ISOLATION OF ALLERGENS OF TIMOTHY GRASS POLLEN

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfilment of the requirements for the degree
of Doctor of Philosophy.

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December, 1967.

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ABSTRACT

The water extract of timothy grass pollen (WSG) was fractionated by a combination of different methods, i.e. salt precipitation, ion-exchange chromatography and gel-filtration on Sephadex. The most active fraction (C-IV) was found to be 100,000 times more potent than WSG and contained all the skin active components of WSG.

Fraction C-IV contained 3 of the 7 antigenic components of WSG, as demonstrated by immunoelectrophoresis. WSG gave two groups of precipitin lines with rabbit anti-WSG antiserum, which were identified as the 'outer' and 'inner' bands on immunodiffusion; the antigenic components of fraction C-IV were shown to belong to the 'inner' groups of antigen of WSG.

The active fraction (C-IV) contained 93% protein and 2.2% carbohydrate, the rest being accounted for as moisture. The protein moiety contained all the naturally occurring amino acids; glutamic acid, alanine and lysine were present in relatively large amounts. The sugar unit of the carbohydrate moiety was identified as arabinose. The sedimentation coefficient of the fraction was 1.5 S.

The dialysable constituents of WSG were further fractionated by gel-filtration on Sephadex G-25 and G-50. The dialysable fraction D-(W)-II possessed properties of à hapten with respect to rabbit anti-WSG antiserum; this haptenic fraction was electrophoretically heterogeneous. D-(W)-II was also shown to possess the capacity of inhibiting P-K reactions elicited by WSG; however, it was slightly skin active when used at the high concentration of 5 mg/ml and the possible reasons for this activity were discussed.

ACKNOWLEDGEMENTS

I wish to thank Professor A.H. Sehon for his guidance and understanding throughout the course of this investigation and for his valuable discussions and criticism during the preparation of this thesis.

I also wish to thank Dr. S.C.R. Meacock for his assistance in introducing me to the field of pollen allergy, and Dr. L. Gyenes for his helpful discussions, criticism and encouragement.

I am indebted to Dr. S.O. Freedman of the Montreal General Hospital for providing the human serum and to his technical staff for their assistance in performing the P-K tests.

I wish to thank my colleagues in our laboratory for their collaboration, and in particular Mr. F.T. Kisil for his many technical aids.

To Miss E. Wilson I extend my thanks for her valued assistance and patience in typing this thesis.

Finally, I wish to express my deep gratitude to the Governments of Canada and Pakistan for the award of a scholarship under the Colombo Plan, which enabled me to come to Canada and to pursue this study.

This investigation was supported by grants provided to Professor A.H. Sehon from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md., the Medical Research Council of Canada, Ottawa, Ontario, and the Life Insurance Medical Research Fund, Rosemont, Pa.

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CHAPTER I

SOME CONCEPTS OF IMMUNOLOGY AND HYPERSENSITIVITY

Introduction

Animals having survived a bacterial infection are found to be resistant to a subsequent infection with the same bacterium. The sera of such animals contain a factor which is capable of agglutinating or dissolving the micro-organism when incubated with it in vitro. This factor is called antibody. Production of antibodies can also be elicited by the injection of foreign macromolecules, known as antigens, into the animals. The antibodies thus confer on animals immunity or protection against subsequent infection. However, an animal may show untoward and often fatal reaction, upon subsequent exposure to certain antigens. This state of altered reactivity, as opposed to immunity, is known as hypersensitivity and is due to the aberration of the immunological mechanism. In 1906, von Pirquet coined the term allergy (from the Greek terms allos-other and ergon-action) for hypersensitivity and the antigen causing hypersensitivity as allergen (1). The term allergy is now used in its broadest sense to include also most of the known immunological diseases.

The first recorded observation of allergy was made by Hippocrates (2) and by Lucretius (3), who noted that 'What food is to one may be fierce poison to others'. In 1565, Botallus (4) observed that certain individuals, when exposed to roses, developed sneezing and itching. The allergic diseases known to-day as hayfever was first described by Bostock (5) in 1819. Elliotson (6) suggested that this condition was associated with the pollination of flowers and the works of Gordon (7), Phobus (8) and Wyman (9)

all pointed towards the pollen as the causative agent. The conclusive evidence of implicating the pollen in hayfever came from Blackley (10,11) and Dunbar (12). The similarity between hayfever and other allergies, such as serum sickness, urticaria and anaphylaxis, was observed by Weichardt (13), Wolf-Eisner (14) and Meltzer (15).

In 1890, Koch (16) reported the first experimental studies in allergy. He observed that guinea pigs, when first injected with tubercle bacilli, did not show any immediate response. However, subsequent injection into the infected guinea pigs caused inflammatory reactions, such as wheal and flare. Flexner (17), in 1894, found that rabbits tolerated the first injection of dog serum but died on reinjection. Von Behring (18), in 1895, noted that guinea pigs could be sensitized with diphtheria toxin. In 1898, Richet (19) observed that dogs previously injected with eel serum exhibited shock symptoms and died on reinjection. Later, Richet and Portier (20) observed similar effects with the poison of sea anemone. The phenomenon was described by them as the antithesis of prophylaxis and, therefore, they coined the term anaphylaxis - meaning without protection.

In 1903, Arthus (21) produced a skin necrotic lesion, known as Arthus reaction, upon successive subcutaneous injections of horse serum into rabbits. A condition known as serum sickness was observed upon the injection of a foreign antiserum into man (22), i.e. about 8 to 12 days following the injection of the antiserum violent reactions, such as rashes, fever, glandular swelling, were noted.

The present-day knowledge of allergy was mostly based on the studies of the immunological mechanisms involved in anaphylaxis, Arthus phenomenon and serum sickness. That antibodies are involved in these phenomena was

suggested as early as the beginning of this century by numerous investigators (23,24,25,26,27).

Antigens

Antigens, by definition, are large molecules, such as proteins, polysaccharides, polypeptides and polynucleotides, which, when injected into the animal, can elicit the formation of antibodies and can combine specifically with the homologous antibodies. The capacity of an antigen to provoke an antibody response is known as immunogenicity (28). In contrast, the capacity of an antigen to react with antibodies, even if the antigen is deprived of the ability to elicit antibody formation, is termed as antigenicity or antigenic specificity. Furthermore, it became apparent that the broad areas of the antigens were required for immunogenicity and antigenic specificity was determined by small portions of these large molecules. The portion responsible for the antigenic specificity is called antigenic determinant, antigenic site, determinant group, or simply hapten. An antigen, in fact, consists of many antigenic determinants and on this basis the phenomenon of cross-reaction has been partially explained. Thus, it is possible, from the consideration of primary structure or steric conformation, for two antigen molecules to have certain determinant groups in common.

To be immunogenic, a molecule requires to be foreign to the host animal. Thus, the proteins of one species will usually be immunogenic when injected into another species. In cases where antibody formation is provoked by the host's own proteins, the self-antigens have undergone slight chemical and physical modification which could no longer be distinguishable from foreign

antigens.

The understanding of antigenic specificity was begun following the work of Landsteiner (29) on the production of antibodies to chemically well defined simple substances of low molecular weights, termed haptens, by coupling them to proteins through covalent bonds. Haptens themselves are non-immunogenic but can react with the antibodies directed against them. This was demonstrated by the ability of the hapten, without being coupled to the protein, to inhibit precipitation of the antigen-antibody system, or by equilibrium dialysis experiments which showed that the simple hapten was bound to antibody.

Since the haptens can be synthesized in vitro and highly specific antibodies can be obtained against the determinant group of known structure, these have been employed to study the immunological responses (discussed in detail in Chapter V).

Antibodies

Antibody activity is associated with globulins, termed immunoglobulins, which display electrophoretic, ultracentrifugal and antigenic heterogeneity. The characteristic feature of immunoglobulins is their ability to react specifically with the homologous antigen. This specificity is a reflection of steric complementarity between the antibody combining sites and the antigenic determinant groups. The combining specificity, however, appears to be unrelated to the structural variability of various immunoglobulins.

Human immunoglobulins are subdivided into five classes. The new nomenclature for immunoglobulins proposed by WHO (30) is used in this text.

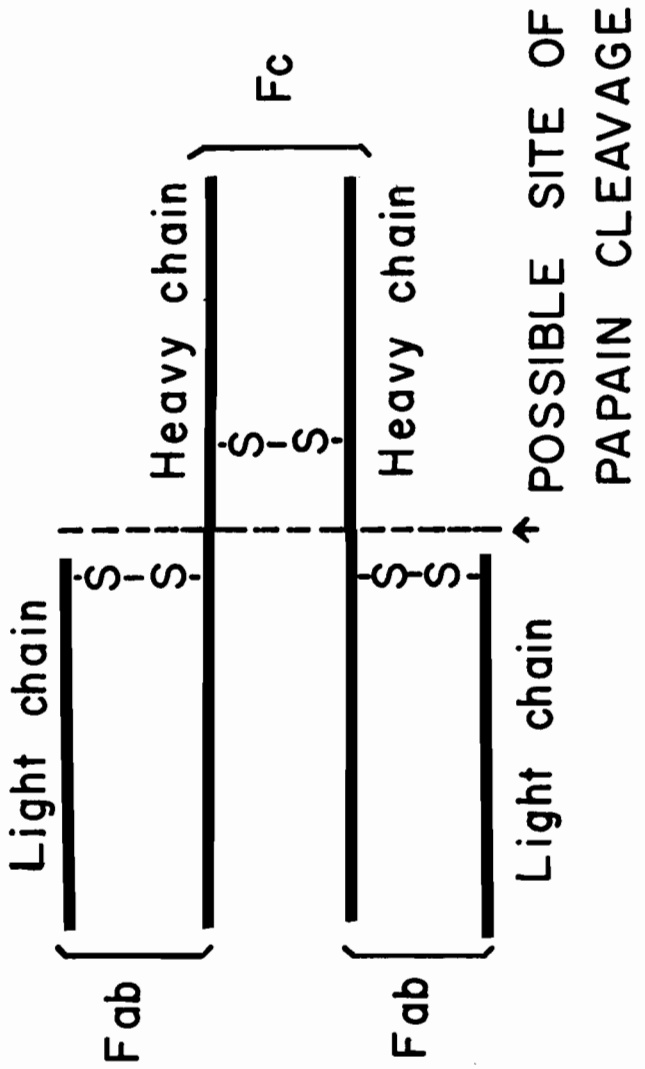
(i) IgG- or γG-globulins. This class represents most of the immuno-

globulins (80%) having a molecular weight of about 150,000 (31) and containing 3% carbohydrate (32). It can be separated in high yields from the other serum proteins by ion-exchange chromatography on DEAE-cellulose (33,34). IgG can be degraded with papain into three fragments (Figure 1), two F_{ab} (I and II) and one F_c (III), which are separable by chromatography on CM-cellulose (35). The F_{ab} fragments each have a molecular weight of 52,000 and contain the antibody combining site. The F_c fragment is devoid of antibody activity, carries most of the antigenic determinants unique to IgG (36,37,38) and can be crystallized under appropriate conditions. On reduction of interchain disulfide bonds by treatment with 0.1M 2-mercapto-ethanol in 6M urea, the IgG molecule dissociates into four polypeptide chains (39). Each of the two longer chains, with molecular weight of the order of 55,000, is termed a heavy (H) chain, and each of the two shorter chains, with molecular weight of about 20,000, is called light (L) chain (40). These chains are separable on the basis of their size by gel-filtration (41). The heavy and light chains differ in their amino acid compositions.

(ii) IgM- or γM-globulins. These immunoglobulins represent 5-10 per cent of the total immunoglobulins, with a molecular weight of about 900,000 and a carbohydrate content of about 10 per cent. IgM can be isolated by preparative ultracentrifugation (42) or by a combination of chromatography and gel-filtration (43). IgM-globulins comprise, in addition to the 19S component(s), two minor components with sedimentation coefficients of the order of 29S and 35S. Exposure of IgM to 0.1M mercaptoethanol leads to dissociation of the parent molecule into sub-units with a sedimentation coefficient of about 7S (44). The sub-units lose all antibody activity but retain the antigenic properties of IgM.

FIGURE 1

Schematic structure of IgG.


Light chain
Heavy chain
Fc
POSSIBLE SITE OF
PAPAIN CLEAVAGE
Heavy chain
Light chain
Fab
Fab

(iii) IgA- or γA-globulins. These immunoglobulins have the electrophoretic mobility of the fast γ- (or slow β-) globulins. They represent 10% of the immunoglobulins and have been isolated by a combination of salt precipitation and preparative electrophoresis (45). Like the IgM-globulins, IgA tends to aggregate into higher molecular weight components; the main component has a sedimentation coefficient of approximately 7S and the minor components (about 15% of the IgA-globulins) have sedimentation coefficients ranging from 9S to 15S (46). The carbohydrate content of IgA is relatively high (about 10%) and the molecular weight of the 7S monomer is about 150,000.

(iv) IgD- or γD-globulins. This class of immunoglobulins was recently discovered by Rowe and Fahey (47) and represents less than 1% of immunoglobulins. IgD has been separated from other immunoglobulins by ion-exchange chromatography on DEAE-cellulose, followed by gel-filtration on Sephadex G-200.

(v) IgE- or γE-globulins. This class of immunoglobulins has been recently proposed to include skin-sensitizing antibodies found in various atopic patients and will be described later under the heading of Antibodies involved in atopic patients.

Recently a human myeloma globulin, termed IgND, has been discovered, which possesses antigenic determinants distinct from those of IgG-, IgA-, IgM- and IgD-globulins, is related to IgE-globulin and is capable of blocking the fixation of reaginic antibodies to normal human skin (48).

The heavy chains of three major classes, IgG, IgA and IgM are structurally distinct and are referred to as γ, α and μ, respectively. In contrast, the light chains of all three classes fall into two antigenically distinct and chemically different forms known as κ and λ. A normal human serum contains

about 70% κ- and 30% λ-chains with smaller amounts of light chains possessing neither antigenic determinant.

The precipitin reaction

Antibody and its homologous antigen, when mixed together in the right proportions, results in the formation of the precipitates of antigen-antibody complexes. The amount of the precipitate formed, on addition of an increasing amount of antigen to a constant amount of antiserum, is represented by the classical precipitin curve (Figure 2). Increased amounts of precipitates are formed through the region of antibody excess to a maximum at the equivalence zone and, on further addition of antigen, the amount of precipitate decreases. This phenomenon has been explained by 'lattice' theory (49).

According to this theory, multivalent antigen molecules combine with bivalent antibodies (precipitating antibodies seem always to be bivalent) to give soluble complexes in the region of antibody excess. These soluble complexes consist of one antigen and two antibody molecules. As the amount of antigen is increased, cross-linked aggregates with the occurrence of alternating and recurring antibody-antigen pattern are formed, making a larger lattice, which eventually becomes a three-dimensional framework and becomes insoluble. In antigen excess, one antibody molecule combines with two antigen molecules to form soluble complexes since no cross-linking can occur.

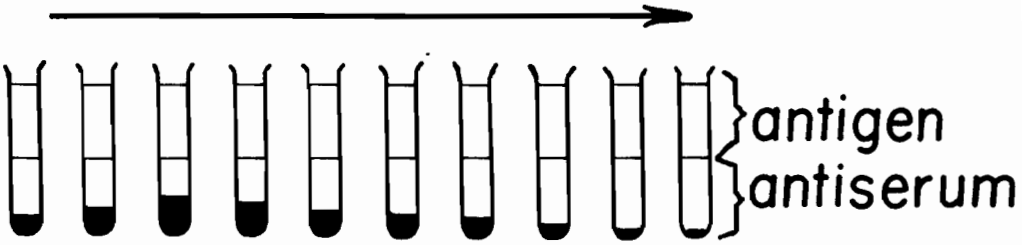
The precipitin reaction involving hapten-protein conjugates and their homologous antibodies can be inhibited by the addition of free hapten, a univalent antigen.

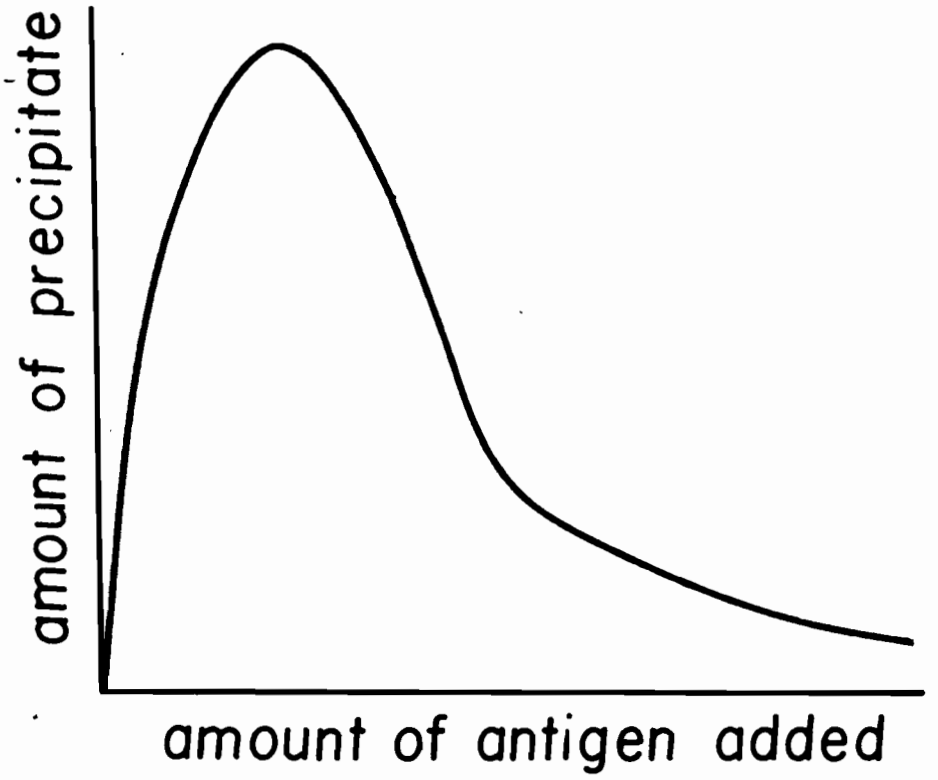
FIGURE 2

Typical precipitin curve.

TYPICAL PRECIPITIN CURVE

Increasing amounts of antigen added to constant amount of antiserum





Hypersensitivity

Hypersensitive states can be divided into two main classes: (i) of the immediate, and (ii) of the delayed type. Immediate type hypersensitivity can be passively transferred with serum into a non-sensitive recipient, and it is characterized by the involvement of humoral antibodies. Anaphylaxis and atopic sensitivity are the examples of immediate type hypersensitivity. With the exception of few opinions claimed (50,51,52), no humoral factors can be demonstrated in the delayed type hypersensitivities, and its passive transfer is usually accomplished with white cells or with extracts of these cells. Among conditions of delayed hypersensitivity are considered tuberculin sensitivity, contact allergy, experimental autoallergies and phenomena related to transplantation immunity.

The manifestations of different forms of immediate type hypersensitivities are described briefly below.

(i) Anaphylaxis. When an animal is sensitized with an antigen, a time interval is required before anaphylactic shock can be produced on reinjection of the antigen into the animal. During this period antibodies are formed and some of these antibodies become fixed to the tissues (shock organs). When the antigen is injected into a sensitized and anaphylactic prone animal, the combination of antigen with tissue-fixed antibodies leads to the release of pharmacologically active substances, such as histamine, serotonine, acetyl choline and kinins responsible for anaphylaxis.

(ii) Atopic sensitivity. The term atopy (meaning strangeness) was introduced by Coca and Cooke (53) to describe a type of hypersensitivity occurring mostly in man. In some instances, however, atopic conditions have been also noted in dogs and other animals (53,54,55,56,57,58,59,60).

Asthma, hayfever, urticaria, angioedema are some of the chief atopic conditions in man. The allergens responsible for this type of hypersensitivity are found in pollens, feathers, animal danders, house dusts, milk, eggs, tomatoes. Simple chemicals, such as common drugs, which may react with the host's proteins, e.g. penicillin, aspirin, can also cause atopic sensitivity.

Atopic sensitivity is considered to be hereditary. Cooke and Van der Veer (61) observed that 48% of their allergic patients and only 12% of non-allergic individuals had a family history of atopic diseases. They also noted that children of allergic patients had the tendency to develop allergies earlier, while children with only one of the parents allergic were inclined to become allergic later in life; those of non-allergic parents, if they developed the disease, tended to do so primarily in their fourth decade. These investigators also noted that children of allergic parents were not born allergic.

Antibodies involved in atopic patients

(i) Reagin. Intradermal injection of the specific allergenic extract into allergic individuals leads to a characteristic reaction, which consists of a wheal surrounded by erythema. In 1921, Prausnitz and Küstner (62) showed that a similar allergic reaction could be elicited by sensitizing the skin of a normal individual with an allergic patient's serum and challenging the sensitized sites, usually 24 hours later, with the homologous allergen. The humoral factor causing the sensitization was designated by Coca as reagin. Reagin meets most of the criteria of an antibody and is, therefore, also known as skin sensitizing antibody.

The experiment of Prausnitz and Küstner has become the recognized test for demonstration of skin sensitizing antibodies in sera of allergic individuals, and the procedure is referred to as the passive transfer P-K test. The P-K test has been widely used for determining the skin activity of various pollen extracts.

Earlier studies (63,64,65) indicated that reaginic activity was associated with 19S immunoglobulins. In a subsequent study (66), reagin was shown to have a sedimentation coefficient higher than 7S and it ranged from 12.4S to 22.5S. Other investigators, however, have assigned a sedimentation coefficient of about 8S to reagin (67,68). Loveless et al (69) reported that skin sensitizing activity was associated with slow moving β-globulins. Sehon et al (70) also obtained similar results with serum protein fractions isolated by starch electrophoresis. It was subsequently claimed that reaginic activity might be associated with IgA-globulins (71,72). Very recently, however, it was demonstrated that reagin did not belong to the IgA class, or to any of the known classes of immunoglobulins; on the basis of their antigenic properties, it was proposed that reagin belonged to a new class of immunoglobulins, designated as IgE-globulins (73,74,75,76,77). This claim was based on the following lines of evidence: (a) the factor(s) responsible for reaginic activity could not be precipitated from allergic sera with antibodies specific for IgG-, or IgM-, or IgA- or IgD-immunoglobulins; (b) reaginic antibodies were coprecipitated with human IgG-globulin on addition of rabbit anti-L chain antibodies; (c) light chains of immunoglobulins were detected in IgE-globulin; (d) skin-sensitizing antibodies in sera of ragweed allergic patients were precipitated with a specific antiserum to IgE-globulins which

did not contain detectable antibodies against IgG-, IgA-, IgM- or IgD-globulins.

As already mentioned, reaginic antibody has the unusual biological property of attaching itself to human skin. Recently it was found that it could also sensitize the skin of the monkey, Macaca irus (78,79). Another biological property of skin-sensitizing antibody is its inability to pass the placenta from maternal to fetal circulation (80,81) and its retention by choroid plexus (82). Skin-sensitizing antibody is thermolabile and its activity is lost by heating at 56°C, the minimal time required for their heat inactivation, varying from serum to serum and ranging from one to ten hours. However, it can be stored in serum proteins at 4°C for long periods without appreciable loss of activity.

Like other immunoglobulins, skin-sensitizing antibody is degraded with papain; however, the digestion period is longer than an hour (83). Unlike immune antibodies, treatment of skin-sensitizing antibody with 0.1M mercaptoethylamine in the absence of papain destroys its biological activity (83,84,68). The inactivation was ascribed to either the destruction of the antibody combining site, or the alteration of the portion of the molecule responsible for skin-fixation.

The immunological characteristic of skin-sensitizing antibody is its inability to precipitate with the homologous allergen in vitro. This was attributed (65,85) to any of the following three possibilities: (a) skin-sensitizing antibody is 'incomplete', i.e. univalent, (b) the affinity of skin-sensitizing antibody for the allergen(s) is very small, or (c) it could be divalent and have a high affinity for the allergen, but its concentration is too low to be detected by the standard precipitin test.

(ii) Blocking antibody. It was recognized (86) that allergic patients receiving hyposensitization treatment showed an increased tolerance towards the offending agent. This tolerance was attributed to the protective capacity of a humoral factor, known as blocking antibody, which was formed during the treatment in addition to the skin-sensitizing antibody. The test for blocking antibody is based on its ability to neutralize the allergen and thus prevent the latter from reacting with skin-sensitizing antibodies in the P-K reaction. Formation of blocking antibody was considered to represent a normal immunological response.

Blocking antibody, in many respects, differs from skin-sensitizing antibody, i.e. (a) it is not inactivated by heat at 56°C (87) or by treatment with 0.1M mercaptoethylamine (83); (b) unlike skin-sensitizing antibody, blocking antibody on excessive concentration was shown by indirect evidence to be precipitated with the homologous allergen in vitro. Blocking antibody is electrophoretically a slow moving γ-globulin with a sedimentation coefficient of 7S (87).

Allergen and skin active component

Allergen. An allergen is defined as the effective antigen present in various pollens, house dusts, foods, etc., which induces the formation of skin-sensitizing antibodies in individuals predisposed to allergies. Combination of allergen with the skin-sensitizing antibody results in sneezing, rashes, itching, etc.

Skin active component. A skin active component is defined as the substance isolated from the above sources, which is capable of eliciting skin reaction in allergic patients or in a passively sensitized site of

a normal recipient. In fact, a skin active component could be a part of the allergen molecule or the allergen itself. Since the mechanism of the in vivo action of an allergen is unknown, the biological activity of the isolated fraction will be referred to hereafter as the skin activity rather than allergenic activity.

CHAPTER II

STUDIES ON THE ALLERGENS IN GRASS AND RAGWEED POLLEN

Introduction

Grass pollens represent a group of about 4,500 species of anemophilous pollens; about 4% of these grasses grow in North America. The most common inducers of hayfever are timothy, June, orchard, redtop, sweet vernal and Bermuda grass.

Pollen to be considered as an important cause of hayfever must fulfil the following requirements (88): (i) the pollen must contain an allergen capable of inducing hayfever; (ii) the pollen must be anemophilous or wind-borne; (iii) the pollen must be sufficiently buoyant to be transferred over a considerable distance; (iv) the plant producing pollen must be widely and abundantly distributed.

Allergenic and antigenic relationships among grass pollens

Freeman (89) first proposed that all grass pollens contained one and the same allergen. Cooke and Van der Veer (61), on performing direct skin tests on twenty patients of mixed grass allergies with six different grass pollen extracts, noticed that an individual reacting to one pollen extract reacted to all. Thommen (88) and Scheppegrell (90) tested patients not only with pollens to which they were clinically sensitive, but also to other varieties of grasses, and observed that the grasses contained common allergens. Coca and Groove (91) concluded that timothy pollen contained all the allergens of orchard grass as well as some additional allergens.

In contrast, Rackemann and Wagner (92) observed that each grass pollen

had certain atopens (allergens) peculiar to itself and certain other atopens common to other grasses. Watson et al (93) obtained no skin reactions on Bermuda grass sensitive patients when tested with timothy pollen extract. Lamson and Miller (94) and Lamson and Alles (95) also observed the specificity of allergens of various grass pollens. Nevertheless, Henderson (96) found that patients who were refractory to treatment with timothy alone got relief with grass mixture. Similarly, Anderson (97) suggested that the use of mixed extracts could alleviate the symptoms of patients allergic to less common pollens.

Using the in vivo cross-neutralization technique, Chobot (98) showed identical allergens in timothy and Bermuda grass pollens. Stull et al (99) also found allergenic identity among grass pollens using cross-neutralization with seven different grasses. On the other hand, using the same technique, Piness and Miller (100) found that 109 patients out of 599 reacted strongly to only one grass pollen and were regarded as negative to 9 others. They arrived at the conclusion that the pollen of each member of the Gramineae had some specific allergen(s) and some which were common to a greater or lesser degree to all members of the botanical family. Similarly, Rakeman and Wagner (92), who tested the skin activity of the extracts of timothy, orchard and redtop, observed that none of the grass extracts ever produced desensitization to the two others. All these investigators, however, performed cross-neutralization with pollen extracts of equal concentration with respect to nitrogen content. Therefore, the results of these studies can lead to erroneous conclusions (101). To establish the allergenic relationships among different grass pollen extracts, it would seem more suitable to use cross-neutralization tests based on a comparison of

of desensitizing doses (102). It was found that a sensitized site could be neutralized with solutions of the allergen(s) of varying concentration by injecting the site at daily intervals with the allergen. However, such a site is still capable of giving a reaction when subsequently challenged with the allergen solution in a much higher concentration. When the allergen solution was used above a certain threshold concentration, all the reagin in the sensitized site could be neutralized by a single injection with the allergen(s) and the site would no longer respond when challenged with the allergen, regardless of the concentration used. Thus, in the latter case, the site had been desensitized and not just neutralized by the initial injection of the allergen. The term 'desensitizing dose' was defined as the concentration of an allergen or allergenic fraction (used in a P-K test) which will neutralize in one single injection a site of normal skin sensitized with appropriate allergic serum.

The antigenic relationships among grass pollen antigens have been mostly established by gel-diffusion (103,104) and hemagglutination (105) using rabbit anti-grass sera. Thus, Augustin (106,107) found that all grass pollens contained one main common protein component. Examining the Ouchterlony patterns of fifteen grass pollen extracts, Augustin (108) noted that timothy and orchard grass shared most of their common antigenic components to a variable extent with pollens of different tribes, and that their antigenic make-up cuts across botanical classifications. She also noted (109) that grass pollens showed two main lines of precipitation, the outer 'A antigens' and the inner 'I antigens', and concluded that the relatively heat stable I-antigens were responsible for the skin activity of grass pollens. Using chromatography on DEAE-cellulose for the isolation

of allergens from timothy and cocksfoot pollens, she observed that the chromatographic patterns of these two pollens were superimposable (110).

Woodehouse (111) analyzed common grass pollens, such as timothy, redtop, orchard grass, June grass, sweet vernal grass, Bermuda grass, by gel diffusion and found that Bermuda grass was antigenically distinct from the rest of the grasses. Furthermore, he concluded that since all the common hayfever grasses, with the exception of Bermuda grass, contained a common major antigen, any one of the grasses could adequately represent the group in the diagnosis and treatment of grass hayfever. However, the use of orchard grass, June grass or timothy was recommended, since these grasses seemed to have the largest complement of minor antigens and yielded the most potent antisera. Gosselin et al (112) analyzed six British grass pollen extracts (including timothy and orchard) by Boyden's tanned cell hemagglutination technique and demonstrated the presence of common antigens in all six extracts. Hemagglutination inhibition method (113) showed an 'antigenic mosaic' for each of the six pollens, suggesting distinct antigens in addition to common ones.

Frick et al (114) carried out extensive studies on the antigenic relationships and skin activities of different grass pollens. They observed that the pollens of timothy, June grass, orchard grass, redtop and sweet vernal contained common antigens which were different from those of Bermuda grass pollen. They also found by the cross-neutralization technique, using desensitizing doses for each pollen, that redtop grass pollen contained all the skin active components common to timothy, June grass, orchard grass and Bermuda grass, and that timothy, June grass, and orchard grass pollens had a complex and, in many respects, similar allergenic composition and

contained additional allergens to those found in Bermuda grass pollen.

Isolation of skin active components from timothy grass pollen

The first chemical studies on a grass pollen were reported by Kamman (115) in 1904. Studying the nature of the constituents of rye grass pollen, a common hayfever plant in Germany, he found that the causative agent was a 'toxalbumin', which was not completely inactivated by heat and resisted proteolytic enzymes, although long contact with trypsin lowered its activity; on the other hand, the globulin fraction was inactive. In 1926, Caulfield et al (116) isolated four fractions from timothy grass pollen by treating the pollen with ether, 95% alcohol, distilled water and 0.144% sodium hydroxide in successive steps, i.e. (i) albumin-proteose was obtained from the aqueous extract of the pollen by precipitation with alcohol, (ii) proteose was the precipitate obtained by heating and acidification of the extract, (iii) glutenin was the alkaline extract of the pollen, and (iv) the alcohol-soluble fraction was isolated from the pollen by extraction with 95% alcohol. On skin-testing it was observed that each patient exhibited a certain degree of sensitivity to these different fractions, which was individually selective and apparently specific. Attention could be drawn to the fact that albumin was not separated from the albumin-proteose fraction, and that some of the fractions were obtained by heating and at extreme pH conditions, which could be the reasons for having observed allergenic specificity of these different fractions.

In the following year, Brenton et al (117) also obtained four fractions, designated as proteose A, proteose B, albumin and glutenin, from a salt (10% sodium chloride) extract of defatted pollen. The albumin fraction

was coagulated from the dialyzed residue by heating. Proteose A and B were prepared from the 10% sodium chloride extract by saturation with ammonium sulfate. The resulting precipitate was dissolved in water, and the solution was freed from salts and globulins by dialysis. The dialyzed extract was then heated, the coagulated albumin was filtered off and the fraction, designated as proteose A, was precipitated from the filtrate with alcohol. The supernatant, remaining after ammonium sulfate precipitation, was dialyzed to eliminate salts, concentrated to a small volume and boiled to remove any coagulable protein, which was removed by filtration. Upon addition of alcohol to the clear filtrate, a precipitate was obtained, which was designated as proteose B. The preparation of glutenin was not described in this study, but probably this was the alkaline extract of pollen as prepared by Caulfield et al (116). By cutaneous tests on grass sensitive patients it was found that 63% of the patients were positive to two or more fractions, 21% only to proteose A and 15% only to the albumin fraction; glutenin was shown to be of negligible importance.

In 1930, Moore et al (118) reported the isolation of an albumin-proteose fraction by electrodialysis of the aqueous extract of defatted pollen, and a globulin fraction by precipitating the dialysate of the aqueous extract after standing for a long period at $4^{\circ}C$. The proteose fraction was prepared by extracting the pollen residue with 0.2% sodium hydroxide after successive extraction with ether, water and 75% alcohol. Using passive transfer test, these investigators found that all fractions were skin active. They also observed that sites neutralized to the proteose, albumin-proteose and globulin fractions gave no further reaction to the whole pollen extract. From these results it was concluded that there were at least three different substances

present in timothy pollen, which were capable of causing specific reactions in allergic individuals or in artificially sensitized rabbits. Since the chemical composition of none of these fractions was reported, although most of the investigators tacitly assumed that these fractions represented different proteins, the results obtained with such fractions would lead to erroneous conclusions regarding the presence of different skin active components in timothy grass pollen. In the same year, Moore and Moore (119) reported that the active substance could be adsorbed onto activated charcoal from an aqueous extract of pollen, and that the activity was related to the amount of nitrogenous material removed from the aqueous extract due to adsorption*.

Moore and Moore (120) isolated from timothy pollen a glucoside in pure form, which was named dactylin, since it was first prepared from orchard grass (Dactylis glomerata L.). Dactylin has an empirical formula of $C_{23}H_{28}O_{15}$. On a weight basis it represents less than 0.5% of the pollen. The fact that dactylin crystals spontaneously precipitate from an aqueous extract of pollen at 2-5°C led these investigators to believe that dactylin was an enzymatic degradation product of some parent substance present in the pollen. Johnson et al (121) found, by paper chromatography, that it was flavonoid and differed from isoquercitrin, quercitrin and quercitin; the carbohydrate moiety of the glucoside was identified as glucose. It was also observed that the glucoside was skin active in some of the timothy sensitive patients tested. Contrariwise, however, Augustin(122) found that

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*A similar selective adsorption of active material from ragweed pollen was also observed in this study, which led the authors to conclude that the active material of each of these two pollens was similar in nature.

the glucoside was devoid of skin activity.

Stull et al (123) first demonstrated that extraction of defatted pollen with distilled water removed practically all skin active substances from the pollen grains. The active substance could then be precipitated from the aqueous extract by saturation with ammonium sulfate. The active fraction, thus obtained, contained 11.3% nitrogen. They also reported that the active substance was nondialysable through a hardened collodion membrane, but dialyzed through ordinary collodion membrane to some extent; however, the pore size of this membrane was not determined. The globulin fraction was shown to be devoid of biological activity. From these results they concluded that the allergenically active substance was an 'albuminous' protein similar to that found in ragweed pollen.

In contrast, Unger et al (124) observed that both the dialysable and nondialysable fractions of timothy pollen extract were equally skin active in timothy sensitive patients. They also found that the active substance dialyzed more freely in a slightly acidic solution. Since these crude pollen extracts may have contained various enzymes which were possibly activated by lowering the pH, it might be visualized that skin active components may have been split up into fragments which diffused freely across the dialysis tubing. Alternatively, it may be suggested that some ester linkages, such as glycosidic bonds, were hydrolyzed, resulting in the cleavage of protein-protein or protein-carbohydrate molecules into smaller units.

In 1932, Gough (125) isolated a polysaccharide fraction from timothy pollen by extraction with 4% sodium hydroxide. To isolate the carbohydrate, the alkaline extract was precipitated with lead acetate. The precipitate

was dissolved in water and the carbohydrate was then precipitated with methyl alcohol. The polysaccharide was shown to be inactive by skin testing; on hydrolysis with acids it yielded l-arabinose, galactose and an unidentified non-reducing acid (126). In 1937, Harley (127) isolated three fractions from timothy grass pollen extract, designated as A, G and C. Fractions A and G were obtained by alcohol fractionation and acid-alkali precipitation of the alkali-saline extract of acetone-defatted pollen. The carbohydrate fraction C was obtained from the supernatant after the removal of all proteins. Fraction A, containing 12.5% nitrogen, was the most skin active, whereas fraction C was only slightly active. The activity of fraction C was attributed to contamination with proteins.

In 1942, Abramson et al (128) fractionated a phosphate buffer extract (pH 7.6) of defatted pollen by the electrophoretic technique of Tiselius. They obtained a slow moving colourless component, a fast migrating pigment and the intermediate pigments. By passive transfer tests, all these fractions were shown to be skin active. From diffusion and ultracentrifugation studies of the colourless component, they concluded that timothy allergens were of small molecular size, with a molecular weight of the order of 5,000. This component was claimed to be the main active component and was named as pratensin (129).

Augustin (130), in 1953, reported the fractionation of timothy pollen extract into 'globulin' and 'albumin' fractions by precipitation with ammonium sulfate. Both fractions were shown to be skin active in allergic patients. She also isolated a strongly pigmented carbohydrate-rich fraction and an almost colourless carbohydrate-poor fraction by isoelectric precipitation, followed by sodium chloride fractionation. These two fractions

had similar skin activities on an equal weight basis. On the basis of the results of ultrafiltration studies, she suggested a molecular weight greater than 10,000 for timothy grass allergens.

Johnson and Thorne (131), in 1958, carried out extensive studies on timothy and rye grass pollens. A single extraction of defatted pollen for 20 hours at $4^{o}C$ with water or aqueous buffers removed the bulk of the soluble pollen constituents, 90-95% of which was lost on dialysis through Visking cellophane tubing. When examined by paper chromatography using n-butanol, acetic acid and water as solvents, the dialysate was found to contain amino acids, small peptides and carbohydrates; the extracts, prior to dialysis, contained a stationary protein band, in addition to these dialysable components. By the moving boundary electrophoresis technique at pH 6.8-7.8, ionic strength 0.1, they detected four components designated as A, B, C and D, all migrating towards the positive pole. The electrophoretic pattern of the whole extract obtained by these authors was similar to that reported by Abramson et al (128). Nucleic acids or their degradation products were not detected in the dialyzed extract, which had a sedimentation coefficient of 2S. In the following year, Augustin (122) obtained a carbohydrate fraction by extracting defatted pollen with organic solvents such as ethylene glycol, diethyl glycol and formamide. The fraction was an arabinose-galactose-pigment complex and was found to contain only 0.25% of the activity usually extracted by aqueous solutions. On the basis of this finding, she concluded that the allergen was not a carbohydrate. By paper chromatography of whole pollen extracts*, she also demonstrated the

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*Pollens were extracted with distilled water, and 'Evans' (0.363 g KH_2PO_4, 1.431 g Na_2HPO_4, 5 g NaCl and 4 g phenol in 1,000 ml distilled water) or 'Coca's' (5 g NaCl, 2.7 g $NaHCO_3$ and 4 g phenol in 100 ml distilled water) fluid for 48 hours.

existence of free amino acids, peptides, carbohydrates, proteins and a number of pigments in timothy pollen extracts. The biologically active material remained at the line of application. This material, on hydrolysis, released the following amino acids (as examined by paper chromatography in butanol acetic acid and pyridine-benzene mixture): cystine, lysine, histidine, aspartic acid, glycine, serine, threonine, glutamic acid, alanine, proline, tyrosine, valine, methionine, tryptophan, phenyl alanine and leucine. Using Visking tubing with an average pore-size of 4 mμ, she showed that the soluble constituents of timothy grass pollen were non-dialysable and suggested that the molecular weight of timothy allergens was over 10,000. However, on prolonged dialysis (over 90 hours), some activity could be found in the dialysate, but this activity accounted for less than 1% of the whole activity. This finding was attributed to the leakage of a small amount of allergenic material through some larger pores which might have been present. The activity of the dialysate could be equally due to the enzymatic degradation by enzymes present in the extract, or due to a change in pH during such long dialysis, possibilities which were not considered by her.

In 1960, Palmstierna (132) reported the use of gel-filtration and ion-exchange chromatography for the isolation of timothy pollen allergens. However, his results ought to be considered with some reservation since the activity of his fractions was tested in sensitized guinea pig and not in man. In this study, pollen grains were disintegrated in a modified Hughes press and then extracted with phosphate buffer. The extract was desalted on Sephadex G-25 and chromatographed on DEAE-cellulose column equilibrated with 0.01M phosphate, pH 7.0. Most of the material passed through the

column and was inactive in sensitized guinea pigs. Skin active material was eluted with 0.5M sodium chloride in 0.01M phosphate buffer, pH 7.0. The active material was then chromatographed on Sephadex G-25 with 0.01M citrate, pH 7.0 as eluant: the active material was not retained on the column and emerged right after the void volume. This fraction was then passed through a column of Sephadex G-75 in equilibrium with 0.01M citrate buffer, pH 7.0, and eluted with the same buffer. The chromatographic pattern showed two major and six minor components. The first component which emerged from the column unhindered was found to be the most potent sensitizer in guinea pigs. From these studies it was concluded that the skin active fraction of timothy pollen resided in a fraction of a rather high molecular weight, probably above 40,000.

In 1962, Augustin and Hayward (110) also reported the isolation of skin active components of timothy and cocksfoot pollens by ion-exchange chromatography on DEAE-cellulose. Pollen, defatted with ether, acetone and petroleum ether, was extracted with Coca's solution. The extract was ultrafiltered and dialyzed through Visking membrane. The dialyzed residue was fractionated by precipitation with sodium chloride in the concentration range of 4-24% NaCl (0.6M-3.8M). All fractions obtained by sodium chloride fractionation were found to be skin active in grass sensitive patients. However, the fraction precipitated at 16% sodium chloride concentration (2.7M) was the most active. This fraction was then chromatographed on DEAE-cellulose with step-wise elution with buffers of increasing ionic strength and decreasing pH. With both timothy and cocksfoot grasses, nine fractions with uneven distribution of skin activity were obtained. Fractions I and VI contained the most skin active components for each of

the grasses. Fraction I of each pollen extract contained two antigens, one of which was identical for both pollens. Fraction VI of timothy and cocksfoot pollens were associated with a very small protein peak, but had the highest skin reactivity in patients. These active fractions were found, on gel-diffusion, to belong to the so-called 'innermost' group of antigens (108,109). On Ouchterlony plates, both fractions were found to contain two antigens.

In 1962, Malley et al (133) also reported the isolation of skin active components from timothy pollen. In this study defatted pollen was extracted with 0.127M phosphate buffer, pH 10.4, and the extract was fractionated by precipitation with ammonium sulfate. The fraction precipitated at 49-55% saturation with ammonium sulphate contained most of the biologically active material. The active fraction was dissolved in water and fractionated by ethanol precipitation; the fraction soluble in 40% ethanol contained the majority of the biological activity. This material was then resolved by chromatography on Sephadex G-75, with saline as eluant, into one minor and one major fraction. On skin testing the subfractions of the latter in grass sensitive patients, these workers observed that there were no patients who reacted to only one of the fractions. Furthermore, on Tiselius electrophoresis in 0.05M citrate buffer at pH 8.0, the major fraction was partly resolved into two overlapping subfractions. These results were interpreted as indicating that the two proteins had slightly different specificities but cross-reacted. However, the behaviour of proteins under electrophoresis is not necessarily the same as under chromatography on Sephadex and, therefore, this type of interpretation can only be speculative. Leitz (134), in 1964, demonstrated that the ethanol soluble fraction of

timothy pollen obtained by Malley et al resolved into six yellow bands on discontinuous gel electrophoresis. However, the allergenic activity was associated with all components and there was no consistency in the activity pattern of the different fractions in different proteins.

Very recently, Malley and Dobson (135) subfractionated further the ethanol soluble fraction by chromatography on DEAE-cellulose into two fractions. The biological activity was associated with both fractions containing common antigenic determinants. On chemical analysis, the fractions were shown to be composed of polypeptides, carbohydrates and pigments.

The most significant study in this field appears to be that of Malley et al (136,137), who reported the isolation of a hapten from the dialysate of timothy pollen extract. Defatted pollen was extracted with 0.15M phosphate buffer, pH 10.4, for 24 hours at $4^{\circ}C$ and the extract was dialyzed through a Visking tube. The dialysate was then chromatographed on Sephadex G-25 with water as eluant. Five distinct components were obtained. The 'haptenic' material, associated with the second fraction,was rechromatographed on Sephadex G-25, G-50 and Amberlite IRC 50. In all these chromatographic procedures the elution patterns showed a single sharp symmetrical peak. The haptenic material was also shown to move as a single band on cellulose-acetate membrane electrophoresis in the pH range of 3-11.25. Chemical analysis showed that the hapten was a polypeptide-carbohydrate-pigment complex. On hydrolysis the peptide moiety yielded all the naturally occurring amino acids with the exception of arginine, hydroxyproline and cystein. The carbohydrate unit was identified as cellobiose and the pigment was shown to have the same spectral characteristics as

'dactylin'. The hapten, on gel-diffusion analysis, was found to inhibit several precipitin lines between whole pollen extract and rabbit anti-timothy sera. On skin testing, however, it had no activity in some patients, whereas in other individuals it was as active as the purified allergen preparation isolated from whole timothy pollen extract. Moreover, more recently, the hapten was shown to be capable of inducing the formation of new reagins in timothy sensitive patients (138). Although the haptenic fraction was able to inhibit the precipitation lines in Ouchterlony plate, the authors did not show whether inhibition of allergic reactions by such fraction could be achieved, which would have established the true haptenic nature of the fraction.

Isolation of skin active components from rye grass pollen

As already mentioned, the first investigation on rye grass pollen (115) was reported in 1904. Extensive and more critical studies on the nature of the allergens of this pollen, however, were started only fifty years later by Johnson and Thorne (131). In these studies it was found that rye grass allergen could be extracted from pollen, either with water or with aqueous buffers. The skin active components, as in the case of timothy, were nondialysable and could be resolved into four components by moving boundary electrophoresis. The electrophoretic pattern of the rye grass pollen extract was very similar to that of timothy pollen. Preliminary ultracentrifugal examination indicated that the sedimentation coefficient of the nondialysable fraction of the extract of rye grass pollen was 2S. In another study (139), the dialyzed extract of rye grass pollen was fractionated with ammonium sulfate. Moving boundary electrophoresis

revealed, however, that none of the ammonium sulfate fractions was homogeneous. Pigments were primarily associated with the fraction precipitated at ammonium sulfate concentrations below 75% saturation, which contained mainly the electrophoretically faster moving components. The fractions precipitated between 75 and 100% saturation with ammonium sulfate gave two slower migrating electrophoretic components in unequal amounts. The slowest component could be isolated from the fraction soluble in saturated ammonium sulfate by adsorption on calcium phosphate gel. This component, and the other constituents of the dialyzed extract, were also isolated readily by electrophoresis-convection. No qualitative difference in the amino acid composition of these electrophoretically separated fractions was observed using two-dimensional paper chromatography. The electrophoretically fastest component was rich in protein, having a 12% nitrogen content. The slowest component, on the other hand, was rich in carbohydrate (21%) with a relatively low nitrogen content and the sugar unit was identified as mannose. All the fractions exhibited a characteristic u.v. absorption spectrum for proteins. In agreement with earlier results, both fractions had a sedimentation coefficient of 2S.

Britton et al (140) found that the dialysate of the rye pollen extract had no or only negligible activity when tested by skin tests on grass sensitive human individuals and that all the activity was associated with the nondialysable components. Ether extraction of the pollen, prior to extraction with aqueous solvents, did not lead to any loss of activity. Skin activity was distributed throughout all the fractions obtained by precipitation with ammonium sulfate, but the fractions precipitated between 50-100% saturation had the highest specific activity. The distribution

of activity among the fractions obtained by electrophoresis-convection was more uniform and there was no enrichment of skin activity of the fractions. From these results it was concluded that there may be more than one allergen in rye grass pollen extract.

Johnson and Marsh (141,142,143,144,145) have recently reported the isolation of rye allergens in 'pure' form. Pollen, defatted with ether, was extracted with 0.001M ammonium bicarbonate and the extract was dialyzed against distilled water. The dialyzed extract was then chromatographed on DEAE-cellulose using distilled water as the initial eluant, followed by Na_2HPO_4 solutions of increasing molarities (0.001, 0.0045 and 0.0075M) within the pH range of 7.7-8.0. Three peaks were obtained under these conditions. The fractions under the second and third peaks, which contained the skin activity, were pooled, concentrated and rechromatographed on Sephadex G-75 using 0.05M ammonium bicarbonate, at pH 8.2, as eluant. Three well resolved components were obtained and the major allergens (alpha and beta) were confined to the second peak, whereas the minor allergen (gamma) was eluted in the third peak. On successive rechromatography of these fractions on Sephadex G-75, the three allergens, alpha, beta and gamma, were obtained in 'pure' form. The allergens were shown to move as single bands on starch-gel electrophoresis in the pH range of 3.1-8.5. However, allergen gamma, in the presence of urea at pH 8.5, was resolved into six bands. Allergens alpha and beta possessed the same electrophoretic mobility at pH 3.1-3.5, but exhibited different mobilities at higher pH. Components alpha and beta gave single precipitin lines with rabbit anti-rye sera, and these lines showed a reaction of identity. On the other hand, component gamma failed to give any precipitin line with these rabbit antisera.

Furthermore, the precipitin reaction between gamma component and the specific rabbit antiserum could not be inhibited by the gamma component, indicating that the alpha and beta allergens did not share common antigenic determinants with the gamma allergen. The ultra-violet absorption spectra of the alpha and beta allergens were identical, but showed marked dissimilarities from that of the gamma component. Molecular weight determination by the sedimentation velocity-diffusion method and by the Archibald technique gave values of 32,000-34,000 for both alpha and beta, but only 9,000-11,000 for the gamma component. These allergens have been shown to be stable towards heating at 100°C at pH 7.3 and also within the pH range of 3-10. The dialysable components of rye pollen were not skin active and did not inhibit the skin reaction elicited by the purified allergens. Components alpha and beta were shown to be glyco-peptides and to contain 4% carbohydrate and to be composed of galactose, mannose, xylose and sedoheptulose.

A glyco-peptide fragment of the component alpha was isolated by successive digestion with trypsin, chymotrypsin and pronase. On gel-diffusion analysis it was found that the glyco-peptide fragment did not give any precipitin band with the specific antiserum and it did not inhibit the precipitin band between the intact alpha component and rabbit anti-rye sera. Moreover, the skin activity remained unaltered when this preparation was treated with enzymes capable of degrading carbohydrates, such as cellulose, β-glucoside, β-galactoside or β-amylose. It was, therefore, concluded that the carbohydrate moiety did not contribute any skin activity to the constituents of rye grass pollen. However, the fact that activity was not lost could not by itself indicate that the carbohydrate was not

important, since it was not shown whether the carbohydrate was completely removed from the allergenic fraction after such enzymatic treatment because a small amount of carbohydrate present might be sufficient to give skin activity. Moreover, the inhibition of precipitin band with respect to rabbit antiserum did not provide any evidence simply because immunological responses in rabbits and man are not identical.

Isolation of skin active components from ragweed pollen

The earlier studies on ragweed pollen, reviewed by Richter and Sehon (146), indicated that, despite painstaking work, the true chemical nature of the allergen(s) in this pollen had not been determined. Nevertheless, the results from most laboratories indicate that the activity is associated with protein or polypeptide components of ragweed pollen.

With the recent development of more refined techniques, such as gel-filtration and ion-exchange chromatography, there has been a renewed attack on the isolation and characterization of allergens from ragweed pollen. Thus, in 1961, Goldfarb et al (147) fractionated an aqueous extract of ragweed pollen by ion-exchange chromatography on DEAE-cellulose. This latter procedure led to the isolation of four active fractions. The most active fraction was further subfractionated by precipitation with ammonium sulfate. The two subfractions, isolated between 0-60% and 61-100% saturation with ammonium sulfate, were analyzed by the Ouchterlony agar gel-diffusion test. One subfraction gave a single precipitin band with a specific rabbit anti-ragweed serum, whilst the other subfraction developed three precipitin bands. The subfractions were antigenically non-identical and both were skin active. The following year these workers (148)

fractionated the subfraction, which precipitated at 0-60% saturation with ammonium sulfate, by chromatography on DEAE-cellulose. During successive rechromatography a group of poorly resolved peaks were obtained; biological activity was found to be associated with all the fractions. The major fraction, designated as C_c, accounted for about 40% of the material and contained 11-12.8% nitrogen; under various conditions of oxidation and reduction it could be cleaved into electrophoretically separable components.

In 1962, Lea and Sehon (149) reported the isolation of a purified active fraction from a dialyzed water soluble ragweed pollen extract (DWSR) by paper block electrophoresis. This fraction, referred to as delta fraction, had a sedimentation coefficient of 3.2S and contained 80% protein and small amounts of carbohydrate, consisting of arabinose and galactose. When examined by passive transfer cross-neutralization tests with sera of untreated ragweed allergic patients, the delta fraction appeared to be the most active fraction containing all the major skin active components of ragweed pollen (150).

King and Norman (151,152), also at the same time, reported the isolation of a highly purified ragweed component, named as antigen E, by the combined methods of salt precipitation, ion-exchange chromatography and gel-filtration. The active fraction contained 99.9% protein and only traces of carbohydrate (arabinose). It gave a single precipitin line with rabbit anti-ragweed sera and 90% of the skin active materials of whole ragweed pollen extract could be precipitated with a rabbit antiserum prepared against antigen E. From these results it was concluded that antigen E contained 90% of the total skin activity of ragweed pollen. In 1967, King et al (153) isolated the second skin active component, designated as antigen K, from ragweed

pollen extract. Antigen K was slightly less skin active than antigen E and shared partial immunological identity with antigen E. Antigen K has a molecular weight of 38,200 and its amino acid composition differs from that of antigen E, which has a molecular weight of 37,800.

Antigen E was stable in the pH range of 6-8.5, but lost its skin activity and antigenic activity outside this range. Lyophilization of antigen E from an aqueous solution containing ammonium bicarbonate led to the formation of higher molecular weight aggregates. Polymerization of antigen E could also be achieved by treatment with 8M urea at pH 7.9. Antigen E was found to be resistant to digestion with proteolytic enzymes, such as trypsin, chymotrypsin and papain, but most of the skin activity was lost on reduction. Antigen E exists in four chemical forms, designated as fractions IV-A, IV-B, IV-C and IV-D, which could be isolated by chromatography on DEAE-cellulose by elution with sodium chloride (linear gradient from 0 to 0.2M). Two of these fractions, i.e. IV-B and IV-C, were isolated in pure form and were shown to have the same amino acid composition. Both fractions have also identical sedimentation coefficients of 3.05S and the same molecular weight of 37,000. However, they differ in charges.

Robbins et al (154) isolated a highly skin active component from ragweed pollen extract by precipitation with ammonium sulfate, followed by ion-exchange chromatography on DEAE-cellulose, gel-filtration on Sephadex G-75, chromatography on DEAE-Sephadex A-50 and gel-filtration on Sephadex G-200. This fraction, designated as A.la$_2$, contained 20% arabinose and possessed multiple antigens as revealed by immunoelectrophoresis.

Cece et al (155) compared the skin activity of several purified ragweed pollen preparations obtained by various investigators. In this study the

passive transfer exhaustion technique was employed using two sera from untreated ragweed sensitive patients and the following purified preparations: A.1 (154), E (152) and C_c (148), were studied. The results of this investigation indicated that none of the purified fractions contained all the skin active components of the whole pollen extract and that all the fractions were antigenically heterogeneous.

Reisman et al (156), in 1964, carried out more detailed studies on the immunochemical properties of various purified ragweed preparations by comparing pool C (148), antigen E (152), delta fraction (149), whole pollen extract and dialyzed extract using the following techniques: (i) immunoelectrophoresis, (ii) gel-diffusion test, (iii) hemagglutination, and (iv) in vitro and in vivo cross-neutralization tests. The results of immunoelectrophoresis analysis indicated that whole ragweed pollen extract had 11-13 components, whereas pool C had 3-4 constituents, the dialyzed residue and antigen E 3 constituents each, and delta fraction 2 components. Gel-diffusion analysis also showed that all purified fractions contained fewer antigens than the whole extract. In the hemagglutination procedure, using Boyden's tanned red cell technique, pool C seemed to be the most potent antigen, in as much as it gave the highest titers with both sera of treated and untreated allergic individuals. On the other hand, fraction delta was the most potent inhibitor of the hemagglutination reaction given by the sera of untreated allergic individuals or of immunized rabbits. However, the results of in vivo cross-neutralization tests indicated that only delta fraction contained all the skin active components present in the whole pollen extract.

Richter et al (157) isolated an allergen (PTP) from the dialysate of

ragweed pollen extract by precipitation with phosphotungstic acid. It was reported earlier (158) that dialysate was skin active in all treated patients, but induced no reaction in over 80% of untreated individuals. PTP, likewise, was found to be skin active mainly in treated patients. The skin active component(s) was found to be resistant to a variety of enzymes, heating and treatment with acid. However, Meacock et al (159) found that dialysable components of WSR were skin active as determined by P-K passive transfer tests with sera of both treated and nontreated individuals. Very recently, Attallah et al (160) have been able to isolate a fraction, devoid of skin activity, from the dialysable components of ragweed, which had the ability of neutralizing the skin activity of WSR in the P-K reaction.

Conclusion

In conclusion, it can be stated that the weight of evidence acquired from studies on the isolation of allergens from various pollens indicated that the active constituents were proteins with varying amounts of carbohydrates. In some cases, however, the amount of carbohydrate present in the purified skin active preparation was insignificant to account for the activity of the preparation. The available finding would, therefore, suggest that the carbohydrate may not be directly involved in the allergenicity of various pollens. The activity of the dialysate reported in some cases may perhaps indicate the presence of a class of allergens of relatively lower molecular weight which might be considered polypeptides rather than proteins, or, as suggested by Lea et al (150), that all proteins may be derived from one or more large, labile molecular species present in the intact

pollen grains, and that the skin activity of dialysable components could be attributed to the relatively stable group(s) present in these proteins. In general, concerning biological activity, ragweed allergens are more labile than grass pollen allergens. In contrast, however, ragweed allergens appear to be more resistant to enzymic hydrolysis than grass allergens. The molecular weight of the allergen(s) isolated in pure form seems to be in the range of 30,000 to 40,000.

Our knowledge on the allergens of grass pollen is still very scanty. None of the investigators has shown by cross-neutralization passive transfer test, based on desensitizing dose, the allergenic relationship of various isolated grass pollen fractions, although most of them, observing the antigenic mosaic of the fractions, tacitly assumed a similar relationship for man.

PURPOSE AND SCOPE OF THE PRESENT INVESTIGATION

The chemical characterization of the allergens in the different grass pollens, which are responsible for the induction of respiratory allergies in man, is of fundamental importance for (i) the understanding of the mechanism of this altered immune response, and (ii) a possible, rational approach to the management of this disease. However, so far, inspite of painstaking efforts in many laboratories, this problem has escaped a satisfactory solution. The purpose of the work reported in this thesis was to isolate the allergen(s) from timothy grass pollen in the hope that some advance could be made towards the elucidation of the nature of allergens in this pollen with the aid of modern techniques available for the isolation and characterization of biopolymers.

The treatment of hayfever, introduced by Noon in 1911 (161), usually involves a series of injections of an aqueous extract of the pollen. Noon thought that hayfever was a type of anaphylaxis and that the allergic individual could be desensitized by multiple injections of small quantities of the allergen preparation at various time intervals, in the same way as one could prevent anaphylactic shock in a sensitized animal by repeated injections of small quantities of antigen prior to administration of the shocking dose. This procedure of "desensitization" or "hyposensitization", introduced by Noon, still constitutes the main treatment offered to clinically allergic individuals. However, some workers (162,163) have noted that a large number of allergic persons do not respond well to this treatment and, in fact, their sensitivity appears to increase. Since the conventional treatment of allergic individuals involves the injection of whole aqueous

extract of the pollen, it may be visualized that they might become sensitized to additional constituent(s) of the pollen to which they were not originally allergic. Such an effect would obviously defeat the purpose of the treatment. To eliminate this possibility, it would be advisable to employ in the desensitization treatment only some of the allergens present in the whole pollen extract, which are actually responsible for the allergic condition.

In Chapter III are described and evaluated different procedures for the extraction of skin active components from timothy grass pollen.

The actual fractionation of the pollen extracts into fractions containing the different skin active components is described in Chapter IV. As will be shown, after many trials and tribulations, a method consisting of a combination of salt precipitation, ion-exchange chromatography and gel-filtration, was found suitable for the isolation of a fairly homogeneous fraction, which was shown to contain all the skin active components and to be one hundred thousand times more active than whole pollen extract on an equal weight basis. Also in Chapter IV are given the biological, immunological and physico-chemical properties of the various fractions of the whole pollen extract. All these results point out the complexity of the chemical nature of grass allergen(s).

Chapter V deals with the isolation and purification of a haptenic fraction, and with the biological properties, particularly in relation to its possible inhibitory effect on the Prausnitz-Küstner passive transfer test.

CHAPTER III

DIFFERENT PROCEDURES FOR THE EXTRACTION OF ALLERGENS FROM TIMOTHY GRASS POLLEN

INTRODUCTION

For the isolation of allergen(s) from various pollens, different extraction procedures involving different media and different methods for the disintegration of the pollen grains, e.g. mechanical disruption by ball-mill grinding (164) and ultrasonic disintegration (165), have been used. Each of these methods has yielded extract(s) of somewhat different composition. In the previous studies (123,131), it was shown that extraction in aqueous media resulted in solubilization of practically all of the skin active materials. However, this conclusion was not arrived at on the basis of cross-neutralization experiments, using the Prausnitz-Küstner passive transfer test. It was, therefore, considered worth evaluating this aspect in the present study by cross-neutralization technique(s) and establishing the physico-chemical and immunochemical properties of these extracts.

METHODS AND MATERIALS

Process of defatting

Timothy pollen* (purchased from either Hollister-Stier Laboratories

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* Pollen samples collected in different years, i.e. over the period of 1963-67, were used during this study. No obvious differences could be detected in the constituents isolated from the various batches.

or Greer Drug and Chemical Corp. U.S.A.) was defatted with diethyl ether, in a Soxhlet extraction apparatus at room temperature, for 24-32 hours, until all the ether soluble pigmented materials were removed. Traces of ether were subsequently evaporated off at reduced pressure in a dessicator or more conveniently by keeping the pollen residue overnight in a fume-hood. In one such extraction process 50 g of pollen could be defatted.

Extraction using the 'Virtis 45' homogenizer (A)

About 3-4 g of defatted pollen was suspended in 30 ml of distilled water and homogenized at 45,000 r.p.m. at 4°C for 15, 30 and 60 minutes; these extracts are designated by the numbers 1, 2 and 3 in Table I. The insoluble pollen residue was removed by filtration under suction and the filtrate was subjected to centrifugation at 10,000 r.p.m. for 15 minutes. The clear supernatant was then concentrated by pervaporation and stored at -20°C for subsequent characterization.

Heat treatment of pollen prior to disintegration with the 'Virtis 45' homogenizer (B)

About 3 g of pollen was suspended in 30 ml of distilled water and heated in a water bath at 60°C for 15, 30 and 60 minutes; these extracts were designated by the numbers 4, 5 and 6. This was followed by homogenization as in (A) for a period of 15 minutes.

Disintegration with Ultrasonic Disintegrator (C)*

Ten g of defatted pollens was suspended in 100 ml of distilled water

*The author is indebted to the Pulp and Paper Research Institute, McGill University, for providing MSE apparatus, operating at 20 kc/s with an output of 60 watts, for this phase of the work.

and divided in 3 equal portions. One portion was disintegrated for 5 minutes and designated as fraction 7; the second portion was disintegrated for 15 minutes and designated as fraction 8. From the third portion, 5 ml volumes were withdrawn and diluted six-fold with distilled water and then disintegrated for 5, 15 and 30 minutes, and were designated as fractions 9, 10 and 11, respectively. The remaining suspension was disintegrated for 5 minutes and was designated as fraction 12.

To avoid excessive temperature rise due to absorption of energy by the medium during disintegration, the suspensions were immersed in crushed ice. The extract was then processed for storage as in (A).

Extraction in aqueous media (D)

The defatted pollen was suspended in distilled water (500 ml water per 100 g of pollen) and extracted with constant stirring for 4 hours at room temperature. It was then filtered through a Buchner funnel using filter paper, Whatman No. 3, under suction. The pollen residue was washed with another small volume of water and filtered in the same way. The combined filtrates, a brown coloured aqueous extract, was either lyophilized or stored in the frozen state as in (A). The aqueous extract was designated as WSG (water soluble grass).

Determination of protein concentration

Different amounts of the lyophilized WSG were weighed in bottles and kept in a desiccator until constant weights were reached. Each of the dry materials was then dissolved in a known volume of distilled water and the optical densities of the solutions were measured at 280 mμ with a

Beckman D.U. spectrophotometer. The optical density at 280 mμ of the solution at different concentrations was then plotted against the concentration expressed in g per cent. The protein concentration in each extract was then deduced from the slope of this calibration line with formula: Concentration = $\frac{\text{O.D.}}{\text{slope}}$, on the implicit assumption that various extracts had identical chemical compositions.

Prausnitz-Küstner passive transfer tests of various extracts

Allergic serum*. The freshly taken blood from a grass sensitive patient (Na) was incubated for 4 hours at room temperature to enhance retraction of the clot. After centrifugation, the serum was separated and passed through a sterilized Seitz filter into sterile vials, and stored at -20°C.

Extracts. The extract was diluted with saline and sterilized by filtration through sterilized Millipore membrane, just prior to being used for injection into human skin.

Methods. The allergic serum was injected into a number of sites in the back of a normal human volunteer (0.05 ml of serum per injection). Control sites contained saline. The sensitized and the control sites were challenged with the extracts (0.025 ml of extract per injection) 24 hours later and the reactions were graded 20 minutes after as -, ±, 1+, 2+, 3+, etc., depending on the size of the wheal and the surrounding erythema.

Cross-neutralization experiments using desensitizing doses of the extracts in the P-K test

The experiments were carried out according to the procedure developed

* i.e. serum from grass-allergic individual.

in this laboratory (102). The "desensitizing dose" of each skin active preparation, i.e. the minimum dose needed to desensitize in one injection a sensitized site so that on rechallenge no further skin reaction can be elicited in the same site, was determined, as illustrated below with respect to WSG. Five sites (I to V) on the back of a normal individual were sensitized with the allergic serum (Na) diluted ten-fold with saline. On the following day, the sites were injected with solutions of WSG containing 12.5, 25, 50, 100 and 200 μg protein per ml, respectively. All the sites gave positive reactions varying from a ± reaction for site I, to 3+ for site V. Twenty-four hours later, all the sites were reinjected with a solution of WSG containing 500 μg protein per ml. Sites I, II, III and IV gave positive reactions. Site V did not react and, therefore, must have been desensitized by the first injection of the extract (200 μg protein/ml). Therefore, the desensitizing dose was considered to be between 100 and 200 μg protein/ml. Since the number of volunteer subjects for P-K tests is limited, and since the accuracy of the test is at best only within a factor of two, a more "exact" evaluation of the desensitizing dose - which would have necessitated more volunteers - was not deemed to be warranted; therefore, the solution of WSG of 200 μg protein/ml was considered to represent the desensitizing dose with respect to this serum diluted ten-fold. In a similar manner, the desensitizing doses of various extracts were determined.

Sixteen sites (I-XVI) on the back of the same individual were sensitized with the same allergic serum (Na) diluted ten-fold with saline (Table IV). On the following day, sites I-IV were challenged with a desensitizing dose of extract # 2, sites V-VIII with a desensitizing dose of extract # 6,

sites IX-XII with a desensitizing dose of extract # 9 and sites XIII-XVI with a desensitizing dose of WSG. On the third day, the sites were rechallenged with the following extracts: sites I, V, IX and XIII with extract # 2, sites II, VI, X and XIV with extract # 6, sites III, VII, XI and XV with extract # 9, and sites IV, VIII, XII and XVI with WSG.

Immunoelectrophoresis (166)

For immunoelectrophoretic analysis, the LKB apparatus, model 6800 A, was used. Six microscope slides, free from dust particles, were coated with 1% agar (Special Agar-Nobel, Difco Laboratories, Detroit, Michigan) solution, heated at 80°C, by spreading a few drops of the solution evenly across the slide, followed by drying in hot air. The agar-coated slides were placed in a frame, mounted on a horizontal levelling table. The contacts between the slides, and between the slides and the frame, were sealed with agar solution. A volume of 10 ml of 1% agar solution in veronal buffer*, pH 8.6 and $\Gamma/2 = 0.075$ containing traces of merthiolate, was poured into each section of the frame. The agar solution was allowed to gel for about 15 minutes. The frame was removed from the table and placed in a closed moistened chamber for an hour. Long narrow troughs and wells were cut in the gel layer with a punch. The gel in the wells was removed with a suction needle connected to an aspirator. The sample was then placed in the wells with a capillary tube.

After application of the sample, the frame was placed on the electrode vessels filled with veronal buffer. Electrical contact between the gel

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*3.684 g of diethyl barbituric acid, 20.6184 g of sodium barbiturate and 8.2038 g of sodium acetate were dissolved in 2 liters of distilled water.

layer and the buffer in the vessels was achieved with rayon wicks, wet with the buffer. For electrophoresis, a D.C. voltage of 250 was applied between the electrodes for 50 minutes. After the electrophoretic run, the gel was removed from each trough with a knife. The appropriate antisera (the production of antisera will be described in Chapter IV) were placed in the troughs and the precipitin arcs allowed to develop by incubating the slides overnight in the moistened chamber. The immunoelectrophoretic pattern was recorded by hand drawing and, in some cases, the slides were stained with bromophenol blue, azocarmine B or ponceau S.

For staining, the slides were first placed in a tank containing saline for about 6 hours to wash off the excess protein. The slides were then put in fresh saline bath for another 16 hours, followed by washing in distilled water for an hour. The slides were covered with filter paper strips and dried at room temperature. The strips were removed from the gel layer and the slides were immersed for 5 minutes in the staining tank. Excess stain was removed by washing the slides with 5% acetic acid several times. The slides were finally washed with distilled water, dried and stored for permanent record.

Immunodiffusion (104)

For immunodiffusion in agar gel, the procedure outlined below was followed.

Four ml of 1% agar in saline at 80°C, containing 0.001% merthiolate, was poured onto a microscope slide and maintained for 15 minutes for gelling. The slides were then placed in a moist chamber for an hour. The wells were subsequently cut out, just prior to the addition of the antigen and

of the antiserum, and the slides were incubated for 20 hours in a moist chamber. The slides were then stained as described earlier.

Ultracentrifugation

The Spinco model E optical ultracentrifuge was used to determine the sedimentation constants of the various extracts. The experiment also served to reveal any heterogeneity of the materials. Since low molecular weight materials were suspected to be present in some of the extracts, the synthetic boundary cell (167), allowing for the formation of a sharp boundary between the solution under observation and the solvent at the start of the experiment, was used instead of the standard cell. All the fractions were dissolved in saline to a concentration of 1-0.6%. The average rotor temperature was 20.5°C and the rotor* speed was 59,780 r.p.m.. Since the extracts were shown by chromatography to be heterogeneous mixtures of multiple substances, the recalculation of the sedimentation coefficients for standard conditions was not deemed necessary.

RESULTS

Skin activity of different extracts

The results illustrated in Table I demonstrate that the extracts prepared by different procedures, when compared in terms of protein concentration, differed in their skin activities.

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*Analytical Rotor An-D, Spinco Model E.

Extraction using the 'Virtis 45' homogenizer (A)

Extract # 2 was found to be significantly more active than extract # 1 and # 3. Thus, a solution of extract # 2, containing 0.1 µg protein/ml, was required to elicit a minimal skin reaction as contrasted to 1 µg protein/ml for extract # 1 and 100 µg protein/ml for extract # 3. More importantly, the skin activity of extract # 3 was smaller by a factor of 1,000 than that of extract # 1. The skin activity of extract # 1 was also found to be less than that of extract # 2 by a factor of 10. In other words, extraction for 15 and 60 minutes yielded materials of smaller skin activity, whilst extraction for 30 minutes yielded materials of largest skin activity.

Heat treatment of pollen prior to disintegration with the 'Virtis 45' homogenizer (B)

In extracts # 4, # 5 and # 6, it was observed that a visible precipitate was formed in the clear filtrate, which was obtained after the removal of pollen residues. The precipitate was found to be insoluble in water. All analyses and the skin activity of these extracts were, therefore, performed after the removal of the precipitate from the filtrate.

Extract # 4 was shown to be slightly more active than extracts # 5 and # 6. All three extracts were shown to elicit minimal skin reactions at a concentration of 10 µg protein/ml.

Disintegration with Ultrasonic Disintegrator (C)

Extract # 9 was shown to be by far the most active since it elicited a skin reaction at a concentration of 0.01 µg protein/ml. Furthermore,

FIGURE 3

Calibration line for the determination of protein concentration.

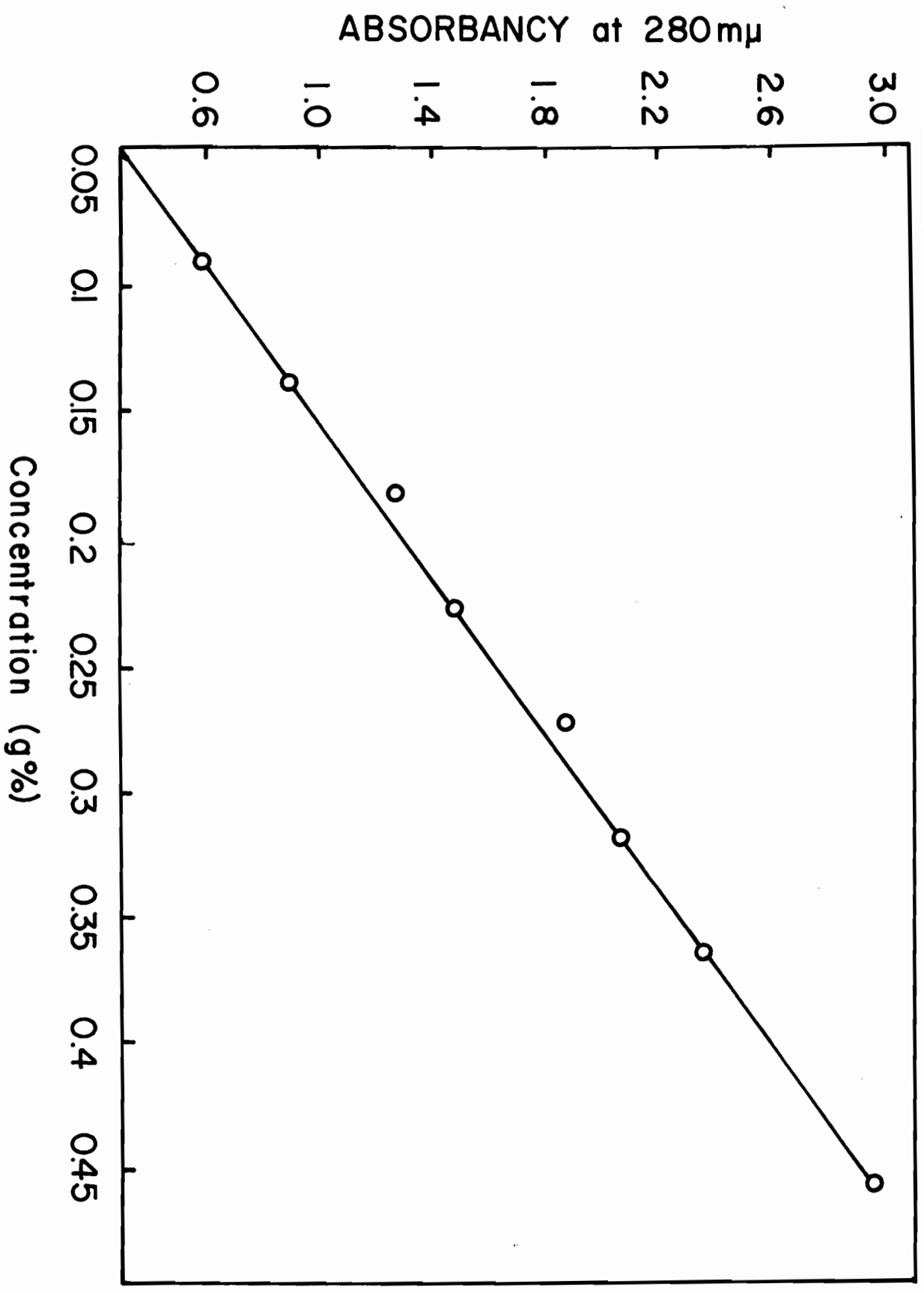

ABSORBANCY at 280mμ
0.6
1.0
1.4
1.8
2.2
2.6
3.0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
0.45
Concentration (g%)

TABLE I

Skin activity of different extracts.

Extract #	Time of extraction in minutes	Extraction method	Skin reactions obtained in sites sensitized with allergic serum of patient 'Na' (ten-fold diluted) and challenged with different extracts Concentration of extracts in µg protein/ml					
			100	10	1.0	0.1	0.01	0.001
1	15	A	3+	1+	±	-	-	-
2	30	A	3+	2+	1+	±	-	-
3	60	A	3+	-	-	-	-	-
4	15	B	2+	1+	-	-	-	-
5	30	B	3+	±	-	-	-	-
6	60	B	2+	±	-	-	-	-
7	5	C	3+	2+	1+	-	-	-
8	15	C	3+	2+	1+	-	-	-
9	5	C	3+	2+	1+	1+	1+	-
10	15	C	3+	2+	1+	±	-	-
11	30	C	3+	2+	1+	-	-	-
12	5	C	3+	2+	1+	-	-	-
WSG	240	D	3+	2+	1+	-	-	-

the activities of extracts # 9 and # 10 were shown to be much higher than those of extracts # 7 and # 8; the main difference in preparing these four extracts was that extracts # 9 and # 10 were obtained by disintegrating a six times diluted suspension of the original pollen suspension from which extracts # 7 and # 8 were obtained.

Extraction in aqueous media (D)

Extract WSG was found to elicit minimal skin reactions at a concentration of 1 µg protein/ml.

Comparison of skin activity of different extracts by different extraction procedures

Table II illustrates a comparison of skin activity of the most active extracts obtained by different extraction procedures. Thus, extract # 9 of ultrasonically disintegrated pollen was more active by a factor of 10 than that of the homogenized pollen (extract # 2) and by a factor of 100 than WSG. The extract of homogenized pollen (# 2) was more active than that of the unhomogenized pollen (WSG) by a factor of 10. The extract obtained by heating the pollen (# 6) was shown to be the least active.

Determination of desensitizing dose of each extract

From the data presented in Table III, it is evident that extract # 2 was capable of eliciting skin reactions even at the low concentration of 12.5 µg protein/ml, and the size of the reaction was determined by the concentration used, i.e. with higher concentrations, bigger reactions were obtained. On rechallenge of the same sites 24 hours later with the same

TABLE II

Comparison of skin activity of different extracts obtained by different extraction procedures.

Extract #	Extraction method	Skin reactions obtained in sites sensitized with allergic serum of patient 'Na' (ten-fold diluted) and challenged with different extracts					
		Concentration of extracts in μg protein/ml					
		100	10	1.0		0.01	0.001
2	A	3+	2+	1+	±	-	-
6	B	2+	±	-	-	-	-
9	C	3+	2+	1+	1+	1+	-
WSG	D	3+	2+	1+	-	-	-

TABLE III

Determination of the desensitizing dose of each extract.

Extract #	Skin reactions obtained in sites sensitized with allergic serum of patient 'Na' (ten-fold diluted) and challenged with different extracts. Concentration of extracts in µg protein/ml				
	200	100	50	25	12.5
2	3+	2+	2+	1+	±
6	3+	2+	1+	1+	±
9	3+	2+	2+	2+	1+
WSG	3+	2+	1+	±	±
24 hours later, sites were challenged with the same extracts at a concentration of 500 µg protein/ml.					
2	-	1+	2+	3+	2+
6	-	1+	2+	3+	2+
9	-	-	1+	2+	3+
WSG	-	1+	2+	3+	3+

extract at a concentration of 500 μg protein/ml, it was shown that while extract # 2 did not elicit further skin reaction in the site injected previously with the same extract at a concentration of 200 μg protein/ml, all the other sites injected on the first occasion with lower concentrations of the extract gave positive reactions. Therefore, 200 μg protein/ml of extract # 2 was considered to represent the desensitizing dose of this preparation. By the same reasoning, it was concluded that the desensitizing dose for either extract # 6 or WSG was also 200 μg protein/ml each, and for extract # 9 the desensitizing dose was 100 μg protein/ml.

Cross-neutralization experiments with different extracts

The results of cross-neutralization experiments illustrated in Table IV demonstrate the allergenic identity of the different extracts.

Immunological analysis

Figure 4 demonstrates the electrophoretic and antigenic heterogeneity of all the extracts, which contained multiple components with cathodic and anodic mobilities. WSG contained a component with a high cathodic mobility, which was absent from extracts # 9 and # 6. However, the two extracts had a component, with a low cathodic mobility, which was absent from WSG and from extract # 2.

The antigenic relationship of the extracts was demonstrated by a gel-diffusion pattern shown in Figure 5. All the extracts gave a strong precipitin band due to rapidly diffusing components and a weak precipitin band closer to the antigen well. The antigenic identity of these extracts, at least with respect to antibodies elicited by immunization with WSG, is

FIGURE 4

Immunoelectrophoretic patterns of extracts; all the troughs were filled with rabbit anti-WSG antiserum and the wells were filled with appropriate extracts; the electrophoresis was carried out in veronal buffer, pH 8.6, for 50 minutes. (The concentration of all extracts was 50 mg/ml.)

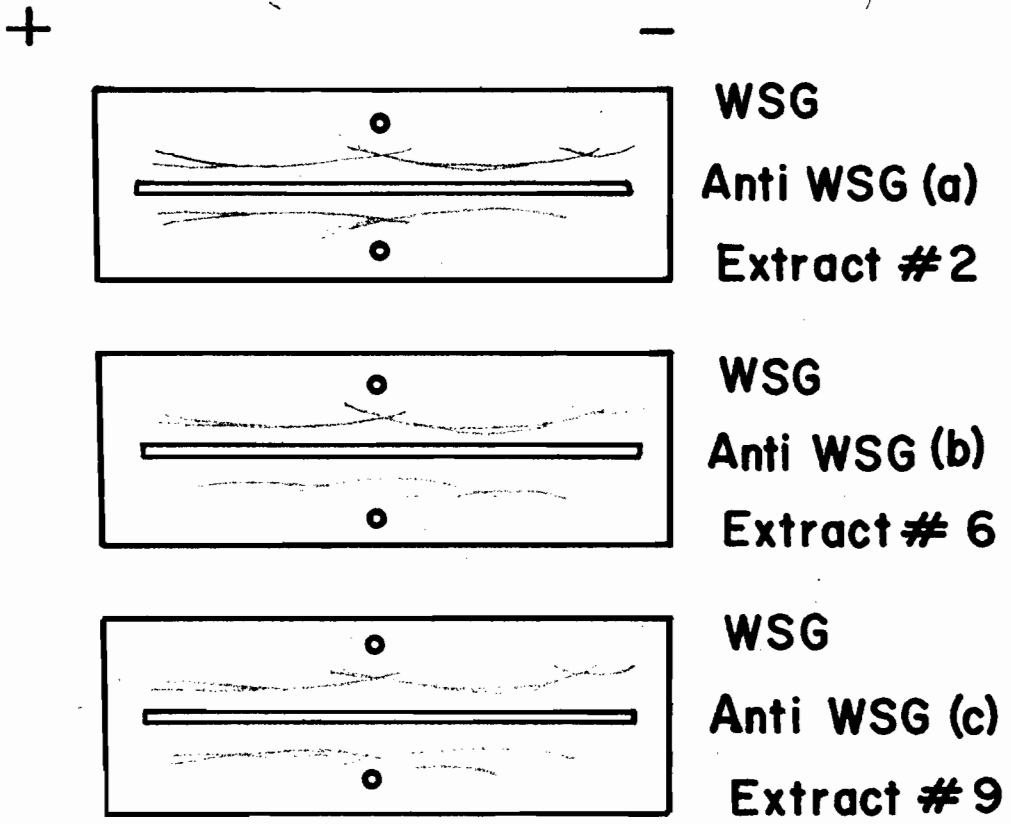
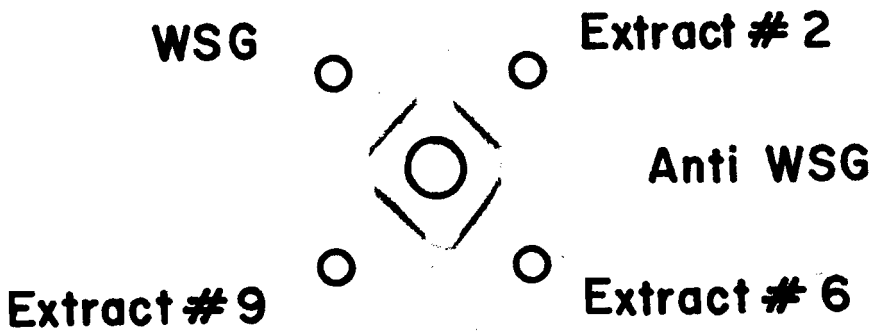

+
−
WSG
Anti WSG (a)
Extract #2
WSG
Anti WSG (b)
Extract # 6
WSG
Anti WSG (c)
Extract # 9

FIGURE 5

Gel-diffusion pattern obtained with extracts # 2, # 6, # 9 and WSG and rabbit anti-WSG antiserum. (The concentration of all extracts was 10 mg/ml.)


WSG
Extract # 2
Anti WSG
Extract # 9
Extract # 6

indicated by the coalescence of the precipitin bands given by all extracts.

Ultracentrifugation

The sedimentation patterns obtained in the analytical ultracentrifuge are shown in Figures 6 and 7. Each extract gave rise to only one main peak, which tended to broaden by diffusion.* Extracts # 2, # 9 and WSG had the same sedimentation coefficient of 1.5S, while extract # 6 had a sedimentation coefficient of 0.76S (Table V).

*The kink in the Schleren pattern was stationary and was due to lens imperfection.

FIGURE 6

Sedimentation patterns for extracts in saline: (a) Extract # 6; (b) Extract # 9 (1%); centrifugation at 59,780 r.p.m. and frames at intervals of eight minutes.

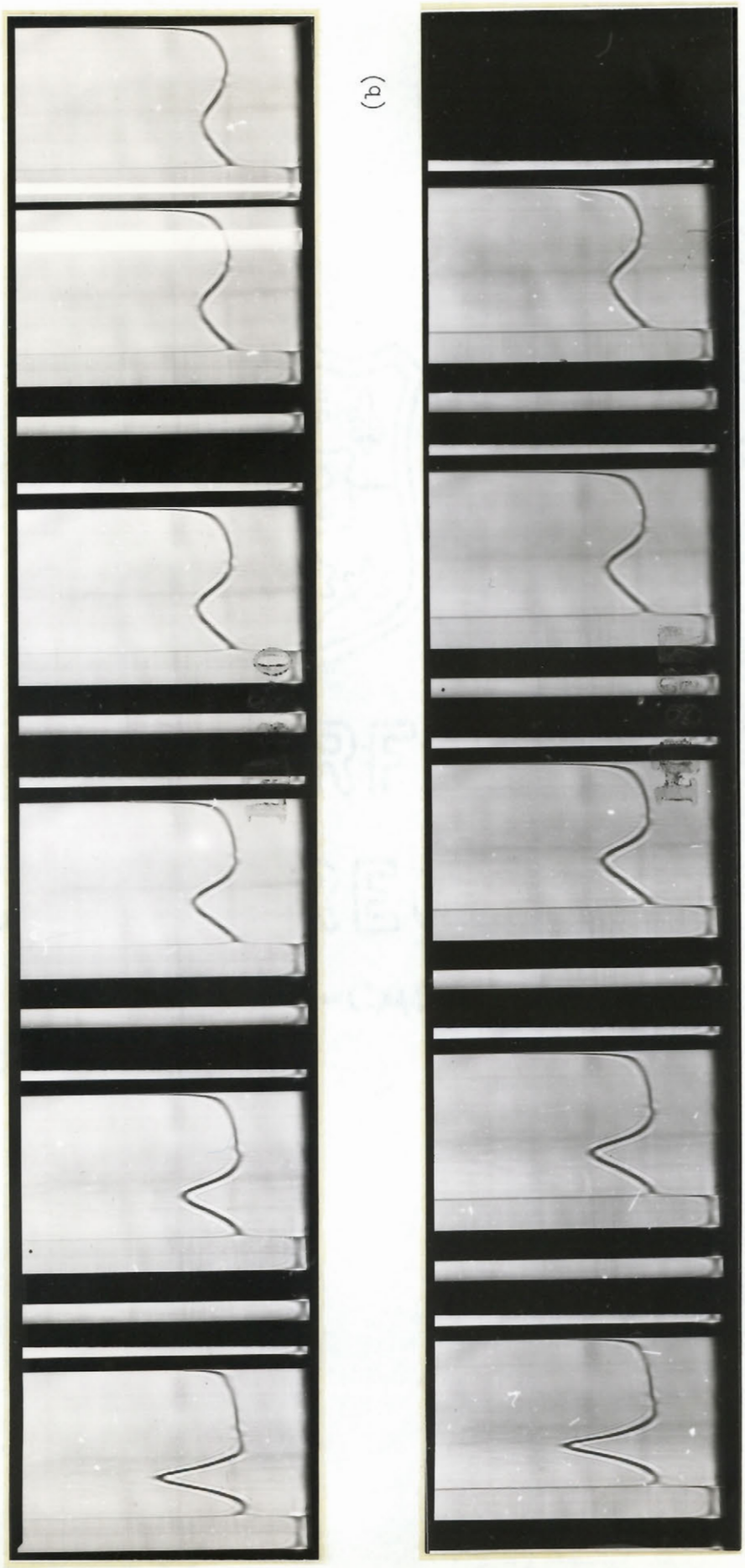

(a)
(b)

FIGURE 7

Sedimentation patterns for extracts in saline: (a) Extract # 2 (1%); (b) Extract WSG (0.6%).

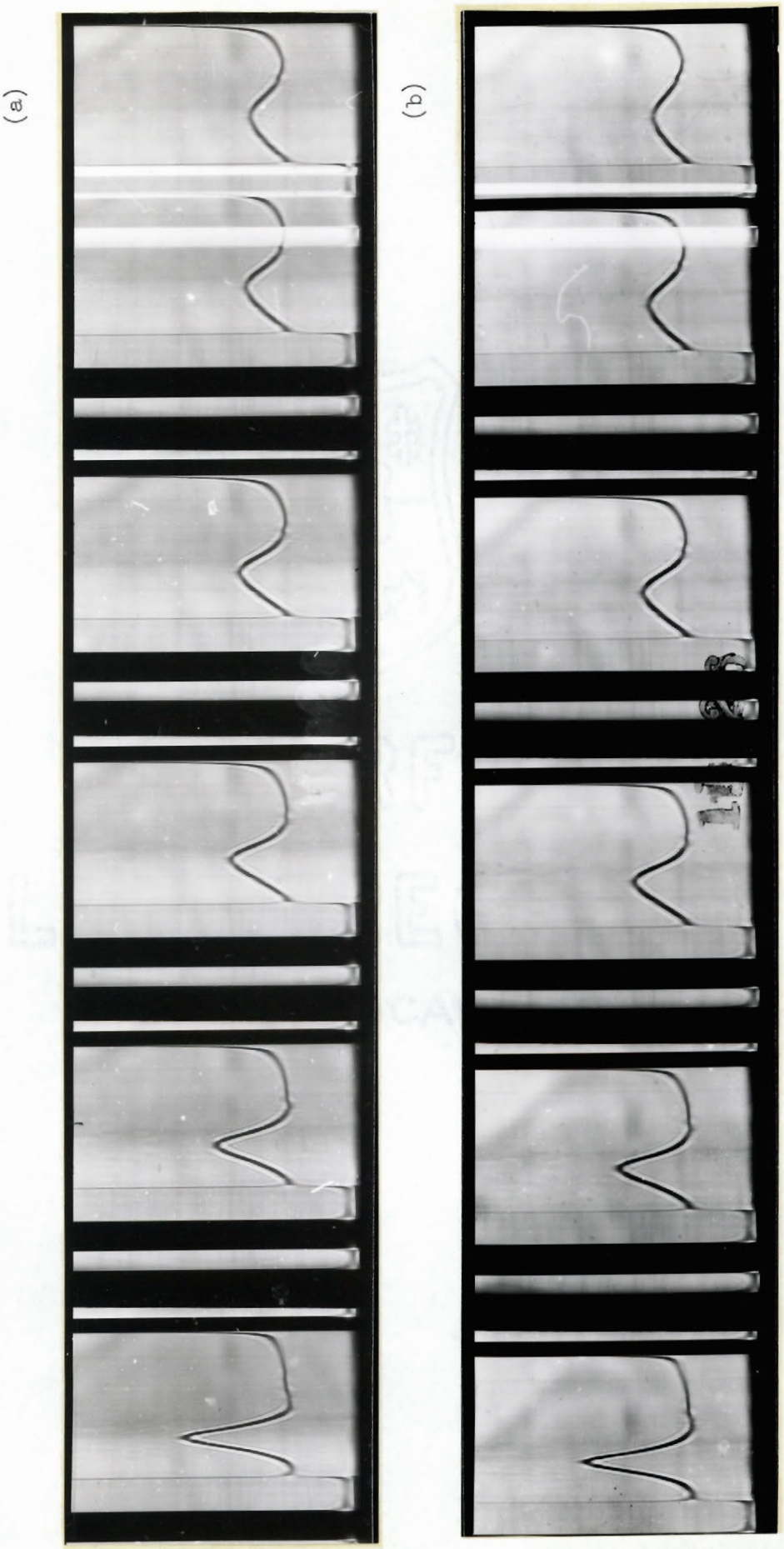

(a)
(b)

TABLE V

Sedimentation data.

Extract #	Sedimentation coefficients*
2	1.5S
6	0.76S
9	1.5S
WSG	1.5S

*Expressed in Svedberg units; 1 Svedberg unit = 10^{-13} sec.

DISCUSSION

The results reported in this chapter demonstrate that the aqueous extract of defatted timothy pollen, which had been ultrasonically or mechanically disintegrated, was more active than the aqueous extract of the whole pollen (WSG) when compared in terms of the same protein concentration. However, it ought to be stressed that this criterion was arbitrary since all these extracts were complex mixtures of different pollen components, whose constituents were not determined.

The aqueous extract of the ultrasonically disintegrated pollen was active at a concentration of 0.01 μg protein/ml (Table I), while the threshold activity of WSG was 1 μg protein/ml. Although the extract of the ultrasonically disintegrated pollen was the most active, the period of disintegration, as well as the concentration of pollen suspension, was shown to affect the activity of the extracts. Thus, disintegration of pollen for more than five minutes resulted in a progressive decrease of the biological activity. The loss in skin activity was shown to be at least in part caused by the heat produced during disintegration. Similar heat inactivation was observed during homogenization. Thus, the extract of pollen homogenized for half an hour was more active than that for an hour.

The extracts of preheated pollen were less active than the extracts of unheated pollen. From these results it may be concluded that the activity of the pollen extract is a function of the treatment used for extraction and that it tends to become reduced under harsher conditions. This observation contradicts the finding of Augustin (122) who stated that

autoclaving of her pollen extract for five minutes had not resulted in the destruction of its skin activity. Ultracentrifugal studies corroborate indirectly this conclusion, since the main components of the extracts of the disintegrated and homogenized pollen, as well as of WSG, had the identical sedimentation coefficient of 1.5S, whereas the main component of the extract of preheated pollen had a sedimentation coefficient of only 0.76S. The lower sedimentation coefficient value of preheated pollen extract would suggest that the allergen(s) in the pollen extract were dissociated into smaller units, which still retained some biological activity, or that some of the high molecular weight, heat labile allergen(s) may have been denatured and removed by centrifugation.

As regards skin activity, this study demonstrated that WSG contained all the active components present in the extracts of the ultrasonically disintegrated or homogenized pollen. Since in these experiments allergic serum from only one patient was used, it may be suggested that the conclusion arrived at may not be of general validity. However, all allergic sera tested (discussed in Chapter IV) gave the same reaction pattern with different chromatographic fractions of the whole pollen extract. Even with weak allergic sera various chromatographic fractions elicited positive reactions. It was, therefore, surmised that a potent allergic serum, such as the one used in this study, would contain all the reaginic activity directed against all the skin active components of the whole pollen extract.

The antigenic identity of the different extracts was demonstrated by immunoelectrophoresis (Figure 4). The extracts of pollen, which had been mechanically or ultrasonically disintegrated shared the main antigenic components of WSG. As demonstrated by immunodiffusion, all extracts gave

the same 'outer' and 'inner' precipitin lines with rabbit anti-WSG antiserum. Furthermore, the immunoelectrophoretic patterns of these extracts (Figure 4), though more complex, were closely similar in their overall appearance.

The asymmetric Schlieren peak, seen in ultracentrifugal studies (Figures 6 and 7), was taken as an indication of the heterogeneity of the extracts. Moreover, the broadening of the peak, which was rather rapid, was attributed to components having high diffusion coefficients, which would be in keeping with the rather low sedimentation coefficients of the order of 1.5S for the main components.

The primary disadvantage of the ultrasonic or mechanical disintegration is the limitation of the technique in handling amounts of pollen larger than about 0.5 g of pollen at one time. Furthermore, it was shown that the skin activity of the extracts obtained by disintegrating dilute suspensions of pollen was higher than that of the extracts obtained by disintegrating a concentrated suspension of pollens. Thus, extracts # 9 and # 10 were more active than # 7 and # 8 (Table I). These experiments demonstrated that for effective disintegration dilute suspensions of pollen were necessary, which limits further the amount of pollen which can be extracted. Also, as pointed out, the temperature control during extraction appeared to be critical. On the other hand, the simple process of extraction with water could handle at least 200 g of pollen at a time and, since WSG contained all the skin active components present in the extracts of the ultrasonically disintegrated pollen, WSG was chosen as starting material for further fractionation of allergen(s).

CHAPTER IV

SECTION A. ISOLATION AND FRACTIONATION OF SKIN ACTIVE COMPONENTS OF WSG

INTRODUCTION

The isolation of allergens in various pollens has received a renewed attention following the introduction of more modern refined techniques, such as gel-filtration through Sephadex or ion-exchange chromatography on cellulose derivatives. Using these techniques a number of recent studies on ragweed, timothy and rye grass pollen allergens has been reported (reviewed in Chapter II). In the present chapters are described methods developed for the isolation, fractionation and purification of allergens in timothy grass pollen, which are similar to those described by King et al (1) for the isolation of ragweed allergen(s). It was demonstrated in Chapter III that WSG contained all the skin active components present in the extracts of mechanically disrupted pollen; therefore, WSG was chosen as the starting material.

METHODS AND MATERIALS

Isolation of the allergens by ammonium sulphate precipitation

Defatted pollen was extracted with distilled water (500 ml water per 100 g pollen) by constant stirring for 4 hours at room temperature. It was then filtered under suction through a Buchner funnel using filter paper Whatman no. 3. The pollen residue was washed with a small volume of water and filtered in the same way. The combined filtrate, a brown coloured

aqueous extract (WSG), was at pH 5.4, which was adjusted to pH 7.0 by adding 3N ammonium hydroxide dropwise. The allergens were precipitated from the filtrate with ammonium sulphate at 90% saturation (by adding 600 g solid ammonium sulphate per 1000 ml of the filtrate) at 4°C under constant stirring for 3-4 hours. The suspension was allowed to remain at 4°C overnight before the removal of the precipitate by centrifugation. The precipitate was washed with ammonium sulphate solution of the same concentration as used for precipitation (i.e. 60 g%). The precipitate was dissolved in a minimum volume of distilled water and the resulting solution was separated from any insoluble materials by centrifugation at 10,000 r.p.m. for 10 minutes. The clear supernatant was dialyzed against distilled water for 3 days with frequent changes of water at 4°C. The dialyzed solution was lyophilized and is referred to as the ammonium sulphate precipitate.

Chromatography on Sephadex G-25 of the ammonium sulphate precipitate

Sephadex G-25, fine grade, was purchased from Pharmacia, Uppsala, Sweden.

Gel-filtration was carried out according to the technique described by Flodin (168). The gel was allowed to swell by pouring the powder into an excess of water with constant stirring; the final equilibrium was achieved at room temperature after 24 hours and the fines were removed from the gel by flotation and decantation. The process was repeated several times till a clear supernatant was obtained. The gel was finally equilibrated with the buffer (0.025M tris (hydroxymethyl) aminomethane and 0.05M HCl, pH 7.9) for 24 hours before pouring it into the column.

A small piece of glass wool was placed in the lower end of a chromatographic column, made from a pyrex glass tube (4 x 124 cm). The column was carefully mounted vertically and the outlet closed. It was filled to one fourth with the buffer. The air bubbles trapped in the glass wool were carefully removed by gently pressing the glass wool with a glass rod. The gel-slurry, made up by mixing equal volumes of the gel and the buffer, was poured into the column slowly. When the gel-slurry had sedimented to a layer of 2.5 cm, the outlet was opened and a slow stream of buffer was allowed to flow out. To ensure uniform packing, the gel-slurry was continuously poured into the column. The pouring was continued until the desired bed length had been obtained. The packed column was then connected to a buffer reservoir and washed (at $4^{\circ}C$) for 24-32 hours to attain stability of the bed.

The buffer above the bed was carefully removed with a Pasteur pipette leaving some buffer above the bed. The remaining buffer was brought to the meniscus by letting it flow from the outlet. The outlet was closed and the sample (70 ml of 6.7% ammonium sulphate precipitate in buffer) was then carefully placed on top of the bed. The sample was allowed to enter the bed by opening the outlet. At the moment when it disappeared through the surface, a small amount of buffer was added to wash the surface with the outlet closed. The outlet was again opened. The washing was repeated 2-3 times. The buffer reservoir was then connected and the elution started. The desired flow rate was obtained by adjusting the hydrostatic pressure head or the stopcock attached to the outlet, or by both. Fractions were collected in 10 ml portions and assayed at an optical density of 280 mμ. Fractions under individual O.D. peaks were pooled together, dialyzed and

freeze-dried.

Chromatography of fraction I on Sephadex G-25

Fraction I, obtained by gel-filtration of the ammonium sulphate precipitate on Sephadex G-25, was rechromatographed on a fresh Sephadex G-25 column under identical conditions, as described above.

Chromatography on DEAE-cellulose of fraction I

DEAE-cellulose (through 325 mesh screen, Brown Company, Berlin, New Hampshire) was washed several times with water and filtered through a Buchner funnel, using filter paper Whatman no. 1. The supporting medium was then suspended in 0.5N NaOH with constant stirring for 3-4 hours, filtered through the Buchner funnel, washed with water on the Buchner funnel and resuspended in 0.5N NaOH. The process was repeated several times until all the colouring materials were removed from DEAE-cellulose; the cellulose was then washed several times with water to remove the alkali and was finally suspended in the first eluting buffer (0.025M tris and 0.015M HCl, pH 7.9) for 24 hours under constant stirring.

A suitable column (50 x 4.5 cm) was packed with this DEAE-cellulose under a nitrogen pressure of 15 lb psi to achieve a constant bed volume of the cellulose. The packed column was flushed for 18-24 hours with the first buffer at $4^{\circ}C$. The sample (3 g) was dissolved in 50 ml of the same buffer and dialyzed against it for 24 hours. The chromatography with the first buffer was then continued.

After elution with the first buffer, the column was washed with the same buffer for 24-48 hours. The buffer on top of the cellulose bed was

withdrawn and replaced with the second buffer of the same pH and increasing ionic strength (0.05M tris + 0.03M HCl, 0.2M NaCl). Chromatography was performed as described above and finally the second buffer was replaced by the third buffer of still higher ionic strength (0.05M tris + 0.03M HCl, 2M NaCl; pH 7.9).

Chromatography on Sephadex G-50, G-75 and G-100 of fraction C

Three columns (110 x 2 cm) were packed with Sephadex G-50, G-75 and G-100, all of fine grade, using 0.05M ammonium acetate buffer, pH 6.85, as equilibrating buffer. In each column, a small amount of Sephadex G-25, coarse, was placed at the bottom of the column to prevent blocking of the filter. The chromatography was continued as described earlier.

The void volume of Sephadex G-75 column was determined by passing a solution of blue dextran through the column under the chosen experimental condition.

Preparation of fraction R

The pollen residue, after water extraction, was further extracted with 4M NaCl for 24 hours at 4°C with continuous stirring and the solution filtered. The filtrate was dialyzed for 7 days with frequent changes of water and finally lyophilized; this fraction was designated as R.

RESULTS

Isolation of the allergens by ammonium sulphate precipitation

The pH of the aqueous extract was 5.4. When the pH was gradually

adjusted to pH 7, the active materials started precipitating, as indicated by the appearance of cloudiness in the solution. The bulk of the active materials was precipitated by the addition of ammonium sulphate. Most of this precipitate could be redissolved in distilled water but a small amount of it, approximately 0.05% of the whole pollen, remained insoluble. During the removal of ammonium sulphate from this fraction by dialysis, additional precipitate was formed and discarded.

Chromatography on Sephadex G-25 of ammonium sulphate precipitate

As shown in Figure 8, the ammonium sulphate precipitate was resolved by chromatography on Sephadex G-25 into several fractions, the major fraction being eluted right after the void volume. The eluates were pooled into 7 fractions, designated by Roman numerals. On lyophilization no residual materials were obtained from any fraction, excepting fraction I. Fraction I was rechromatographed through a fresh Sephadex G-25 column (Figure 9).

During chromatography some pigments were shown to be strongly adsorbed onto the Sephadex, forming a yellow coloured band just below the bed volume of the gel in the column. The pigments could be liberated by treating the gel with 1N acetic acid for a few hours. In an acidic solution the pigment was colourless, while in an alkaline solution it gave a yellow colour.

Chromatography on DEAE-cellulose of fraction I

Figure 10 shows the chromatographic pattern of fraction I on DEAE-cellulose column at pH 7.9 with step-wise elution using buffers of increasing ionic

FIGURE 8

Chromatography on Sephadex G-25 (110 x 4 cm) of the "ammonium sulphate precipitate". Eluant: 0.025 M tris x 0.015 M HCl, pH 7.9; flow rate = 65 ml/hr; vol. of fractions = 10 ml; sample applied = 4 g dissolved in 70 ml eluant.

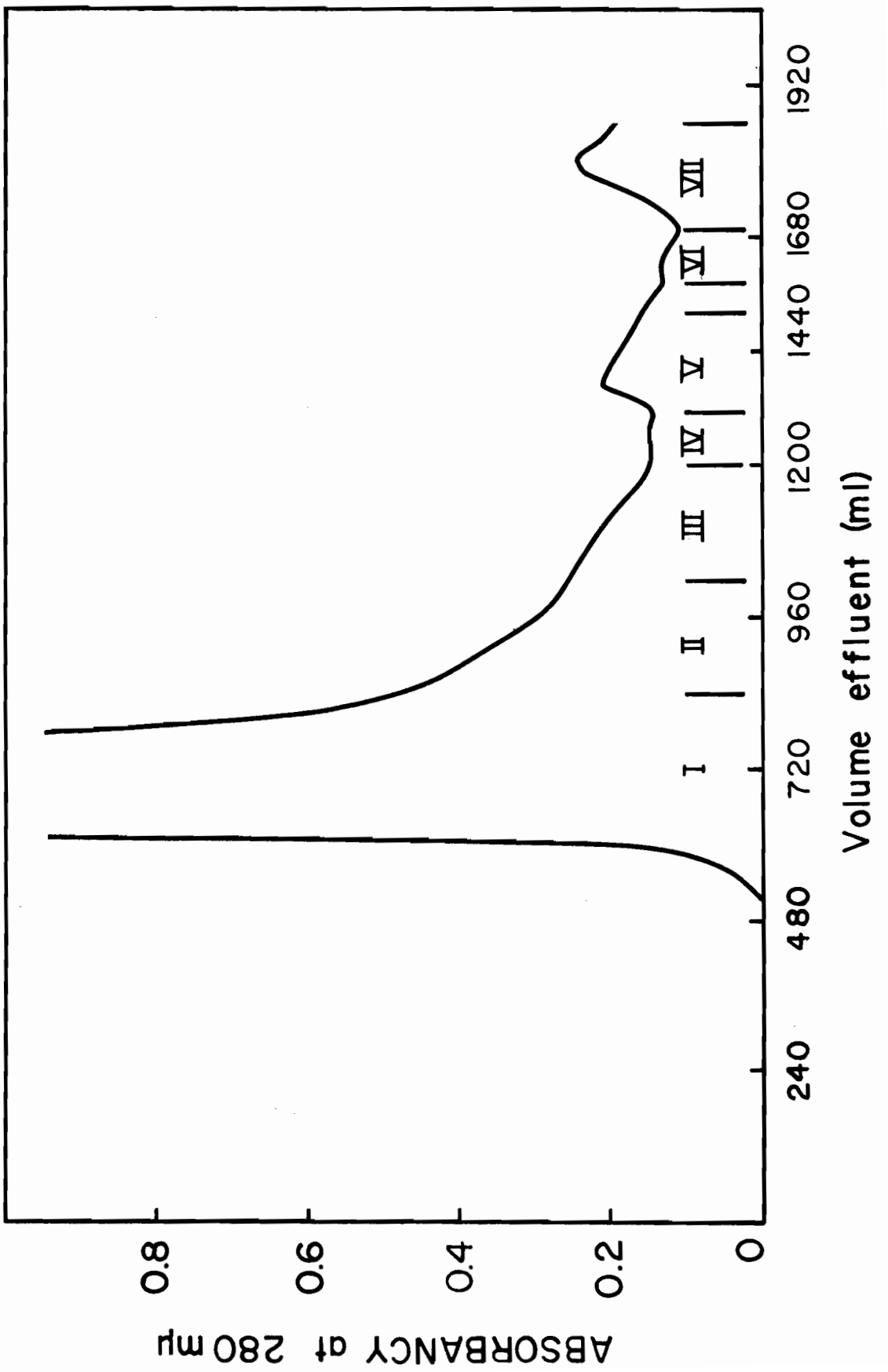

ABSORBANCY at 280 mμ
0
0.2
0.4
0.6
0.8
240
480
720
960
1200
1440
1680
1920
Volume effluent (ml)
I
II
III
IV
V
VI
VII

FIGURE 9

Chromatography of fraction I on Sephadex G-25 under conditions identical to those used for isolation of fraction I (see page 73).

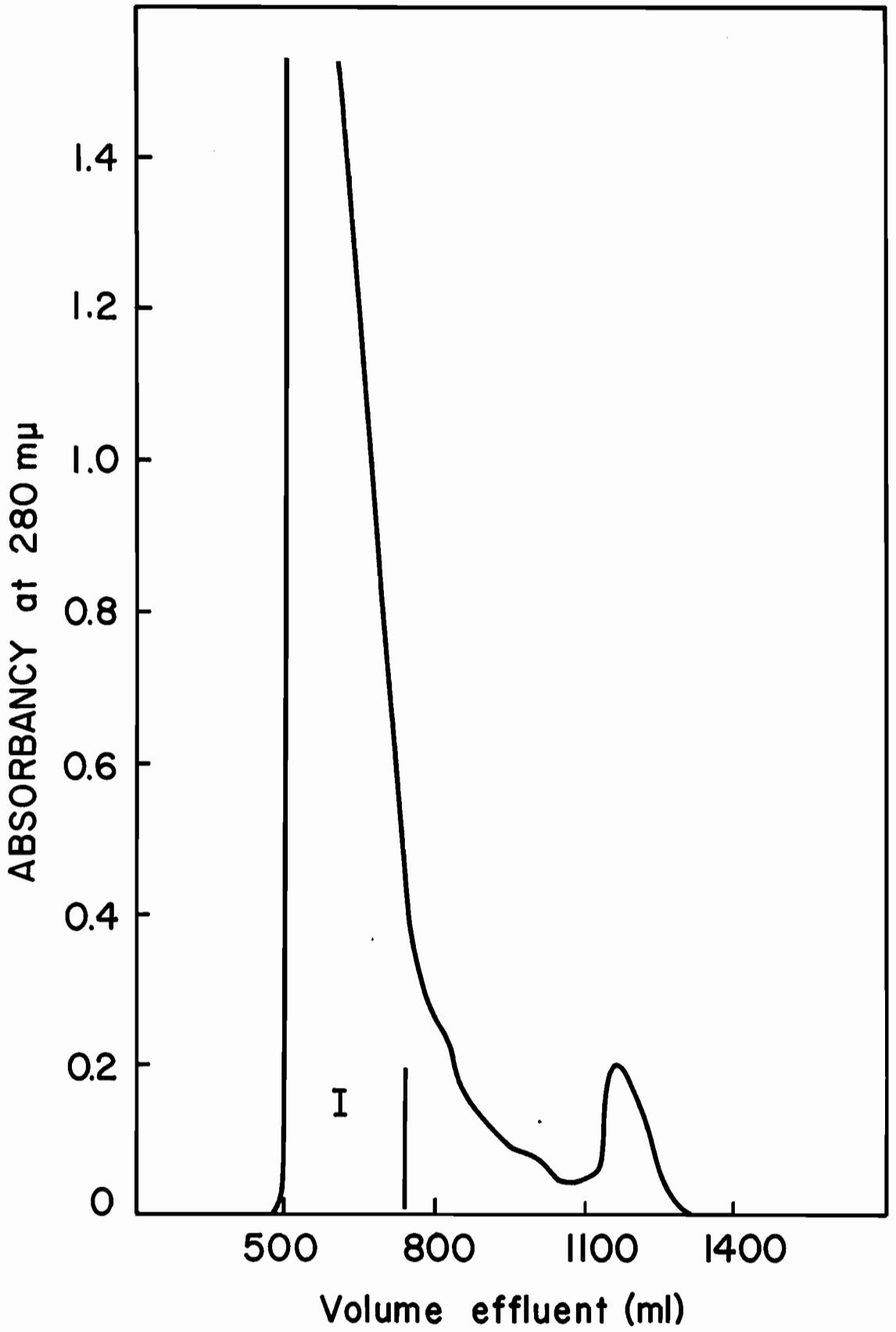
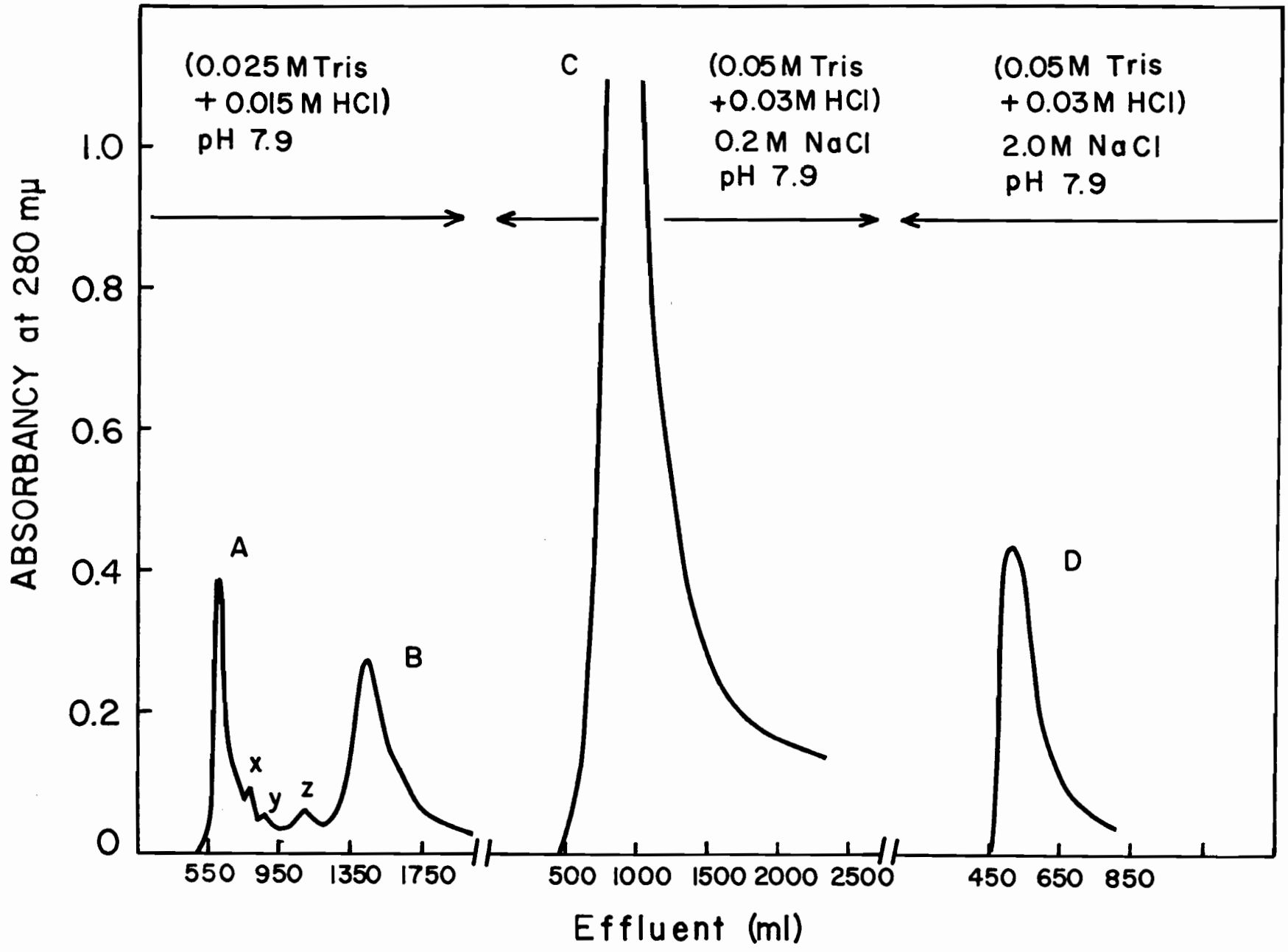

ABSORBANCY at 280 mμ
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
I
500
800
1100
1400
Volume effluent (ml)

FIGURE 10

Chromatography on DEAE-cellulose (4 x 45 cm) of fraction I using step-wise elution with increasing ionic strength of the buffer at pH 7.9; flow rate = 50 ml/hr; vol. of fractions = 10 ml; sample applied = 3 g dissolved in 50 ml of the initial buffer and dialyzed against the same buffer for 24 hrs at 4°C.


(0.025 M Tris
+ 0.015 M HCl)
pH 7.9
C
(0.05 M Tris
+0.03M HCl)
0.2 M NaCl
pH 7.9
(0.05 M Tris
+0.03 M HCl)
2.0 M NaCl
pH 7.9
ABSORBANCY at 280 mμ
1.0
0.8
0.6
0.4
0.2
0
A
B
x
y
z
D
550 950 1350 1750
500 1000 1500 2000 2500
450 650 850
Effluent (ml)

strength. The subfractions designated as A, x, y, z and B were eluted with the first buffer which contained no sodium chloride. The minor peaks x, y and z were not resolved unless the cellulose had been pretreated with alkali. Fraction A had a characteristic pink colour; the colour could also be observed during the chromatographic run in the form of a pink band.

Only one fraction was eluted with the second buffer. In contrast to the pink colour of fraction A, this fraction, designated as C, was yellow in colour. Fraction D was eluted with the third buffer.

Chromatography on Sephadex G-50, G-75 and G-100 of fraction C

The components present in fraction C were further separated on the three different columns used. On Sephadex G-50 and G-100 fraction C was resolved poorly into four overlapping peaks. The best resolution of fraction C into more clearly separated subfractions was achieved by chromatography on Sephadex G-75 (Figure 11); these subfractions were designated arbitrarily as C-I, C-II, C-III, C-IV and C-V. Fraction C-IV contained most of the components of fraction C.

Chromatography of fraction C-IV on Sephadex G-75

Figure 12 shows the chromatographic pattern of fraction C-IV on Sephadex G-75. The symmetry of the peak was considered to indicate that fraction C-IV was fairly well purified fraction. Fraction C-IV was found to be retained on Sephadex G-75 column. Thus, fraction C-IV came off at an elution volume of 173 ml, while the void volume, as determined by blue dextran, of the column was 93 ml.

FIGURE 11

Chromatography on Sephadex G-75 (110 x 2 cm) of fraction C. Eluant: 0.05 M ammonium acetate buffer, pH 6.85; flow rate = 8 ml/hr; vol. of fractions = 4 ml. Sample: 200 mg dissolved in 3 ml of the eluting buffer.

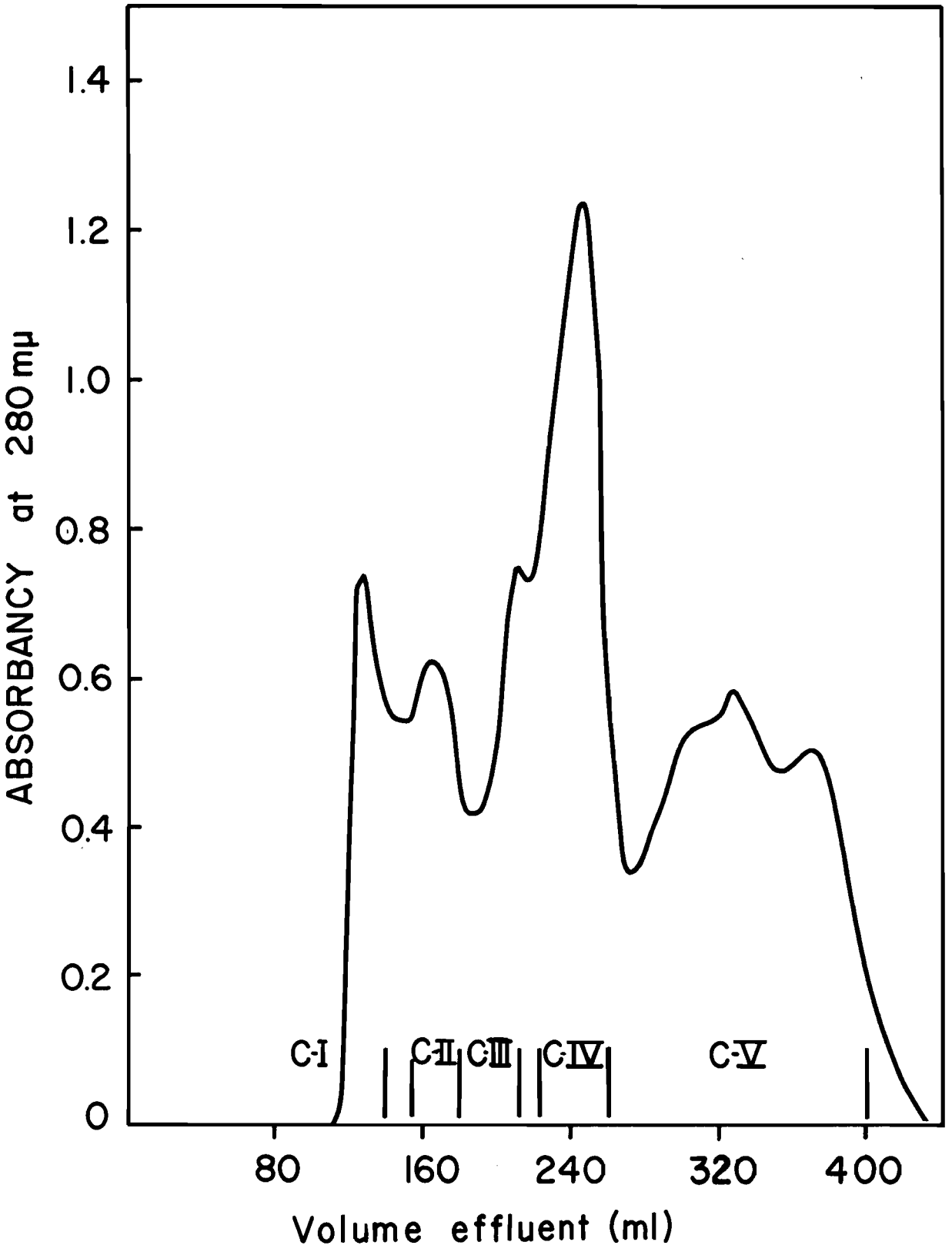
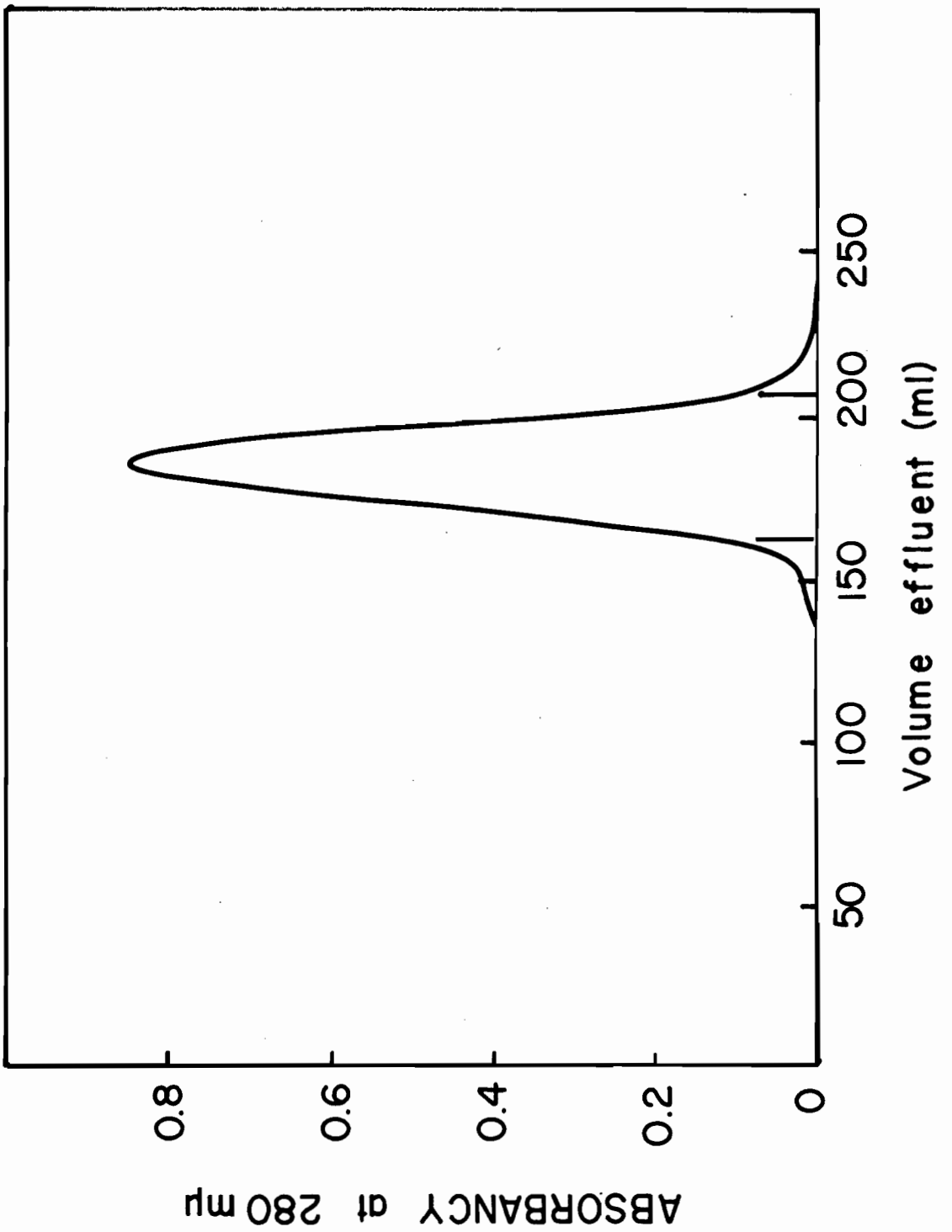

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
ABSORBANCY at 280 mμ
C-I
C-II
C-III
C-IV
C-V
80
160
240
320
400
Volume effluent (ml)

FIGURE 12

Chromatography on Sephadex G-75 of fraction C-IV under conditions identical to those used for isolation of fraction C-IV (see page 77).

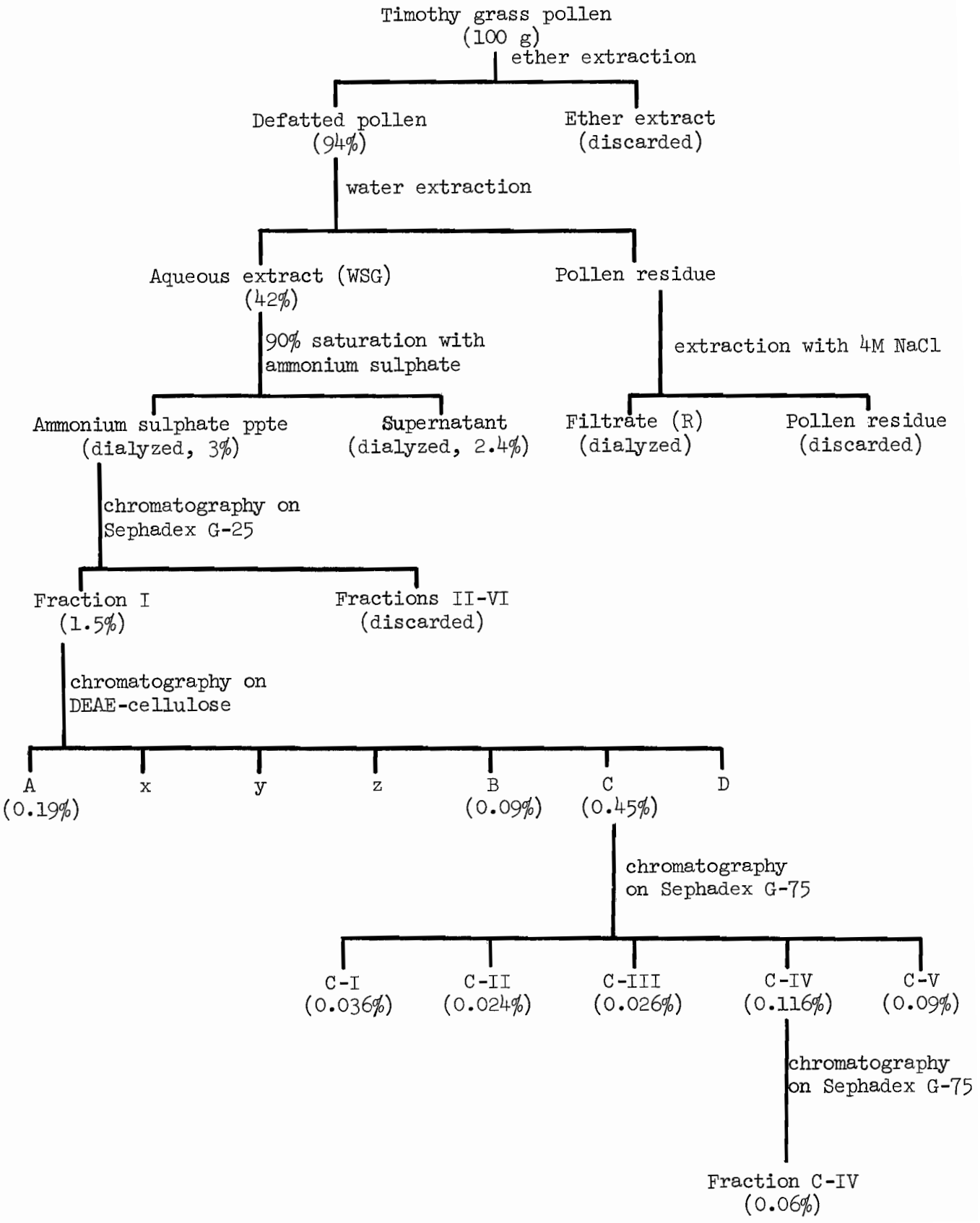

ABSORBANCY at 280 mμ
0
0.2
0.4
0.6
0.8
50
100
150
200
250
Volume effluent (ml)

In Figure 13 is given the overall flowsheet for the isolation, fractionation and purification of non-dialysable constituents of WSG, together with the yields of various fractions expressed as percentages of the weight of the whole pollen. As can be seen,this procedure resulted in the removal of 42% of the weight of the whole pollen in the form of an aqueous extract (WSG). However, 89% of WSG was lost during dialysis.

During chromatography on DEAE-cellulose only 50% of the material applied could be recovered. On the other hand, about 70% of the original sample was recovered by chromatography on Sephadex G-75.

FIGURE 13

Flow-sheet for the extraction and fractionation of timothy grass pollen. Weights given in brackets refer to lyophilized materials and are expressed as percentages of the whole pollen.


Timothy grass pollen
(100 g)
ether extraction
Defatted pollen
(94%)
Ether extract
(discarded)
water extraction
Aqueous extract (WSG)
(42%)
Pollen residue
90% saturation with
ammonium sulphate
extraction with 4M NaCl
Ammonium sulphate ppte
(dialyzed, 3%)
Supernatant
(dialyzed, 2.4%)
Filtrate (R)
(dialyzed)
Pollen residue
(discarded)
chromatography on
Sephadex G-25
Fraction I
(1.5%)
Fractions II-VI
(discarded)
chromatography on
DEAE-cellulose
A
(0.19%)
x
y
z
B
(0.09%)
C
(0.45%)
D
chromatography
on Sephadex G-75
C-I
(0.036%)
C-II
(0.024%)
C-III
(0.026%)
C-IV
(0.116%)
C-V
(0.09%)
chromatography
on Sephadex G-75
Fraction C-IV
(0.06%)

SECTION B. BIOLOGICAL PROPERTIES OF DIFFERENT FRACTIONS

INTRODUCTION

In the present section will be described the evaluation of the skin activities of WSG and of its fractions in terms of Prausnitz-Küstner passive transfer tests.

METHODS AND MATERIALS

Determination of the P-K titer of the allergic sera of grass sensitive patients

Sera from grass sensitive patients were processed as described in Chapter III and multiple skin sites of normal volunteers were sensitized with these sera at different dilutions and challenged with WSG at a concentration of 100 μg/ml.

Determination of the skin activities of various fractions

Lyophilized fractions were dissolved in saline to the desired concentrations and the skin activity of the solution was determined by Prausnitz-Küstner passive transfer test as described in the preceding chapter.

Cross-neutralization experiments

The experiment was carried out as described in Chapter III.

RESULTS

Skin activity of different fractions of WSG

Table VI shows the titration of the five allergic sera. The weakest serum was that of the treated patient 'Ba', which even on ten-fold dilution failed to give a positive P-K reaction. The serum of the untreated patient 'Na' appeared to be the strongest, giving a positive reaction at a dilution of 1:1000. The sera of patients 'Ma', 'Sa' and 'St' were equally potent, giving positive reactions at dilutions of 1:100.

The results, illustrated in Table VII, demonstrate that different fractions differed in their skin activities when tested with respect to the allergic serum from patient 'Sa'. Thus, WSG and each of its fractions, R, A, C and D, elicited a still detectable skin reaction at a concentration of 100 μg/ml, whereas B and x were more active by a factor of 10. Thus, one may conclude that the combined methods of isolation, fractionation and purification resulted only in a ten-fold enrichment of fractions B and x with respect to the skin activity of the WSG.

Table VIII shows the different skin activities of different fractions with respect to the serum from patient 'St'. Thus, fractions R, A, B, C and D were shown to be active at a concentration of 100 μg/ml as contrasted to the activity of the whole pollen extract which gave a skin reaction at a concentration of 500 μg/ml. Moreover, fraction x was shown to be by far the most active in as much as it could elicit a skin reaction at a concentration of 10 μg/ml and was more active than whole pollen extract (WSG) by a factor of 50. It is evident from these results that the skin active components in WSG relative to this particular serum were concentrated in

TABLE VI

Determination of the titer of allergic sera of grass sensitive patients.

Patients	Reactions obtained in skin sites sensitized with allergic sera and challenged with WSG (100 µg/ml) Dilutions of the sera			
	1:10	1:100	1:500	1:1,000
Ba (treated)	-	-	-	-
Ma (untreated)	3+	1+	-	-
St (untreated)	3+	1+	-	-
Sa (untreated)	3+	1+	-	-
Na (untreated)	3+	2+	1+	±

TABLE VII

Determination of skin activity of various fractions of WSG using the serum of patient 'Sa'.

Fractions	Skin reactions obtained in sites sensitized with the allergic serum at ten-fold dilution and challenged with different fractions of WSG at different concentration.				
	Concentration of fractions in µg/ml				
	1,000	500	100	10	1.0
WSG	2+	2+	±	-	-
R	2+	2+	2+	-	-
A	2+	1+	1+	-	-
B	2+	2+	2+	±	-
x	2+	2+	2+	1+	-
C	2+	1+	1+	-	-
D	2+	2+	1+	-	-

Fractions y and z were omitted due to their low yields.

TABLE VIII

Determination of skin activity of various fractions of WSG using the serum of patient 'St'.

Fractions	Skin reactions obtained in sites sensitized with the allergic serum at ten-fold dilution and challenged with different fractions				
	Concentration of fractions in µg/ml				
	1,000	500	100	10	1.0
WSG	2+	2+	-	-	-
R	3+	2+	2+	-	-
A	4+	4+	3+	-	-
B	2+	2+	2+	-	-
x	3+	3+	2+	2+	-
C	2+	2+	2+	-	-
D	2+	2+	2+	-	-

Fractions y and z were omitted due to their low yields.

fraction x.

The data presented in Table IX demonstrate that different pollen fractions differed in their skin activities with respect to the serum from patient 'Na'. Fractions R, A, B and x were equally active and were able to elicit a minimal skin reaction at a concentration of 10 μg/ml, whereas WSG gave a minimal skin reaction at a concentration of 1 μg/ml, i.e. these fractions were less active than the whole pollen extract by a factor of 10. Fraction D was, on the other hand, more active than the whole pollen extract by a factor of 10. Fraction C was shown to be by far the most active in as much as it elicited a minimal skin reaction at a concentration of 0.001 μg/ml. These results indicate that fraction C was rich in skin active components relative to this particular serum, and also suggest that a thousand-fold purification of the skin active components, corresponding to fraction C, has been achieved by the methods of isolation and purification reported in the preceding section.

The skin activity of fraction C and its subfraction is shown in Table X. Since the serum from patient 'Na' was the most potent and also due to the limited number of P-K subjects available, the serum from this patient only was used in this determination. The results illustrated in this table demonstrate that some of the subfractions of fraction C were more active and others less active than the parent fraction. Thus, while fraction C elicited a minimal skin reaction at a concentration of 0.001 μg/ml subfraction C-IV could elicit a minimal skin reaction at a concentration of 0.00001 μg/ml. On the other hand, subfractions C-I, C-II and C-V elicited minimal skin reactions at concentrations of 0.1 μg/ml, 0.01 μg/ml and 1.0 μg/ml, respectively, and subfraction C-III was shown to be as active

TABLE IX

Determination of skin activity of various fractions of WSG using the serum from patient 'Na'.

	Skin reactions obtained in sites sensitized with the allergic serum at ten-fold dilution and challenged with different fractions					
	Concentration of fractions in μg/ml					
	100	10	1.0	0.1	0.01	0.001
WSG	3+	1+	1+	-	-	-
R	3+	2+	-	-	-	-
A	3+	2+	-	-	-	-
B	3+	2+	-	-	-	-
x	4+	3+	-	-	-	-
C	3+	3+	2+	2+	1+	1+
D	3+	3+	2+	1+	-	-

Fractions y and z were omitted due to their low yields.

TABLE X

Determination of skin activity of fraction C and its sub-fractions using the serum from patient 'Na'.

Fractions	Skin reactions obtained in sites sensitized with the allergic serum at ten-fold dilution and challenged with different fractions					
	Concentration of fractions in µg/ml					
	1.0	0.1	0.01	0.001	0.0001	0.00001
C	2+	2+	1+	±	-	-
C-I	1+	±	-	-	-	-
C-II	2+	1+	±	-	-	-
C-III	2+	1+	1+	±	-	-
C-IV*	3+	2+	2+	2+	2+	1+
C-V	±	-	-	-	-	-
	Concentration of fractions in µg/ml			
	100	10	1.0	0.1
WSG	3+	2+	1+	-

*The fraction was rechromatographed on Sephadex G-75 before its analysis.

as the parent fraction. Consequently, it can be concluded that fraction C-IV was more active than the whole pollen extract by a factor of 100,000 and more than its parent fraction by a factor of 100. The marked increase in the specific biological activity of fraction C-IV suggests that this fraction contained highly purified components of WSG.

Determination of desensitizing dose

From the results illustrated in Table XI, it is evident that fraction A was capable of eliciting skin reactions even at the low concentration of 12.5 μg/ml. On rechallenge of the same sites 24 hours later with the same fractions at a concentration of 500 μg/ml, it was shown that while fraction A did not elicit further skin reaction in the site challenged previously with this fraction at a concentration of 200 μg/ml, all the other sites injected with lower concentrations of the fraction gave positive reactions. Therefore, 200 μg/ml of fraction A was considered to represent the desensitizing dose of this fraction. By the same reasoning, it was concluded that the desensitizing dose for fractions C and C-IV was 50 μg/ml each, and for fractions R and WSG 200 μg/ml each.

Cross-neutralization experiments using Prausnitz-Küstner passive transfer tests

The results of cross-neutralization experiments, illustrated in Table XII, demonstrate the allergenic identity of fractions C, C-IV, R and WSG as contrasted with that of fraction A. Thus, sites I-V, injected with the desensitizing dose of fraction A, gave positive reactions, and when these sites (II-V) were reinjected with other fractions they gave further positive reactions. This was considered to indicate that fraction A lacked some

TABLE XI

Determination of the desensitizing dose of fractions WSG, R, A, C and C-IV.

Fractions	Skin reactions obtained in sites sensitized with allergic serum of patient 'Na' (ten-fold diluted) and challenged with different fractions. Concentrations of fractions in µg/ml				
	200	100	50	25	12.5
A	2+	2+	2+	2+	2+
C	2+	2+	2+	2+	2+
C-IV*	2+	2+	2+	2+	2+
R	2+	2+	2+	2+	2+
WSG	2+	2+	2+	2+	2+
24 hrs later, sites were rechallenged with the same fractions of 500 µg/ml.					
A	-	±	1+	2+	3+
C	-	-	-	1+	1+
C-IV*	-	-	-	±	1+
R	-	1+	2+	2+	2+
WSG	-	1+	2+	2+	3+

*The fraction was rechromatographed on Sephadex G-75 before its analysis.

TABLE XII

Cross-neutralization experiments with different fractions.

Fractions	All sites had been sensitized with allergic serum of patient 'Na' (ten-fold diluted) and challenged with the desensitizing dose of the appropriate fraction									
	S#	SR	S#	SR	S#	SR	S#	SR	S#	SR
A	I	3+	II	3+	III	3+	IV	3+	V	3+
C	VI	3+	VII	3+	VIII	3+	IX	3+	X	3+
C-IV*	XI	3+	XII	3+	XIII	3+	XIV	3+	XV	3+
R	XVI	3+	XVII	3+	XVIII	3+	XIX	3+	XX	3+
WSG	XXI	3+	XXII	3+	XXIII	3+	XXIV	3+	XXV	3+
24 hrs later, sites were rechallenged with desensitizing dose of the same fractions.										
A	I	-	VI	-	XI	-	XVI	-	XXI	-
C	II	1+	VII	-	XII	-	XVII	-	XXII	-
C-IV*	III	1+	VIII	-	XIII	-	XVIII	-	XXIII	-
R	IV	1+	IX	-	XIV	-	XIX	-	XXIV	-
WSG	V	1+	X	-	XV	-	XX	-	XXV	-

*The fraction was rechromatographed on Sephadex G-75 before its analysis.

S = Site; SR = Skin Reaction.

of the active components of other fractions. Sites VI-XXV, when injected with fractions C, C-IV, R and WSG in their desensitizing doses, did not give any further reactions on rechallenge with a higher dose, indicating thus the presence of all skin active components of WSG in fractions C, C-IV and R.

SECTION C. IMMUNOLOGICAL PROPERTIES OF DIFFERENT FRACTIONS

INTRODUCTION

In this section are described the antigenic properties as deduced from immunodiffusion and immunoelectrophoretic experiments.

METHODS AND MATERIALS

Production of rabbit anti-sera

Antigens. WSG, the dialyzed "ammonium sulphate precipitate", and the dialyzed phosphate buffer extract of timothy grass pollen (the last preparation is described in Chapter V) were used as antigens. The antigens were dissolved in saline (10 mg/ml) and the solutions were sterilized by filtration through sterile Millipore membranes. Equal volumes (5 ml) of each antigen solution and of complete Freund's adjuvant (Difco Laboratories, Detroit, Michigan) were transferred to a previously sterilized emulsifying syringe (MulsiJet, Inc., Elmhurst, Illinois) and the mixture was emulsified for half an hour. The emulsion was kept overnight at $4^{\circ}C$ to ensure the stability of the emulsion. The stable emulsion was then used for immunization.

Immunization schedule. Three groups of three rabbits each were immunized with each of the three different antigens according to the following schedule. Each rabbit received a dose of 0.4 ml of the emulsified antigens per foot pad. Animals were then allowed to rest for at least two weeks. The presence of antibodies in the sera of these animals was established by ring test and/or hemagglutination. When antibodies were

detected in sufficiently high titer (about 2,000), 40 ml of blood was collected from the appropriate rabbit from the marginal ear vein. Five days later another 40 ml of blood was collected in the same way. The animals were then allowed to rest for 3-4 weeks and given a second course of immunization consisting of a series of intramuscular injections (1 ml per rabbit per week for three weeks). All rabbit antisera were sterilized by filtration through sterile Seitz pads and stored at 4°C.

Ring test

The "ring test" was performed in 6 x 50 mm tubes. The antiserum was placed in the bottom of the tube and the antigen was carefully layered on top of the serum; a white precipitate in the form of a filled "ring" formed at the interface if the antiserum contained sufficient antibodies.

The hemagglutination test

Erythrocytes. Rabbit erythrocytes were used for the hemagglutination technique. For this purpose blood was collected from the marginal vein into an equal volume of Alsever's solution (169) from rabbits which had not been previously bled or which had been rested for at least six weeks after an earlier bleeding. If the erythrocytes were obtained from rabbits bled within a shorter interval, false hemagglutination occurred. For storage of erythrocytes the blood in the Alsever's solution was kept at 4°C for as long as 10 days.

Diluent. The serum used as diluent was separated from the blood of the same rabbit from which the erythrocytes were obtained. After inactivation of complement by heating at 56°C for 30 minutes, it was diluted

100-fold with phosphate buffer at pH 7.3.

Bis-diazotized-benzidine (BDB). This was prepared according to the method used previously in this laboratory (170). For this purpose, 0.46 g of benzidine was dissolved in 90 ml of 0.2N HCl and brought to $0^{\circ}C$ in an ice bath. Similarly, 0.35 g of $NaNO_2$ in 10 ml of distilled water was brought to $0^{\circ}C$, and added dropwisely to the benzidine solution (over a period of 5 minutes), care being taken to keep the temperature under $5^{\circ}C$. This solution was checked at several intervals over a period of 30 minutes with starch iodide paper for excess nitrous acid, indicated by the blue colour. The almost colourless or occasionally pale yellow BDB preparation was divided into a set of vials (1 ml per vial) at $0^{\circ}C$, and then quickly frozen at $-78^{\circ}C$ in acetone-dry ice bath and stored at about $-20^{\circ}C$ until use.

Before coupling the antigen to erythrocytes, the vial of the frozen BDB was thawed in the palm and added to 14 ml of cold phosphate buffer at pH 7.3. This solution will be referred to hereafter as BDB-phosphate solution; this solution must be used as soon as possible because it deteriorates quickly, resulting in the formation of a brown product.

Sensitization of the erythrocytes. The rabbit red blood cells were washed three times with cold physiological saline and the packed cells were resuspended in an equal volume of saline. The optimal quantity of the antigen was then placed into a 15 ml centrifuge tube and mixed with 0.1 ml of the 50% red cell suspension. Finally, the optimal amount of BDB-phosphate solution (freshly prepared) was added, and the mixture inverted several times to ensure even mixing. The reaction was allowed to proceed at room temperature for 15 minutes with occasional stirring. "Sensitized"

cells were separated by centrifugation and the brownish supernatant was discarded. The cells were washed with 3.5 ml of diluent and resuspended to a final volume of 2.5 ml. This will be referred to as sensitized cell suspension.

Standardization of the method. To establish the optimal ratio of each batch of BDB for each of the pollen extracts, the following procedures were used. With a constant amount of erythrocytes, (i) the BDB-phosphate volume was varied keeping the antigen concentration constant, and (ii) the antigen concentration was varied and the BDB-phosphate volume was kept constant. In addition, two controls were used, viz. each batch of sensitized cells was tested (i) with normal rabbit serum and (ii) with the appropriate rabbit antisera. The ratio of BDB-phosphate solution to that of antigen was considered optimal when the highest titer was obtained with a given immune serum and when no reaction occurred with normal sera.

If insufficient antigen was used, the erythrocytes became coupled to each other by BDB and gave false positive patterns in the diluent. On the other hand, if too much antigen was used, the sensitivity of the method was lowered; presumably in this case the antigen molecules were polymerized by reaction with BDB and were not available for attachment to the erythrocytes.

Performance of the hemagglutination test. The test was performed in a micro-hemagglutination plate supplied by Cooke Engineering Co., Medical Research Division, Alexandria, Virginia. To remove non-specific agglutinins, all sera were absorbed out with an equal volume of packed, non-sensitized rabbit erythrocytes (washed three times with cold saline) for 1-2 hours at room temperature or overnight in the cold. The cells used for absorption and sensitization were always from the same rabbit.

The absorbed antisera were serially diluted in halving dilutions with diluent, each well of the hemagglutination plate containing 0.05 ml of the diluent.

A volume of 0.025 ml of the sensitized cell suspension was added to each well. The hemagglutination plate was gently shaken to disperse the cells. The reaction proceeded at room temperature and could be read after a minimum of 3 hours. Usually they were read after 12 hours, though the patterns remained unchanged for at least 30 hours, or for several days in the cold. The hemagglutination patterns were considered negative if a discrete compact red button, with or without a clear dot at the center was formed, and as positive if a gelatinous layer spreading across the bottom of the tube was obtained.

The titer of the antiserum was expressed as the reciprocal of the highest dilution of antiserum which gave positive pattern. Because the end-point of the agglutination is somewhat subjective, each reading was compared against a control row of sensitized cells in diluent.

Immunodiffusion. This was performed as described in Chapter III.

Immunoelectrophoresis. It was carried out as described in the preceding chapter.

RESULTS

The data presented in Table XIII illustrate the optimal ratio of BDB-phosphate and antigen concentration needed for successful hemagglutination. As expected, high amounts of BDB-phosphate gave no hemagglutination patterns. Similarly, too high and too low a concentration of antigen solution gave no hemagglutination. Thus, WSG at concentrations of 2.25 mg/3.6 ml and

TABLE XIII

Standardization of hemagglutination test using WSG as antigen.

50% RBC	BDB-phosphate	Antigens	Hemagglutination titer
0.1 ml	1.5 ml	1.5 mg in 2 ml saline	nil
0.1 ml	1.5 ml	1.0 mg in 2 ml saline	nil
0.1 ml	1.5 ml	0.5 mg in 2 ml saline	nil
0.1 ml	1.0 ml	1.5 mg in 2.5 ml saline	nil
0.1 ml	1.0 ml	1.0 mg in 2.5 ml saline	nil
0.1 ml	1.0 ml	0.5 mg in 2.5 ml saline	nil
0.1 ml	0.5 ml	1.5 mg in 3 ml saline	nil
0.1 ml	0.5 ml	1.0 mg in 3 ml saline	nil
0.1 ml	0.5 ml	0.5 mg in 3 ml saline	nil
0.1 ml	0.25 ml	2.25 mg in 3.25 ml saline	nil
0.1 ml	0.25 ml	2.0 mg in 3.25 ml saline	526
0.1 ml	0.25 ml	1.75 mg in 3.25 ml saline	2048
0.1 ml	0.25 ml	1.50 mg in 3.25 ml saline	4096
0.1 ml	0.25 ml	1.25 mg in 3.25 ml saline	263
0.1 ml	0.25 ml	1.00 mg in 3.25 ml saline	nil

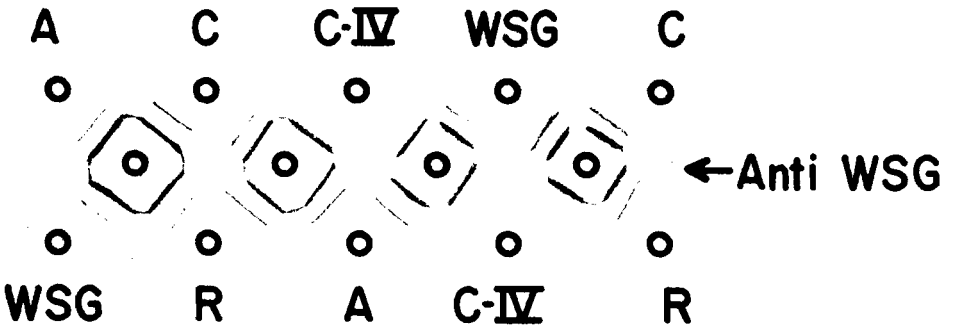
1.0 mg/3.6 ml gave no hemagglutination reactions with the antisera. WSG at a concentration of 1.5 mg/3.6 ml gave the highest titer of 2^{11} (i.e. 4096) for the antisera used in the standardization procedure and, therefore, this concentration of antigen was considered to be optimal in conjunction with 0.25 ml BDB-phosphate.

It was found that during the first course of immunization, the antisera obtained from rabbits immunized with WSG failed to give visible "ring tests" and the hemagglutination titers were only between 200-1000. On the other hand, the antisera obtained from rabbits immunized with dialyzed phosphate buffer extract or ammonium sulphate precipitate gave a high hemagglutination titer ranging from 11,000-22,000. During the second course of immunization, however, similar high titers were reached with antisera obtained from rabbits immunized with WSG. The results presented here are in good agreement with those of Campbell et al (171) who found that best antisera were obtained by immunizing the rabbits with dialyzed residues of pollen extracts.

Figure 14 shows a typical Ouchterlony precipitin pattern of different fractions developed against rabbit anti-WSG antiserum. All fractions excepting C-IV reacted with the antiserum to develop the typical pattern of precipitin bands which was always observed with the whole pollen extract, namely the thin outer line and the thick inner line. Fraction C-IV, however, gave only the thick inner line which merged with the inner line of the other fractions. This result demonstrates that although fraction C-IV had contained all the skin active components of the whole pollen extract, it contained only some of the antigenic component(s) of the whole pollen extract.

FIGURE 14

Immunodiffusion patterns in agar gel. The central wells were filled with rabbit anti-WSG antiserum. The outer wells were filled with different fractions at 1%.


A
C
C-Ⅳ
WSG
C
Anti WSG
WSG
R
A
C-Ⅳ
R

A more detailed analysis of the antigenic make-up of these fractions was provided by their immunoelectrophoretic pattern, as shown in Figure 15. Six to seven antigenic components were readily demonstrated in WSG. In general only six antigenic components were detected in WSG. The seventh component had zero mobility in the buffer used and was observed near the antiserum trough only if the distance between the well and trough was increased; however, under such conditions, some weakly reacting antigenic components (the component near the antigenic well and the component having the highest cathodic mobility) did not show up. The separation of the antigenic components of WSG was poorer when the electrophoresis was done in tris-HCl buffer at pH 7.9.

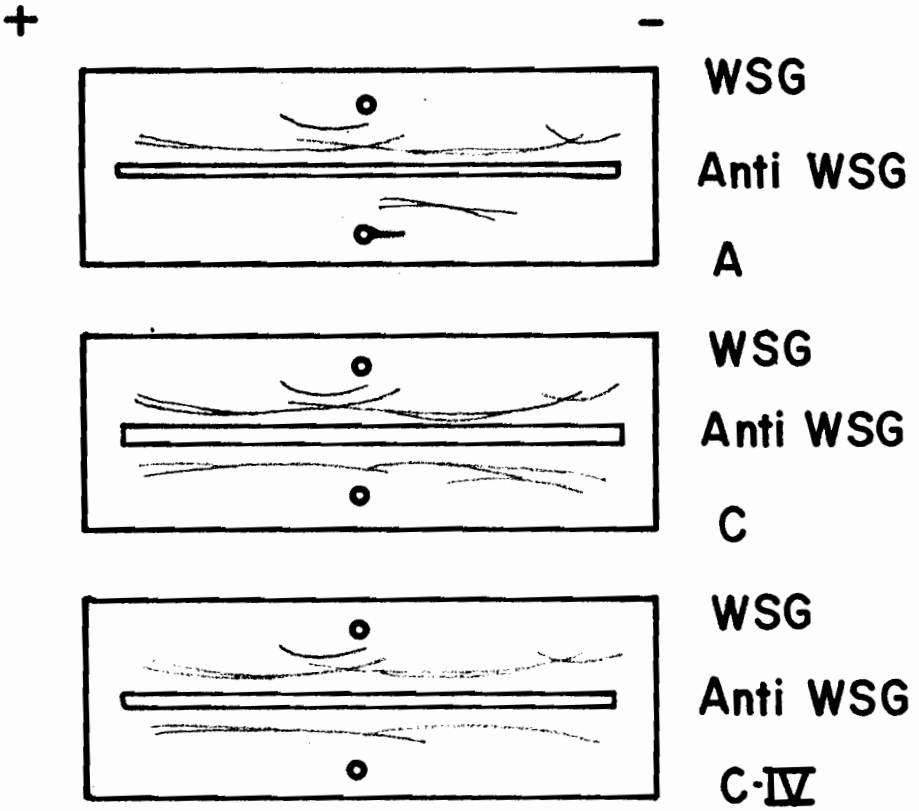
For immunoelectrophoretic analysis various concentrations of the different fractions were used and it was observed that, excepting WSG, all other fractions exhibited the same pattern throughout the concentration range investigated. For WSG, the minimum concentration required to give a clear immunoelectrophoretic pattern was 7.5% (w/v). The reason for such a high concentration was considered to be due to dialysable components which constituted 89% of WSG, and which were shown to inhibit the precipitin bands (discussed in Chapter V).

Fraction A was shown to have 2-3 antigenic components all moving towards the cathode. Fraction C was shown to have at least 5 antigenic components with three moving towards the cathode and two moving towards the anode. Like fraction A, fraction C-IV contained three antigenic components but unlike fraction A, two of them moved towards the anode.

From the results of immunodiffusion and immunoelectrophoretic analysis it was concluded that fraction C-IV contained only some of the antigenic

FIGURE 15

Immunoelectrophoretic patterns of WSG (7.5% w/v) and of its chromatographic fractions (1% w/v). Immunoelectrophoretic analysis was done in Veronal buffer, pH 8.6, for 50 minutes and the patterns were developed with rabbit anti-WSG antiserum (H.A. = 22,072).


+
-
WSG
Anti WSG
A
WSG
Anti WSG
C
WSG
Anti WSG
C-IV

components of the whole pollen extract and that these were highly heterogeneous as demonstrated by their wide range of electrophoretic mobilities.

SECTION D. PHYSICOCHEMICAL CHARACTERIZATION OF DIFFERENT FRACTIONS

INTRODUCTION

In the preceding sections the isolation, fractionation and the biological and immunological properties of the fractions were reported. In this section the further characterization of the fractions by physico-chemical techniques, such as paper chromatograpny, absorption spectroscopy and ultracentrifugation, will be described.

METHODS AND MATERIALS

Carbohydrate analysis

Detection of carbohydrate by spot tests (172). The test involves the following set of reactions. Carbohydrates are hydrolysed by heating with strong mineral acids or with oxalic acid, which leads to the formation of monosaccharides. On further heating, the monosaccharides are partially dehydrated to furfural or similar aldehydes such as hydroxy methyl furfural in the case of hexoses. These aldehydes are volatile on steam distillation and react with aniline to give violet products.

Procedure. A pinch of the sample was placed in a microcrucible. One drop of syrupy phosphoric acid was added, and the crucible was covered with a disc of filter paper moistened with aniline acetate solution (10% aniline in 10% glacial acetic acid). A watch glass was used as paper weight. The bottom of the crucible was cautiously heated for 30 seconds with a microflame.

Identification of the sugar by paper chromatography (173). A portion of 10 mg of the sample was treated with 2 ml of 72% H_2SO_4 at 40°C for 30 minutes and then diluted 25 times with water and heated for 1 hour under 15 lb pressure. After the hydrolysis, solid $BaCO_3$ was added to neutralize the acidic solution. The hydrolysate was then filtered through celite. The filtrate thus obtained was shaken with cation exchange resin (Rexyn (H) 101, about 10 g) and then filtered. The filtrate was concentrated in vacuo and the concentrated solution was examined by descending paper chromatography at room temperature as follows. Two sheets of chromatographic paper (70 x 20 cm) were used:- one chromatography was performed with the solvent system: ethyl acetate: acetic acid: water (9:2:2) for 18 hours, which allows separation of certain sugars, such as xylulose and ribulose, and the other chromatography was performed for 22 hours with the solvent system: ethyl acetate: pyridine: water (8:2:1), which allows the separations of sugars like glucose, fructose, galactose and mannose, but the fast moving sugars such as xylulose run off the paper.

For each experiment, the hydrolysed sample and a standard mixture of known sugars, were applied side by side. After the completion of the chromatographic separation, the paper was dried and then sprayed with O-aminodiphenyl (3.0 g of O-aminodiphenyl was dissolved in acetic acid, 100 ml, to which was added 1.3 ml of 85% reagent grade phosphoric acid). The unknown sugar could be thus identified by reference to the standards.

Quantitative estimation of carbohydrate by orcinol method (174). (i) The test solution was prepared by dissolving 0.1 mg of the sample in 1 ml distilled water. (ii) The detecting reagent was made by dissolving 2.0 g of orcinol (commercially available orcinol was crystallized

from benzene before use) and 1.359 g of ferric ammonium sulphate in 25 ml distilled water, to which 415 ml HCl was added and made up to a final volume of 500 ml with distilled water.

Procedure. A volume of 3 ml of the reagent was added to each Kjeldahl flask containing 1 ml of the test solution. The mixture was heated in a boiling water bath for 20 minutes and then cooled to room temperature; the optical density of the mixtures was determined at 660 mμ. A blank containing 3 ml of the reagent and 1 ml distilled water was treated under identical conditions. The optical densities of the mixtures were obtained by subtracting the reading of the blank from the observed optical densities of the mixtures. Similarly, a standard curve for the orcinol reaction of arabinose was prepared by plotting the optical densities against various amounts of arabinose. The carbohydrate content of the sample was then calculated by converting its optical density into the amount of arabinose.

Determination of protein content

The protein content of each fraction was determined by the micro-Kjeldahl procedure adopted by McKenzie et al (175). Each sample was digested with 0.75 ml of concentrated H_2SO_4 containing about 700 mg of K_2SO_4 plus 0.25 ml $HgSO_4$ (20 g of HgO dissolved in 200 ml of 4N H_2SO_4), the protein nitrogen being converted to $(NH_4)_2SO_4$. To each Kjeldahl flask was added 5 ml of 40% NaOH and the ammonia liberated was steam distilled into a solution of boric acid containing methyl red and methylene blue (6 g of boric acid + 10 ml of 0.2% methyl red: 0.2% methylene blue (2:1) per 1 liter) as an indicator. Titration was performed with 0.0003M $KH(IO_3)_2$ solution.

Absorption spectroscopy

Each sample was dissolved in 0.01M phosphate buffer, pH 7.0, to a concentration of 0.05% and its absorption spectrum was determined in the U.V. region.

Ultracentrifugation

Ultracentrifugation was performed as described in the preceding chapter.

RESULTS

Carbohydrate analysis

The spot tests revealed the presence of carbohydrate in all fractions. Thus, WSG, fraction I, ammonium sulphate precipitate and fraction A gave a pink colour within 30 seconds; in other fractions, particularly in C and C-IV, the colour appeared only after 90 seconds and was usually faint.

The sugar unit of the carbohydrate moiety of the various chromatographic fractions was identified as arabinose.

Carbohydrate and protein contents of various fractions

The data in Table XIV give the carbohydrate and protein contents of various fractions. Thus, fractions A and C-IV were shown to contain the highest and the least amounts of carbohydrate, respectively, but the latter contained the highest amount of protein. From these data it was concluded that fractions A and B had the properties of glycoproteins and fractions C and C-IV could be considered as proteins.*

*As will be shown (discussed in Section E), fraction C-IV was found to have a higher protein content than obtained by the method described in this section.

FIGURE 16

Standard curve for the orcinol reaction for arabinose.

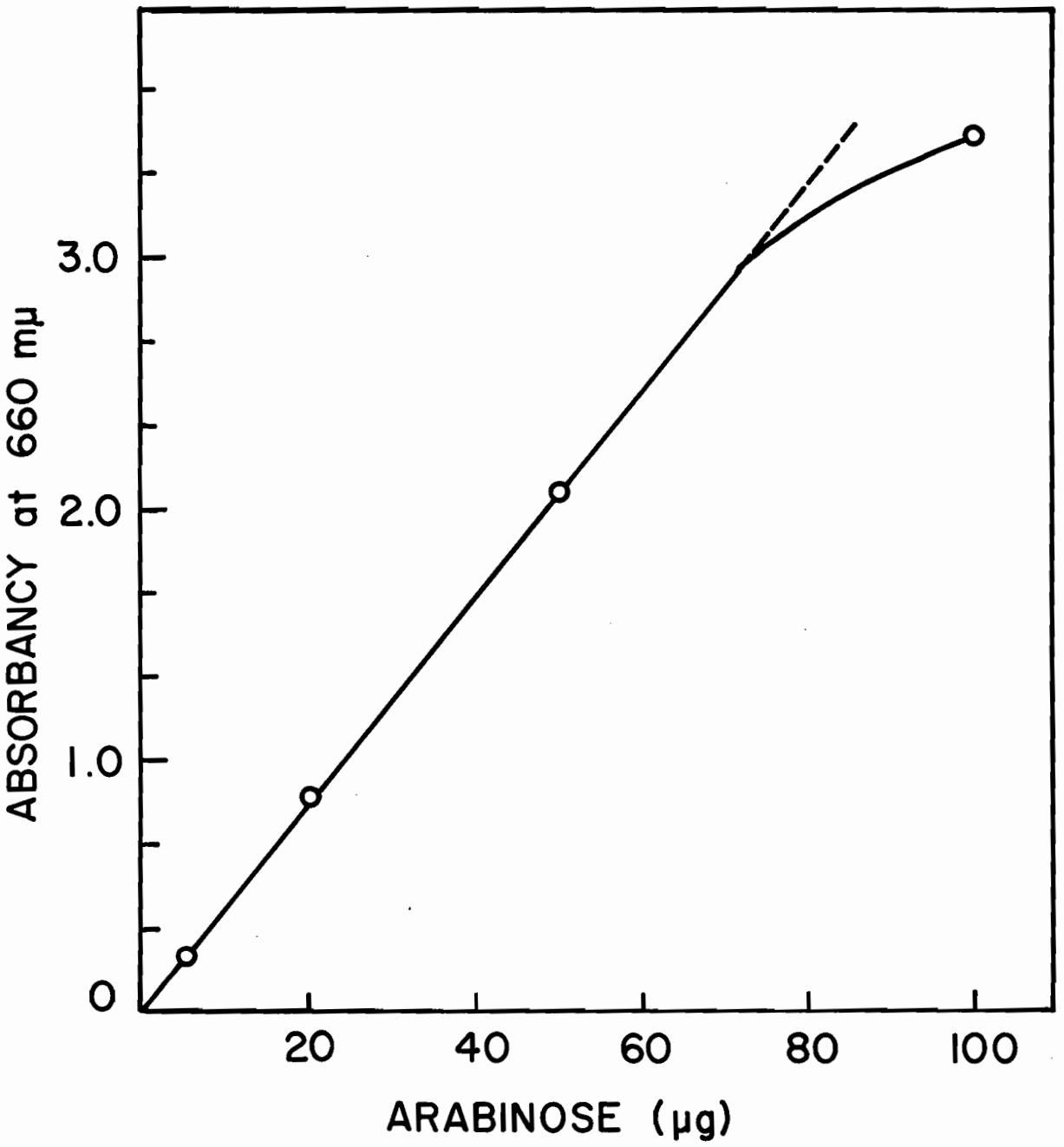

ABSORBANCY at 660 mμ
3.0
2.0
1.0
0
20
40
60
80
100
ARABINOSE (μg)

TABLE XIV

Fraction	%Nitrogen	%Protein*	%Carbohydrate
WSG	2.6	-	-
Ammonium sulphate precipitate	11.25	-	-
I	9.6	62.4	-
A	5.6	36.4	35.0
B	8.6	55.6	18.0
C	10.7	69.55	4.1
D	9.6	62.4	-
C-IV	13.1	85.2	2.2

*The nitrogen values were multiplied by the factor of 6.5.

Absorption spectra

Figure 17 shows the absorption spectra of some of the purified fractions and of α-chymotrypsin. It is evident from these spectra that both the glyco-protein and protein fractions had characteristic absorption patterns of proteins with an absorption maximum at 280 mμ.

Ultracentrifugation

Figures 18-21 represent the typical sedimentation patterns of the fractions. Only fraction C-IV was shown to be fairly homogeneous. Thus, after 48 minutes of centrifugation the sedimentation pattern of fraction C-IV (Figure 20) was still symmetrical.

The sedimentation coefficients of various fractions were found to lie in the region of 1.5-2.0S. (Table XV).

FIGURE 17

U.V. absorption spectra of fractions at concentrations of 0.05% in 0.01M phosphate buffer, pH 7.3.

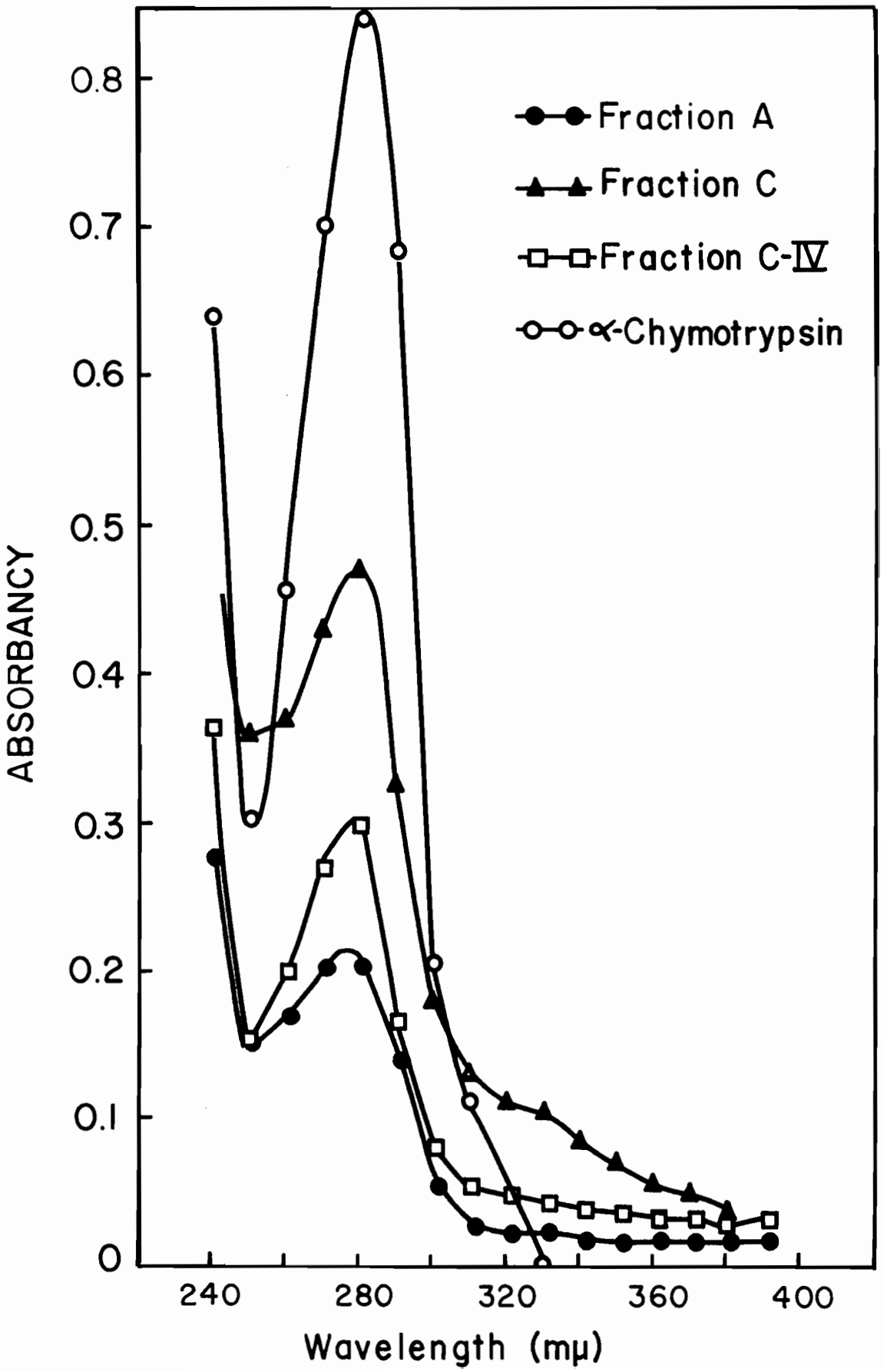

Fraction A
Fraction C
Fraction C-Ⅳ
α-Chymotrypsin
ABSORBANCY
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
240
280
320
360
400
Wavelength (mμ)

TABLE XV

Sedimentation coefficients of fractions.

Fraction	Sedimentation coefficients
WSG	1.5 S
R	1.9 S
A	2.0 S
B	1.7 S
C	1.5 S
C-IV	1.5 S

FIGURE 18

Sedimentation patterns for fractions A and B in saline; concentration = 0.6%; centrifugation at 59,780 r.p.m.; frames taken at intervals of 8 minutes.

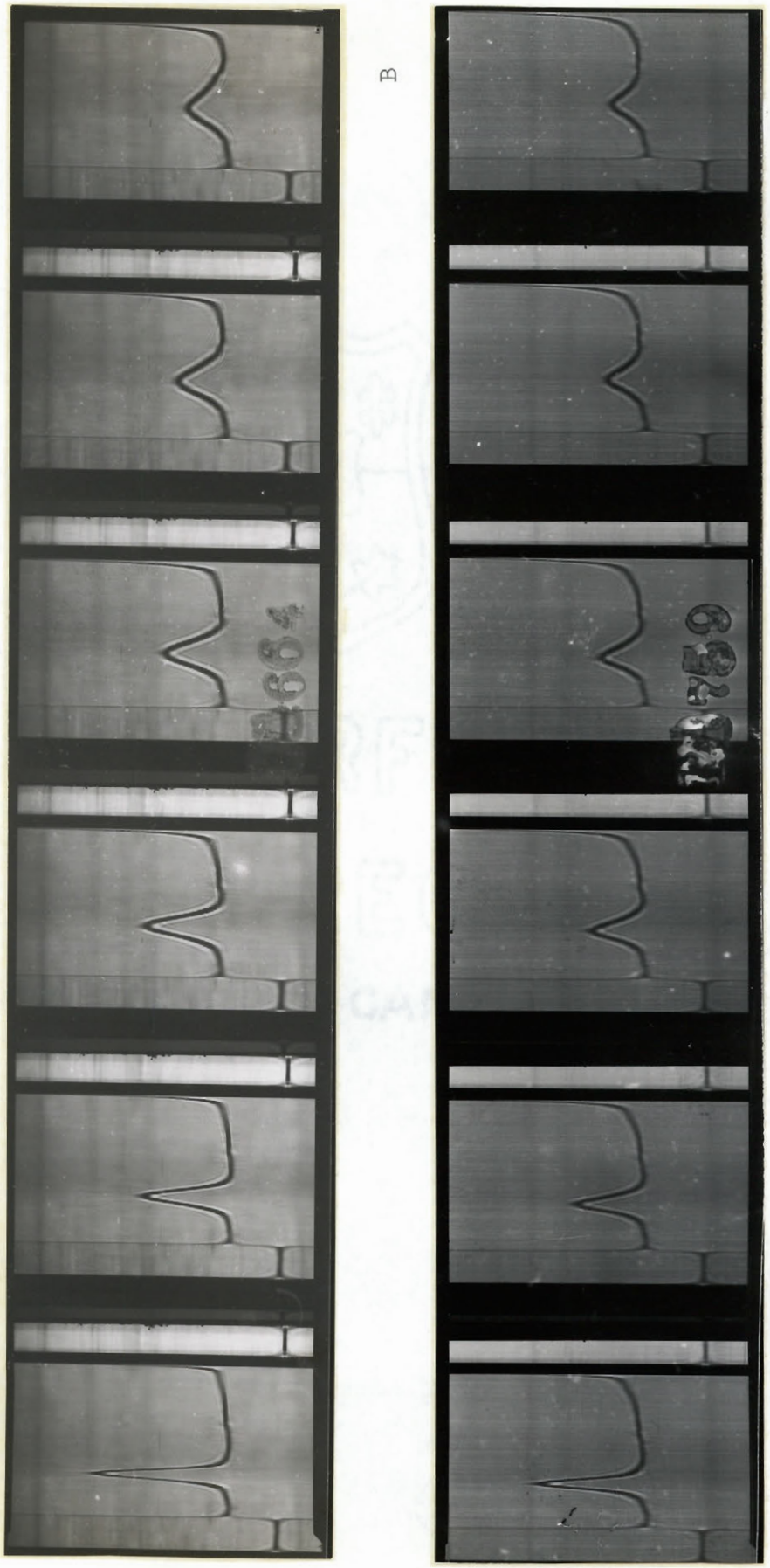
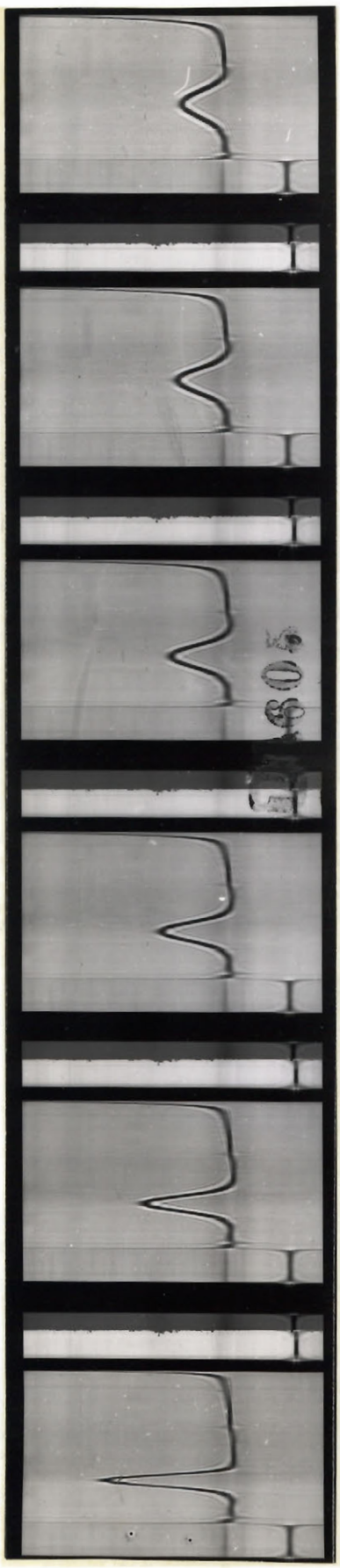

A
B
B664
B679

FIGURE 19

Sedimentation pattern for fraction C (0.6%); centrifugation at 59,780 r.p.m.; frames at intervals of 8 minutes.


605

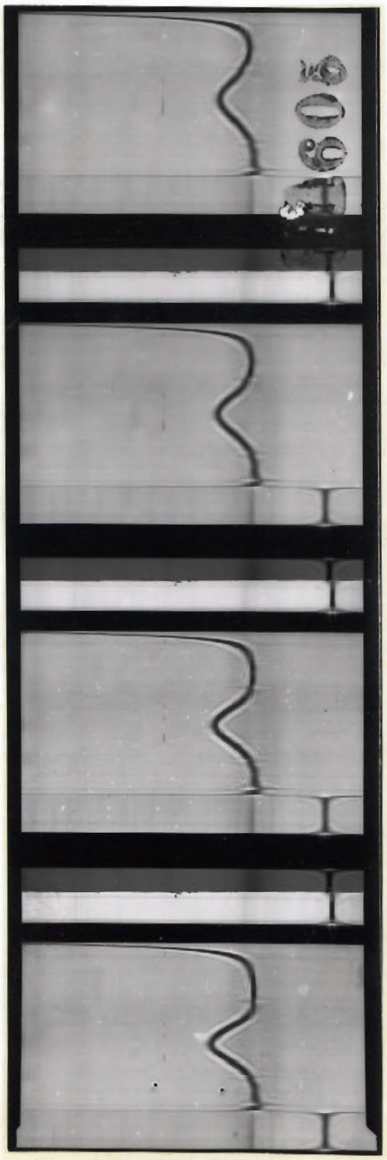

605

FIGURE 20

Sedimentation pattern for fraction C-IV (0.8%); centrifugation at 59,780 r.p.m.; frames at intervals of 8 minutes.

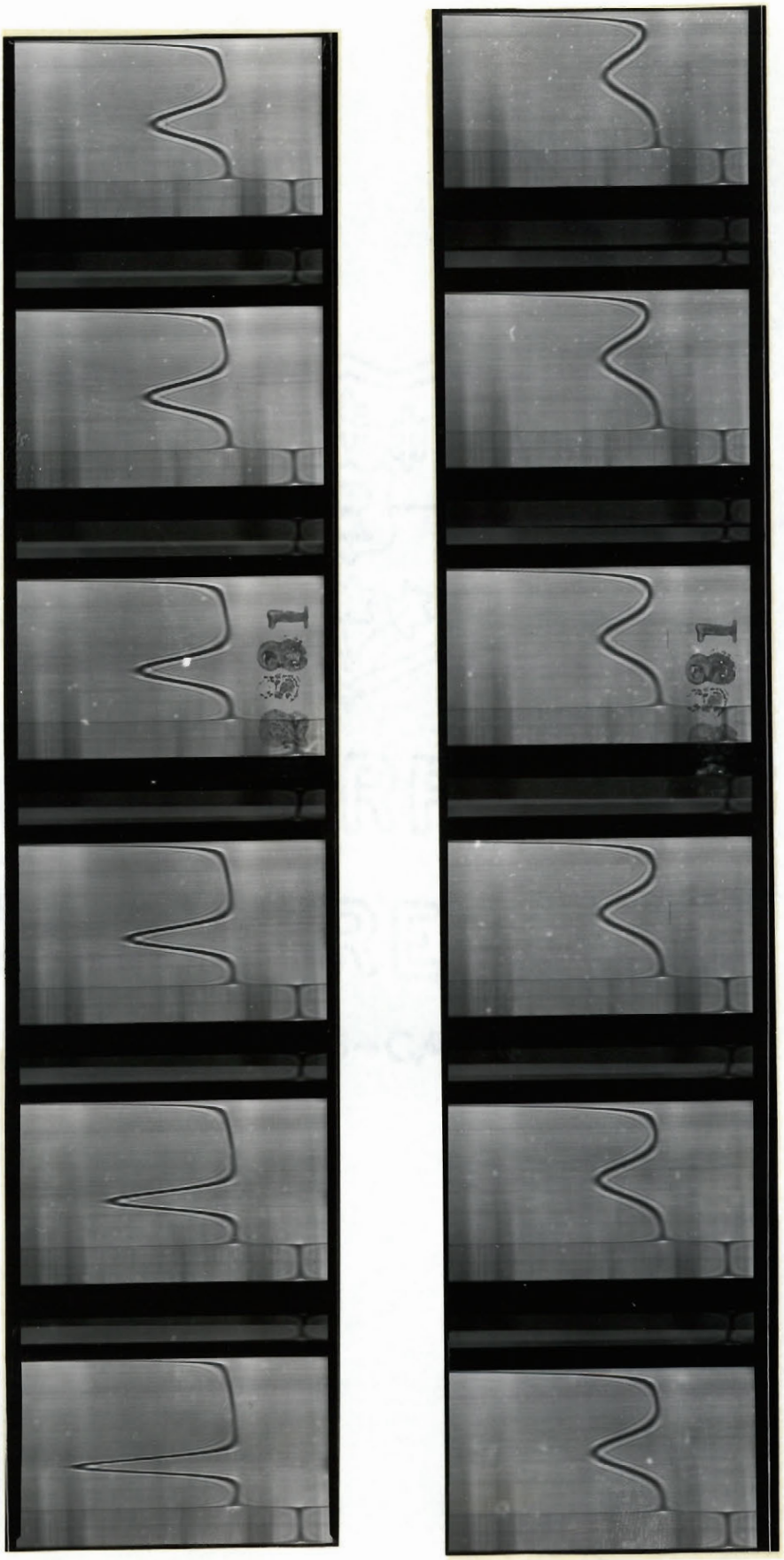
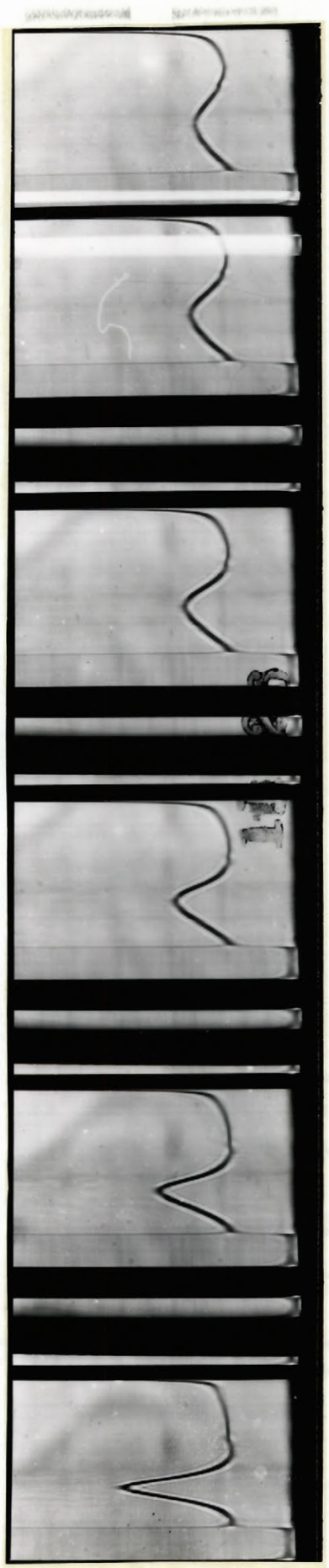


FIGURE 21

Sedimentation pattern for WSG (0.6%); centrifugation at 59,780 r.p.m.; frames at intervals of 8 minutes.



SECTION E. AMINO ACID COMPOSITION OF THE FRACTIONS

INTRODUCTION

In this section is described the analysis of amino acid composition of the fractions employing the automated procedure developed originally by Moore et al. (176).

METHODS AND MATERIALS

A brief description of the analyser

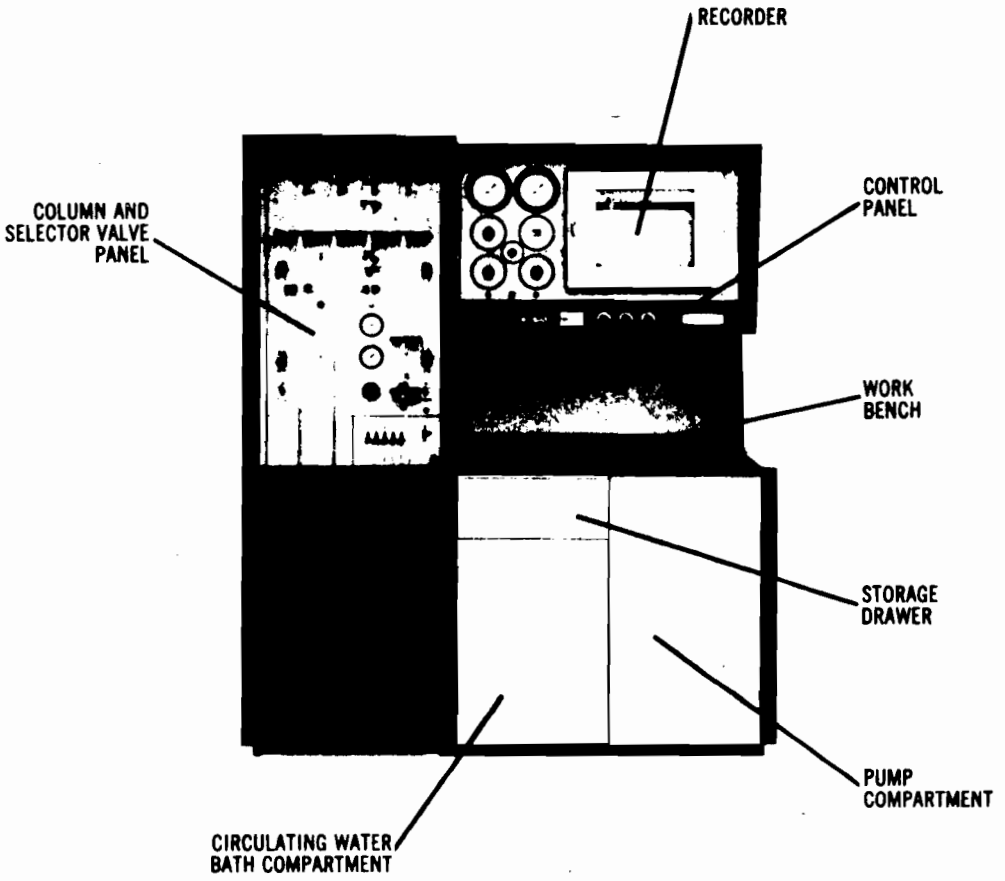
All amino acid analyses were done with the Beckman automatic amino acid analyser (Model 120 B), shown in Figure 22. The instrument includes storage facilities for all reagents used during the analytical run, complete apparatus for the automatic separation and for the quantitative analysis, a multipoint recorder, the pumping system, and controls for selecting and establishing the operating and timing parameters for a complete variety of types of analyses.

Principle of separation of amino acids

The separation of the amino acids in a sample is effected by chromatography on a column of the sodium salt of a polysulphonic acid resin, the cation exchanger. When an amino acid is placed on top of the column, ion exchange takes place between the Na^+ of the resin and the positively charged amino group of the amino acid. This is a reversible reaction and equilibrium is established. Under a given set of conditions (such as

FIGURE 22

Beckman Automatic Amino Acid Analyser, Model 120 B (from Beckman manual).


RECORDER
CONTROL PANEL
COLUMN AND SELECTOR VALVE PANEL
WORK BENCH
STORAGE DRAWER
PUMP COMPARTMENT
CIRCULATING WATER BATH COMPARTMENT

chemical composition, resin particle size and resin pore size, diameter and length of the packed column, pH and ionic strength, rate of flow of the eluting buffer and temperature), the amount of a given quantity of an amino acid which is bound to the ion-exchange resin, relative to that remaining in solution at equilibrium, is usually expressed by a distribution coefficient, K. The value of K for a given amino acid depends on the structure of the individual amino acid. As the eluting buffer is pumped onto the column, the amino acid in solution filters down the column and consequently the equilibrium on the top of the column is disturbed, resulting in the release of more of the amino acid from the resin and in the re-establishment of a new equilibrium. This process repeats itself as the amino acid travels down the column. Providing the capacity of the resin is not exceeded with an appropriate set of conditions, the amino acids in a sample are separated from each other by the time they emerge from the column.

Preparation of buffers

All buffers were prepared in deionized water according to the recipes recommended by Beckman Instrument Co. (Table XVI). Prior to use, portions of the appropriate buffers were filtered and then placed in the reservoirs of the Model 120 B Analyser. Buffers were usually prepared on a large scale (40 liters) and stored at room temperature by adding octanoic acid as a preservative. The presence of thiodiglycol (TG) in the buffers of pH 2.2, 3.28 and 4.25 helped to minimize the conversion of small amounts of methionine to methionine sulphoxide during the addition of the sample to the column. For uniform drop size polyoxyethylene lauryl alcohol

TABLE XVI

Sodium citrate buffers.

Reagents	pH 2.2(±0.03) sample dilutor	3.28(±0.01) long column	4.25(±0.02) long column	5.28(±0.02) short column	3.28(±0.01) filling buffer
Sodium conc.	0.2N	0.2N	0.2N	0.35N	0.20N
Sodium Citrate	19.6 g	784.3 g	784.3 g	1372.6 g	19.6 g
Concentrated HCl	16.5ml	493 ml	335 ml	260 ml	12.3 ml
Thiodiglycol (TG)	20 ml	200 ml	200 ml	-	20 ml
BRIJ-35 solution	2 ml	80 ml	80 ml	80 ml	2 ml
Octanoic acid	0.1 ml	4 ml	4 ml	4 ml	0.1 ml
Final volume	1 liter	40 liters	40 liters	40 liters	1 liter

(BRIJ-35) (50 g of the solid detergent dissolved in 100 ml of hot water) was added to the buffers.

Preparation of the ninhydrin solution

The 4N sodium acetate buffer was prepared by dissolving 1,008 g of $NaOAc.3H_2O$ in one liter of de-ionized water in a 2-liter volumetric flask by stirring overnight. Then 200 ml of glacial acetic acid was added and the flask was filled with water almost to the mark. The pH of the solution was expected to be at 5.5 ± 0.03. For final adjustment of the pH, pellets of NaOH were added (5 g of NaOH corresponded to about 0.04 pH units) and the solution was made up to 2 liters.

Because peroxides quantitatively destroy the reducing power of stannous chloride, their presence in methyl cellosolve was checked by mixing 3 ml of this solvent with 3 ml of a 4% aqueous solution of potassium iodide. In absence of peroxides, the resulting solution should be colourless. If there was a slight yellow colour observed, the methyl cellosolve was discarded.

Three liters of filtered peroxide-free cellosolve was added to one liter of filtered 4N sodium acetate buffer. The resulting mixture was stirred magnetically while N_2 was bubbled through for 15 minutes. Eighty grams of ninhydrin and 1.60 g of $SnCl_2.2H_2O$ were added; stirring was continued until complete dissolution of reagents. The ninhydrin solution thus prepared was transferred to the reservoir, a 4-liter bottle, which was painted black to eliminate photochemically induced reactions.

Hydrolysis of the sample for amino acid analysis

To hydrolyze pollen fractions, a hydrolytic method was developed previously in this laboratory (149) to avoid the loss of certain amino acids which is caused by the formation of insoluble humin, a complex product of sugar and amino acid. However, in the present study, the standard method of hydrolysis was used, any loss of amino acids due to this cause being accounted for by extrapolation of the concentrations of the amino acids determined for different times of hydrolysis back to zero time.

Five mg of sample was weighed in a standard 16 x 150 mm Pyrex test tube which had previously been cleaned with chromic acid and rinsed thoroughly with de-ionized water and dried. The sample was then dissolved in 0.5 ml of de-ionized water and 0.5 ml of reagent grade of concentrated HCl was added. To remove any dissolved air, the tube was sealed onto the manifold of a vacuum system provided with a mechanical pump. The solution was then frozen by immersing it into a Dewar flask containing a mixture of dry ice and alcohol and then evacuated to a pressure less than 50 micron Hg. The sample maintained under this low pressure was thawed to release the air trapped in the frozen state. The solution was frozen again, evacuated and sealed under low pressure. The hydrolysis was continued in an oven at 110-112°C for 24, 48 and 72 hours with a duplicate for each hydrolysis period. After the hydrolysis, the tubes were chilled and opened. Hydrochloric acid was removed during lyophilization. To ensure that all HCl was completely removed, the residue was redissolved twice in one ml of distilled water and subjected again to lyophilization. Finally, the residue was dissolved in 5 ml of sodium citrate buffer of

pH 2.2. Before applying the sample on the column, the solution was centrifuged to remove any insoluble materials if present.

Preparation and operation of columns*

For the chromatographic analysis of hydrolysates two grades of sulphonated styrene-8% divinyl benzene copolymer resins: (i) particle size of 19-25 microns, and (ii) particle size of 31-41 microns, were used. For the separation of the neutral and acidic amino acids a large column (65 x 1 cm) was packed with the resin of the larger size, whilst a short column (25 x 1 cm) was packed with the resin of smaller size for the separation of the basic amino acids. Before packing, the resins were freed from fines (these are usually present due to the grinding operation in the process of manufacture of the resin, resulting in the fracture of the particles which on subsequent handling break off) by free sedimentation in the appropriate buffer subsequently to be used in the elution, except that BRIJ-35 and TG were left out. After packing the column, the flow rate was adjusted to 65 ml/hr. Before use, the columns were washed with 0.2N NaOH and then equilibrated with the appropriate buffer.

To save operational time, the analysis was first carried out in a short column. The buffer above the disc was removed and one ml of the sample solution was introduced into the column from a bent tip pipette; at the end of the delivery tube the tip was slightly touched against the wall of the column immediately above the solution surface. The sample

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* The author is indebted to Dr. N.A. Attallah for his help in operating the Automatic Amino Acid Analyser.

was forced into the column under an air pressure of 15 psi. Application of air pressure was discontinued as soon as the solution disappeared from the disc. At no time was the air bubble forced into the resin. Then it was washed in with three aliquots of 0.2 ml buffer, pH 2.2. The column was filled with the citrate buffer of pH 5.28 and the chromatography was continued. Under these conditions, all acidic and neutral amino acids were eluted as one peak. Prior to the elution of the basic amino acids, the base lines of the multipoint recorder were adjusted. The total operational time was approximately 60 minutes. As no amino acids were irreversibly retained by the column, regeneration could be easily achieved by passing the buffer through for an additional half hour.

When the short column was in operation, the sample was applied to the long column with care and, after entering the column material, the surface of the column was washed gently three times with 0.2 ml volumes of the sodium citrate buffer of pH 3.28. The column was then connected to the pump. The buffer was passed through only for two minutes and the column was allowed to stand at 56°C, ready for analysis. For the analysis of the acidic and neutral amino acids, the automatic timer was set to 90 minutes for change of buffers from the first at pH 3.28 to the second at pH 4.25.

After separation, the amino acid in the effluent was mixed with ninhydrin and the reaction was allowed to proceed in a bath system, maintained at the boiling point of water in order to ensure constant environmental factors. During the reaction, ninhydrin, with α-amino acid, participated in a deaminative oxidative decarboxylation and then condensed further to give a blue compound; the colour formed in the reaction with proline and

hydroxyproline was yellow. Using a colorimeter in conjunction with a photovoltaic cell, the colour developed was proportionally converted into electric current, which was made to drive a conventional multipoint recorder to plot the results of the analysis as absorbance versus time. To allow for two different colours developed, two separate colorimeter readings were recorded simultaneously in different colours on the chart. One photometer unit of the colorimeter measures the absorbance of proline and hydroxyproline at a wavelength of 440 mμ and the second photometer unit measures at 570 mμ for the rest of the amino acids.

Estimation of amino acids

Each amino acid had its own characteristic elution time under constant environmental factors. Thus, by comparing an unknown chromatogram with that previously obtained with a standard mixture of known amino acids, every amino acid present in the sample could be identified. In addition, the elution rate of the column was kept constant at a constant value of 68 ml/hr, while the chart travelled at a speed of 6 inches per hour. Furthermore, to facilitate the measurement along the time scale, the recorder printed a dot every 6 seconds for each curve on the chart, which was calibrated across in absorbance on a logarithmic scale from zero to infinity. Therefore, the area, which was proportional to the amount of a given amino acid, could be readily integrated on the absorbance-time graph by the height-width method. The area under the peak was determined, as recommended in the Beckman manual, by multiplying the height (H) of the peak by the width (W) which was measured at half height. The peak height could be read from the chart directly while the width was measured by

counting the dots above the width at the half height (i.e. the total time for the chart to travel along the width). The concentration of each amino acid was calculated by the following equation.

$$C_u = \frac{H_u \times W_u}{H_c \times W_c} \times C_c ,$$

where H_c, W_c and C_c are the height (absorbance), width (time) and concentration (micromole) of the standard mixture, and H_u, W_u and C_u the corresponding terms for the sample of unknown composition.

Since certain amino acids, such as serine and threonine were likely to be destroyed during the hydrolysis, their concentrations were corrected by extrapolating back to zero time for the hydrolysis; on the other hand, for the amino acids which were released later, the concentrations of these amino acids were based on the average of 48-hour hydrolysates.

Determination of tryptophan by spectrophotometric method (177)

Since tryptophan was destroyed under acid hydrolysis, it was determined spectrophotometrically. Thus, the optical densities of the sample solution (0.538 mg of the sample was dissolved in 1 ml of 0.1N NaOH) in the range of 280-360 mμ were measured. A base line was plotted through the optical densities at 320-260 mμ and was projected to the optical density at 280 mμ and the optical densities at 280 and 294.4 mμ were then obtained by subtracting the observed value from the base line plot. This eliminated the absorption due to the cloudiness of the solution. Knowing the molarity of tyrosine from the automatic amino acid analysis, the concentration of tryptophan was determined by the following formula (177):

$$\frac{\text{Molar concentration of tyrosine}}{\text{Molar concentration of tryptophan}} = \frac{0.592 \times \text{O.D.}_{\text{at } 294.4} - 0.263 \times \text{O.D.}_{\text{at } 280}}{0.263 \times \text{O.D.}_{\text{at } 280} - 0.170 \times \text{O.D.}_{\text{at } 294.4}}$$

RESULTS

Amino acid analysis revealed that serine, threonine, glycine, phenylalanine, proline, arginine and half cystine were partly destroyed during hydrolysis and, therefore, their concentrations were extrapolated to zero time of hydrolysis (Figure 23). On the other hand, the concentrations of lysine, histidine, alanine, valine, methionine, isoleucine, leucine, tyrosine, and aspartic and glutamic acids were higher after 48 hours of hydrolysis than either at 24 or 72 hours. Consequently, the reported concentrations of these amino acids were based on the average of duplicate determinations at 48 hours of hydrolysis.

Table XVII shows the amino acid composition of fractions C and C-IV. As expected, these fractions differed in their amino acid composition. The protein contents of these fractions were calculated from amino acid analyses and it was found that fraction C contained 77.5% protein and fraction C-IV contained 93.1%. These values are higher than those obtained by Kjeldahl nitrogen estimation (cf. Table XIV).

The amino acid analysis of fraction C-IV is compared with those of rye grass and ragweed pollen allergens (Table XVIII). As expected, each of three different pollen fractions was shown to have a unique amino acid composition. Most significantly, glutamic acid and aspartic acid were shown to be present in high amounts in all three different allergens.

FIGURE 23

Calculation of the concentrations of amino acids, by extrapolation to zero time, of values determined for 24, 48 and 72 hours of hydrolysis.

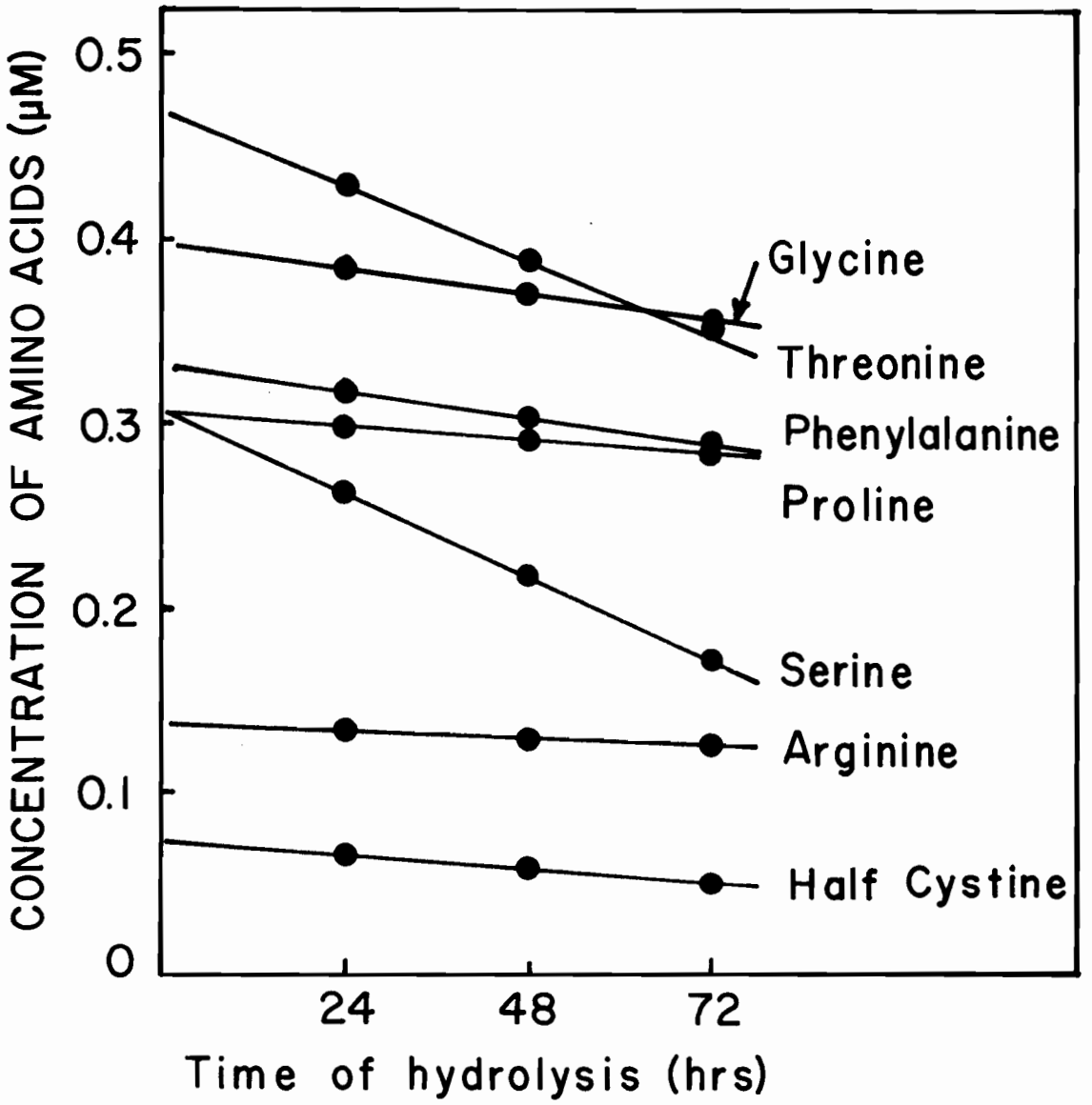
CONCENTRATION OF AMINO ACIDS (μM)
0.5
0.4
0.3
0.2
0.1
0
Glycine
Threonine
Phenylalanine
Proline
Serine
Arginine
Half Cystine
24
48
72
Time of hydrolysis (hrs)

TABLE XVII

Amino acid compositions of fractions C and C-IV.

Amino acid	Fraction C g/100 g sample	Fraction C-IV g/100 g sample
Lysine	7.3	8.8
Histidine	1.2	0.9
Arginine	3.5	2.9
Aspartic acid	8.6	7.1
Threonine	4.3	6.2
Serine	3.4	3.6
Glutamic acid	10.9	14.3
Proline	3.6	3.8
Glycine	3.1	3.0
Alanine	7.6	12.9
Half cystine	2.5	1.0
Valine	4.5	6.9
Methionine	1.3	0.6
Isoleucine	2.6	3.8
Leucine	3.8	4.7
Tyrosine	3.0	3.5
Phenylalanine	4.6	6.5
Tryptophan	1.7	2.6
Totals	77.5	93.1*

*Fraction C-IV was shown to contain 2.2% carbohydrate and the rest (i.e. 5%) may be accounted for its moisture content.

TABLE XVIII

Amino acid compositions of three different pollen allergens.

Amino acid	Timothy allergen C-IV	Rye allergen[a] α (or β)[c]	Ragweed allergen[b] IV-B (or IV-C)[c]
	g/100 g total a.a.	g/100 g total a.a.	g/100 g total a.a.
Lysine	9.5	12.8	6.1
Histidine	1.0	1.6	2.2
Arginine	3.1	3.5	6.5
Aspartic acid	7.7	11.6	15.3
Threonine	6.7	6.8	4.8
Serine	3.9	4.2	6.4
Glutamic acid	15.4	9.9	8.6
Proline	4.1	5.0	4.1
Glycine	3.2	7.1	6.5
Alanine	13.9	5.4	6.5
Half cystine	1.1	2.4	2.0
Valine	7.5	5.5	6.6
Methionine	0.7	1.0	2.5
Isoleucine	4.1	4.4	6.2
Leucine	5.0	4.0	6.5
Tyrosine	3.8	5.5	1.7
Phenylalanine	7.0	4.4	4.7
Tryptophan	2.8	4.1	2.7
Totals	100.5	99.2	99 .9

[a] based on the results of Johnson et al (141).

[b] based on the results of King et al (152).

[c] amino acid compositions of α and β and of IV-B and IV-C are identical.

SECTION F. PREPARATION OF INSOLUBLE α-CHYMOTRYPSIN AND THE EFFECT OF CHYMOTRYPTIC DIGESTION OF FRACTION C ON THE SKIN ACTIVITY

INTRODUCTION

In a parallel study in this laboratory (178) insolubilized α-chymotrypsin was prepared, as originally suggested by Katchalski (179), by coupling the enzyme to ethylene maleic anhydride copolymer (EMA). The advantage of this technique is that the insoluble enzyme can be readily separated by centrifugation from the digestion mixture containing the substrate. In this section is reported an attempt to degrade enzymatically fraction C with the insoluble α-chymotrypsin.

METHODS AND MATERIALS

Preparation of insoluble α-chymotrypsin by coupling it to ethylene-maleic-anhydride copolymer (EMA)*

A saturated solution of EMA (DX-840-91, Monsanto Canada Ltd., Lasalle, Quebec) was made by dissolving EMA in acetone with constant stirring for one hour at room temperature; the resulting solution was filtered. For this purpose, 200 mg of α-chymotrypsin was dissolved in 50 ml of saline and the pH of the solution was raised to 8.15 by adding a few drops of 1M Na_2HPO_4. Saturated EMA solution was added dropwise to the enzyme solution with vigorous stirring at $4^{\circ}C$. The precipitation of EMA-α-

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*The author is indebted to Dr. E.R. Centeno for his advice in the preparation of the insoluble enzyme.

chymotrypsin conjugate was continued for one hour. The precipitate was collected by centrifugation and washed repeatedly with saline until the optical density of the washing was zero and was finally washed with water. The precipitate was then lyophilized and stored at -20°C.

Determination of the activity of the insoluble α-chymotrypsin

The activity of the insoluble enzyme was determined according to the method of Kunitz (180) using casein as the substrate. Thus, 100 mg of casein was dissolved in 10 ml of 0.1M borate buffer, pH 8.4, and the resulting solution was filtered. The temperature of the solution was maintained at 37°C in a water bath. A suspension of insoluble α-chymotrypsin (1 mg/ml of the buffer) was made in 25 ml of the same buffer and kept stirring for 2 hours at 4°C. Different volumes of this suspension were then transferred into a series of 12 ml centrifuge tubes and the temperature was raised to 37°C as before. One ml portion of the casein solution was pipetted into each of these tubes. Different volumes of the buffer were added so that each tube contained the same volume of buffer. The reaction was then allowed to proceed for 20 minutes. After the incubation period, undigested protein was precipitated by adding 3 ml of 5% trichloroacetic acid (TCA) and the precipitates along with the insoluble enzyme were immediately removed by centrifugation. The optical densities of the supernatant at 280 mμ were read and plotted against the amount of enzyme added to the reaction mixture. The specific activity (mg of casein degraded/mg of insoluble enzyme) of the enzyme was then determined by the following equation,

$$\text{Specific Activity} = \frac{D}{E \times 20},$$

where E is the mg of enzyme added to the reaction mixture and D is the corresponding optical density of the supernatant which was read from the tangent to the curve of the enzyme activity (Figure 24) and 20 is the reaction time in minutes.

Degradation of fraction C

Equal volumes (5 ml) of enzyme suspension (2 mg/ml borate buffer, pH 8.4) and fraction C (2 mg/ml borate buffer, pH 8.4) were incubated together for 30 and 60 minutes at 37°C. Under identical conditions two controls, consisting of (1) 5 mg of fraction C in 5 ml buffer and (2) 5 mg of the insoluble enzyme in 5 ml buffer, were used. The enzyme was then removed by centrifugation and the supernatant was sterilized by passing through a sterilized Millipore membrane. The skin activity of the mixtures was determined by passive transfer tests as described earlier.

RESULTS

Figure 24 demonstrates that the insoluble enzyme preparation retained its enzymatic activity and that the extent of casein degradation depended on the amount of insolubilized enzyme added. The specific activity of the enzyme preparation was found to be 0.33 units (mg of casein degraded per mg of insoluble enzyme).

The results illustrated in Table XIX demonstrate that the skin activity of fraction C was unaffected by treatment with the insolubilized enzyme. It was also found that the use of higher amounts of this enzymatic preparation did not lead to any loss of the skin activity of fraction C.

Furthermore, incubation of the mixture for 24 hours at 37°C did not result in the reduction of the original activity.

FIGURE 24

The enzymatic activity of insoluble α-chymotrypsin with casein as substrate.

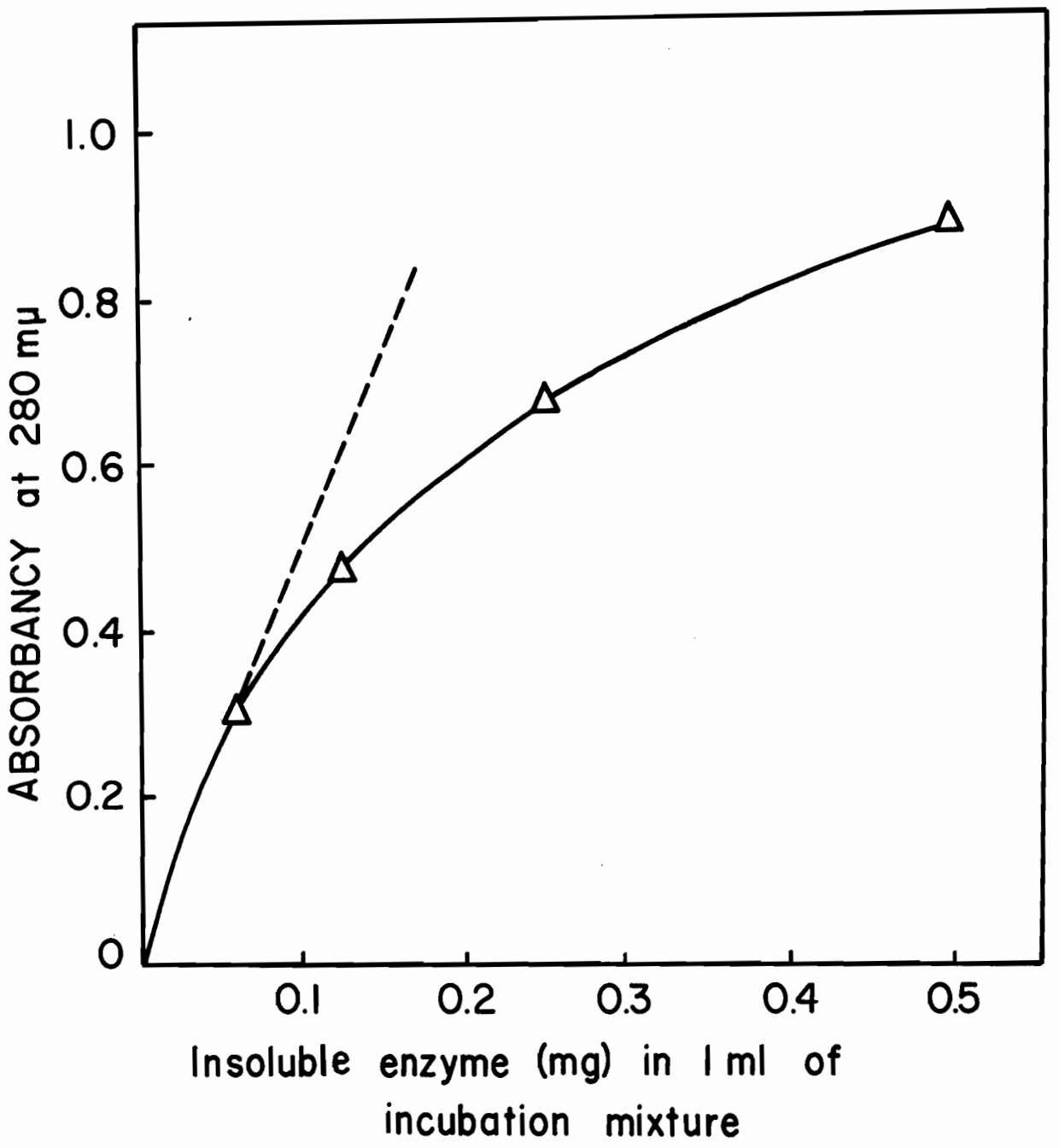
1.0
0.8
0.6
0.4
0.2
0
ABSORBANCY at 280 mμ
0.1
0.2
0.3
0.4
0.5
Insoluble enzyme (mg) in 1 ml of
incubation mixture

TABLE XIX

The enzymatic effect on the skin activity of fraction C.

Incubation mixture	Skin reactions obtained in sites sensitized with allergic serum from patient 'Na'(ten-fold diluted) and challenged with the incubating mixture. Original concentration of fraction C in the incubation mixture (µg/ml)				
	1000	100	10	1.0	0.1
Enzyme + fraction C	3+	2+	1+	-	-
Fraction C	3+	2+	1+	-	-
Enzyme	-	-	-	-	-

DISCUSSION

This study has demonstrated that it is possible to isolate the skin active component(s) of timothy grass pollen in a fraction having a considerable degree of homogeneity from the complex mixture of substances found in the whole extract of timothy pollen. An apparent 100,000-fold purification of the skin active component(s) was achieved by a combination of the methods of salt precipitation, gel-filtration and ion-exchange chromatography. Previous studies by other workers (110,133) had also resulted in the isolation of active fractions from timothy pollen, but no attempt had been made in those studies to compare the relative activities of the fractions with the activity of the whole pollen extract by the cross-neutralization technique using the passive transfer test.

As already mentioned in Chapter II, during "hyposensitization" treatment of hayfever patients with crude extracts, the patients are injected with extracts containing some pollen components, to which they may not have been originally sensitive and to which they may become sensitized during treatment. Therefore, it would seem highly desirable to use purified fraction(s) devoid of components to which the patient was not originally sensitive. The active fraction described here was 100,000-fold more potent in terms of its skin activity and contained all the skin active determinants of WSG. Thus, fraction C-IV, by comparison with WSG, would appear to possess all the desired attributes of a preparation which could be adopted for clinical use. Furthermore, it was observed in this investigation that even weakly allergic sera from untreated patients gave the same reaction pattern with different chromatographic fractions and, therefore,

it is conceivable that a large majority of timothy sensitive allergic individuals are sensitive to the same number of skin active components present in the whole pollen extract. Consequently, it was believed that this fraction would also be useful in the treatment of the majority of patients.

Obviously, it might not be advisable to treat a patient, who is sensitive only to some of the skin active components present in the crude extract with fraction C-IV since the patient might be sensitized by other skin active components of this fraction to which he had not been originally sensitive. In this particular case, it would have been useful to separate the skin active components of this fraction so that the patient could be treated only with the particular skin active components to which he was actually sensitive. However, since the amount of the active fraction is exceedingly low, i.e. 0.06% of the whole pollen, and since the molecular and electrophoretic properties of the components of C-IV are closely related to each other, the separation of these active components was not attempted. However, the use of fraction C-IV in desensitization treatment would be preferable to WSG since one does not know the effect of injecting the inactive components of WSG.

Although fraction C-IV was 100,000 times more active than WSG for eliciting minimal skin reaction, in desensitization experiment it proved to be only four times as potent as WSG, i.e. the corresponding desensitizing doses were 50 and 200 μg/ml for C-IV and WSG, respectively. This is an apparent paradox and is attributed to the following: (i) the concentration needed for minimal skin reaction is indeed very low, because it reflects the most active component; (ii) the concentration needed to desensitize a site

might be disproportionately larger since it would require the participation of all skin active components in amounts corresponding to their respective "desensitization" doses.

It was established that a site desensitized with respect to fraction A gave still a positive reaction to fraction C-IV, whereas fraction A did not elicit any skin reaction in a site previously desensitized with respect to fraction C-IV. This is interpreted as indicating that A contains fewer skin active components than fraction C-IV. This evidence, taken together with the fact that fractions A and C-IV behaved differently on chromatography, suggests that fraction A had only some of the allergenic determinant(s) of the skin active components of fraction C-IV and that these determinants were associated with different molecular species present in the two fractions. Alternatively, one may suggest that the activity of fraction A was due to some fragments of the components of C-IV, which might have arisen through the degradation of some of the active components by enzymes present in the pollen grain and which became activated during the procedure of extraction.

In the present study it was demonstrated that the strongest rabbit antisera to the pollen constituents were obtained by immunizing rabbits with the dialyzed residues of the pollen extract containing high molecular weight components. This observation is in line with the generally accepted view in immunology that macromolecular antigens are more immunogenic than low molecular weight antigens.

Although fraction C-IV was shown to contain all the skin active components of WSG, it possessed fewer (a minimum 3 out of 7) antigenic components than WSG as demonstrated by immunoelectrophoresis (Figure 15). This observation may be discussed in the light of the following three possibilities regarding

the relationship of allergenicity in man and antigenicity in rabbits: (i) identical antigenic and allergenic determinants are present on the same molecule; (ii) different groups responsible for antigenic and allergenic properties are on the same molecule; (iii) the antigenic and allergenic determinants are on the different molecules.

The first possibility could not be examined at this stage since it would require the availability of more monodisperse components, which could be used for the elucidation of the chemical structure of the allergen. The second and third possibilities could have been examined in principle by incubating fraction C-IV with a potent rabbit antiserum against whole pollen extract in the antibody excess region, and testing the supernatant for skin activity. If the activity of the supernatant was identical to that of the original solution of C-IV, one would have concluded that the antigenic and allergenic determinants were on different molecules. On the other hand, if all skin active materials could be absorbed out, one would conclude that the same molecule(s) carry the antigenic and allergenic determinants, i.e. possibilities (i) or (ii) would be plausible. Since volunteers who would be willing to be injected with solutions containing rabbit serum proteins were not available, this type of experiment could not be attempted.

Since C-IV contains all the allergens and gives rise only to inner precipitin band in immunodiffusion experiments, as compared to the two sets of inner and outer precipitin bands obtained with WSG, one may conclude that antigens responsible for the outer precipitin bands are not allergenic in man. However, as stated earlier, one cannot, on the basis of the available evidence, identify categorically the allergens with the antigenic components

of C-IV. Therefore, the conclusion of Augustin that the antigens responsible for inner precipitin bands are allergens cannot be taken too seriously (109).

The whole pollen extract gave a relatively simple pattern on immunodiffusion analysis, whereas in immunoelectrophoresis it appeared to consist of a complex mosaic of antigens. This difference in the complexity of the two patterns was due to the fact that in the latter procedure the components of WSG were separated prior to diffusion. These observations demonstrate again that simple immunodiffusion reveals only the minimum number of components; in this particular case it is clear that both the "outer" and the "inner" precipitin zone were composed of several unresolved precipitin bands.

Amino acid analysis revealed that the minimum protein content of fraction C-IV was 93%, as compared to the lower value of 85% determined by Kjeldahl digestion. This difference is not considered to be significant and could be due to incomplete digestion in the latter procedure. For amino acid analysis the hydrolysis of the protein was carried out at different periods; the loss of certain amino acids which are destroyed during hydrolysis and the possibility of missing out some other amino acids, which are released after longer hydrolysis, was minimized by extrapolating the concentration of amino acids back to zero time of hydrolysis and by performing the hydrolysis for different lengths of time. Consequently, the estimate of 93% for the protein content by amino acid analysis is considered to be a minimum value.

The properties of fraction C-IV, such as the absorption spectrum in the u.v. region (Figure 17) and the amino acid content, qualify it as a

protein, with small amounts of carbohydrate. Some active timothy grass pollen fractions obtained by other workers were reported to be composed of polypeptide, carbohydrate and pigments but not enough chemical data of these fractions had been obtained for comparison with the present observations. However, some of the allergenic constituents of ragweed and rye pollens, which had been chemically characterized, were shown to be proteins with small amounts of carbohydrate. Thus, the active fraction of rye was shown to contain 5% carbohydrate (141) and even the purest antigen of ragweed, i.e. antigen E, contains only 0.9% carbohydrate (151), as compared to fraction C-IV, which contained 2.2% carbohydrate. Fraction A, which was shown to contain most of the carbohydrates (35%), was as active as the crude extract, whereas fraction C-IV with only 2.2% carbohydrate was 100,000-fold more active than the crude extract. Furthermore, fraction A contained only 36% protein but fraction C-IV contained 93% protein. Consequently, it is reasonable to conclude that the increase in the specific skin activity of fraction IV was due to the enrichment of the protein content rather than due to its carbohydrate content. It might, therefore, be suggested that the contribution of the carbohydrate moiety to the skin activity of fraction C-IV might be minimal, if any at all.

The amino acid analysis of fraction C-IV clearly demonstrates that timothy grass pollen allergen is distinct from the botanically related grass pollen. Moreover, glutamic acid was present in large amounts in fraction C-IV, whereas lysine and aspartic acid were present in large amounts in the active fractions of rye and ragweed pollens, respectively (Table XVIII). However, the two amino acids, glutamic and aspartic acids, were found to be present in large amounts in these different pollen allergens.

The sedimentation coefficient of fraction C-IV was 1.5S. Although the diffusion coefficient and the partial specific volume of this material were not determined, the low S-value and the retarded elution of the fraction on Sephadex G-75 would suggest a molecular weight in the region of 15-20,000. As shown by immunoelectrophoresis, fraction C-IV was not a single component. Therefore, further efforts to obtain molecular weights more precisely did not seem warranted at this stage.

The insoluble α-chymotrypsin was shown to possess enzymatic activity when tested for its ability to degrade casein; therefore, the susceptibility of fraction C towards the insoluble enzyme was considered to be the same as that towards the free enzyme. The insoluble enzyme can be readily separated by centrifugation from digestion mixture containing the substrate as compared to soluble chymotrypsin, which would have to be removed by chromatography on Sephadex or some other more complicated procedure. For the simplicity of the technique, the insoluble enzyme was, therefore, used to digest the fraction. However, it was found that skin active components of this fraction were resistant to the digestion by the insoluble enzyme. The failure of the insoluble chymotrypsin to degrade fraction C might be due to the non-accessibility of regions of C-IV containing phenylalanine and tyrosine to the enzyme. As is known, chymotrypsin acts efficiently on a protein which has been denatured by heating. However, as demonstrated in Chapter III, the skin activity of the fraction would be destroyed on heating and, therefore, further studies using this approach were not deemed appropriate.

Fraction C-IV did not lose activity on lyophilization and retained its activity on storage for a year in powder form at $4^{\circ}C$. On re-running

the stored fraction C-IV on Sephadex G-75, a single peak was observed in the chromatographic pattern, indicating that no chemical degradation had occurred during storage.

Although fraction C-IV has all the properties required to be used for desensitization treatment of patients, the laborious process for its isolation represents the main limitation of the method. Moreover, in spite of these many efforts, fraction C-IV was chemically still complex and, therefore, could not be used for the elucidation of the detailed chemical nature of skin active components. Consequently, it was deemed desirable to concentrate further efforts on the low molecular weight components of the pollen extract, which could be obtained by dialysis of the crude extract. It is clear that if an active fraction could be isolated by this procedure, it might represent one of the actual haptenic components, which could then be used more profitably for further chemical characterization and, moreover, one might also envisage that such compounds would be useful in an alternate method for treatment of patients, since these haptens might be capable of blocking antibody active sites.

CHAPTER V

STUDIES ON THE NATURE OF THE DIALYSABLE CONSTITUENTS OF THE AQUEOUS EXTRACT OF TIMOTHY GRASS POLLEN (WSG)

INTRODUCTION

Landsteiner (29) introduced the term hapten to designate a chemically well defined molecule, which by itself is non-immunogenic but can acquire the immunogenicity when coupled to a larger "carrier" molecule. In cases where haptens appear to give inflammatory reactions by themselves, it has been shown that they become coupled in vivo to the host's proteins through covalent bonds. Thus, benzyl-penicillin, through its metabolic intermediates, combines in vivo with proteins to elicit a hypersensitive response (181).

Because of the simplicity in the chemical structure of the haptens as compared to macromolecules, they have been widely used for the elucidation of immunological responses at molecular level. Although haptens cannot provoke the production of antibodies, they can combine specifically with the homologous antibodies giving rise to soluble complexes. This property has been exploited for the study of the mechanisms and energetics of antigen-antibody interactions and for the definition of the size of the antibody-combining site.

The conjugates formed as a result of chemical combination of simple haptens with proteins are effective in eliciting immediate type allergic responses primarily because they are polyfunctional, i.e. they carry two or more antigenic groups per molecule. It has been shown that injection of 2,4-dinitrophenyl-protein conjugates, consisting of about 40 dinitrophenyl

groups per molecule of protein, into human skin previously sensitized with rabbit anti-dinitrophenyl antibodies elicited an immediate skin response (182). However, when peptide derivatives possessing only one dinitrophenyl group per molecule were injected, no response was obtained.

In contrast, Schlossman et al (183) showed that guinea pigs could be sensitized with α,N-DNP-oligo-L-lysyl peptides consisting of at least seven lysyl residues and that the DNP-octapeptide derivative could induce the formation of both delayed and immediate sensitivity in the same animal.

There is fairly good agreement among various investigators (184,185, 186,187,188,189,190) that the haptenic portion of the immunizing molecule is responsible for eliciting the immediate skin response, whereas the carrier portion of the immunizing antigen is the determinant for the delayed type response. Karush and Eisen (191), working on bovine DNP-gamma globulin system, have suggested that the hydrophobic character of the carrier molecule, which provides sufficient affinity of the immunogen to react with antibody in aqueous media, is required for delayed type sensitivity. On the other hand, Schlossman et al (192) observed that the difference between the immunogenicity and non-immunogenicity in the α,N-DNP-oligo-L-lysine series was, at most, two lysyl residues (i.e. from octamer to hexamer). The results were interpreted as due to the presence of two additional ε-amino groups with resultant increase in positive charge to provide sufficient stability of electrostatic binding to some specific molecule or receptor site.

More recently, chlorogenic acid was found to be one of the allergenic constituents of green coffee bean (193) and the skin activity elicited by it was shown to be greatly enhanced by coupling it to a carrier molecule, such as HSA (194). Furthermore, the monosubstituted chlorogenic acid

derivative failed to elicit skin reactions when tested by direct or passive transfer P-K test. Consequently, it was suggested that the skin reactions, elicited by apparently free chlorogenic acid, were due to its conjugates produced in vivo with host's proteins. More recently (195), these conclusions were further substantiated inasmuch as the quinic acid residue was shown to be the immuno-dominant locus of chlorogenic acid; for this purpose, quinic acid-HSA conjugates, consisting of 15-18 quinic acid residues per HSA molecule were synthesized and proven active.

The possible presence of similar haptenic materials in various pollens was suggested by observations of Campbell et al (171), who found that the best precipitating rabbit antisera against the soluble components of olive and Bermuda grass pollen antigens were obtained by immunization with non-dialysable constituents of extracts of these pollens. Later, Malley et al (136) reported the actual isolation of a hapten from the dialysable constituents of timothy pollen, which did not give a precipitin reaction with rabbit anti-timothy antiserum and which, more importantly, had the ability of inhibiting the precipitin reaction between this antiserum and dialyzed extracts; however, this fraction had some skin activity when tested directly on timothy sensitive patients.

The understanding of the mechanism involved in allergic reactions could greatly be advanced by studying a model system in which the allergenic determinant is a simple hapten. In addition to a theoretical value of such a study, it can be visualized that univalent haptens could be used as desensitizing agents, since the intravenous administration of non-toxic, univalent haptens to allergic patients might be expected to result in the blocking of all antibody sites (196) and thus lead to the protection

of the patients from the allergens by inhibition of reactions between skin-sensitizing antibodies and the multivalent allergens present in the inhaled air. The study reported in this chapter represents an attempt to isolate the hapten in pure form, using chromatography on Sephadex G-25 and G-50, and the immunological and biological evaluation of the isolated fractions of the dialysable constituents.

METHODS AND MATERIALS

Preparation of dialysates

Timothy grass pollen was defatted as described in Chapter III. The defatted pollen was extracted with water (100 g pollen in 500 ml water) by constant stirring at room temperature for four hours. The pollen residue was removed by filtration and the clear filtrate was concentrated by pervaporation to 90 ml. The concentrated solution was dialyzed against 4 liters of distilled water through Visking tubing (Seamless cellulose tubing, Union Carbide Canada Ltd., Visking Division, Lindsay, Ontario) for 24 hours at $4^{o}C$. The dialysate, thus obtained, was lyophilized and designated as D-(W).

In another set of experiments, the pollen was extracted according to the method of Malley et al (136), i.e. the defatted pollen was treated with 0.15M sodium phosphate buffer, pH 10.6 (100 g pollen in 500 ml buffer) by constant stirring at $4^{o}C$ for 24 hours and the clear solution obtained, after filtration and centrifugation, was dialyzed against water. The dialysate was then concentrated by pervaporation and designated as D-(Ph).

Chromatography on Sephadex G-25

A column (40 x 2 cm) of Sephadex G-25 was made according to the procedure described in Chapter IV. The column was equilibrated and eluted with distilled water. Two ml of a solution containing 10-12% of the dialysate was applied onto the column. The flow rate was usually adjusted to 6-8 ml per hour and 3-4 ml fractions were collected. The optical densities at 280 mμ of the fractions were recorded manually with a D.U. spectrophotometer. The fractions were either lyophilized or concentrated by pervaporation, and stored at -20°C. For rechromatography, fresh Sephadex G-25 columns were prepared and used under identical conditions.

Chromatography on Sephadex G-50

A column (40 x 2 cm) was packed with Sephadex G-50 (medium), equilibrated with water or 0.2M ammonium acetate, pH 6.85. Before packing the column with Sephadex G-50, a small amount of Sephadex G-25 (coarse) was placed at the bottom of the column to avoid clogging of the pores in the sintered disc. Two ml of a 6% sample, obtained from rechromatography on Sephadex G-25, was applied. The chromatography was performed as described above.

Immunological studies

Ring test. This was performed as described in the preceding chapter.

Immunodiffusion. The central well was filled with antiserum and the outer wells were filled with fractions of D-(W) and D-(Ph) in various concentrations (1-10%). The pattern was developed as described in Chapter III.

Inhibition experiment. The antiserum and the haptenic fraction at

a concentration of 2% were incubated together for an hour at room temperature. The mixture was placed in the central well, while the outer wells were filled with 1% WSG in saline. In a control plate, the central well was filled with antiserum without the haptenic fraction. For specificity of inhibition, the antisera against BSA (bovine serum albumin) and WSR (water soluble ragweed) were incubated with the haptenic fraction and the pattern was developed against the homologous antigens.

Determination of skin activity of the dialysate of WSG and its various chromatographic fractions

This was performed as described in Chapter III.

Neutralization of P-K reactions to WSG*

One volume of a ten-fold diluted allergic serum (from patient 'Na') was incubated with 1 ml of haptenic fraction in varying concentrations at room temperature for 20 hours, followed by incubation at 37°C for an hour (the final dilution of the serum was ten-fold). Multiple sites were then sensitized with 0.05 ml of these mixtures and challenged, 24 hours later, with 0.025 ml of whole pollen extract (WSG) at a concentration of 100 µg/ml. As controls, skin sites containing ragweed allergic serum (1:10 diluted), pre-incubated with the highest concentration of the haptenic fraction in the neutralization tests, were challenged with WSR at a concentration of 100 µg/ml.

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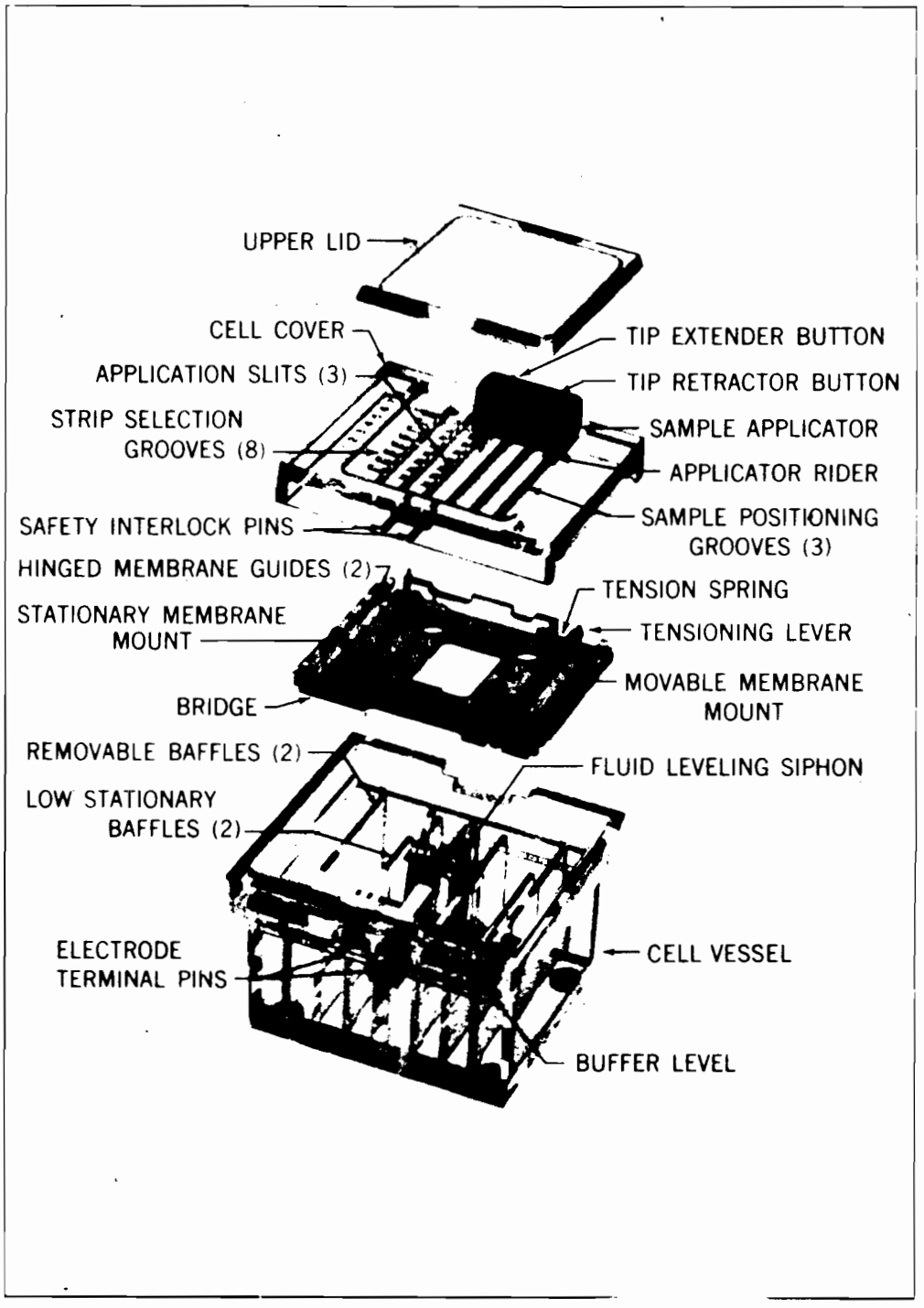
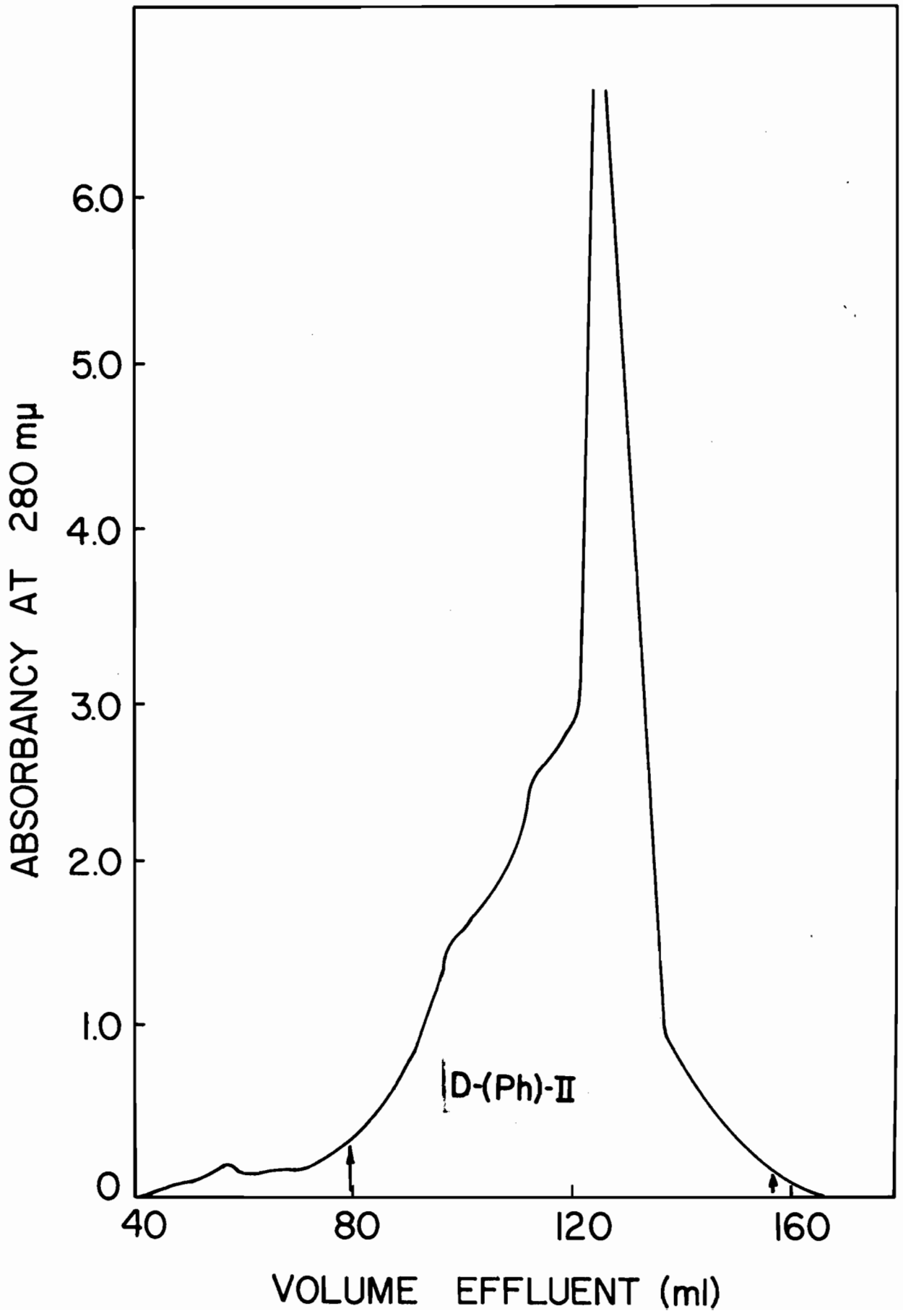
* All solutions were sterilized by passing through a sterile Millipore membrane and the serum was sterilized by passing through a sterile Sietz pad.


UPPER LID
CELL COVER
APPLICATION SLITS (3)
STRIP SELECTION GROOVES (8)
SAFETY INTERLOCK PINS
HINGED MEMBRANE GUIDES (2)
STATIONARY MEMBRANE MOUNT
BRIDGE
REMOVABLE BAFFLES (2)
LOW STATIONARY BAFFLES (2)
ELECTRODE TERMINAL PINS
TIP EXTENDER BUTTON
TIP RETRACTOR BUTTON
SAMPLE APPLICATOR
APPLICATOR RIDER
SAMPLE POSITIONING GROOVES (3)
TENSION SPRING
TENSIONING LEVER
MOVABLE MEMBRANE MOUNT
FLUID LEVELING SIPHON
CELL VESSEL
BUFFER LEVEL


ABSORBANCY AT 280 mμ
6.0
5.0
4.0
3.0
2.0
1.0
0
40
80
120
160
VOLUME EFFLUENT (ml)
D-(Ph)-II

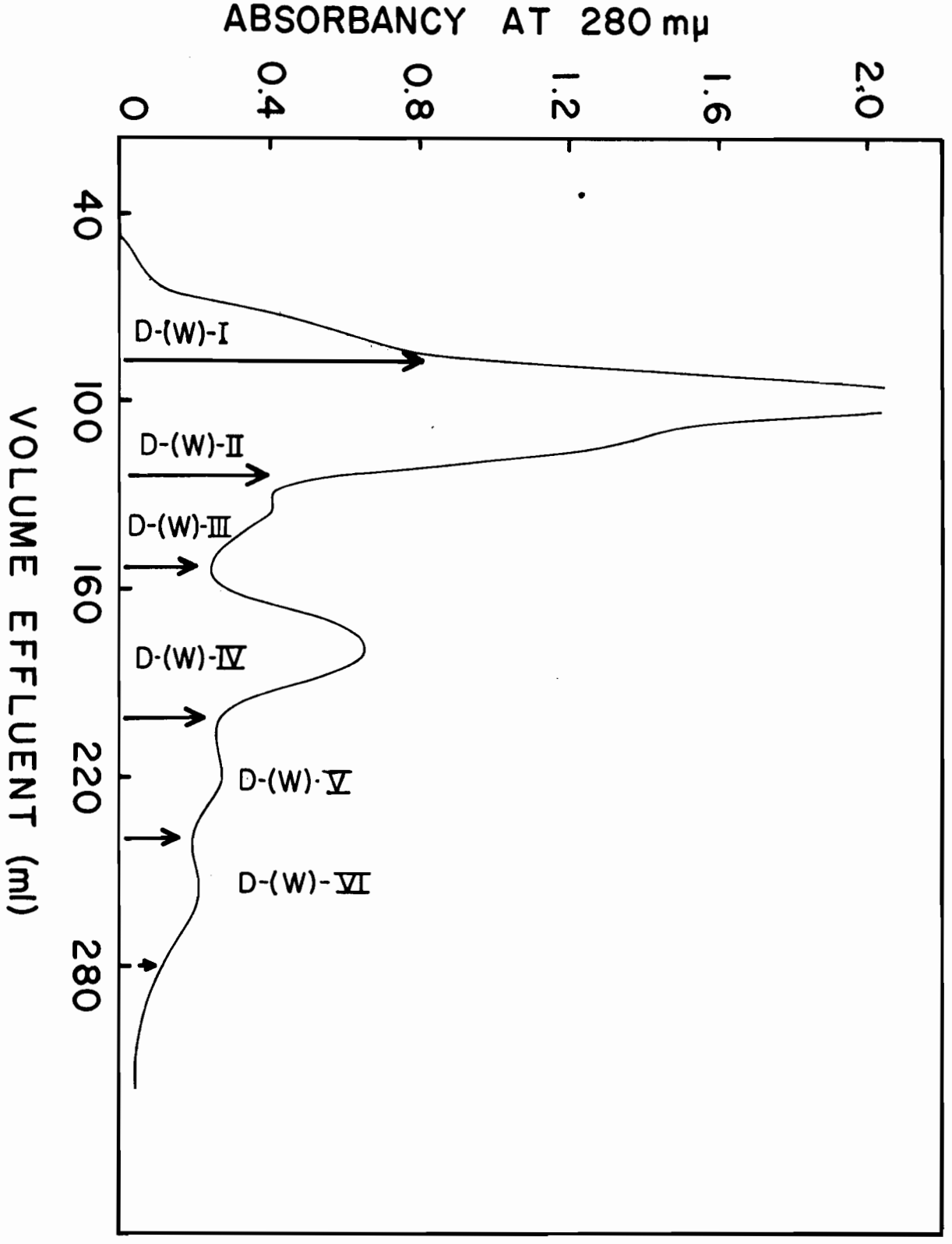

ABSORBANCY AT 280 mμ
0
0.4
0.8
1.2
1.6
2.0
VOLUME EFFLUENT (ml)
40
100
160
220
280
D-(W)-I
D-(W)-II
D-(W)-III
D-(W)-IV
D-(W)-V
D-(W)-VI

FIGURE 29

Rechromatography on Sephadex G-25 (40 x 2 cm) of fraction D-(W)-II.
Eluant: water; flow rate = 8 ml/hr; vol.of fractions collected = 4 ml;
sample applied: 120 mg dissolved in 2 ml water.

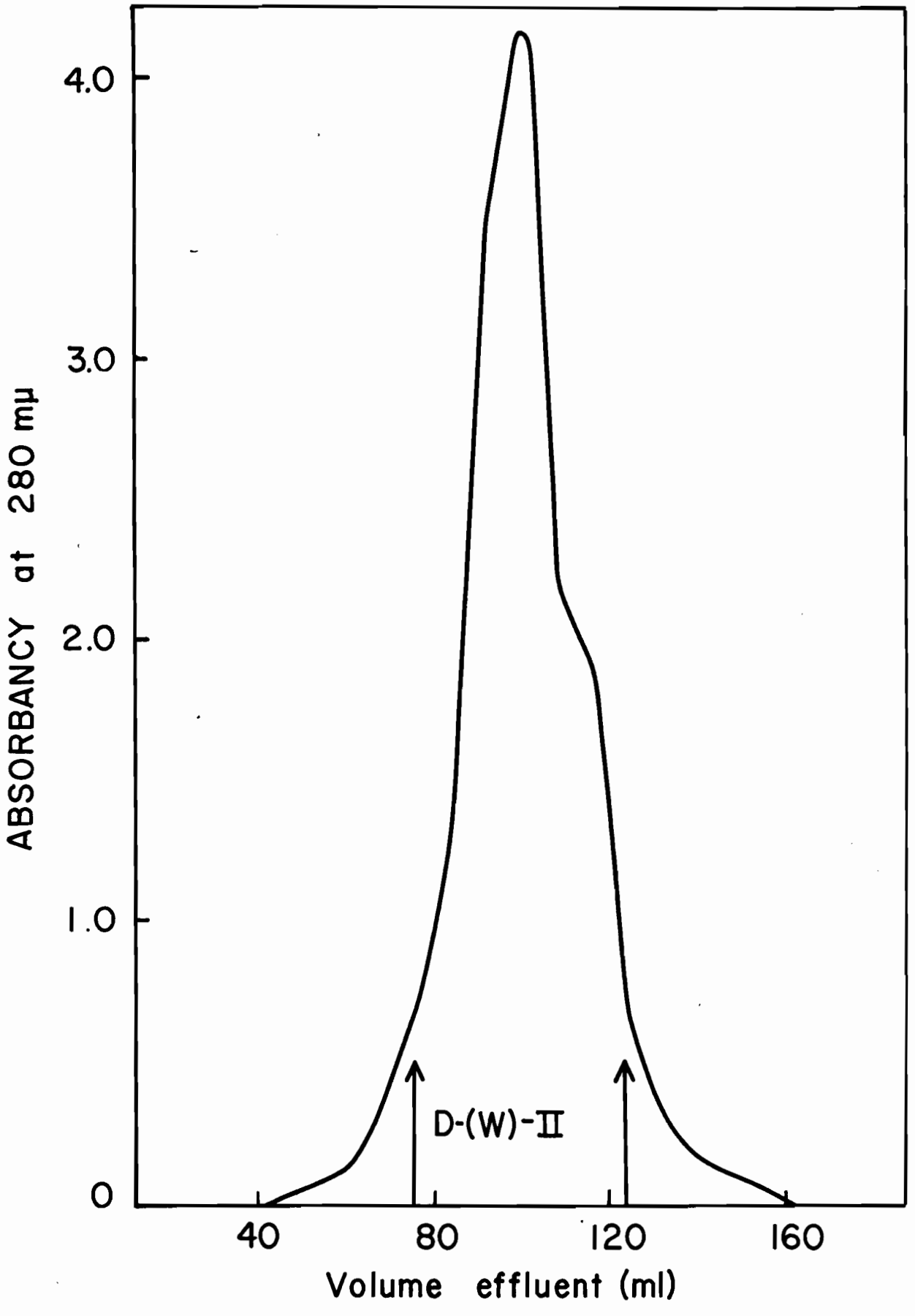
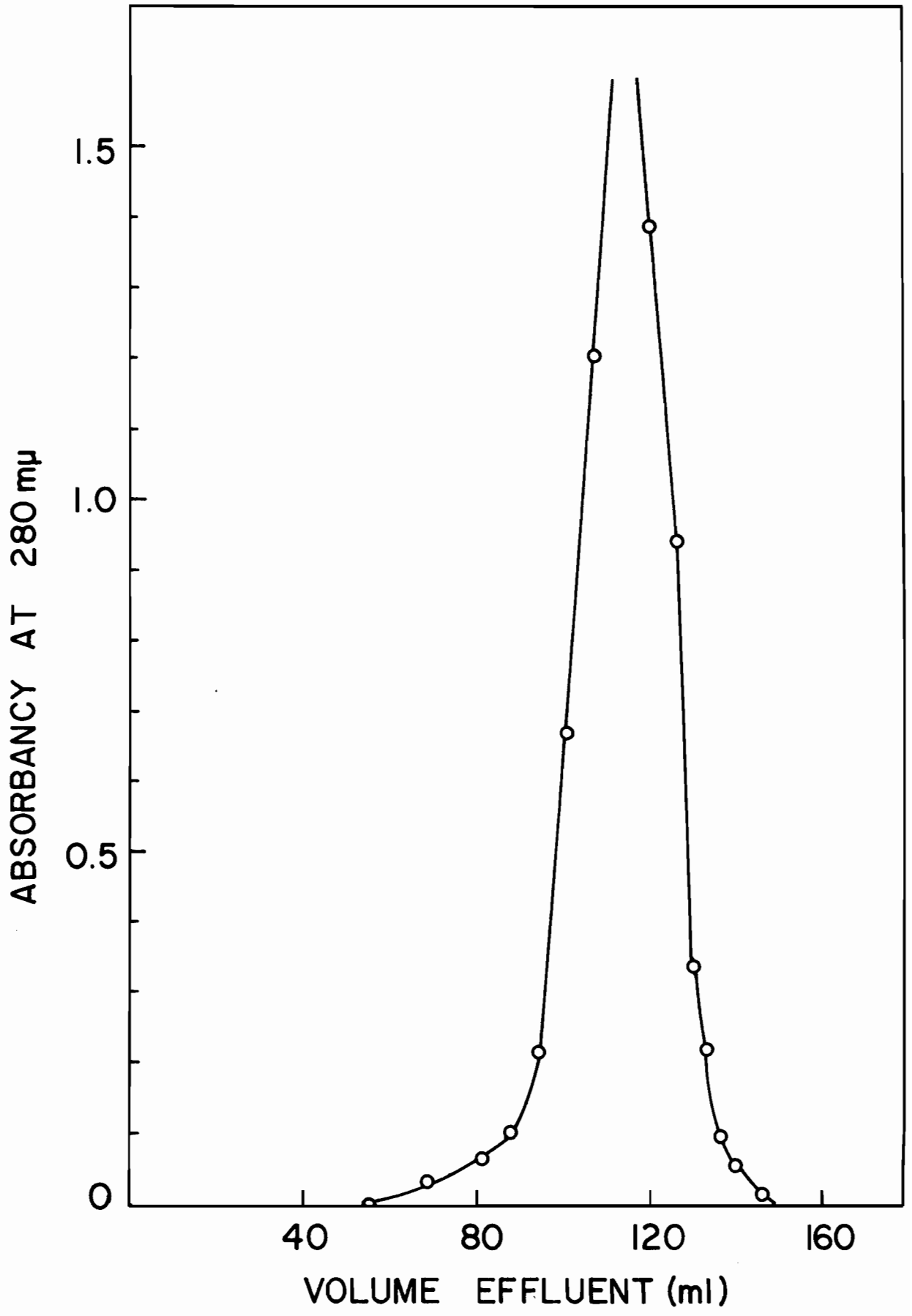

4.0
3.0
2.0
1.0
0
ABSORBANCY at 280 mμ
D-(W)-II
40
80
120
160
Volume effluent (ml)

FIGURE 30

Chromatography on Sephadex G-50 (40 x 2 cm) of fraction D-(W)-II.
Eluant: water; flow rate = 7.5 ml/hr; vol. of fractions collected = 3.25 ml;
sample applied: 2 ml (about 4%).


ABSORBANCY AT 280 mμ
1.5
1.0
0.5
0
40
80
120
160
VOLUME EFFLUENT (ml)

the same buffer, was resolved into two major components, D-(W)-II-A and D-(W)-II-B (Figure 31). The elution volume of D-(W)-II-A was 100 ml. Furthermore, on rechromatography of fraction D-(W)-II-A (Figure 32), this fraction was eluted as a single peak at the same elution volume of 100 ml.

Microzone electrophoresis

It is evident from Figure 33 that fraction D-(W)-II is a heterogeneous mixture of at least seven electrophoretically separable components. Components a, b, c and d moved towards the positive electrode, whereas components f and g moved towards the negative electrode. Each component gave a characteristic colour with ninhydrin. Thus, component a was blue, b was yellow, c was pink, d was grey, e was violet, f was orange and g was grey. The appearance of colour for bands a, b, d, e and f was complete within three hours, whereas the colour of bands c and g appeared only after 24 hours of development. It was observed in a preliminary experiment that bands a, c and g did not develop with 0.1% ninhydrin while the colour of the other bands was very faint.

Fraction D-(W)-II-A, rechromatographed on Sephadex G-50, was analysed by electrophoresis under identical conditions. This fraction was found to contain at least four of the components (a, b, e and f) of D-(W)-II. Bands e and f developed immediately after spraying with ninhydrin, while bands a and b developed much later. The colour of the bands a and b was very weak and unstable. The results suggested that rechromatography of fraction D-(W)-II on Sephadex G-50 had resulted in the elimination of three components (c, d and g). These results also suggest that fraction D-(W)-II-A contains mainly components e and f with trace amounts of a and b.

FIGURE 31

Chromatography on Sephadex G-50 (40 x 2 cm) of fraction D-(W)-II. Eluant: 0.2M ammonium acetate, pH 6.85; flow rate = 6 ml/hr; vol. of fractions collected: 3 ml; sample applied: 2 ml (about 4%).

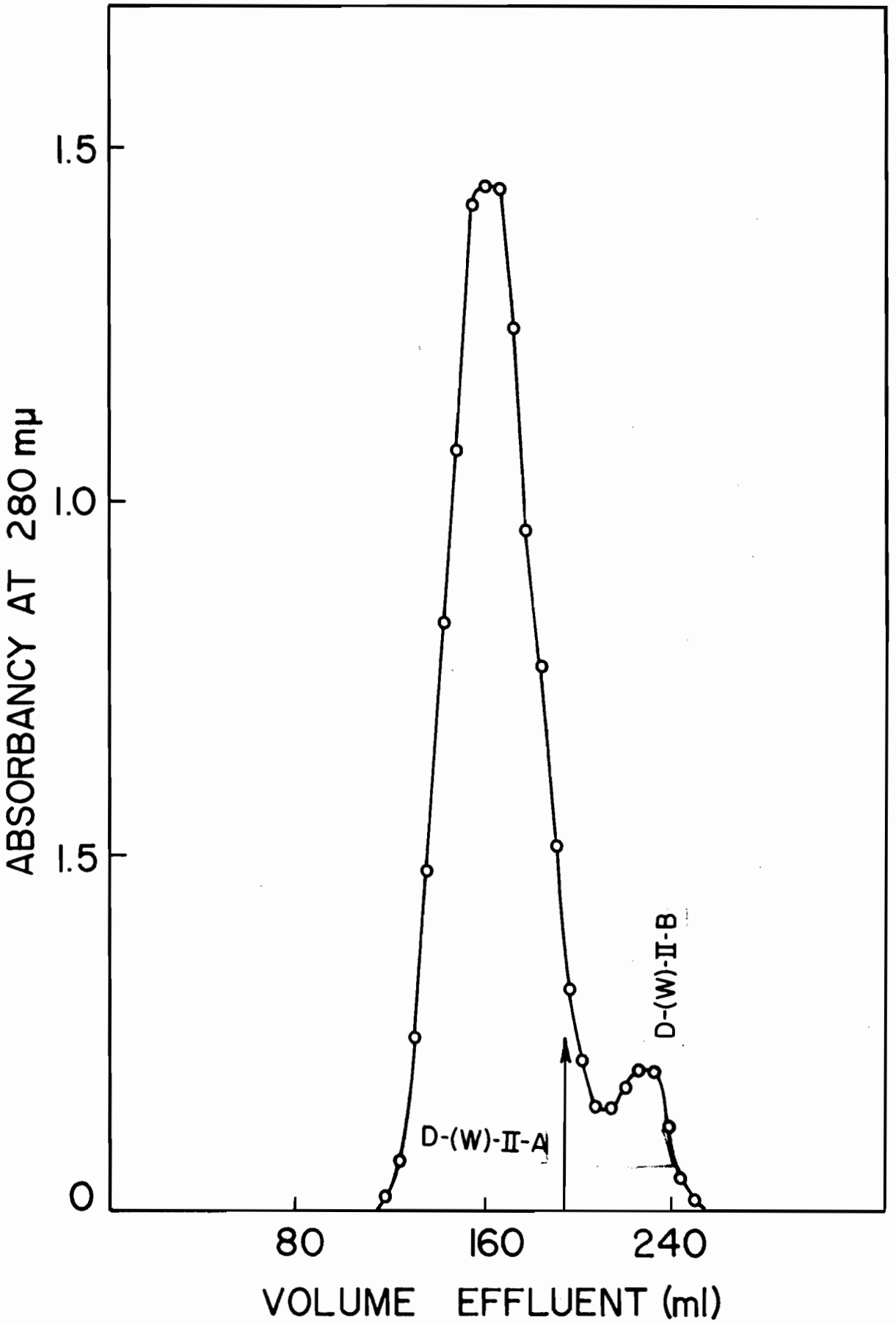

1.5
1.0
1.5
0
ABSORBANCY AT 280 mμ
D-(W)-II-A
D-(W)-II-B
80
160
240
VOLUME EFFLUENT (ml)

FIGURE 32

Rechromatography on Sephadex G-50 (40 x 2 cm) of fraction D-(W)-II-A. Eluant: 0.2M ammonium acetate, pH 6.85; flow rate = 8 ml/hr; vol. of fractions collected: 4 ml.

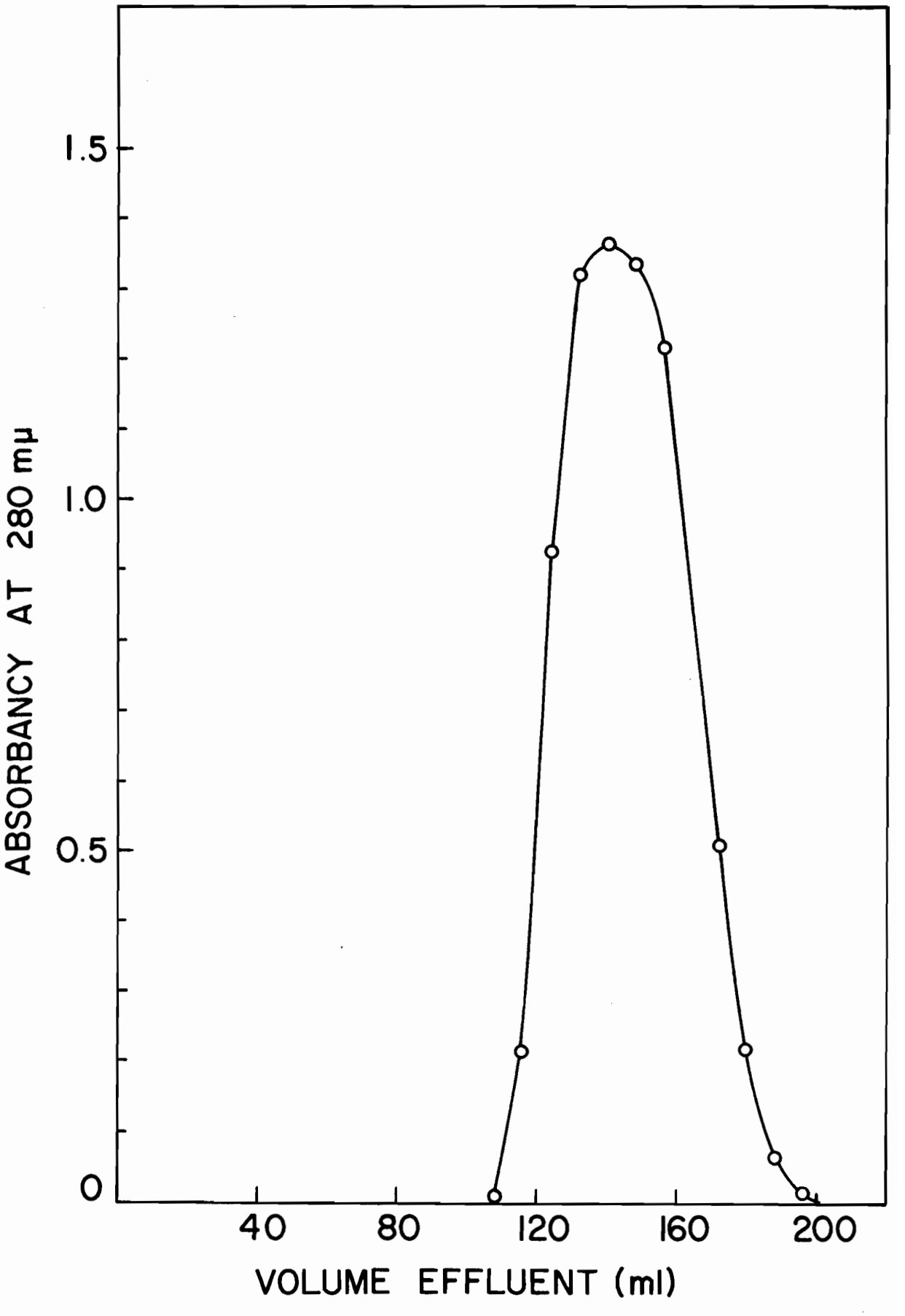
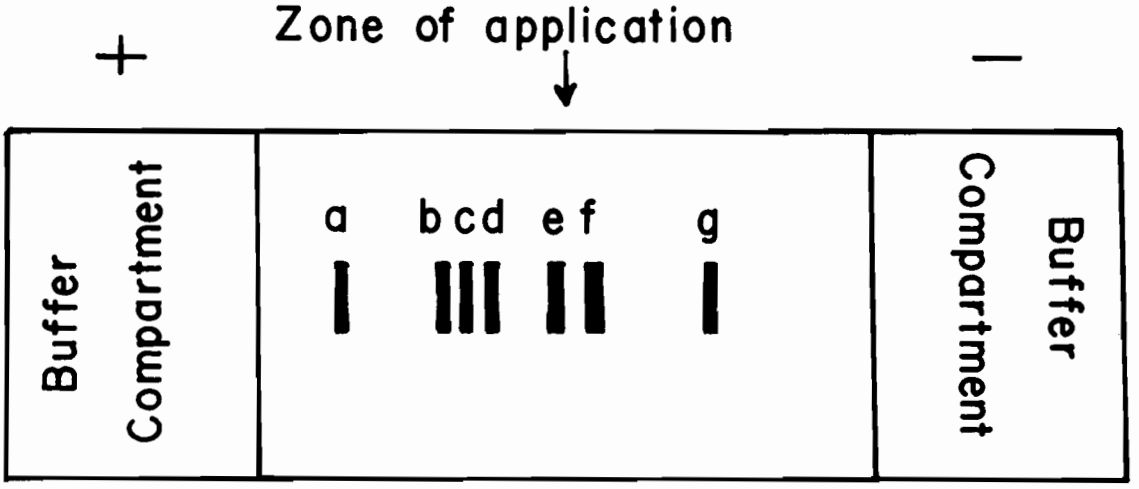

1.5
1.0
0.5
0
ABSORBANCY AT 280 mμ
40
80
120
160
200
VOLUME EFFLUENT (ml)

FIGURE 33

A typical electrophoretic pattern of fraction D(W)-II. The electrophoresis was carried out in barbital buffer, pH 8.6, $\Gamma/2 = 0.075$. The duration of the run was 30 min under a constant current (3 ma and 250 V). The cellulose-acetate membrane strip (14.5 x 5.7 cm) was dried and sprayed with 1% ninhydrin in n-butanol.


Zone of application
+
−
Buffer Compartment
a
b c d
e f
g
Buffer Compartment

Immunological studies

With rabbit anti-WSG antiserum, none of the fractions D-(W)-I, D-(W)-II, D-(W)-II-A gave a visible ring test, whereas WSG gave - as would be expected - a readily visible precipitin band.

The failure of these fractions to form precipitin bands with antibodies against whole pollen extract was further confirmed by immunodiffusion analysis on Ouchterlony plate, as shown in Figure 34. Thus, as can be seen, two clearly visible groups of precipitin bands - a typical pattern for WSG and anti WSG system - were formed (wells # 1, # 3, # 5, # 8, # 6 and # 10), whereas no precipitin bands were observed with fractions D-(W)-I, D-(W)-II and D-(W)-II-A (wells # 2, # 7, # 9 and # 4). Identical results were obtained with higher concentrations (up to 10%) of these fractions.

Fraction D-(Ph)-II obtained by chromatography of the dialysate of the phosphate buffer extract also failed to give any precipitin bands. Fraction D-(Ph)-I of the same dialysate was not examined due to the low amount of material available at the time.

The results of inhibition experiment illustrated in Figure 35 demonstrate that fractions D-(W)-II and D-(W)-II-A inhibited specifically the inner group of precipitin lines developed between WSG and rabbit anti-WSG antiserum. The inhibition was shown to be highly specific, since these fractions failed to inhibit any of the precipitin lines developed between BSA and anti-BSA or between WSR and anti-WSR (Figure 35, f-i and j-m). Fraction D-(Ph)-II of the phosphate buffer extract behaved also in the same way. The inhibitory capacity of fraction D-(W)-II was not lost during successive stages of chromatography.

FIGURE 34

The central wells (a, b, c, d) were filled with rabbit anti-WSG antiserum. Wells # 1, # 6, # 3, # 8, # 5 and # 10 were filled with WSG (0.5% in saline). Wells # 2 and # 7 were filled with fraction D-I (1%). Well # 4 was filled with fraction D-IIA (1%) and well # 9 with fraction D-II (1%).

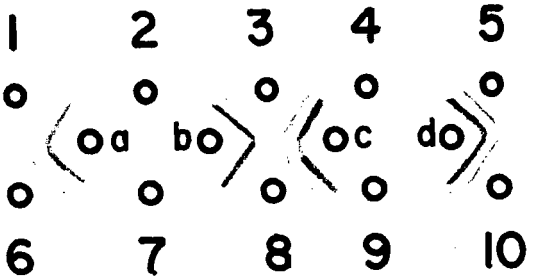

1
2
3
4
5
a
b
c
d
6
7
8
9
10

FIGURE 35

a. The central well was filled with preincubated mixture of 0.1 ml rabbit anti-WSG antiserum and 0.2 ml saline. Outer wells were filled with WSG (1%).

b. The central well was filled with preincubated mixture of 0.1 ml anti- WSG antiserum and 0.3 ml saline. Outer wells were filled with WSG (1%).

c. The central well was filled with preincubated mixture of 0.1 ml anti- WSG antiserum and 0.2 ml rechromatographed fraction D-(W)-II (2%). Outer wells were filled with WSG (1%).

d. The central well was filled with preincubated mixture of 0.1 ml anti- WSG antiserum and 0.3 ml rechromatographed fraction D-(W)-II (2%). Outer wells were filled with WSG (1%).

e. The central well was filled with preincubated mixture of 0.1 ml anti- WSG antiserum and 0.2 ml rechromatographed fraction D-(W)-II-A (2%). Outer wells were filled with WSG (1%).

f. The central well was filled with preincubated mixture of 0.1 ml rabbit anti-BSA antiserum and 0.2 ml saline. Outer wells were filled with BSA (1%).

g. The central well was filled with preincubated mixture of 0.1 ml anti- BSA antiserum and 0.3 ml saline. Outer wells were filled with BSA (1%).

h. The central well was filled with preincubated mixture of 0.1 ml anti- BSA antiserum and 0.2 ml rechromatographed fraction D-(W)-II (2%). Outer wells were filled with BSA (1%).

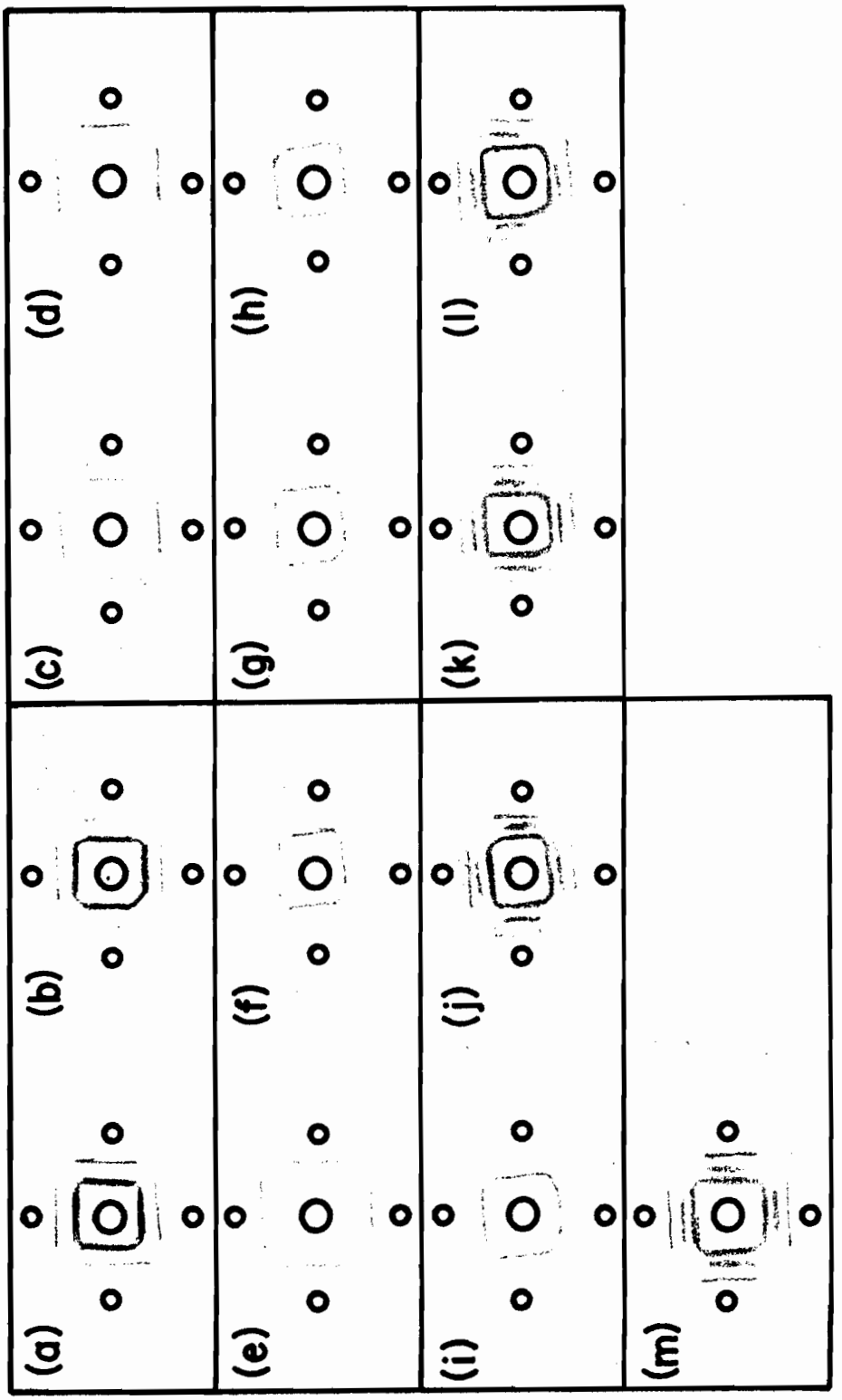
i. The central well was filled with preincubated mixture of 0.1 ml anti- BSA antiserum and 0.3 ml rechromatographed fraction D-(W)-II (2%). Outer wells were filled with BSA (1%).

j. The central well was filled with preincubated mixture of 0.1 ml rabbit anti-WSR antiserum and 0.2 ml saline. Outer wells were filled with WSR (1%).

k. The central well was filled with preincubated mixture of 0.1 ml anti- WSR antiserum and 0.3 ml saline. Outer wells were filled with WSR (1%).

l. The central well was filled with preincubated mixture of 0.1 ml anti- WSR antiserum and 0.2 ml rechromatographed fraction D-(W)-II (2%). Outer wells were filled with WSR (1%).

m. The central well was filled with preincubated mixture of 0.1 ml anti- WSR antiserum and 0.3 ml rechromatographed fraction D-(W)-II (2%). Outer wells were filled with WSR (1%).


(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
(m)

Skin activity of dialysate and of the corresponding chromatographic fractions

Table XX shows the skin activity of the dialysate of the aqueous extract and of the various fractions of the dialysate and of fraction D-(Ph)-II. The dialysate (D-(W)) was found to elicit readily detectable skin reactions (2+) only at concentrations higher than 5,000 µg/ml. The chromatographic fractions D-(W)-I, D-(W)-II, D-(Ph)-II and D-(W)-II-A also gave small reactions at concentrations above 5,000 µg/ml. This was considered to indicate that the fractionation of the dialysate by gel-filtration on Sephadex did not result in an increase of the biological activity of the dialysate. Furthermore, successive steps of purification of fraction D-(W)-II did not lead to any detectable change in the biological activity of the fraction. Fractions D-(W)-III and D-(W)-IV did not elicit skin reactions at the high concentration of 1,000 µg/ml; these fractions could not be used in higher concentrations due to the low yields of the fractions. It is to be pointed out, however, that the skin activity of the dialysate and of its chromatographic fractions was insignificant when compared to the skin activity of the whole pollen extract (WSG), which elicited minimal skin reactions at a concentration of 1 µg/ml, i.e. the skin activity of the dialysate and of its chromatographic fractions was at least 1/5,000 of that of WSG. It is worth noting that none of these fractions elicited skin reactions in the control sites which had been injected with saline instead of allergic serum, indicating thus that the high concentration of these fractions did not lead to any nonspecific reaction.

Neutralization of P-K reaction to WSG

The results illustrated in Table XXI demonstrated that fraction D-(W)-II

TABLE XX

Skin activity of dialysate and of its various chromatographic fractions.

Fractions	Skin reactions obtained in sites sensitized with a ten-fold diluted allergic serum from patient 'Na' and challenged with different fractions. Concentration of fractions in µg/ml						
	10,000	5,000	1,000	100	10	1.0	0.1
WSG				3+	2+	1+	1+
D-(W)	2+	2+	-	-	-	-	-
D-(W)-I	1+	1+	-	-	-	-	-
D-(W)-II	1+	1+	-	-	-	-	-
D-(Ph)-II	1+	1+	-	-	-	-	-
D-(W)-III			-	-	-	-	-
D-(W)-IV			-	-	-	-	-
D-(W)-II-A	1+	1+	-	-	-	-	-

TABLE XXI

Neutralization of P-K reactions to WSG.

Sites sensitized with mixture of allergic serum incubated with fraction D-(W)-II in various concentrations.	Skin reactions obtained on challenge with WSG 100 µg/ml.
1 ml of five-fold diluted allergic serum + 1 ml of fraction D-(W)-II (10 mg/ml)	±
1 ml of five-fold diluted allergic serum + 1 ml of fraction D-(W)-II (5 mg/ml)	1+
1 ml of five-fold diluted allergic serum + 1 ml of fraction D-(W)-II (2.5 mg/ml)	2+
1 ml of five-fold diluted allergic serum + 1 ml saline	3+

was capable of neutralizing almost completely the allergic serum when incubated with it in vitro. Thus, the size of the reaction obtained by challenging the sites with WSG was found to decrease gradually with higher concentrations of fractions D-(W)-II incubated with the allergic serum. In a control experiment (not shown in the table) it was shown that WSR elicited the expected full-size reaction in sites sensitized with a mixture of allergic serum from ragweed patients which had been preincubated with fraction D-(W)-II (10 mg/ml). This result demonstrates that the high concentration of fraction D-(W)-II did not have any deleterious nonspecific effect on the skin.

It is to be noted that complete neutralization (if any) of the allergic serum by fraction D-(W)-II was not attempted because it would have required the use of much higher concentrations of fraction D-(W)-II, which irritated severely the skin.

Determination of highest dilution of allergic serum and lowest concentration of WSG which could still give a positive P-K reaction

The data presented in Table XXII shows the relationship between the concentration of WSG and the dilution of allergic serum regarding readily detectable skin reactions. Thus, when the sites were sensitized with allergic serum at ten-fold dilution, WSG could elicit a positive skin reaction at a concentration of 5 μg/ml; on the other hand, with more diluted serum, i.e. with twenty-, forty-, eighty-, one hundred-, two hundred-fold diluted allergic serum, the lowest concentration of WSG which elicited a positive reaction was 10 μg/ml, 25 μg/ml, 50 μg/ml, 100 μg/ml, respectively.

There was no significant difference in the size of reactions elicited

TABLE XXII

Determination of highest dilution of allergic serum and lowest concentration of WSG required to elicit a skin reaction.

Serum dilution for sensitizing the sites	Reactions obtained in sensitized sites on challenging with WSG. Concentration of WSG in µg/ml				
	100	50	25	10	5
1/10	3+	3+	2+	1+	1+
1/20	3+	2+	2+	1+	±
1/40	3+	2+	1+	±	-
1/80	2+	2+	1+	±	-
1/100	3+	2+	±	±	-
1/200	2+	±	-	-	-
1/400	-	-	-	-	-

on sensitization of the skin sites with ten- and twenty-fold diluted sera or with forty- and eighty-fold diluted sera. A slight difference was, however, observed in the size of reactions elicited by sensitization with allergic serum at eighty- and hundred-fold dilutions. More importantly, the higher the dilution of the allergic serum, the higher was the concentration of WSG in order to elicit skin reactions. The forty- or eighty-fold dilution of allergic serum and the concentration of 25 μg/ml of WSG were considered to be the highest dilution of the allergic serum and the lowest concentration of WSG which would still be able to elicit skin reaction.

In vivo inhibition of P-K reactions to WSG

Table XXIII shows the reactions obtained in sites sensitized with forty-fold diluted allergic serum on challenge with mixtures consisting of WSG at a concentration of 25 μg/ml and fraction D-(W)-II in varying amounts. Thus, a mixture containing 25 μg of WSG and 2.5 mg of fraction D-(W)-II per ml elicited a 2+ reaction. A similar reaction was observed when the concentration of fraction D-(W)-II was increased up to 10 mg/ml. WSG at a concentration of 25 μg/ml also elicited a skin reaction of 2+, whereas fraction D-(W)-II at concentration of 10 mg/ml elicited 1+ reaction. These results, therefore, demonstrate that the presence of fraction D-(W)-II did not interfere with the skin activity of the whole pollen extract (WSG), i.e. there was no inhibition of P-K reactions to WSG by fraction D-(W)-II.

Ultracentrifugal studies

Analytical ultracentrifugation revealed only a single peak in both fractions D-(W)-I and D-(W)-II with a sedimentation coefficient of 0.58 S.

TABLE XXIII

In vivo inhibition of P-K reactions to WSG.

Sample used for challenge of sensitized sites	Skin reactions obtained*
(25 µg of WSG + 2.5 mg of D-(W)-II)/ml	2+
(25 µg of WSG + 5 mg of D-(W)-II)/ml	2+
(25 µg of WSG + 10 mg of D-(W)-II)/ml	2+
25 µg of WSG/ml	2+
10 mg of D-(W)-II	1+

*The sites had been sensitized with a forty-fold diluted serum of patient 'Na' 24 hrs prior to challenge.

DISCUSSION

The two dialysates, D-(W) and D-(Ph), of the aqueous extract and of the phosphate buffer extract of timothy grass pollen, respectively, were shown to differ in the number of their constituents by chromatography on Sephadex G-25. Each of these dialysates contained a component (D-(W)-II and D-(Ph)-II, respectively) with identical immunological and biological properties. However, for an efficient fractionation of the constituents of the dialysate, D-(W) was a more convenient starting material than D-(Ph), since the latter contained a large amount of salts that had to be removed prior to any further fractionation.

By successive chromatography on Sephadex G-25 and G-50, a fraction with haptenic properties was isolated. It was observed that fraction D-(W)-II could be further resolved into two sub-fractions, D-(W)-II-A and D-(W)-II-B, by gel-filtration on Sephadex G-50 when the column was equilibrated and eluted with 0.2M ammonium acetate buffer instead of water. In contrast, Malley found that his haptenic fraction was eluted as a single component from either Sephadex G-50 or Amberlite IRC-50 column.

The purity of the haptenic fraction was studied by microzone electrophoresis. It was claimed by Malley et al (136) that the haptenic fraction moved as a single band on electrophoresis on cellulose acetate membrane. On the other hand, the haptenic fraction D-(W)-II isolated in this study was found to contain at least seven electrophoretically separable components (Figure 33). Moreover, fraction D-(W)-II-A was shown to contain four components and had the same biological activity as the parent fraction D-(W)-II. It was also observed that all the components of fraction D-(W)-II

were stained with ninhydrin provided the concentration of ninhydrin was 1%, as compared to the usual concentration of 0.1% employed in the analysis of Malley et al. Malley's observation of having detected only a single electrophoretic band in his haptenic material could be attributed to the following reasons: (i) the ninhydrin concentration used by him, as shown in the present study, might have been too low to detect some weakly reacting components; (ii) some of the bands develop only after 24 hours; (iii) the faster moving components, during his longer electrophoretic runs, may have been allowed to run off the membrane. To resolve the discrepancy between the results reported here and those of Malley, the author pointed out these possibilities recently, in a private communication*, to Malley. In response, Malley agreed that his original haptenic fraction, which had been purified by passing through Sephadex G-25, medium, had been subsequently resolved into 2-6 components, depending on the type and grade of Sephadex gel used. Moreover, Malley states that more recently he has purified further his haptenic preparation by chromatography on Sephadex G-50 (fine grade) and that he has detected by electrophoresis on cellulose acetate membrane, as done in this present study, two components in his purified fraction. As mentioned in the section of results, fraction D-(W)-II-A contained two strongly ninhydrin stainable bands in addition to the two other weakly stainable bands, which appeared much later but faded away within a couple of hours. These two bands might have escaped Malley's detection in his analysis of the purified haptenic fraction. This alone would have justified the repetition of the original work of Malley, because of the published works

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*The author wishes to acknowledge his gratitude for free exchange of views with Dr. A. Malley.

of Malley, which lead one to believe that he had actually succeeded in isolating a "pure" component.

In spite of this criticism of Malley's work, the findings presented in this thesis support his original claim that the dialysable constituents of WSG possess haptenic properties with respect to rabbit anti-WSG antiserum. Thus, the dialysable constituents did not give any precipitin bands with WSG antiserum and, moreover, inhibited the 'inner' precipitin band developed between WSG and anti-WSG antiserum. Two possible reasons for the inhibition of precipitin lines may be invoked: (i) a non-immunological effect, such as neutralization of electrostatic forces holding antigen-antibody molecules together, by the components of fraction (D-(W)-II) leading to the dissociation of antigen-antibody complexes, or (ii) direct and specific combination of the fraction with antibodies. The first possibility can be ruled out since the haptenic fraction failed to inhibit any of the precipitin lines between BSA-anti BSA and WSR-anti WSR systems and, therefore, the author is inclined to propose that this inhibition was due to specific combination of the fraction with antibodies. If this explanation is accepted, one would also accept the univalency of the fraction.

The haptenic fraction was found to elicit minimal skin reactions only when used at very high concentrations (5,000 μg/ml); in contrast, as shown in Chapter IV, the active fraction elicited skin reactions at 0.00001 μg/ml. Consequently, it would seem reasonable to conclude that any skin activity of the haptenic fraction might have been due to a slight contamination with one of the polyvalent skin active components. Alternatively, one might envisage that at such high concentration of hapten, a multimolecular aggregate behaving like a complete polymeric antigen might be formed and

give positive skin reaction.

A partial neutralization of the P-K reaction to WSG was observed when a ten-fold diluted allergic serum was incubated with the haptenic fraction at high concentrations. To observe complete neutralization (if this were indeed possible) by using higher concentrations was not considered practical because the haptenic fraction was shown to irritate the skin at higher concentrations than used for neutralization experiment. Neutralization experiment using more diluted allergic serum was not attempted because (i) at higher serum dilution, some of the reagins, which are present in low concentrations, might have been below the threshold of detection and might have been missed; (ii) it was shown in the present study that for a positive skin reaction the concentration of the allergen needed was higher at increasing dilutions of the allergic serum; (iii) limitation of the available P-K subjects.

In spite of the slight skin activity of the haptenic fraction at high concentration, i.e. 5 mg/ml, as compared to the activity of fraction C-IV detectable at 10^{-8} mg/ml, the results of the neutralization experiments with the haptenic fraction indicated that the bulk of the neutralization was due to the inhibitory nature of the haptenic fraction. This conclusion is based on the fact that the size of the reaction (3+) elicited by WSG at 10^{-3} mg/ml was shown to decrease when the allergic serum was mixed with the haptenic fraction at a concentration of 1.25 mg/ml, whereas the threshold concentration of the haptenic fraction by itself, which could elicit only a 1+ reaction, was 5 mg/ml. Furthermore, a small reaction ($\pm$) was elicited by WSG at a concentration of 10^{-3} mg/ml in site sensitized with the allergic serum preincubated with the haptenic fraction at a concentration of 5 mg/ml;

this slight reaction could be attributed to the higher affinity of WSG for the combining site of reaginic antibody than that of the haptenic fraction.

To examine further the inhibitory nature of the haptenic fraction it was thought that the haptenic fraction when mixed in excess with small amount of WSG should be able to inhibit competitively the P-K reaction to WSG. However, the results of such experiment showed no inhibition. This observation might have been due to the higher affinity of WSG for the combining site of reagins than that of the haptenic fraction; the reaction with WSG might thus have been elicited in preference to any inhibition by the haptenic fraction.

CHAPTER VI

GENERAL DISCUSSION

Skin active non-dialysable fraction C-IV

In the present investigation a combination of different methods, such as salt precipitation, ion-exchange chromatography and gel-filtration on Sephadex, were used to isolate skin active fraction (C-IV) from the whole aqueous pollen extract of timothy grass pollen. Since fraction C-IV elicited a minimal skin reaction in a passively sensitized site at a concentration of 10^{-8} mg/ml, as compared to WSG which elicited a minimal skin reaction at a concentration of 10^{-3} mg/ml, it would appear at first sight that a 100,000-fold purification of the allergen has been accomplished. However, in desensitization experiments, the active fraction proved to be only four times as potent as WSG. To explain this discrepancy one must consider critically the difference in the factors determining the sensitivity of the two procedures.

As discussed earlier, the elicitation of a minimal reaction in the P-K test by a skin active pollen preparation is a reflection of the most active component present in the preparation, while desensitization of a sensitized site by the same preparation is due to the participation of all the skin active components in appropriate concentrations which were present in the preparation. The assay method based on the ability of a multi-component fraction to elicit a minimal reaction in the skin of an allergic individual or in a site passively sensitized cannot, therefore, be used unequivocally to compare the biological potency of various preparations.

Fraction C-IV was primarily a protein containing trace amounts of

carbohydrate. It had a U.V. absorption spectrum characteristic of proteins and its protein content determined by amino acid analysis was 93%. The amino acid analysis revealed the presence of all the naturally occurring amino acids, and glutamic acid, alanine and lysine were present in relatively larger amounts. The amino acid composition of fraction C-IV was shown to be different from that of its parent fraction C, indicating thus that the latter fraction was not simply a polymeric product of the former. Moreover, the amino acid composition of the active fraction (C-IV) differed from that of the active fraction of rye grass pollen, which is considered to be a direct demonstration that the two active fractions of timothy and rye grass pollens are chemically different entities. This would explain why some grass sensitive patients fare better when treated with extracts of mixed grasses rather than with a single grass extract.

The fact that glutamic and aspartic acids are present in large amounts in the active fractions of the three different pollens, i.e. in timothy, rye and ragweed pollens, might suggest the possible participation of these amino acids in the appropriate allergenic determinant groups of these pollens.

The carbohydrate moiety of fraction C-IV was shown to contain arabinose. Stanworth (197) and Berrens et al (198) have suggested that the carbohydrate moieties of allergenic substances, such as horse dandruff and house dust allergens, might be responsible for the allergenicity of these substances; no such conclusion seems to apply to pollen allergens. Thus, neither alder (199) nor ragweed (151) pollen allergens contain significant amounts of carbohydrates; moreover, it was shown that the higher the protein content of individual ragweed pollen fractions, the higher was the specific activity (149). In this connection, one may also cite the results of Augustin (108) who showed

that several carbohydrases did not effect the skin activity of cocksfoot and timothy pollens extracts. In the present study the active fraction of timothy grass pollen was found to contain only 2.2% carbohydrate. Furthermore, the increase in specific skin activity was parallelled by an increase in protein content of the active fractions, C-IV being the most active and having the lowest carbohydrate content. The weight of all this evidence supports the view that the carbohydrate moiety of WSG is not directly responsible for the allergenicity of grass pollen.

Although the molecular weight of fraction C-IV was not determined, the low sedimentation coefficient (1.5S) and its retarded elution volume on Sephadex G-75 would suggest a molecular weight of the order of 15-20,000, which is in accord with the conclusion of Augustin, based on her ultra-filtration studies of timothy pollen preparation, that the molecular weight of timothy pollen allergens was 14,000. On the other hand, a higher molecular weight (32-37,000) was ascribed to the main active components of rye grass (141) and ragweed pollens (152), though the presence of some low molecular weight (9-11,000) allergens were detected in both rye grass (141) and ragweed (151) pollen extracts. The presence of lower molecular weight skin active components was demonstrated in the extracts of timothy grass pollen since some skin activity was found in a chromatographic fraction eluting after fraction C-IV. Fraction C-IV was shown to contain all the skin active components of the whole pollen extract and, therefore, one may postulate that the lower molecular weight allergens had arisen from the degradation of the high molecular weight components, probably by enzymatic digestion during the process of extraction.

Hjorth (200) observed that allergen when stored in solution lost their

skin activity due to the adsorption of the allergens to the glassware. This effect could, however, be minimized by storing the solution in silicone coated glassware (133). During the present investigation, the author also observed loss in the activity of allergen solutions on storage. This loss was shown to be more pronounced in crude and dilute solutions than in pure and concentrated solutions. Consequently, all the pollen fractions were lyophilized and stored in powder form at $4^{o}C$. It was found that lyophilized materials retained their biological activity after storage for four years; one can, therefore, recommend the process of lyophilization for storing the various pollen preparations. Although skin active fractions were shown to be stable on lyophilization, their activity seemed to be reduced on heating.

The active fraction (fraction C) was shown to be resistant to enzymatic digestion with insoluble α-chymotrypsin preparation. Antigen E of ragweed (152) was also shown to be resistant to chymotrypsin, trypsin and papain. In contrast, the skin activity of timothy and cocksfoot pollen extracts was destroyed by enzymatic digestion of pepsin and trypsin (109) and that of the active fraction of rye grass was lost by enzymatic digestion with chymotrypsin and trypsin (143). This difference in reactivity with respect to digestion by enzymes might be due to differences observed in the amino acid composition and/or structure of the different pollen allergens.

The water soluble grass extract (WSG) was shown to contain all the skin active components of extracts of pollen which had been ultrasonically or mechanically disintegrated. This is considered to indicate that the skin active components present in the pollen are readily dissolved out on contact of the pollen with the wet mucous membrane in the respiratory tract or in the eyes of individuals exposed to pollen.

The results of desensitization experiment demonstrated the multiplicity of the allergenic determinants in timothy grass pollen. At present, there is no experimental evidence demonstrating that the allergenic determinant could be identified with the antigenic determinant inducing antibody formation in rabbits, although many investigators have tacitly assumed that this was the case. King et al (152) showed that he could remove 90% of the skin activity of ragweed pollen extract by precipitation with rabbit anti-E antiserum, which can be taken as evidence only for the presence of antigenic and allergenic determinants on the same molecule(s), but not for their identity. This criticism is substantiated by the observation that the antigens responsible for the "outer" precipitin band formed in immunodiffusion studies in this investigation were not allergens.

Hapten-like properties of dialysable fraction D-(W)-II

Using serum of an untreated allergic individual in P-K tests, it was demonstrated that the dialysable constituents had only slight skin activity when used at high concentrations. However, since the most active fraction elicited skin reactions at 10^{-8} mg/ml, as compared to the dialysable constituents which elicited skin reaction at 5 mg/ml, it would appear that the activity of dialysable constituents might have been due to slight contamination with the polyvalent skin active components. Alternatively, the activity of dialysable constituents could be attributed to the formation of multimolecular aggregates of the constituents behaving like a polymeric, complete antigen and thus giving positive skin reaction.

Previous investigators (123,124,122) reported conflicting results on the skin activity of dialysable constituents of timothy grass pollen. More

recently, Malley et al (136) found that his haptenic fraction had some activity in some patients, in a few patients no activity, and two patients tested had the same degree of reactivity to hapten as to the purified allergen. However, there was no report on whether the patients tested by Malley et al were treated or untreated. This is very important since Malley himself reported recently (138) that the haptenic fraction isolated by him from the dialysable constituents led to the production of new reaginic antibodies in man; one would thus expect that treated patients exposed to dialysable constituents during 'hyposensitization' treatment might give strong positive skin reactions with the dialysable constituents.

It was shown that the haptenic fraction D-(W)-II could partially neutralize the P-K reaction to WSG when the allergic serum was pre-incubated with the haptenic fraction. The size of the reaction (3+) elicited by WSG at 10^{-3} mg/ml was found to decrease when the allergic serum was mixed with the haptenic fraction at a concentration of 1.25 mg/ml, and a small reaction ($\pm$) was elicited by WSG at a concentration of 10^{-3} mg/ml in a site sensitized with the allergic serum pre-incubated with the haptenic fraction at a concentration of 5 mg/ml. Since the threshold concentration of the haptenic fraction by itself, i.e. that capable of eliciting a 1+ reaction, was 5 mg/ml, it would seem that the observed neutralization was due primarily to the inhibitory nature of the haptenic fraction.

The results presented in this thesis confirm the original claim of Malley regarding the haptenic nature of dialysable constituents with respect to rabbit anti-timothy pollen antiserum. It was demonstrated that the haptenic fraction inhibited specifically the 'inner' precipitin band of WSG and anti-WSG rabbit antiserum. Since it was also found that the

active fraction (C-IV) gave rise only to the 'inner' precipitin band, one can speculate that the haptenic fraction might have arisen by some degradation process (possibly due to enzymes present in pollens) of the antigenic components of fraction C-IV.

In contrast to the published works of Malley, it was unequivocally demonstrated that the haptenic fraction consisted of at least two electrophoretically separable components.

Attempts may be made in future investigations to obtain univalent fragments by enzymatic or chemical degradation of fraction C-IV which would inhibit specifically an allergic reaction to WSG. This would involve the selection of proper enzymes or chemical reagents which would lead to the production of haptenic, univalent fragments capable of inhibiting the allergic reaction; one could subsequently isolate and characterize chemically the inhibitor(s) from the pool of digested products.

SUMMARY

1. Defatted timothy grass pollen was extracted by procedures involving the disintegration of pollen grains mechanically and ultrasonically and the extracts were shown by immunoelectrophoresis, immunodiffusion and ultracentrifugation to be heterogeneous mixtures of multiple constituents. The extracts of pollen, which had been mechanically or ultrasonically disintegrated, shared the main antigenic components of the water soluble grass extracts (WSG), as demonstrated by immunodiffusion, all extracts giving the same 'outer' and 'inner' precipitin lines with rabbit anti-WSG antiserum. The immunoelectrophoretic patterns of these extracts, though more complex, were closely similar in their overall appearance.

2. All extracts were shown to be skin active and no qualitative difference as to the number of skin active components could be detected among the various extracts.

3. The biological activity of the extracts was reduced on heating.

4. The water soluble grass extract (WSG) was fractionated into various fractions by a combination of different methods, i.e. salt precipitation, ion-exchange chromatography on DEAE-cellulose and gel-filtration on Sephadex G-25 and G-75. The skin activity was shown to be distributed among different chromatographic fractions.

5. The most active fraction was shown, by cross-neutralization in Prausnitz-Küstner passive transfer tests, to contain all the skin active components of WSG. A 100,000-fold purification of at least the most active component of fraction C-IV has been achieved.

6. The active fraction (C-IV) was found to contain 93% protein and 2.2% carbohydrate, and the rest accounted for its moisture content. The amino acid analysis revealed the presence of all the naturally occurring amino acids with glutamic acid, alanine and lysine being present in large amounts. The carbohydrate monomeric unit was identified as arabinose.

7. The active fraction (C-IV) was shown to contain at least three out of the seven antigenic components of WSG, as determined by immuno-electrophoresis. The immunodiffusion analysis revealed that the antigenic components of fraction C-IV belonged to the 'inner' groups of antigens of WSG.

8. The whole dialysate (D-(W)) was skin active and dialysable constituents fractionated by gel-filtration on Sephadex G-25 and G-50 were only slightly skin active when used at high concentration (5 mg/ml). This activity was attributed to either slight contamination by polyvalent skin active components which could elicit a skin reaction at a concentration of 10^{-8} mg/ml or the formation of polymeric aggregates at high concentration behaving like a polyvalent skin active component.

9. The haptenic fraction D-(W)-II isolated from dialysable constituents was shown to inhibit specifically the 'inner' precipitin band developed between WSG and anti-WSG serum in immunodiffusion experiments.

10. This haptenic fraction could also partially neutralize the P-K reaction to WSG when the allergic serum was preincubated with the haptenic fraction.

11. It was shown by microzone electrophoresis on cellulose acetate membrane that the haptenic fraction consisted of at least two electrophoretically separable components.

CLAIMS TO ORIGINALITY

1. The cross-neutralization technique using the Prausnitz-Küstner test was adopted to demonstrate that the water soluble grass extract (WSG) contained the same number of skin active components as that of extracts of pollen which had been mechanically or ultrasonically disintegrated.

2. All the skin active components of WSG were isolated by a combination of different methods, i.e. salt precipitation, ion-exchange chromatography and gel-filtration on Sephadex, in a single fraction C-IV.

3. A 100,000-fold purification of at least one of the most skin active components of fraction C-IV was achieved.

4. It was shown that the assay methods currently used to determine the skin activity are not adequate.

5. The most active fraction was shown to contain 93% protein and 2.2% carbohydrate; the sugar unit was identified as arabinose while all the naturally occurring amino acids were detected in the hydrolysate of the protein moiety, glutamic, alanine and lysine being in preponderance.

6. It was discovered that the ninhydrin concentration had to be increased to 1% from the usual concentration of 0.1% for the

demonstration of multiple electrophoretically separable constituents of the haptenic fraction by microzone electrophoresis.

7. The haptenic fraction of timothy grass pollen was shown to consist of at least two electrophoretically separable components.

8. It was demonstrated that the haptenic fraction was able to neutralize partially the P-K reaction to WSG when the allergic serum was incubated with the haptenic fraction prior to sensitizing the skin site.

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