

STUDIES ON THE NATURE OF ALLERGENIC MATERIAL IN ANIMAL DANDERS AND POLLENS

A PRELIMINARY REPORT

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ONE of the greatest obstacles to progress in allergy is our limited understanding of the nature of antigens. Inherent in this problem is the inadequate measure of composition and uniformity. Recent developments in physicochemical techniques now permit a more reliable assessment of these questions. It has been our purpose, by utilizing such methods as the ultracentrifuge and electrophoresis on filter paper, to pursue this type of investigation and to submit this preliminary report. We have fractionated four selected antigens and have attempted to relate these fractions to clinical sensitivity in an effort to accomplish further resolution of basic questions.

The recent literature reflects the trend toward employment of new techniques of analysis. Abramson and his co-workers^{1, 2} established the place of electrophoretic and centrifugal techniques in the field of allergy. The studies of Spies and those of Coulson^{3, 5, 6} on the chemical nature of cottonseed and castor bean pointed to the necessity of continued chemical studies of antigens. Friedmann⁴ amplified this subject and stressed the relationship to standardization. Loveless⁷ made significant contributions to the understanding of antigen fractionation using the Nielson-Kirkwood electrophoresis-convection apparatus. Dankner and his associates⁸ and Perlman,⁹ working separately, evolved paper chromatographic techniques for fractionation into more basic units of pollen antigens. Bookman and Wax¹⁰ utilized ion exchange resin to fractionate ragweed. Squire¹¹ investigated the relationships between horse dander and horse serum by free boundary electrophoresis. Becker^{12, 13} showed the presence of at least five antigens in ragweed pollen extracts, using agar diffusion technique. Stull and his co-workers¹⁴ were able to fractionate ragweed pollen on the basis of differing water solubility constants. Scherago, Berkowitz, and Reitman¹⁵ studied molecular size of antigens and were unable to relate this to clinical activity. Rimington and Maunsel¹⁶ found evidence for the polypeptide-carbohydrate nature of house dust antigen in their basic chemical studies, while Campbell, Silver, and Vannier¹⁷ utilized paper electrophoresis and chromatography in their studies with dust extracts. Stevens and his associates¹⁸ fractionated ragweed, utilizing the free boundary electrophoresis apparatus. These investigations serve to point out the recent emphasis upon a more fundamental approach to the study of allergenic substances, using newer physicochemical methods. Each represents a step forward in our comprehension of the nature of the materials used in clinical allergy.

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MATERIALS

Four antigens were used in the experiments to be described. Two of these antigens, horse dander and cattle dander, are environmental types and two, *Cynodon dactylon* (Bermuda grass) and *Ambrosia psilostachya* (Western ragweed), represent important pollens in the Southern California area. The environmental material was procured by vacuuming groups of animals, using a clean, new bag for each species. The powdery epidermal material obtained was mixed in Coca's solution¹⁹ and frozen and thawed several times to fragment any intact cellular substance. The solution obtained after simple filtration was precipitated in the cold in 70 per cent acetone according to known techniques.²⁰ The resulting precipitate was dried in vacuo, used in these experiments on a weight by volume basis, and referred to as pure extract. The unprecipitated but clarified extracts served as controls and were referred to as raw dander. The pollen was collected, cleaned by fine-screen sifting, and extracted by grinding 2 grams in 25 ml. of saline in a glass homogenizer. The final extract, after clarification by centrifugation and filtration, was used as concentrated pollen antigen.

METHODS

Nitrogen.—Nitrogen determinations were performed by direct nesslerization on each solution and on each fraction obtained. No attempt was made to differentiate protein nitrogen from nonprotein nitrogen in this study, since it was felt that this would contribute no additional information as to the degree of antigenicity of each fraction.^{6, 10}

Sedimentation.—All sedimentation data were obtained through the use of a Spinco Model L analytical ultracentrifuge. The antigens under observation were suspended in a 0.2 molar solution of sodium chloride, which was found to give optimal results. Under the conditions of these experiments, it was not possible to obtain a Schlieren pattern of the pollen suspensions because of their low molecular weight. This is in accord with previous investigations.^{18, 21} As a result, no sedimentation data are reported for these.

Electrophoretic Fractionation.—Our apparatus was essentially that described by Kunkel²² for filter paper work, with minor modifications dictated by experience. A Spinco-Durum unit²³ was also used on several occasions, but none of the patterns derived are used in this report. After a run, the paper strips were stained and read in a continuous scanning device developed in this laboratory, the details of which will be reported elsewhere.²⁴ Essentially, this consisted of a Beckman Model B spectrophotometer, in which a specially designed unit continuously scanned a series of paper strips and inscribed the changing optical density on an Esterline-Angus strip chart recorder. In a typical experiment, Whatman #3MM paper was used. After the paper had been allowed to equilibrate with the buffer solution, 50 Lambda of allergen, representing 0.5 mg. of nitrogen, was placed at the starting point of each of two strips, which were then superimposed. The buffer solution was Veronal

at pH 8.6, 0.1 ionic strength. It was found, after repeated attempts, that a run of six hours at 200 volts and 12 milliamperes produced satisfactory separation. All runs were made using the double thickness of paper described. One layer was saved for clinical trial, while the other was dried and stained for reading. The two epidermal materials were stained with Brom-Phenol-Blue as described by Block, Durrum, and Zweig.²³ The pollen strips were dried and sprayed with 1 per cent ninhydrin in water-saturated butanol to which pyridine was added to 5 per cent of the final volume. This method was used since it was not possible to prevent spontaneous elution of the pollen material from the paper, despite attempts to fix the antigen by various means. The treated pollen strips were dried in an oven at 100° C. for ten minutes to develop color. It was found that the color was further intensified and stabilized after remaining in total darkness for twenty-four hours. The strips to be used for skin testing were dried and cut into half-inch (12.7 mm.) segments. Drying did not affect results, yet simplified manipulation. Each segment was eluted overnight in the cold in 2 ml. of saline solution. A positive control for each antigen, consisting of routine test material, was eluted from filter paper but not otherwise treated. All eluates were placed in capped vials under sterile conditions and intradermal testing was performed on skin-sensitive persons. All samples were checked for sterility by bacteriologic culture in peptone broth prior to testing. The test samples were coded so as not to prejudice the results. The skin reactions were graded from 0 to a maximum of 4, which represented a wheal at least 1 cm. in diameter. Only Grade 3 and 4 reactions were accepted as valid, since the electrolyte buffer could conceivably induce small wheals as the result of nonspecific irritation.

RESULTS

Epidermal Antigens.—Both the cow and horse dander antigens, raw or acetone-precipitated, yielded an intensely colored dark brown solution when dissolved in saline. This color could not be removed by dialysis against saline for forty-eight hours. The color seemed to be related to protein components in that, after precipitation, the supernate became clear. Unfortunately, this color interfered with the transmission of light through the optical system of the analytical ultracentrifuge unless the sample was greatly diluted. As a result, it is possible that some components were present in concentrations too small to be resolved as peaks in the Schlieren patterns. Contrariwise, this same color intensity enabled us to follow the progress of migration on the filter paper strips in electrophoretic studies for the greater part of the run. At the end of the run, the color would become diffused due to binding, etc. This colored component, however, did not necessarily correspond to the area of the paper electrophoretic strips which would take up the Brom-Phenol-Blue stain, nor did it correspond to areas which gave the greatest skin test activity. Actually, our observations tended to associate those components in the strip immediately preceding the naturally colored areas with maximum response in skin test trials.

Another interesting general observation noted in the results of nitrogen analysis was that the nitrogen content of the precipitated epidermal antigens was always about three times that of the raw, unprecipitated material when compared on a basis of original weight (Table I). The significance of this will be discussed later.

TABLE I

ANTIGEN PREPARATION	TOTAL NITROGEN	NUMBER OF COMPONENTS			ESTIMATED MOLECULAR WEIGHT
		ANALYTICAL ULTRA-CENTRIFUGE	PAPER ELECTRO-PHORESIS	SKIN TEST ACTIVITY	
Acetone-precipitated horse dander	1.5 mg./ml.	1	2 major	1 active	40,000
Raw extract of horse dander	0.59 mg./ml.	2 plus	Multiple diffuse		
Acetone-precipitated cattle dander	2.3 mg./ml.	1	2 major	1 active	32,000
Raw extract of cattle dander	0.83 mg./ml.	2 plus	Multiple diffuse		
<i>Cynodon dactylon</i> extract	0.24 mg./ml.	Diffuse	6 with ninhydrin stain	At least 3 active	Less than 10,000
<i>Ambrosia psilostachya</i> extract	0.33 mg./ml.	Diffuse	7 with ninhydrin stain	At least 3 active	Less than 10,000

In the analytical ultracentrifuge studies, the raw cow dander gave evidence of at least two major peaks only one of which could be demonstrated in the precipitated cow dander solution. The color density previously referred to can be seen sedimenting with the major component (Fig. 1) as a dark area occupying the right side and greater portion of the photographic film. The less intensely colored horse dander antigen shows only one clearly defined peak in both the raw and precipitated states (Fig. 1).

Through calculation of the sedimentation constants for the precipitated epidermal antigens, the average molecular weight was approximately 32,000 for cattle dander and 40,000 for horse dander. This cannot be regarded as much more than theoretical inasmuch as the calculations make assumptions as to molecular size, density, and a spherical shape which is rather unlikely in the case of large molecular weight proteins. Due to lack of a separatory cell, we were not able to obtain the individual components for skin test correlation studies.

A series of experiments were carried out using paper electrophoretic techniques. The results contained in this article cannot reflect the time and trials required before it was possible to obtain controlled behavior of the materials under study. The number of variables is considerable; these range from the type of apparatus itself to variations in current, buffer, time, etc. Various dyes were tried in order to obtain the most clear-cut definition. Even fluorescence was attempted, with interesting but not constant results. The automatic scanning device previously mentioned converted the stained strips

into curves of intensity against distance with more rapid interpretation of results. Under the conditions used, at least three major peaks were obtained with the precipitated cattle dander, while the precipitated horse dander showed two major components. Eluates of one-half inch segments of the strips were tested for skin reactivity in sensitive patients. It was found that positive skin test activity could be correlated with the eluates corresponding with the slowest moving major fraction (Figs. 2 and 3). No electrophoretic data are presented for the raw epidermal material, since patterns obtained with these were diffuse and undifferentiated.

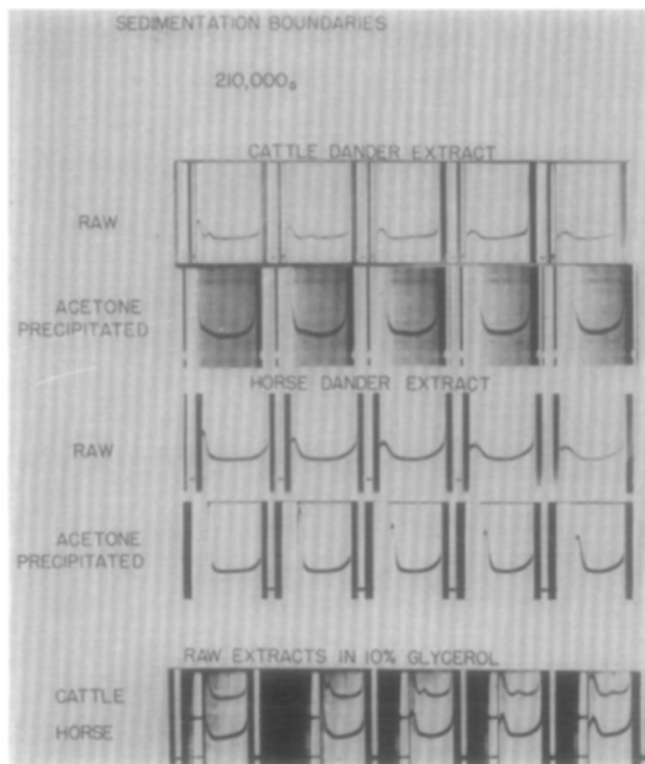


Fig. 1.

During the course of this study, the question of a possible correlation between the precipitated epidermal antigens and the whole serum of both cattle and horse concurrently arose (Figs. 2 and 3). It can be seen that the precipitated antigens tend to follow the migration rate of albumin components of the respective sera.

Pollen Antigens.—Our results indicate that pollen antigens are quite different from the epidermal type. The protein nitrogen content of the pollen extracts was found to be much lower than the nitrogen content in an equal weight of epidermal antigen. Thus, the extract of *Ambrosia psilostachya* contained 0.33 mg. nitrogen per milliliter and *Cynodon dactylon* 0.24 mg. nitrogen

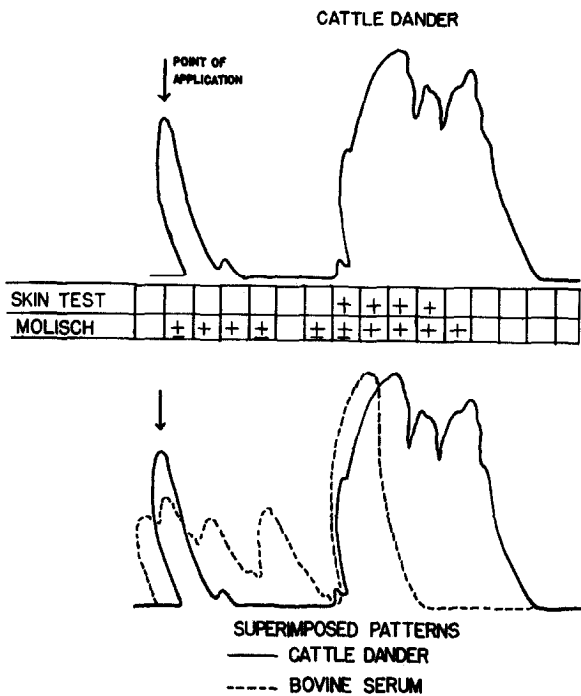


Fig. 2.

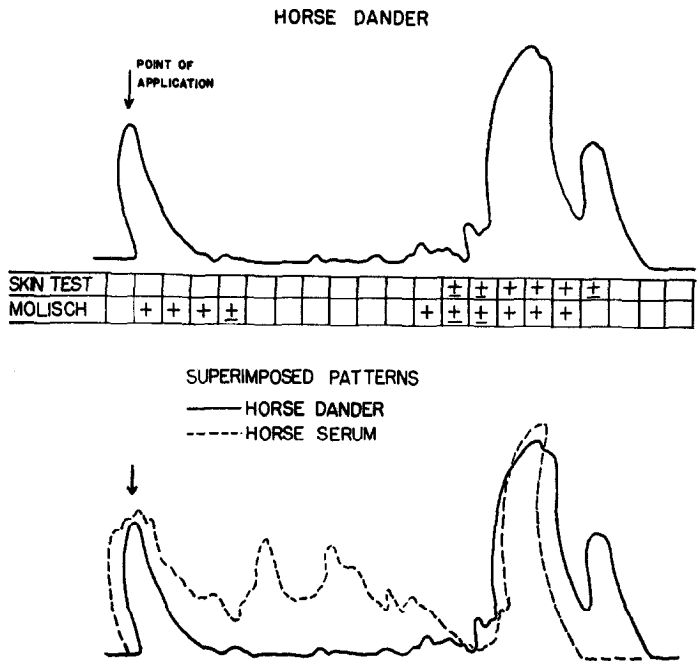


Fig. 3.

per milliliter of solution as compared with 1.5 to 2 mg. nitrogen per milliliter in the epidermal antigen extracts. These and subsequent studies were carried out on undialyzed material, since we were interested in the whole substance and its behavior upon paper electrophoresis.

Using paper electrophoretic techniques, we obtained six components with extracts of *Ambrosia psilostachya* and seven components with extracts of *Cynodon dactylon*. Several of the more slowly migrating components which remained near the original point of application showed similar rates of migration in the case of both pollens. This similarity was not reflected in antigenicity of the eluted fractions. On skin testing of the fractions, however, it was found that no single electrophoretic component was clearly implicated

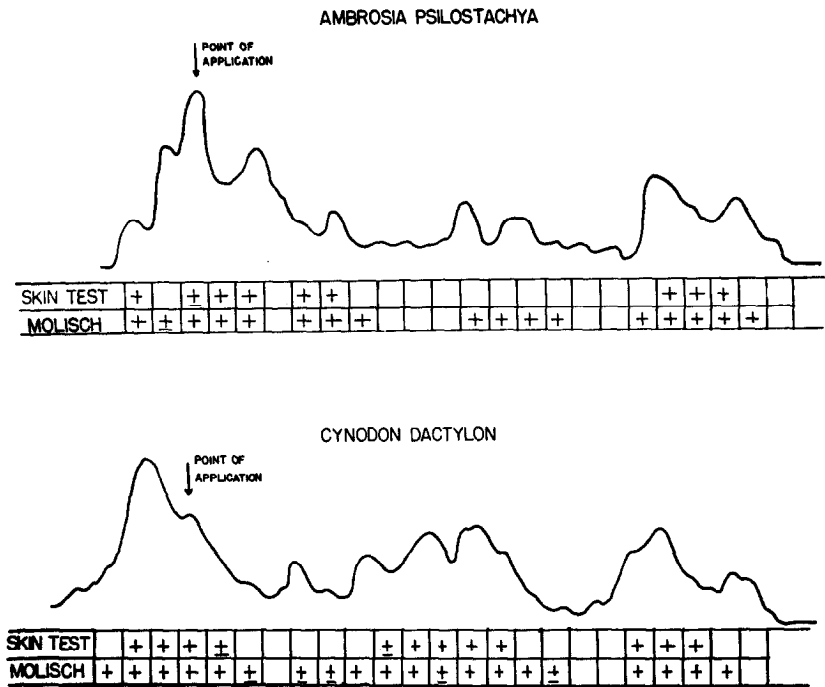


Fig. 4.

as a location of major antigenicity. Usually, two or more strip segments showed skin test activity (Fig. 4). Since considerable attention has been focused upon the existence of polysaccharide complexes in these materials, we routinely performed a Molisch test on all eluates. Generally, strongly positive Molisch reactions occurred in material which showed skin test activity or which showed as stained components on the paper strip. Significant reactions did not occur in either antigenically inactive or unstained areas of the strips. It should be noted that such tests were similarly positive when applied to the active areas of epidermal antigen paper strips.

DISCUSSION

The experimental procedures and the results we have described permit us to form some definite, although limited, opinions regarding the character of the several antigens which were studied. In the case of the epidermal antigens, the purification by precipitation resulted in more homogeneous material. These purified substances appear to be protein in nature with a molecular weight and an electrophoretic migration suggesting that they resemble serum albumin. Comparisons of the electrophoretic fractions with skin tests show that activity resides primarily in one component. However, presence of other major but relatively inactive components in the purified extracts points out the failure of complete isolation of a specific skin-reactive fraction. As noted in our results using the ultracentrifuge, it was necessary to dilute the material in solution in order to reduce the optical density. This procedure may have diluted certain components beyond the resolving power of the Schlieren optics. While we do not believe that this has resulted in the loss of significant fractions of the antigen, the possibility does exist. The threefold increase in nitrogen concentration in the purified epidermal extracts is more apparent than real, since the raw material contained much insoluble matter, such as hair, dirt, etc., which probably contained relatively little nitrogen. The main skin-reactive fractions in the purified epidermal extracts represent only a portion of the total nitrogen content, as can be deduced from examination of the electrophoretic curves, since other main nitrogenous components were not skin reactive. This raises the question of the value of nitrogen as a standard of potency without specific reference to procedures and interpretation.

We are satisfied that filter paper electrophoresis is a valuable tool in this type of study. However, certain features of the final patterns require explanation. It should be noted that all the curves presented are not a truly linear representation of the stained strips. This is due to the mechanical nature of the recorder in which the pen writer describes an arc instead of a straight line. Thus, there is a displacement of the upper portions of the curves to the left. The initial peak at the point of application represents pigmented, insoluble material which was clinically inactive in all instances. This fraction, therefore, may be regarded as an artifact. Also, it is necessary to realize that the patterns we have obtained were produced under highly specific circumstances which gave most clear-cut results in our hands. The electrophoretic patterns of all antigens studied could be reproduced quite consistently. In view of the relatively simple procedure involved, it suggests a better approach to standardization. The same material should yield the same pattern under standardized conditions in the hands of different workers.

Our studies with pollen antigens were limited to filter paper electrophoresis primarily. The value of the ultracentrifuge in this instance was unfortunately limited to more easily resolved substances which have molecular weights of the order of 10,000 or more. On electrophoretic separation, the pollens characteristically possessed more fractions than the epidermal antigens. This is a quite logical result of the use of crude pollen extracts, whereas the epidermal

antigens were subjected to purification procedures. There appeared to be no component specificity in that positive skin tests could be elicited with several fractions. This would support the validity of using crude pollen extracts clinically with our present limited knowledge of their composition. These observations suggest that there are inherent pitfalls in applying purification procedures to pollen extracts. Alteration or loss of the multiplicity of active components can occur; yet this loss may conceivably be masked by the remaining active fractions.

As a supplement to these observations, we have begun an investigation of the role of polysaccharides as factors in antigenicity. The stimulus for these supplementary studies is derived from the observation that skin test activity was as intimately related to carbohydrate content of electrophoretic fractions as it was to protein.

SUMMARY AND CONCLUSIONS

1. Two epidermal antigens and two pollen antigens in common clinical use were studied with particular emphasis upon ultracentrifuge and filter paper electrophoresis.

2. Fractionation of the epidermal antigens was achieved by ultracentrifuge, but the low molecular weight of the pollen antigen did not permit fractionation by this method. The molecular weight of horse dander antigen was estimated to be approximately 40,000 and that of cattle dander antigen 32,000. Molecular weights for the pollen antigens were found to be less than 10,000.

3. Electrophoretic separation was accomplished with all four antigens, the epidermal type showing fewer components than the pollen type. In relating skin sensitivity to electrophoretic components, activity appeared to be concentrated in a rather narrow area in the epidermal antigens, whereas the activity of the pollen antigens was more widely distributed.

4. The significance of these findings is discussed.

REFERENCES

1. Abramson, H. A.: Chemical, Physical and Immunological Properties of Electrophoretically Purified Extracts, *Ann. Allergy* 5: 19, 1947.
2. Abramson, H. A., Moore, D. H., and Gittner, H. H.: Electrophoretic and Ultracentrifugal Analysis of Hay-Fever-Producing Component of Ragweed Pollen Extract, *J. Phys. Chem.* 46: 192, 1942.
3. Spies, J. R., and Coulson, E. J.: The Chemistry of Allergens. VIII. Isolation and Properties of an Active Protein-Polysaccharide Fraction, CB-IA, From Castor Beans, *J. Am. Chem. Soc.* 65: 1720, 1943.
4. Friedmann, R. Augustin: Chemical and Immunological Properties of Timothy Grass Pollen Extracts and Problems of Standardization, *Quart. Rev. Allergy* 6: 290, 1952.
5. Coulson, E. J., Spies, R. S., Jansen, E. F., and Stevens, H.: The Immunochemistry of Allergen. VIII. Precipitin Formation and Passive Transfer Reactions With Allergenic Proteins From Cottonseed and Castor Beans, *J. Immunol.* 52: 259, 1946.
6. Coulson, E. J., Spies, R. S., and Stevens, H.: The Immunochemistry of Allergens. IX. The Relationship of Carbohydrate to the Antigenic Properties of the Allergenic Protein From Cottonseed, *J. Immunol.* 62: 171, 1949.
7. Loveless, M. H.: Fractionation of Ragweed Pollen by Dialysis and Electrophoresis-Convection (Nielsen-Kirkwood Apparatus), *J. ALLERGY* 21: 262, 1950.
8. Dankner, A., Bukantz, S., Johnson, M. C., and Alexander, H. L.: Fractionation of Pollen Extracts by Chromatography. I. Preliminary Studies With Ragweed Pollen, *J. ALLERGY* 22: 437, 1951.

9. Perlman, E.: Chromatographic Analysis of Ragweed Pollen Extracts, *Bull. New York Acad. Med.* **27**: 586, 1951.
10. Bookman, R., and Wax, H.: Fractionation of Ragweed Antigens by the Use of Ion Exchange Resins, *J. ALLERGY* **25**: 12, 1954.
11. Squire, J. R.: The Relationship Between Horse Dandruff and Horse Serum Antigens in Asthma, *Clin. Sc.* **9**: 127, 1950.
12. Becker, E. L., and Munoz, J.: Multiplicity of Antigens in Ragweed Pollen as Demonstrated by the Technique of Oudin, *Proc. Soc. Exper. Biol. & Med.* **72**: 287, 1949.
13. Becker, E. L.: Quantitative Studies in Skin Testing; The Assay of Ragweed Extracts by Means of Scratch Test Utilizing an All or None Response, *J. ALLERGY* **19**: 108, 1948.
14. Stull, A., Sherman, W. B., and Hampton, S. F.: Antigenic Fractions in Ragweed Pollen; Water-soluble Fractions, *J. ALLERGY* **12**: 117, 1941.
15. Scherago, M., Berkowitz, B., and Reitman, M.: Standardization of Dust Extracts. I. Standardization on the Basis of Equal Molecular Size, *Ann. Allergy* **8**: 437, 1950.
16. Rimington, C., and Maunsel, K.: A New Approach to the Problem of Dust Allergy, *Internat. Arch. Allergy and Appl. Immunol.* **1**: 115, 1950.
17. Campbell, D. H., Silver, D. Z., and Vannier, W.: Immunochemical Studies of House Dust Allergen, *J. ALLERGY* **25**: 73, 1954.
18. Stevens, F. A., Moore, D., and Gelston, T.: The Major Electrophoretic Component of Giant Ragweed Extracts: Molecular Weight, Chemical and Biological Characteristics, *J. ALLERGY* **22**: 356, 1951.
19. Coca, A. F.: Studies in Specific Hypersensitiveness. V. The Preparation of Fluid Extracts and Solutions for Use in the Diagnosis and Treatment of the Allergies With Notes on the Collection of Pollens, *J. Immunol.* **7**: 163, 1922.
20. Cavelti, P. A.: Complement Fixation Studies in Allergy, *J. ALLERGY* **21**: 532, 1950.
21. Abramson, H. A., and Moore, D. H.: A Discussion of Electrophoresis With Special Reference to Serum and Allergens, *J. Lab. & Clin. Med.* **26**: 174, 1940.
22. Kunkel, H.: "Zone Electrophoresis" in *Methods of Biochemical Analysis*, David Glick, Ed. New York, 1954, Interscience Publishers, Inc., vol. 1, pp. 141-170.
23. Block, R. J., Durrum, E. L., and Zweig, G.: *A Manual of Paper Chromatography and Paper Electrophoresis*, New York, 1955, Academic Press, Inc., Part II, Paper Electrophoresis, pp. 333-409.
24. Silver, D. Z., and Bookman, R.: A Rapid Automatic Scanning Device for Paper Electrophoresis and Chromatography. (Accepted for publication in *Analytical Chemistry*.)