammonium sulfate at increasing concentrations and secondly, by ion-exchange chromatography on DEAE Sephadex A 50. These procedures were suitable for providing fractions of allergic serum highly enriched with reaginic activity. In addition, antibodies to ragweed of IgG class were separated from reagins. Since blocking antibodies are known to be capable of combining with allergenic constituents of ragweed pollen which are either identical or adjacent to the allergenic determinants for reagins (245), the presence of blocking antibodies, whether in whole sera or in serum fractions, would mean that a competition with reagins for these allergenic determinants would occur. Moreover, the results of that study suggested that the affinity of blocking antibodies for ragweed

immunosorbent was greater than that of reagins. Obviously under such conditions the amount of reagins which could be adsorbed onto the immunosorbent would be greatly reduced. It is worth mentioning that since reagins were subsequently isolated only from serum fractions highly enriched with reagins, the conclusions arrived at as to the nature of reagin may not be strictly applicable to the total reaginic population present in whole allergic sera.

The presence of skin-sensitizing activity in eluates recovered from immunosorbents prepared with amino-cellulose demonstrated that these immunosorbents might be used for the isolation of reagins.

By radio-immunodiffusion procedures using I^{131}-DWSR, the presence of IgE antibodies was demonstrated in both the NaI and the gly-HCl eluates. Since it had been previously demonstrated that immunodiffusion techniques were inadequate in detecting trace amounts of proteins, the eluates were used to immunize guinea pigs in which immune paralysis to the majority of human serum proteins other than reagins had been induced by prior administration of cord serum. This latter procedure was used in order to suppress the production of antibodies to normal serum proteins and to focus the antibody response to reagins. On subsequent Ouchterlony analysis of the guinea pig antiserum with allergic serum and I^{125}-ragweed antigen E, the presence of antibodies to IgE was revealed in this antiserum. These results are in accord with Ishizaka's demonstration that reagins are associated with IgE globulins (42,43). The essential difference between the present study and that of Ishizaka's is that in the present investigation anti-IgE antisera were obtained by immunization of guinea pigs with

reagins isolated specifically with the aid of an immunosorbent, rather than by immunization with serum fractions enriched with reagins followed by extensive absorptions with the immunoglobulins of the other four classes. In view of the difficulties in obtaining immunochemically pure immunoglobulin preparations, the isolation procedures developed in the present study are preferable to the experimental approach used by Ishizaka in rendering antisera specific to reagins by absorption with the other classes of immunoglobulins.

A modification of the method used for the induction of immune paralysis to human serum proteins would be the use of supernatants obtained after thorough absorption of an allergic serum with immunosorbent. Such preparations would be devoid not only of reagins but also of the whole spectrum of antibodies to allergenic constituents and would, therefore, provide an ideal preparation for the induction of immune paralysis to normal serum proteins in experimental animals. Since only a small portion of the total immunoglobulin population (IgG, IgA, IgM) possesses antibody activity and would therefore become adsorbed by the immunosorbent, it can be assumed that a sufficient amount of the normal immunoglobulins would still be present in the supernatant for the induction of immune paralysis. On the other hand, the concentration of reaginic or normal IgE is too low to be effective for the same purpose. Upon subsequent immunization of such pre-treated animals with proteins eluted from immunosorbent, it may be anticipated that, the animals would produce antibodies specifically to reagins.

In addition to the presence of antibodies to IgE, the guinea pig antiserum was shown, by immunoelectrophoretic analysis with allergic sera, to contain antibodies to a human serum protein constituent with the mobility of an α-globulin. However, in another guinea pig antiserum produced by immunization with a non-reaginic eluate in normal guinea pig (i.e. not pre-treated with NCS) antibodies to the α-globulins were not detected. One possible explanation for this divergence in results is that during fractionation of the allergic serum the α-globulin constituent was completely removed from the IgG fraction subsequently used to saturate the immunosorbent. The other possibility is that the α-globulin components of the allergic serum had been fractionated simultaneously with reagins and had been adsorbed non-specifically onto the immunosorbents.

To establish whether the α-globulin components were uniquely and specifically adsorbed from allergic sera, normal human sera would have to be employed for the saturation of ragweed immunosorbents in control experiments. Then, if the presence of the α-globulin components were detected by the immunization procedures described, it would indicate that these proteins had indeed been selectively adsorbed onto the immunosorbent. An alternative explanation for the presence of the α-globulins is that they serve some function in combination with ragweed, e.g. facilitating the elimination of pollen by an individual (normal or allergic) after its inhalation. Evidence to support this latter interpretation was obtained from <u>in vitro</u> experiments in which it was demonstrated that a constant amount of protein had been adsorbed from normal human sera by ragweed EMA immunosorbents.

In addition, it was found in another study (250) that both normal rabbit and normal human sera were capable of agglutinating (i.e. combining with) ragweed pollen. The clarification of the role of serum proteins other than antibodies in the reaction with ragweed components must await future experimentation.

A common observation made throughout the various phases of the present study was the heterogeneity of reagins. Thus, during attempts to isolate IgA by physicochemical procedures, it was revealed that the distribution of skin-sensitizing activity in eluates from DEAE-Sephadex A 50 was different for the various allergic sera. These experiments demonstrated that reagins possessed a range of net electrical charge. Differences in the charge of reagins were also demonstrated in other experiments designed to investigate the relationship in reactivity of electrophoretic fractions of DWSR with differently charged reaginic serum fractions. Moreover, it was shown in these studies that a positively charged electrophoretic fraction elicited greater skin reactions when it was used to challenge skin sites sensitized with negatively charged reaginic serum fraction, while the converse was also true.

The electrophoretic fractions of DWSR themselves possessed a broad range of electrical charge. Although all fractions were skin active, a general feature observed in all skin tests with the fractions was that the skin activity, per unit weight of electrophoretic fraction, was lower for fractions migrating to the cathode, whereas fractions with anodal mobility possessed higher but more variable skin activities. It remains to be determined whether these differences in skin activity

result from differences in: (i) the quantity(s) of allergenically active constituent(s), and/or (ii) the allergenic activity of the constituents per se. The presence of at least five different allergenic components was revealed by the cross neutralization tests with six electrophoretic fractions using three allergic sera. Moreover, the allergenic composition of each fraction was different. It was also determined from these experiments that each of the three allergic sera contained reagins against a certain number of these allergens. Thus for the different variations in allergenic composition of the electrophoretic fractions of DWSR a corresponding variation in the number of different specificities of reagins was detected. However, no two sera possessed reagins of identical specificity.

In addition to differences in the specificities of reagins a heterogeneity in their affinity for the various allergens was noted during elutions from immunosorbent. The effectiveness of the elution in terms of the recovery of reagin was in the order of 2M NaI > gly-HCl > urea. Moreover, two groups of reagins which differed in their elutability with respect to gly-HCl and 2M NaI were demonstrated to be present in the two ragweed allergic sera studied. A novel procedure was for the immunospecific isolation of reagins by their displacement from immunosorbent with the homologous haptens. The recovery of only a small portion of reagins, in relation to the amount of skin-sensitizing activity eluted subsequently with NaI, was attributed, in part, to the displacement only of those reagins with a low affinity for the allergens which is in conformity with

with the concept of heterogeneity of antibodies with respect to their avidity for the antigen, and, in part, to the low concentrations of the haptenic constituents used for the elution. Since it was shown that one allergic serum contained two groups of reagins, one with a higher skin-fixation capacity than the other, it may be concluded that the portion of the IgE (reaginic) molecule which is responsible for skin sensitization is heterogeneous.

All of the results described provide ample demonstration of the great diversity and complexity and heterogeneity of reagins with respect to (i) net electrical charge, (ii) antibody specificity, (iii) affinity for the allergens and (iv) skin-fixation.

SUMMARY

1. A simple procedure was developed for isolating IgA from normal human serum using salt precipitation with ammonium sulfate in a concentration corresponding to a saturation of 45% and ion-exchange chromatography on DEAE-cellulose using step-wise elution with phosphate buffer at constant pH 8.0 and increasing ionic strength. The maximum amount of IgA was recovered with buffers at 0.035M. The IgA fraction possessed a sedimentation coefficient of 7S and appeared to be pure on examination by immunoelectrophoresis and by vertical starch gel electrophoresis with subsequent immunodiffusion analysis in agar. However, the presence of trace contaminating serum proteins, chiefly IgG and transferrin, was revealed by homologous antibodies in an antiserum produced by hyper-immunization of a rabbit with the IgA preparation.

2. The fractionation of human ragweed allergic sera was achieved using a concentration of ammonium sulfate corresponding to 45% saturation, or by a step-wise increase in ammonium sulfate concentration corresponding to 30%, 30-35% and 35-45% saturations, followed by ion-exchange chromatography on DEAE-Sephadex A 50. The results of these studies revealed that the distribution of the maximum reaginic activity did not parallel the distribution of the highest concentration of immuno-globulins of the types IgG, IgA or, IgM, and suggested that immunoglobulins with ion-exchange properties intermediate to those of IgG and IgA were the actual carriers of reaginic activity.

3. In a model system rabbit antiserum to DWSR was adsorbed with immunosorbents prepared by insolubilizing DWSR with ethylene-maleic anhydride, and it

was found that the antibodies could be recovered by non-specific elution with 2M NaCl and with the dilaysable constituents of a chymotryptic digest of casein. The immunochemically specific displacement of rabbit antibodies from the immunosorbent with low molecular weight, hapten-like components prepared from the dialysable fraction of ragweed pollen extract, resulted in enrichment of antibody activity. The application of these procedures for the isolation of reagins from ragweed allergic sera was successful in isolating allergen-reagin complexes only.

4. Reagins from sera of ragweed allergic individuals were adsorbed specifically onto immunosorbents prepared by coupling DWSR to amino-cellulose. Reaginic activity could be recovered by elution with glycine-HCl at pH 2.5, or 2M NaI at pH 9.1, or with urea at pH 7.4, in presence of a mixture of proteins as in (i) normal human serum or, (ii) the globulins of normal human serum or, (iii) the globulins of cord serum, or of a single well defined class of proteins as those of human or rabbit serum albumin; the effectiveness of the elution in terms of recovery of reagins was in the order of 2M NaI > glycine-HCl > urea. With a given eluting agent, maximum reaginic activity was recovered at a protein concentration of 7 mg/ml, about 50% of the activity at 0.7 mg/ml, and no activity was detected in eluates containing protein below the concentration of 0.07 mg/ml. All these protein preparations had a comparable stabilizing effect on reaginic activity. The specific elution of a portion of reagins was also accomplished by their displacement with haptenic constituents of ragweed pollen extracts. The heterogeneity of reagins was demonstrated

by the fact that one group of reagins was eluted with glycine-HCl and the other with NaI, irrespective of the order of addition of the eluting agent to the immunosorbents.

5. By radio-immunodiffusion procedures, using I^{131}-DWSR, the presence of IgE antibodies was demonstrated in both the NaI and the glycine-HCl eluates. These eluates were used to immunized guinea pigs in which immune paralysis had been induced by the intravenous admininstration of normal cord serum. On subsequent immunoelectrophoretic analysis of the guinea pig antiserum with allergic serum, the presence of antibodies to a human serum protein constituent with the mobility of an α-globulin was revealed in this antiserum. Moreover, by the Ouchterlony procedure with I^{125}-antigen E of ragweed pollen, the guinea pig antiserum was shown to contain antibodies to IgE.

6. (a) The non-dialysable aqueous extract of ragweed pollen (DWSR) was subfractionated by zone electrophoresis on Sephadex G-25 into fractions differing in their electrophoretic mobility. The electrophoretic fractions contained at least two common antigenic components in addition to at least 5 other antigenic components. The cross-neutralization of P-K reactions by two electrophoretic fractions with three allergic sera revealed (i) the presence of at least five distinct allergenic constituents, and (ii) a different spectrum of reagins for the three allergic sera directed against one or more of the allergenic components.

(b) The skin activities of the electrophoretic fractions of DWSR were shown to be a function of both the allergic serum and the

volunteer used for the P-K tests. From a comparison of P-K reactions obtained with electrophoretic fractions of DWSR and three allergic sera tested in two different volunteers it was inferred that reagins differed in their affinity for normal skin, i.e. they differed in their skin-fixation.

(c) Maximal wheal and flare reactions were obtained in P-K tests on interaction of fractions of allergic serum, separated by ion-exchange chromatography on DEAE-Sephadex A 50, with electrophoretic fractions of DWSR of opposite electrical charge.

7. The inactivation of reagins in vivo with the mercaptan "Acthiol", was shown to be partially successful in a model system using monkeys sensitized intradermally with human reaginic serum.

CLAIMS TO ORIGINALITY

1. By the application of physicochemical procedures e.g. salting-out and ion-exchange chromatography, which were employed to fractionate human serum, it was possible to isolate pure IgA. However, by a hyperimmunization technique, the presence of contaminating serum proteins in the "pure" IgA preparation was revealed.

2. It was revealed that reagins were not associated (at least not exclusively) with IgG, IgA or IgM. The chromatographic distribution of reaginic activity suggested that reagins were associated with an immunoglobulin(s) with elution properties intermediate to those of IgG and IgA.

3. Antibodies to DWSR were isolated by adsorption of rabbit antiserum to DWSR with immunosorbents, prepared by the insolubilization of DWSR with ethylene-maleic anhydride. The adsorbed antibodies were specifically eluted by their displacement from the immunosorbent with dialysable hapten-like constituents of the aqueous extract of ragweed pollen.

4. An allergen-reagin complex was isolated from immunosorbent, which had been saturated with ragweed allergic serum, by elution with dialysable constituents of ragweed pollen.

5. Reagins adsorbed to immunosorbents prepared by coupling DWSR to amino-cellulose could be recovered by elution with 2M NaI at pH 9.1.

6. The effectiveness of the elution from immunosorbent, in terms of recovery of reagins, was in the order of 2M NaI, at pH 9.1>glycine-

HCl, at pH 2.5> 6M urea at pH 7.4.

7. It was shown that the presence of a single well defined class of serum protein, such as human or rabbit serum albumin with physico- and immunochemical properties distinct from those of reagin(s), could replace whole serum as the stabilizing proteins for reaginic activity during the elutions with buffers at low pH from immunosorbent.

8. The heterogeneity of reagins with respect to their elutability from immunosorbent was demonstrated by the fact that one group of reagins was eluted with glycine-HCl and the other with NaI, irrespective of the order of the addition of the eluting agent to the immunosorbent.

9. The specific elution of a portion of reagins was accomplished by their displacement from immunosorbent with haptenic constituents of ragweed pollen extracts.

10. Reagins isolated by immunospecific methods from human ragweed allergic sera were identified as IgE.

11. The presence of at least 5 distinct allergenic constituents was demonstrated in fractions of DWSR obtained by electrophoresis on Sephadex G-25. Moreover, a different spectrum of reagins against one or more of these allergenic constituents was found in each of the allergic sera studied.

12. The skin activity of the electrophoretic fractions of DWSR was shown to be a function of both the allergic serum and the volunteer used for the P-K tests. Moreover, evidence was obtained to suggest that reagins differed in their skin fixation ability.

13. It was demonstrated that maximal skin reactions were obtained in P-K tests on the interaction of fractions of allergic serum with electrophoretic fractions of DWSR of opposite electical charge.

14. It was demonstrated that reagins could be inactivated in part *in vivo* by the intravenous administration of the mercaptan "Acthiol".

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