STUDIES ON THE ALLERGENS OF RAGWEED POLLEN

A THESIS

by

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EXPERIMENTAL*

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CHAPTER I

GENERAL INTRODUCTION *

Although allergy and immunity may appear, on superficial examination, to be two unrelated phenomena, studies have revealed that the mechanisms of the reactions involved in the manifestations of the immune and allergic states have common features. A brief survey of the fundamental aspects of immunology would therefore appear to be essential prior to a discussion of the mechanisms involved in the allergic reaction.

The existence of immunity can be observed in an animal which, having recovered from a bacterial infection, is found to be resistant to a second infection with the pathogenic organism. The serum obtained from such an animal is capable of causing agglutination or lysis of the organism when incubated with it in vitro. The factor in the serum responsible for this reaction is called the antibody and the substance which induced its formation, in this case the bacteria,is referred to as the antigen. Antibodies have been isolated in pure form and appear to possess the same properties as normal serum gamma globulins, i.e., similar electrophoretic mobility, sedimentation constant, amino acid composition and solubility properties. Antibodies can be differentiated from normal gamma globulins only by their ability to combine specifically with the antigen. Not only virulent organisms but also toxins and innocuous substances such as

* More detailed descriptions of the concepts presented in this chapter can be found in the references given at the end of the chapter.

red blood cells and serum proteins of another animal species can induce the formation of antibodies. Incubation of each of these antigens with its respective antiserum results in a specific combination of the antigen and antibody. This reaction manifests itself in neutralization and often in precipitation of the toxin in the case of toxins, in agglutination (in the absence of complement) or hemolysis (in the presence of complement) in the case of the red blood cells and in precipitation in the case of the serum protein antigens. In order for precipitation or agglutination to take place, it has been generally accepted that both the antigen and the antibody must each have at least two reactive groupings, i.e.,they must be "multivalent". It has been shown that the reaction between the antigen and the antibody is highly specific and that even closely related antigens do not cross-react. This specificity must therefore be attributed to a configurational complimentariness between the antigen and the antibody and may be compared to the specificity of an enzymatic reaction.

There are substances, usually of low molecular weight, which, by themselves, cannot induce the formation of antibodies but which are antigenic if they are conjugated to large carrier molecules (i.e., serum proteins). Such substances are capable of reacting with the antibody once it has been formed. Landsteiner named such substances haptens.

Usually no visible reaction occurs when a hapten is incubated with its respective antiserum whereas incubation of the antiserum with the conjugated hapten results in precipitate formation. Combination of the

hapten with the antibody can be demonstrated to have taken place, however, since the specific antiserum incubated with hapten is no longer capable of precipitating with the conjugated hapten. Since the free hapten can not precipitate with the antiserum, it can possess only one chemical grouping capable of reacting with the antibody. Such a hapten (i.e., benzoic acid, sulphanilic acid) is referred to as a univalent hapten. There are, however, haptens which appear to be multivalent. Heidelberger and Avery isolated a soluble specific carbohydrate substance from pneumococcus which reacted with the antiserum to give a precipitate but which was unable, by itself, to stimulate antibody formation.

The formation of antibodies by an animal in response to antigenic stimulation does not always result in an immunity. In 1902, Portier and Richet reported that the injection of a toxic extract of the tentacles of certain sea anemones into a dog which had recovered from a previous sublethal dose caused violent illness and often death even if the dose injected was so small that it did not produce symptoms in normal animals. To characterize this apparently increased susceptibility to the toxin, they coined the term anaphylaxis or anaphylactic shock to differentiate this condition from the immune state. It has since been shown that substances capable of provoking anaphylaxis need not be toxic in themselves but can also be innocuous materials such as serum proteins of a foreign species. It is only necessary to "sensitize" the animal by one or more injections of the material and, after an appropriate time interval,

to administer the "shocking dose". If the produced shock is not fatal, the animal, after recovery, is temporarily refractory to the substance.

Anaphylactic shock appears to be due to contraction of smooth muscle and to increased capillary permeability. Anaphylaxis can be induced most easily in the guinea pig. Immediately after injection of the "shocking dose", the animal becomes restless, coughs and rubs its nose. Respirations, which were at first increased in frequency, become slower and laboured and the animal begins to gasp and makes tremendous inspiratory efforts. After a few minutes, the animal dies. In the guinea pig, anaphylaxis appears to be caused by contraction of the smooth muscle in the bronchioles causing the latter to constrict thus preventing the animal from breathing.

There can be no doubt that anaphylaxis is essentially the result of an antibody-antigen reaction since it can be produced only with the same substance that was used for the sensitization of the animal, the induction period for anaphylactic sensitivity is of similar length to that for antibody production and sensitivity can be passively conferred to a normal animal by transfer of serum from a sensitized animal. However, there is still uncertainty as to where the reaction causing anaphylaxis occurs. Two different theories have been offered. According to one view, the antibody-antigen reaction occurs in the circulation (humoral theory), whereas according to the other view, the

antibody-antigen reaction occurs with antibodies present in, or fixed to, the tissues (cellular theory). In both cases, the antibody-antigen reaction appears to cause the release of histamine or histamine-like substances which act on the shock organs. The fact that anaphylaxis can be induced easily in a sensitized animal only after the concentration of circulating antibody has decreased to a low level or when antibody has completely disappeared from the circulation would support the view that the antibody-antigen reaction causing anaphylaxis occurs in the tissues. Specific desensitization of a sensitive animal by successive injections of small, sublethal doses of the antigen is difficult to explain except by the assumption that antibodies present in the animal are slowly neutralized with the antigen so that little free antibody is present in the tissues when the shocking dose is administered.

Anaphylaxis is classified as a hypersensitivity by many immunologists, since the animal has become sensitive, rather than immune, to the antigen. However, the period of time during which anaphylactic sensitivity is most prominent appears to be only a phase of the immune state when the concentration of antibody has decreased or when antibody has entirely disappeared from the circulation although there is still antibody fixed to the tissues. Anaphylactic shock, as experimentally induced in animals, is essentially an artificial state. However, there are conditions that occur in human beings (i.e., serum sickness, tuberculin sensitivity, allergy) which resemble anaphylaxis

in certain respects. After it was discovered that antisera obtained in animals against certain bacterial toxins had great therapeutic effects when injected into persons suffering from bacterial infections, the injection of foreign antisera, especially horse antisera, into humans became relatively common. It was soon found that some individuals became ill and sometimes developed violent reactions about 8 to 16 days after they had been injected with the antisera. This illness, which is characterized by the occurrence of rashes, fever, pains in the joints, edema and glandular swelling, was given the name of serum sickness. At first it was thought that the reaction was due to the antitoxin content of the injected antiserum, but it was observed that the injection of normal serum from the same animal could also produce serum sickness. Von Pirquet and Schick offered an explanation for the etiology of serum sickness. They suggested that the patient forms antibodies against the injected foreign serum proteins. The reaction of these antibodies with the antigens which have still persisted in the circulation or which may have been fixed to the tissues causes the symptoms. The antibodies formed by the patient to the harmless serum proteins have therefore made him hypersensitive to the injected proteins.

Simultaneous to the discovery of serum sickness, it was noted that tuberculous animals reacted to the injection of tuberculin in a manner characteristic of the hypersensitive state. It was also observed that individuals who had serum sickness or tuberculosis were capable

of giving skin reactions when injected intradermally with the foreign serum proteins and tuberculin, respectively. Von Pirquet introduced the term allergy (from the greek allos-altered and ergeia-reactivity) in order to distinguish a condition of acquired sensitivity to a foreign substance from a condition of tolerance or immunity to it. He noted that the symptoms of hay fever were similar in many respects to those of tuberculin hypersensitivity and serum sickness, two conditions which he defined as allergic states, and thus hay fever was classified as an allergy. Von Pirquet also proposed the term allergen for the causative agent in allergy to differentiate it from the antigen, which, by definition, is responsible for the production of antibodies characteristic of the immune state.

It has been known since the 1870's that a reaction could be obtained in the skin of an allergic person upon intradermal injection of the specific allergen. In 1921, Prausnitz and Küstner reported that a site in the skin of a normal individual which had been sensitized with the serum of an allergic individual was capable of giving a cutaneous reaction (wheal and erythema) when challenged with the allergen to which the donor person was sensitive. This reaction was specific and was similar in appearance to that produced in the skin of the allergic individual. They thereby demonstrated that there exists a substance in the blood of the allergic person capable of reacting specifically with the allergen. This factor was named "reagin" or skin sensitizing antibody to differentiate it from the antibodies nor-

mally formed by antigenic stimulation. Reagin is not a normal type antibody since it can not give a precipitate nor can it be inactivated or neutralized when the serum of the allergic person is incubated with the allergen in vitro. It can be inactivated by heating at 56° C for several hours whereas this treatment does not appear to affect ordinary antibodies. The failure of reagin to give a precipitate when incubated with the allergen preparation may be attributed to the possibility that it is "polyvalent" but present in a very low concentration in the blood or that it or the allergen are "univalent" molecules. The fact that allergen preparations can give precipitates with rabbit antisera does not necessarily imply that the allergen is polyvalent. It is possible that the allergen and the antigen(s) in such preparations are separate entities. The concentration of reagin in the blood of an allergic person appears to be extremely low, since the electrophoretic patterns of the sera of allergic persons are indistinguishable from those of sera of normal individuals and we have succeeded, in this laboratory, in isolating fractions of allergic sera devoid of detectable protein but containing reagin. The allergic state and anaphylactic sensitivity are therefore both characterized by low concentration of circulating antibody.

Many substances, such as pollen, house dust, danders, fungi, foods, drugs and simple chemicals, have been found to be capable of inducing allergy. Hay fever is a predominant type of allergy on the Amer-

ican continent and affects millions of people during the spring and summer months. It is characterized by watery exudation from the mucous membranes of the upper respiratory tract and conjunctiva, which results in violent and often protracted sneezing, nasal discharge and lacrimation. Many pollens of trees, grasses and weeds have been shown to be responsible for hay fever and ragweed pollen appears to affect more people than any other pollen.

The purpose of the work reported in this thesis was to isolate and characterize the allergen(s) in ragweed pollen. The isolation of the allergen is not only important as an academic achievement but is necessary in order for further progress to be made in the study of the nature and treatment of allergies as typified by ragweed pollen sensitivity. The treatment of hay fever usually involves a series of injections of an aqueous extract of the pollen. This type of treatment was introduced by Noon in 1911. He thought that hay fever was a type of anaphylaxis and that one could desensitize the allergic individual to the pollen by injecting him with small quantities of the allergen preparation at various time intervals in the same way as one can prevent anaphylactic shock in a sensitized animal by injecting it with small quantities of the antigen prior to the administration of the shocking dose. This procedure of "desensitization" or "hyposensitization" instituted by Noon still constitutes the main treatment offered to clinically allergic individuals. However, it has been

noted that a large percentage of allergic persons do not respond well to this treatment and, in fact, become even more sensitive to the allergen. Since these individuals are injected with the whole aqueous extract of the pollen, they may become sensitive to a constituent(s) of the pollen to which they were not originally allergic. Such an effect would counteract any desensitization to the original allergen which may have taken place during the treatment. The isolation of the allergen would allow only its injection during the treatment which might lead to more effective desensitization of the allergic individual.

Many attempts have been made during the past 30 years to isolate the allergen or allergens in ragweed pollen but almost all have met with little success. To understand the difficulties encountered by the many investigators, it is necessary to examine the structure and the composition of ragweed pollen.

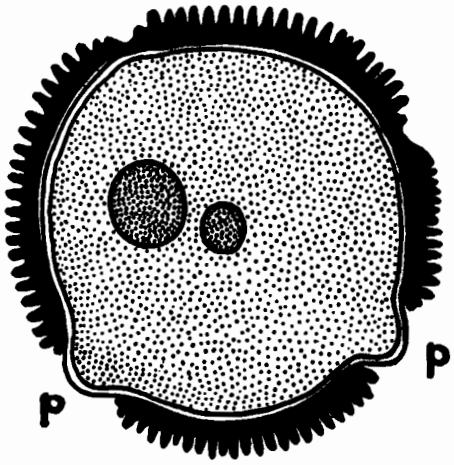
Short and giant ragweeds are the most commonly found ragweeds on this continent. Short or dwarf ragweed (Ambrosia artemisiaefolia) is an annual herb not more than 4 feet high, frequently much branched, with rough, fern-like leaves (Fig 1a). Giant or tall ragweed (Ambrosia trifida) is a coarse branching annual herb, with rough stems and leaves, often reaching a height of 15 feet. Both ragweeds pollinate (release their pollens into the air) from about the first week in August to the first week in October.

Fig. 1.

(a) Short Ragweed (Ambrosia Artemisiaefolia).

(b) Ragweed Pollen. Several pores (P) can be seen protruding through the surface of the pollen grain.

(a)



(b)

The pollen grains of both ragweeds are very similar in structure (Fig 1b). They are small spherical structures, 16 to 20 microns in diameter, and are bright yellow in colour. The outer cell wall or exine forms a very conspicuous portion of the cell (about 65 per cent of the weight of the pollen) and is composed of a hard, seemingly inert material. Short, blunt spines, numerous furrows and pores are found on the surface of the pollen. The inner cavity of the pollen consists of cytoplasm, cytoplasmic granules and two nuclei, all of which are separated from the outer cell wall by a thin inner cell wall or intine, which is composed chiefly of cellulose. One of the two nuclei is referred to as the tube nucleus (it controls the growth of the pollen tube during the process of fertilization) and the other is the generative nucleus, which undergoes a single mitotic division forming two sperms or male cells which are directly concerned with the fertilization of the ovule. The pollen is a very complex structure, endowed with all the processes of a living cell and therefore contains all the chemical constituents present in living cells, such as enzymes, nucleoproteins, carbohydrates, proteins, lipids, peptides and, in addition, pigments. The difficulty then of isolating an allergen from the pollen is similar to that of isolating an enzyme from a cell preparation.

The numerous reports of attempts at isolating the allergen from ragweed pollen which have appeared during the last three decades

are very confusing and often contradictory. There have been claims that the allergen is a protein and that it is non-dialyzable. Some investigators have claimed that the allergen is not a protein and that it is dialyzable. Still others have associated allergenic activity with carbohydrate, lipid and pigment constituents of the pollen. In some cases, the allergen has been found to be destroyed by degradation with proteolytic enzymes while in other cases it was found not to be affected by the same treatment. Therefore, rather than presenting all the reports in the order of their chronological appearance, the historical summary has been divided into numerous sections, each section containing all the data relevant to a single aspect of the work done on ragweed pollen.

REFERENCES

1. Cushing, J.E., and Campbell, D.H.,
Principles of Immunology,
McGraw Hill Book Company, Inc., Publisher.

2. Boyd, W.C.,
Fundamentals of Immunology, Third Edition,
Interscience, Publisher.

3. Kabat, E.A., and Meyer, M.M.,
Experimental Immunochemistry,
Charles C. Thomas, Publisher.

4. Campbell, D.H., and Lanni, F.,
The Chemistry of Antibodies. Chapter XI in
D.M. Greenberg, Editor,
Amino Acids and Proteins,
Charles C. Thomas, Publisher.

5. Carpenter, P.L.,
Immunology and Serology,
W.B. Saunders Company, Publisher.

6. Landsteiner, K.,
The Specificity of Serological Reactions,
Harvard University Press, Publisher.

7. Fuller, H.J., and Tippo, O.,
College Botany,
Henry Holt and Company, Publisher.

8. Wodehouse, R.P.,
Hay Fever Plants,
Chronica Botanica Company, Publisher.

CHAPTER II

HISTORICAL SUMMARY

Early Studies.

The first recorded observation of the condition referred to today as hay fever was made by Botallus in 1565 (1) who noted that certain individuals began to sneeze when exposed to certain flowers.

In 1819, Bostock (2) described the symptoms of the disease and it was subsequently known as"Bostock's catarrh". Evidence that this catarrh might be due to pollen was presented by Elliotson in 1831 (3), who observed that the period of worst symptoms coincided with the maturing of grasses (pollination) in the haying season. Because of its apparent relationship with the season of the year, the disease became known as hay fever. The work of Gordon (4), Phoebus (5), Salter (6) and Wyman (7) all implicated pollen as the causative agent. Blackley, in 1865 (8), performed the first diagnostic skin test for hay fever and also showed that the pollen of grasses was capable of inducing conditions identical to hay fever in allergic individuals upon their inhalation of the pollen. Dunbar (9), who accepted the view that the pollen of grasses was responsible for hay fever, repeated the main experiments of Blackley and established beyond doubt the role of pollen as the causative agent. In 1910, Meltzer (10) showed that a patient suffering from hay fever was sensitive to a constit-

uent of the pollen.

Studies on the Chemical Composition of Ragweed Pollen.

In 1914, Heyl commenced a series of analyses on ragweed pollen. In his initial communication (12), he reported the composition of the pollen to be as follows: moisture, 5.3 per cent; phosphorus, 0.37 per cent; alcohol-soluble material, 42.9 per cent; crude fiber (mostly cellulose), 12.2 per cent; pentosans, 7.3 per cent; dextrin, 2.1 per cent; ash, 5.4 per cent; protein, 24.4 per cent. He assumed that all the nitrogen-containing material was protein. The ether soluble fraction, obtained on defatting the ragweed pollen, amounted to about 12.5 per cent of the dry weight of the pollen. In a subsequent study (13), he showed that the water soluble extract of the pollen, obtained after the pollen was defatted with ether and extracted with 95 per cent alcohol, contained peptones, adenine, guanosine, histidine, arginine, lysine and several other not well characterized constituents. In addition to these components, his extract contained two different proteins; an albumin, which coagulated at 45 to 50° C in the presence of small amounts of acetic acid and a proteose, which could not be coagulated by heat but which could be precipitated by saturating the solution with ammonium sulphate. Extraction of the pollen with dilute alkali after its extraction with water yielded a glutelin fraction which contained histidine whereas the albumin and proteose fractions did not (14). All three protein fractions were

allergenically active in ragweed sensitive individuals. Heyl also demonstrated (15) that the yellow pigments in ragweed pollen are flavanols which are constituents of many flowers. Two of the pigments which he isolated were isoquercitrin, which is a quercetin-glucoside, and isorhamnetin, which is a monomethyl ether of quercetin. In a study of the ether soluble fraction of the pollen (16), he demonstrated the presence of formic, acetic, valeric, lauric, oleic, palmitic and myristic acids. Heyl concluded that none of the substances which he showed to be present in ragweed pollen (he identified twenty-seven substances) "appear to represent any chemical specialization of the cell". He did not attempt to correlate allergenic activity with all the fractions which he isolated.

A similar but less comprehensive study on the chemical composition of ragweed pollen was carried out at the same time by Koessler (17). This investigator determined the total nitrogen of the whole pollen and its distribution between the different proteins isolated and also showed that ragweed pollen does not contain starch.

These studies are important in that they demonstrate the complexity of ragweed pollen and of the aqueous extract of the pollen. However, they do not offer any aid in elucidating the nature of the allergen or allergens present in the pollen.

Allergenic Identity of Pollens of Giant and Short Ragweed.

Since many investigators used pollen of either giant or short ragweed and some made no mention of the type of pollen used, the

allergenic relationship between the two pollens must be pointed out. Spain and Hopkins (18), using the method of cross-neutralization* of sites sensitized with sera of individuals allergic to both pollens, failed to find any specific differences in the allergens of the two pollens. Similar results were obtained by Stull et al (19) who also showed that the extract of short ragweed pollen was as effective as that of giant ragweed pollen in the treatment of hay fever. Brown (20) reported that many patients suffering from hay fever were equally sensitive to extracts of giant and short ragweed pollen. After treating such allergic individuals with the extract of one of the pollens, the injection of an equivalent or larger quantity of the other pollen extract into any of these individuals was not followed by a constitutional reaction which would have occurred had the allergens of the two pollens not been identical. Coca and Grove (21), employing the method of passive transfer, found that sensitized sites neutralized to an extract of low ragweed pollen would not react to an extract of giant ragweed pollen and vice versa. The sera used in their experiments were of patients allergic to both types of ragweed pollen.

Thus, it would appear that the allergens of giant and short ragweed pollen are identical.

*A critical discussion of the cross-neutralization test is presented in Chapter V .

Antigenicity of Ragweed Pollen.

Although precipitating antibodies to ragweed pollen extracts have never been found in sera of allergic individuals, ragweed pollen has been found to be antigenic in both rabbits and guinea pigs, precipitating antibodies consistently being formed by both these animals (23-37, 40). A large number of investigations have demonstrated that ragweed pollen contains several antigens (26, 28-32, 37). For example, Wodehouse has demonstrated the existence of at least seven antigens in extracts of ragweed pollen using the gel diffusion method (30, 31).

Harrison and Armstrong (38) and Walzer and Grove (39) concluded that the antigens in giant and short ragweed pollens are identical insofar as their antigenicity in guinea pigs is concerned. They found that the uterus of a guinea pig sensitized to an extract of giant ragweed pollen was sensitive to both pollen extracts and that after the uterus was desensitized with either pollen extract, it was found to be insensitive to both. However, in order to correlate the antigenicity of ragweed pollen with its allergenicity, it must be ascertained that the antigens in ragweed pollen which induce the formation of precipitating antibodies in laboratory animals are identical to the allergens responsible for hay fever in man.

Black (41) showed that ragweed pollen extracts digested with trypsin were no longer able to act as antigens in complement fixation tests (using human allergic serum) although they were allergeni-

cally as active as before digestion. Armstrong and Harrison (42) could find no relation between the complement binding capacity of the pollen extract (using rabbit antisera) and its ability to produce skin reactions in allergic individuals. Mosko et al (23), Roth and Nelson (24) and Hecht et al (25) all succeeded in separating extracts of the pollen into fractions which were either allergenically active or which were able to give positive precipitin tests with rabbit anti-ragweed pollen sera. The available evidence would therefore suggest that the allergens and antigens in ragweed pollen are distinct entities.

Evidence in Support of the Protein Nature of the Allergen(s) in Ragweed Pollen.

In 1906, Wolff-Eisener (43) discussed the possibility that hay fever might be the result of a sensitivity to a protein component of the pollen and that the condition might be similar to anaphylactic sensitivity which can be induced in animals. In 1911, the theory of hay fever as a protein sensitivity was further advanced when Noon (44), assuming that hay fever was a type of anaphylactic sensitivity, announced that he had obtained active desensitization of patients suffering from hay fever by the injection of water soluble extracts of the pollen. Since only proteins were then thought of as being capable of inducing the formation of antibodies and since desensitization was interpreted as a neutralization of antibodies by the injected pollen extract, it was generally accepted that hay fever was a sensitivity to

a protein constitutent of the pollen. As a confirmation of this belief, Koessler (45), in 1914, stated that pollen preparations in which no protein could be detected were devoid of allergenic activity.

Bernton et al (46), Rappaport and Johnson (47) and Moore et al (49) isolated protein fractions from ragweed pollen extracts by precipitation with either ammonium sulphate or alcohol. All the fractions were found to be active by skin test in allergic individuals, although variations in the sensitivity of the individuals to the protein fractions was noted (46, 47).

Spain and Newell (48) demonstrated that they could remove completely the non-protein nitrogen from ragweed pollen extracts by ultrafiltration without appreciably diminishing the activity.

Unger et al (50) prepared a protein and a carbohydrate fraction from an aqueous extract of the pollen. They found that passively sensitized sites neutralized to the protein failed to react to the carbohydrate, whereas sites neutralized to the carbohydrate were still capable of reacting to the protein. The opinion of these investigators was that the allergen was a protein.

Stull et al have been the most active proponents of the theory that the allergen is a protein. In 1931, they reported the isolation of an active protein from an extract of giant ragweed pollen (81). It contained 13.52 per cent nitrogen, 50.37 per cent carbon

and 6.40 per cent hydrogen and gave a negative Molisch test. They also isolated an active material of similar composition from an extract of short ragweed pollen (19).

In subsequent publications (52-54), Stull et al reported the fractionation of extracts of ragweed pollen by precipitation with ammonium sulphate. One of the active fractions contained 12 per cent nitrogen and appeared to be free from carbohydrate while a second fraction contained only 2 per cent nitrogen and a considerable quantity of carbohydrate (53). They also described the preparation of an active alkali-soluble protein fraction (glutelin) from ragweed pollen (54). The active material in each of the fractions was found to be non-dialyzable and, therefore, was considered to be a protein by these investigators.

Other evidence in favour of the protein nature of the allergen was presented by Cohen and Friedman (56). They obtained active fractions from a pollen extract by fractional precipitation with ammonium sulphate. Freezing and thawing of these preparations resulted in a separation of pigment from unpigmented, protein material which was allergenically active.

Bukantz et al (58, 59) studied the dialyzable and non-dialyzable components of ragweed pollen extract. The non-dialyzable residue contained most of the allergenic activity of the original extract and displayed properties characteristic of proteins. After hydrolysis, the non-dialyzable residue was found to contain fifteen amino acids as well as arabinose.

Evidence in Favour of the Non-Protein Nature of the Allergen.

Until the middle nineteen-twenties, it was widely accepted that the allergenically active substance in ragweed pollen was a protein. Then Grove and Coca (60, 61) announced that they had been able to eliminate all nitrogen from a pollen extract without causing a diminution in its allergenic activity. The extract was digested with trypsin and subjected to exhaustive dialysis. The dialyzed residue was found to be just as active allergenically as the original extract, although no nitrogen attributable to the pollen extract could be detected. Black (41) repeated the experiments of Grove and Coca and obtained identical results. Black and Moore (62) subsequently reported that a protein-free preparation (negative biuret and xanthoproteic tests, no precipitation with picric acid and sodium tungstate) gave good clinical results when it was used in the treatment of allergic patients.

Long and Teller (63) obtained an ultrafiltrate of a pollen extract which did not contain protein but which possessed all the allergens present in the original extract as it was capable of neutralizing sites to the whole extract.

Abramson et al (64-69, 77) isolated an active fraction from an aqueous extract of ragweed pollen by free electrophoresis. It was colourless, did not coagulate on boiling, did not precipitate with trichloracetic, nitric and sulphosalicylic acids and gave a

negative Molisch test. They claimed this material was a high molecular weight polypeptide.

Hecht et al (25) observed that incubating an extract of ragweed pollen at pH 4.0 and 60° C for one hour resulted in the formation of a precipitate. Only the precipitate was capable of giving a positive precipitin test with rabbit anti-ragweed sera. However, the supernatant was found to be allergenically as active as the original whole extract. The authors claimed that the precipitate was composed of high molecular weight material and the supernatant was composed of low molecular weight material although they did not present any evidence to support their claims. Mosko et al (23) repeated the experiments of Hecht et al and extended the study to include more immunologic experiments. The material which did not precipitate on heating to 60° C at pH 4.0 contained essentially all the allergenic activity of the extract, but was incapable of eliciting anaphylactic shock in guinea pigs sensitized to whole ragweed pollen extract and of precipitating with rabbit anti-ragweed serum. The material which precipitated at pH 4.0 and 60° C possessed slight allergenic activity, induced anaphylactic shock in guinea pigs sensitized to the whole extract and precipitated with rabbit anti-ragweed serum. Roth and Nelson (24) demonstrated that they could separate the allergenically-active material from the constituents capable of reacting with the rabbit anti-ragweed serum. They extracted ragweed pollen for about twenty days and the extracting solvent was

examined each day for allergenic activity, nitrogen content, and ability to precipitate with rabbit antiserum. The eighth extract no longer possessed allergenic activity whereas all the extracts up to the twentieth were able to precipitate with rabbit antiserum. These investigators concluded that the allergenic activity is a property of the low molecular weight material only.

Rockwell (70-72) reported the isolation of active material from a pollen extract by precipitation with HCl. The precipitated material, which contained about 90 per cent of the allergenic activity of the whole extract, had a minimum molecular weight of 4,500 as determined by its sulphur content and had the properties of a high molecular weight polypeptide. The precipitated material was not dialyzable, gave a positive biuret reaction, did not lose any activity on digestion with pepsin and was not coagulated by heat. In addition to peptide material, the precipitate also contained arabinose and a pigment which Rockwell identified as isorhamnetin. From his data, Rockwell postulated that each allergen molecule contained one molecule of isorhamnetin, one molecule of arabinose and two polypeptide chains containing a total of twenty-eight amino acids. The material precipitated with HCl also gave good therapeutic results when used instead of whole extract in the treatment of allergic individuals (71-73). Subsequently, Rockwell reported that the allergenically active material precipitated with HCl could be separated

into five active fractions by fractional precipitation of the whole extract with acid and salt solutions (74). All the fractions were of different composition and their minimal molecular weights varied from 650 to 4,500 as calculated from elemental analyses. All the fractions contained pigment, polypeptide and carbohydrate components.

Brown and Benotti (75) treated ragweed pollen extract with decolorizing carbon (Norite A) at pH 6.6 and found that the supernatant after centrifugation was very active by skin test although the charcoal had taken up over 60 per cent of the total nitrogen of the extract. The supernatant gave a negative xanthoproteic test but gave positive Molisch and ninhydrin tests and was found to contain free amino groups. They (76) also precipitated the ragweed pollen extract with phosphotungstic acid and found that only 50 per cent of the nitrogen was precipitated. The supernatant did not contain detectable protein and produced large reactions when injected in the skin of allergic individuals. However, this preparation also produced reactions, though of lesser intensity, in the skin of normal, non-allergic persons.

In 1948, Robbins et al (57) described the isolation of a heat-stable, allergenically active substance from an aqueous extract of the pollen. The extract was boiled for one hour at pH 4.0

which resulted in precipitation of the protein. The active supernatant was absorbed on colloidal aluminum hydroxide cream from which the active material was eluted with M/15 phosphate buffer, pH 7.4. The active material was then precipitated with alcohol at 0°C. The isolated material contained a carbohydrate component (composed of hexose, pentose and hexuronic acid), flavanol pigment and a component referred to by Robbins et al as "a protein component". This latter constituent, on hydrolysis with HCl, yielded 14 amino acids (arginine, cystine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, tryptophane, tyrosine and valine). This isolated material was allergenically more active than the whole extract containing the same amount of nitrogen. In view of the fact that the material was dialyzable and did not precipitate with either picric or trichloracetic acid, it is very possible that the "protein component" was a polypeptide.

Allergenicity of the Carbohydrate Constituents of Ragweed Pollen Extract.

The finding of Grove and Coca (60, 61) that all the nitrogen of a pollen extract could be eliminated without any accompanying loss of cutaneous activity influenced many investigators to turn their attention to the possibility that the allergen(s) in ragweed pollen might be a carbohydrate. In 1931, Black (78) isolated an active substance of carbohydrate nature by precipitation of the pollen extract with alcohol at pH 4.4. The active material did not pre-

cipitate with picric, tannic and phosphotungstic acids and the biuret, Millon and xanthoproteic tests were negative. The Molisch test was positive. The material, when made up to a volume equal to that of the original extract, was more active than the latter. Hydrolysis of this carbohydrate fraction yielded 55 per cent reducing sugar and the nitrogen content was 6 per cent. Black concluded that the material was not a protein but a complex carbohydrate. He did not explain what the nitrogen content of the material might represent. Conceivably, it could have represented up to 37 per cent protein or peptide (assuming a conversion factor of 6.25).

Caulfeild et al (79) also isolated an active material rich in carbohydrate. The method used for its isolation was very harsh and it is most surprising that the activity was not destroyed in the process of its isolation. The protein in the extract was precipitated at pH 4.2 and the carbohydrate was then precipitated with lead acetate at alkaline pH. The precipitate was dissolved by grinding it in cold, dilute sulphuric acid. The lead sulphate was removed by centrifugation and the excess sulphuric acid was removed by the addition of barium hydroxide. The material which remained in solution was dialyzed, reprecipitated with alcohol, redissolved in trichloracetic acid and finally reprecipitated with alcohol and dried. The final product contained only 1.5 per cent nitrogen and after hydrolysis yielded 58 per cent reducing sugar. It was active by both direct skin test and by passive transfer (79,80). The investigators felt that protein could not have

been present as a contaminant since this carbohydrate fraction was unable to elicit anaphylactic shock in guinea pigs sensitized to the whole pollen extract. However, if there would have been protein present in their preparation which was not antigenic in the guinea pig, it would not have been detected. These investigators did not make any suggestion concerning the composition of the nitrogen-containing material present in the active fraction.

Evidence has also accumulated which suggests that the carbohydrate constituents of the pollen extract are not allergenically active.

Baldwin et al (27) isolated several fractions from an aqueous extract of the pollen by fractional precipitation with alcohol. One of the fractions contained 60 per cent carbohydrate, only 1.4 per cent nitrogen and gave a negative ninhydrin test. However, this fraction was allergenically not as active as the other preparations nor was it very antigenic in rabbits and guinea pigs. These workers found that the allergenic activity of the fractions diminished as the nitrogen concentration decreased.

In 1931, Stull et al (81) isolated two active fractions from the pollen extract, one of which contained a large amount of carbohydrate as well as some nitrogen while the other consisted of protein and appeared to be devoid of carbohydrate as it gave a negative Molisch test. Since both fractions were able to neutralize sensitized sites to each other, these workers concluded that the

carbohydrate was not active but that it had been contaminated with the protein which was allergenically active. Johnson and Rappaport (82) reported the isolation of a fraction composed largely of carbohydrate which was very active by skin test. However, they found that the active material could be precipitated by saturation of the carbohydrate fraction with ammonium sulphate, leaving the relatively inert carbohydrate in solution. They concluded that the active principle was not a carbohydrate.

Unger et al (50) isolated a carbohydrate and a protein fraction from ragweed pollen. The carbohydrate fraction gave a very strong Molisch test and negative Millon and biuret tests. The nitrogen content of this fraction was 5.28 per cent. Sites passively sensitized with allergic serum failed to react to the carbohydrate solution after being neutralized to the protein fraction. On the other hand, all sites neutralized to the carbohydrate fraction gave positive reactions when challenged with the protein fraction. Unger et al concluded that the carbohydrate in the pollen extract was not allergenically active.

Allergenicity of the Pigment Constituents in Ragweed Pollen.

Abramson et al (64-69, 77) separated pigmented material from other constituents of the pollen extract by free electrophoresis and noted that the most rapidly migrating pigment was almost as active as the main, colourless, slow-moving fraction (67-77). Cohen

and Friedman (56) succeeded in isolating pigment fractions which possessed allergenic activity. However, these fractions contained nitrogen, which would suggest that the fractions may have been contaminated with a small quantity of protein or peptide material.

Robbins et al (57) reported that the pigment constituent(s) in the pollen extract was inactive and a similar observation was made by Perlman (84) who noted that very little allergenic activity was associated with the pigments when they were separated from the other components of the extract by paper chromatography.

Stevens et al (83) noted that a pigmented material precipitated from an aqueous extract of the pollen on standing for several days at 4° C. Purification of this precipitate resulted in the isolation of the pigment isoquercitrin, which on hydrolysis yielded two compounds, glucose and the pigment quercetin (a pentahydroxy-flavone). This preparation was allergenically inactive and contained no nitrogen. Preparations of isoquercitrin obtained by mild methods of fractionation of the precipitate were allergenically active but they also contained nitrogen which would suggest that the activity may have been due to nitrogen-containing contaminants.

Goldfarb et al (85) removed most of the pigment from ragweed pollen by extracting the pollen with 90 per cent methyl

alcohol prior to its extraction with water. This pigment-deficient aqueous extract was allergenically as active as the aqueous extract prepared from the untreated pollen. These investigators felt that the pigment possessed no allergenic properties.

Allergenic Activity of the Lipid Fraction of Ragweed Pollen.

Milford (86), in 1930, demonstrated that the ether soluble fraction of the pollen possessed allergenic activity. The ether was evaporated from the ether extract of the pollen leaving a residue with a semisolid consistency which was dissolved in a non-polar solvent to exclude water soluble material and Seitz filtered to remove pollen granules which might have been present in the extract. Qualitative tests showed the absence of nitrogen. The ether soluble material gave positive cutaneous reactions in over 60 per cent of allergic individuals tested, all of whom reacted to an aqueous extract of the pollen.

Moore et al (87) dialyzed an ether soluble extract against ether, using a membrane made of thin rubber, and found that the allergenic activity was not dialyzable. They do not state the pore size or permeability properties of the membrane. The dialyzed residue contained nitrogen and carbohydrate and was allergenically active in 50 per cent of allergic individuals tested.

The ether soluble extract obtained by Johnson and Rappaport (82) contained 0.2 to 0.3 per cent nitrogen and elicited posit-

ive skin tests in 19 of 21 allergic patients.

Dialyzability of the Allergen.

Unger et al (88) observed that allergenically active material could dialyze through three different membranes (collodion, cellophane and parchment) and that dialysis was more rapid with solutions at pH 3.2 that at pH 8.6.

Johnson and Rappaport (82) reported that the dialysate (they do not state the type of dialyzing membrane used) was active by skin test in 41 of 42 patients and Long and Teller (63), using ultrafiltration through cellophane membranes, observed that the ultrafiltrate contained less activity than the original pollen extract.

Allergenically active dialysates of pollen extracts were also obtained by Stone et al (90) and Bukantz et al (58, 59), who both used Visking tubing as the dialyzing membrane. Stone et al (90) found that 50 per cent of the allergenic activity in the extract was dialyzable whereas Bukantz et al (58, 59) observed that the dialysate was allergenically not as active as the dialyzed residue.

There have also been reports to the effect that the allergenically active material is not dialyzable. Grove and Coca (60, 61) and Black (41) showed that active material was not dialyzable through cellophane tubing either before or after the whole extract had been thoroughly digested with trypsin. Spain and New-

ell (48) demonstrated that the degree of diffusibility of allergenically active material through cellophane membranes depended upon the type of membrane used. A "thin" cellophane membrane (presumably a membrane of large pore size) allowed a large quantity of active material to diffuse through whereas a very "dense" cellophane membrane (presumably a membrane of small pore size) allowed all the non-protein nitrogen but not the allergenically active material to diffuse through. Stull et al (52) could find no activity in their dialysates of the pollen extract using Visking tubing. Unfortunately, they tested their dialysates with the serum of only one allergic individual.

More recently, Rockwell (70) reported that the active material obtained by precipitation of the pollen extract was non-dialyzable.

The discrepancy in the observations pertaining to the dialyzability of the allergen(s) may be due to the fact that membranes of different composition and most probably of different pore size were used by the investigators. The reports do not state whether treated or untreated allergic individuals were used for testing the dialysate for its allergenic activity. This is a most important point as will be emphasized in Chapter VII of this thesis.

Evidence for the Multiplicity of Allergens in Ragweed Pollen Extract.

From the findings of these many investigations, one could

arrive at the logical conclusion that there are several allergens of different composition in ragweed pollen. Much evidence has accumulated in support of such a view.

Milford (86) observed that reactions could still be elicited by injecting an aqueous extract of the pollen into passively sensitized sites previously neutralized to the ether soluble fraction of the pollen. Moore et al (49, 87) obtained similar results and claimed that there exist at least three distinct allergens in ragweed pollen.

Caulfied et al (79) obtained a number of active fractions from an extract of the pollen and each was made up to the same volume as the original extract. From results of cross-neutralization tests with the fractions on an equal volume basis, these workers concluded that there were several allergens in ragweed pollen. They also found that a fraction which was very active in the skin of one allergic individual would sometimes be completely inactive in the skin of another.

Stull et al (53) observed that two fractions obtained from an aqueous extract of ragweed pollen by precipitation with ammonium sulphate would not neutralize to each other. Cohen and Friedman (56) performed cross-neutralization tests with three active fractions isolated from a pollen extract by precipitation with ammonium sulphate at pH 4.0 and found that each fraction failed to neutralize to the other two. The results of their experiments suggested that rag-

weed pollen contained at least three different allergens.

Loveless et al (91) separated a pollen extract by dialysis and then fractionated the dialysate by free electrophoresis. Results of cross-neutralization experiments with the fractions thus obtained suggested that ragweed pollen contained at least three allergens, two in the dialysate and a third in the non-dialyzable portion of the extract. In a subsequent study, Loveless and Timasheff (92) fractionated a pollen extract by electrophoresis-convection and were able to demonstrate the presence of at least two allergens by cross-neutralization tests.

In most of these studies, the allergen fractions were tested on an equal nitrogen basis. It has, however, been shown (41, 48, 95, 115, 116) that there exists no correlation between the nitrogen content of a fraction and its allergenic activity. There would therefore appear to be no rationale for comparing allergen fractions on the basis of their nitrogen content which, as will be shown in Chapter V of this thesis, permits the drawing of fallacious conclusions as to the number of allergens involved.

Effects of Heat and Hydrogen Ion Concentration on the Allergenic Activity.

Gay (93), in 1926, reported that heating an extract of ragweed pollen for as short a time as one minute at 100° C produced a decrease in the allergenic activity. Although boiling the extract at 100° C for one hour did not completely abolish the activity, auto-

claving it for twenty minutes at fifteen pounds pressure resulted in complete inactivation. Gay found that heat destroyed the activity of the pollen extract regardless whether the pH of the solution was 4.0, 7.4 or 8.4. Arbesman and Eagle (94) reported that 75 per cent of the allergenic activity of an aqueous extract of ragweed pollen was destroyed within fifteen minutes at 56°C, 90 per cent within one hour and more than 95 per cent within four hours. Hampton et al (96) also observed loss of allergenic activity in extracts heated at 56°C for 30 minutes.

Stull et al (95) reported that heating a neutral aqueous solution caused a diminution in activity, but they did not specify the length of time of heating nor the temperature. They found that heating the whole pollen extract at 70° C for one hour resulted in the destruction of most of the activity (53).

However, evidence suggesting that heat does not destroy the activity of the pollen extract has been presented. Grove and Coca (61) and Black (41) showed that heating the extract for one hour at 56° C or boiling it for ten minutes did not affect its allergenicity. Long and Teller (63) observed that heating an aqueous extract at 100° C for one hour resulted in only a slight decrease in activity.

Rappaport (97) heated the whole pollen, prior to its

extraction, at 140° C for two hours. The allergenicity of the pollen extract as determined by the size of the skin reactions it induced in allergic persons and by its effectiveness in the treatment of hay fever, appeared to be unaffected by this treatment. Stull et al (95) also claimed that heating the pollen in the dry state at 150° C did not destroy its activity.

Stone et al (90) heated the dialysate of ragweed pollen extract for one hour at 100° C and were unable to detect any loss of activity and Robbins et al (57) isolated a very active component from an extract of ragweed pollen after first boiling the extract for one hour at pH 4.0.

Loveless (91) separated the aqueous extract of ragweed pollen into dialyzable and non-dialyzable fractions. Heating the dialysate in a water bath at 100° C for one hour had no observable effect on its allergenicity, whereas exposure of the dialyzed residue to 60° C for one hour resulted in the loss of 50 per cent of its original activity.

Brown et al (98), in 1931, reported that the allergenic activity of a ragweed pollen extract was rapidly destroyed if kept in 0. 1 N NaOH for a short period of time. Batchelder and Gonder (99) found that maintaining an aqueous extract of the pollen at 37° C for four hours at any pH outside the range 4.5 to 7.5 resulted in 75 per cent loss of activity and that the pH region for maximum stability of activity was approximately 5.5 to 6.5. It is interesting to note,

however, that Rockwell (70-72) was able to isolate highly active material by precipitation with concentrated HCl.

Effect of Enzyme Digestion on Allergenic Activity.

Enzymatic digestion of ragweed pollen extracts has been carried out on numerous occasions in attempts at defining the chemical nature of the pollen allergen(s).

Although various enzymes, such as amylase, invertase, catalase, reductase, pectinase, phosphomonoesterase, adenosinetriphosphatase and pyrophosphatase have been shown to be present in ragweed pollen (100, 101), their relation to, or effect on, the allergenic activity of the pollen extract has not been investigated.

Grove and Coca (60, 61) and Black (41) reported that digestion of either the whole pollen or the aqueous extract of the pollen with trypsin did not result in destruction of activity.

On the other hand, Harsh and Huber (55) observed that digestion of ragweed pollen extract with pepsin followed by prolonged dialysis resulted in marked loss in allergenic activity which could not be accounted for by dialysis of the extract alone. They did find a small but constant amount of activity in the pollen extract unaffected by proteolytic digestion.

Rockwell (103) suspended whole, defatted pollen in hydrochloric acid at pH 1 and digested the pollen with pepsin. The digestion of the pollen resulted in only a small loss of allergenic activity in the aqueous extract which he claimed was not due to digestion

of the active material but rather to a decrease in its solubility.

There have been a number of reports that the allergen can be absorbed through the gastro-intestinal tract when the whole pollen is taken by mouth (104-106) and that it can be absorbed through the mucous membrane lining the nose and throat when the pollen is sprayed into the nostrils (107, 108). These findings would suggest that the allergen is not destroyed by the enzymes present in the respiratory tract, stomach and intestine. Hecht et al (104) demonstrated that the absorption of the allergen through the gastrointestinal tract was dependant on the acidity of the fluids in the stomach. They demonstrated that a decrease in gastric acidity tended to facilitate absorption of the allergen from orally administered pollen extract while an increase in gastric acidity prevented its absorption. They did not determine whether the latter effect was due to enzymatic digestion of the allergen or to alteration in the permeability of the membranes lining the digestive tract.

Physico-Chemical Studies on Ragweed Pollen Extract.

(i). Electrophoretic Studies.

In a series of papers, Abramson et al (64-69, 77) described the separation of ragweed pollen extract by free electrophoresis into as many as six pigmented components and a colourless material which appeared to be present in greater concentration than any of the other components. They were able to test both the col-

ourless material as well as the most rapidly migrating pigment for allergenic activity by withdrawing them from the cell at the termination of the experiment. The active, colourless material which was referred to by these investigators as the "immobile fraction" migrated very slowly to the positive pole (in phosphate buffer, pH 7.4) and had an electrophoretic mobility of 0.5×10^{-5} cm^2/volt/sec (68). The diffusion coefficient of this material was found to be $1.7 \pm 0.5 \times 10^{-6}$ cm^2/sec (64). From the electrophoretic pattern of the whole extract, they calculated that the immobile component constituted as much as 75 per cent of the total material in the extract. However, they did not take into account the possible contribution of the salt boundary which normally forms at the interface between the buffer and the solution and which also manifests itself as a stationary or immobile peak in the electrophoretic pattern. The immobile material was found to be present in both dialyzed and undialyzed preparations of the pollen extract. This finding was later substantiated by Loveless et al (91) who also showed that the dialysate of the pollen extract had a greater proportion of electrophoretically immobile material than the dialyzed residue.

In 1943, Newell (109) repeated much of the work of Abramson et al and extended the study by performing electrophoretic analyses on fractions obtained by various procedures from ragweed pollen.

He found that none of the fractions isolated previously by other workers using precipitation methods was electrophoretically homogeneous. All fractions were complex mixtures of materials and a stationary peak having almost no net charge was found in every preparation at pH 4.0 and pH 7.0. Newell pointed out that the stationary peak could not be correlated with allergenic activity since it was also present in inactive preparations. The stationary peak could have been due, at least in part, to a salt boundary although Newell made no mention of this possibility.

Although Newell was unable to prepare a homogeneous fraction from the pollen, Loveless and Best (111) reported the isolation of a colourless, immobile fraction by electrophoresis-convection which appeared to be homogeneous and which was allergenically active. However, Loveless and Timasheff (92) later reported that this essentially immobile material separated into two components when subjected to prolonged electrophoresis in phosphate buffer at pH 5.5. Although Loveless and Timasheff separated the pollen extract into a number of fractions by electrophoresis-convection, one cannot attach too much significance to their work since more than 60 per cent of their preparation was dialyzable and the method of electrophoresis-convection can be applied only to non-dialyzable materials.

Stevens et al (83) identified one of the rapidly migrating pigments as isoquercitrin by comparing the electrophoretic pattern of the whole extract with that of a purified preparation of isoquercitrin. They also analyzed the dialyzed residue of the pollen extract and found that it displayed a single, slow-moving peak (electrophoretic mobility = 0.6×10^{-5} cm^2/volt/sec)in buffer at pH 8.4 (110). They did not state the composition of the buffer. The diffusion coefficient of this apparently homogeneous material was 7.1×10^{-7} cm^2/sec in M/15 phosphate buffer, pH 7.4, which is about one-half the value obtained by Abramson et al.

In 1954, Goldfarb et al (85) reported the preparation of an aqueous extract which was obtained by extracting the pollen with water after it had been extracted with 90 per cent methyl alcohol. This preparation appeared to be less intensely pigmented than the aqueous extract of the untreated pollen. These workers claimed that this material was a purified extract as compared to the whole water soluble extract of the pollen. Electrophoretic examination of this preparation in phosphate buffer, pH 7.4, revealed the presence of one main, slow-moving peak and a second peak which was a new component not present in the aqueous extract of the untreated pollen.

(ii). Ultracentrifugal Studies and Molecular Weight Determinations.

Sanigar (112) was the first to analyze ragweed pollen extract by ultracentrifugation and he obtained a value of 1.0×10^{-13}

sec^{-1} for the sedimentation constant of his active extract. He suggested that this value indicated a molecular weight of less than 17,000.

Abramson et al (64, 69, 77) found that the electrophoretically immobile and colourless material isolated from the pollen extract had a sedimentation constant of $1.5 \pm 0.2 \times 10^{-13}$ sec^{-1} which was identical to that of the whole extract. From a knowledge of its sedimentation constant and diffusion coefficient ($1.7 \pm 0.5 \times 10^{-6}$ cm^2/sec), they calculated a molecular weight of about 5,000 for this allergenically active material.

Stevens et al (110) found that the dialyzed residue of the pollen extract had a sedimentation constant of 1.3×10^{-13} sec^{-1} in phosphate buffer, pH 7.4, and on the basis of this value and the diffusion coefficient (7.1×10^{-7} cm^2/sec), they calculated a molecular weight of 17,800. On the other hand, Loveless and Timasheff (92) estimated the molecular weight of the dialyzed residue to be between 2,500 and 4,000 on the basis of the sedimentation constant of 0.5×10^{-13} sec^{-1} calculated for this active material in phosphate buffer, pH 5.5.

However, in only two of the above investigations was the diffusion coefficient determined and in none was the partial specific volume estimated. A molecular weight calculated on the basis of the sedimentation constant and the assumed values for the

diffusion coefficient and partial specific volume is, at the very best, only a rough approximation of the true molecular weight.

Rockwell calculated minimum molecular weights of 650, 950, 1117, 1168, and 4800 for his five active fractions on the basis of data obtained by a method independent of the physico-chemical properties of the materials (74). He calculated the molar concentrations of all the elements in each preparation and divided all the values by that of the element present in the lowest concentration. He thereby found the minimum number of atoms of each element which must be present in a molecule of each fraction. However, Rockwell had to make the assumption that the fractions were homogeneous and he offered no evidence that they were.

(iii). Chromatographic Studies on Ragweed Pollen Extracts.

Bukantz et al (58, 59) fractionated the dialysate obtained from an aqueous extract of ragweed pollen by descending paper chromatography. In their initial study (58) they used lutidine as the solvent which, they later discovered, was capable of eliciting positive skin reactions in allergic individuals, although not in normal persons. (Winkenwerder et al (113) and Sherman (114) noted that

many allergic individuals reacted by skin test to nucleic acids and to purified purine salts, such as adenine sulphate and guanine chloride, the molecular structures of which bear some resemblance to that of lutidine). In a subsequent study, Bukantz et al (59) used methyl cellosolve as the solvent and were able to separate the dialysate of the pollen extract into one carbohydrate and eight peptide fractions all of which possessed allergenic activity. The free carbohydrates present in the dialysate were shown to be glucose and fructose. The dialyzed residue, after hydrolysis followed by chromatography, was found to consist of fifteen amino acids and arabinose. Most of the allergenic activity of the whole extract resided in the dialyzed residue. They were, however, unable to fractionate this material.

Perlman (84) separated the whole water soluble extract of ragweed pollen by ascending paper chromatography and detected seven peptide, three carbohydrate and two pigment bands. He did not stain for protein. Most of the fractions eluted from the paper were found to be allergenically active. It is interesting to note that activity was also detected in segments of the paper devoid of pigment or stainable material.

Bernstein et al (115) chromatographed a water soluble extract of the pollen through a column of alumina. All the pigmented mat-

erial was absorbed at the top of the column and only colourless material, amounting to 60 per cent of the weight of the solids in the extract, passed through. Distilled water was used as the eluting agent and the eluates were collected in 15 ml quantities. Eleven such aliquots were collected and most were found to be allergenically active by skin test.

Bookman and Wax (116) used ion exchange resins in an attempt to isolate the allergen from the pollen extract. They passed extracts of the pollen through columns of various resins (Dowex-2, Dowex-50 and Amberlite IRC-50) and eluted them with buffers of different pH. The majority of the eluates were allergenically active, but the investigators did not determine the composition of any of these fractions.

Summary.

Different investigators presented conflicting points of view with respect to the composition and properties of the allergen or allergens present in ragweed pollen. Although many workers claimed to have isolated and identified the allergen(s), none proved that he was dealing with a pure material. In fact, electrophoretic studies by Newell (109) on several fractions of ragweed pollen isolated by methods used by other investigators (up to the year 1942) revealed that no fraction was homogeneous.

Extracts and fractions of ragweed pollen have been examined

in the ultracentrifuge in attempts at determining the molecular weight of the allergen. However, in no study was the material under investigation shown to be homogeneous and in no case were all the parameters essential for a calculation of the molecular weight determined. The calculated molecular weights can therefore be accepted as only rough approximations of the true molecular weight of the allergen.

Electrophoretic and chromatographic studies demonstrated that these methods could be utilized for the fractionation of ragweed pollen extract. The chromatographic studies on the dialyzable fraction of the pollen extract revealed that allergenically active fractions could be obtained containing only peptide or carbohydrate materials. Unfortunately, no attempts were made to fractionate the non-dialyzable fraction of the extract which contains the bulk of the allergenic activity. Since almost every fraction obtained by the chromatographic separation (paper and column chromatography) of the extract possessed allergenic activity, it would appear that all the fractions were "contaminated" with the active material which escaped detection due to its low concentration.

Thus one cannot arrive at any definite conclusions as to the composition and properties of the allergen(s) on the basis of work done in the past.

EXPERIMENTAL

CHAPTER III

PRELIMINARY FRACTIONATION OF RAGWEED POLLEN

INTRODUCTION

In 1954, Goldfarb et al (85) reported the preparation of an aqueous extract from ragweed pollen which had been freed of much of the water soluble pigments. This preparation, which they referred to as MLL, was as active as the aqueous extract of the defatted pollen (on an equal nitrogen basis). MLL was therefore selected as the starting material for our attempts to isolate the allergen(s) and the physicochemical and allergenic properties of this material were compared to those of other fractions obtained from ragweed pollen.

METHODS AND MATERIALS

Short ragweed pollen was defatted with diethyl ether in a Soxhlet extraction apparatus for 3 days. The defatted pollen was extracted with 90 per cent methyl alcohol (50 ml of alcohol per 5 grams of defatted pollen) and the extracting medium was changed every hour until it was completely colourless. The alcohol extracts were combined and the alcohol was distilled under reduced pressure. The residue was lyophilized and is referred to as ROH SOL.

The pollen, after the alcohol treatment, was extracted with

water (100 ml of water per 5 grams of the alcohol extracted pollen) for 72 hours. The yellow-coloured aqueous extract was lyophilized and constitutes MLL. A portion of the insoluble residue of the pollen was then extracted with water for 12 more days and the residue remaining after this treatment is referred to as MLL INSOL. All extractions were performed at 4°C.

To determine the specific effect, if any, of alcohol on the composition and allergenicity of the constituents of ragweed pollen, MLL was compared with a water extract of the defatted pollen which had not undergone preliminary alcohol extraction. The defatted pollen was extracted with water (100 ml of water per 5 grams of defatted pollen) for 72 hours. The suspension was then centrifuged at 20,000 rpm for 20 minutes in a Spinco Model L preparative ultracentrifuge. The yellow-coloured supernatant was slightly turbid and this opalescence was attributed to some lipoidal material still present in the pollen after the extensive defatting procedure. The solution was lyophilized, dissolved in 20 ml of water and recentrifuged at 20,000 rpm for 20 minutes. A white material rose to the surface of the liquid and the clear, yellow-coloured solution below it was drawn off and lyophilized. This material is referred to as WSR (water soluble ragweed). The insoluble pollen residue which remained after this extraction was further extracted for 16 days with water, the extracting fluid being changed every 2 days. The insoluble residue which remained after this treatment was lyophilized and is referred to as WSR INSOL. Portions of this material were extracted

with normal human serum, saline (0.9 per cent NaCl) and phosphate buffer, pH 7.6, for 72 hours.

Both WSR and MLL were dialyzed through Visking tubing against water, the ratio of the volume of the solution within the Visking tubing to that outside being 1:5. In each case, the aqueous solution of the extract was dialyzed for 36 hours and the dialysate was subsequently lyophilized. The residue remaining within the sac was further dialyzed for 10 days against running tap water to insure complete removal of dialyzable material and was lyophilized.

To determine whether extractable allergenically active material was still associated with MLL INSOL, a portion of this material was extracted with 0.05N NaOH for 72 hours. The insoluble residue was lyophilized and is referred to as NaOH INSOL. The yellow-coloured supernatant was acidified to pH 6.0 with 0.1N HCl at which point a white, fluffy precipitate separated out. The supernatant was separated from this fine precipitate by centrifugation at 25,000 rpm for 20 minutes and is referred to as pH 6 SOL. The precipitate is referred to as NaOH SOL. Both fractions were lyophilized. Fig. 2 is a flowsheet of the procedure used in the fractionation of ragweed pollen.

The physico-chemical and allergenic properties of the extracts were compared as follows:

Free Electrophoresis:

MLL, WSR and ROH SOL were analyzed by free electrophoresis in the Spinco Tiselius apparatus. The solutions for electrophoresis were

prepared by dissolving the extracts in the following buffers:

(a) Veronal buffer, pH 8.6, ionic strength 0.1;

(b) Borate buffer, pH 8.6, ionic strength 0.1;

(c) Phosphate buffer, pH 7.80, total ionic strength 0.17 (contribution of NaCl 0.15);

(d) Glycine buffer, pH 10.6, total ionic strength 0.17 (contribution of NaCl 0.15);

(e) Glycine buffer, pH 3.5, total ionic strength 0.17 (contribution of NaCl 0.15).

Each extract was found to contain both dialyzable and non-dialyzable components. Therefore, when each was analyzed by electrophoresis for all its constituents, initial dialysis against the respective buffer was dispensed with.

The conductivities of the buffer and the solution of the extract in the respective buffer were equalized prior to each electrophoretic experiment. The pH of the solution was raised or lowered to that of the buffer by the addition of either 0.1N NaOH or 0.1N HCl; the conductivities were then equalized by adding solid NaCl to the buffer. These steps were necessary since, in each case, the solution had a higher conductivity than, as well as a different pH from, its corresponding buffer.

Paper Electrophoresis:

The aqueous solution of the material to be separated by paper electrophoresis was applied at the center of the strip of Whatman No. 1 filter paper (55 cm x 4 cm), which had previously been immersed in the buffer and semi-dried between sheets of filter paper, as a narrow band

about 3 cm long. The paper was then layered onto a horizontal plexiglass* plate which was used as the supporting surface. This, in turn, was placed onto the buffer vessels, the two extremities of the papers dipping into the buffer to a depth of about 3 cm. The buffer vessels were filled to equal levels in order to prevent chromatographic effects from disturbing the resolution of the material under observation. For similar reasons, the plate supporting the filter paper strips was leveled both longitudinally and vertically.

Heating, caused by passage of current through the paper strips, results in evaporation of water from the filter papers saturated with buffer. To minimize evaporation from the papers during electrophoresis, a glass plate supported by a foam rubber frame was placed over the papers, forming a closed system. A sheet of Whatman No. 3 mm filter paper was placed between the glass plate and the foam rubber frame. Its function was to absorb any water vapor which might condense on the glass plate during electrophoresis. Due to the lower resistance of this buffer-saturated filter paper, as compared to that of the Whatman No. 1 filter paper strips, more heat was dissipated from it. Consequently, the electrophoretic chamber was saturated with vapor emanating mainly from the Whatman No. 3 mm filter paper. The buffer vessels were then connected by paper

* The upper surface of the plexiglass plate was provided with small, closely spaced pyramidal projections, extending to a height of 0.3 cm above the surface of the plate. This arrangement prevented the paper from sagging and the formation of a surface-liquid film between the plexiglass and the paper. The plexiglass plate was 40 cm long and 24 cm wide.

wicks with the electrode vessels which had been filled to equal levels with 20 per cent KCl solution. The electrode vessels contained silver-silver chloride electrodes* which were used in preference to the conventional platinum or carbon and steel electrodes to insure constancy of pH during electrophoresis.

One hour was allowed for the buffer to migrate up the paper strips towards the zones of application (equilibration time) and a potential of 110 volts DC was applied. The duration of an electrophoretic experiment never exceeded 14 hours but varied with the fraction being analyzed. Electrophoresis was carried out at room temperature.

Upon completion of the experiment, the paper strips were dried at 120°C and stained for carbohydrates, proteins, amino acids and peptides. The following staining reagents were used:

(a) One per cent ninhydrin solution in 95 per cent ethyl alcohol to detect amino acids and peptides (117);

(b) A metaperiodate-permanganate solution to detect carbohydrates (118);

(c) Amido-black (10 B) to stain for proteins (119);

The papers were sprayed with reagents (a) and (b) and were immersed in reagent (c).

For the sake of brevity, all bands revealed by ninhydrin staining are referred to as peptide bands.

* The silver-silver chloride electrodes were prepared from silver strips 0.6 cm wide and 0.08 cm thick.

The extracts were analyzed in the following buffers:

(a) Veronal buffer, pH 8.6, ionic strength 0.10;

(b) Borate buffer, pH 8.6, ionic strength 0.10;

(c) Phosphate buffer, pH 7.8, ionic strength 0.17 (contribution of NaCl 0.15);

(d) Citrate buffer, pH 4.56, ionic strength 0.10;

Ultracentrifugation:

The Spinco Model E optical ultracentrifuge was used to determine the sedimentation constants of the various fractions. The experiments also served to disclose any heterogeniety of the materials.

The synthetic boundary cell, as devised by Pickels et al (120), was used instead of the standard cell. This cell allows for the formation of a sharp boundary between the solution under observation and the solvent at the start of the experiment. The average rotor temperature was 20.5°C and the rotor rate was 59, 780 rpm. The sedimentation constants were not recalculated for standard conditions.

All the fractions, with the exception of ROH SOL, were dissolved in 0.9 per cent NaCl. The ROH SOL fraction was dissolved in glycine buffer, at pH 10.6 due to its low solubility in solutions of lower alkalinity.

Allergenic Activity:

Solutions of all the fractions were made up in saline to concentrations of 1,000 units* per ml. Nitrogen values were determined by a modification of the method of Lanni et al (121). The Nessler

* 1 unit = 10^{-5} mg nitrogen.

reagent was prepared according to the specifications of Koch and McKeekin (122). All the fractions were tested on ragweed sensitive persons by scratch test and the reactions were graded after 20 minutes as 0, 1+, 2+, 3+ and 4+, depending on size and extent of the wheal and the surrounding erythema.

RESULTS

As shown in Table I, initial extraction of the pollen with methyl alcohol followed by extraction with water resulted in a lower yield of extractable material as compared with the amount of material extracted from the pollen with water only. The data in this table were arrived at by assuming that the lyophilized fractions were completely dehydrated.

Free Electrophoresis:

WSR, MLL and ROH SOL were all found to be electrophoretically heterogeneous with at least three distinct peaks identified in the electrophoretic pattern of each of these extracts (Figs. 3-5). An essentially immobile peak was present in all the electrophoretic patterns of the extracts. The most striking electrophoretic separation occurred with WSR in glycine buffer at pH 3.5. At least 5 well-characterized, small peaks were seen migrating to the negative pole at a rapid rate (Fig. 3).

Paper Electrophoresis:

Preliminary experiments indicated that maximum resolution of any of the extracts occurred in borate buffer, pH 8.6. Therefore, results obtained with this buffer are the only ones reported.

It is evident from Figs. 6 and 7 that WSR and MLL are distinct materials, containing different numbers of electrophoretically separable components. WSR (Fig. 6) was resolved into 4 pigment, 1 protein, 1 carbohydrate and 5 peptide bands. It ought to be mentioned that all 5 peptide bands were detected only in the early stages of electrophoresis (6 hours) of WSR (Fig. 6). In experiments of longer duration (12 to 14 hours), the 2 rapidly migrating cathodic peptide bands had completely run off the paper.

On the other hand, MLL (Fig. 7) separated into 1 diffuse pigment, 1 protein, 1 carbohydrate and 3 peptide bands.

ROL SOL gave rise to 3 pigment bands (Fig. 8). No carbohydrate or protein stainable material could be detected while a faintly stained peptide band was noted at the cathodic side of the point of application.

The pH 6 SOL fraction (Fig. 9) could not be resolved into more than 1 component by paper electrophoresis in any of the buffer.

A comparison of the electrophoretic patterns of the dialysate, dialyzed residue and whole extract of WSR and MLL, respectively (Figs. 10 and 11), suggested that both WSR and MLL could be fractionated by dialysis against water through Visking tubing. The dialysate of WSR (Fig. 10) contained 4 pigment, 1 carbohydrate and 5 peptide bands. Only 3 of the peptide bands are shown in Fig. 10 since the 2 rapidly migrating peptide bands had run off the paper. The dialyzed residue of WSR consisted mainly of a protein-stainable material which migrated slowly to the anode. A slightly visible pigment band and a weakly stain-

able carbohydrate band appeared to be superimposed on the protein band. In addition, a faint peptide band was detected which migrated slowly to the cathode.

The dialysate of MLL (duration of electrophoresis was 5 hours) resolved itself into 4 peptide, 1 carbohydrate and 2 pigment bands. (Fig. 11). In addition, a faintly stained protein band was also detected. Three of the four peptide bands migrated at a faster rate than any of the peptide bands present in undialyzed MLL. The dialyzed residue of MLL (Fig. 11) (duration of electrophoresis was 12 hours) consisted of 1 protein, 1 pigment, 1 carbohydrate and 3 peptide bands. The total number of electrophoretically separated bands in the dialysate and dialyzed residue of MLL was greater than the number detected in undialyzed MLL (duration of electrophoresis was 12 hours).

Ultracentrifugation:

Each extract gave rise to only 1 diffuse peak in the analytical ultracentrifuge. Fig. 12 is a photograph of a typical experiment. Sedimentation constants were determined for the majority of the fractions (Table II). The sedimentation constants* varied from 0.45 to 2.54S. WSR had a sedimentation constant of 0.92S, while its dialysate and dialzed residue had sedimentation constants of 0.61 and 2.54S, respectively.

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

Allergenic Activity:

The results of skin tests are shown in Table III. WSR and MLL appeared to exhibit comparable allergenic activity and ROH SOL and pH 6 SOL slightly less. The activity of the ROH SOL fraction diminished rapidly on standing and completely disappeared after 4 weeks of storage in aqueous solution at 4°C. On the other hand, the activity of the other fractions did not appear to diminish even after 3 months of storage in aqueous solution at 4°C.

In the present study, the ether soluble fraction of ragweed pollen was not tested for allergenic activity. However, it has been shown in the past to be active (82, 86, 87).

WSR INSOL, even after 16 days of continuous extraction with water, still possessed cutaneous activity, whereas the extracting fluid (water) at this point possessed no allergenic activity. Extraction of WSR INSOL with normal human serum, saline or phosphate buffer at pH 7.6 resulted in liberation of allergenically active material into solution.

MLL INSOL was not active when tested by scratch. However, the pH 6 SOL fraction extracted from this apparently inert ragweed pollen residue was quite active. NaOH INSOL and NaOH SOL displayed essentially no activity.

DISCUSSION

Analysis by free electrophoresis demonstrated that none of the preparations was homogeneous. A stationary component was distinguished in each of the electrophoretic patterns of the WSR, MLL and ROH SOL extracts in the various buffers. One possible interpretation

for these results is that the extracts might contain a component having no ionizable groups, i.e. a closed-ring polypeptide devoid of free amino or carboxyl groups. The large, diffuse, stationary peak might also represent the contribution of a salt boundary.

Paper electrophoresis permits the fractionation of a solution containing several materials without preliminary adjustment of its pH and conductivity to those of the buffer. It proved to be a far more effective tool than free electrophoresis for the analysis of ragweed pollen fractions. The technique yielded more distinct and reproducible patterns and allowed for simultaneous detection and identification of pigment, carbohydrate, peptide and protein bands by visual observation and selective staining.

WSR resolved itself into eleven components in borate buffer whereas MLL resolved itself into a lesser number of components. Regardless of the buffer used, MLL was always resolved into a smaller number of components than WSR, thus substantiating the claim of Goldfarb et al (85) that MLL was a "purified" extract as compared to WSR.

Alcohol extraction, prior to water extraction, appeared to free the ragweed pollen of much of its water soluble pigments which is in accordance with the findings of Goldfarb et al (85). The 3 rapidly migrating pigments present in WSR were absent in MLL but were present in ROH SOL.

The dialysate of WSR appeared to contain all the components

present in whole WSR with the exception of the protein constituent which was present in the dialyzed residue. Since all the components identified in WSR were accounted for in the dialysate and dialyzed residue, it is highly unlikely that ragweed pollen constituents are constantly breaking down during dialysis as has been suggested by Loveless et al (91).

There is, however, a suggestion of some alteration occurring in WSR on dialysis. The dialyzed residue stained for carbohydrate, although very weakly, in the area of the protein band, whereas the original WSR extract contained no carbohydrate stainable material associated with the protein constituent. There are at least two explanations to account for this observation:

(1) Some configurational change might have taken place within the protein moiety of WSR during the extensive dialysis liberating carbohydrate stainable groups.

(2) The fact that carbohydrate stainable material was detected in the dialyzed residue of WSR and not in WSR itself may be due to a concentration effect. Since the dialyzed residue was found to constitute less than 5 per cent of the weight of WSR, the concentration of the carbohydrate in a given weight of the dialyzed residue would be many times that present in an equal weight of WSR.

The presence of detectable pigment migrating with the protein in the dialyzed residue and its apparent absence in the whole WSR can be explained in the same manner.

The total number of electrophoretically distinct components present in the dialysate and dialyzed residue of MLL exceeded the number found in undialyzed MLL and several of the peptides in the dialysate migrated at a more rapid rate than any in undialyzed MLL. These findings suggest that degradation of larger into smaller molecules did occur in MLL on dialysis. As in the case with the dialyzed residue of WSR, pigment was present in the dialyzed residue of MLL and it migrated with the protein. These data imply that a pigment is either specifically bound to the protein or has similar electrophoretic properties.

The pH 6 SOL fraction could not be resolved into more than one component by paper electrophoresis. One could interpret this finding in terms of a one-component system, the protein, carbohydrate, peptide and pigment all being part of one and the same molecule which is resistant to alkaline hydrolysis.

On the basis of the sedimentation constants found for the various extracts, molecular weights conceivably could be calculated provided that the diffusion coefficient and partial specific volume were known for each fraction. However, diffusion coefficients and partial specific volumes are significant only when calculated for one-component systems. Since none of our fractions, except possibly the pH 6 SOL, complied with this requirement, molecular weight determinations were not attempted.

There is no doubt that a shift of low molecular weight material occurred through the Visking tubing on dialysis of WSR against water. This was reflected by the great discrepancy in the sedimentation constants of the dialysate and dialyzed residue of WSR. This finding serves as evidence for the presence of some large molecular weight material in ragweed pollen extract which might have been masked by the relatively greater concentration of low molecular weight material when the whole extract was examined in the ultracentrifuge. This assumption seems reasonable in view of our finding that about 95 per cent, by weight, of WSR dialyzed through Visking tubing.

The sedimentation constant of MLL was considerably greater than that of WSR, suggesting that some low molecular weight material had been removed by the preliminary alcohol extraction. The finding of a low sedimentation constant for ROH SOL supports this interpretation.

Since some of the fractions were insoluble (MLL INSOL, WSR INSOL), scratch tests, rather than intracutaneous injections, were used in order to compare the various preparations for their allergenic activity. It is worth noting that ragweed pollen powder displayed cutaneous activity even after 16 days of extraction with water. Since the extracting fluid at the end of the 16 days extraction displayed no activity, it would appear that the allergenically active groups present in the pollen residue were not easily extract-

able. However, since further extraction of the pollen residue with normal human serum, saline or phosphate buffer, pH 7.6, resulted in a release of allergenically active material into solution, it would appear that these allergenically active constituents possess solubility properties different from the components in the water soluble extract (WSR) of the pollen.

MLL INSOL, which exhibited no activity by scratch test, nevertheless was able to release active material when extracted with 0.05 N NaOH for 72 hours. As it is highly inconceivable that NaOH could convert an inert material into an allergenically active one, a possible explanation for this finding would be that there exist active components in the MLL INSOL fraction which are shielded in some way from direct contact with water and alcohol during the extraction with these solvents and which cannot elicit a skin reaction when tested in the skin of an allergic person as they may reside within the pollen grain and cannot react with the reagin in the skin prior to their extraction with the NaOH.

Since each sensitive person appeared to display his own pattern of reactivity to different fractions, it was impossible to evaluate, on an absolute basis, the allergenic activity displayed by each fraction.

Fig. 2

Flowsheet of the Fractionation of Ragweed Pollen.

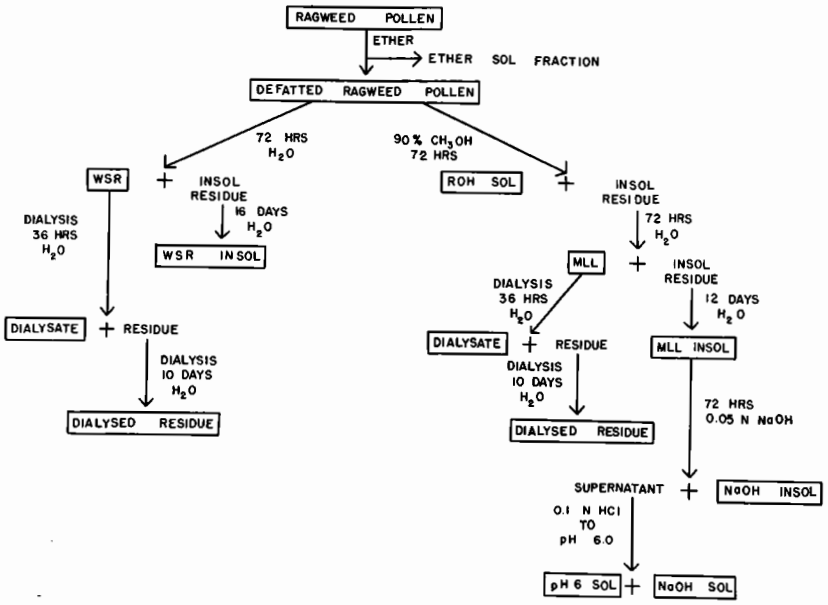


Fig. 3

Free Electrophoresis of WSR

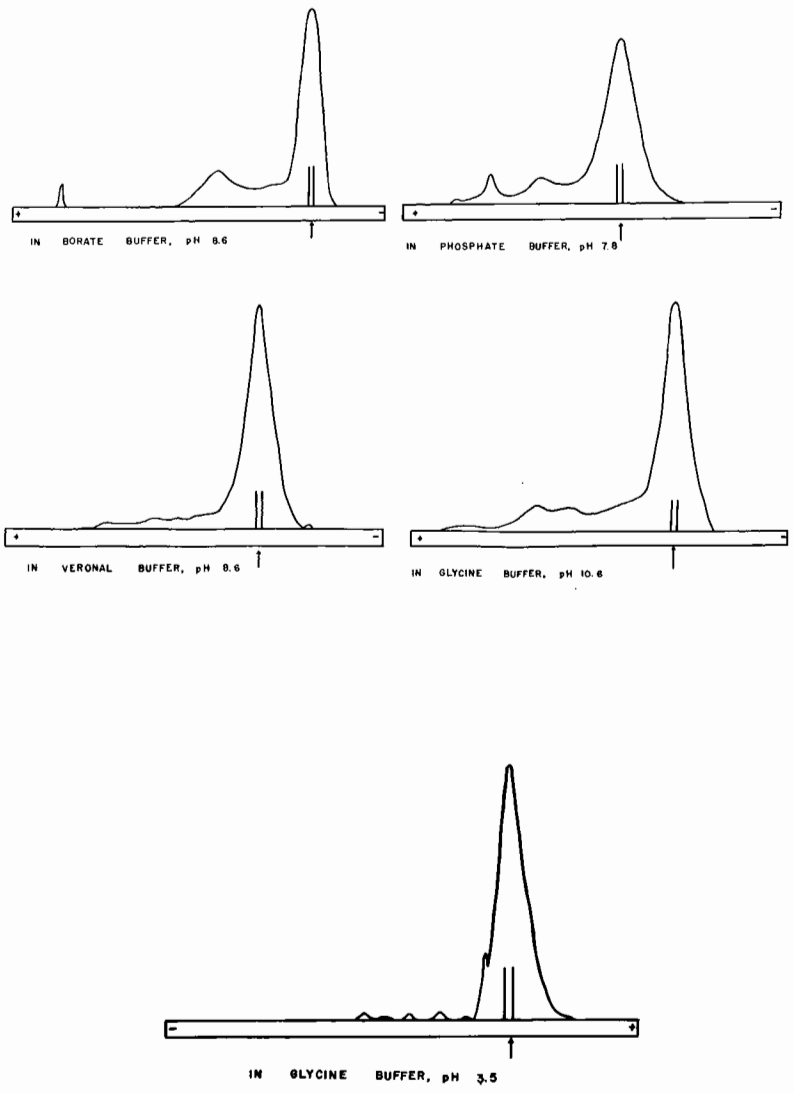


Fig. 4.

Free Electrophoresis of MLL

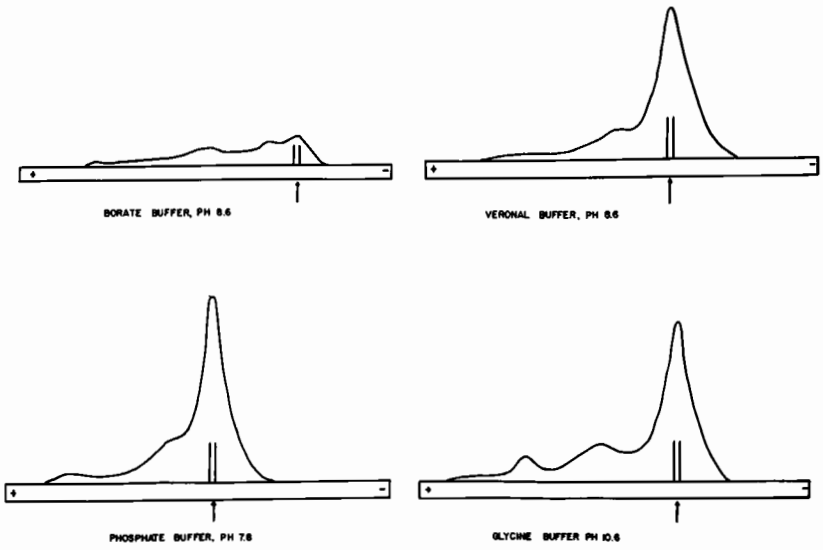


Fig. 5.

Free Electrophoresis of ROH SOL in Glycine Buffer, pH 10.6.

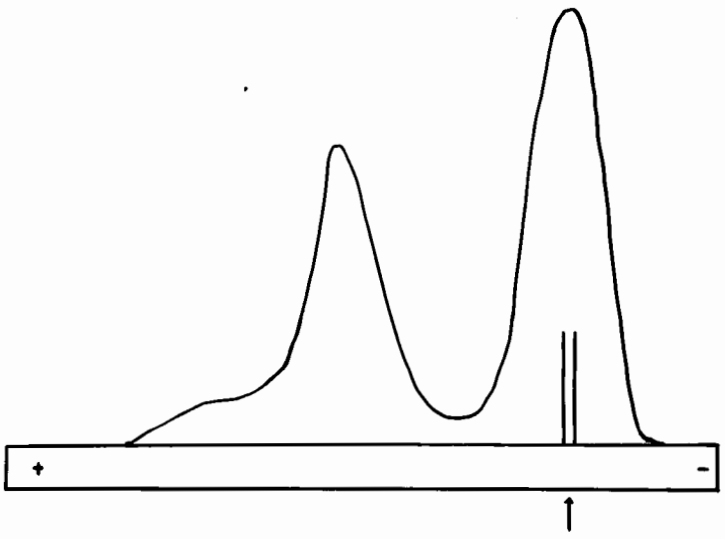


Fig. 6.

Paper Electrophoresis of WSR in Borate Buffer, pH 8.6. The upper four diagrams represent the resolution of the constituents after six hours of electrophoresis. The lower four diagrams represent the separation of the components after twelve hours of electrophoresis.

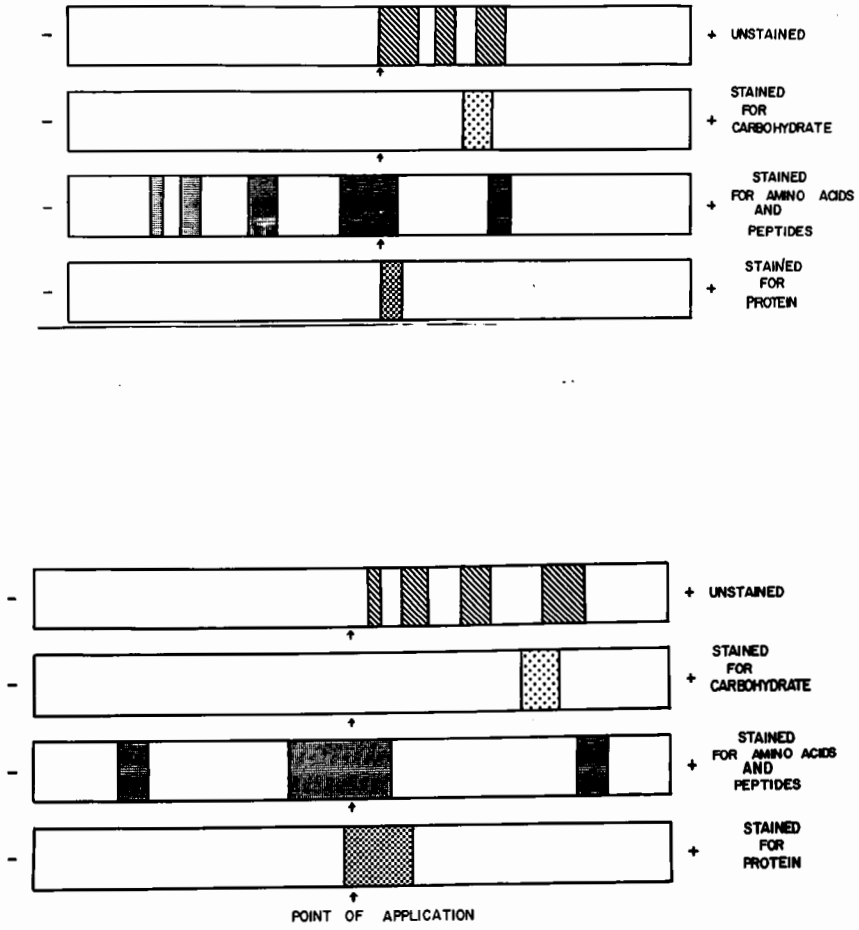


Fig. 7.

Paper Electrophoresis of MLL in Borate Buffer, pH 8.6, for Ten Hours.

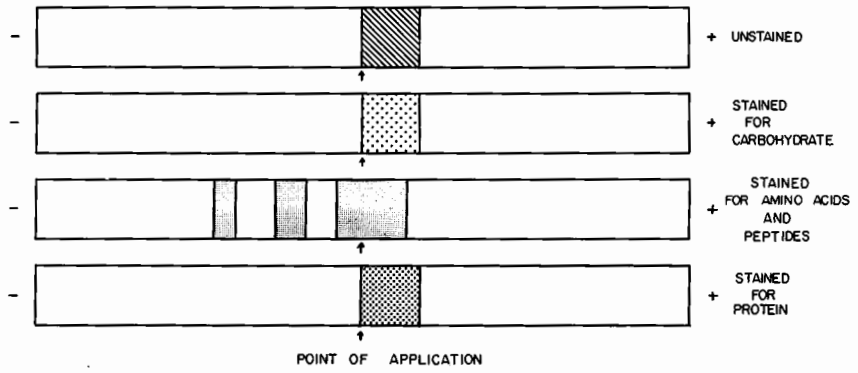


Fig. 8.

Paper Electrophoresis of ROH SOL in Borate Buffer, pH 8.6, for Twelve Hours.

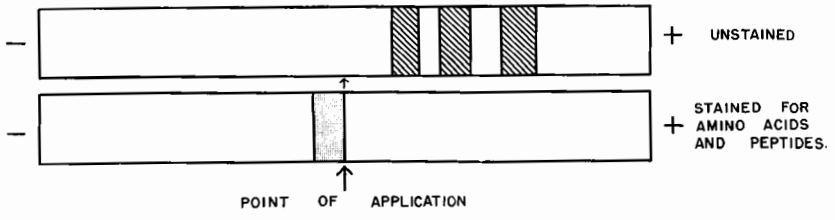


Fig. 9.

Paper Electrophoresis of pH 6 SOL in Borate Buffer, pH 8.6, for Twelve Hours.

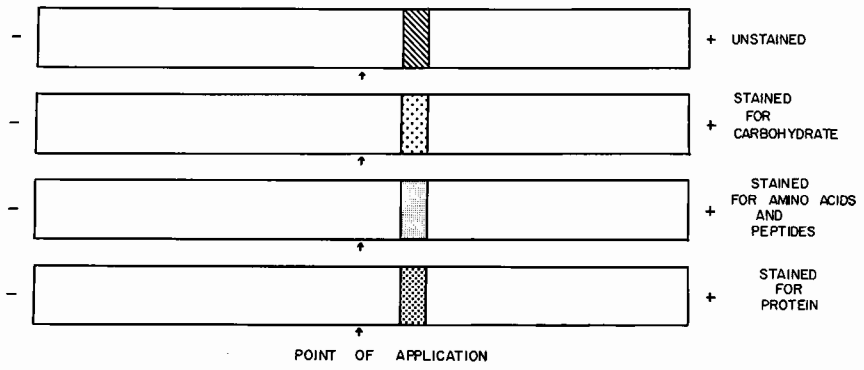


Fig. 10

Paper Electrophoresis of WSR (Upper Four Diagrams), the Dialysate of WSR (Middle Three Diagrams) and the Dialyzed Residue of WSR (Lower Four Diagrams). Duration of electrophoresis was twelve hours for each of the preparations.

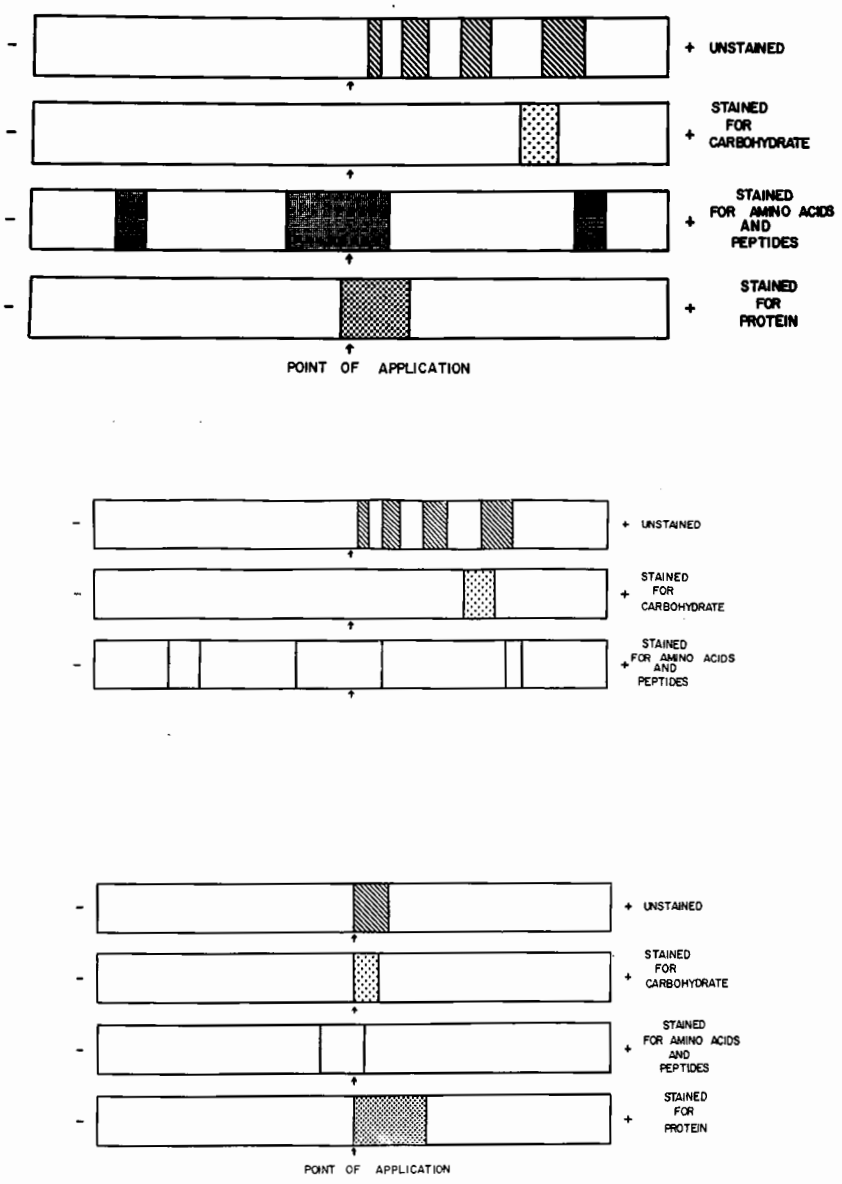
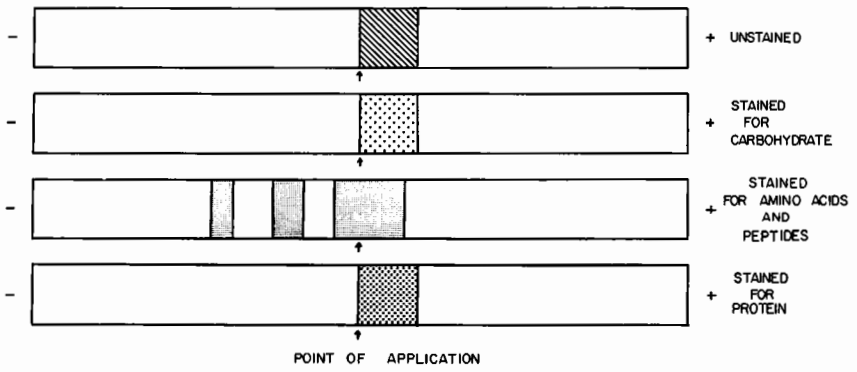
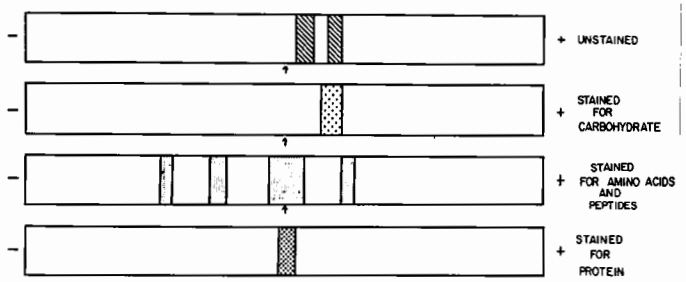


Fig. 11.

Paper Electrophoresis of MLL (Upper Four Diagrams), the Dialysate of MLL (Middle Four Diagrams) and the Dialyzed Residue of MLL (Lower Four Diagrams).





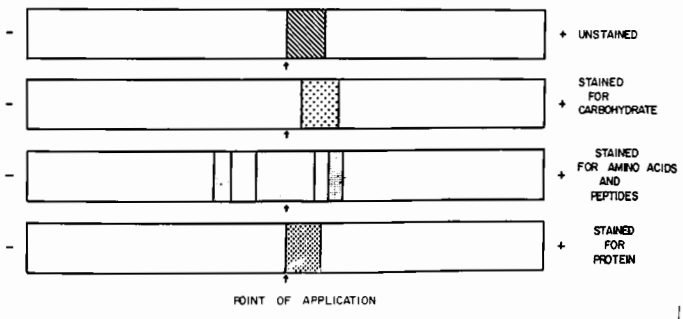
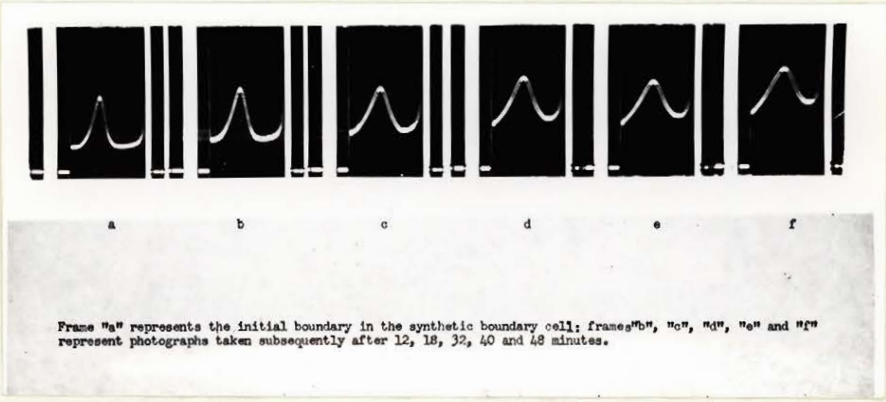


Fig. 12.

Sedimentation Photographs of WSR Analyzed in the Synthetic Boundary Cell



Frame "a" represents the initial boundary in the synthetic boundary cell; frames"b", "c", "d", "e" and "f" represent photographs taken subsequently after 12, 18, 32, 40 and 48 minutes.

TABLE I

Fraction	Percentage of whole defatted pollen
WSR.....	30
ROH SOL	12
MLL	6
pH 6 SOL	20
NaOH SOL	8
NaOH INSOL	52

TABLE II

Ultracentrifugal Analyses of the Allergenically Active Fractions Isolated from Ragweed Pollen*

Fraction	Sedimentation constant**
WSR	0.92
WSR - dialysate	0.61
WSR - dialyzed residue	2.54
ROH SOL+.....	0.45
MLL	1.34
pH 6 SOL	1.17

* Performed in 0.9 per cent NaCl, except where indicated otherwise.
** Expressed in Svedberg units; 1 Svedberg unit = 10^{-13} sec^{-1}
\+ Experiment performed in glycine buffer, pH 10.6

TABLE III

Allergenic Activity of the Fractions of Ragweed Pollen as Observed by Scratch Test.

FRACTION*	SUBJECT TESTED								
	L.K.	M.G.	S.T.	A.M.	M.L.	D.K.	B.L.	B.K.	M.B.
pH 6 SOL	2+	±	0	4+	2+	±	±	3+	3+
NaOH SOL	±	0	0	0	0	0	0	0	0
NaOH INSOL	0	0	0	0	0	±	0	0	0
LSR	4+	2+	4+	4+	2+	2+	±	4+	-
LSR INSOL EXTRACTED WITH WATER - 5 DAYS	3+	0	±	4+	1+	±	-	3+	-
LSR INSOL EXTRACTED WITH WATER - 14 DAYS	-	-	-	-	2+	-	±	3+	4+
TOH SOL	4+	0	4+	0	0	±	-	0	0
TLL	4+	-	-	4+	3+	2+	-	4+	4+
TLL INSOL	±	0	0	0	1+	±	-	0	-

*Each fraction made up to 1000 units/ml.
1 unit = 10^{-5} mg nitrogen.

CHAPTER IV

FRACTIONATION OF THE WATER SOLUBLE EXTRACT OF RAGWEED POLLEN (WSR) AND THE CHARACTERIZATION OF THE FRACTIONS

A. FRACTIONATION OF WSR BY PAPER ELECTROPHORESIS

INTRODUCTION

In the preceding chapter, it was demonstrated that fractions of ragweed pollen obtained by extracting the pollen with various solvents were all allergenically active and heterogeneous when examined by paper and free electrophoresis. Since the water soluble extract of the defatted pollen (WSR) was obtained under the least harsh conditions, its constituents were accepted as most closely resembling the naturally-occurring materials present in the pollen. The results of experiments in chapter III also suggested that paper electrophoresis would be suitable for the fractionation of WSR. In the present study, WSR was fractionated by paper electrophoresis in various buffers in order to determine the conditions for optimal resolution of its constituents.

METHODS AND MATERIALS

WSR was fractionated by electrophoresis on paper in the following buffers: acetate, pH 3.8; citrate, pH 4.6; phosphate, pH

6.0and 7.8; veronal, pH 8.6 and borate, pH 8.6. The ionic strength of all the buffers was 0.10. The staining reagents used for the detection of protein, carbohydrate and peptide were the same as those used in chapter III. To determine whether carbohydrate material not stained by the metaperiodate permanganate reagent was present in WSR, ten other carbohydrate-staining reagents were employed. These are indicated in Fig. 16 and were prepared according to reference 117. In all staining procedures, glucose and sucrose were used as controls.

Preparative electrophoresis was carried out using the same apparatus as was described in the preceding chapter. Three sheets of Whatman No. 3 mm filter paper (55 cm x 15 cm) were layered one above the other on the plexiglass plate and one sheet of Whatman No. 1 filter paper was layered above these three, care being taken not to trap air between the papers. Whatman No. 3 mm filter paper was used for preparative electrophoresis in preference to the thinner Whatman No. 1 filter paper due to its greater absorption capacity. The function of the No. 1 filter paper, after being appropriately stained, was to provide a "print" showing the resolution of WSR.

WSR was dissolved in water (25 to 30 per cent, W/V) and 0.3 ml was applied as a straight line transversely along the center of the papers. One hour was allowed for the buffer to migrate up the papers towards the zone of application and a potential of 110 volts DC was then applied. At the termination of the experiment, the Whatman No. 1 filter

paper was removed and dried at 120°C. It was then cut longitudinally into three narrow strips, one of which was stained for peptides, one for proteins and one for carbohydrates. These strips served as guides for the isolation of the various constituents. The three Whatman No. 3 mm filter papers (still layered one above the other) were then cut into segments corresponding to the constituents (peptide, carbohydrate, protein and pigment). The segments of filter paper were then rolled and transferred into plastic tubes provided with perforated bottoms. Each plastic tube was placed in a 40 ml standard centrifuge tube on three glass projections protruding from the sides toward the center of the centrifuge tube about 2.5 cm from the bottom of the latter. The tubes were centrifuged for 5 minutes at 2,000 rpm. Four ml of distilled water were then added to each plastic tube and centrifugation was repeated a second time to insure complete elution of the material. The eluates were brought to pH 7.0 to 7.5 and to 0.9 per cent with respect to NaCl and were tested for allergenic activity by the method of passive transfer.

For the passive transfer test, only individuals who gave no skin reactions on intradermal injection of a solution of WSR containing 1,000 units per ml were accepted as test subjects. The reaginic serum* was injected into several sites in the back of the subject (0.05 ml of serum per injection). Control sites contained saline or normal human serum. The sensitized and control sites were challenged with the aller-

* Serum obtained from a clinically allergic individual is referred to as reaginic or allergic serum.

gen solutions (0.05 ml of allergen solution per injection) 18 to 24 hours later and the reactions were noted 20 minutes after injection of the allergen. All solutions used for skin tests were sterilized by Seitz filtration and stored in sterile vials at $4^{o}C$.

RESULTS AND DISCUSSION.

Electrophoresis of WSR in the citrate (pH 4.6) and phosphate (pH 7.8) buffers resulted in an almost identical separation of the components even though the buffers differed greatly in pH (Figs. 13 and 14). In both these buffers as well as in the acetate (pH 3.8) and phosphate (pH 6.0) buffers, however, the carbohydrate, protein, pigment and several of the more intensely stained peptide bands were not well separated from each other and remained in the central area of the electropherogram. The electrophoretic separation of WSR in veronal buffer (pH 8.6) was more satisfactory as the pigments separated from ninhydrin stainable material. The duration of electrophoresis in the citrate and phosphate buffers varied from 6 to 8 hours and in the acetate and veronal buffers from 12 to 15 hours. Even prolonged electrophoresis (up to 20 hours) in all these buffers did not lead to more satisfactory resolution.

When the electropherograms of WSR obtained in acetate, citrate, veronal and phosphate buffers were cut into segments and eluted, the allergenic activity was found to be located, in each case, around the zone of application. Since most of the constituents of WSR were also located in this area of the electropherogram, the resolution of

WSR was not considered to be satisfactory in these buffers.

The optimal resolution of the components of WSR with respect to allergenic activity occurred in borate buffer, pH 8.6, after 16 hours of electrophoresis. As illustrated in Fig. 15, WSR was resolved into 4 pigment (P_1, P_2, P_3 and P_4), 1 carbohydrate (CHO), 1 protein (Pt) and 3 peptide (AA_1, AA_2 and AA_3) bands. Two rapidly migrating peptide bands, not shown in Fig. 15, were allowed to run of the paper in order to obtain an optimal resolution of the other constituents of the extract.

Fig. 16 shows that 8 of the carbohydrate-staining reagents produced positive reactions in the region of the paper designated CHO. In addition, two of the carbohydrate reagents, the alkaline potassium permanganate and the ammoniacal silver nitrate, elicited positive reactions in the P_1 and P_2 regions of the paper. Since the pigment bands are due most probably to flavanol pigments which have been shown to be present in ragweed (15, 70, 83), it is conceivable that the reactions elicited in the P_1 and P_2 regions of the paper were caused by free phenolic groups present in these pigments. This interpretation is supported by the fact that a positive "carbohydrate" reaction was obtained with phenol itself using the latter two carbohydrate staining reagents. Three of the carbohydrate reagents (Fig. 16) produced faintly stained bands in the cathodic region of the AA_1 band and two reagents gave barely stainable bands in the AA_2 region of the paper electropherogram. Therefore, the

material present in the area of the paper designated CHO (Fig. 15) is considered to be the only free carbohydrate present in WSR.

The allergenic activity was found not to be centrally located (as was found with the other buffers) but extended into the anodic region of the paper up to the edge of the most rapidly migrating pigment (P_1) band. The loss of the two rapidly migrating peptide bands is of no consequence to this study as they were found not to possess any allergenic activity.

Since the AA_1, P_4 and Pt components did not separate from each other (Fig. 15), they were eluted as one fraction which is referred to as AA_1+Pt. For the same reason, components P_1, AA_3 and CHO were eluted as one fraction designated P_1+C. Components AA_2, P_3 and P_2 were considered to be homogeneous fractions. The five fractions thus isolated were designated AA_2, AA_1+Pt, P_3, P_2 and P_1+C (Fig. 15). Solutions of fractions P_3, P_2 and P_1+C were light yellow, deep yellow and amber in colour, respectively. A solution of fraction AA_1+Pt has a straw-yellow colour and that of fraction AA_2 was colourless.

The entire procedure, electrophoresis of WSR in borate buffer and elution, was repeated about 25 times and the individual eluates of each fraction were combined and lyophilized.

Fig. 13.

Paper Electrophoresis of WSR in Citrate Buffer, pH 4.6

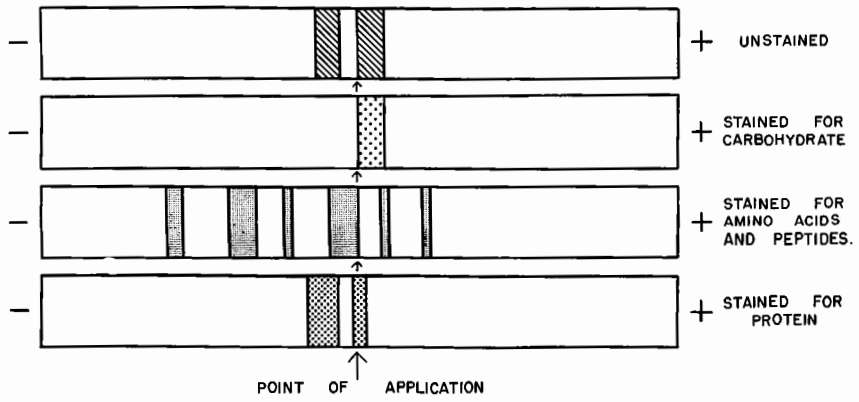


Fig. 14.

Paper Electrophoresis of WSR in Phosphate Buffer, pH 7.8.

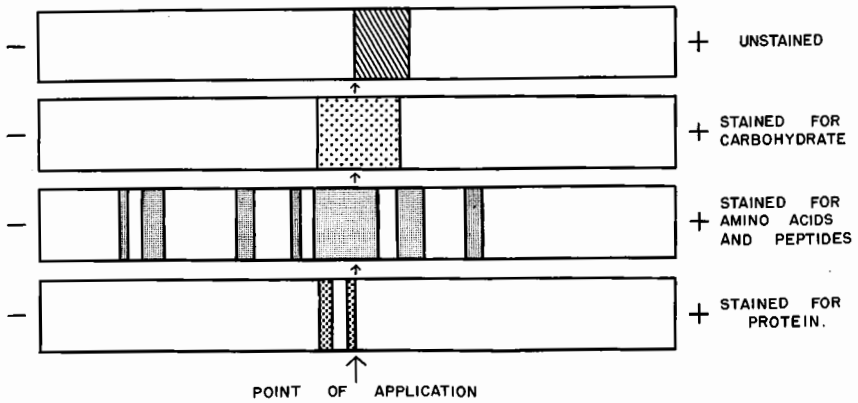
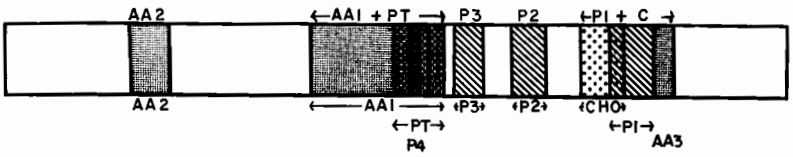


Fig. 15.

Electrophoretic Separation (on Paper) of WSR in Borate Buffer, pH 8.6, and the Designation of the Fractions Isolated.



AA REFERS TO THE BANDS REVEALED BY NINHYDRIN STAINING.

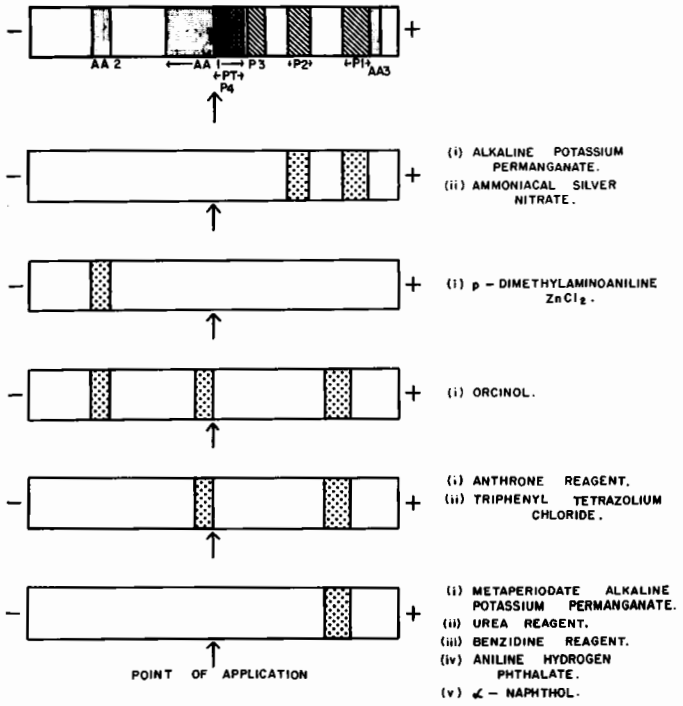
PT REFERS TO THE BANDS REVEALED BY AMIDO – BLACK.

C REFERS TO THE BANDS REVEALED BY THE CARBOHYDRATE REAGENT.

P REFERS TO THE VISIBLE PIGMENT BANDS.

Fig. 16.

The Materials in WSR Detected by the Carbohydrate Staining Reagents. The uppermost diagram represents the paper electropherogram of WSR analyzed in borate buffer, pH 8.6, stained for proteins and peptides only. The visible pigment bands are also shown. The lower five diagrams represent the bands obtained with the various carbohydrate staining reagents.



B. ALLERGENIC AND PHYSICO-CHEMICAL PROPERTIES OF THE FRACTIONS.

INTRODUCTION

The isolation of five fractions (AA_2, AA_1+Pt, P_3, P_2 and P_1+C) from the water soluble extract of ragweed pollen (WSR) was described in the preceding section. In the present study, these fractions were analyzed for their allergenic and physico-chemical properties. The relationship between the nitrogen and carbohydrate content and the allergenicity of the fractions was also investigated.

METHODS AND MATERIALS

Allergenic Activity of the Fractions:

The fractions were dissolved in saline and made up to concentrations of 1,000 units per ml. The pH of the solutions was 8.6, since each fraction contained the constituents of the borate buffer. The fractions were tested for allergenic activity by both the scratch test and the passive transfer technique. The scratch test was performed in the skin of clinically allergic individuals. The passive transfer test was performed in the manner as described in chapter IV-A. Reactions were graded 20 minutes after injection of the allergen fractions as 0, 1+, 2+, 3+, 4+, etc....., depending on the size of the wheal and the surrounding erythema.

Ultracentrifugal Analyses:

The following preparations were analysed in the Spinco Model E optical ultracentrifuge.

(i) Undialyzed fractions. Fractions AA_1+Pt, P_3, P_2 and P_1+C, each containing the constituents of the borate buffer, were dissolved in water to concentrations of 1 per cent. In addition, a 1 per cent solution of WSR in borate buffer was also prepared.

(ii) Dialyzed fractions. About 0.5 gram of each lyophilized fraction was dialyzed through Visking tubing against running tap water for 48 hours in an attempt to eliminate all easily dialyzable constituents. The material which remained within the Visking sac was lyophilized and dissolved in borate buffer to a concentration of 1 per cent.

WSR was also dialyzed for 48 hours and portions of the dialyzed residue, after being lyophilized, were dissolved in saline, the pH of which was adjusted to 8.6, and borate buffer, pH 8.6.

The preparations were analyzed in the synthetic boundary cell (120) and the solvent layer consisted of borate buffer, pH 8.6, in the same concentration as present in the various preparations. For the analysis of the dialyzed residue of WSR dissolved in saline, pH 8.6, the solvent layer consisted of saline adjusted to pH 8.6. The average rotor temperature for all the runs was 20.5°C and the rotor rate was 59, 780 rpm. The sedimentation constants were not recalculated for standard conditions and their dependence on concentration was not determined.

Relation Between Allergenic Activity and the Nitrogen or Carbohydrate Content of the Fractions.

WSR was fractionated by paper electrophoresis in borate buffer, pH 8.6, on Whatman No.3 mm filter paper. The electropherograms were then cut transversely into segments varying from 2 to 4 cm in width which were eluted with water by the technique described in chapter IV-A.

The nitrogen content of each eluate was determined by the technique of Lanni et al (121). The carbohydrate content or reducing power of each eluate was determined by the Somogyi method (123) and was expressed as glucose equivalents. Glucose was chosen as the standard since it has been shown to be one of the free carbohydrates in WSR (59, 83, 136). The allergenic activity of the eluates was demonstrated by the passive transfer technique.

RESULTS

Allergenic Activity of the Fractions.

Only two of the fractions, AA_1 + Pt and P_3, displayed cutaneous activity when examined by scratch test on allergic individuals (Table IV). However, both these fractions displayed allergenic activity inconsistently as at times they elicited no reactions at all. Fractions AA_2, P_2 and P_1 + C gave no reactions when examined by scratch test.

On the other hand, by the passive transfer method, fractions AA_1 + Pt, P_3, P_2 and P_1 + C were all shown to be active and only

fraction AA_2 was inactive (Table V). Tested on an equal nitrogen basis (1,000 units per ml), fractions AA_1+Pt, P_3 and P_2 displayed almost equal allergenicity with fraction P_3 being slightly more active than the other two. Fraction P_1+C consistently elicited the smallest reaction of all the fractions.

Ultracentrifugal Analyses.

Each of the undialyzed fractions gave rise to only one peak in the ultracentrifuge and they all had low sedimentation constants (Table VI). A sedimentation constant of 0.56S was calculated for undialyzed WSR. Three of the fractions (P_3, P_2 and P_1+C) had sedimentation constants lower than this value and fraction AA_1+Pt had a considerably higher sedimentation constant.

The sedimentation constants of the dialyzed residues were much higher than those of the respective undialyzed fractions (Table VII). The dialyzed residue of fraction AA_1+Pt was the only fraction to be resolved into two components, their sedimentation constants being 1.81 and 4.51S.

The dialyzed residue of WSR had sedimentations constants of 1.80 and 1.33S when analyzed in saline (pH 8.6) and borate buffer (pH 8.6), respectively.

Relation Between the Allergenic Activity and the Nitrogen or Carbohydrate Content of the Fractions.

The nitrogen and carbohydrate (reducing power) content of the eluates is shown in Fig. 17.

The findings of an extremely high concentration of reducing material in the P_1+C fraction suggests the presence of carbohydrate, which is in agreement with the results obtained with the carbohydrate-staining reagents (Fig. 16). As can be seen from Fig. 18, there exists no correlation between the nitrogen content and the allergenic activity of the eluates. No correlation was found to exist between the carbohydrate content and the allergenic activity of the eluates as well.

DISCUSSION

The results obtained with the scratch tests (Table IV) were inconsistent and erratic. The fractions which elicited reactions in some individuals (fractions AA_1+Pt and P_3) gave no reactions in other allergic individuals. There are at least two possible explanations to account for this observation:

(1) Allergic individuals may react differently to various fractions, thus displaying their individual patterns of reactivity.

(2) The scratch test may be subject to too many variables to be considered as a dependable tool. The depth of the scratch may govern the intensity of the observed reaction. Furthermore, there may exist no correlation between the quantity and specificity of reagin bound in the skin and that circulating in the blood of the allergic individual.

The results obtained with the passive transfer method would

appear to lend support to the latter assumption. The sera of allergic individuals when tested for reaginic activity to the fractions by the passive transfer method all yielded almost identical reaction patterns (Table V) although the reagin content of the sera varied markedly. It was also found that the passive transfer test demonstrated the presence of reagins to fractions P_2 and P_1+C in sera of individuals who gave no skin reactions to these fractions. One must therefore conclude that the passive transfer test, for reasons as yet not too well understood, is a far more sensitive method than the scratch test in testing materials for allergenic activity and that the results obtained by the passive transfer method are more consistent and reproducible.

The lack of correlation between allergenic activity and nitrogen content has been reported by others (41, 48, 95, 115, 116). There therefore appears to be no rationale for testing ragweed pollen fractions for allergenic activity on an equal nitrogen basis. Nevertheless, due to lack of knowledge of the precise chemical constitution of the allergenic entity or entities in ragweed pollen, the nitrogen unit has been generally accepted as the basis for comparing fractions of the pollen for their allergenic activity.

Results of ultracentrifugal experiments suggest that most of the constituents of WSR are of low molecular weight since the sedimentation constant of WSR was only 0.56S. Since the dialyzed residue

of WSR had a lower sedimentation constant when it was analyzed in borate buffer, pH 8.6 (1.33S), as compared to when it was analyzed in saline, pH 8.6 (1.80S), it would appear that the borate ions are capable of interacting with the WSR constituents and of impeding their rate of sedimentation. The values of the sedimentation constants of the different fractions are, therefore, not very significant since all the fractions were analyzed in borate buffer. However, the values can nevertheless be used as a guide for a comparison of the relative molecular weights of the fractions. Since the sedimentation constant of the dialyzed residue of each fraction was significantly higher than that of the respective undialyzed fraction, it must be concluded that all the fractions are heterogeneous and that there exist materials of different molecular weight in each. It is interesting to note the presence of a rapidly sedimenting peak in the dialyzed residue of fraction AA_1+Pt. Its sedimentation constant of 4.51S is indicative of high molecular weight; however, no suggestion as to the actual molecular weights of the components of WSR is made since the other parameters necessary for the determination of the molecular weight (partial specific volume, diffusion coefficient) were not determined due to the heterogeniety of the fractions.

Fig. 17.

Analysis of the Electropherogram of WSR for Total Nitrogen and Carbohydrate Content.

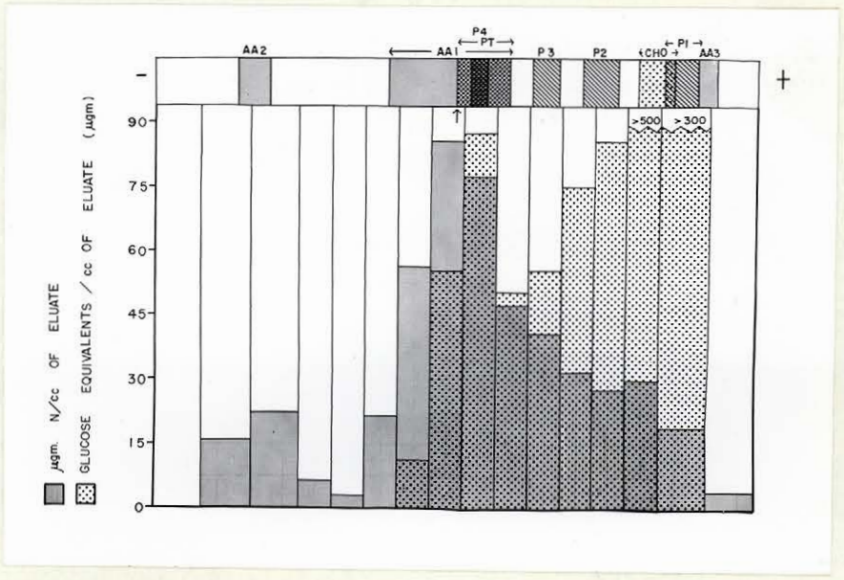


Fig. 18.

Correlation of Allergenic Active with Nitrogen Content.

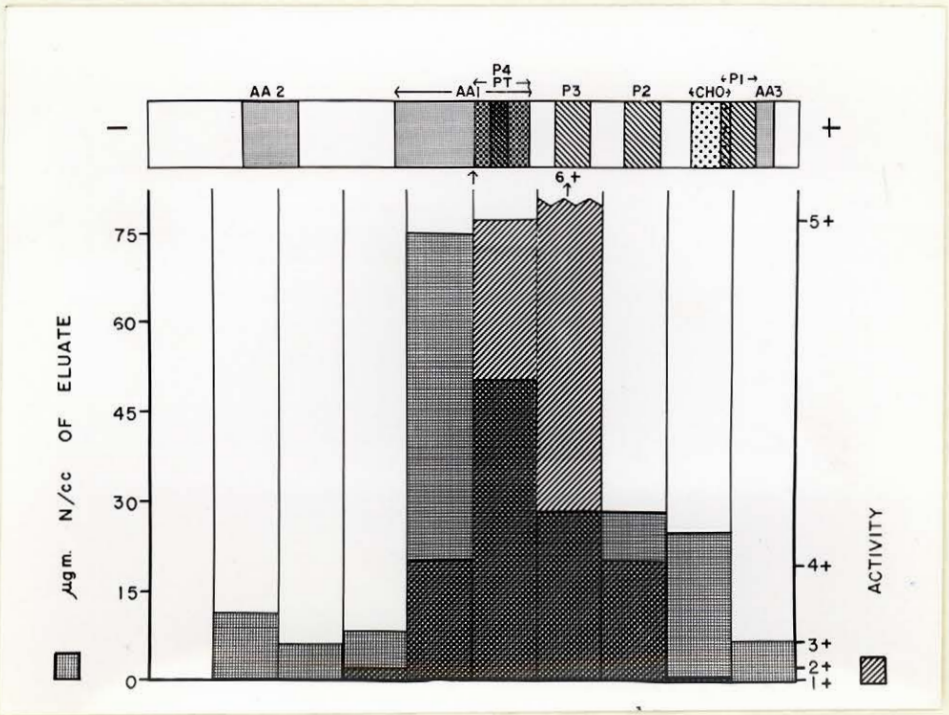


TABLE IV

Allergenic Activity of the WSR Fractions as Observed by Scratch Test.

SUBJECT TESTED	REACTION ELICITED BY FRACTION*				
	AA_2	AA_4 + Pt	P_3	P_2	P_1+C
A.P.	0	1+	0	0	0
G.K.	-	-	0	-	-
L.G.	-	-	0	-	-
F.T.	0	4+	0	0	0
C.P.	0	0	0	0	0
B.C.	0	1+	1+	0	0
A.G.	0	0	0	0	0
A.J.	0	4+	2+	0	0
C.G.	0	3+	1+	0	0

* EACH FRACTION MADE UP TO 1000 UNITS / CC

1 UNIT = 10^{-5} mg NITROGEN

TABLE V

Allergenic Activity of the WSR Fractions as Observed by the Passive Transfer Method.

ALLERGIC SERUM USED	REACTION ELICITED BY FRACTION*					
	AA_2	AA_4 + Pt	P_3	P_2	P_1+C	WSR
B.K.	0	3+	4+	3+	2+	3+
B.L.	±	3+	4+	2+	2+	4+
B.P.	0	5+	6+	6±	3+	5+
J.S.	0	3+	4+	3+	1+	3+
B.W.	0	4+	5+	4+	2+	4+

* ALL FRACTIONS ARE MADE UP TO 1000 UNITS / CC

1 UNIT = 10^{-5} mg NITROGEN

TABLE VI

Ultracentrifugal Analyses of the Undialyzed Fractions of WSR.*

Fraction	Sedimentation constant **
WSR	0.56
AA_1+Pt	1.20
P_3	0.28
P_2	0.19
P_1+C	0.16

* Performed in borate buffer, pH 8.6

** Expressed in Svedberg units.

TABLE VII

Ultracentrifugal Analyses of the Dialyzed Residues of the Fractions of WSR.*

Fraction	Sedimentation constant **
WSR - Dialyzed residue	1.33
AA_1 + Pt - Dialyzed residue	1.81 and 4.51
P_3 - Dialyzed residue	1.47
P_2 - Dialyzed residue	0.82
P_1 + C-Dialyzed residue	0.95

* Performed in borate buffer, pH 8.6

** Expressed in Svedberg Units.

CHAPTER V

DEMONSTRATION OF TWO ALLERGENS IN WSR AND A CRITICAL EVALUATION OF THE NEUTRALIZATION TECHNIQUE

INTRODUCTION

The results of previous investigations have suggested the presence of at least two allergens in ragweed pollen (49, 53, 79, 86, 87, 91, 92). In the majority of these studies, the conclusions were based on results of cross neutralization experiments carried out with the allergen preparations used on an equal nitrogen basis of 1,000 units per ml. The present study was undertaken to determine whether the four active fractions isolated from WSR (AA_1+Pt, P_3, P_2 and P_1+C) contained identical or different allergens.

A. CROSS NEUTRALIZATION EXPERIMENTS WITH THE FRACTIONS USED ON AN EQUAL NITROGEN BASIS.

METHODS AND MATERIALS

A cross neutralization experiment, as currently carried out, involves the following steps:

(a) Sensitization of a site in the back of a non-allergic subject with the allergic serum.

(b) Injection of an allergen preparation into the sensitized site every 24 hours until it no longer elicits a reaction, i.e. the

site is neutralized with respect to the allergen preparation.

(c) Injection of a second allergen preparation into the site.

If a reaction is obtained in step (c), then it can be assumed that the allergen preparation used to effect neutralization does not possess all the allergens present in the second allergen preparation. If no reaction is obtained in step (c), then it can be assumed that the allergen preparation used in step (b) contains all the allergens present in the preparation used in step (c).

The active fractions (AA_1+Pt, P_3, P_2 and P_1+C) and WSR were made up in saline to a concentration of 1,000 units per ml. The pH of the solutions of the fractions was 8.6 since they contained the constituents of the buffer (boric acid-sodium borate) used in the electrophoretic fractionation of WSR.

Preliminary experiments demonstrated that three to four daily injections of any of fractions AA_1+Pt, P_3 or P_2 were required to effect neutralization with respect to any of these fractions of a site sensitized with undiluted allergic serum whereas six to seven daily injections of fraction P_1+C were required to effect neutralization of a sensitized site to this fraction. In order to keep the time factor the same for all the sites, only fractions AA_1+Pt, P_3 and P_2 were used in the following experiments.

The cross neutralization experiments were performed in duplicate with each of three sera obtained from untreated allergic

individuals. These three sera were used for all the experiments recorded in this chapter. The procedure for the cross neutralization experiment is illustrated in Fig. 19. The allergic sera were used in the undiluted state. The interval of time between successive injections was 24 hours and each injection consisted of 0.05 ml of solution. Reactions were graded 20 minutes after injection of the allergen fractions. Controls consisted of unsensitized sites and sites "sensitized" with saline and normal human serum which were injected with the allergen fractions.

RESULTS

Sites 1 to 3 and 4 to 6 were neutralized to fractions AA_1+Pt and P_3, respectively, in three days, while it took four days to neutralize sites 7 to 9 to fraction P_2 (Stage II, Table VIII). The initial reactions observed in the sites injected with fractions AA_1+Pt and P_3 were about equal in intensity, while those observed in the sites injected with fraction P_2 were somewhat smaller.

The results of stage III of the experiment revealed that after being neutralized in stage II to fraction AA_1+Pt, site 2 gave a small reaction when challenged with fraction P_3 and site 3 gave no reaction when injected with fraction P_2. Sites 4 and 6, which had been neutralized to fraction P_3 in stage II, no longer reacted when challenged with fractions AA_1+Pt and P_2, respectively. Sites 7 and 8, which had been neutralized to fraction P_2 in stage II, gave quite large reactions when challenged with fractions AA_1+Pt and P_3, respectively

(Table VIII).

No reactions were induced in stage IV of the experiment, thus demonstrating that all the sites had been neutralized by the injections in stages II and III.

The results of stage V demonstrate that the sites which had given no reactions in stage IV were also neutralized to WSR. Site 5, which had been neutralized only to fraction P_3, did not react when challenged with WSR, while sites 1 and 9, which had been neutralized to fractions AA_1+Pt and P_2, respectively, gave reactions when challenged with WSR.

The results were identical for the three allergic sera used.

INTERPRETATION OF RESULTS.

The results suggest that WSR contains a minimum of three allergens. Fraction P_3 neutralized to fractions AA_1+Pt and P_2 and to WSR, fraction AA_1+Pt neutralized to fraction P_2 but not to fraction P_3 nor to WSR and fraction P_2 neutralized to neither fractions AA_1+Pt and P_3 nor to WSR. Therefore, if one assumes that fraction P_2 contains a minimum of one allergen, then fraction AA_1+Pt must contain a minimum of two allergens and fraction P_3 a minimum of three allergens. Since fraction P_3 neutralized to WSR, it can be concluded that there exist a minimum of three allergens in WSR.

B. DEMONSTRATION OF THE UNRELIABILITY OF THE STANDARD CROSS NEUTRALIZATION TEST.

The experiment presented in Table IX demonstrates the

fallacy of the cross neutralization test in the manner as it is currently carried out. Sites in the back of a normal individual were sensitized with an undiluted allergic serum and were neutralized to solutions of fraction AA_1+Pt varying in concentration from 10 to 3000 units per ml. As can be seen in Table IX, different sites required different quantities (2.5, 20, 100 and 150 units) of the allergen fraction in order to be neutralized. When the neutralized sites were injected with a solution of fraction AA_1+Pt containing 4000 units per ml, only the site which had been neutralized with the AA_1+Pt fraction in a concentration of 3000 units per ml did not react. The other sites had therefore been only apparently neutralized to their respective allergen solutions. This experiment demonstrates that one can differentiate between two states of non-reactivity of sensitized sites, neutralization and desensitization. Neutralization of a sensitized site is defined as the inactivation of the site to an allergen preparation but whether the neutralized site will react to the same allergen preparation when used in a higher concentration depends on the concentration of the allergen used to effect neutralization. Desensitization of a site is defined as the inactivation of all the reagin in the sensitized site so that the site can no longer react when challenged with the allergen fraction, regardless of the concentration used. Thus, for a constant quantity of reagin, the neutralization titer of the allergen is not an absolute value and may vary depending on the

concentration of the allergen solution used. The desensitization titer corresponds to the minimum concentration of the allergen capable of effecting desensitization of a sensitized site. A solution of the allergen capable of effecting desensitization of a site in one injection is considered to contain one desensitizing unit per ml.

The desensitizing titers of the active fractions and of WSR were determined in the following manner, using fraction AA_1+Pt as an example. Five sites on the back of a normal individual were sensitized with one of the allergic sera diluted 15 fold with saline. On the following day, the sites were injected with solutions of fraction AA_1+Pt containing 100, 200, 400, 800 and 1600 units per ml, respectively. All the sites gave large reactions varying from a 4+ reaction for site 1 to a 6+reaction for site 5. Twenty-four hours later, all the sites were injected with a solution of fraction AA_1+Pt containing 6,400 units per ml. Only sites 1, 2 and 3 gave positive reactions. Sites 4 and 5 did not react and therefore must have been desensitized by the first injection of the allergen (800 and 1600 units per ml, respectively). Therefore, a solution of fraction AA_1+Pt in a concentration of 800 units per ml contains 1 desensitizing unit per ml for this particular serum diluted 15 fold. In a similar manner, fractions P_3, and P_2 and WSR were standardized for their desensitizing equivalents which were found to be 1600, 6400 and 3000 units per ml, respectively.

C. CROSS NEUTRALIZATION EXPERIMENTS WITH THE FRACTIONS USED ON AN EQUAL DESENSITIZING BASIS.

INTRODUCTION

If the allergens present in the four active fractions (AA_1+Pt, P_3, P_2 and P_1+C) are identical but differ only in their concentration per nitrogen unit, the fractions should be able to desensitize sites to each other. On the other hand, if the fractions contain identical as well as different allergens, it should not be possible to achieve desensitization to all the fractions using only one of the fractions as the desensitizing agent. The following experiments were performed to ascertain which of these two conditions apply to the allergen fractions.

METHODS AND MATERIALS

(i) Experiments with the allergic sera diluted 15 fold

Fractions AA_1+Pt, P_3 and P_2 and WSR were dissolved in saline to a concentration of one desensitizing unit per ml (800, 1600 6400 and 3000 units per ml, respectively). A solution of fraction P_1+C in a concentration of 12,800 units was also prepared although this concentration was below the desensitizing concentration for this fraction. However, the injection of a solution of fraction P_1+C in a concentration exceeding 12,800 units per ml consistently resulted in an immediate irritation (formation of an erythema) and in the formation of a blister due to the high concentration of the borate buffer constituents (90 per cent, by weight, of the fraction). This

effect would mask any specific reaction which might occur between the allergen and the reagin.

The procedure and results of the cross neutralization experiment are shown in Fig. 20.

(ii) Experiments with the allergic serum diluted 200 fold.

Cross neutralization experiments were performed with the allergic sera diluted 200 fold and fraction P_1+C used in a concentration of 1600 units per ml as it was observed that this concentration of fraction P_1+C was sufficient to desensitize a site prepared with the allergic serum diluted 200 fold. Fractions AA_1+Pt, P_3 and P_2 and WSR were used in the same concentrations as in (i). In addition, solutions of fraction AA_1+Pt containing 50 and 200 units per ml were also prepared.

RESULTS.

(i) Experiments with the allergic sera diluted 15 fold.

The reactions observed in stage II of the experiment were all about equal in intensity as indicated in Fig. 20.

Stage III of the experiment produced some very interesting results. None of the sites desensitized to fraction AA_1+Pt, P_3 or P_2 in stage II reacted when challenged with any of the fractions in stage III. However, large reactions were obtained when fractions AA_1+Pt, P_3 and P_2 were injected into sites incompletely desensitized to fraction P_1+C.

No reactions were produced when WSR was injected into sites desensitized to fractions AA_1+Pt, P_3 and P_2. Only the site incompletely desensitized to fraction P_1+C reacted when injected with WSR (Fig. 20).

Similar results were obtained when the experiment was repeated with the two other allergic sera.

(ii) Experiments with the allergic sera diluted 200 fold.

Sites which were desensitized to fraction P_1+C in stage II (Table X) were still capable of giving large reactions when injected with fractions AA_1+Pt, P_3 and P_2 (stage III). Furthermore, fraction AA_1+Pt used in its desensitizing concentration with respect to the serum diluted 200 fold (200 units per ml) or in one-fourth its desensitizing concentration (50 units per ml) also induced large reactions when injected into sites desensitized to fraction P_1+C.

INTERPRETATION OF RESULTS

As one would expect, the desensitizing titers of the allergen fractions varied with different allergic sera. For this reason, sera possessing about equal quantities of reagin were selected for these experiments.

The quantity of allergen required to desensitize a site prepared on the back of a normal individual with any of the undiluted sera was so large that reactions invariably occurred in other unchallenged sites in the back. This effect was probably due to the absorption of the allergen from the injected site by the lymphatic

system and in its dissemination over the entire back of the subject. Such an effect would obviously render the results of a cross neutralization experiment ambiguous. On the other hand, using the allergic sera in too high a dilution would prevent the detection of reagins of different specificities which might exist in low concentration in the undiluted allergic sera. In consequence, the sera were arbitrarily diluted 15 fold for the cross neutralization experiments. The experiments with the sera diluted 200 fold were necessitated due to the fact that we were unable to prepare a solution of fraction P_1+C in a concentration high enough so that it would be capable of desensitizing a site prepared with the allergic serum diluted only 15 fold.

Since fractions AA_1+Pt, P_3 and P_2 were able to desensitize to each other and to WSR, it can be concluded that these fractions are allergenically identical and that each of them possesses all the allergens present in the whole extract. In view of the fact that fraction P_1+C was unable to desensitize to any of the other fractions or to WSR, it can be concluded that WSR as well as each of fractions AA_1+Pt, P_3 and P_2 contains a minimum of two allergens.

D. EXPERIMENTS WITH HEATED ALLERGIC SERA.

INTRODUCTION.

In 1940, Loveless (124) stated that reagin in sera of ragweed sensitive individuals could be inactivated by heating the sera for 1 to 2 hours at 56°C. If we assume that there are at least two allergens in WSR, then it may also be assumed that there are at least

two specific reagins in sera of allergic individuals, each formed in response to a specific allergen. It might therefore be possible to inactivate one of the reagins without completely inactivating the others by heating the serum for different periods of time.

METHODS AND MATERIALS

Aliquots of an allergic serum were Seitz-filtered into sterile bottles which were placed in a water bath at 56°C and heated for ½, 1, 2, 4, 6 and 8 hours, respectively.

Four sites on the back of a non-sensitive individual were sensitized with each of the above heated serum preparations and the sites were challenged 24 hours later with the four allergen fractions (AA_1+Pt, P_3, P_2 and P_1+C in concentrations of 800, 800, 1600, and 6400 units per ml, respectively).

In addition, sensitized sites were prepared with the allergic serum, undiluted, and with the serum diluted 10 fold, 100 fold, 1000 fold, and 10,000 fold. These sites were also challenged with the four allergen fractions 24 hours later.

The entire experiment was performed in duplicate with each of the three allergic sera used in this study.

RESULTS

As can be seen in Table XI, the activity of the reaginic serum diminished equally and uniformly with respect to the four allergen fractions with increased dilution of the serum. Sites sensitized

with the allergic serum diluted 10,000 fold were no longer able to react to fraction P_1+C although they were still capable of eliciting small reactions to fractions AA_1+Pt, P_3 and P_2.

In contrast to these results, the serum after having been heated for various intervals of time at 56°C behaved quite differently (Table XII). Heating the allergic serum for 2 hours almost completely abolished its capacity to give a skin reaction to fraction P_1+C while its activity to the other allergen fractions appeared not to be diminished. Heating the serum for 4 hours resulted in a negative reaction to fraction P_1+C although the three other fractions (AA_1^+ Pt, P_3 and P_2) were still capable of eliciting large reactions.

A site sensitized with the serum heated for 4 hours and which no longer gave a reaction when challenged with fraction P_1+ C was injected with fraction AA_1^+ Pt in a concentration of only 50 units per ml. The reaction produced was equal to that elicited by the injection of fraction AA_1^+ Pt in a concentration of 800 units per ml.

Similar results were obtained when the experiments were repeated with the two other allergic sera.

INTERPRETATION OF RESULTS.

It was demonstrated that the reagin(s) directed against the allergen(s) in fraction P_1+C was inactivated by heat while the reagin(s) directed against the allergen(s) present in fractions

AA_1+Pt, P_3 and P_2 was still active. This finding suggests the presence of at least two reagins in the allergic sera. If it can be assumed that an allergen can induce the formation of only one reagin in the allergic individual, then we can conclude that there exist at least two allergens in ragweed pollen, one allergen being common to all four active fraction and the other one being present in fractions AA_1+Pt, P_3 and P_2 only.

DISCUSSION

From the data presented in this chapter, there can be no doubt that ragweed pollen extract(WSR) contains at least two allergens.

Results of cross neutralization experiments with the fractions on an equal nitrogen basis (section A) suggested that there are at least three allergens in WSR. Fraction P_3 appeared to contain all three allergens, fraction AA_1+Pt two allergens and fraction P_2 only one allergen. Fraction P_1+C was not used in these experiments. However, the results were not conclusive as it was shown that a sensitized site could be neutralized without actually inactivating all the reagin in that site. Sites which appeared to have been neutralized to fractions AA_1+Pt, P_3 and P_2 nevertheless gave reactions when further challenged with the respective allergen fractions in concentrations higher than those used for neutralization. Neutralization of all the reagin in a sensitized site or desensitization of the site required the injection of a solution of

the allergen fraction in a definite concentration which had to be determined experimentally and which varied with different sera. It was found that sensitized sites would be only neutralized to an allergen solution used in a concentration below a certain threshold value; in order to effect desensitization of the site, the concentration of the allergen had to be above this threshold value (section B).

On the basis of the results of experiments with the allergen fractions used on an equal desensitizing basis (section C) and of experiments with heated allergic sera (section D), it would appear that fraction P_1+C contains only one allergen, which is common to all the fractions, and that fractions $^{AA}_1$+Pt, P_3 and P_2 contain at least one additional allergen, which is identical in the three fractions.

In all cross neutralization experiments, it is essential to ascertain unequivocally that sensitized sites are at no time refractory to give a reaction. This would imply that sensitized sites are at no time depleted of factors (i.e. histamine or histamine-like substances) whose absence would prevent a wheal and flare reaction. Three different experiments demonstrated that under the conditions of our experiments, the sites were never refractory:

(i) A single site in the back of an allergic individual injected at intervals of 24 hours for 28 days with WSR in con-

centrations varying from 100 units per ml (during the first week of injections) to 5,000 units per ml (during the last week of injections) gave reactions of equal intensity at all times.

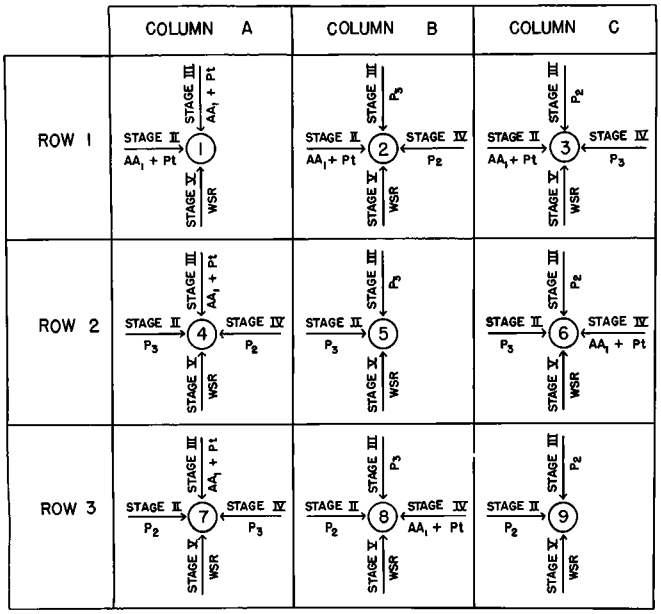
(ii) Sites sensitized with the serum of an allergic individual containing reagins to ragweed pollen and grass could be desensitized with respect to WSR without incurring any loss of activity to grass.

(iii) Sites desensitized to fraction P_1+C gave further reactions when challenged with the other fractions.

The findings suggest that results derived from cross neutralization experiments using solutions of the allergen fractions containing an equal number of units per ml may give rise to misleading and often fallacious conclusions. One must always be aware of the possibility that desensitization of the sites may not have taken place in the various phases of the experiment when interpreting the data of a cross neutralization experiment.

Fig. 19.

Scheme of Injections for the Cross Neutralization Experiment with the WSR Fractions Used on an Equal Nitrogen Basis.



STAGE I : — SENSITIZATION OF ALL SITES WITH ALLERGIC SERUM.

Fig. 20.

Results of the Cross Neutralization Experiment with the WSR Fractions Used on the Basis of Equal Activity Units and the Allergic Serum Diluted 15 Fold.

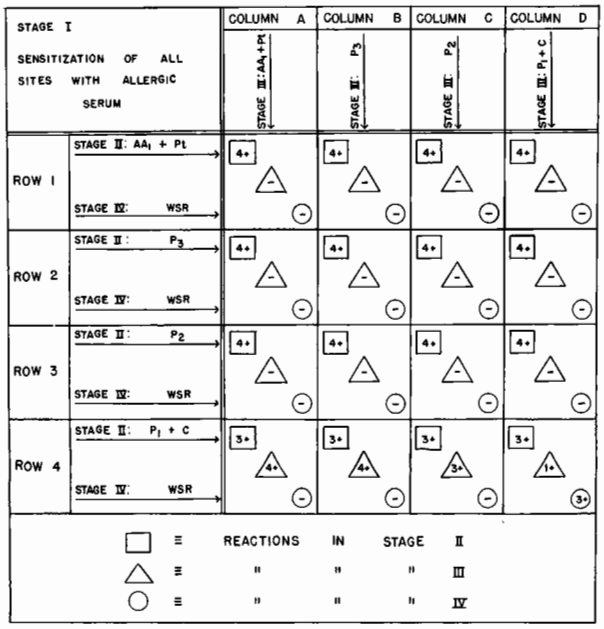


TABLE VIII

Results of the Cross Neutralization Experiment with the Fractions of WSR Used on an Equal Nitrogen Basis.

	Sites								
	1	2	3	4	5	6	7	8	9
Stage II*									
Fraction injected	$AA_1 + Pt$	$AA_1 + Pt$	$AA_1 + Pt$	P_3	P_3	P_3	P_3	P_3	P_3
Size of reaction, first injection stage II	7+	6+	7+	7+	7+	7+	5+	5+	5+
Stage III									
Fraction injected	$AA_1 + Pt$	P_3	P_3	$A_1 + Pt$	P_3	P_3	$AA_1 + Pt$	P_3	P_3
Size of reaction, first injection stage III	—	±	—	—	—	—	3±	3+	—
Stage IV									
Fraction injected	X†	P_3	P_3	P_3	X	$AA_1 + Pt$	P_3	$AA_1 + Pt$	X
Size of reaction, first injection stage IV	X	—	—	—	X	—	—	—	X
Stage V									
Material injected	WSR	WSR	WSR	WSR	WSR	WSR	WSR	WSR	WSR
Size of reaction	1+	—	—	—	—	—	—	—	3+

* Stage I comprises the sensitisation of all sites with allergic serum.
† X denotes site not challenged.

TABLE IX

Neutralization Experiment with Solutions of Fraction AA_1+Pt in Different Concentrations.

	Sites			
	1	2	3	4
Concentration of solution of fraction AA_1+Pt used to effect neutralization of sensitized site*	10**	100	1000	3000
Reaction observed after first injection	2+	4+	6+	6+
Number of daily injections[x] required to effect neutralization	5	4	2	1
Number of nitrogen units required to effect neutralization	2.5	20	100	15
Reactions observed on injecting the neutralized sites with a solution of fraction AA_1+Pt containing 4000 units/ml	4+	3+	1+	0

* Concentrations expressed as units per ml.
** 1 unit = 10^{-5} mg. nitrogen.
x Each injection = 0.05 ml.

TABLE X

Cross Neutralization Experiment with the Fractions of WSR Used in Desensitizing Concentrations and the Allergic Serum Diluted 200 Fold.

	Sites							
	1	2	3	4	5	6	7	8
Stage II*								
Injection of fraction $P_1 + C$ in concentration of (units/ml)	1600†	1600	1600	1600	3200	3200	3200	3200
Reactions observed in stage II	2+	2+	2+	2+	2+	2+	2+	2+
Stage III								
Fractions injected	$AA_1 + Pt$‡	P_3	P_2	$P_1 + C$	$AA_1 + Pt$	P_3	P_2	$P_1 + C$
Units/ml	800	1600	6400	6400	800	1600	6400	6400
Reactions observed in stage III	3+	3+	3+	—	3+	3+	3+	—

* Stage I comprises the sensitization of all sites with allergic serum.

† 1 unit = 10^{-5} mg nitrogen.

‡ Identical reactions were obtained with a solution of fraction $AA_1 + Pt$ in a concentration of 200 units/ml. which was its desensitizing concentration for the 200-fold diluted serum.

TABLE XI

Reactions Obtained with the WSR Fractions Injected into Sites Sensitized with the Allergic Serum Used in Ten Fold Dilutions.

Dilution of allergic serum injected into the sites.	Reactions obtained with fraction			
	AA_1+Pt	P_3	P_2	P_1+C
1:1	5+	5+	5+	5+
1:10	5+	5+	5+	5+
1:100	4+	4+	3+	3+
1:1,000	3+	3+	3+	2+
1:10,000	1+	1+	1+	0

TABLE XII

Reactions Obtained With the WSR Fractions Injected Into Sites Sensitized With the Allergic Serum Heated at 56°C for Various Intervals of Time.

Allergic serum (hours heated)	Reactions obtained with fraction			
	AA_1 + Pt	P_3	P_2	P_1 + C
½	5+	5+	5+	4+
1	5+	5+	5+	2+
2	5+	5+	4+	±
4	3+	3+	2+	0
6	2+	2+	1+	0
8	1+	1+	1+	0

CHAPTER VI

FURTHER STUDIES ON THE WSR FRACTIONS.

INTRODUCTION

Three of the active fractions isolated from WSR by paper electrophoresis, AA_1^+Pt, P_3 and P_2, were shown to possess at least two allergens each (chapter V). In this study, the fractions were analyzed by paper electrophoresis in various buffers in attempts at separating the allergens from inert constituents which may be present in the fractions. Since more than 90 per cent of the lyophilized fractions consisted of the constituents of the borate buffer, several attempts were made to eliminate the borate from the fractions.

METHODS AND MATERIALS.

Electrophoresis.

Fractions AA_1^+Pt, P_3 and P_2 were dissolved in saline and analyzed by paper electrophoresis(using Whatman No. 3 mm filter paper) in the following buffers: (a) acetate, pH 6.0; (b) phosphate, pH 7.4; (c) borate, pH 8.6 and (d) carbonate, pH 8.8. The ionic strength of all the buffers was 0.10. The duration of electrophoresis varied from 10 to 15 hours at 110 volts DC. The pH of each allergen solution was adjusted to that of the buffer prior to electrophoresis. The electropherograms were eluted (method described in chapter

IV-A) and the eluates were tested for allergenic activity by the passive transfer test.

Removal of Borate.

The separation of the borate constituents from the pollen components of the fractions was attempted by the following methods.

(i) Dialysis. An aliquot of each fraction was dissolved in saline and dialyzed through Visking tubing against running tap water for 48 hours. The dialyzed residue of each fraction was lyophilized and tested for allergenic activity.

Dialysis of the fractions was also performed through collodion membranes of pore size sufficiently small to allow only the borate ions to diffuse through. Four grams of collodion were dissolved in 100 ml of a solution of diethyl ether and ethyl alcohol (1:1) and 10 ml of the collodion solution were applied onto the surface of clean mercury in a Petri dish 10 cm in diameter. The ether and alcohol were allowed to evaporate and the collodion membrane was peeled off from the surface of the mercury. The membranes were swelled in ethyl alcohol according to the method of Carr et al (137). The apparatus for dialysis consisted of three cylindrical compartments made of lucite. The center compartment was open on both ends and the two side compartments were each open on only one side. The collodion membranes were placed between the compartments.

The center compartment was provided with an opening through which the allergen solution could be introduced or withdrawn. The solution in this compartment was stirred during the dialysis and distilled water was circulated continuously in the side compartments.

(ii) Removal of borate as trimethylborate by distillation. The addition of methyl alcohol to an aqueous solution of boric acid results in the formation of trimethylborate which distills at 68.5°C at 760 mm of mercury and at much lower temperatures under reduced pressure (138). Since the formation of trimethylborate proceeds only with the free boric acid, the pH of the aqueous solutions of the fractions was lowered to about 4.0 by the addition of HCl. Four volumes of redistilled methyl alcohol were added to each volume of allergen solution and the distillation of the trimethylborate was performed at 0°C under reduced pressure. The distillation took about 30 minutes. If the residue was found to contain borate (by the method of Smith (139), the whole procedure was repeated. One portion of the borate-free residue was dissolved in saline and the pH of the solution was adjusted to 8.6 by the addition of NaOH. The other portion was dissolved in saline-borate, pH 8.6. The allergenic activity of these solutions was compared to that of the untreated fractions. The following were used as additional controls: (a) solutions of the fractions maintained at pH 3.5 to 4.0 for 7 hours at 25°C and then adjusted to pH 8.6 and (b) solutions

of the fractions to which methyl alcohol had been added and which had been distilled for several hours at pH 8.6 and 0°C.

All solutions tested for allergenic activity were made up to a concentration of 1000 units per ml.

RESULTS.

Electrophoresis.

Since electrophoresis of the fractions in all the buffers resulted in almost identical separations, only the results obtained in the carbonate buffer are presented diagramatically (Fig. 21). Fractions P_3 and P_2 gave ninhydrin stainable bands in all the buffers except borate. In none of the fractions was the peptide constituent found to possess allergenic activity. In each case, allergenic activity was distributed over a wide area of the electropherogram (Fig. 21) and was located in areas of the paper devoid of detectable materials.

Removal of Borate.

(i) Dialysis. More than 95 per cent of the material in each fraction was found to be dialyzable. The dialyzed residues were found to be free from borate and were allergenically active. However, it was found that allergenically active material could dialyze through this membrane quite readily(Visking tubing).

Collodion membranes swollen in 84 to 85 per cent ethyl alcohol retained all allergenically active components whereas about 80 per cent of the borate was removed over a period of 6 to 7 days

of dialysis. However, the allergenic activity of the dialyzed residues of the fractions was found to be greatly diminished as compared to that of the undialyzed fractions.

(ii) Removal of borate as trimethylborate by distillation. Complete removal of borate from the fractions was achieved by distillation with methyl alcohol at pH 4.0. However, the allergenic activity of the fractions after this treatment was consistently found to be greatly diminished or completely destroyed (Table XIII). Solutions of the fractions which had been incubated at pH 4.0 for 7 hours at 25°C displayed no diminution in activity (Table XIV). Fractions to which methyl alcohol had been added and from which the alcohol was subsequently distilled at pH 8.6 and 0°C showed no decrease in activity except for fraction P_1+C which became inactive in most instances (Table XIV). The addition of borate buffer to the residues of the fractions distilled at pH 4.0 did not restore the allergenic activity (Table XV).

DISCUSSION

Since the fractions had been obtained by the electrophoretic fractionation of WSR in borate buffer, pH 8.6, it was most surprising to find that the activity in each fraction analyzed in this buffer was spread over an area of the electropherogram as wide as that observed with whole WSR. Since similar separations occurred with the fractions in all the buffers, this diffuse distribution

of the allergenically active constituents cannot be attributed to the effects of the buffer ions. A possible explanation for this observation would be that active material is constantly breaking down in both WSR and in the fractions of WSR. Since the allergenic activity was found in eluates of segments of the electropherogram devoid of carbohydrate, peptide, protein or pigment, it would appear that the active material is either none of these or that it is present in a concentration toolow to be detected by the staining reagents.

The finding that fractions P_3 and P_2 both displayed ninhydrin stainable material in all the buffers except the borate buffer suggests that borate exerts an inhibiting influence on the ninhydrin reaction.

Attempts to remove the borate buffer constituents from the fractions without a concomitant loss of allergenic activity were unsuccessful. Although dialysis through Visking tubing permitted the rapid elimination of borate, allergenically active material was found to dialyze through this membrane just as readily. Unfortunately, the allergic sera used for testing the dialysates for allergenic activity were obtained from treated allergic patients. As is shown in the following chapter, the dialysate of WSR reacts with sera of treated allergic individuals only.

Since allergenically active material was never found in

the dialysates of the fractions using the collodion membranes, the loss of activity noted in the dialyzed residues can only be attributed to a spontaneous inactivation of the allergens during the dialysis.

The author offers no explanation as to why distillation of the fractions in methyl alcohol at pH 4.0 resulted in almost complete loss of allergenic activity since neither the pH of 4.0 nor the methyl alcohol by itself had any deleterious effect on the activity.

Fig. 21.

Electrophoretic Analyses of Fractions AA_1+Pt, P_3 and P_2 in Carbonate Buffer, pH 8.8.

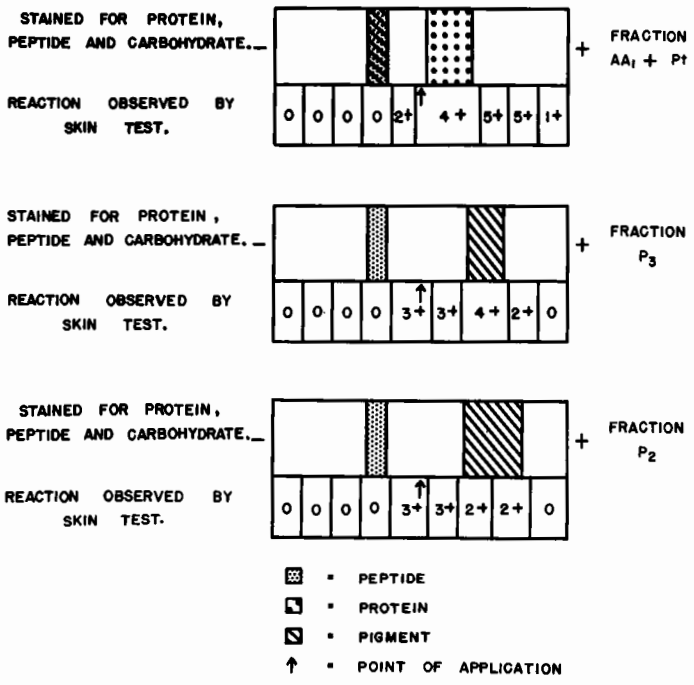


TABLE XIII

Allergenic Activity of the WSR Fractions After Being Dissolved in Methanol and Distilled at pH 4.0

Fraction tested *	Reaction elicited by the fraction	
	Before distillation	After distillation
AA_1 + Pt	6 +	2 +
P_3	5 +	1 +
P_2	5 +	3 +
P_1 + C.....	2 +	±

*All fractions made up to a concentration of 1000 units/ml.

TABLE XIV

Allergenic Activity of the WSR Fractions After Being Dissolved in Water and Distilled at pH 8.6 and After Standing at pH 4.0 for 7 hours.

Fraction tested*	Reaction elicited by the fraction		
	Untreated fraction	After distillation at pH 8.6	After standing at pH 4.0 for 7 hours
AA_1+Pt	4+	5+	4+
P_3	5+	3+	4+
P_2	4+	4+	4+
P_1 + C	2+	0	2+

* All fractions made up to a concentration of 1000 units/ml.

TABLE XV

Effect of the Addition of Borate Buffer on the Allergenic Activity of the WSR Fractions After Distillation with Methanol at pH 4.0

Fraction tested*	Untreated fraction	Reactions elicited by the fraction after distillation with methanol at pH 4.0	
		Made up to volume in saline to pH 8.6	Made up to volume in borate buffer, pH 8.6
AA_1+ Pt	5+	1+	2+
P_3	6+	1+	2+
P_2	5+	3+	3+
P_1+C	3+	1+	1+

* All fractions made up to a concentration of 1000 units/ml.

CHAPTER VII

NON-ALLERGENICITY OF THE DIALYSATE OF WSR IN UNTREATED ALLERGIC PERSONS

INTRODUCTION

In 1911, Noon (44) observed that patients suffering from hay fever showed clinical improvement after they had received a series of graded injections of pollen extract. Vander Veer et al (11), continuing the work of Noon, reported that 90 per cent of more than 500 ragweed sensitive patients treated with the extract of ragweed pollen obtained clinical relief from hay fever. In 1935, Cooke et al (51) demonstrated that a new factor appears in the serum of an individual who had undergone this procedure of "desensitization". This factor, referred to as "blocking antibody", is not found in sera of untreated allergic individuals and is capable of reacting with the allergen when the two are mixed in vitro and of preventing this incubated allergen from reacting with reagin in the skin of an allergic person or in a site sensitized with serum of an allergic individual. The presence of blocking antibody in sera of treated allergic individuals has been confirmed by Loveless (124), Frank and Gelfand (125), Sherman (126), Cooke et al (127) and more recently by Sehon et al (128).

Since the sera of treated allergic individuals*contain blocking antibodies, they might also contain new reagins of a specificity different from those found in sera of untreated allergic persons.

The following experiments were performed to test this possibility.

METHODS AND MATERIALS

Three allergen preparations of ragweed pollen were used: (a) WSR, the whole water soluble extract of the defatted pollen, (b) DIALYSATE, the fraction of WSR which dialyzed through Visking tubing during the first 24 hours and (c) DIALYZED RESIDUE, the fraction of WSR which remained within the Visking sac after 21 days of dialysis against running tap water.

Blood was drawn from treated and untreated ragweed sensitive patients and the sera were separated by centrifugation and sterilized by Seitz filtration. In addition, sera were obtained from 5 allergic patients both before and after they had undergone desensitization treatment. Each of the undiluted sera was injected (0.05 ml per injection) into two sites in the back of a normal non-allergic individual. Twenty-four hours later, the sensitized sites were challenged with WSR

* All ragweed sensitive patients who had undergone at least one series of injections of ragweed pollen extract are referred to as treated or desensitized individuals. Sera obtained from such persons are referred to as treated sera as compared to untreated sera obtained from untreated allergic individuals.

and DIALYSATE, respectively, used in concentrations of 1000 units per ml, and the reactions were graded depending on the size of the wheal and the surrounding erythema.

Whenever it was feasible, intradermal skin tests were performed on untreated and treated allergic individuals with both WSR and DIALYSATE.

Sites in the back of a normal individual were injected with eight treated sera, diluted 10 fold, and the sites were desensitized to DIALYZED RESIDUE. Similarly sensitized sites were desensitized to DIALYSATE and all the sites were then challenged with WSR.

In another experiment, sera from two treated individuals containing reagin to DIALYSATE were tested for their capacity to block or inactivate the allergens in WSR and in DIALYSATE. The blocking experiment was conducted in the following manner. Several sites in the back of a non-allergic person were injected with an allergic serum containing reagin to DIALYSATE (obtained from a treated patient) in a dilution such that the sites could be desensitized with a known concentration of WSR not exceeding 200 units per ml. A solution containing twice the desensitizing concentration of WSR was incubated for 24 hours at $4^{\circ}C$ with an equal volume of one of the two test sera previously heated at $56^{\circ}C$ for 8 to 10 hours to destroy its reaginic activity. An aliquot of this incubated solution was then injected into each of these sensitized sites. Twenty-four hours later, the sites were challenged with WSR. Solutions of WSR incubated with saline and normal human serum

instead of with the test serum served as controls for the experiment.

The blocking capacity of the two test sera with respect to DIALYSATE was determined by substituting DIALYSATE for WSR in the entire experiment. All injections were performed at 24 hour intervals and each injection consisted of 0.05 ml of solution, regardless of its nature or concentration.

RESULTS

As can be seen in Tables XVI and XVII, all sera reacted to WSR. All sera were also found to react to DIALYZED RESIDUE. On the other hand, only 6 of 36 untreated sera and 27 of 32 treated sera reacted to DIALYSATE by the passive transfer test. Only 11 of 26 untreated individuals as compared to all of the 10 treated individuals reacted to DIALYSATE by direct skin test whereas they all reacted to WSR (Tables XVI and XVII).

As can be seen in Table XVIII, none of the sera obtained from five individuals prior to desensitization treatment possessed reagins to DIALYSATE. However, the sera obtained from four of these same individuals after they had undergone desensitization treatment possessed reagins to DIALYSATE as demonstrated by the passive transfer test. The fifth individual became reactive to intradermal injection of DIALYSATE although no reagin directed toward DIALYSATE could be detected in his serum. Sera obtained from one of the five individuals (B.P.) one month and eighteen months after his last ragweed pollen injection were equally reactive to DIALYSATE (Table XVIII).

None of the sites sensitized with the eight treated sera and desensitized to DIALYZED RESIDUE was able to react when subsequently

challenged with WSR. On the other hand,all sites desensitized to DIALYSATE gave large reactions when challenged with WSR.

Neither of the two sera,obtained from treated individuals,used in the blocking experiments demonstrated any ability to inactivate DIALYSATE although both were able to block WSR in serum dilutions as high as 1:32.

DISCUSSION

Since 86 per cent of all treated and only 17 per cent of all untreated sera possessed reagin(s) to DIALYSATE, it can be concluded that reagin(s) of a new specificity was formed by ragweed sensitive patients undergoing desensitization treatment with ragweed pollen extract. The demonstration of the absence of this reagin(s) in the sera of allergic individuals prior to their undergoing desensitization treatment and the presence of this reagin(s) in the sera obtained from the same individuals after they had undergone desensitization treatment supports this conclusion. From these results, it appears that the "true" allergens, i.e. those which react with either the sera or the skin of untreated allergic individuals, are present only in the non-dialyzable fraction of WSR, which constitutes only 2 to 4 per cent, by weight, of WSR and only 0.5 to 1.0 per cent of the whole pollen.

The significance of the new reagin(s) detected only in the sera of treated allergic persons is not clear at this time. It is possible that it may be responsible for the increased clinical sensitivity

observed in some treated individuals. Patients B.P. and B.W. both claimed to have had more pronounced clinical symptoms after treatment although their sera had blocking antibody titers to WSR of 1:32. Although Loveless (129) claims that blocking antibody is the factor responsible for the clinical improvement shown in treated allergic patients suffering from hay fever, other attempts at correlating blocking antibody titers with clinical results have not been very rewarding (126, 130-132). Although the sera of both these treated patients (B.P. and B.W.) contained reagin to DIALYSATE in high titer, this does not imply that this reagin was the cause for the increased clinical sensitivity since we have detected reagin to WSR in the skin and sera of many individuals who have never had symptoms of clinical allergy. We have also been able to demonstrate that formation of reagin can be induced in some non-allergic individuals without evoking conditions of clinical allergy (see appendix).

In view of the fact that sites sensitized with treated sera and desensitized to DIALYZED RESIDUE were no longer capable of reacting when injected with WSR, it would appear that DIALYZED RESIDUE possesses all the allergens present in WSR. The "true" allergens must be of large molecular size or act as haptens linked to large carrier molecules since they do not dialyze through the Visking membrane.

An observation which requires explanation is why some untreated ragweed sensitive individuals possessed reagins to the allergens in the DIALYSATE.. It may be significant that all of these untreated individuals who reacted by skin test to DIALYSATE also reacted to some allergens other than ragweed (i.e. dust, horse dander and grass). Since it has been demonstrated that cross-reactivity exists between different allergens, it is possible that the reagins reacting with the allergens in the DIALYSATE in these individuals were formed on stimulation with other allergen preparations. However, one cannot exclude the possibility that these individuals were inherently capable of forming these reagins after exposure to all the allergens in ragweed pollen during the ragweed season. Five untreated allergic individuals gave positive skin reactions to DIALYSATE whereas their sera possessed no reagin to this allergen preparation detectable by passive transfer. Two possible explanations for this observation are: (1) The intradermal skin test is more sensitive than the passive transfer test to detect small quantities of reagin. This explanation alone, however, seems doubtful in this case since reactions in these individuals induced by direct skin tests with WSR were not significantly larger than the reactions obtained with their sera by passive transfer. (2) These individuals may have formed reagins in very low concentration to the allergens in the DIALYSATE during

the ragweed season. These reagins may have been "bound" to the skin while their concentration in the blood diminished when further stimulation for their formation ceased. Loveless (133) showed that reagin has a very marked affinity for the skin and is capable of being "bound" to it. Since four of the five individuals gave only small skin reactions to DIALYSATE, it can be concluded that reagin(s) directed toward DIALYSATE was present in only negligible quantity in their skin.

TABLE XVI

Reaginic Activity in Sera and Skin of Untreated Allergic Individuals to DIALYSATE and WSR

Serum or skin of patient tested	Reaction by passive transfer to:		Reaction by direct skin test to:	
	DIALYSATE	WSR	DIALYSATE	WSR
B.P.	0	5 +	x	x
A.L.	0	5 +	x	x
A.P.	0	5 +	x	x
L.M.	0	2 +	0	4 +
M.G.	0	3 +	0	6 +
D.G.	0	4 +	0	6 +
S.R.	0	4 +	0	4 +
P.G.	0	4 +	0	6 +
A.W.	0	1 +	0	3 +
# 9	0	3 +	x	x
#10	0	3 +	0	2 +
#11	0	2 +	0	2 +
#18	0	5 +	x	x
#22	0	2 +	x	x
#23	0	3 +	x	x
#48	0	2 +	x	x
#49	0	1 +	0	3 +
#50	0	3 +	x	x
#51	0	3 +	0	3 +
#53	0	4 +	1 +	4 +
#54	0	4 +	1 +	5 +
#57	0	4 +	4 +	5 +
#58	0	4 +	x	x
#59	0	4 +	x	x
#62	0	4 +	1 +	4 +
#64	0	2 +	x	x
#65	0	2 +	x	x
#66	0	2 +	1 +	3 +
#68	0	4 +	x	x
#69	0	3 +	0	5 +
H.L.	1 +	5 +	x	x
R.A.	2 +	2 +	x	x
#52	3 +	3 +	2 +	4 +
#61	2 +	2 +	x	x
#63	3 +	2 +	2 +	4 +
#67	2 +	4 +	x	x
P.M.	x	x	0	5 +
R.N.	x	x	1 +	4 +
R.S.	x	x	0	6 +
R.K.	x	x	0	4 +
F.J.	x	x	0	2 +
#60	x	x	2 +	4 +
#71	x	x	6 +	6 +
#73	x	x	3 +	6 +
#74	x	x	0	2 +

x Test not performed.

TABLE XVII

Reaginic Activity in Sera of Treated Allergic Individuals to DIALYSATE and WSR

Serum tested	Reaction by passive transfer to: DIALYSATE	WSR
B.P.	5 +	5 +
F.W.	4 +	4 +
A.S.	3 +	3 +
R.D.	5 +	4 +
# 3	2 +	2 +
# 4	1 +	4 +
# 5	1 +	3 +
# 7	3 +	4 +
#10	1 +	4 +
#11	1 +	2 +
#12	2 +	3 +
#14	4 +	4 +
#13	1 +	1 +
#25	4 +	4 +
#28	2 +	3 +
#29	2 +	3 +
#35	4 +	4 +
#36	2 +	3 +
#37	1 +	2 +
#38	4 +	4 +
#40	3 +	5 +
#41	2 +	3 +
#42	4 +	3 +
#43	4 +	4 +
#45	4 +	4 +
#55	3 +	3 +
#56	2 +	4 +
#22	0	3 +
#24	0	3 +
#33	0	3 +
#44	0	2 +
#46	0	4 +

In addition, 10 individuals who had undergone treatment reacted without exception by direct skin test to DIALYSATE and WSR. All reactions to DIALYSATE were comparable to reactions evoked by the injection of WSR (i.e. 3+or greater).

TABLE XVIII

Reaginic Activity in Sera of Allergic Individuals Before and After Treatment.

Serum tested		Reaction by passive transfer to: DIALYSATE	WSR
B.P.	Pre*	0	5+
	Post**	5+	5+
	Post 2 x	5+	5+
10	Pre	0	3+
	Post	1+	4+
11	Pre	0	2+
	Post	1+	2+
18	Pre	0	5+
	Post	2+	4+
22 xx	Pre	0	2+
	Post	0	3+

* Pre refers to serum obtained before treatment.

** Post refers to serum obtained from the treated individual after the ragweed season.

x This serum was obtained 18 months after B.P. received his last injection of ragweed pollen.

xx Patient 22 reacted by skin test to DIALYSATE (2+) after undergoing treatment although his serum contained no detectable reagin by the passive transfer method.

CHAPTER VIII

ISOLATION AND CHARACTERIZATION OF AN ALLERGEN FROM RAGWEED POLLEN

INTRODUCTION

The four allergenically active fraction isolated from WSR by paper electrophoresis (AA_1+Pt, P_3, P_2 and P_1+C) were all found to be heterogeneous, each containing at least two chemically distinct constituents (pigment, carbohydrate, peptide or protein). Each fraction was also found to contain materials with different sedimentation constants (chapter IV-B). It was therefore impossible to determine the chemical composition of the allergen(s) in any of the fractions without their further fractionation.

In experiments in chapter VI, it was demonstrated that allergenic activity could be isolated from areas of the electropherograms of the fractions devoid of any detectable material. This finding was interpreted as an indication that the allergenically active material might be present in an exceedingly low concentration in the fractions. It was shown in chapter VII that a large quantity of inactive material could be eliminated from WSR by dialysis through Visking tubing without a concomitant loss of allergenic activity with respect to sera of untreated allergic individuals. In the present study, the protein component(s) was removed from WSR by heating

the pollen extract at 60°C prior to its electrophoretic fractionation and an allergen was isolated by dialysis of one of the fractions isolated.

METHODS AND MATERIALS

An aqueous solution of WSR was heated for 15 minutes at 60°C and the precipitate which formed was centrifuged off. The supernatant, which is referred to as deproteinated WSR (Fig. 22), was fractionated by electrophoresis on paper in borate buffer, pH 8.6, and the fractions eluted from the electropherogram are referred to as AA_2, AA_1^-, AA_1^+, P_3, P_2 and P_1+C (Fig.23). These fractions were compared for their allergenicity with the fractions of whole WSR obtained in the same manner.

Fraction AA_1^- was dialyzed through Visking tubing against running tap water for 14 days (Fig. 22) to insure complete elimination of dialyzable material. The dialyzed residue, referred to hereafter as fraction AA_1^-D, was lyophilized and solutions of it varying in concentration from 1 mg per ml to 10^{-8} mg per ml were tested for allergenic activity by the passive transfer method.

Fraction AA_1^-D was hydrolyzed with 6N, 1N, 0.1N, 0.01N and 0.001N HCl in sealed glass tubes at 100°C for periods of 15 minutes, 2 hours, 8 hours and 14 hours. Fraction AA_1^+ and the dialyzed residue of WSR were also hydrolyzed with 6N HCl at 100°C for 14 hours. A solution of fraction AA_1^-D in 6N HCl was incubat-

ed at 25°C for 8 hours. The HCl was removed by drying the solutions in vacuo. The residues were dissolved in water and the pH of the solutions was adjusted to 7.0 to 7.5 by the addition of sodium bicarbonate. Solutions of fraction AA_1^-D, at pH 7.0, were heated in a boiling water bath for 15 minutes, 2 hours, 8 hours and 14 hours. All solutions were sterilized by Seitz filtration and tested for allergenic activity by the passive transfer method.

The fractions (AA_1^-D, AA_1^+ and the dialyzed residue of WSR) were examined by ascending paper chromatography using Whatman No. 1 filter paper (30 cm x 30 cm) both before and after they were hydrolyzed. For chromatography in one dimension, the following solvent systems were used; (a) 77 per cent ethyl alcohol (134), (b) phenol saturated with water (134), (c) 80 per cent pyridine (134) and (d) butanol:acetic acid: water = 4:1:5 (V/V/V) (134). The fractions were also examined by ascending paper chromatography in two dimensions, using the following combinations of solvent systems; (a) 77 per cent ethyl alcohol followed by butanol:acetic acid: water and (b) 80 per cent pyridine followed by phenol saturated with water. After chromatography had proceeded sufficiently in each solvent system, the papers were dried at room temperature and sprayed with ninhydrin to detect the presence of peptides and amino acids and with the metaperiodate-permanganate and the ammon-

iacal silver nitrate reagents to detect the presence of carbohydrates.

In order to determine whether allergenic activity was associated with stainable material, the following experiments were performed. Unhydrolyzed fraction AA_1^-D was applied as a straight line 6 cm long about 1 cm above the bottom edge of the Whatman No. 1 filter paper. One dimensional chromatography was performed in 77 per cent ethyl alcohol and in butanol: acetic acid: water. The papers were dried at room temperature and a strip was cut from each paper vertical to the line of application and sprayed with ninhydrin. This strip served as a guide for the localization of the constituents on the unstained portion of the filter paper. The latter was cut into segments which were eluted individually with saline. The eluates were tested for allergenic activity by the passive transfer method.

Precipitating antibodies to fraction AA_1^-D detected in both rabbit and goat anti-WSR sera were coupled to diazotized polystyrene*. A solution of fraction AA_1^-D in a concentration of 0.001 mg per ml was incubated with each of the polystyrene-antibody conjugates. Two hours later, the suspensions were centrifuged and the

* The author wishes to thank Mr. L. Gyenes for performing these experiments. A complete description of the preparation of the polystyrene-antibody conjugates will be presented in a forthcoming report from this laboratory (135).

supernatants were drawn off (supernatants A). The polystyrene-antibody conjugates were washed five times with saline and then suspended in saline. The pH of the suspensions was adjusted to 3.0 and incubation was carried out for two hours at 25°C. The suspensions were then centrifuged and the supernatants were decanted (supernatants B). Supernatants A and B were tested for allergenic activity by the passive transfer method..

Fraction AA_1-D, in a concentration of 1 per cent, was analyzed in the Spinco Model E optical ultracentrifuge using the synthetic boundary cell (120). Saline was used as the solvent and the pH of the solution was adjusted to 7.0. The rotor temperature was 20.5°C and the rotor speed was 59, 780 rpm. The sedimentation constant was not recalculated for standard conditions and its dependence on concentration was not determined.

RESULTS.

The precipitate which resulted on heating the aqueous solution of WSR at 60°C for 15 minutes was slightly yellow in colour and was found to stain only for protein. The supernatant gave the same pattern on electrophoresis in borate buffer, pH 8.6, as did the whole WSR except that the former did not stain for protein (Fig. 23). Fraction AA_1^+ had a faint yellow colour and fraction AA_1^- had a slight straw-yellow colour after its elution from the electropherogram although no pigment could be seen in the

region of the paper containing this fraction.

Fraction AA_1^- and the dialysate of this fraction gave intense stains with ninhydrin. Fraction AA_1-D gave only a faint colour reaction with ninhydrin whereas it gave an intense stain with ninhydrin after it had been hydrolyzed with 6N HCl at 100°C for as short a period of time as 15 minutes. No carbohydrate was detected in the unhydrolyzed material nor in the 15 minute, 2 hour and 14 hour hydrolysates of fraction AA_1-D.

The fractions eluted from the electropherogram of the deproteinated WSR were able to desensitize sites prepared with allergic sera (obtained from untreated allergic individuals) to the respective fractions obtained from the electropherogram of whole WSR. Fraction AA_2, obtained from the deproteinated WSR, possessed no allergenic activity. The dialysate of fraction AA_1- reacted only with sera of treated allergic individuals whereas the dialyzed residue of this fraction, AA_1^-D, reacted with sera of both treated and untreated allergic persons. Fraction AA_1-D was capable of inducing a 2^+ reaction in an amount as small as 10^{-7} mg. This fraction lost all allergenic activity after it was hydrolyzed with 6N HCl at 100°C for only 15 minutes or with 1N and 0.1N HCl for 14 hours (Table XIX). There was no loss of activity when fraction AA_1-D was incubated in 6N HCl at 25°C for 8

hours or when it was heated at 100°C at pH 7.0, or in 0.01N and 0.001N HCl for periods as long as 14 hours.

When fraction AA_1-D was analyzed by paper chromatography, the ninhydrin stainable material was found to be located about the zone of application in both solvent systems. The allergenic activity was associated only with the ninhydrin stainable material.

Both one and two dimensional chromatography of fraction AA_1-D hydrolyzed for 14 hours with 6N HCl at 100°C revealed only 8 spots after spraying with ninhydrin corresponding to the amino acids lysine, arginine, hydroxyproline, glycine, valine, alanine, norleucine and glutamic acid (Fig. 24). On the basis of the intensity of the stained spots, arginine appeared to be present in the lowest and lysine in the highest concentration of the 8 amino acids. Solutions of fraction AA_1-D heated at 100°C in 6N HCl for 15 minutes and in 1N HCl for 14 hours appeared to be completely hydrolyzed whereas all the solutions of fraction AA_1-D which had been heated in lower concentrations of HCl appeared to be incompletely hydrolyzed since the chromatograms of these preparations all gave less than 8 spots, and some of the spots did not correspond to any of the 8 amino acids detected in the completely hydrolyzed material. Neither the solution of fraction AA_1-D boiled at pH 7.0 for 14 hours nor that incubated at 25°C in 6N HCl for 8 hours appeared to have under-

gone any hydrolysis as they stained only faintly with ninhydrin and each gave only 1 spot on the chromatogram. Fourteen to sixteen ninhydrin stainable spots, corresponding to known amino acids, were detected on the two dimensional chromatograms of the hydrolysates of fraction AA_1^+ and of the dialyzed residue of WSR. Lysine could be detected only on the chromatogram of the hydrolysate of fraction AA_1^+.

Results of the experiments using the polystyrene-antibody conjugates are summarized in Table XX. Since supernatant A from the polystyrene-rabbit antibody conjugate gave a negative reaction by passive transfer, it can be assumed that the allergenically active material present in fraction AA_1-D was taken up by the antibodies coupled to the polystyrene. Supernatant B of this system gave a 2+ reaction by the passive transfer test, indicating that the allergen had been dissociated from the antibody coupled to the polystyrene. The allergenically active material was not taken up by the polystyrene-goat antibody conjugate.

An interesting observation was the loss of the ability of fraction AA_1-D to give a positive ring test with either goat or rabbit anti-WSR sera after fraction AA_1-D was heated in a concentration of 1 mg per ml at 100°C at pH 7.0 for 8 hours, although a solution of fraction AA_1-D in a concentration of only 0.05 mg per ml was capable of giving a positive ring test with these antisera.

This heated preparation of fraction AA_1-D could not inhibit the reaction between untreated fraction AA_1-D and the rabbit and goat antisera. This treatment did not appear to cause a diminution in the allergenic activity of the fraction as observed by the passive transfer test with various dilutions of the heated allergen solution.

The sedimentation constant of the unhydrolyzed fraction AA_1-D was found to be 1.20S.

DISCUSSION

Although heating an aqueous solution of WSR at 60°C for 15 minutes may not have caused complete precipitation of the protein, no stain for protein was obtained with the deproteinated WSR in a concentration as high as 50 grams per cent. This precipitation of protein was found to occur at 4°C as well but only very slowly and did not reach completion after even one month. The deproteinated WSR did produce a slight opalescence on saturation with ammonium sulphate and a precipitate with absolute ethyl alcohol. However, neither of these tests is an absolute criterion for the detection of protein. In fact, the dialyzable fraction of WSR contained material precipitable with both absolute ethyl alcohol and ammonium sulphate.

Fraction AA_1- was selected for the studies reported here since it contained only ninhydrin stainable material and some

pigment. Assuming that a negligible amount of protein may still have been present in the deproteinated WSR, it would have migrated as a broad bandextending from the zone of application to the P_3 region of the electropherogram, as it does in whole WSR. Therefore, it could not have been present as an undetected contaminant in fraction AA_1-. Since the dialysate of fraction AA_1- reacted only with sera of treated allergic individuals and the dialyzed residue (AA_1-D) reacted with sera of both treated and untreated allergic persons, only the latter preparation was investigated since only it was considered to contain the "true" allergens (those allergens capable of reacting with the sera or in the skin of untreated allergic individuals).

On the basis of the finding that the ninhydrin stain obtained with fraction AA_1-D was very faint but increased greatly in intensity after fraction AA_1-D was hydrolyzed, it would appear that the material in its native state is a polypeptide. Since unhydrolyzed fraction AA_1-D gave only one spot on the chromatogram whereas hydrolyzed fraction AA_1-D gave eight spots, it can be concluded that all eight amino acids were incorporated into the polypeptide.

Since the allergenic activity was associated with only the ninhydrin stainable material when fraction AA_1-D was analyzed by paper chromatography and since the material lost all aller-

genic activity after it had been hydrolyzed with HCl, it would appear that the polypeptide is the active material. The fact that the allergenic activity was not lost when the treatment of fraction AA_1-D did not result in hydrolysis of the polypeptide supports this belief. The pigment does not appear to be allergenically active since it could be destroyed (by boiling fraction AA_1-D in 0.01N HCl for several hours) without a resulting diminution in the allergenicity of fraction AA_1-D.

The results would appear to conflict with those obtained in experiments in chapter VI when it was found that the activity was not associated with the ninhydrin stainable material in fraction AA_1+Pt of which fraction AA_1-D is a part. However, the constituent(s) of fraction AA_1-D may not have been present in a concentration high enough in fraction AA_1+Pt to be detected by the staining reagents.

It was very surprising to find that fraction AA_1-D was capable of giving a precipitate with rabbit and goat anti-WSR sera since it has previously been reported that only the protein constituent(s) of WSR possessed this property (23-25). The allergenically active material in fraction AA_1-D was taken up by the rabbit antibodies, but not by the goat antibodies, coupled to the polystyrene. Since the goat anti-WSR serum was capable of "blocking"

the reaction between reagin (using the serum of an untreated allergic individual) and WSR in a passively sensitized site, it must be concluded that the goat antiserum possessed antibodies to the allergen(s). Other investigations in this laboratory demonstrated that red blood cells to which were coupled goat anti-WSR serum proteins were not agglutinated in the presence of WSR whereas red blood cells to which were coupled rabbit anti-WSR serum proteins did agglutinate in the presence of WSR. Since antibody molecules are believed to contain only two groupings capable of reacting with the antigen, the possibility of steric hindrance of the antibody groupings by the polystyrene or by the surface of the red blood cell when the goat antibody was coupled to either of these supporting media cannot be ignored.

Fraction AA_1-D lost its ability to give positive ring tests with rabbit and goat anti-WSR sera but not its allergenicity after being heated in a boiling water bath for 8 hours at pH 7.0. This observation suggests that the groupings on the polypeptide molecule responsible for allergenic activity are less susceptible to being inactivated or denatured than are the groupings responsible for eliciting positive ring tests with the rabbit and goat precipitating antibodies.

It was interesting to note that lysine, one of the amino acids identified on the chromatogram of the hydrolysate of fraction AA_1-D, could not be detected on the chromatogram of the

hydrolysate of the dialyzed residue of WSR. It would therefore appear that the material detected in fraction AA_1-D is present in too low a concentration in the dialyzed residue of WSR to be detected by staining reagents when the latter is used as test material. Since the dialyzed residue constitutes less than 5 per cent of the weight of WSR, it is not surprising that the active material contained in fraction AA_1-D could not be detected in whole WSR.

The sedimentation constant for fraction AA_1-D was found to be 1.20S. However, since the diffusion coefficient and the partial specific volume for this material were not determined, due to the small quantity of fraction AA_1-D available at the time, a determination of the molecular weight was not attempted.

Fig. 22.

Flowsheet of the Procedure for the Isolation of Deproteinated WSR and Fraction AA_1-D.

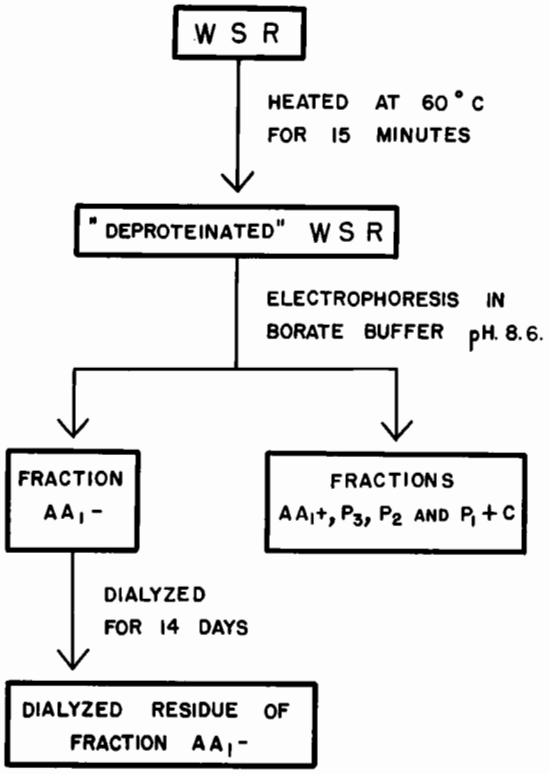
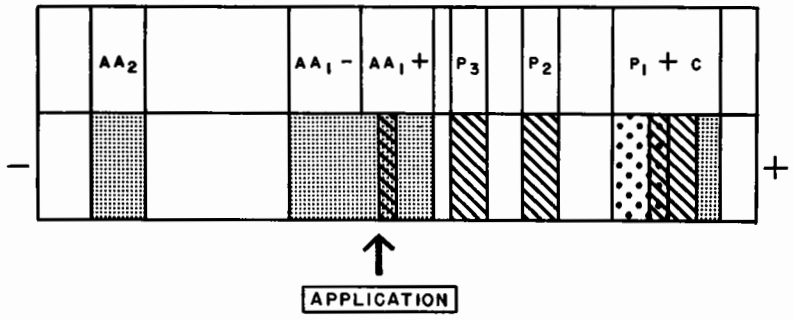


Fig. 23.

Electrophoretic Fractionation (on Paper) of Deproteinated WSR and the Designation of the Fractions Isolated.



AA REFERS TO THE BANDS REVEALED BY NINHYDRIN STAINING.

C REFERS TO THE BANDS REVEALED BY THE CARBOHYDRATE REAGENT.

P REFERS TO THE VISIBLE PIGMENT BANDS.

Fig. 24.

Two Dimensional Chromatogram of the Hydrolysate of Fraction AA_1-D. a, lysine; b, arginine; c, glycine; d, glutamic acid; e, hydroxyproline; f, alanine; g, valine; h, norleucine.

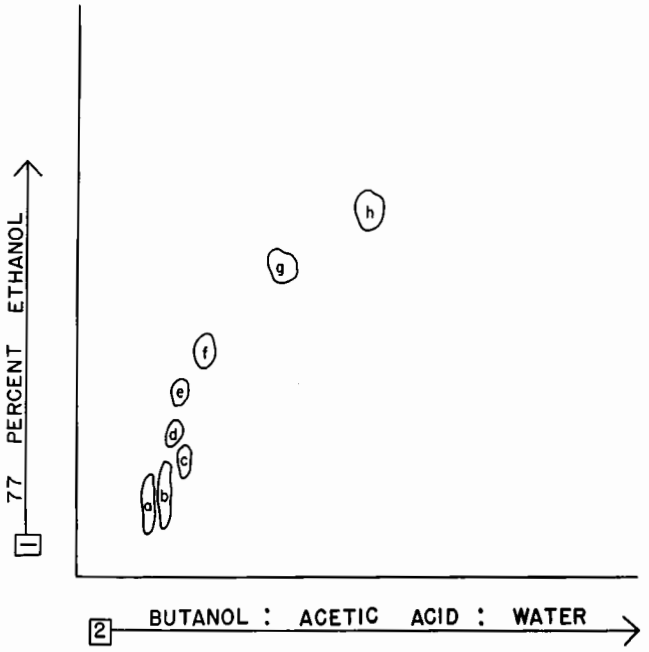


TABLE XIX

Effect of Heat and/or Acid on the Allergenic Activity of Fraction AA_1-D

Fraction AA_1-D treated in the following manner.	Concentration of solution: mg/ml.	Reaction by passive transfer.
Not treated	0.001	4 +
14 hours at 100°C, pH 7.0	0.001	4 +
8 hours at 25°C, 6N HCl	0.001	4 +
15 minutes at 100°C, 6N HCl	0.10	0
14 hours at 100°C, 1N HCl	0.10	0
14 hours at 100°C, 0.1N HCl	0.001	0
14 hours at 100°C, 0.01N HCl	0.001	4 +
14 hours at 100°C, 0.001N HCl	0.001	4 +

TABLE XX

Allergenic Activity of Fraction AA_1-D on Treatment With the Polystyrene-Antibody Conjugates.

Material tested	Reaction by passive transfer
Polystyrene-rabbit antibody system	
Supernatant A	0
Supernatant B	2+
Polystyrene-goat antibody system	
Supernatant A	2+
Supernatant B	0

CHAPTER IX

GENERAL DISCUSSION

From the results which have been presented in this thesis, one can understand the difficulties which were encountered by previous investigators who attempted to isolate the allergen(s) from ragweed pollen. It was shown that an allergenically active material (fraction AA_1-D) could be isolated by dialyzing a fraction (AA_1-) obtained by the electrophoretic separation of the water soluble extract of ragweed pollen (WSR) which had been depleted of its protein component(s). Since the injection of 10^{-7} mg of fraction AA_1-D into the skin of an allergic individual elicited a reaction as large as the injection of 10^{-4} mg of WSR, it would appear that a 1000 fold purification of the allergen has been accomplished. Fraction AA_1-D consisted of a straw-coloured pigment and a peptide composed of eight amino acids-arginine, lysine, glutamic acid, glycine, alanine, hydroxyproline, valine and norleucine. Since the pigment could be destroyed without a concomitant loss of allergenic activity of the preparation whereas hydrolysis of the peptide resulted in a complete loss of allergenic activity, it would appear that the peptide is the allergen. The fact that the allergenic activity of fraction AA_1-D was associated with the peptide when the preparation was analyzed by paper chromatography would tend to support this belief. Although it cannot be stated catagorically that the nitrogen-contain-

ing material in fraction AA_1-D is not a protein, its low sedimentation constant (1.20S), its failure to stain for protein and the fact that it is composed of only eight different amino acids suggests that it is a peptide. However, the peptide possessed several properties characteristic of proteins as it could be precipitated with ammonium sulphate and ethyl alcohol used in the same concentrations as were necessary to precipitate the protein constituent(s) of the pollen extract. Since ammonium sulphate and ethyl alcohol were the reagents most commonly used by previous investigators in attempts at isolating the allergen(s) from ragweed pollen (46, 47, 49, 52-54, 56), there can be no doubt that the active peptide was precipitated together with the protein component(s) in WSR upon the addition of either ammonium sulphate or ethyl alcohol to the aqueous extract of the pollen. Since the bulk of such a precipitate has been found to consist of protein with the active peptide present in only a trace amount, it is not surprising that the allergenic activity was attributed to protein constituents of the pollen by previous investigators. However, those investigators who used heat to precipitate the protein component(s) of WSR (23, 25) found that the precipitated protein possessed only slight allergenicity. From the results presented in this thesis, it would appear that heat causes precipitation of only the protein

fraction of WSR, leaving the active peptide in solution. However, it is possible that allergenically active groupings on the protein molecule may have been inactivated by the heating. Our finding that the deproteinated WSR was allergenically as active as the whole WSR would indicate that the protein, in its native state, possesses at most only slight allergenicity. Heating the pollen extract at 60°C is therefore a suitable method for separating the protein component(s) from the extract without destroying the allergenic activity.

It is interesting to note that investigators who claimed to have prepared active carbohydrate fractions from WSR (27, 50, 78, 79, 82) were never able to eliminate the nitrogen-containing material from their active fractions without an accompanying loss of allergenic activity. In most cases, the nitrogen-containing material was regarded as an inactive contaminant. It apparently did not occur to these workers that the "contaminant" in their active preparations might be the allergen. Due to its high biological potency, only a minute quantity of the allergen would have had to be present in any of the preparations in order to give the impression that the material identified as carbohydrate was the allergen.

It was demonstrated that the dialyzable fraction of WSR was allergenically active with respect to sera and skin of trea-

ted (desensitized) allergic individuals only whereas the non-dialyzable fraction of WSR was allergenic when tested with the sera or in the skin of both treated and untreated allergic persons. It therefore appears that during the course of the desensitization treatment, the allergic individual forms a new reagin(s) with a different specificity from any found in the sera or skin of untreated allergic patients. Since such a finding has never been reported previously, it must be concluded that investigators in the past did not discriminate between the allergic sera or individuals as to whether they were treated or not when testing the allergen fractions for cutaneous activity. It is probable that the investigators who reported that the allergen was dialyzable (58, 59, 63, 82, 88, 90, 91) tested the dialysate for allergenic activity with the sera of treated allergic persons. This is probably true in the cases of Loveless et al (91) and Bukantz et al (58, 59) who confined most of their investigations to the dialyzable constituents of the pollen extract. However, one cannot exclude the possibility that the dialyzing membranes used by some of the previous investigators were actually permeable to the allergen(s) which reacts with the sera and skin of untreated allergic individuals and which has been designated as the "true" allergen(s) in this thesis.

It was very surprising to find that fraction AA_1-D gave a precipitate with rabbit and goat anti-WSR sera since it has been

previously claimed that only constituent(s) of WSR is capable of reacting with the precipitating antibodies formed in experimental animals (23-25). Since fraction AA_1-D contained only peptide and pigment, it can be assumed that the rabbit and goat antibodies were directed toward the peptide. It would therefore appear that the allergen isolated is antigenic in experimental animals although it may be that different chemical groupings on the peptide molecule are responsible for its allergenicity and its antigenicity.

It was demonstrated that the cross neutralization test with the allergen preparations used on an equal nitrogen basis is unreliable insofar as the determination of the number of allergens present in the various active fractions is concerned. It was shown that a passively sensitized site could be neutralized by successive injections of an allergen solution and yet still give a reaction if further challenged with a solution of the same allergen in a higher concentration. A possible explanation for this finding is that only a small quantity of unreacted reagin is left in the apparently neutralized site and in order to obtain a visible reaction in the site it is necessary to inject a large amount of the allergen so that all the free reagin molecules can react with the allergen simultaneously. A site which has been injected with a quantity of the allergen capable of neutralizing all the reagin

in the site at one time is regarded as desensitized, rather than neutralized. When cross neutralization experiments were performed with sera of untreated allergic persons and with the WSR fractions (AA_1+Pt, P_3 and P_2) used on an equal nitrogen basis, the results of the experiments suggested that fraction P_2 contained one, fraction AA_1+Pt two and fraction P_3 three allergens. When the cross neutralization experiments were performed with sera of untreated allergic individuals and the WSR fractions used on an equal desensitizing basis, it was shown that fractions AA_1+Pt, P_3 and P_2 were allergenically identical and that there must exist a minimum of two allergens in ragweed pollen. The results of the experiments with the heated allergic sera corroborated this view. On the basis of our findings, the interpretations of results of cross neutralization experiments performed by previous investigators who used ragweed pollen fractions on an equal nitrogen basis must be re-evaluated. At least two important factors were not taken into consideration by previous workers. They did not discriminate between the sera which they used for their cross neutralization experiments as to whether they were obtained from treated or untreated allergic persons nor were they aware of the fact that failure of a sensitized site to give a visible reaction does not necessarily imply that all the reagin in the site is neutralized. It is therefor surprising that previous investigators concluded, on the basis of their cross neutralization experiments, that ragweed pollen con-

tains a minimum of only two (53, 79, 86, 92) or three (49, 56, 91) allergens.

Since fraction AA_1-D was capable of desensitizing a passively sensitized site to whole WSR, it must contain a minimum of two allergens, both of which must be situated on the peptide molecule. However, it is possible that only one of the allergens is located on the peptide and that the other is present in too low a concentration to be detected by staining reagents even after the extensive purification which has been performed. It is also possible that fraction AA_1-D contains several peptides possessing similar physico-chemical properties and that the allergens are situated on different peptide molecules.

CHAPTER X

SUMMARY

1. Defatted ragweed pollen was extracted with various solvents and the fractions were analyzed by free and paper electrophoresis and ultracentrifugation and shown to be of different composition.

2. All the extracts of the pollen displayed allergenic activity.

3. The water soluble extract of defatted ragweed pollen (WSR) was separated into four allergenically active fractions by paper electrophoresis in borate buffer, pH 8.6.

4. The WSR fractions were shown to be heterogeneous by ultracentrifugation and were found to contain materials with sedimentation constants varying from 0.16 to 4.51S. These sedimentation constants were determined for the fractions examined in borate buffer, pH 8.6, and no inference is made as to the actual molecular weights of the materials.

5. The passive transfer technique was found to be far superior to the scratch test in defining the allergenic activity of the WSR fractions.

6. The classic cross neutralization test was shown to be inadequate for defining the number of allergens present in different ragweed pollen fractions. A variation of the method, based on the

ability of the allergen solutions to "desensitize" sites prepared with allergic serum, rather than to "neutralize" them, was presented.

7. It was shown that ragweed pollen must contain at least two allergens and that the sera of allergic individuals contain at least two reagins.

8. Sera (and skin) of untreated allergic individuals were found to possess reagins directed only toward the constituents in the non-dialyzable fraction of WSR whereas sera and skin of treated allergic persons were found to contain reagins directed toward constituents in the dialyzable and non-dialyzable fractions of WSR.

9. The dialyzed residue of one of the fractions obtained by the electrophoretic fractionation of deproteinated WSR was found to be allergenically active in an amount as small as 10^{-7} mg. It consisted of a pigment and a peptide composed of eight amino acids - arginine, lysine, glycine, alanine, valine, hydroxyproline, glutamic acid and norleucine. Results of studies with this material suggest that the peptide is one of the allergens in ragweed pollen.

APPENDIX

INDUCED FORMATION OF REAGIN IN NON-ALLERGIC PERSONS TO EXTRACTS OF RAGWEED POLLEN

At the time when the cross neutralization experiments with the WSR fractions (AA_1+Pt, P_3 and P_2), used on an equal nitrogen basis, were performed (see chapter V-A), it was noted that some of the non-allergic volunteers used for the passive transfer experiments developed reagins to the pollen fractions. Positive reactions to WSR could be elicited in unsensitized portions of the backs and arms of these subjects within four days after the initial injections of the allergen fractions. Fraction P_1+C, which was not even used in these cross neutralization experiments, consistently elicited the largest reactions and fraction P_2 produced reactions of slightly lesser intensity. Surprisingly, no reactions were produced with fractions AA_1+Pt and P_3 except in one subject (M.D.) who gave a moderate reaction to fraction P_3. All of these persons gave positive reactions to WSR (Table XXI).

A comparison of the reactions obtained in these individuals with those obtained using the sera of allergic persons (Table V) suggests that the newly acquired reaginic activity by each of these subjects used for the passive transfer experiments was not due to the absorption of the injected reagin and in its distribution over

the entire surface of the subject but was formed de novo by the individual. If this reaginic activity had been due to absorbed reagin, one would have expected that the subject and the allergic serum used in the passive transfer experiment would have exhibited the same relative degree of activity to the four allergen fractions. However, the acquired sensitivity of the subjects was greater to fraction P_1+C than to fraction P_2 and was non-existent to fractions AA_1+Pt and P_3 (except in subject M.D.), whereas the relative degree of activity of the allergic sera to the fractions was in the order of AA_1+Pt = P_3 = P_2 > P_1+C (Table V). This finding suggests that the allergens to which these subjects had formed reagins were different from those responsible for the induction of clinical allergy. Furthermore, it is interesting to note that the sera of two subjects (M.D. and A.L.) were capable of passively transferring their activity. The reactions observed in sites sensitized with these sera and challenged with the allergen fractions were identical to those observed on intradermal injection of the fractions into these two individuals (Table XXI). The sera of the other subjects were not tested by the passive transfer method. It is of interest that none of these subjects complained of having developed clinical hay fever or asthma during the following two years. One subject (M.D.) was tested with the WSR fractions 10 months after initially showing sensitivity and the reactions were identical to those observed originally (Table XXI).

The rapidity with which this newly acquired sensitivity

was developed by these individuals is most striking. In the case of one subject (R.H.), the sensitivity was noticed only two days after the initial injections of the allergen fractions, while in the other subjects the sensitivity was demonstrated within 3 to 4 days. Assuming that the formation of reagin is similar to the formation of protecting or precipitating antibodies, the rapid occurrence of the reaginic activity in the subjects after the initial injection of the allergen fractions would suggest a more rapid "primary response" than has ever been reported (140). None of the subjects had previously been injected with extracts of ragweed pollen or any other pollen. However, they all had been subjected to airborne ragweed pollen and it has been shown that the allergen constituents of the pollen can be absorbed through the nasal mucous membrane, the respiratory tract and the gastro-intestinal tract (22, 89, 104-108). Under these circumstances, these persons could have become "subclinically" sensitized to the allergens in ragweed pollen prior to our having used them as subjects for the passive transfer experiments. Therefore, the possibility of our having evoked an "anamnestic response" in these subjects cannot be ruled out.

CONCLUSIONS

1. Some normal, non-allergic persons are capable of forming reagins in response to injections of extracts of ragweed pollen without becoming clinically allergic.

2. The reagins formed by these persons are detected within only two to four days after initial injections of the ragweed pollen

fractions and they can be passively transferred.

3. The constituents of the ragweed pollen which induced the formation of reagins in the non-allergic individuals appear to be different from those which elicit reagins in clinically allergic persons.

TABLE XXI

Allergenic Activity of the WSR Fractions as Observed by Scratch Test in Non-Allergic Individuals Who Had Acquired Reaginic Activity.

Person tested	Reaction elicited by fraction				
	AA_1 + Pt	P_3	P_2	P_1 + C	WSR
D.K.	-	-	1+	3+	3+
M.D.	-	2+	3+	4+	3+
A.S.-B	-	-	1+	3+	2+
A.L.	-	-	2+	3+	3+
R.H.	-	±	1+	2+	2+

CLAIMS TO ORIGINALITY

1. The technique of paper electrophoresis was used for the first time as a means for fractionating ragweed pollen extract. It was shown that the aqueous extract of ragweed pollen contains at least 5 peptide, 1 protein, 1 carbohydrate and 4 pigment components.

2. It has been shown that the cross neutralization test as currently carried out (with allergen preparations tested on an equal nitrogen basis) is unreliable as far as the determination of the number of allergens present in the active preparations is concerned. The fallacies of the test have been pointed out.

3. Cross neutralization experiments with the fractions of ragweed pollen used on an equal desensitizing basis demonstrated the presence of two allergens in ragweed pollen.

4. It has been demonstrated that there exist at least two reagins or skin sensitizing antibodies in sera of allergic individuals.

5. It was shown that sera and skin of ragweed sensitive individuals who had never been treated (desensitized) for their allergy contained reagins directed only toward constituents in the non-dialyzable fraction of the aqueous extract of ragweed pollen.

On the other hand, sera and skin of treated allergic individuals possessed reagins to the dialyzable and non-dialyzable fractions of the pollen extract. This finding suggests that the desensitization treatment induces the formation of a reagin of a new specificity not formed by untreated allergic patients.

6. It was demonstrated that reagin formation can be induced in some normal, non-allergic individuals without evoking conditions of clinical allergy.

7. An allergenically active substance has been isolated from the aqueous extract of ragweed pollen. It was non-dialyzable and was composed of a pigment and a peptide composed of eight amino acids-lysine, arginine, glycine, alanine, glutamic acid, valine, norleucine and hydroxyproline. The injection of 10^{-7} mg of this material into the skin of an allergic individual was capable of eliciting a cutaneous reaction. From results of experiments with this material it would appear that the peptide is one of the allergens in ragweed pollen.

BIBLIOGRAPHY

1. Botallus, L.,
Commentariole Duo, Lugdino, 22, 1565, cited in
Rockwell, G.E., and Rockwell, E.M.,
Chapter 15, in
Abramson, H.A. Editor.
Treatment of Asthma, Baltimore, 1951, Williams and Wilkins.

2. Bostock, J.,
Med. Chirurg. Trans., London, 14: 437, 1828, cited in
Hurwitz, S.H.,
J. Allergy, 1: 245, 1929-30.

3. Elliotson, J.,
The Lancet, 2: 370, 1830-31, cited in
Hurwitz, S.H.,
J. Allergy, 1: 245, 1929-30.

4. Gordon, W.,
London Med. Gaz., 4: 266, 1829, cited in
Hurwitz, S.H.,
J. Allergy, 1: 245, 1929-30.

5. Phoebus, P.,
Der Typische Frühsommer Katarrh oder das Sogenannte Heufieber, Heuasthma, Giessen, 1862, cited in
Hurwitz, S.H.,
J. Allergy, 1: 245, 1929-30.

6. Salter, H.H.
On Asthma; Its Pathology and Treatment, Philadelphia, 1864, Blanchard and Lea, cited in
Hurwitz, S.H.,
J. Allergy, 1: 245, 1929-30.

7. Wyman, M.,
Autumnal Catarrh. New York, 1876, Hurd and Houghton, cited in
Hurwitz, S.H.,
J. Allergy, 1: 245, 1929-30.

8. Blackley, C.H.,
Hay Fever; Its Causes, Treatment and Effective Prevention, Second Edition, London, 1880, Baillière, Tindall and Cox, cited in
Newell, J.M.,
J. Allergy, 13: 177, 1941-42.

9. Dunbar, W.P.,
Zur Ursache und Specifichen Heilung des Heufiebers, München, 1903, cited in
Hurwitz, S.H.,
J. Allergy, 1: 245, 1929-30.

10. Meltzer, S.J.,
J.A.M.A. 55: 1021, 1910.

11. Vander Veer, Jr., A., Cooke, R.A., and Spain, W.C.,
Am. J. Med. Sc., 174: 101, 1927.

12. Heyl, F.W.,
J.A.C.S., 39: 1470, 1917.

13. Heyl, F.W.,
J.A.C.S., 41: 670, 1919.

14. Heyl, F.W., and Hopkins, H.H.,
J.A.C.S., 42: 1738, 1920.

15. Heyl, F.W.,
J.A.C.S., 41: 1285, 1919.

16. Heyl, F.W.,
J.A.C.S., 44: 2283, 1922.

17. Koessler, J.H.,
J. Biol. Chem., 35: 415, 1918.

18. Spain, W.C., and Hopkins, M.,
J. Allergy, 1: 209, 1929-30.

19. Stull, A., Cooke, R.A., and Chobot, R.,
J. Allergy, 3: 120, 1931-32.

20. Brown A.,
J. Immunol., 13: 73, 1927.

21. Coca, A.F., and Grove, E.F.,
J. Immunol., 10: 445, 1925.

22. Strömme, O.,
Acta Allergologica, 8: 283, 1955.

23. Mosko, M., Hecht, R., and Weil, H.,
J. Allergy, 13: 149, 1941-42.

24. Roth, R.R., and Nelson, T.,
J. Allergy, 13: 283, 1941-42.

25. Hecht, R., Rappaport, B.Z., and Welker, W.H.,
Proc. Soc. Exper. Biol. and Med., 39: 588, 1938.

26. Sherman, W.B., and Stull, A.,
J. Allergy, 18: 287, 1947.

27. Baldwin, H.S., Moyer, A.W., and de Gara, P.F.,
J. Allergy, 18: 296, 1947.

28. Goldfarb, A.R., Libretti, A., Kaplan, M., Abramson, H.A., and Aaronson, A.,
Int. Arch. Allergy and Applied Immunol., 8: 243, 1956.

29. Wodehouse, R.P.,
Int. Arch. Allergy and Applied Immunol., 5: 337, 1954.

30. Wodehouse, R.P.,
Int. Arch. Allergy and Applied Immunol., 5: 425, 1954.

31. Wodehouse, R.P.,
Ann. Allergy, 13: 39, 1955.

32. Becker, E.L., and Munoz, J.,
Proc. Soc. Exper. Biol. and Med., 72: 287, 1949.

33. Caulfeild, A.H.W., Cohen, C., and Eadie, G.S.,
J. Immunol., 12: 153, 1926.

34. Eagle, H., Arbesman, C.E., and Winkenwerder, W.,
J. Immunol., 36: 425, 1939.

35. Kulka, A.M., and Hirsch, D.,
J. Immunol., 50: 127, 1945.

36. Kulka, A.M., and Hirsch, D.,
J. Immunol. 53: 391, 1946.

37. Cebra, J.J.,
J. Immunol., 79: 118, 1957.

38. Harrison, W.T., Armstrong, C.,
U. S. Public Health Rep., 39: 1261, 1924.

39. Walzer, M., and Grove, E.F.,
J. Immunol., 10: 483, 1925.

40. Parker, J.T.,
J. Immunol., 9: 515, 1924.

41. Black, J.H.,
J. Lab. and Clin. Med., 10: 378, 1924-25.

42. Armstrong, C., and Harrison, W.T.,
U. S. Public Health Rep., 39: 2422, 1924.

43. Wolff-Eisener, A.,
Das Heufieber, sein Wesen und seine Behandlung, München, 1906, Lehmann, cited in
Newell, J.M.,
J. Allergy, 13: 177, 1941-42.

44. Noon, L.,
The Lancet, 1: 1572, 1911.

45. Koessler, K.K.,
The specific Treatment of Hay Fever (Pollen Disease), in Forcheimer's Therapeusis of Internal Diseases, New York, 1914, D. Appleton and Co.,

46. Bernton, H.S., Jones, D.B., and Csonka, F.A.,
Southern Med. J., 20: 257, 1927.

47. Rappaport, B.Z., and Johnson, C.A.,
Proc. Soc. Exper. Biol. and Med., 26: 771, 1928-29.

48. Spain, W.C., and Newell, J.M.,
J. Allergy, 5: 455, 1933-34.

49. Moore, M.B., Cromwell, H.W., and Moore, E.E.,
J. Allergy, 2: 85, 1930-31.

50. Unger, L., Moore, M.B., Cromwell, H.W., and Seeber, C.H.,
J. Allergy, 5: 115, 1933-34.

51. Cooke, R.A., Barnard, J.H., Hebald, S., and Stull, A.,
J. Exper. Med. 62: 733, 1935.

52. Stull, A., and Sherman, W.B.
J. Allergy, 10: 130, 1938-39.

53. Stull, A., Sherman, W.B., and Hampton, S.F.,
J. Allergy, 12: 117, 1940-41.

54. Stull, A., Sherman, W.B., and Wing, W.M.,
J. Allergy, 13: 537, 1941-42.

55. Harsh, G.F., and Huber, H.L.,
J. Allergy, 14: 121, 1942-43.

56. Cohen, M.B., and Friedman, H.J.,
J. Allergy, 14: 368, 1942-43.

57. Robbins, K. C., Samuels, A., and Mosko, M.M.,
J. Allergy, 19: 35, 1948.

58. Dankner, A., Bukantz, S.C., Johnson, M.C., and Alexander, H.L.,
J. Allergy, 22: 437, 1951.

59. Frankel, S., Johnson, M.C., Schiele, A.W. Bukantz, S.C.,
and Alexander, H.L.,
J. Allergy, 26: 33, 1955.

60. Grove, E.F., and Coca, A.F.,
Proc. Soc. Exper. Biol. and Med. 21: 48, 1923-24.

61. Grove, E.F., and Coca, A.F.,
J. Immunol.,10: 471, 1925.

62. Black, J. H., and Moore, M.C.,
J.A.M.A., 86: 324, 1926.

63. Long, W.L., and Teller, I.,
J. Allergy, 9: 433, 1937-38.

64. Abramson, H.A., Moore, D.H., and Gettner, H.H.,
Proc. Soc. Exper. Biol. and Med. 46: 153, 1941.

65. Abramson, H.A., and Moore, D.H.,
J. Lab. and Clin. Med., 26: 174, 1940-41.

66. Abramson, H.A., Moore, D.H., Gettner, H., Gagarin, J., and
Jennings, L.,
Proc. Soc. Exper. Biol. and Med. 44: 311, 1940.

67. Abramson, H.A.,
Ann. Allergy, 5: 19, 1947.

68. Abramson, H.A., Moore, D.H., Gettner, H., Gagarin, J., and Jennings, L.,
J.A.C.S., 62: 1627, 1940.

69. Abramson, H.A., Moore, D.H., and Gettner, H.H.,
J. Phys. Chem., 46: 192, 1942.

70. Rockwell, G.E.,
Ann. Allergy, 1: 43, 1943.

71. Rockwell, G.E.,
Ohio State Med. J., 37: 651, 1941

72. Rockwell, G.E.,
J. Immunol., 43: 259, 1942.

73. Rockwell, G.E.,
Ohio State Med. J., 38: 433, 1942.

74. Rockwell, G.E.,
Ohio State Med. J., 39: 128, 1943.

75. Brown, E.A., and Benotti, N.,
Ohio State Med. J., 38: 1011, 1942.

76. Brown, E.A., and Benotti, N.,
J. Immunol. 47: 89, 1943.

77. Abramson, H.A.,
Chapter 11 in
Abramson, H. A., Editor,
Treatment of Asthma, Baltimore, 1951,
Williams and Wilkins Company.

78. Black, J.H.,
J. Allergy, 2: 161, 1930-31.

79. Caulfeild, A.H.W., Brown, M.H., and Waters, E.T.,
J. Allergy, 7: 1, 1935-36.

80. Caulfeild, A.H.W.,
Proc. Soc. Exper. Biol. and Med., 31: 573, 1933-34.

81. Stull, A., Cooke, R.A., and Chobot, R.,
J. Biol. Chem., 92: 569, 1931.

82. Johnson, C.A., and Rappaport, B.Z.,
J. Infect. Dis., 50: 290, 1932.

83. Stevens, F.A., Moore, D., and Baer, H.,
J. Allergy, 22: 165, 1951.

84. Perlman, E.,
Bull. N.Y. Acad. Med., 27: 586, 1951.

85. Goldfarb, A. R., Moore, D.H., Rapaport, H.G., Sklarofsky, B., Gettner, H.H., and Abramson, H.A.,
Proc. Soc. Exper. Biol. and Med., 85: 255, 1954.

86. Milford, E.L.,
J. Allergy, 1: 331, 1929-30.

87. Moore, M.B., Cromwell, H.W., and Moore, E.E.,
J. Allergy, 2: 6, 1930-31.

88. Unger, L., Cromwell, H.W., and Moore, M.B.,
J. Allergy, 3: 253, 1931-32.

89. MacQuiddy, E.L.,
J. Allergy, 13: 41, 1941-42.

90. Stone, G.C.H., Harkavy, J., and Brooks, G.,
Ann. Allergy, 5: 546, 1947.

91. Loveless, M.H., Wright, I., and Ryan, A.,
J. Allergy, 22: 120, 1951.

92. Loveless, M.H., and Timasheff, S.N.,
Arch. Biochem. and Biophys., 58: 298, 1955.

93. Gay, L. N.,
J. Immunol., 11: 371, 1926.

94. Arbesman, C.E., and Eagle, H.,
J. Allergy, 11: 18, 1939-40.

95. Stull, A., Chobot, R., and Cooke, R.A.,
J. Allergy, 1: 470, 1929-30.

96. Hampton, S.F., Bukantz, S.C., and Johnson, M.C.,
J. Allergy, 20: 19, 1949.

97. Rappaport, B.Z.,
J. Allergy, 4: 1, 1932-33.

98. Brown, A., Milford, E.L., and Coca, A.F.,
J. Allergy, 2: 301, 1930-31.

99. Batchelder, A.C., and Gonder, Jr., T.A.,
J. Allergy, 12: 425, 1940-41.

100. Paton, J.B.,
Proc. Soc. Exper. Biol. and Med., 17: 60, 1919-20.

101. Nakamura, G.R., and Becker, E.L.,
Arch. Biochem. and Biophys., 33: 78, 1951.

102. Bernstein, Jr., C., and Kirsner, J.B.,
J. Allergy, 8: 221, 1936-37.

103. Rockwell, G. E.,
J. Immunol. 41: 225, 1941.

104. Hecht, R., Mosko, M.M., Lubin, J., Sulzberger, M.B.,
and Baer, R.L.,
J. Allergy, 15: 9, 1944.

105. Thiberge, N.F.,
J. Allergy, 15: 298, 1944.

106. Walzer, M.,
J. Immunol., 14: 143, 1927.

107. Cohen, M.B., Ecker, E.E., and Rudolph, J.A.,
J. Allergy, 1: 529, 1929-30.

108. Cohen, M.B., Ecker, E.E., Breitbart, J.R., and Ruldolph, J.A.,
J. Immunol., 18: 419, 1930.

109. Newell, J.M.,
J. Allergy, 14: 444, 1942-43.

110. Stevens, F.A., Moore, D., and Gelston, T.,
J. Allergy, 22: 356, 1951.

111. Loveless, M.H., and Best, R.,
Fed. Proc. 8: 407, 1949.

112. Sanigar, E.B.,
J. Franklin Institute, 230: 781, 1940.

113. Winkenwerder, W.L., Buell, M.V., and Howard, J.E.,
Science, 90: 356, 1939.

114. Sherman, W.B.,
J. Allergy, 14: 1, 1942-43.

115. Bernstein, T.B., Mosher, A.L., and Lariella, R.P.,
Science, 113: 377, 1951.

116. Bookman, R., and Wax, H.,
J.Allergy, 25: 12, 1954.

117. Block, R.J., Durrum, E.L., and Zweig, G.,
A Manual of Paper Chromatography and Paper Electrophoresis,
New York, 1955, Academic Press.

118. Lemieux, R.U., and Bauer, H.F.,
Analytical Chem., 26: 920, 1954.

119. Pučar, Z.,
Hoppe-Seyler's Ztschr. Physiol. Chem., 296: 62, 1954.

120. Pickels, E.G., Harrington, W.F., and Schachman, H.K.,
Proc. Nat. Acad. Sc., 38: 943, 1952.

121. Lanni, F., Dillon, M.L.,and Beard, J.W.,
Proc. Soc. Exper. Biol. and Med., 74: 4,1950.

122. Koch, F.C., and McMeekin, T.L.
J.A.C.S., 46: 2066, 1924.

123. Somogyi, M.,
J. Biol. Chem., 160: 61, 1945.

124. Loveless, M.H.,
J. Immunol., 38: 25, 1940.

125. Frank, D.E., and Gelfand, H.H.,
J. Allergy, 14: 273, 1942-43.

126. Sherman, W.B.,
J. Immunol., 40: 289, 1941.

127. Cooke, R.A., Loveless, M.H., and Stull, A.,
J. Exper. Med., 66: 689, 1937.

128. Sehon, A.H., Hollinger, H.Z., Harter, J.G., Schweitzer, A.E., and Rose, B.,
J. Allergy, 28: 229, 1957.

129. Loveless, M.H.,
J. Immunol. 47; 165, 1943.

130. Gelfand, H.H., and Frank, D.E.,
J. Allergy, 15: 332, 1944.

131. Scully, M.A., and Rackemann, F.M.,
J. Allergy, 12: 549, 1940-41.

132. Alexander, H.L., Johnson, M.C., and Bukantz, S.C.,
J. Allergy, 19: 1, 1948.

133. Loveless, M.H.,
J. Immunol., 41: 15, 1941.

134. Block, R.J., and Weiss, K.W.,
Amino Acid Handbook, 1956, Charles. C. Thomas.

135. Sehon, A.H., Gyenes, L., and Rose, B.,
To be published.

136. Brown, E.A., and Benotti, N.,
Ann. Allergy, 1: 208, 1943.

137. Carr, C.W., Anderson, D., and Miller, I.,
Science, 125: 1245, 1957.

138. Bor, Gmelins Handbuch der Anorganischen Chemie, systemnummer 13: 228, Verlag Chemie, G.M.B.H., Weinheim (Bergstrasse), 1954.

139. Smith, G.S.,
The Analyst, 60: 735, 1935.

140. Edsall, G.,
Ann. Rev. Microbiol. 9: 347, 1955.