1985

Isolation and Characterization of the Crossactive Antigenic and Allergenic components in CALLISTEMON CITRINIS and MELALEUCA QUINQUENERVIA Pollen by Immunochemical Methods

Brett E. Stanaland
University of Central Florida

ISOLATION AND CHARACTERIZATION OF THE CROSSREACTIVE ANTIGENIC AND ALLERGENIC COMPONENTS IN *CALLISTEMON CITRINIS* AND *MELALEUCA QUINQUENERVIA* POLLEN BY IMMUNOCHEMICAL METHODS

BY

BRETT ERIC STANALAND
B.S., University of Central Florida, 1983

THESIS

Submitted in partial fulfillment of the requirements
for the Master of Science degree in Microbiology
in the Graduate Studies Program of the College of Arts and Sciences
University of Central Florida
Orlando, Florida

Summer Term
1985

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to all those who assisted in this research. To Dr. Robert Nash Gennaro who suffered the most, by giving his blood whenever asked and his assistance during research and thesis preparation. To Dr. Rudy Wodzinski for his assistance and encouragement during research and thesis preparation. To Dr. M. G. Bausher and the USDA for the use of their equipment and assistance in research materials. To Dr. S. D. Klotz for his professional assistance and guidance and the Allergy Clinic for the use of their equipment and assistance in research materials. To Steven Shivers for all his valuable assistance. And very special thanks to my co-chairmen and major professors, Dr. Michael J. Sweeney and Dr. Roseann S. White, for all their invaluable help and assistance in research, research presentations, and the preparation of this thesis.

Finally, I would like to thank both my parents, Mr. and Mrs. Billy Dow Stanaland, with special thanks to my mother to whom I am dedicating this thesis, for without her unselfish support and inspiration, none of this work could have been possible.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LITERATURE REVIEW

I. Clinical Allergy

Immediate hypersensitivity is one of the major types of allergic reactions diagnosed and treated by the clinical allergist.

The Mechanism of Immediate Hypersensitivity

Classical immediate hypersensitivity reactions are mediated by antibody effector mechanisms. The mechanism consists of reactions mediated by immunoglobulin E (IgE), a class of immunoglobulins with unique biological properties. An allergen is the moiety in an allergenic preparation which induces the production of IgE antibodies. An antigen is any moiety capable of stimulating the production of immunoglobulins, irrespective of which class. In 1966 IgE was demonstrated to be a new class of immunoglobulins and, subsequently, its role in immediate hypersensitivity disorders has been intensely investigated [20].

Immediate hypersensitivity produces clinical allergic symptoms via the following sequence of reactions: 1) exposure to allergen; 2) development of an IgE antibody response to the allergen; 3) binding of the IgE antibodies to mast cells or basophils via a site in the Fc region; 4) reexposure to the allergen; 5) interaction of allergen with the allergen-specific IgE antibodies bound to the surface membrane of mast cells or basophil; 6) release of potent

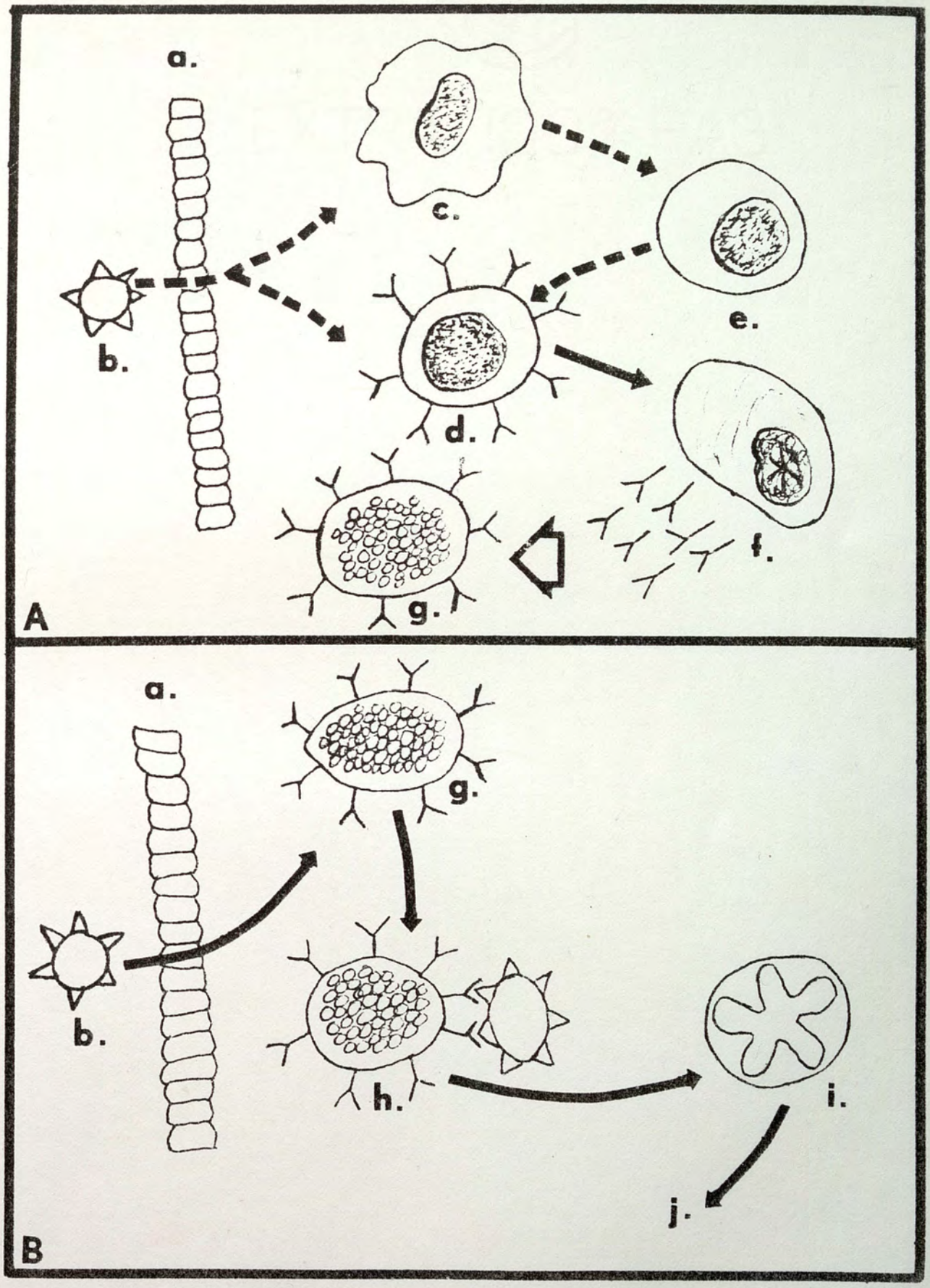
chemical mediators from sensitized mast cells or basophils; and 7) action of these mediators on various organs (Fig. 1) [20].

Methods of Allergy Detection

Many methods are used clinically for in vivo and in vitro detection of specific allergenic reactivity. Examples of in vivo diagnostic techniques are skin testing and provocative testing, whereas, examples of in vitro tests are the radioallergosorbent test (RAST), fluoresence allergosorbent test (FAST), etc. The paper radioimmunosorbent test (PRIST) can be used to determine total IgE as an aid to assessment of overall patient reactivity [20].

Skin testing, the direct introduction of an allergen into the skin of a patient, provides a simple and efficient technique for determining the presence of IgE antibodies to specific allergens [29]. IgE is the antibody responsible for the wheal and flare reaction of a positive skin test. The clinical significance of the skin test depends upon a correlation of the reaction itself (wheal and flare) with the history, physical examination findings, and other laboratory tests obtained using patient sera [20]. Three different methods of skin testing are used clinically: 1) prick test; 2) scratch test; and 3) intradermal skin testing. Each method varies in the degree of sensitivity with the scratch test being the least sensitive and intradermal testing, the most sensitive [20].

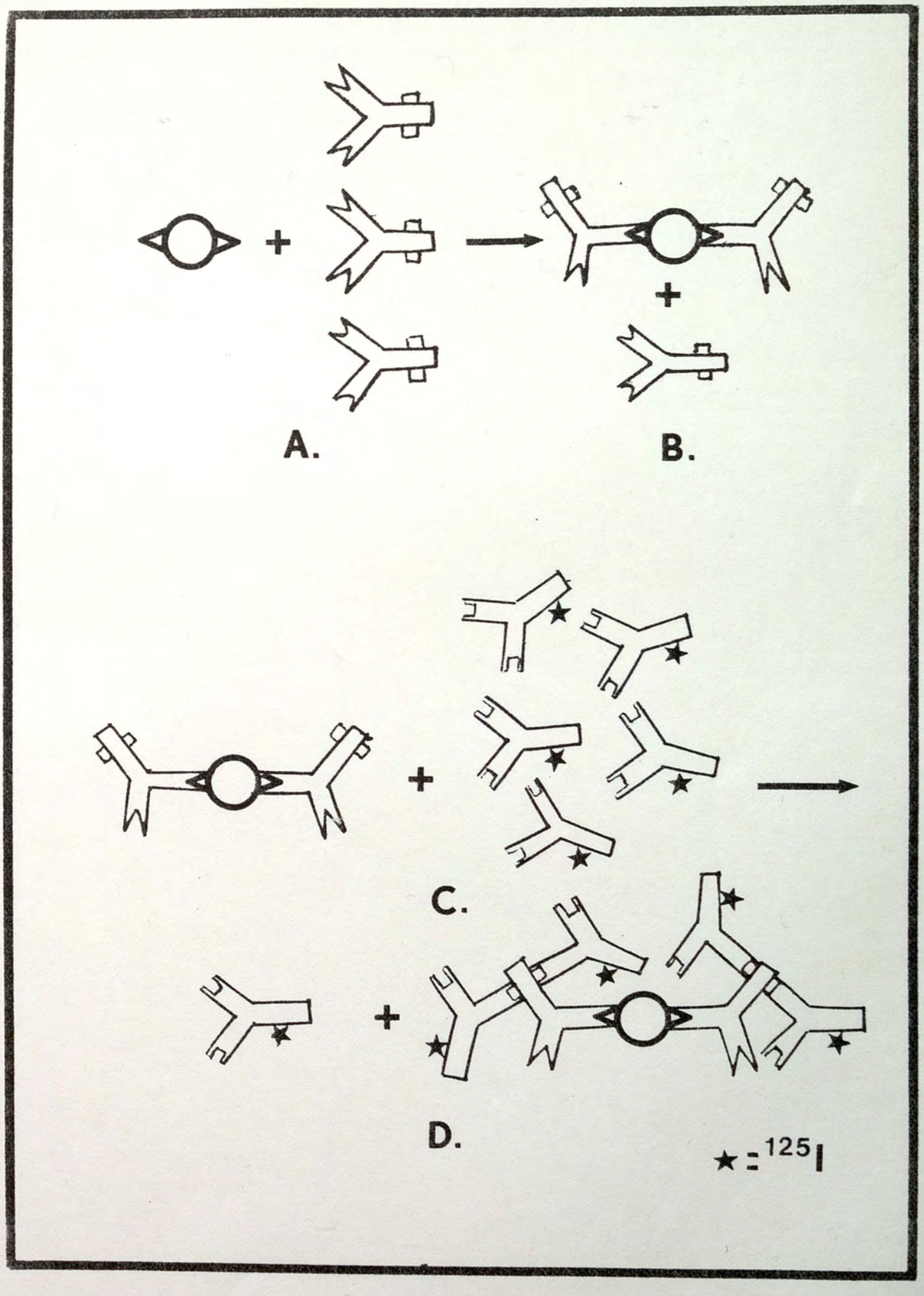
Fig. 1: The sequence of reactions which result in immediate hypersensitivity. A. Allergen sensitization. Allergen (b) is absorbed across skin, gastric, or respiratory mucosa (a). A macrophage (c) takes up the allergen, processes it, and presents it to the T-lymphocyte (e). A B-lymphocyte (d) exposed to allergen is influenced by the T-lymphocyte to mature into an allergen-specific IgE immunoglobulin-secreting plasma cell (f). Allergen-specific IgE antibodies are absorbed onto the surface of a mast cell (g) (sensitization). B. Allergen stimulation of mediator release. Allergen is absorbed and reacts with the sensitized mast cell (g) causing the cross-linking of IgE antibody. The mast cell degranulates (i) and mediators are released (j).


a.
b.
c.
d.
e.
f.
g.
A
a.
b.
g.
h.
i.
j.
B

Provocative testing, the direct administration of the allergen to the respiratory mucosa in the case of respiratory allergy (nasal, conjunctiva, and bronchial), and subsequent observation of target-organ response, is sometimes used as an adjunct to skin testing in selected patients. A major advantage of this technique over skin testing is that it allows more precise identification of clinically important allergens, especially in patients with a large number of positive skin tests [20]. Major disadvantages of this technique include the limitation of one allergen per test session, the imprecise quantification of response, the difficulty in standardization of each allergen, and the possible production of severe symptoms such as a marked bronchospasm following bronchial challenge in patients with severe asthma [20]. Provocative testing may be used to confirm cases of food allergy, where double-blind-controlled food challenges are performed to supplement unsatisfactory food skin tests.

The RAST test is a radioimmunoassay which measures allergen-specific IgE [10]. The assay consists of three major steps: 1) the allergen is coupled to a cyanogen bromide activated solid phase support; 2) the serum is added, and IgE, if present, binds to the allergen on the solid phase support; 3) radiolabeled ^{125}I-antihuman-IgE globulin is then added, and the amount of allergen-specific IgE is determined from the amount of radioactivity remaining after washing the unbound radioactive antihuman-IgE away from the solid support (Fig. 2) [10].

Fig. 2. Radioallergosorbent test (RAST). The allergen of interest, covalently coupled to a paper disc, is reacted with patient sera (A). The specific IgE to the allergen of interest in the patient's sera binds to the disc (B). After nonspecific IgE is washed away, radiolabeled anti-IgE antibodies are added (C) which form a complex (D), which after washing away excess radiolabeled antibody can be measured in a gamma counter in counts per minute (cpm). High concentrations of specific IgE antibody in patient's sera would result in high cpm measured.


A.
B.
C.
D.
★ = 125I

A patient's IgE level is often determined using the PRIST assay [20]. This test is used mainly by clinicians to monitor the patient's potential for immediate hypersensitivity, and it may also reflect the effectiveness of desensitization [20]. The PRIST test is a radioimmunoassay which measures IgE binding to anti-human IgE antibodies which are covalently bound to a paper disc.

Basic Principles of Therapy for Allergic Disease

The treatment of patients with allergic disease should be individualized, based upon the general principles of avoidance of allergens and irritants, judicious use of pharmacological therapy, and, if indicated, the administration of immunotherapy (hyposensitization) [20]. This individual approach can be adjusted according to the intensity, discomfort, and severity of the allergic disease, as well as the inconvenience, cost, sources, possible adverse effects, and prognosis for success of treatment.

Conventional hyposensitization treatment of allergic patients suffering from hay fever to a given pollen consists of a series of injections of the whole aqueous extract of the pollen [13]. A problem reported with this form of treatment is the injection of a complex antigen preparation which may result in the sensitization of patients to some component(s) of the extract to which they may not have been sensitive prior to treatment [13]. The most desirable form of hyposensitization treatment would be to treat the patient with a purified and well-characterized fraction(s) of the extract that

is (are) devoid of components to which the patients are not sensitive [1].

Another proposed form of therapy reported is the administration of hapten like fragments to inhibit multivalent allergen cross-linking of cell-fixed IgE [12,13]. The hapten fragment would contain only a single allergenic determinant of the complete allergenic molecule. These univalent compounds would then be expected to bind to the Fab regions of the IgE antibodies and thus inhibit the combination of IgE antibodies with the offending multivalent allergen [12,13].

II. Methods for the Characterization and Standardization of Allergenic Extracts

Allergenic extracts have been in use for over 70 years for the diagnosis and therapy of various allergic disorders [28,33]. These extracts are obtained from a large number of substances believed to contain antigens thought to be allergenic. Although allergenic extracts have long been in use, many questions remain unanswered about the safety, quality, and effectiveness of all but a few extracts [1]. The need for characterization and standardization of allergenic extracts used for clinical diagnosis and treatment is obvious and undisputable [1,2,12,28,33]. Only limited attempts at achieving standardization has as yet occurred. Problems with standardization include differences in materials and diagnostic procedures, terminology, and principles for the evaluation and recording of results.

A standard extract is defined as one which contains a measured amount of specific allergen, the allergenic potency of which can be reproduced with lot-to-lot consistency [1,28]. One problem in standardization is the availability of a reference extract for comparison with each new lot of extract prepared.

The use of standardized extracts may improve the accuracy and reliability of diagnosis and treatment of allergic disease [1]. Such extracts would aid in the identification of skin reactive individuals versus those with allergic disease [28]. Immunotherapy (hyposensitization) may improve due to accurate quantification of constituents responsible for allergenic reactivity. Stability studies would be more meaningful with standardized extracts and would enable better expiration dating. The purification and characterization of the allergenic components in extracts would contribute not only to the safety of allergenic extracts but also provide a reference to compare and standardize extract preparations [28,29].

Separation of Antigenic Components of Pollen Extracts

Allergenic extracts are purified using protein chemistry techniques: isoelectric focusing, gel filtration, ion exchange chromatography, two-dimensional electrophoresis, dialysis, filtration, etc.; or immunochemical techniques: Ouchterlony analysis (double diffusion), crossed immunoelectrophoresis, tandem-crossed immunoelectrophoresis, affinity chromatography, etc. [30,34].

The desired components of allergenic extracts are all of the major and minor allergens of the original material present in a

chemically defined system with all non-allergic material and irritants being absent [1]. The use of many different separation techniques may be required to achieve the stated level of characterization.

Detection of the Allergenic Components in Pollen Extracts

Allergenic components in pollen extracts can be detected by utilizing allergic patient sera and techniques to detect the binding of IgE to the specific allergens. Very sensitive techniques are required to identify allergenic components due to the low concentration of IgE in human serum (Table 1) [20]. Crossed radioimmunoelectrophoresis (CRIE), autoradiography of isoelectric focusing gels, etc., are techniques which allow the identification of the allergenic components in a complex mixture of antigens [2,3,12,14,18,25,27,33,35]. These techniques utilize an anti-human IgE antibody which is labeled with radioactive ^{125}I (^{125}I-anti-IgE). Autoradiographic methods are time-consuming. The resulting patterns are often blurred and difficult to read. There is a need for a faster technique with high resolving power to detect and quantitate allergens in allergenic extracts.

Detection of Cross-reactivity Between Pollen Extracts of Different Species

Antigenic and allergenic cross-reactivity has been reported with many different types of allergens [12,15,17,31]. Antigenic cross-reactivity can be determined by various immunochemical techniques: Ouchterlony analysis (double diffusion), rocket

TABLE 1

IMMUNOGLOBULIN COMPOSITION OF HUMAN SERUM

IMMUNOGLOBULIN (Ig)	SERUM CONC. (mg/dl)	% TOTAL Ig
IgG	1000	85%
IgA	200	10-15%
IgM	120	5-10%
IgD	3	0.2%
IgE	0.05	0.002%

Reference: Lawlor, G. J.; Fischer, T. J. Manual of allergy and immunology. Waltham, MA: Little, Brown and Company; 1981.

immunoelectrophoresis, tandem-crossed immunoelectrophoresis, etc. These techniques are based upon a precipitation reaction which occurs in an agar gel if antisera produced against one antigen comes in contact with a cross-reactive antigen.

The detection of allergenic cross-reactivity can also be determined by the RAST inhibition or polystyrene tube radioimmunoassay (PTRIA) [31]. These assays both use competitive inhibition to evaluate if the specific allergen-antibody reaction is cross-reactive. This inhibition by the cross-reactive allergen reduces the binding of the original allergen to which the antibody was made.

SECTION I
ISOLATION AND CHARACTERIZATION OF THE CROSS-REACTIVE ANTIGENIC AND ALLERGENIC COMPONENTS IN *CALLISTEMON CITRINIS* AND *MELALEUCA QUINQUENERVIA* POLLEN BY IMMUNOCHEMICAL METHODS I

I. Abstract

Antigenic extracts obtained from Bottlebrush (*Callistemon citrinis*) and Melaleuca (*Melaleuca quinquenervia*) pollen were analyzed by crossed immunoelectrophoresis (CIE), tandem-crossed immunoelectrophoresis (TCIE), and trans-blot enzyme-linked crossed immunoelectrophoresis (TECIE). CIE analysis detected 14 and 12 antigenic components in Bottlebrush and Melaleuca, respectively. TCIE analysis of Bottlebrush pollen, employing rabbit anti-Melaleuca serum in the intermediate gel, demonstrated that all of the antigenic components detected were cross-reactive. Similar analysis of Melaleuca using rabbit anti-Bottlebrush serum in the intermediate gel also showed that all of the antigenic components were cross-reactive. At least three Bottlebrush and two Melaleuca cross-reactive pollen components were demonstrated to be allergenic by TECIE analysis.

II. Introduction

Increased interest in the study of allergen extracts and the determination of which components of these extracts are allergenic has occurred during the past 10 years [28]. This interest has been stimulated by the development of new immunochemical techniques to facilitate the separation of antigens and the determination

of which antigenic components are actually allergenic [6]. Unfortunately, the majority of current research in allergen characterization has been restricted to the major allergens which are widely distributed [5,6,8,22]. Allergens found only in specific areas and antigens not commonly thought to be responsible for allergic reactions have not been well studied.

Allergenic cross-reactivity among pollens of related species of trees and grasses is documented [6]. Individuals allergic to one grass or tree pollen may also suffer from allergic reactions following exposure to other grasses or trees not indigenous to the locale where the sensitizations occurred [12].

Callistemon citrinis and Melaleuca quinquenervia, more commonly known as Bottlebrush and Melaleuca, respectively, belong to the family Myrtaceae and are found throughout Florida but are concentrated in the southern regions of the state since they are sensitive to cold. There are literally forests of Melaleuca trees in the Florida Everglades. The Melaleuca trees were originally transported to Florida from Australia [26].

It has previously been reported that an aromatic chemical substance (not pollen) of Melaleuca is responsible for "causing widespread misery" [26]. It is not known whether the pollen or other substances contain the allergenic reactivity. The need for characterization of the allergenic components in Melaleuca is obvious.

For many years, allergists have speculated that Bottlebrush and Melaleuca pollens might have very similar or cross-reactive allergenic

components. This speculation is based upon the clinical observation that most patients who were skin test reactive to Melaleuca pollen were also found to be reactive to Bottlebrush pollen. A very high degree of cross-reactivity between the allergenic components of these two pollens would provide an explanation for the clinical observation.

Allergen extracts are used clinically for allergen-specific diagnostic tests performed either in vitro or in vivo [3,22]. Examples of in vitro testing include RAST and FAST analysis, etc. Examples of in vivo diagnostic tests include both skin and provocative testing. An allergen extract in which all the allergenic components are known and accurately quantified is needed for clinical use to ensure quantification of allergen testing and treatment. A highly specific extract would also eliminate the injection of extraneous materials during desensitization [19].

Crossed radioimmunoelectrophoresis (CRIE) has been used for the determination of the allergenic bands detected in CIE analysis [2,3,12,14,18,27,33,35]. CRIE requires the use of autoradiography which involves a lengthy development time after incubation with sera obtained from allergic patients and ^{125}I labeled anti-human IgE antibodies. Distinct precipitates which produce radiostaining in CRIE may represent distinct allergens [24,37]. The use of trans-blot electrophoresis applied to CIE has also been used for this purpose [16]. A modification of this technique, called trans-blot enzyme-linked crossed immunoelectrophoresis (TECIE), identifies

allergenic bands in CIE patterns in minutes after incubation with a horseradish peroxidase enzyme-linked anti-human IgE antibody.

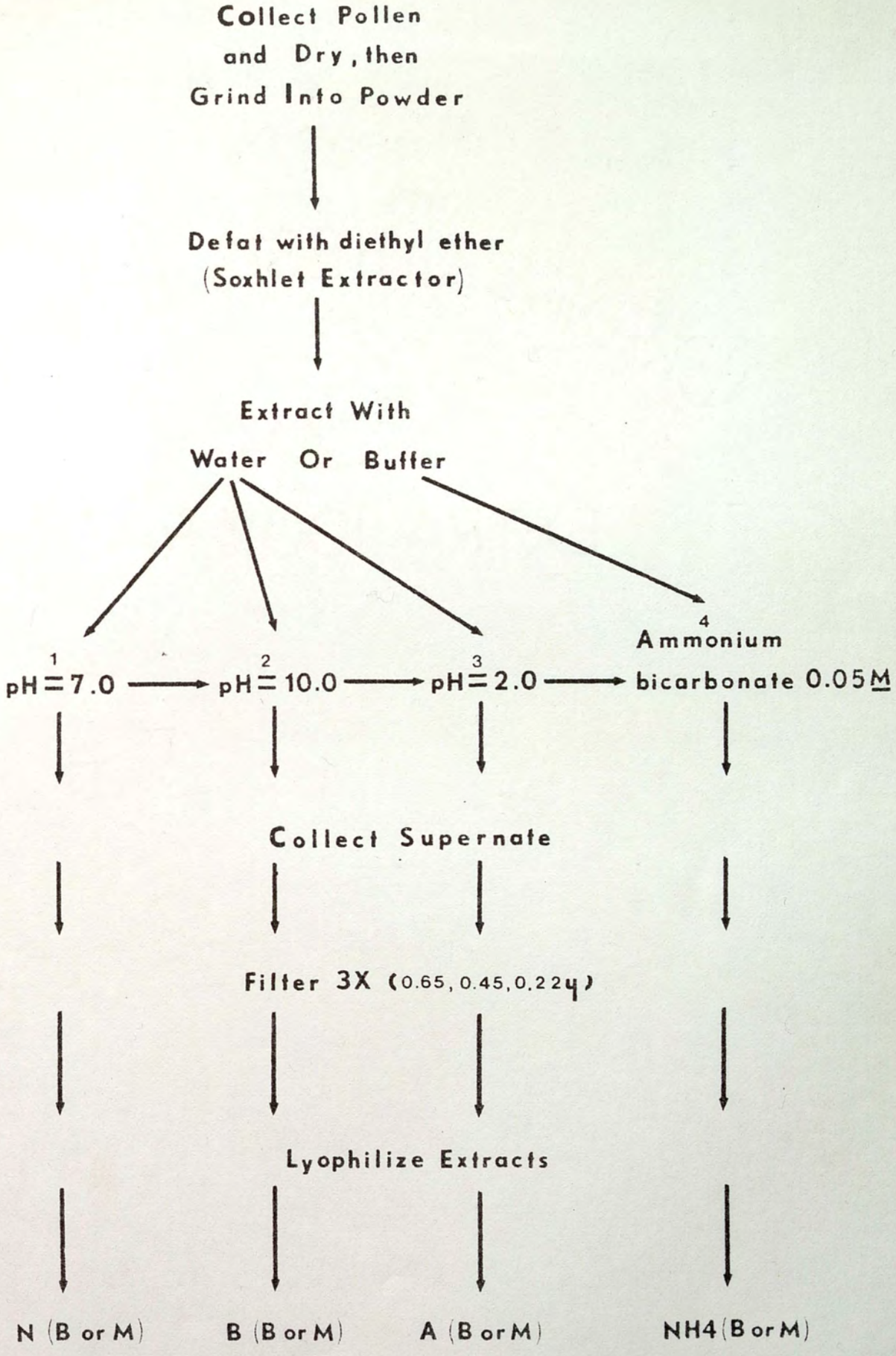
The purpose of this study was to evaluate, with CIE and TCIE, the cross-antigenicity between Bottlebrush and Melaleuca pollen. TECIE analysis was utilized to determine which, if any, of the cross-reactive antigenic components are allergenic.

III. Materials and Methods

Allergen Extracts

Pollen from Melaleuca and Bottlebrush trees was obtained by removing and collecting the anther portion of the whole flower. Five grams of pollen material were dried, ground into a very fine powder in a Wiley Mill grinder (50 mesh), defatted in a Soxhlet extractor with 200 ml diethyl ether, and air-dried for 24 hours. Four individual extractions were performed on the defatted pollens for 48 hours at 4 degrees C (Fig. 3). The first three extractions were water extractions and used 200 ml distilled water at pH 7.00, 10.00, and 2.00, respectively, adjusted with 1.0 M HCL and 1.0 M NaOH. The fourth extraction was performed in 200 ml 0.005 M ammonium bicarbonate at pH 7.8. The incubated extracts were centrifuged at 40,000 x g for 20 minutes. The resultant supernates were filtered using 0.65, 0.45, and 0.22 micron millipore filters. Filtered material was lyophilized and stored at -20 degrees C until use. Four different extracts of each pollen were produced (Fig. 3).

Fig. 3. Preparation of antigenic extracts from Bottlebrush and Melaleuca pollen. Extracts labeled as follows: N = neutral, B = basic, A = acidic, and NH_4 = ammonium bicarbonate.


Collect Pollen
and Dry, then
Grind Into Powder
Defat with diethyl ether
(Soxhlet Extractor)
Extract With
Water Or Buffer
1
pH = 7.0
2
pH = 10.0
3
pH = 2.0
4
Ammonium
bicarbonate 0.05M
Collect Supernate
Filter 3X (0.65, 0.45, 0.22μ)
Lyophilize Extracts
N (B or M)
B (B or M)
A (B or M)
NH4 (B or M)

Rabbit Antisera

Antisera toward Bottlebrush and Melaleuca pollens were prepared in rabbits. Intramuscular injections were performed in the flanks every two weeks for six weeks, then once a month thereafter. The first injection contained 20 mg of the crude defatted pollen triturated into a solution of 1 ml of phosphate buffered saline (PBS). All subsequent injections contained 2 mg of the ammonium bicarbonate extracted material (NH4B or NH4M) in a volume of 1 ml containing equal parts of Freund's complete adjuvant and PBS. Subcutaneous injections, performed at various times between adjuvant injections, used 0.01 ml of a solution containing 2 mg of extracted antigen per ml PBS. Rabbits were bled seven-nine days following each adjuvant injection. Sera for each antigen were collected from at least two rabbits and pooled for immunochemical analysis.

Human Serum

Human serum samples were collected from patients who were designated as allergic or non-allergic based upon clinical testing. Patients were considered to be allergic if skin test results using Bottlebrush and Melaleuca pollen extracts were 2+ or greater using a grading system presented in Table 2. Patients were considered non-allergic if skin test results were 0.

RAST Analysis

Allergosorbent discs (solid-phase allergen) were prepared by coupling each of the four pollen extracts of Melaleuca and Bottlebrush

TABLE 2

GRADING SYSTEM FOR SKIN TESTS

GRADE	ERYTHEMA (mm)	WHEAL (mm)
0	< 5	< 4
±	5-10	5-10
1+	11-20	5-10
2+	21-30	5-10
3+	31-40	10-15[a]
4+	> 40	> 15[b]

[a] or pseudopods.

[b] or with many pseudopods.

Reference: Rose, N. R.; Friedman, H. Manual of clinical immunology, 2d ed. Washington, D. C.: American Society for Microbiology; 1980.

to cyanogen bromide activated Whatman #44 filter paper discs and used for RAST testing [10]. Activated discs were incubated with 100 microliters of either allergic or nonallergic patient sera for 3 hours, washed, and incubated overnight in 50 microliters of ^{125}I labeled goat anti-human IgE. Counts per minute were then measured in a Picker (Spectroscaler 4R) gamma counter. All discs were analyzed in duplicate.

Crossed Immunoelectrophoresis

Crossed immunoelectrophoresis (CIE) was performed using 7.5 x 7.5 cm glass plates covered with Gel-Bond (Bio-Rad), and 1% agarose dissolved in 0.02 M Veronal buffer, pH 8.6, containing 0.005 M calcium lactate and 0.05 M sodium azide [23,36]. Five microliter samples of either crude Bottlebrush or Melaleuca pollen extract (10 mg/ml) were subjected to electrophoresis in the first dimension at room temperature for 25 minutes at 10 V/cm. The second dimension electrophoresis was performed at room temperature for 16-20 hours at 2 V/cm with 20 microliters of rabbit antiserum/cm^2 gel. Gels were deproteinized for 24 hours in PBS, pressed under filter paper, dried, and stained with Coomassie Brilliant Blue R-250 (Eastman) [13].

Tandem-crossed Immunoelectrophoresis

Tandem-crossed immunoelectrophoresis (TCIE) was performed with two gel sections containing different antisera [4,19]. This technique allowed the comparison of these antisera. This technique was used to

determine which antigens were cross-reactive. TCIE was performed, as previously described, for CIE with the addition of 20 microliters of antiserum/cm^2 in the intermediate gel.

Trans-blot Enzyme-linked Crossed Immunoelectrophoresis

Trans-blot enzyme-linked crossed immunoelectrophoresis (TECIE) is trans-blot electrophoresis applied to CIE and tandem-CIE gels, as previously described [25], with the following modifications: washed nitrocellulose membranes, which were previously incubated in a 1 to 5 dilution of allergic human serum for 15-18 hours, were incubated for 3 hours in a 1 to 3000 dilution of goat anti-human IgE (Epsilon chain specific) horseradish peroxidase labeled serum. The sheets were developed according to the Bio-Rad Immuno-blot assay [7].

IV. Results

RAST Analysis

RAST analysis of Bottlebrush and Melaleuca extracts demonstrated binding of IgE to all eight extract types using allergic patient sera (Fig. 4). The acid extracts of Bottlebrush and Melaleuca bound between 1700 and 1800 counts per minute (cpm) using serum from allergic patients. These cpm values for the acid extracts were 400-1200 cpm less than any other extract type. Ammonium bicarbonate extracts of both Bottlebrush and Melaleuca pollen bound between 2800 and 3200 cpm which were as much as or greater cpm than any other extract type. RAST analysis of the pollen extracts using non-allergic

patient sera showed very low levels of IgE binding because cpm values ranged between 500-700 for all extract types.

Crossed Immunoelectrophoretic Analysis

The patterns obtained using hyperimmune rabbit antisera to the respective pollen in CIE analysis of NH4B and NH4M antigenic extracts are in Figures 5A and 6A. In all cases, the anode is represented on the left side of the gel while the cathode is on the right. Many antigenic components were detected after protein staining of the CIE-plates. Schematic drawings of the CIE patterns with individually numbered precipitates show the presence of at least 14 and 12 antigenic components in NH4B and NH4M extracts, respectively (Figs. 5B and 6B).

Tanden-crossed Immunoelectrophoretic Analysis

Patterns obtained with TCIE analysis of NH4B and NH4M are in Figures 7A and 8A. All of the visualized antigenic components of NH4B precipitated in the intermediate gel which contained antiserum to Melaleuca pollen (Fig. 7). TCIE analysis of NH4M also detected precipitin bands in the intermediate gel when analyzed with antisera to Bottlebrush pollen in the tandem gel (Fig. 8).

Trans-blot Enzyme-linked Crossed Immunoelectrophoretic Analysis

Analysis of the CIE patterns of NH4B and NH4M extracts utilizing TECIE are in Figures 9 and 10. IgE from allergic patient sera was shown to be reactive to precipitin bands 2, 3, and 4 of the CIE of NH4B since these were the only bands that developed after incubation

with horseradish peroxidase labeled goat anti-human IgE (Fig. 9). Similar analysis of the CIE of NH4M demonstrated that precipitin bands 1 and 2 bound IgE from allergic patient sera (Fig. 10).

Analysis by TECIE of the TCIE pattern of NH4B extract demonstrated that precipitin bands 2, 3, and 4, in the intermediate gel developed with rabbit anti-Melaleuca serum, also bind IgE (Fig. 11 A and B). Similar analysis of NH4M, detected that precipitin bands 1 and 2, which occurred in the intermediate gel containing rabbit anti-Bottlebrush serum, bound IgE from allergic patient sera (Fig. 12 A and B).

Fig. 4. RAST analysis of Bottlebrush and Melaleuca pollen extracts. Solid bars represent allergens analyzed with allergic patient sera (black: NH4B and NH4M, white: all other extracts). Cross hatched bars represent allergens analyzed with non-allergic patient sera.

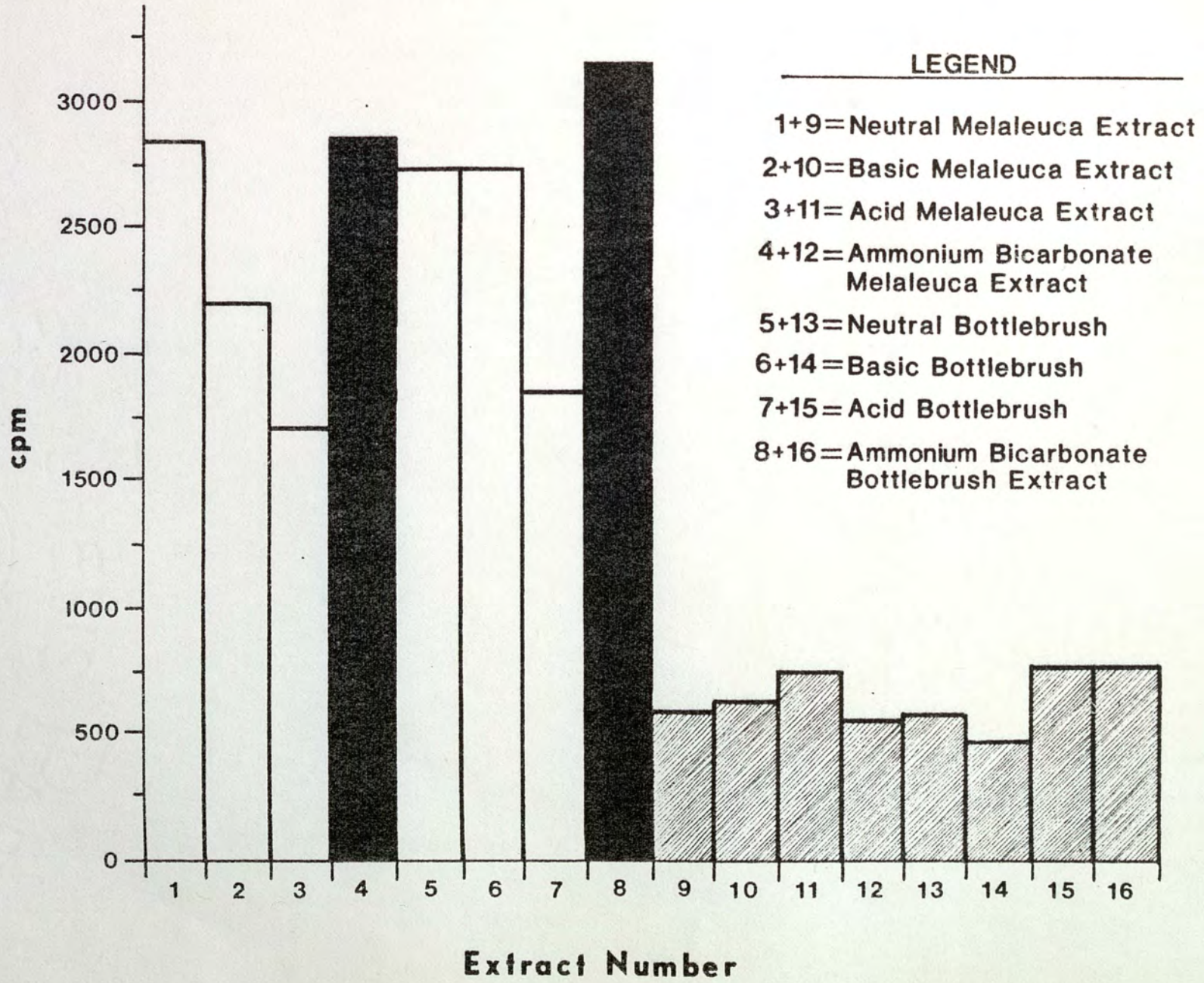
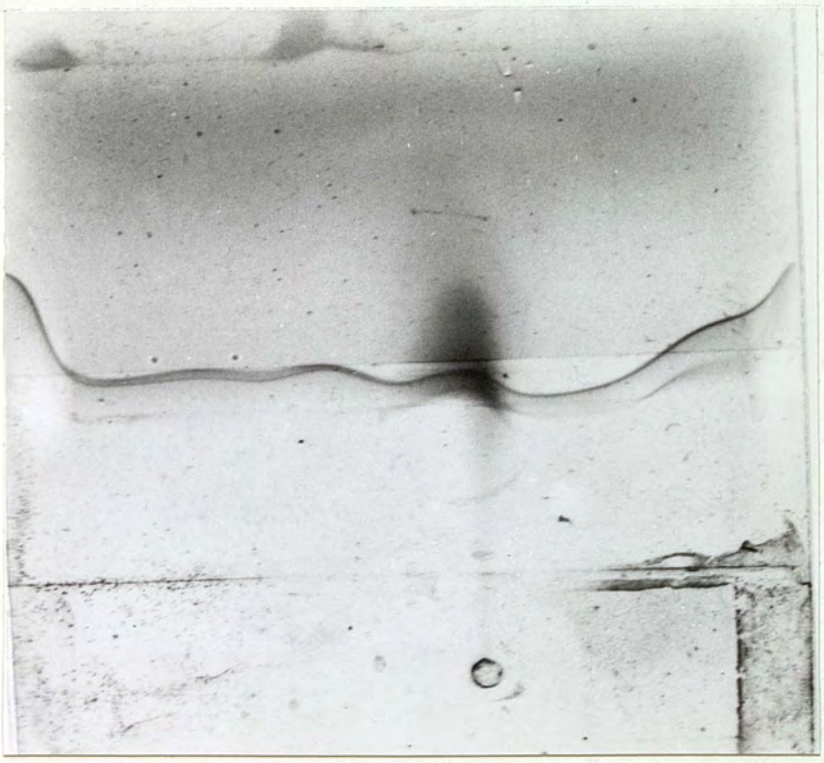

LEGEND
1+9=Neutral Melaleuca Extract
2+10=Basic Melaleuca Extract
3+11=Acid Melaleuca Extract
4+12=Ammonium Bicarbonate Melaleuca Extract
5+13=Neutral Bottlebrush
6+14=Basic Bottlebrush
7+15=Acid Bottlebrush
8+16=Ammonium Bicarbonate Bottlebrush Extract
cpm
3000
2500
2000
1500
1000
500
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Extract Number

Fig. 5. Crossed immunoelectrophoresis of the ammonium bicarbonate extract of Bottlebrush (NH_4B) against hyperimmune rabbit antisera to Bottlebrush.
A. Photograph. B. Schematic representation of NH_4B CIE.

A.



B.

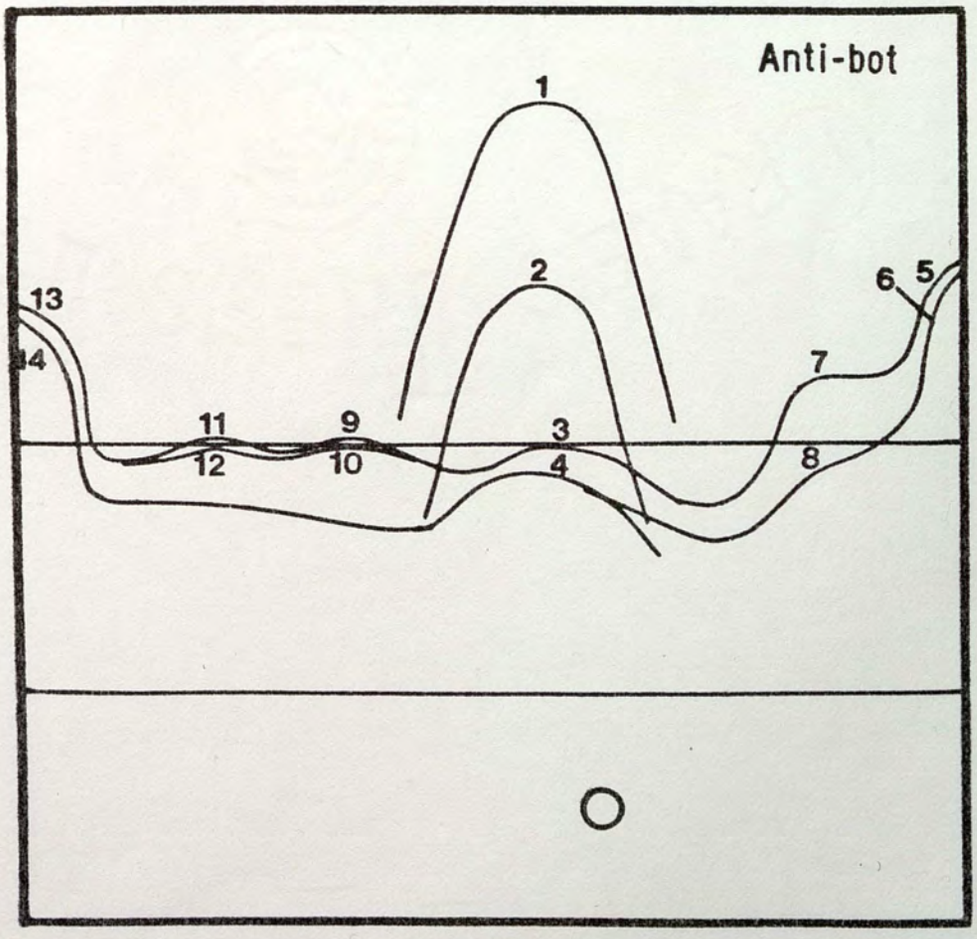
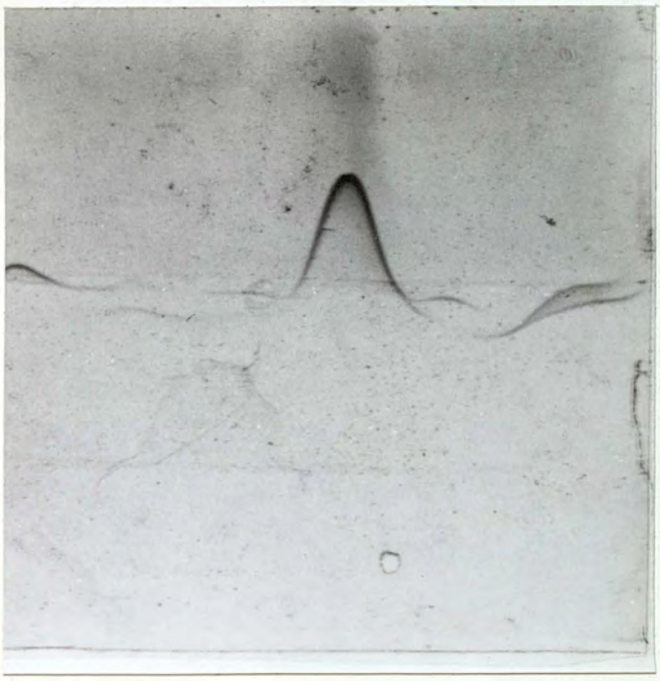


Fig. 6. Crossed immunoelectrophoresis of the ammonium bicarbonate extracts of Melaleuca (NH4M) against hyperimmune rabbit antisera to Melaleuca.
A. Photograph. B. Schematic representation of NH4M CIE.

A.



B.

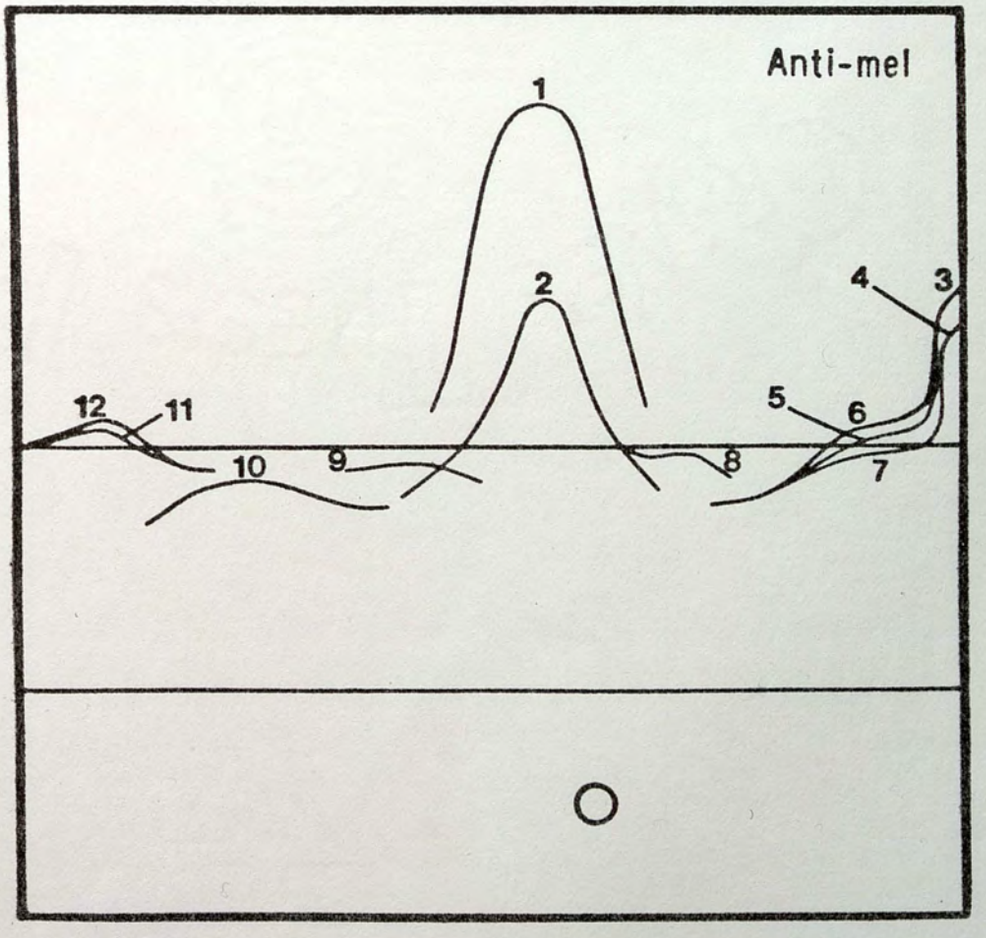
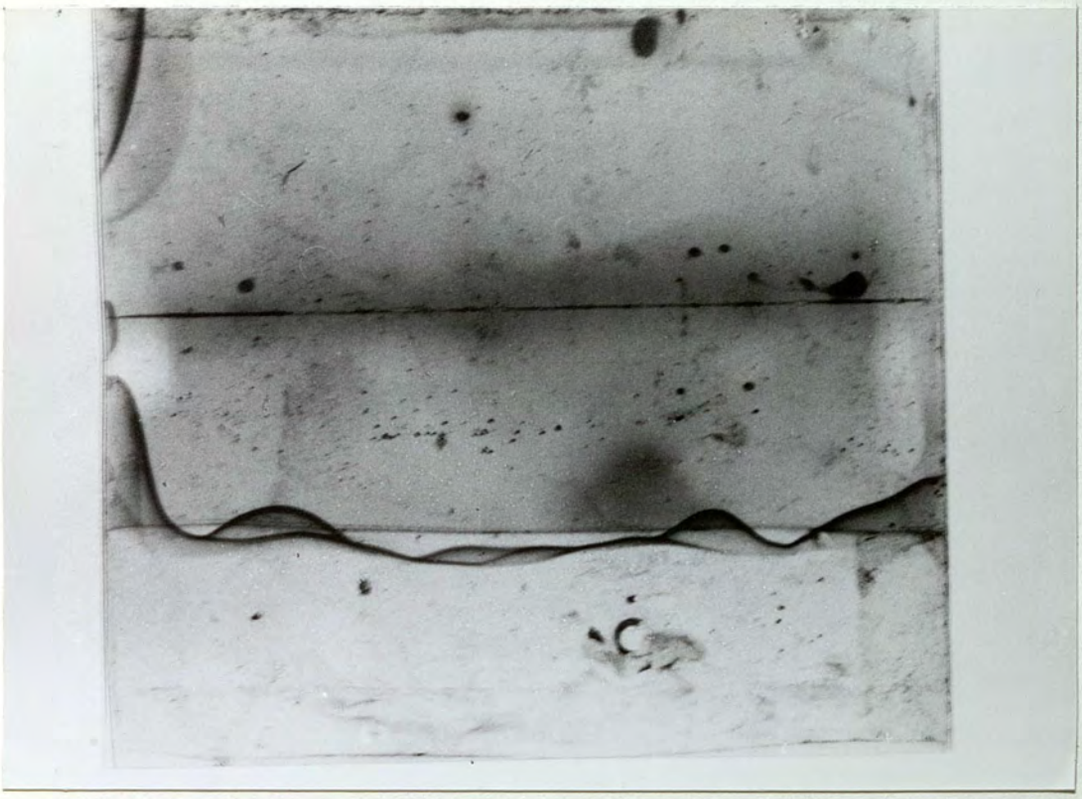


Fig. 7. Tandem-crossed immunoelectrophoresis of the ammonium bicarbonate extract of Bottlebrush (NH4B) against hyperimmune rabbit antisera to Melaleuca in the tandem gel and Bottlebrush in the second dimension gel. A. Photograph. B. Schematic representation of TCIE of NH4B with numeration as in Figure 5.

A.



B.

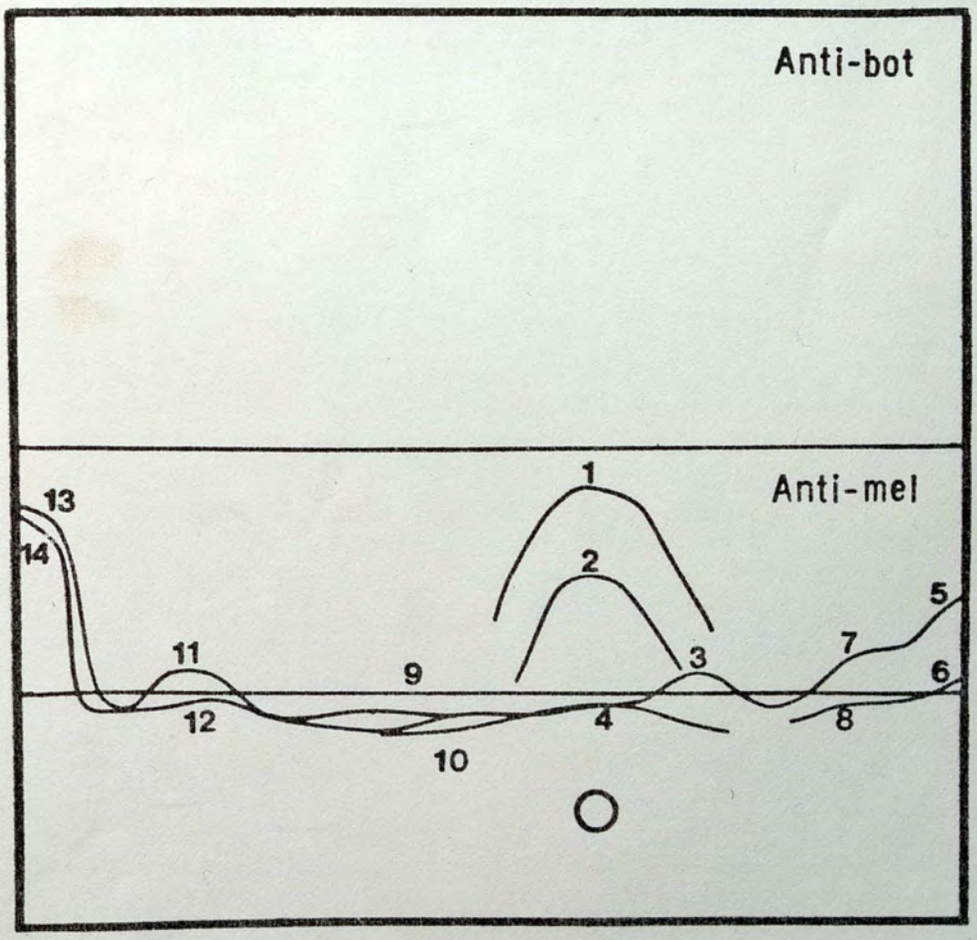
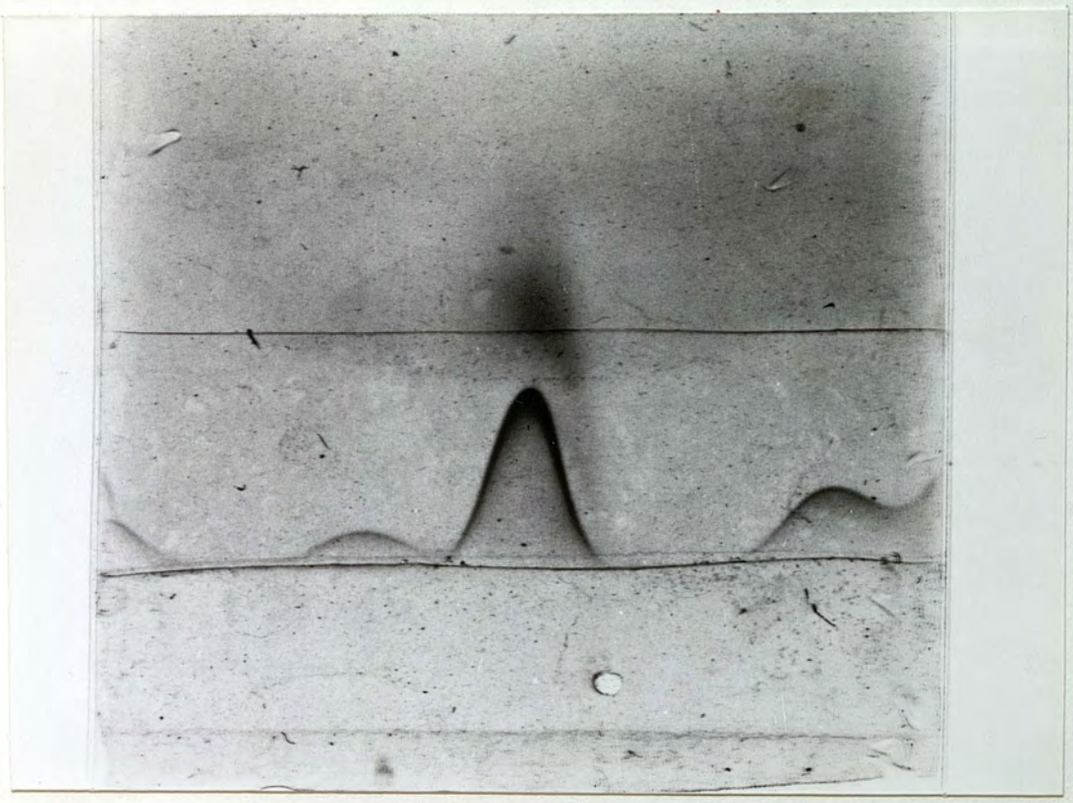


Fig. 8. Tandem-crossed immunoelectrophoresis of the ammonium bicarbonate extract of Melaleuca (NH4M) against hyperimmune rabbit antisera to Bottlebrush in the tandem gel and Melaleuca in the second dimension gel. A. Photograph. B. Schematic representation with numeration as in Figure 6.

A.



B.

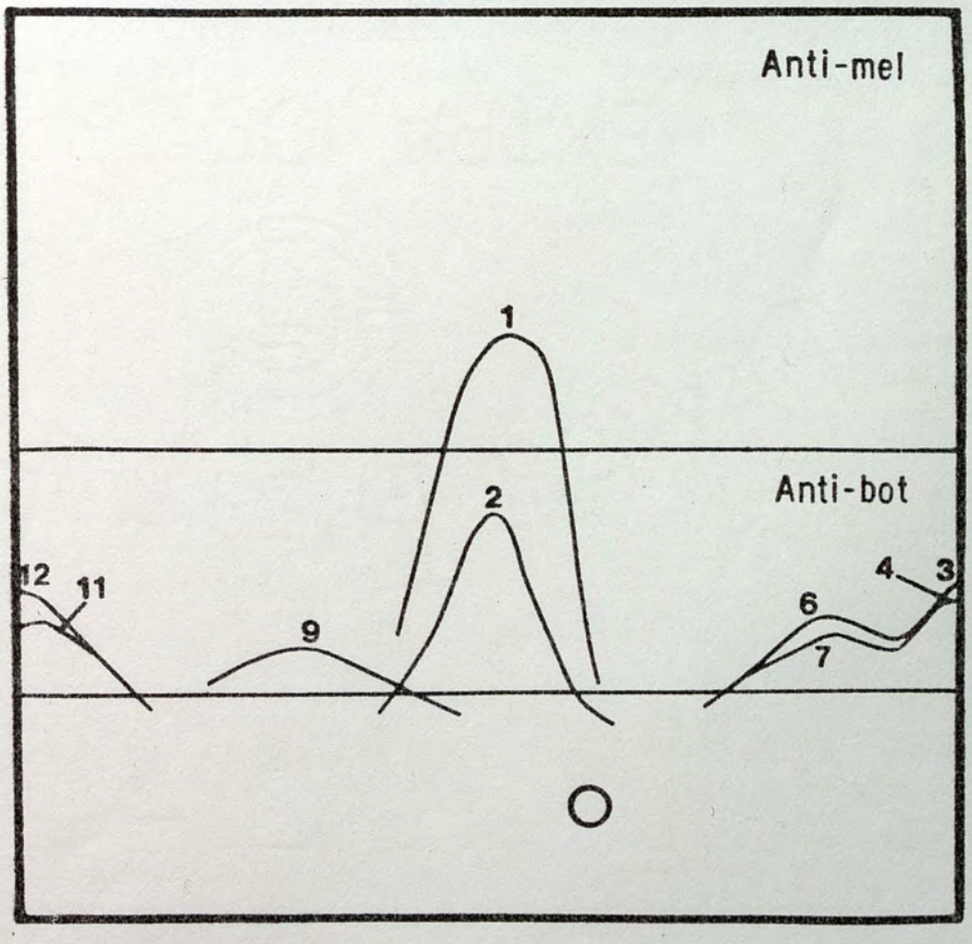
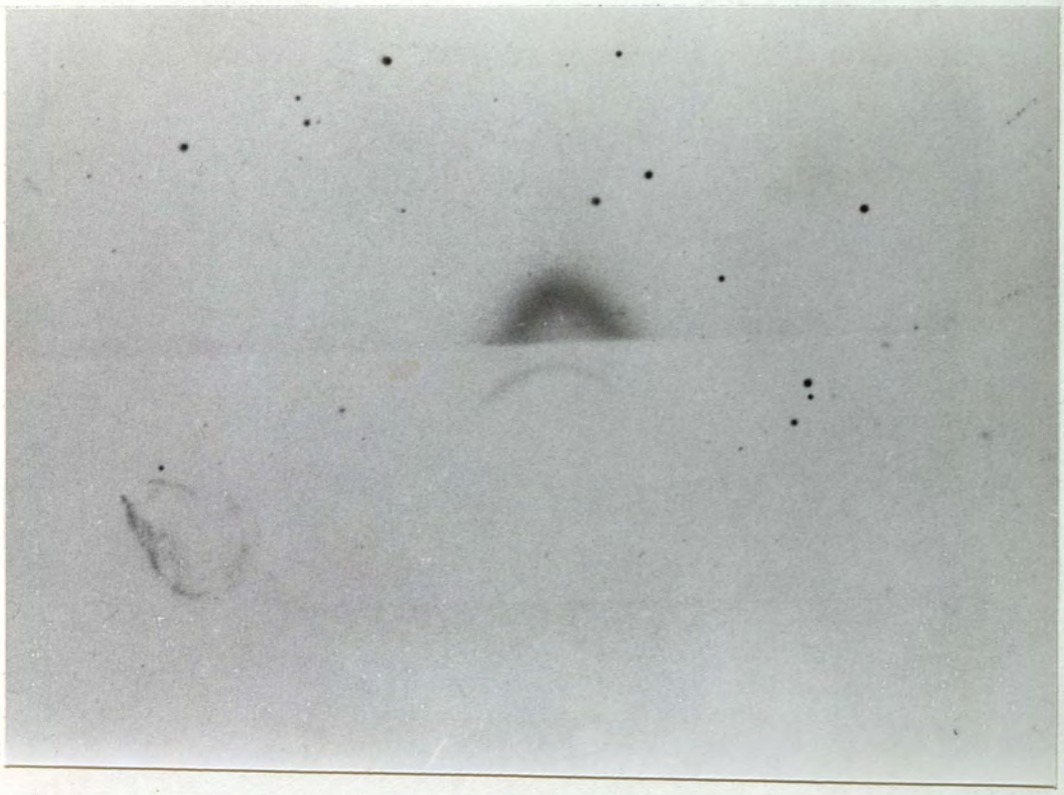


Fig. 9. Trans-blot enzyme-linked crossed immunoelectrophoresis of the ammonium bicarbonate extract of Bottlebrush (NH_4B).
A. Photograph. B. Schematic representation with numeration as in Figures 5 and 7.

A.



B.

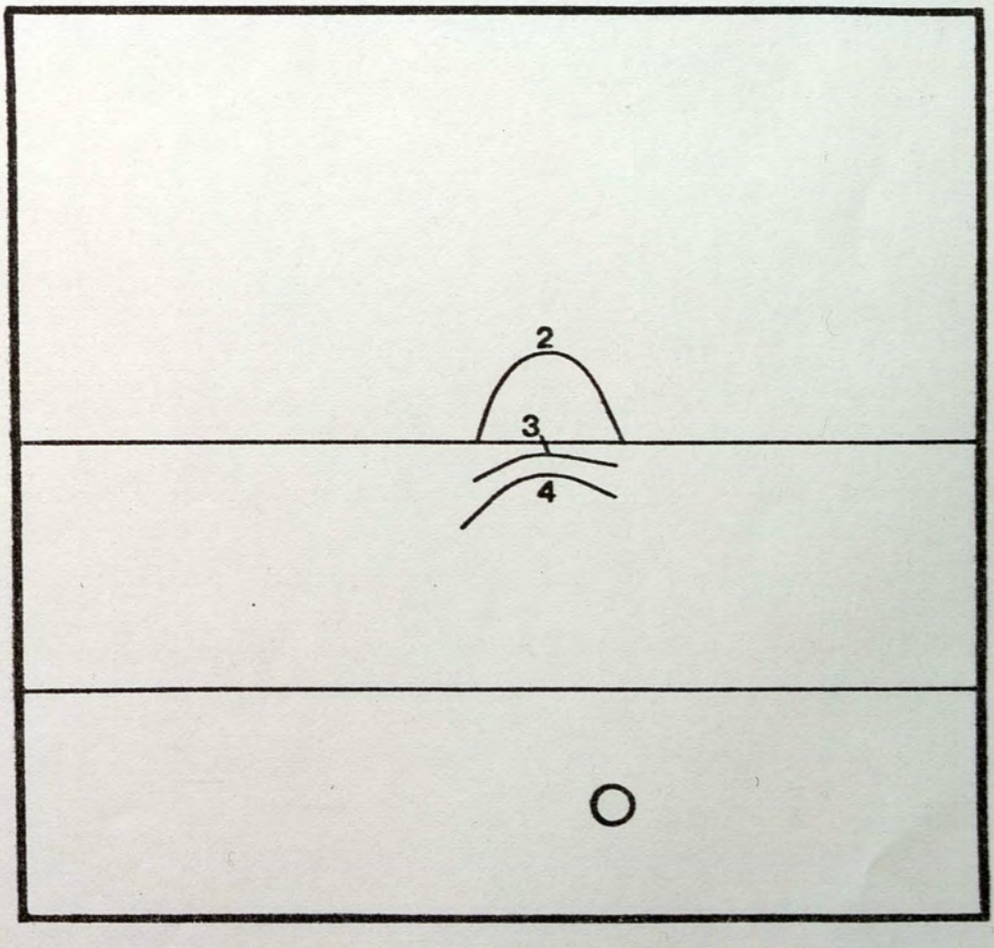
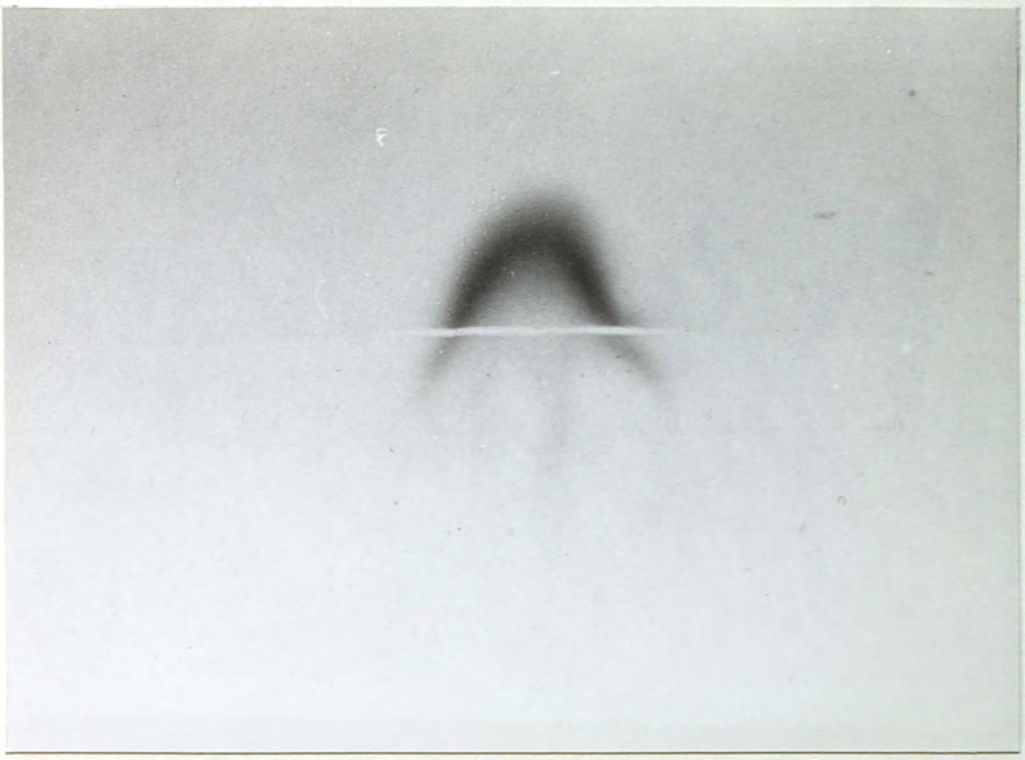


Fig. 10. Trans-blot enzyme-linked crossed immunoelectrophoresis of the ammonium bicarbonate extract of Melaleuca (NH4M).
A. Photograph. B. Schematic representation with numeration as in Figures 6 and 8.

A.



B.

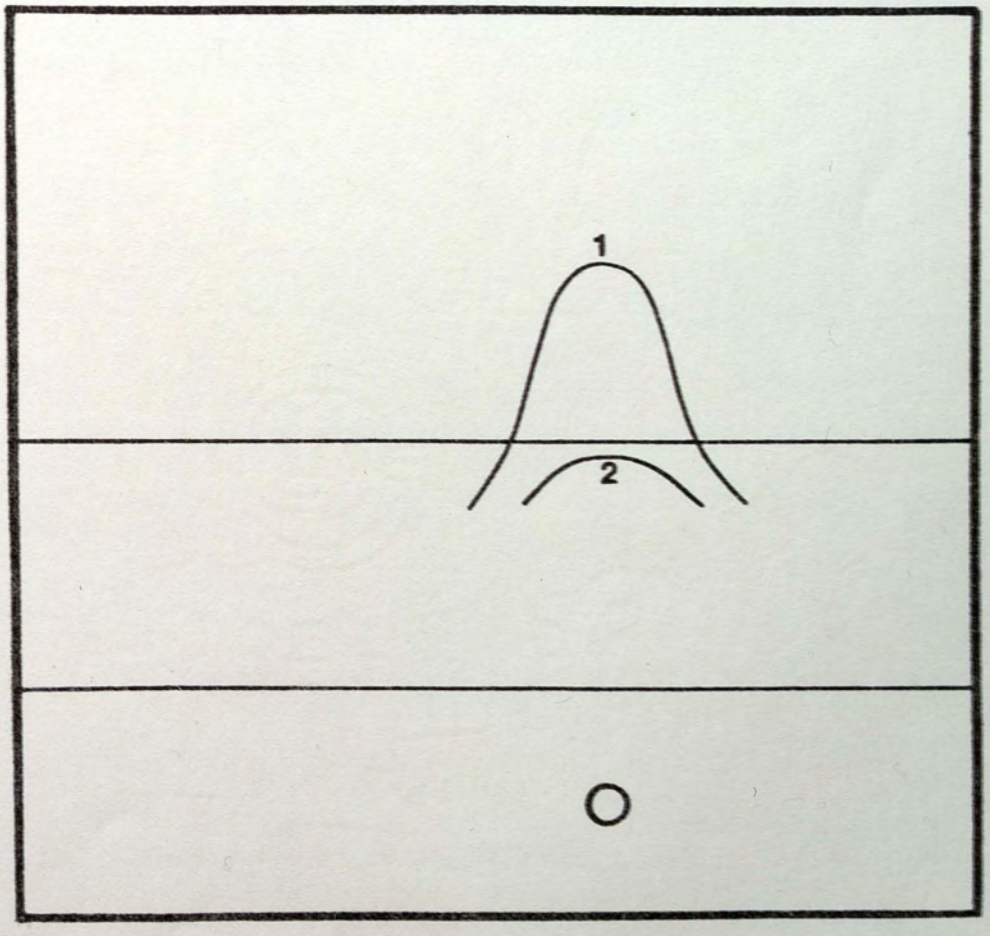
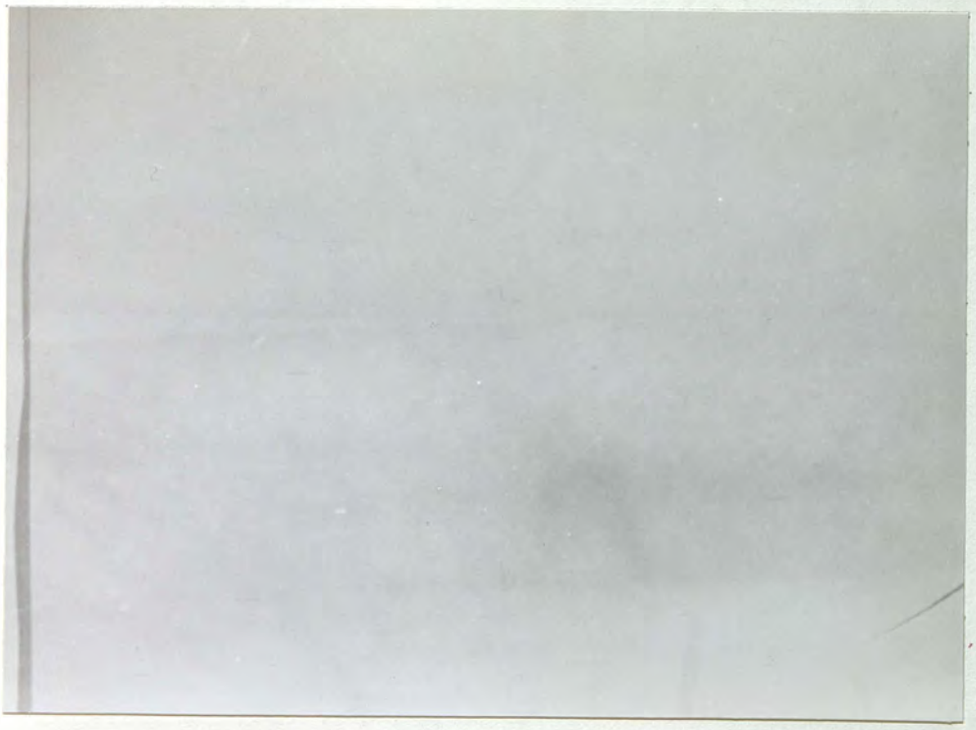


Fig. 11. Trans-blot enzyme-linked crossed immunoelectrophoresis of TCIE of the ammonium bicarbonate extract of Bottlebrush (NH_4B).
A. Photograph. B. Schematic representation with numeration as in Figures 5 and 7.

A.



B.

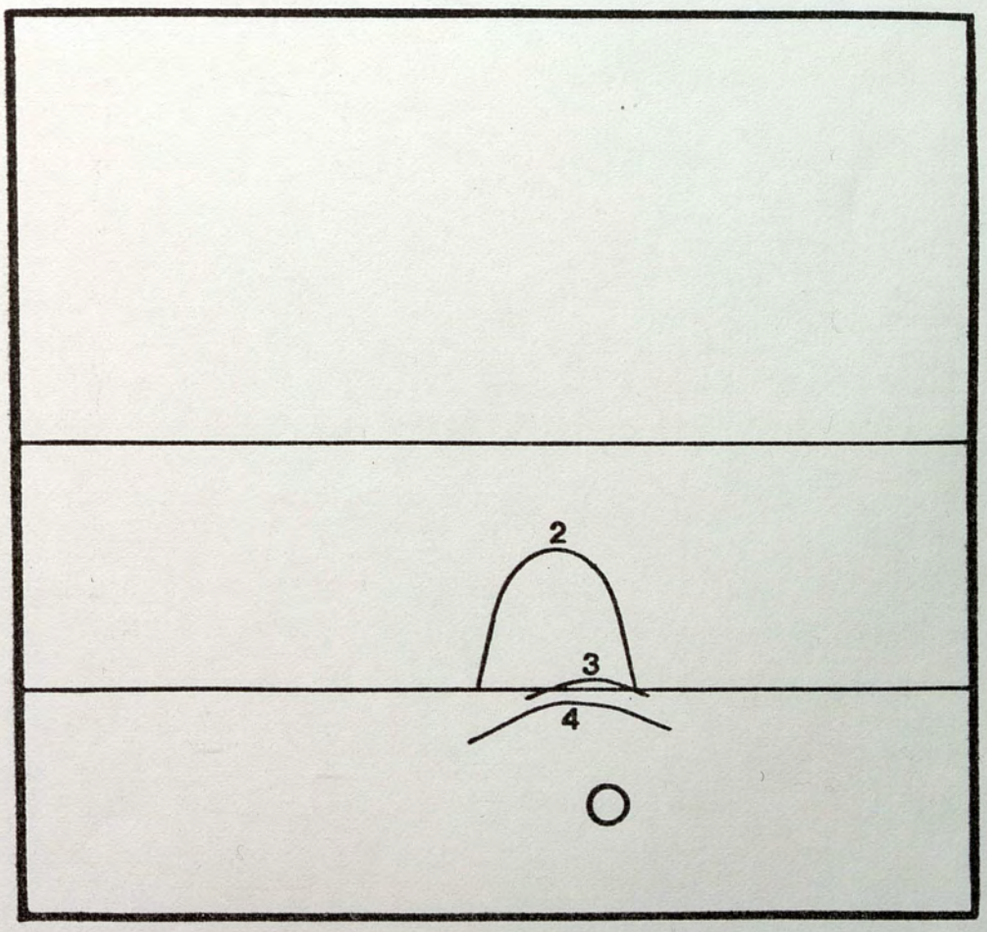
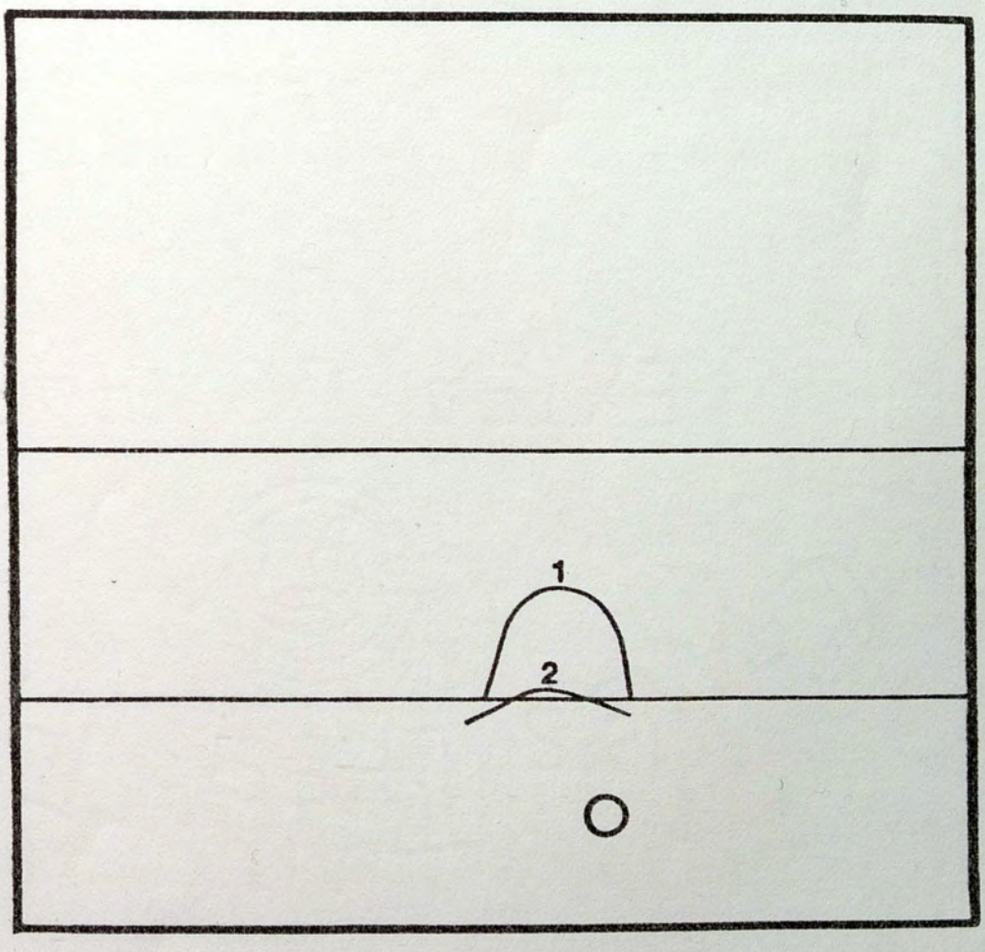


Fig. 12. Trans-blot enzyme-linked crossed immunoelectrophoresis of TCIE of the ammonium bicarbonate extract of Melaleuca (NH4M).
A. Photograph. B. Schematic representation with numeration as in Figures 6 and 8.

A.

B.



V. Discussion

RAST analysis of pollen extracts demonstrated that the ammonium bicarbonate extracts of both Bottlebrush (NH4B) and Melaleuca (NH4M) pollen contained as much or greater allergenic reactivity than any of the other extract types when using specific allergic patient sera. These extracts, NH4B and NH4M, were therefore selected for further characterization.

The identification of antigens within a complex mixture of components, such as pollen extracts, has been greatly facilitated by CIE and variations of this technique [12]. CIE analysis of the NH4B and NH4M extracts utilizing antibodies raised in rabbits demonstrated that the two pollens contain multiple antigenic components. TCIE analysis indicated that these pollen antigens are cross-reactive. This conclusion is reached because precipitin bands were detected in the tandem gel containing sera to the opposite pollen demonstrating a strong reaction with antisera raised to the other antigen.

The use of an enzyme linked anti-human IgE in detection of allergic components in CIE patterns trans-blotted to nitrocellulose membranes has not been previously reported. This technique is very sensitive and has a 50 picogram detection limit [7]. TECIE detects allergenic bands in immunoprecipitates faster than CRIE. Development time after incubation with anti-human IgE horseradish peroxidase labeled antibodies is less than an hour. The potential of this technique for aid in rapid detection of allergenic components in CIE analysis is obvious.

Problems have been reported to occur with the detection of allergenic components in precipitin bands. It has been previously demonstrated that precipitates may contain trace amounts of allergens due to coprecipitation if they are positioned in areas enclosed by precipitates of these allergens [3]. Therefore, precipitin bands 3 and 4 using TECIE of the NH4B extract might contain enough trace amounts of precipitin band 2 to bind IgE and, therefore, appear allergenic. Similar examination of NH4M trans-blot patterns indicated that band 2 might contain trace amounts of band 1 and, therefore, bind IgE and appear allergenic.

Distinct antigenic components of two different pollen antigenic extracts may carry identical allergenic determinants giving rise to IgE cross-reactivity [3]. These antigenic components of Bottlebrush and Melaleuca appeared to have a different overall structure when analyzed by CIE; but, due to the extensive cross-reactivity observed by TCIE, the actual antigenic determinants which make up the antigenic components of these two pollen extracts appear to be very similar, if not identical. The question still remains whether or not the allergenic components identified by TECIE are actually cross-reactive. RAST inhibition analysis, which is presently under study, could possibly provide an answer to this question.

SECTION II

ISOLATION AND CHARACTERIZATION OF THE CROSS-REACTIVE ANTIGENIC AND ALLERGENIC COMPONENTS IN CALLISTEMON CITRINIS AND MELALEUCA QUINQUENERVIA POLLEN BY IMMUNOCHEMICAL METHODS II

I. Abstract

Aqueous extracts of both Bottlebrush (Callistemon citrinis) and Melaleuca (Melaleuca quinquenervia) were analyzed for allergenic cross-reactivity. RAST inhibition analysis was performed on ammonium bicarbonate extracts of Bottlebrush (NH4B) and Melaleuca (NH4M) pollen. RAST inhibition analysis demonstrated that the extracts contained allergenically cross-reactive components. Sephadex G-100 column chromatography of NH4B and NH4M extracts resulted in at least four distinct peaks for each extract analyzed. These fractions were designated NH4B1 thru NH4B4 and NH4M1 thru NH4M4. Utilizing a modified dot-blot assay for detection of allergenic components, the first elution peaks of Bottlebrush and Melaleuca, NH4B1 and NH4M1, respectively, were found to contain allergenic components. The allergenic components of NH4B1 and NH4M1 had estimated molecular weights of 50,000 and 35,000 daltons, respectively.

II. Introduction

Antigenic cross-reactivity between Bottlebrush (Callistemon citrinis) and Melaleuca (Melaleuca quinquenervia) has been demonstrated (SECTION I). Also, trans-blot enzyme-linked crossed immunoelectrophoresis (TECIE) demonstrated that the visualized

precipitin bands obtained by crossed immunoelectrophoresis (CIE) and tandem-crossed immunoelectrophoresis (TCIE) contained allergenic activity (SECTION I).

Allergenic cross-reactivity among pollen allergens has been well documented [6,12,17,21,22,32]. Cross-reactivity between allergens can be demonstrated using RAST inhibition assays [6]. RAST inhibition studies have previously indicated that clustering of hypersensitivities to plant material exists due to the presence of IgE antibodies which cross-react with several antigens [17].

The research reported here further substantiates the allergenic cross-reactivity between the ammonium bicarbonate extracts of Bottlebrush (NH4B) and Melaleuca (NH4M) pollen and further characterizes the allergenic components.

III. Materials and Methods

RAST and RAST Inhibition

Antigen extracts, rabbit antisera, and specific patient sera were obtained as outlined in SECTION I. RAST inhibition was performed using the method of Gleich et al. [16] with the following modifications. Both NH4B and NH4M allergen extracts were bound to Sepharose-4B before incubation with allergic sera [21]. Five 2-fold dilutions were made of a mixture of 1 ml of packed Sepharose-antigen (Sepharose-ag) in 1 ml of RAST diluent (1 to 2 - 1 to 32) of both Sepharose-Melaleuca (Sepharose-MEL) and Sepharose-Bottlebrush (Sepharose-BOT). After centrifugation at 700 x g for 10 minutes,

the diluent was removed from the Sepharose-ag mixtures and 1 ml of allergic sera diluted 1 to 2 was added to each dilution of Sepharose-ag. The tubes containing the Sepharose-ag and human serum mixtures were rotated, incubated at 4 degrees C for 24 hours and centrifuged at 5,000 x g for 5 minutes. The sera was collected and RAST analysis was performed [10]. The percent inhibition of IgE binding in RAST analysis was calculated according to the following formula:

$$\% \text{ Inhibition} = \frac{\text{cpm (0)} - \text{cpm (x)}}{\text{cpm (0)} - \text{cpm (n)}} \times 100$$

where cpm (0) = the cpm obtained with uninhibited sera; cpm (n) = the radioactivity bound to the discs incubated with a normal control serum containing no IgE antibodies to the allergens, and cpm (x) = cpm obtained with inhibited sera [21]. Phadabas RAST Unit calculations (PRUs) were calculated (Pharmacia Diagnostics, Uppsala, Sweden).

Sephadex G-100 Chromatography

Gel filtration was performed at room temperature using Sephadex G-100 equilibrated with 0.025 M Tris buffer pH 7.5 and packed into a glass column (2.5 x 90 cm). The void volume was calibrated using blue dextran. Twenty mg of either the NH4B or NH4M lyopholized extract mixed with 2 ml of Tris buffer was applied to the column. Eighty 3 ml fractions were collected at a flow rate of 10 ml/hr and monitored at 277 nm.

Molecular Weight Determinations

Approximate molecular weights were calculated using a Sephadex G-100 column calibrated with the following standards: bovine gamma globulin (158,000 daltons), bovine serum albumin (67,000 daltons), myoglobin (17,000 daltons), and vitamin B-12 (1,350 daltons). Molecular weights were estimated from the elution positions of the antigens as compared to the elution positions of the standards.

Protein Blotting

Immuno-Blotting was performed as by Bio-Rad [7] except for the following modification. A modification of Immuno-Blotting, called Allergo-blot, was used to detect which of the NH4B and NH4M pollen extract fractions obtained with Sephadex G-100 chromatography was (were) allergenic. Allergo-blot analysis of gel filtration fractions was performed by blotting 1 microliter of each G-100 fraction onto a nitrocellulose membrane previously lined with a 6 x 6 mm grid. Unbound sites on the membrane were blocked with 3% gelatin and Tris buffered saline (TBS) solution and incubated for 18-20 hours in a 1 to 4 dilution of allergic patient sera in 1% gelatin and TBS. Two 10-minute washes using 100 ml TBS were performed on the membranes before incubating for 3 hours in sheep anti-human IgE (epsilon chain specific) peroxidase labeled antibodies (United States Biochemicals, Cat. No. 1177H) diluted 1 to 3000 in 1% gelatin and TBS. Cord blood (human fetal cord serum) was obtained and used as a negative control.

IV. Results

Competitive Inhibition Studies Using RAST Analysis

The ability of Sepharose bound Melaleuca and Bottlebrush antigens to compete with NH4B and NH4M RAST discs for binding of allergic patient IgE was studied using the RAST inhibition assay (Table 2). The % inhibition of IgE binding to NH4M and NH4B RAST discs decreased from 69 to 6% and 82 to 1%, respectively, with decreasing concentrations of Sepharose-MEL (Figs. 13 and 14). Similar decreases in the 9% inhibition were observed for varying concentrations of Sepharose-BOT. These decreases in % inhibition of IgE binding ranged from 84% to 18% using NH4B discs and 70% to 3% using NH4M discs (Figs. 13 and 14). Sepharose-BOT and Sepharose-MEL appeared to have almost equivalent ability to inhibit the binding of allergic patient IgE to either NH4B or NH4M RAST discs and, as the dilution of these Sepharose-ag(s) increases, the % inhibition decreases (Figs. 13 and 14). The maximum inhibitions were observed at the highest concentrations of Sepharose-ag(s) and were approximately 70% for RAST analysis using NH4M discs and 80% using NH4B discs. In contrast, when Sepharose bound human serum albumin (Sepharose-HSA) was used as a control, the RAST analysis using NH4M and NH4B discs showed only 25% and 29% maximal inhibition, respectively.

TABLE 3

COMPETITIVE INHIBITION OF NH4M OR NH4B BINDING TO IgE
BY VARYING CONCENTRATIONS OF SEPHAROSE BOUND ANTIGENS

SERA	ALLERGEN DISCS NH4M cpm	score	pru	% inhib	NH4B cpm	score	pru	% inhib
Neg. Control	438	0	0.1		606	0	0.1	
Sepharose-MEL								
2	1089	1/0	0.23	69%	1042	1/0	0.21	82%
4	1313	1/0	0.34	58%	1461	1	0.41	65%
8	1606	1	0.46	44%	1599	1	0.46	59%
16	2347	2	1.00	08%	1685	1	0.50	55%
32	2393	2	1.02	06%	3006	2	1.08	01%
Sepharose-BOT								
2	1052	1/0	0.22	70%	996	1/0	0.19	84%
4	1373	1	0.37	55%	1479	1	0.41	64%
8	1670	1	0.50	41%	1519	1	0.43	62%
16	2340	2	0.93	09%	1665	1	0.49	56%
32	2446	2	1.08	03%	2583	2	1.60	18%
Sepharose-HSA								
2	2007	1	0.72	25%	2316	2	0.96	29%
4	2085	1	0.79	21%	2513	2	1.12	21%
8	2168	1	0.84	17%	2880	2	1.46	06%
16	2432	2	1.07	04%	2947	2	1.55	03%
32	2508	2	1.11	01%	3005	2	1.60	01%
Unabsorbed	2517	2	1.00		3027	2	1.62	

Fig. 13. Inhibition of patient IgE binding to NH4B RAST discs by varying concentrations of Sepharose bound antigens. Sepharose-BOT (Squares), Sepharose-MEL (Triangles), and Sepharose-HSA (Circles).

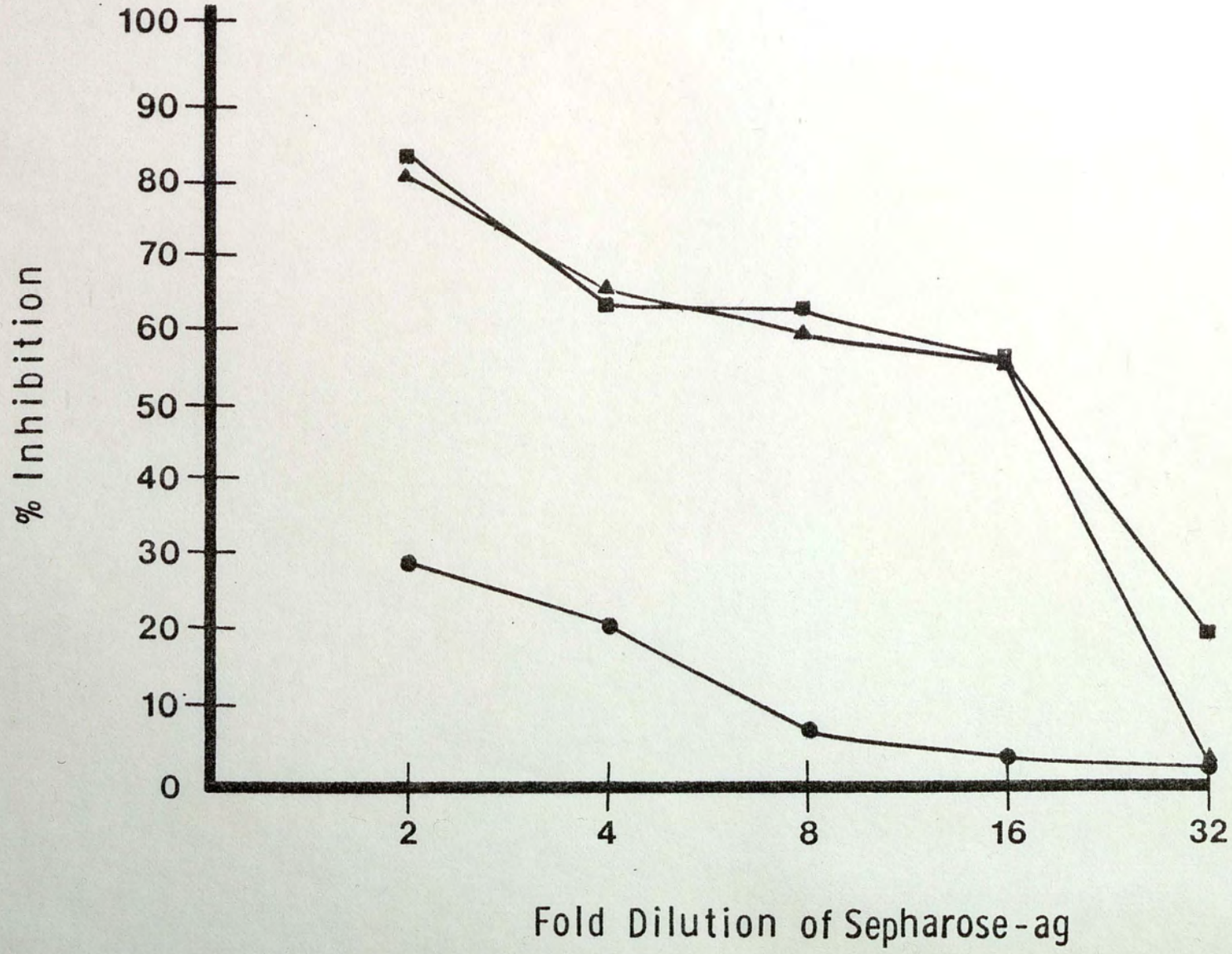
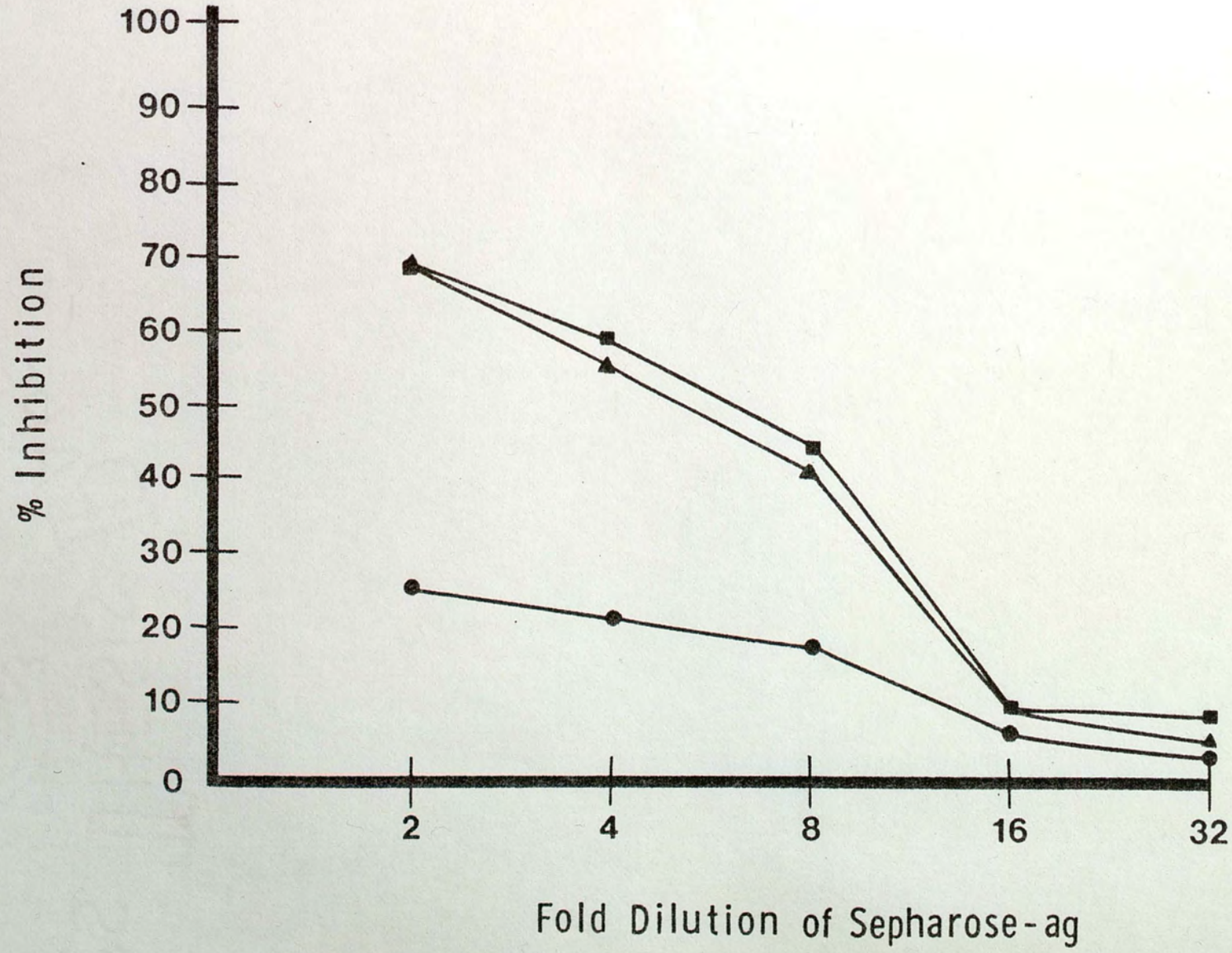

100
90
80
70
60
50
40
30
20
10
0
% Inhibition
2
4
8
16
32
Fold Dilution of Sepharose-ag

Fig. 14. Inhibition of patient IgE binding to NH4M RAST discs by varying concentrations of Sepharose bound antigens. Sepharose-MEL (Squares), Sepharose-BOT (Triangles), and Sepharose-HSA (Circles).


100
90
80
70
60
50
40
30
20
10
0
% Inhibition
2
4
8
16
32
Fold Dilution of Sepharose-ag

Separation of Antigenic Components by Sephadex G-100 Chromatography

Four peaks from each of the elution profiles, termed NH4B1 thru NH4B4 and NH4M1 thru NH4M4, respectively, were separated by gel chromatography and detected by monitoring absorbence at 277 nm (Figs. 15 and 16).

Protein Blotting of Sephadex G-100 Fractions

In the schematic representations of the immuno-blots of the G-100 fractions of NH4B and NH4M using rabbit antisera to the respective pollen, each square represents a fraction obtained from Sephadex G-100 chromatography. Those squares designated with a circle indicate fractions containing antigenic reactivity to either rabbit anti-Bottlebrush or anti-Melaleuca serum detected with goat anti-rabbit IgG. Peaks NH4B1, NH4B2, NH4B3, and NH4B4 were shown to have antigenic activity when incubated with rabbit anti-Bottlebrush serum (Fig. 17). Peaks NH4M1 and NH4M2 were also shown to contain antigenic activity when incubated with rabbit anti-Melaleuca serum (Fig. 18).

Schematic representation of the allergo-blots of the G-100 fractions of NH4B and NH4M using allergic patient sera and goat anti-human IgE demonstrated that the NH4B1 and NH4M1 peaks contain allergenic reactivity (Figs. 18 and 19).

Molecular Weight Determinations

The molecular weights of the allergenic peaks were estimated by comparing them with standards run on the same column. The peaks,

NH4B1 and NH4M1, were absorbed on the column. They were eluted from the column between the bovine serum albumin (67,000 daltons) and myoglobin (17,000 daltons) standards. The estimated molecular weights of NH4B1 and NH4M1 peaks are 50,000 and 35,000 daltons, respectively.

Fig. 15. Separation of proteins in the ammonium bicarbonate extract of Bottlebrush (NH4B) by Sephadex G-100 column chromatography. Arrows indicate the elution fractions of the following standards: bovine gamma globulin (BGG), bovine serum albumin (BSA), myoglobin (MG), and vitamin B-12 (B-12).

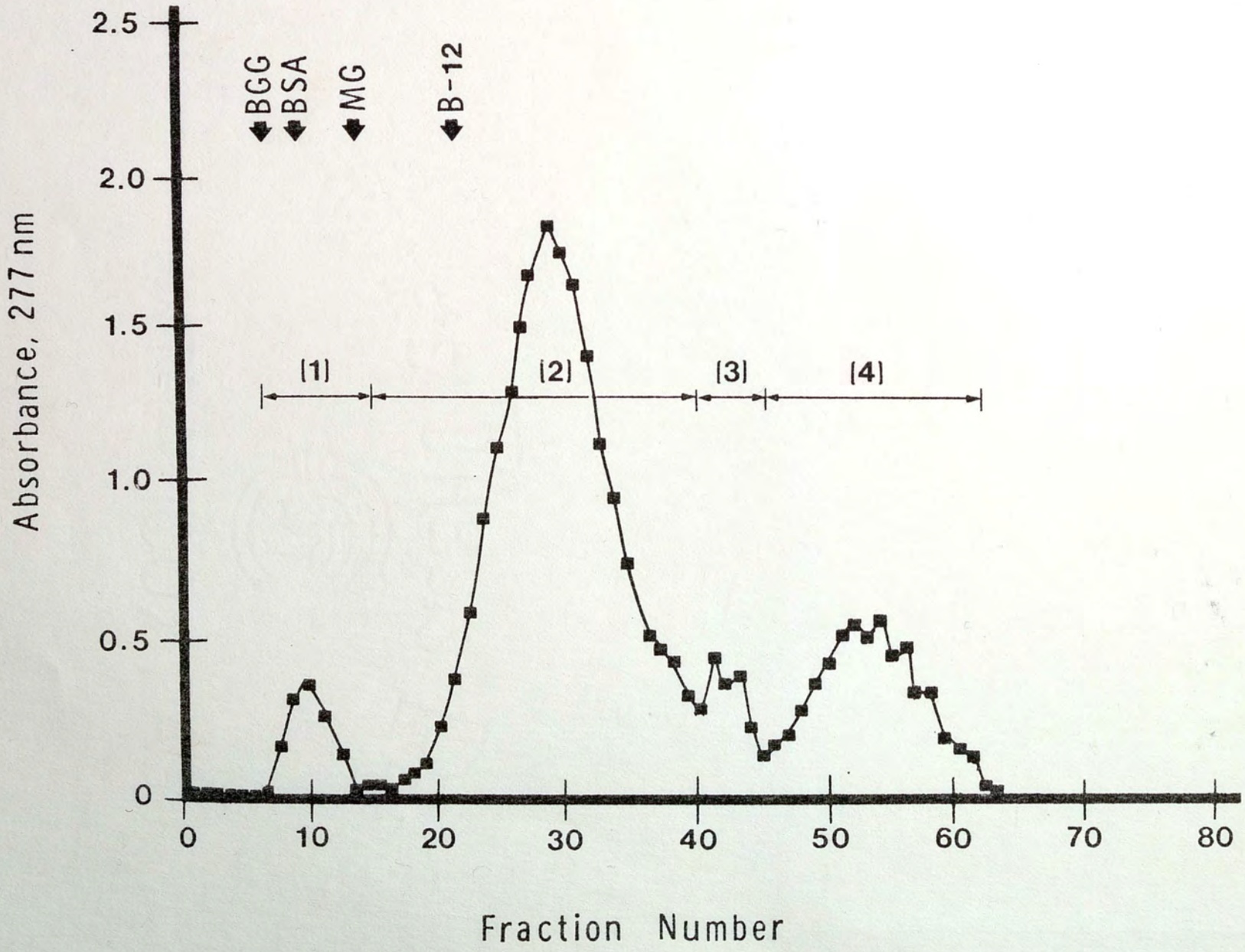

2.5
2.0
1.5
1.0
0.5
0
Absorbance, 277 nm
BGG
BSA
MG
B-12
[1]
[2]
[3]
[4]
0
10
20
30
40
50
60
70
80
Fraction Number

Fig. 16. Separation of proteins in the ammonium bicarbonate extract of Melaleuca (NH4M) by Sephadex G-100 column chromatography. Arrows indicate the elution fractions of the same standards presented in Fig. 15.

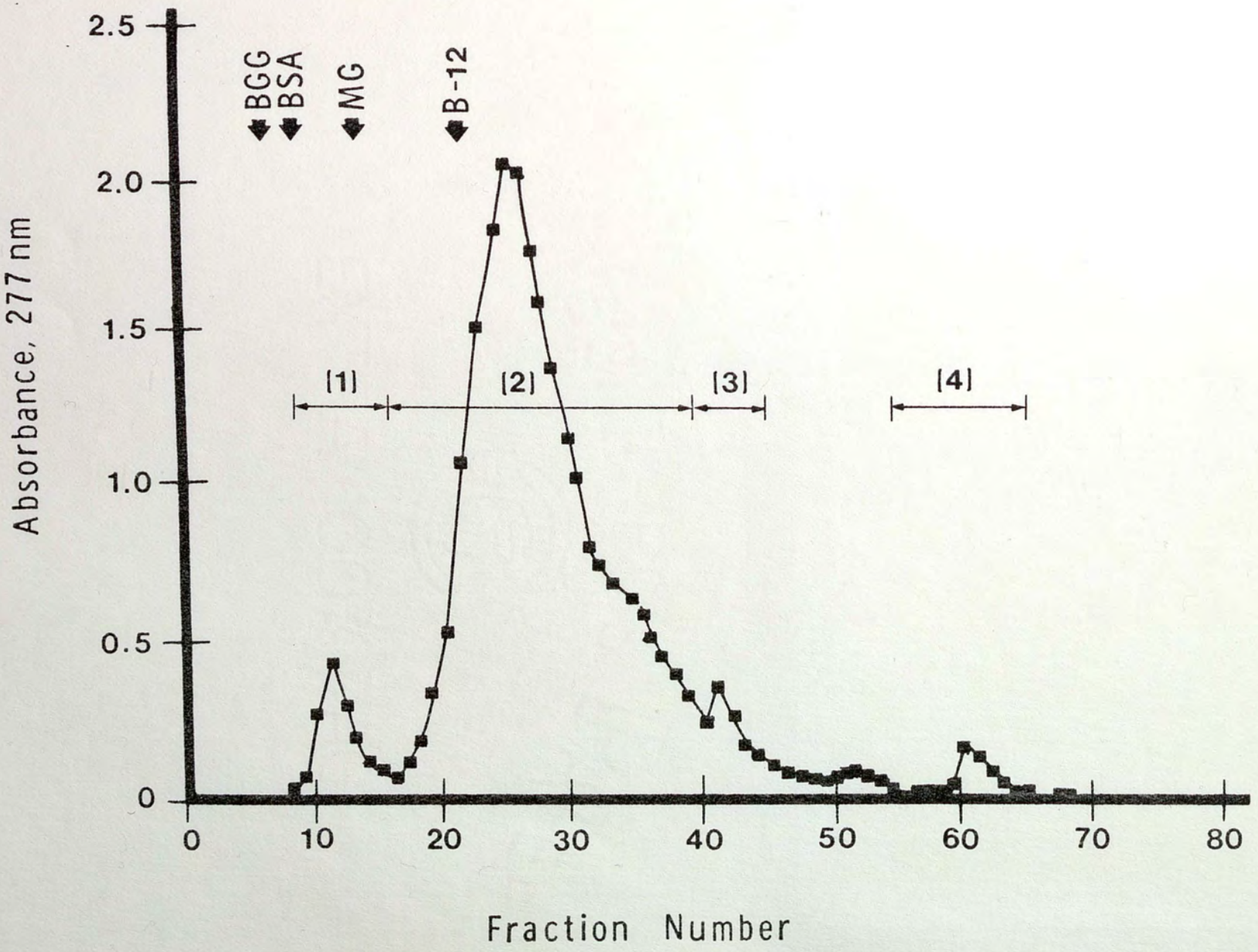
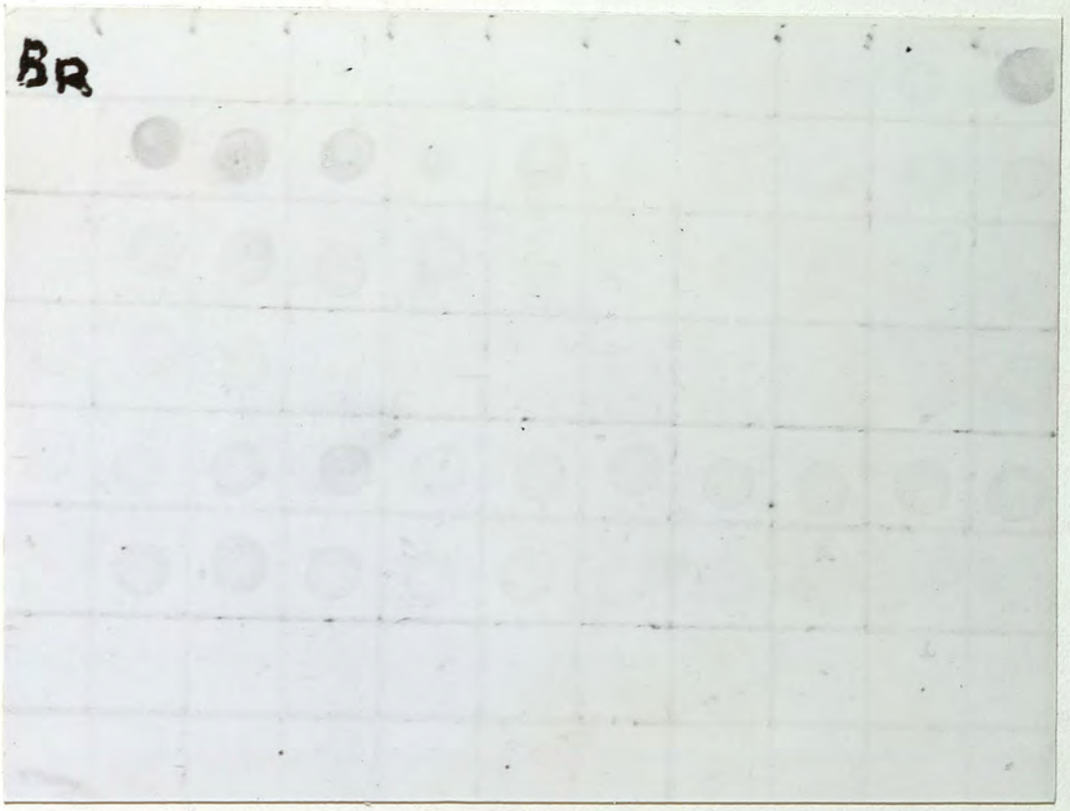

2.5
2.0
1.5
1.0
0.5
0
Absorbance, 277 nm
BGG
BSA
MG
B-12
[1]
[2]
[3]
[4]
0
10
20
30
40
50
60
70
80
Fraction Number

Fig. 17. Immuno-blot analysis of the Bottlebrush (NH_4B) fractions obtained with gel filtration.
A. Photograph. B. Schematic representation of A with intensity of color reactions designated for each fraction as following: ○ very light dot, ◔ light dot, ◐ medium dot on left side of elution peak, ◑ medium dot on right side of elution peak, ● dark dot.

A.

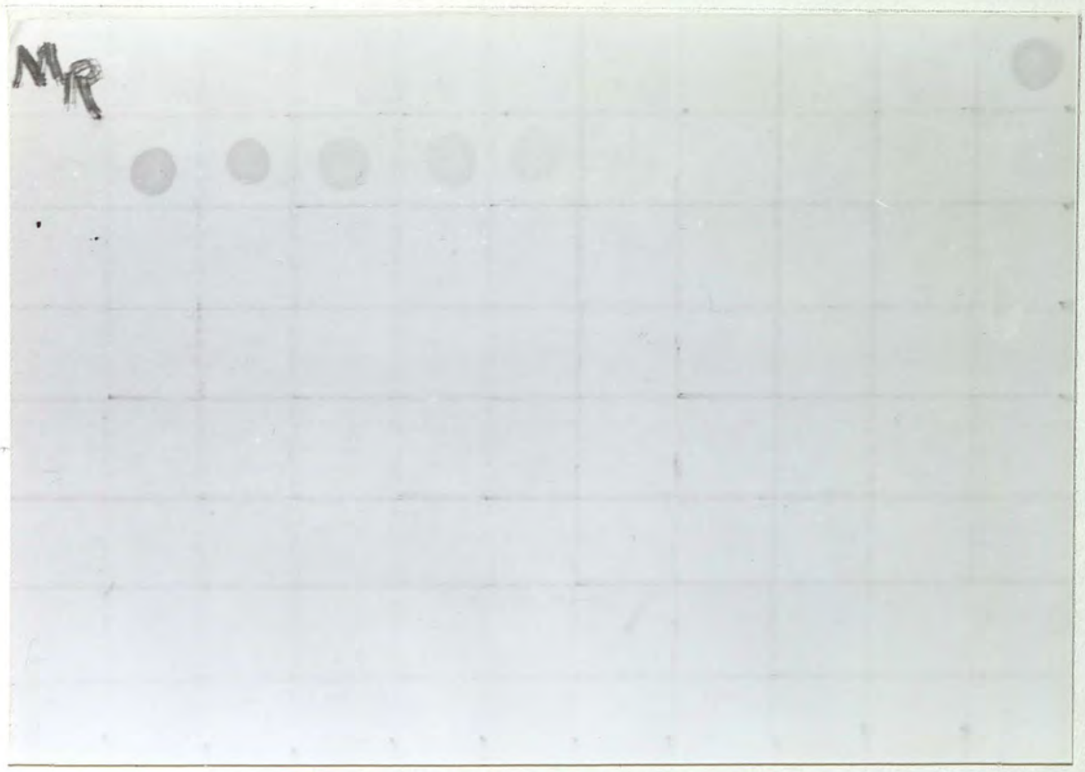


B.

B_R	1	2	3	4	5	6	7	○	◐	●
	●	●	●	◑	◑	○	○	○	◫	◑
	◐	◑	◑	◫	○	26	27	28	29	30
	31	32	33	34	35	36	37	38	39	○
	○	◐	●	◑	◫	○	○	○	◐	◑
	◐	◑	◑	○	55	56	57	58	59	60
	61	62	63	64	65	66	67	68	69	70
	71	72	73	74	75	76	77	78	79	80

Fig. 18. Immuno-blot analysis of the Melaleuca (NH4M) fractions obtained with gel filtration. A. Photograph. B. Schematic representation using symbols as in Fig. 17.

A.

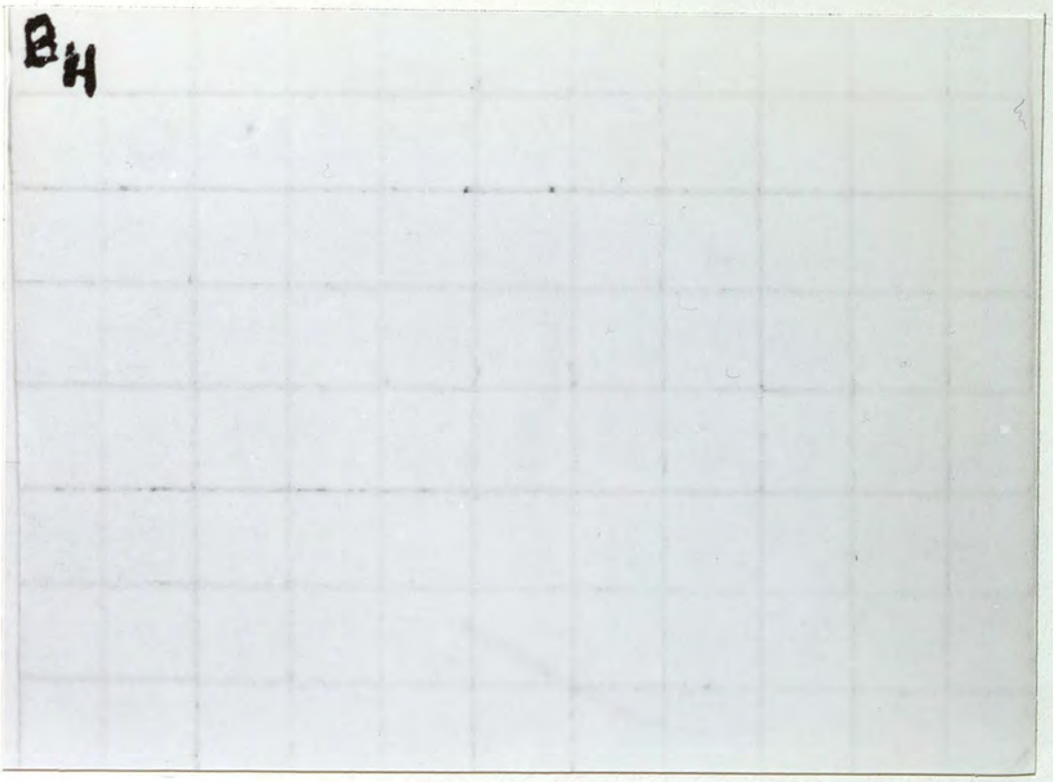


B.

M_R	1	2	3	4	5	6	7	8	◫	◐
	●	●	◑	◑	◑	◫	○	○	○	◫
	○	22	23	24	25	26	27	28	29	30
	31	32	33	34	35	36	37	38	39	40
	41	42	43	44	45	46	47	48	49	50
	51	52	53	54	55	56	57	58	59	60
	61	62	63	64	65	66	67	68	69	70
	71	72	73	74	75	76	77	78	79	80

Fig. 19. Allergo-blot analysis of the Bottlebrush (NH4B) fractions obtained with gel filtration.
A. Photograph. B. Schematic representation using symbols as in Fig. 17.

A.

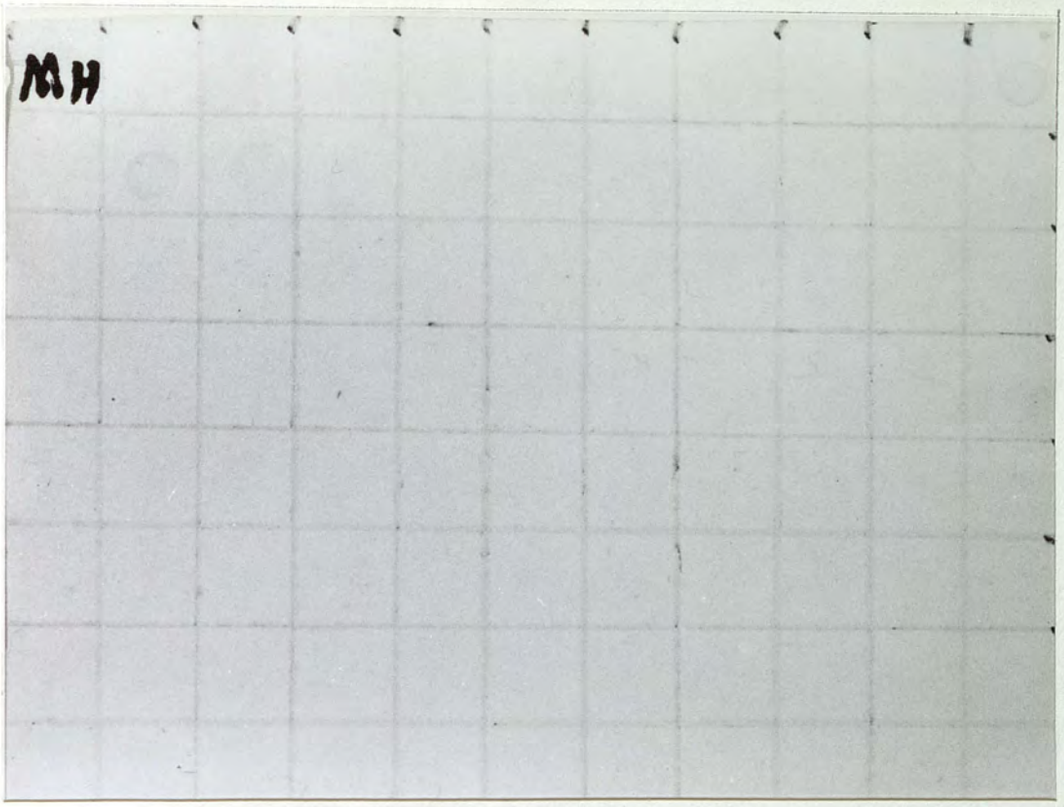


B.

B_H	1	2	3	4	5	6	7	8	9	◯
	◐	◐	13	14	15	16	17	18	19	20
	21	22	23	24	25	26	27	28	29	30
	31	32	33	34	35	36	37	38	39	40
	41	42	43	44	45	46	47	48	49	50
	51	52	53	54	55	56	57	58	59	60
	61	62	63	64	65	66	67	68	69	70
	71	72	73	74	75	76	77	78	79	80

Fig. 20. Allergo-blot analysis of the Melaleuca (NH4M) fractions obtained with gel filtration.
A. Photograph. B. Schematic representation using symbols as in Fig. 17.

A.



B.

M_H	1	2	3	4	5	6	7	8	9	○
	◐	◫	13	14	15	16	17	18	19	20
	21	22	23	24	25	26	27	28	29	30
	31	32	33	34	35	36	37	38	39	40
	41	42	43	44	45	46	47	48	49	50
	51	52	53	54	55	56	57	58	59	60
	61	62	63	64	65	66	67	68	69	70
	71	72	73	74	75	76	77	78	79	80

V. Discussion

Various techniques have been used to characterize Bottlebrush and Melaleuca pollen. These pollens were identified to be allergenically cross-reactive. TCIE analysis determined that the NH4B and NH4M extracts contain multiple antigenic components which were extensively antigenic and cross-reactive (SECTION I). TECIE analysis identified some of these components to be allergenic and also antigenically cross-reactive (SECTION I).

RAST inhibition analysis has greatly enhanced the detection of allergenic cross-reactivity among pollen allergens [7]. Sepharose bound antigens, either Bottlebrush or Melaleuca, were able to competitively inhibit the binding of allergic patient IgE to NH4B and NH4M RAST discs. The variations in % inhibition of IgE binding to NH4B or NH4M discs was in the same range of variability as previously reported for duplicate samples in RAST when Sepharose-BOT or Sepharose-MEL was used as the competitor (Ventrex Laboratories, Portland, ME). These RAST analyses, therefore, indicate the antigenic components of the NH4B and NH4M extracts apparently contain very similar, if not identical, allergenic determinants.

Gel filtration analysis of the NH4B and NH4M pollen extracts identified four peaks. Allergo-blot analysis of gel filtration fractions found the NH4B1 and NH4M1 peaks to contain allergenic reactivity and have estimated molecular weights of 50,000 and 35,000 daltons, respectively. The three peaks not found to be allergenic

would not be expected to be, because they eluted past the molecular weight range of most allergens, which is 10,000-60,000 daltons [11].

The Allergo-blot that we developed was used to detect allergen specific IgE in patient sera. Allergo-blot can detect IgE binding to allergens which are in very low concencentration, such as gel filtration fractions. RAST has previously been reported to be the most convenient technique to identify these fractions [3]. Allergo-blot may be performed more rapidly and less expensively than RAST techniques without loss of sensitivity. By utilizing many extracts from different allergens, this technique can be used as an initial screen for detecting possible allergic reactivity of patients before administration of skin tests, thereby avoiding the risk of possible adverse reactions.

GENERAL DISCUSSION

Allergen characterization for clinical use has become more important within the past few years. The use of different immunochemical techniques in various combinations has facilitated the identification and separation of allergenic components in complex allergenic extracts. These techniques have enhanced the standardization of allergy detection and therapy.

The use of CIE analysis and TCIE analysis in SECTION I demonstrated that NH4B and NH4M extracts contain multiple antigenic components. CIE analysis of the NH4B and NH4M extracts demonstrated the precipitin patterns to be similar in number, although different in the actual position of the bands. TCIE analysis in SECTION I confirmed that Bottlebrush and Melaleuca pollen extracts contain antigenically cross-reactive components. These demonstrations of extensive antigenic cross-reactivity, together with the differences observed in precipitin patterns between the pollens, indicate structural differences of the antigenic components which are formed by very similar or possibly identical antigenic determinants. The use of TECIE for detection of allergenic components in precipitin patterns obtained with CIE and TCIE appears to offer many distinct advantages over CRIE. The shorter development time of TECIE i.e., minutes instead of days, is probably the most important advantage over CRIE. Many TECIEs could be performed within the time it takes to perform one

CRIE, enabling faster identification of allergenic components and greater potential for allergen research. Another advantage is the clarity of CIE pattern development with the use of TECIE. CRIE can often result in blurred development of CIE patterns due to variability in both incubation time of photographic plates and radioactivity of the ^{125}I-anti-IgE antibodies bound to the bands. With a 50 picogram detection limit, TECIE can easily detect all major and possibly most minor allergenic components in precipitin bands with clear development of precipitation patterns. Since the concentration of anti-human IgE peroxidase labeled antibody is 1 to 3000 and this antibody is diluted in a buffer of 1% gelatin, the binding of this antibody to precipitin bands containing bound IgE antibodies is very specific. TECIE is a very sensitive, specific, fast, and inexpensive method to analyze precipitation patterns for allergenic reactivity.

TECIE analysis detected at least three and two allergenic components in the NH4B and NH4M extracts, respectively. These precipitin bands of both CIE and TCIE analysis having allergenic reactivity were among those precipitin bands which had little electrophoretic migration toward the anode. This would indicate then that at pH 8.6 these allergenic components are slightly negative in charge. These same precipitin bands were antigenically cross-reactive since they precipitated in the intermediate gel containing antisera to the cross-reactive pollen.

RAST inhibition studies in SECTION II indicated that the NH4B and NH4M pollen extracts are allergenically cross-reactive. This

cross-reactivity appears to be quite extensive as verified by the similar inhibition curves for each antigen. This information lends credence to the assumption that aerosolized pollen from one type of tree could sensitize an individual to allergenic components to another type of tree without direct contact. Once sensitization has occurred, a direct secondary exposure to a cross-reactive non-aerosolized tree pollen could result in a reaction due to the extensive allergenic cross-reactivity. The extensive cross-reactivity may be used to advantage, since it may be expected that the preparation of suitable tolerogenic derivatives of the allergens of one tree pollen would result also in effective suppression of the IgE antibody responses to the cross-reactive allergen(s) present in the other pollen [12]. Allergenic cross-reactivity between allergens from many species of trees, weeds, and grasses should be characterized if this phenomenon is to be fully understood.

The data presented here demonstrate that there is (are) component(s) of the NH4B and NH4M extracts with molecular weights greater than 35,000 which contain allergenic reactivity. These apparently high molecular weights might be attributable to a high carbohydrate content, which has been shown to vitiate molecular weight determinations of some allergenic components which were calibrated with proteins of known molecular weight [13]. These types of components would not be expected to be a chemical substance like that which has been reported to be the cause of morbidity with Melaleuca trees [26]. It is quite possible that it is a combination of chemical

substance(s) along with pollen components which cause allergic problems to Bottlebrush and Melaleuca pollen.

Studies aimed at isolating pure allergens, utilizing ion exchange chromatography, affinity chromatography, and isoelectric focusing are proceeding.

LITERATURE CITED

1. Aas, K. Clinical and experimental aspects of standardization and purification of allergens. Int. Archs Allergy Appl. Immun. 49:44-54; 1975.

2. Anderson, M. C.; Baer, H. Antigenic and allergenic changes during storage of a pollen extract. J. Allergy Clin. Immunol. 69:3-10; 1982.

3. Aukrust, L.; Grimmer, O.; Aas, K. Demonstration of distinct allergens by means of immunological methods. Int. Archs Allergy Appl. Immun. 57:183-192; 1978.

4. Axselsen, N. H. Intermediate gel in crossed and in fused rocket immunoelectrophoresis. A manual of quantitative immunoelectrophoresis. Methods and applications. Scand. J. Immunol. 2:71-77; 1973.

5. Baldo, B. A.; Chensee, Q. J.; Howden, M. E. H.; Sharp, P. J. Allergens from Plantain (Plantago lanceolata). Int. Archs Allergy Appl. Immun. 68:295-304; 1982.

6. Bernstein, I. L.; Perera, M.; Gallagher, J.; Michael, G.; Johansson, S. G. O. In vitro cross-allergenicity of major aeroallergenic pollens by the radioallergosorbent technique. J. Allergy Clin. Immun. 57:141-152; 1976.

7. Bio-Rad Immun-Blot Assay Kit Instruction Manual GAR-HRP (Goat anti-Rabbit IgG Horseradish Peroxidase Conjugate). Richmond, CA: Bio-Rad Laboratories.

8. Blanchard, G. C.; Gardner, R. The characterization of some of the antigens and allergens in ragweed pollen. Annals of Allergy. 39:253-262; 1977.

9. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt. Biochem. 72:248-254; 1976.

10. Ceska, M.; Eriksson, R.; Varga, J. M. Radioimmunosorbent assay of allergens. J. Allergy Clin. Immunol. 49:1-9; 1972.

11. Chakrabarty, S.; Ekramoddoullah, A. K. M.; Kisil, F. T.; Sehon, A. H. Allergenic relationships of two different antigens of Kentucky Bluegrass pollen. Int. Archs Allergy Appl. Immun. 66:13-20; 1981.

12. Chakrabarty, S.; Lowenstein, H.; Ekramoddoullah, A. K. M.; Kisil, F. T.; Sehon, A. H. Detection of cross-reactive allergens in Kentucky Bluegrass pollen and six other grasses by crossed radioimmunoelectrophoresis. Int. Archs Allergy Appl. Immun. 66:142-157; 1981.

13. Ekramoddoullah, A. K. M.; Chakrabarty, S.; Kisil, F. T.; Sehon, A. H. Isolation of a purified allergen from Kentucky Bluegrass pollen. Int. Archs Allergy Appl. Immun. 63:220-235; 1980.

14. Florvaag, E.; Elsayed, S. Comparative studies on tree pollen allergens VIII. Int. Archs Allergy Appl. Immuno. 75:300-308; 1984.

15. Florvaag, F.; Elsayed, S.; Apold, J. Comparitive studies on tree pollen allergens II. Int. Archs Allergy Appl. Immun. 67:49-56; 1982.

16. Gleich, G. J.; Larson, J. B.; Jones, R. T.; Baer, H. Measurement of the potency of allergy extracts by their inhibitory capacities in the radioallergosorbent test. J. Allery Clin. Immunol. 53:158; 1974.

17. Halmepuro, L.; Vuontela, K.; Kalimo, K.; Bjorksten, F. Cross-reactivity of IgE antibodies with allergens in Birch pollen, fruits and vegetables. Int. Archs Allergy Appl. Immun. 74:235-240; 1984.

18. Ipsen, H.; Lowenstein, H. Isolation and immunochemical characterization of the major allergen of Birch pollen (Betula verrucosa). J. Allergy Clin. Immunol. 72:150-159; 1983.

19. Kroll, J. Tandem-crossed immunoelectrophoresis. Scand. J. Immunol. 2:57-59; 1973.

20. Lawlor, G. J.; Fischer, T. J. Manual of allergy and immunology. Waltham, MA: Little, Brown and Company; 1981.

21. Leiferman, K. M.; Gleich, G. J. The cross-reactivity of IgE antibodies with pollen allergens I. J. Allergy Clin. Immunol. 58:129-138; 1976.

22. Leiferman, K. M.; Gleich, G. J.; Jones, R. T. The cross-reactivity of IgE antibodies with pollen allergens II. J. Allergy Clin. Immunol. 58:140-148; 1976.

23. Lowenstein, H. Quantitative immunoelectrophoretic methods as a tool for the analysis and isolation of allergens. Prog. Allergy. 25:1-62; 1978.

24. Lowenstein, H.; Markussen, B.; Weeke, B. Identification of allergens in extract of horse hair and dandruff by means of crossed radioimmunoelectrophoresis. Int. Archs Allergy Appl. Immun. 51:38-47; 1976.

25. Lum, M. A.; Reed, D. E. Electrophoretic protein transfer applied to immunoelectrophoresis. J. Immunological Methods. 64:377-382; 1983.

26. Morton, J. F. Melaleuca tree causes respiratory distress. Asthma Allergy Almanac. Fall edition; 1980.

27. Renck, B.; Einarsson, R. Crossed radioimmunoelectrophoretic analysis of cultivated rye (Secale cereale) pollen allergens. Int. Archs Allery Appl. Immun. 73:193-197; 1984.

28. Schaeffer, M.; Sisk, L. C. Allergenic extracts: a review of their safety and efficacy. Annals of Allergy. 52:2-11; 1984.

29. Shaflee, A.; Yunginger, J. W.; Gleich, G. J. Isolation and characterization of Russian thistle (Salsola pestifer) pollen allergens. J. Allergy Clin. Immunol. 67:472-481; 1981.

30. Thompson, R. A. Techniques in clinical immunology. Oxford, England: Blackwell Scientific Publications; 1977.

31. Vela, C.; Plates, C.; Gurbindo, C.; Tricas, L.; Subiza, E.; Garcia, R.; Lahoz, C. Fractionation and biological characterization of Olea europea pollen extract. Int. Archs Allergy Appl. Immun. 68:289-294; 1982.

32. Viander, M.; Valovirta, E.; Vanto, T.; Koivikko, A. Cross-reactivity of cat and dog allergen extracts. Int. Archs Allergy Appl. Immuno. 71:252-260; 1983.

33. Vik, H.; Florvaag, E.; Apold, J.; Paulsen, B. S.; Elsayed, S. Comparative studies on tree-pollen allergens IV. Int. Archs Allergy Appl. Immun. 68:371-376; 1982.

34. Vik, H.; Elsayed, S.; Apold, J. Comparative studies on tree-pollen allergens V. Int. Archs Allergy Appl. Immun. 71:40-46; 1983.

35. Vik, H.; Elsayed, S.; Apold, J.; Paulsen, B. S. Comparative studies on tree-pollen allergens. Int. Archs Allergy Appl. Immuno. 68:70-78; 1982.

36. Weeke, B. Crossed immunoelectrophoresis. Scand. J. Immunol. 2:47-56; 1973.

37. Weeke, B.; Lowenstein, H. Allergens indentified in crossed radioimmunoelectrophoresis. Scand. J. Immunol. 2:149-153; 1973.