STUDIES ON RAGWEED POLLEN ALLERGENS

A THESIS

by

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CHAPTER I

INTRODUCTION

The production in vivo of antibodies to pathogenic antigens, such as influenza virus, polio virus and diphtheria, or to non-pathogenic substances, such as serum proteins, constitutes the basis of immunity (1). The antibodies produced to pathogenic antigens serve as a specific defense against these antigens, and the immunized host, if reexposed to the same pathogens resists their harmful effects (1). In contrast to immunity, some animals and humans develop hypersensitivity to either a toxic or innocuous antigen: i.e., following the initial formation of antibodies to an antigen, the host reacts in an abnormal manner if reexposed to the same substance (1). The well known condition of hay fever represents just such a hypersensitivity reaction to the pollens of trees, grasses and weeds. Of the pollen sensitivities occurring on the North American continent, that caused by ragweed is the most prevalent. The present study forms a continuation of investigations made in this laboratory on the chemical nature of constituents of ragweed pollen involved in eliciting ragweed hypersensitivity. A brief survey of work done in this and other laboratories on the

isolation and characterization of these constituents is presented in Sections C and D of this chapter. It is appropriate to preface this survey with a discussion of immune and hypersensitive reactions (Section A) and of hypersensitivity to ragweed pollen (Section B).

A. Immune and Hypersensitive Reactions

The reactions between antigens and antibodies constitute the fundamental basis of immune and hypersensitive phenomena (1). In general, antigens are substances which when administered parenterally to animals or humans, stimulate the formation of antibodies after a certain interval. The latent period, during which no antibody is detectable in the serum, varies depending on the nature of the antigen and animal species (2). Particulate antigens, for example, bacteria and erythrocytes, induce the formation of antibodies in rabbits within 2 to 5 days after an intravenous injection, while soluble antigens, such as serum proteins, require a longer latent period (2).

It has been established that, to be antigenic, a substance must be foreign to the circulation of the host and possess macromolecular dimensions (3). Antigens are

usually protein in nature, although some carbohydrates and lipids have also been found capable of inducing antibody formation (4).

Antibodies belong to the gamma globulin fraction of the serum proteins (5). The only firmly established property which differentiates antibodies from normal gamma globulins is the capacity of specific combination with the inducing antigen. Specificity of combination is the basic feature of antigen-antibody reactions (6) and signifies that antibodies produced to a specific antigen discriminate in their combination with a variety of substrates. The parameters of specificity were elucidated to a great extent by Landsteiner (7) who coupled small organic molecules (haptens), non-antigenic in themselves, to proteins and showed that immunization of rabbits with these conjugates led to the formation of antibodies specific to the haptenic moieties. The specificity of the antibodies produced were shown to depend upon the chemical nature and spatial configuration of the haptens.

Studies on hapten-antibody interaction have provided the conceptual framework for elucidating the nature of immunological reactions. A protein antigen is considered to possess different antigenic (haptenic) determinants, each

composed of 2 to 4 amino acid residues (8). The antigenic determinants combine with complimentary sites on the antibody molecule (1). Antigen-antibody reactions take place in 2 stages. The first stage involves the rapid interaction of the combining sites of antigen and antibody. This stage merges into the relatively slower second stage of visible reaction. The form of the visible reaction is mainly dependent on whether the reaction occurs in vitro or in vivo, and on the nature of the antigen and antibody.

The precipitin, hemagglutination and complement-fixation reactions are examples of the second stage of antigen-antibody reactions occurring in vitro. The mechanism of the second stage of the precipitin reaction has been elucidated on the basis of Marrack's lattice hypothesis (9). According to this hypothesis, a multivalent antigen (one with several combining sites) and divalent antibody combine to form a lattice of antigen-antibody aggregates which precipitate out of solution.

Anaphylactic shock, serum sickness and allergic reactions are visible manifestations of the second stage of antigen-antibody reactions occurring in vivo (1).

(a) Anaphylaxis (10) or anaphylactic shock has been classified as a hypersensitivity condition in humans and

animals. Anaphylactic shock is most easily induced in guinea pigs. The animal is sensitized with antigen and the shocking injection of the same substance is administered 10 to 21 days later. Usually within a minute, the guinea pig becomes restless, its hair bristles, it coughs, scratches its face and its respiration becomes slow and laboured. Death generally follows within 2 to 4 minutes. Should the animal recover from shock it is temporarily refractory to further injections of the specific antigen.

Anaphylactic shock involves antigen-antibody reactions as in the immune state, since: (i) substances which act as sensitizers have been found to function as antigens; (ii) a period of induction comparable to that observed for antibody production must elapse before the animal is challenged with the shocking injection of the same antigen; (iii) the same specificity is observed in both the anaphylactic and immune conditions; (iv) sensitivity can be passively transferred to normal recipients by sera of sensitized animals; (v) desensitization may be achieved by administering small doses of antigen at frequent intervals.

A major factor in anaphylactic shock is the combination of antigen with cell-fixed antibody; as a

result pharmacologically active reagents including histamine (11), serotonin (12) and acetylcholine (13) are released which contract the bronchial muscles, leading to death by asphyxiation.

(b) Serum sickness (10) may be cited as another hypersensitivity condition. Upon inoculation with foreign antisera (particularly that of the horse), some humans become ill and occasionally develop violent reactions 8 to 16 days following inoculation. The symptoms of the illness are rashes, fever, pain in the joints, edema and swelling of lymph nodes. Von Pirquet and Schick (14) suggested that the patient forms specific antibodies which complex with the injected serum proteins. Evidence has been obtained that the symptoms of serum sickness are due to soluble antigen-antibody complexes in the circulation (15, 16).

(c) Allergy (10) is a condition in which the host develops a hypersensitive response to foreign substances such as pollens, mold spores, house dust, fungi, foods, drugs, and simple chemicals. The offending antigens in this case are called allergens.

Blackley (17) in 1880 was the first to demonstrate that a skin reaction ("wheal and erythema") could be

produced in an allergic person upon intradermal injection of the specific allergen. In 1921, Prausnitz and Kustner (18) demonstrated that the same reaction may be obtained by passively transferring the allergic patient's serum into the skin of a normal individual and, 24 to 48 hours later, challenging with the allergen (Prausnitz-Kustner or P-K test). It was thereby shown that a factor in the serum of an allergic person reacts specifically with the allergen to give a cutaneous wheal and erythema reaction. This serum factor is termed reagin or skin sensitizing antibody. In contrast to human immune antibodies, reagins have the ability to become fixed to human skin (10). The cutaneous reaction is considered to be due to an allergen-reagin reaction which causes the release of vasodilatory substances such as histamine, serotonin and bradykinin at the injection site (10). The reaction develops in 3 successive phases (2): (i) erythema caused by local dilatation of capillaries; (ii) a spreading flare resulting from widespread arteriolar dilatation, produced by a local nerve reflex; and (iii) formation of a wheal due to increased permeability of the endothelium of small blood vessels.

B. Hypersensitivity to Ragweed Pollen

The allergy commonly referred to as hay fever is caused by the pollens of trees, grasses and weeds. In susceptible patients, the allergic condition results from absorption of the pollen allergens through exposed mucous membranes (10). The symptoms are recognized by watery exudation from the mucous membranes of the upper respiratory tract and conjunctivae and violent, often protracted sneezing and nasal discharge. As noted earlier, of the pollen allergies occurring in North America, that caused by ragweed is the most widespread.

Ragweed sensitive patients are usually treated by Noon's method of desensitization (19) which was introduced in 1911 and which consists of a series of subcutaneous injections of the aqueous extract of the pollen in graded doses at frequent intervals. It has been found that such inoculations of aqueous extracts of ragweed pollen in ragweed allergic patients produce specific antibodies which do not fix to skin but combine with ragweed allergens to block the allergen-reagin reaction (blocking antibodies) (20, 21). Contrary to expectations, a poor correlation has been found between the degree of desensitization of ragweed sensitive patients and blocking antibody titers (22). Many patients with low titers of blocking antibody have

been clinically desensitized to the same degree as those with high blocking antibody titers. Furthermore, it has been noted (23) that some allergic persons do not show improvement of symptoms but develop a greater sensitivity to the allergen following desensitization therapy. It would appear that such patients become allergic to one or several constituents of the pollen to which they were originally not sensitive (23).

The foregoing underlines the importance of studying the allergens in ragweed pollen. Since new reagins in allergic patients may be formed upon treatment with crude extracts of the pollen, a more effective desensitization might be obtained by treatment with an isolated specific allergen. Such studies might also be of academic value. Since allergens induce the formation in humans of reagins which have properties distinct from ordinary immune antibodies, it would be of interest to determine the chemical nature of the allergenic and antigenic moieties of the allergen. More insight might thereby be gained into the nature of the in vivo and in vitro reactions of ragweed allergens and their corresponding reagins.

C. Chemical Nature of Ragweed Pollen Allergens

The isolation of allergens present in ragweed pollen has posed a difficult problem because of the pollen's complexity. The pollen possesses all of the components and chemical constituents of living cells (24). The two types of ragweed, Ambrosia artemisiaefolia ("dwarf") and Ambrosia trifida ("giant"), produce bright yellow pollen grains that are spherical and 16 to 20 microns in diameter. The outer cell wall or exine constitutes about 65% of the weight of the pollen. The inner cavity of the pollen grain is separated from the outer cell wall by a thin inner cell wall or intine and contains cytoplasm, cytoplasmic granules and two nuclei. One nucleus is the tube nucleus which controls the development of the pollen tube in the process of fertilization. The other is the generative nucleus, which through a single mitotic division, forms two sperms which fertilize the ovule.

Ragweed is thus a complex material composed of proteins, nucleoproteins, peptides, carbohydrates, lipids and pigments. During the past four decades numerous attempts have been made to isolate and characterize the allergens in ragweed pollen. Some workers have claimed the allergens to be protein, while others have associated allergēnicity with carbohydrate, lipid and pigment components.

Early Studies

Evidence for the protein nature of the allergens in ragweed pollen was provided by Bernton et al (25), Rappaport and Johnson (26), and Moore et al (27) who isolated protein fractions using ammonium sulphate and alcohol precipitation methods. All of the fractions were found to be active by skin tests in allergic individuals. Similarly, Stull et al (28) isolated by ammonium sulphate precipitation allergenically active protein material from an extract of giant ragweed pollen; the protein was free of carbohydrate as evidenced by a negative Molisch test. Cohen and Friedman (29) also isolated active protein fractions free of pigment. However, these workers did not report on the carbohydrate content of their preparation.

Other workers have presented data in favour of the carbohydrate nature of ragweed pollen allergens. Black (30) succeeded in isolating an active carbohydrate substance by precipitating the pollen extract with alcohol at pH 4.4. Reagents such as picric acid, tannic acid and phosphotungstic acid which normally precipitate proteins did not precipitate the active material. In addition, the Millon and xanthroproteic tests for tyrosine were found to be negative, while the Molisch test for carbohydrate was positive. Hydrolysis

of this carbohydrate fraction yielded 55% reducing sugar and 6% nitrogen. Similarly, Caulfield et al (31) have isolated allergenically active carbohydrate material which, on hydrolysis, yielded 58% reducing sugar and 1.5% nitrogen. In view of the appreciable nitrogen content of the carbohydrate preparations of Black and Caulfield et al, the possibility cannot be excluded that activity was due to contamination of the carbohydrates with protein.

Abramson et al (32 - 38) separated pigmented from non-pigmented material of the pollen by free electrophoresis. The pigment with the greatest electrophoretic mobility was allergenically active. In their studies mentioned above, Cohen and Friedman (29) isolated allergenically active pigment components. Since the pigment constituents contained nitrogen, a possibility arises that they were contaminated with small amounts of allergenically active protein or peptide material.

Lipid components of ragweed pollen have also been shown to possess allergenic activity. Milford (39) prepared an ether soluble fraction of the pollen which was allergenically active; qualitative tests showed the absence of nitrogen. Cutaneous reactions were obtained with ether soluble material in over 60% of the allergic individuals tested. Johnson and Rappaport (40) isolated an ether

soluble fraction which contained 0.2 to 0.3% nitrogen and which gave skin tests in 19 of 21 allergic patients.

Recent Studies

Robbins et al (41) have fractionated extracts of dwarf ragweed using diethyl aminoethyl (DEAE)-cellulose chromatography and have found at least 7 allergens, 4 major and 3 minor. The major allergens seemed to be protein as determined by optical density measurements at 280 mμ. Practically all patients tested reacted to 1 major allergen, whereas only about 50% reacted to the other 3 major allergens.

Goldfarb et al (42) fractionated dwarf ragweed by DEAE-cellulose column chromatography and obtained a highly active fraction("Pool Cc"). The fraction was homogeneous by electrophoretic and gel diffusion criteria.

Lea and Sehon (43) prepared dialyzed residue of ragweed pollen by dialysis of water soluble ragweed and obtained a fraction ("delta fraction") by paper electrophoresis of the residue. Further purification of this fraction by chromatography on Sephadex G-75 yielded a major component which had a sedimentation coefficient of 3.5 S and contained small amounts of arabinose and

galactose. Lea and Sehon have observed that various allergenically active fractions isolated by zone electrophoresis and by chromatography on dextran gels underwent decomposition and suggested that the fractions were derived from labile high molecular weight components of the intact pollen grain.

King et al (44) appear to have isolated the purest allergenic preparation from ragweed pollen extract by ammonium sulphate precipitation and chromatography on DEAE-cellulose and Sephadex G-75. The allergen had a molecular weight of approximately 32,000 and contained 16.0% nitrogen, 2.4% arabinose, 0.3% hexosamine and 0.6% hexuronic acid. One mole of leucine per 32,000 grams of the allergen was obtained by carboxypeptidase degredation of the fraction indicating that the allergen consisted of a single protein. King et al found that the carbohydrate content decreased while the allergenic activity increased at each stage of purification and concluded that the allergenic activity was due to protein.

D. Dialyzability of the Allergens of Ragweed Pollen

The dialyzability of the allergens of ragweed pollen has also been studied over the past four decades. Grove and Coca (45) were the first to undertake such studies. These workers dialyzed pollen extracts in cellophane tubing and reported a loss of two-thirds of the nitrogenous material across the membrane. The dialyzed residue still retained approximately the same amount of allergenic activity as the undialyzed extract in ophthalmic and cutaneous tests. In a later study, Grove and Coca (46) digested pollen extracts with trypsin for 24 hours and subjected the digest to dialysis. The nitrogen content within the sac decreased to almost the same level as that of the enzyme control to which no pollen had been added, indicating extensive degredation. The dialyzed residue that remained in the tubing retained the same allergenic activity as non-digested, non-dialyzed pollen extract. Grove and Coca concluded that the allergen is a non-dialyzable and perhaps non-nitrogenous substance. The results of Grove and Coca were confirmed by Black (47).

Rockwell (48) purified a ragweed pollen extract by hydrochloric acid precipitation and found the allergenic activity of the precipitate to be non-dialyzable.

None of the above mentioned investigators have

reported on the activity of the dialysate. Johnson and Rappaport (40) were the first to note that ragweed dialysates gave skin reactions in allergic patients. These workers fractionated aqueous pollen extracts by ammonium sulphate and dialyzed the precipitated fractions against water. The dialysates were treated with ammonium sulphate to give precipitates which proved highly allergenic.

The findings of Johnson and Rappaport were confirmed by Unger et al (49) who found that materials dialyzable through collodion, cellophane and parchment membranes were allergenically active.

Spain and Newell (50) using cellophane membranes of various sizes demonstrated that the dialyzability of allergenically active material through these membranes depended on the type of membrane used. Dense membranes of small pore sizes retained all of the allergenic activity, while only the non-protein nitrogen (not precipitable with phosphotungstic acid) was able to diffuse through. Less dense membranes of greater pore sizes allowed the passage of a relatively large quantity of active proteins (phosphotungstic acid precipitable) through the cellophane membranes.

Stull et al (51) prepared dialysates of pollen extract using Visking tubing. Their preparations displayed

no allergenic activity by either a neutralization technique or direct intracutaneous tests on allergic individuals.

Loveless et al (52) found, in contrast to Stull et al, that material dialyzing through Visking tubing was allergenically active in approximately 80% of allergic persons tested whereas the non-dialyzable components gave cutaneous reactions in all ragweed allergic individuals. It is noteworthy that these workers did not specify whether treated or untreated allergic subjects were used.

Richter et al (53) have demonstrated the importance of making a distinction between treated and untreated patients in studying the dialyzability of ragweed pollen allergens. They showed that all treated and untreated allergic persons reacted when challenged with water soluble ragweed and dialyzed residue either by direct or P-K skin tests. On the other hand, only 17% of all untreated and 86% of all treated sera reacted to dialysate. Richter et al attributed these findings to the formation of reagin(s) of new specificity in the sera of treated allergic subjects and not present in untreated ones.

Richter et al (54) have studied the physicochemical properties of the dialyzable fraction of WSR and have attempted to purify the allergens therein. They employed phosphotungstic acid to precipitate the allergenically

active material of the dialysate, which they called phosphotungstic acid precipitate (PTP). Paper electrophoresis of PTP revealed a single ninhydrin positive band which was found to be allergenically active by P-K test. Prolonged heating at 100°C, strong acid and treatment with various enzymes did not appreciably affect the allergenic activity of PTP.

The experimental work presented in this thesis represents a study on the chemical nature and homogeneity of the allergens of phosphotungstic acid precipitate (PTP) obtained from dialysate of water soluble ragweed (WSR). An auxiliary study was also made to determine some of the immunological properties of PTP.

CHAPTER II

MATERIALS AND METHODS

Water Soluble Ragweed Preparations

Water soluble ragweed and its derivatives were prepared according to the methods of Richter et al (54). Dwarf ragweed pollen was defatted with diethyl ether in a Soxhlet apparatus for 36 hours. The defatted pollen was dried in air for 24 hours to remove the ether and was then extracted with distilled water (100 ml of water per 5 gm of ragweed pollen) for 72 hours. The resulting suspension was filtered through a Buchner funnel and the filtrate centrifuged at 22,500 rpm for 20 minutes in a Spinco model L preparatory ultracentrifuge. The clear supernatant (water soluble ragweed, WSR) was placed in a Visking sac and dialyzed against 40 volumes of distilled water for 72 hours.

The dialysate and dialyzable residue were lyophilized and stored at 4°C.

Preparation of Phosphotungstic Acid Precipitate (PTP) (54)

Phosphotungstic acid (0.62%) was added to a solution of dialysate of 20 mg/ml concentration. The volume ratio of acid to dialysate was 1.6:1 and the final concentration

of phosphotungstic acid was 0.38%. The resulting suspension was centrifuged at 22,500 rpm for 20 minutes in a Spinco preparatory ultracentrifuge (Model L). The supernatant was discarded and the precipitate washed 3 times with a solution of 0.38% phosphotungstic acid. The final precipitate was resuspended in distilled water and lyophilized.

Paper Electrophoresis

The materials to be fractionated were spotted on Whatman No 1 paper strips (each 4 cm x 55 cm) with a Lang-Levy pipette. The strips were wetted with appropriate buffer, (see below) and the latter allowed to diffuse uniformly into the spotted areas. The strips were semi-dried with blotting paper and placed on a plexiglass plate. A foam rubber frame was placed over the plate which in turn was overlayed by a glass plate. To absorb any moisture due to evaporation and condensation during electrophoresis, a sheet of Whatman No 3 MM filter paper was placed between the foam rubber frame and the glass plate. The end of the strips were then immersed into buffer vessels which had been filled to equal levels. The buffer solutions were connected to 5% KCl solutions by paper wicks. A potential difference ranging from 150 to 350 volts DC was applied to

Ag-AgCl electrodes in the KCl solutions.

The following buffers were employed for paper electrophoresis:

(a) ammonium hydroxide-acetic acid buffer, pH 8.6, ionic strength 0.1;

(b) formic-acetic acid buffer, pH 2.1, ionic strength 0.05.

Isolation of Electrophoretic Fractions

The strips containing the fractions to be eluted were dried and cuts were made corresponding to stained areas of guide strips (see below). The cut segments were eluted overnight at 4°C with 0.2 M acetic acid or with the buffer used in electrophoresis. The eluates were dried down either by rotory evaporation or lyophilization, and the residues dissolved in appropriate volumes of distilled water or 0.2 M acetic acid. Aliquots of these solutions were serially diluted with borate-saline[1] and Seitz filtered for the determination of allergenic activity by P-K tests.

1 Borate-saline was prepared by mixing borate buffer, pH 8.6, ionic strength 0.1 with 1.8% NaCl in a 1:1 ratio (v/v).

Descending Paper Chromatography (55)

An aliquot of the material (usually in a volume of 100 µl) was applied as a spot 3 inches from one end of a filter paper strip (Whatman No 1). The end of the paper, nearest the spot, was folded, looped over a glass rod and inserted into a glass trough housed in a cylindrical chromatography chamber. After equilibration for 1 to 2 hours with the solvent employed (see below), the latter was added to the trough and chromatography allowed to proceed for 18 hours. The strip was then removed and dried in a chromatography oven. The dried strips were dipped in or sprayed with an appropriate staining reagent.

The solvent system:

pyridine:butanol:acetic acid:water (7:10:2:8 v/v)

was used for the chromatography of amino acids and peptides. For chromatographic analysis of carbohydrates, the solvent system:

n-butanol:acetic acid:water (4:1:5 v/v)

was employed.

Detection of Amino Acids and Peptides

(a) Strips developed by chromatography or electrophoresis were dried for 15 minutes at 100°C and dipped in 0.1% solution of ninhydrin in acetone made 5% in pyridine (55).

(b) The paper strips were dried at 60°C for 15 to 20 minutes and then sprayed with 20% sodium hypochlorite. After drying at room temperature, the strips were dipped in absolute ethanol and dried. The strips were then sprayed with a solution of starch-iodide (1% starch and 1% KI, 1:1 v/v) (55).

Quantitative Amino Acid Analysis

The material to be analyzed (1 mg) was placed in a glass ampule, 2 ml of 6N HCl were added and the ampule was sealed in vacuo. Hydrolysis was allowed to proceed for 18 hours at 110°C. The hydrolysate was dried down in a rotory evaporator to expel the HCl and the residue was analyzed in a Spinco Amino Acid Analyzer by the method of Spackmen et al (56).

Detection of Carbohydrates

(a) The Lemieux-Bauer reagent (57) was employed to detect carbohydrates on paper. The reagent was composed of 2% sodium metaperiodate, 2% potassium permanganate and 4% sodium carbonate. The chromatography strips were sprayed with the reagent and dried at room temperature.

(b) Ortho-aminodiphenyl was another spray reagent used for the detection of carbohydrates. It was prepared by

adding 1.3 ml of 85% phosphoric acid to a 3% solution of ortho-aminodiphenyl in acetic acid (58). After spraying, the spots were developed by heating the strip at 90°C.

(c) The orcinol reaction was employed for the detection of pentoses. The stock reagent consisted of 13.5 grams of ferric sulphate and 20 grams of orcinol dissolved in 500 ml of distilled water. The solution was kept at 4°C. To 25 ml of the stock reagent were added 415 ml of concentrated HCl and the volume was brought to 500 ml with distilled water to form the test reagent (59). To 3 ml of the test solution were added 9 ml of the test reagent and the final solution was heated for 20 minutes in a boiling water bath. The development of a green colour indicated the presence of pentose.

Carbohydrate Analysis

For the analysis of carbohydrates, 2 mg of the material were dissolved in 2 ml of 2N HCl and the solution transferred to a glass ampule. The ampule was sealed in vacuo and hydrolysis was allowed to proceed for 8 hours at 100°C. The hydrolysate was dried down by rotory evaporation,

the residue dissolved in 0.2 ml of 0.2 M acetic acid and analyzed by descending paper chromatography. The component sugars were identified by their Rf values (55), obtained by dividing the distance of migration of sugars by that of the solvent front. Identification was confirmed by parallel chromatography of known sugar standards.

N-Terminal Analysis

N-terminal amino acid analysis was made by the 2,4-dinitrofluorobenzene (DNFB) method of Sanger and Thompson (60). To a solution of 3 mg of PTP in 0.2 ml of 2% N-methylmorpholine were added 20 µl of DNFB dissolved in 0.2 ml of ethanol. After shaking overnight, 0.5 ml of distilled water and 4 to 5 drops of N-methylmorpholine were added to the suspension: the excess DNFB was extracted with diethyl ether and the residue rotory evaporated to dryness. To insure the complete removal of dinitrophenol, the dried residue was rotory evaporated over a "cold finger" for 1 hour at 60°C. The residue was then dissolved in 2 ml of 6N HCl and hydrolyzed for 16 hours at 110°C in a sealed, evacuated glass ampule. To minimize the destruction of glycine and proline, an alternative method of hydrolysis was employed in which the residue was dissolved in 2 ml

of 12N HCl and hydrolyzed as above for 4 hours at 110°C. Following hydrolysis, the sample was rotory evaporated and the residue redissolved in 1 ml of 1N HCl. The N-terminal dinitrophenyl (DNP)-amino acids were extracted from the hydrolysate with 5 ml volumes of diethyl ether (4 extractions) and the (combined) ether extracts rotory evaporated to dryness. The residue was dissolved in acetone and applied as a spot to Whatman No 1 filter paper for ascending paper chromatography (61), using the solvent system, toluene:pyridine:ethylene monochlorhydrin:0.8N ammonia (5:1:3:3 v/v).

The Rf values obtained for the DNP-amino acid spots were compared with those of the literature (61).

P-K Test

For the P-K test, only normal subjects were used, i.e., subjects who did not give a wheal and erythema reaction to an intracutaneous injection of 0.05 ml of WSR in borate-saline (2.5 mg/ml).

An appropriate number of sites were prepared on the backs of normal subjects by intracutaneous injections of 0.05 ml of serum from treated[1] allergic subjects.

1 "Treated" refers to ragweed allergic individuals who had received therapeutic injections of ragweed.

The serum was diluted 1:10 with borate-saline and Seitz filtered prior to skin testing. The sensitized sites were challenged 18 to 24 hours later with intracutaneous injections of 0.05 ml volumes of test allergenic and control solutions. After 20 minutes, the cutaneous reactions were observed and graded as 0 to 3+ depending on the size of the wheal and erythema; reactions showing pseudopod formation were graded as 4+. The titer of an allergenic solution was taken as the reciprocal of the highest dilution of the solution giving a 1+ cutaneous reaction.

Controls consisted of: (a) saline injected into a sensitized serum site; (b) a sensitized site left unchallenged with an allergenic solution; (c) a saline site challenged with test allergen (to rule out the possibility of non-specific reaction due to allergen alone); (d) sensitized sites challenged with buffers used in the preparation of the various allergenic fractions; the buffers were diluted with an appropriate volume of borate-saline.

Preparation of Rabbit Antiserum to WSR

Rabbits weighing 3 to 5 kg were immunized with an emulsion of WSR in complete Freund's adjuvant (final

concentration of WSR: 50 mg/ml). Two weekly subcutaneous injections of 2 ml each were given for 3 weeks. The animals were bled from the marginal ear veins, 1 week following the last injection. After clotting at room temperature, the blood was allowed to stand at 4°C for 24 hours. The serum was then separated from the clot by centrifugation and stored at -10°C until used.

Ring Test (62)

Undiluted rabbit anti-WSR (0.2 ml) was added to a series of tubes (each 0.3 cm i.d.) and overlayered with aqueous solutions of various ragweed pollen fractions (5 mg/ml). The tubes were allowed to stand 1 hour for the development of a precipitate at the interface. For controls, normal rabbit serum was used in place of antiserum.

Ouchterlony Plates (63)

A suspension of 1% agar in saline containing 1:10,000 merthiolate was heated to 80°C until the agar dissolved. Six ml of the liquified agar were added to a Petri dish (5 cm diameter) to form an even layer 2 to 3 mm thick. The Petri dish was covered, the agar allowed to solidify for 2 hours at room temperature. Circular wells were cut out, separated by a distance of 2.5 cm.

Antisera and antigen solutions were added to the appropriate wells, the plates were incubated in a humidifying chamber and examined periodically for the development of precipitin lines.

Hemagglutination-Inhibition (64)

Rabbit erythrocytes, collected in Alsever's solution, were washed 3 times in physiological saline (0.9% NaCl) and a 2.5% suspension of the washed cells was prepared in saline. To 6 ml of this suspension were added an equal volume of 1:20,000 tannic acid, prepared in physiological buffer solution (PBS: consisting of equal volumes of saline and 0.17 M phosphate buffer, pH 7.2). The suspension was incubated at 37°C for 10 minutes and centrifuged to collect the cells. The tanned cells were washed 3 times in PBS and resuspended in 6 ml of the same buffer.

The tanned cells were sensitized by the addition of a 1:6 solution of WSR (0.75 mg/ml) prepared in PBS. The sensitized cells were washed twice in diluent (1:100 normal rabbit serum prepared in PBS) and the cells were resuspended in 6 ml of diluent. Doubling dilutions of rabbit anti-WSR (1:10) were made in 1 ml volumes of diluent in a series of test tubes, and 0.1 ml

of sensitized tanned cells were added to each tube. The tubes were shaken and allowed to stand at room temperature. Patterns of agglutination were read 6 hours later: the titer of the antiserum was taken as the reciprocal of the highest dilution to give a positive hemagglutination. Controls consisted of: (a) sensitized cells in diluent; (b) tanned non-sensitized cells in antiserum; (c) tanned non-sensitized cells in diluent; (d) erythrocytes neither tanned nor sensitized in antiserum.

For inhibition studies, twofold serial dilutions of the test inhibitor in 0.5 ml volumes were prepared in diluent. To each tube was added 0.5 ml of rabbit anti-WSR diluted to one-tenth that of the highest dilution giving positive hemagglutination. After incubation of the test inhibitor with the diluted antiserum for 1 hour at room temperature, 0.1 ml of sensitized rabbit cells was added to each tube. Inhibition of agglutination was taken as evidence for the presence of inhibiting antigen. The minimal amount of antigen required for complete inhibition was calculated from the number of tubes showing no hemagglutination.

CHAPTER III

EXPERIMENTS AND RESULTS

Experiment 1. Paper Electrophoresis of PTP at Alkaline pH

The phosphotungstic acid precipitate of the dialysate of WSR, prepared as described in Chapter II, was subjected to paper electrophoresis, using ammonium hydroxide-acetic acid buffer, pH 8.6, ionic strength 0.1. One mg of PTP was applied per strip and, in initial experiments, electrophoresis was allowed to proceed for 3 hours, at 150 volts. After drying, the strips were stained with starch-iodide for the detection of peptide constituents. No fast moving components were observed (Fig 1a) and the pigment and peptide components were poorly resolved. The duration of electrophoresis was therefore prolonged to 18 hours (Fig 1b), after which adequate separation of the single pigment and peptide components was achieved. No carbohydrate components were detected upon staining with the Lemieux-Bauer reagent.

Experiment 2. Paper Electrophoresis of PTP at Acidic pH

Electrophoresis of PTP was carried out in formic-acetic acid buffer, pH 2.1, ionic strength 0.05 for 10 hours

at 350 volts. One pigment component was observed identical to that obtained at alkaline pH. In contrast to the single peptide spot obtained with alkali electrophoresis, 5 ninhydrin positive components which stained equally with starch-iodide were observed displaced toward the cathodic pole(Fig 2).

Experiment 3. Paper Chromatography of PTP

Prior to analysis by descending paper chromatography, PTP was treated as follows. PTP was suspended in distilled water, the pH adjusted to 3.2[1] with dilute acetic acid in a pH stat (Radiometer), and the resulting suspension centrifuged at 22,500 rpm for 20 minutes. The supernatant was discarded; the sediment was suspended in distilled water and solubilized by raising the pH to 6.1 with dilute sodium hydroxide. The same procedure was repeated 2 additional times. An appropriate aliquot of solubilized sediment prepared after each washing was analyzed by descending paper chromatography for 18 hours using the solvent system: pyridine:butanol:acetic acid:water (7:10:2:8 v/v). For

1 The addition of phosphotungstic acid to the dialysate of WSR results in a precipitation of allergenically active material at pH 3.2.

comparison, the dialysate of WSR was similarly chromatographed. The chromatograms of all the sediment preparations were similar and showed multiple ninhydrin positive components. The number of components obtained with the sediments (Fig 3a) were fewer than that obtained with dialysate (Fig 3b).

Experiment 4. N-Terminal Analysis of PTP

From the results obtained by electrophoretic and chromatographic analyses, it was uncertain whether PTP is composed of multiple peptides or a single peptide component which decomposes in an acidic medium into smaller peptidic fragments or amino acids. In an attempt to resolve this problem, an N-terminal amino acid analysis of PTP was made using the DNFB method of Sanger and Thompson (60). In one experiment, the 2,4-dinitrophenyl (DNP)-derivatives of PTP were hydrolyzed in 6N HCl for 16 hours at 110°C, while in another, hydrolysis of the DNP-derivatives was carried out in 12N HCl for 4 hours at 110°C. In both cases, a single DNP-amino acid was obtained having an Rf value of 0.08 corresponding to that of DNP-aspartic acid (61).

Experiment 5. Carbohydrate Analysis of PTP

As noted in Experiment 1, no carbohydrate components were revealed on staining the paper electropherograms of PTP with the Lemieux-Bauer reagent. Additional methods for the detection of carbohydrates were employed in order to insure that PTP was free of carbohydrate constituents.

(a) The orcinol reaction was found to be positive for PTP, indicating the presence of pentoses.

(b) PTP (2 mg) was hydrolyzed in 2N HCl, and the hydrolysate analyzed for simple sugars by descending paper chromatography in the solvent system: n-butanol: acetic acid:water (4:1:5 v/v). The chromatograms were stained with ortho-aminodiphenyl reagent and revealed 3 sugars having Rf values corresponding to those of D-arabinose, D-galactose and D-xylose (Table 1).

Experiment 6. Quantitative Amino Acid Analysis of PTP

For this purpose, 1 mg of PTP was hydrolyzed in 6N HCl at 110°C for 18 hours and the hydrolysate analyzed in an automatic Spinco Amino Acid Analyzer by the method of Spackman et al (56). The results of the analysis are

shown in Table 2. No attempt was made to assay the tryptophan content of PTP. Table 2 shows also the minimum number of amino acid residues in PTP calculated on the assumption that 0.004 μM obtained for tyrosine represents 1 amino acid residue. On this basis, PTP is composed of a minimum of 91-95 amino acid residues.

Experiment 7. Isolation of the Active Peptide Fraction of PTP

An aqueous solution of PTP (10 mg/ml) was spotted on Whatman No 1 paper (1 mg per strip) and subjected to electrophoresis at pH 8.6 as described in Experiment 1. Guide strips were stained with starch-iodide and cuts corresponding to pigment, peptide as well as non-stainable regions were made on unstained strips. The components were eluted and tested for allergenic activity by the P-K test. Only the peptide constituent was active and gave a titer of 4,000 compared to 2,000 for PTP (Table 3).

Experiment 8. Re-electrophoresis of the Active Peptide Fraction at Alkaline pH

The homogeneity of the isolated peptide fraction of PTP observed on electrophoresis at alkaline pH was confirmed

by re-electrophoresis of the active peptide fraction in the same buffer employed for isolation of this fraction (Experiment 7). A single starch-iodide positive component was observed in the cathodic region.

Experiment 9. Paper Electrophoresis of the Active Peptide Fraction of PTP at Acidic pH

As noted in Experiment 2, paper electrophoresis of PTP in formic-acetic acid buffer at pH 2.1, for 10 hours at 350 volts revealed multiple ninhydrin and starch-iodide positive components. Similar results were obtained on electrophoresis in the formic-acetic acid buffer of the active peptide fraction derived from PTP (Fig 4).

Experiment 10. Paper Chromatography of the Active Peptide Fraction of PTP

The allergenically active peptide fraction isolated by alkali electrophoresis was examined for homogeneity by descending paper chromatography for 18 hours using the solvent system: pyridine:butanol:acetic acid:water (7:10:2:8 v/v). Two starch-iodide positive components were observed on the chromatogram (Fig 5).

Experiment 11. Precipitin Reactions

PTP, dialysate, dialyzed residue and WSR were tested for antigenicity by reaction with rabbit anti-WSR in ring tests. All the ragweed preparations except PTP gave positive reactions. In addition, no precipitin reaction was observed for PTP by the Ouchterlony technique when tested against rabbit anti-WSR (Fig 6), while WSR gave a precipitin band with the antiserum both before and after absorption with PTP.

Experiment 12. Hemagglutination-Inhibition Studies

Since PTP showed no antigenicity by the ring test or Ouchterlony technique, the more sensitive tanned cell hemagglutination-inhibition method was employed. The concentration of WSR required for optimal sensitization of the tanned rabbit erythrocytes was found to be 0.75 mg/ml. The rabbit anti-WSR antiserum used was found to have a titer of 10,240.

PTP, dialysate, dialyzed residue and WSR served as test inhibitors in a concentration of 5 mg/ml in each case. From Table 4, it may be seen that PTP inhibited the hemagglutination of the WSR-rabbit anti-WSR system to the same

extent as dialysate, while dialyzed residue was most active in this respect.

From the number of tubes showing inhibition (Table 4), it was calculated that a minimal amount of 0.625 mg of PTP, 0.625 mg of dialysate, 0.0049 mg of dialyzed residue and 0.0098 mg of WSR were required to completely inhibit hemagglutination of the WSR-rabbit anti-WSR system under the conditions employed. Thus, on a weight basis, the dialyzed residue showed approximately 100 times the inhibitory activity of dialysate or PTP.

CHAPTER IV

DISCUSSION

The present studies were designed to investigate the chemical nature and antigenic properties of an allergenically active fraction present in the dialysate of water soluble ragweed pollen. The active fraction was isolated from the dialysate by phosphotungstic acid precipitation and is referred to as phosphotungstic acid precipitate (PTP).

Paper electrophoresis of PTP at pH 8.6 revealed one pigment and one peptide staining component. All of the allergenic activity was found to be localized in the peptide moiety, in agreement with the findings of Richter et al (54). Therefore, the pigment may be considered as an allergenically inactive impurity which co-precipitates with the allergen. By P-K test, the peptide component gave a titer of 4,000 compared to 2,000 for PTP, indicating that the conditions of isolation did not appreciably affect the allergenic activity.

As noted above, a single peptide staining component was observed by paper electrophoresis of PTP at pH 8.6. Similarly, re-electrophoresis of the isolated peptide at the same pH showed a single electrophoretic component. Further evidence for homogeneity of the peptide constituent of PTP was obtained by N-terminal amino acid analysis: only

dinitrophenyl-aspartic acid was isolated from the acidic and neutral fraction of the acid hydrolysate of dinitrophenylated PTP. It should be noted, however, that the single dinitrophenyl-amino acid obtained indicates the minimum number of peptide chains present: it is possible that PTP contained one or more peptides having N-terminal amino acids unreactive with 2,4-dinitrofluorobenzene.

The appearance of multiple ninhydrin and starch-iodide positive components on paper chromatography and on paper electrophoresis at pH 2.1 of PTP and its peptide moiety, indicates that the latter substances are heterogeneous or extremely labile under the conditions employed. Since Richter et al (54) obtained a single peptide component on paper electrophoresis of PTP at pH 3.7, it is possible that at a pH lower than 3.7, such as that employed in the present studies, cleavage of susceptible amide bonds occurs, resulting in fragmentation of the initially homogeneous peptide.

The present studies have shown that PTP contains carbohydrate consisting of D-arabinose, D-galactose and D-xylose units. In contrast, Richter et al (54) reported the absence of carbohydrate in PTP. It is possible that the method of hydrolysis used by Richter et al (54) caused extensive destruction of the carbohydrate. In addition,

the Lemieux-Bauer reagent employed by these workers may be insufficiently sensitive to detect sugars at the level of concentration present in PTP. The presence of carbohydrate in PTP, raises the possibility that the allergen is glycopeptide in nature.

As pointed out in the section on "Experiments and Results", quantitative amino acid analysis was made on 1 mg of PTP. It was anticipated that acid hydrolysis of this amount of PTP would result in recoveries of the constituent amino acids in 0.1 micromolar amounts, assuming a molecular weight for PTP of 10,000. The molecular weight estimation was based on the sedimentation coefficient of 0.7 S found for PTP by Richter et al (54). The low levels of amino acids obtained, of the order of 0.01 micromoles, was therefore surprising. It is likely that inordinately large amounts of pigment are present in PTP, but the possibility cannot be excluded that extensive destruction of the amino acids occurred during hydrolysis. In view of these considerations, the amino acid composition obtained for PTP in the present study must be considered as preliminary only.

Assuming the presence of a single tyrosine residue, it was shown that PTP contains 91-94 amino acid residues

per molecule. On this basis, PTP has a molecular weight of 9-10,000. The relatively high content (16 residues) of lysine and arginine is of interest, in view of the finding of Richter et al (54) that the allergenic activity of PTP was unaffected by treatment with trypsin, an enzyme specific to these two basic amino acids.

Immunochemical studies were carried out to determine the antigenicity of PTP and to establish the antigenic relationship of PTP with various ragweed fractions. No visible reaction occurred between PTP and rabbit anti-WSR antiserum either by ring test or Ouchterlony technique. The antigenicity of PTP was assayed by the hemagglutination technique in view of the relatively high sensitivity of this method. PTP inhibited the hemagglutination of the WSR-rabbit anti-WSR system to the same extent as dialysate. In contrast, no inhibition was observed when PTP was used as a test inhibitor in the heterologous system: guinea pig liver-rabbit anti-guinea pig liver, indicating that PTP specifically inhibited the WSR-rabbit anti-WSR system. The dialyzed residue inhibited the hemagglutination of the WSR-rabbit anti-WSR system to a much greater extent than dialysate, demonstrating that the dialyzed residue is the principal antigen of WSR.

Since dialyzed residue constitutes 5% of the weight of WSR, it may be further concluded that appreciable amounts of non-antigenic components are removed by dialysis of WSR. Similar results with regard to the antigenic relationship between dialysate, dialyzed residue and WSR were reported by Perelmutter (65), using the micro-Ouchterlony and bis-diazotized-benzidene hemagglutination-inhibiton techniques.

SUMMARY

1. The phosphotungstic acid precipitate (PTP) prepared from the dialysate of water soluble ragweed was examined by paper electrophoresis in ammonium hydroxide-acetic acid buffer, pH 8.6, ionic strength 0.1, and was found to contain one pigment and one peptide constituent.

2. Paper electrophoresis of PTP using formic-acetic acid buffer, pH 2.1, ionic strength 0.05, revealed five ninhydrin and starch-iodide positive components.

3. Preparations of PTP obtained by three successive precipitations at pH 3.2 showed similar multiple ninhydrin positive components on descending paper chromatography in: pyridine:butanol:acetic acid:water (7:10:2:8 v/v).

4. N-terminal amino acid analysis of PTP by the dinitrofluorobenzene method showed the presence of only aspartic acid as N-terminal.

5. PTP was shown to contain one or several carbohydrates consisting of the simple sugars: D-arabinose, D-galactose and D-xylose.

6. Amino acid analysis of PTP showed the presence of sixteen different amino acids and a total of ninety one to ninety five amino acid residues.

7. The allergenic activity of PTP was found to be associated with a peptide fraction isolated by paper electrophoresis in ammonium hydroxide-acetic acid buffer, pH 8.6, ionic strength 0.1.

8. Re-electrophoresis of the isolated peptide fraction in ammonium hydroxide-acetic acid buffer, pH 8.6, ionic strength 0.1 showed a single peptide component in the cathodic region.

9. Electrophoresis of the isolated peptide fraction in formic-acetic acid buffer, pH 2.1, ionic strength 0.05 revealed five ninhydrin and starch-iodide constituents.

10. Analysis of the active peptide fraction of PTP by descending paper chromatography in pyridine:butanol: acetic acid:water (7:10:2:8 v/v) revealed two starch-iodide positive components.

11. PTP was tested for antigenicity by the ring, Ouchterlony and hemagglutination-inhibition techniques. While no precipitin reaction occurred between PTP and rabbit anti-WSR, PTP inhibited the hemagglutination of the WSR-rabbit anti-WSR system, indicating the presence of a single antigenic determinant in PTP.

TABLE 1

Rf Values Obtained for Carbohydrate Components of PTP

	Rf	
Carbohydrate	PTP	Literature[1]
D-galactose	0.16	0.16
D-arabinose	0.21	0.21
D-xylose	0.28	0.28

1 Data taken from Block, R.J., Durrum, E.L., Zweig, G.,(55).

TABLE 2

Quantitative Amino Acid Analysis of PTP[1]

Amino Acids	Micromoles	No of Residues[2]
lysine	0.048	12
histidine	0.008	2
arginine	0.017	4
cysteic acid	0.028	7
aspartic acid	0.034	8 - 9
threonine	0.019	5
serine	0.030	7 - 8
glutamic acid	0.040	10
proline	0.020	5
glycine	0.039	10
alanine	0.030	7 - 8
valine	0.018	4 - 5
isoleucine	0.011	3
leucine	0.016	4
tyrosine	0.004	1
phenylalanine	0.008	2
Number of Residues		91 - 95

1 PTP was hydrolyzed in 6N HCl for 18 hours at 110°C and the hydrolysate analyzed in an automatic Spinco Amino Acid Analyzer by the method of Spackman et al (56).

2 Based on 0.004 μM ≡ 1 residue; the number of residues have been taken to the nearest integer.

TABLE 3

Allergenic Activity of PTP and Peptide Fraction of PTP

	P-K Activity							
Dilution[1]	1:5	1:50	1:100	1:500	1:1000	1:2000	1:4000	1:10000
Sample								
PTP	3+	3+	3+	2+	2+	1+	0	0
Active Peptide Fraction	3+	3+	3+	2+	2+	1+	1+	0

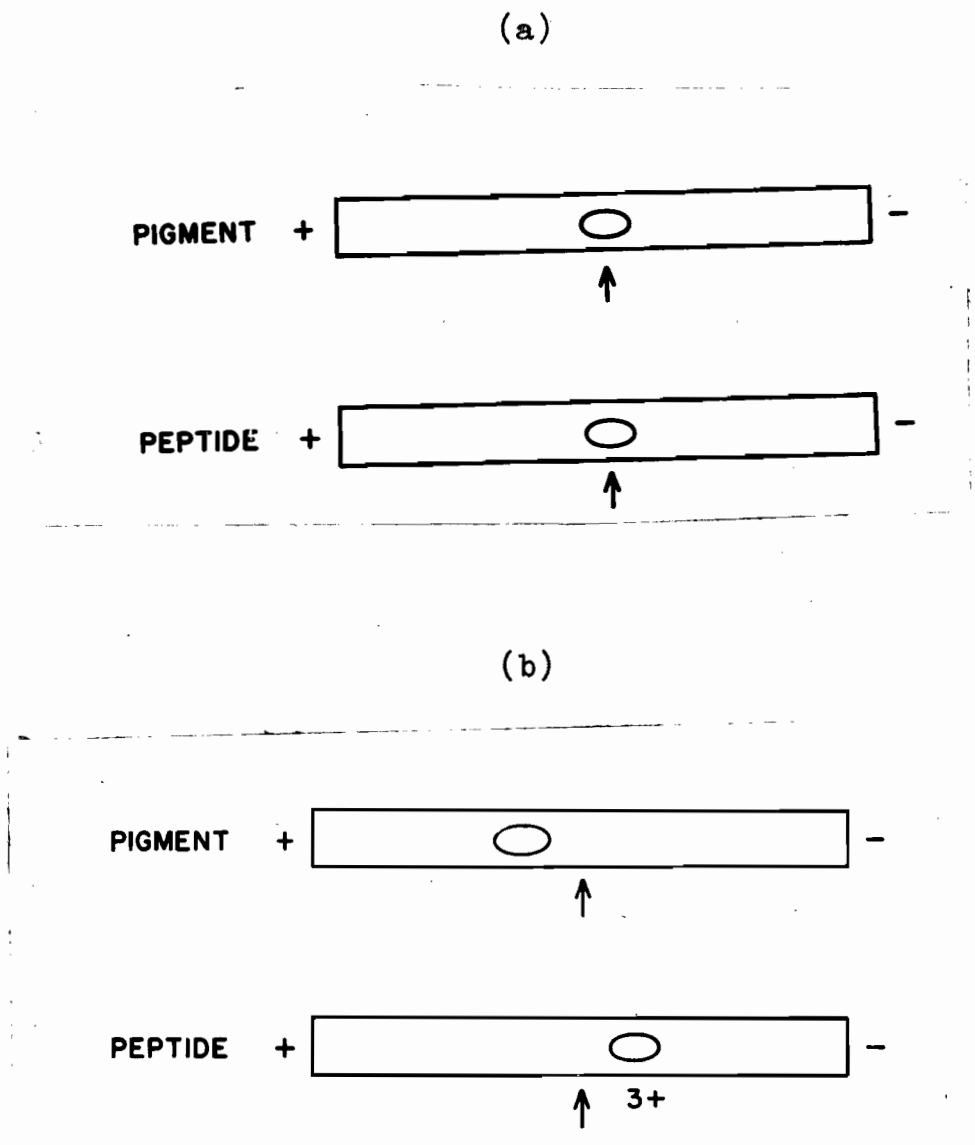
1 The lyophilized materials were dissolved in borate-saline to an initial concentration of 1 mg/ml and appropriate dilutions were made with borate-saline.

TABLE 4

Antigenicity of PTP, Dialysate, Dialyzed Residue and WSR by the Hemagglutination-Inhibition Technique

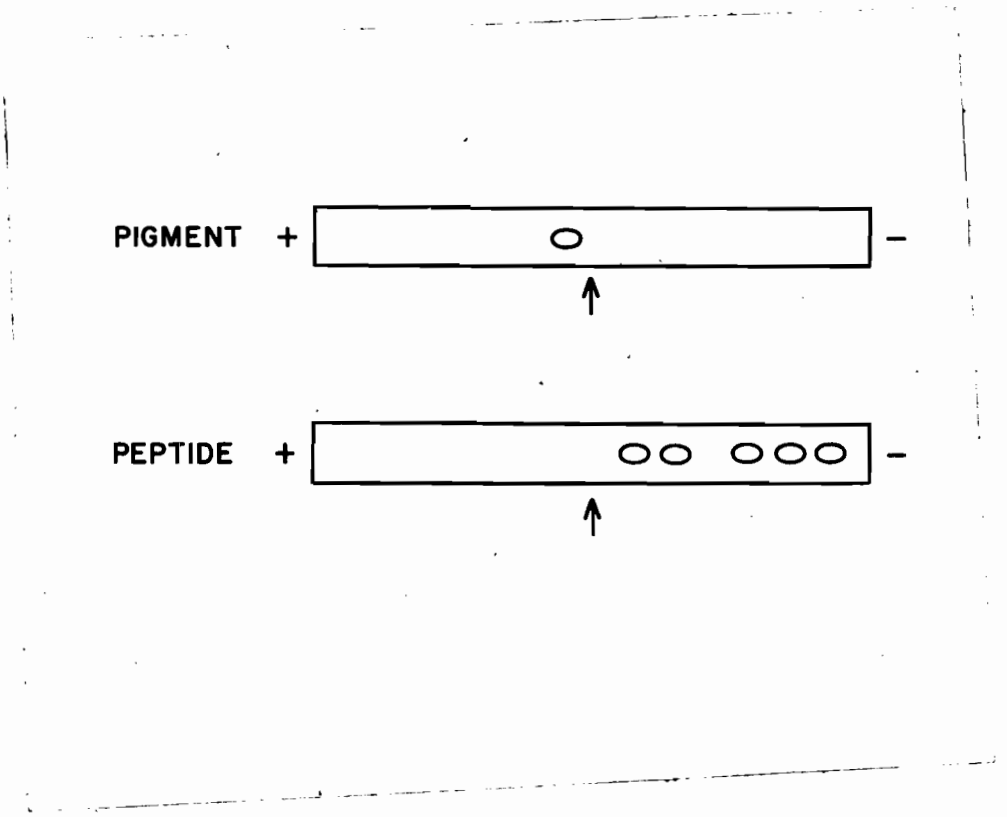
Test Inhibitor	No of Tubes Showing Complete Inhibition
PTP	3
Dialysate	3
Dialyzed Residue	10
WSR	9

FIGURE 1



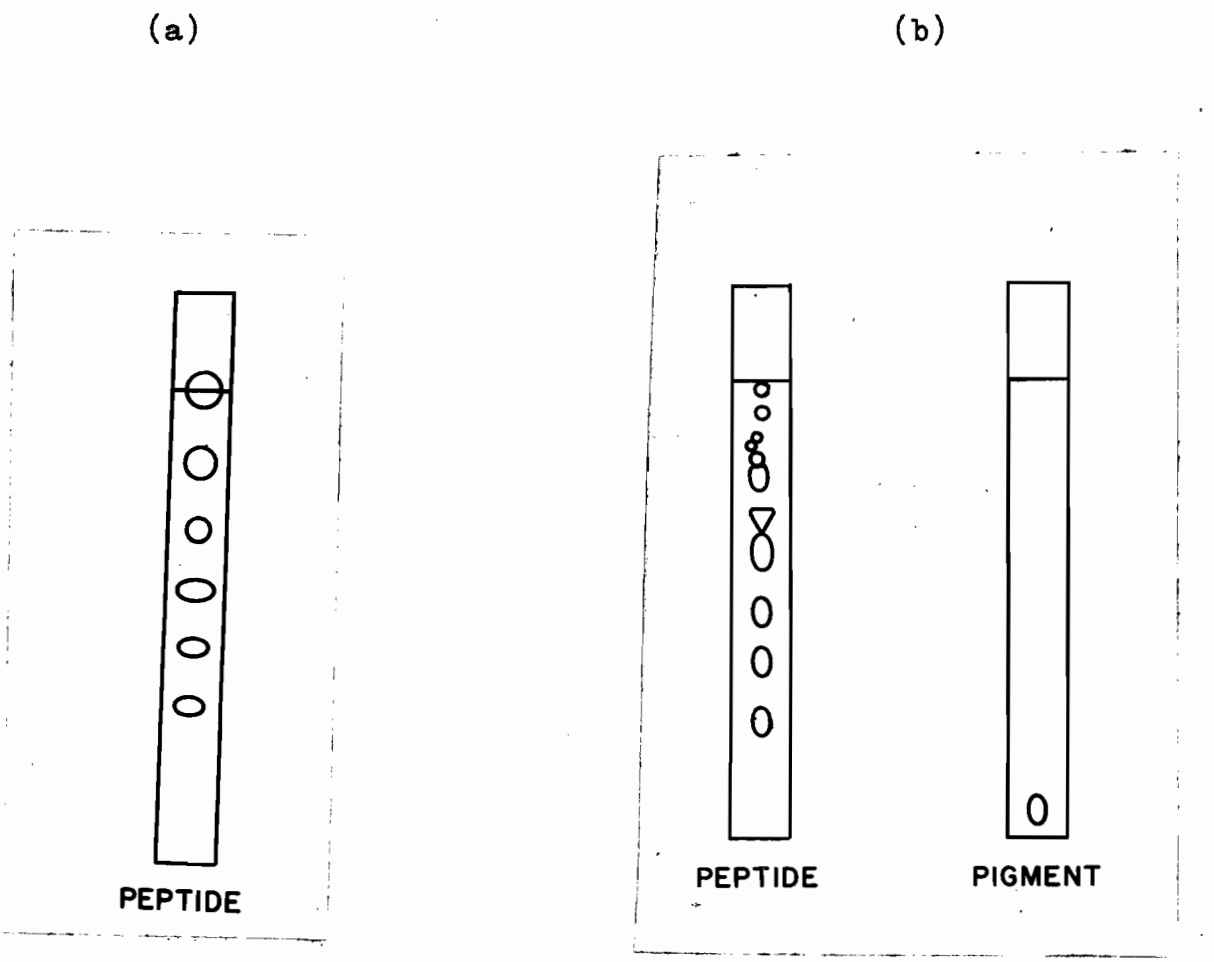
Paper electrophoresis of PTP in ammonium hydroxide-acetic acid buffer, pH 8.6, ionic strength 0.1. Time of electrophoresis: (a) 3 hours (b) 18 hours. Voltage: 150 V.

FIGURE 2



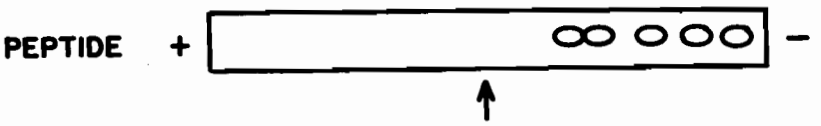
Paper electrophoresis of PTP in formic-acetic acid buffer, pH 2.1, ionic strength 0.05 for 10 hours. Voltage: 350 V.

FIGURE 3



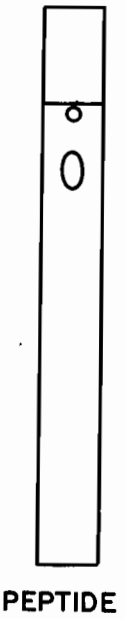
(a) Descending paper chromatography of reprecipitated PTP in pyridine:butanol:acetic acid:water (7:10:2:8 v/v). Duration of chromatography: 18 hours.
(b) Descending paper chromatography of dialysate of WSR in pyridine:butanol:acetic acid:water (7:10:2:8 v/v). Duration of chromatography: 18 hours.

FIGURE 4



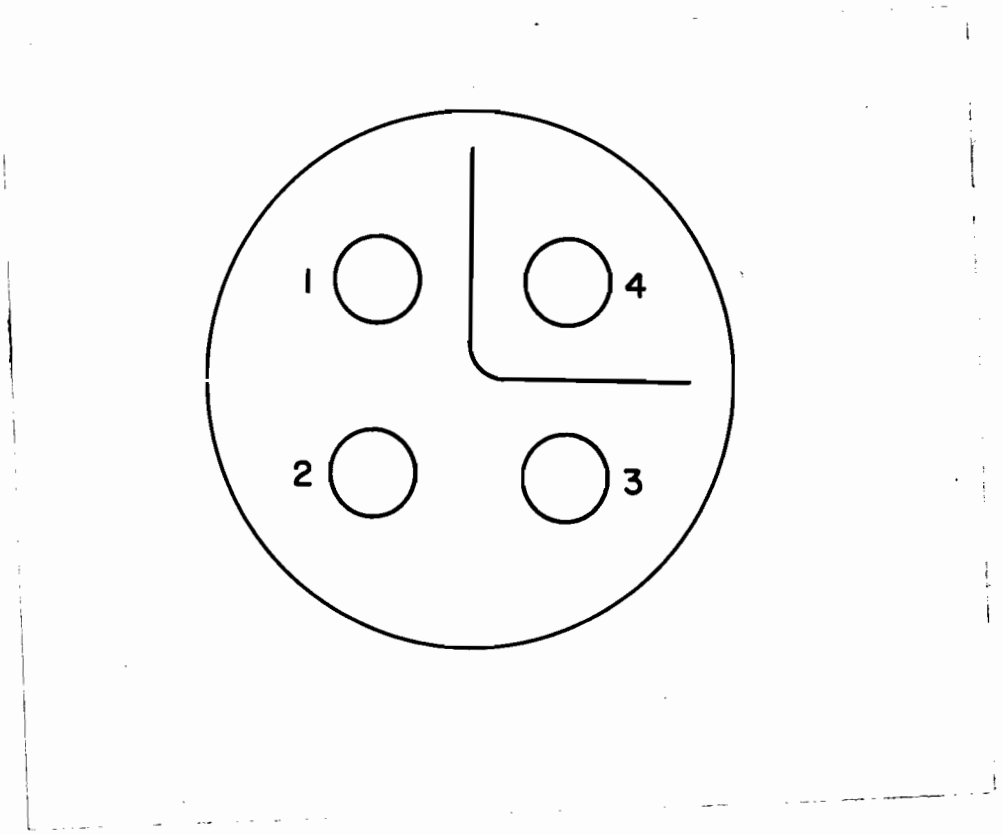
Paper electrophoresis of the active peptide fraction of PTP in formic-acetic acid buffer, pH 2.1, ionic strength 0.05 for 10 hours. Voltage: 350 V.

FIGURE 5



Descending paper chromatography of the active peptide fraction of PTP in pyridine:butanol:acetic acid:water (7:10:2:8 v/v). Duration of chromatography: 18 hours.

FIGURE 6



Agar gel diffusion analysis of PTP and WSR. Well 1: rabbit anti-WSR absorbed with PTP; for the absorption, 2 mg of PTP were added to 0.5 ml of the rabbit anti-serum and the precipitate which formed was removed by centrifugation. Well 2: solution of PTP in saline (1 mg/ml); well 3: rabbit anti-WSR; well 4: solution of WSR in saline (1 mg/ml).

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