

ALLERGENIC FRACTIONS OF LOW RAGWEED POLLEN. II. SOME IMMUNOLOGIC, ELECTROPHORETIC, AND CHEMICAL CHARACTERISTICS OF DIFFUSATES*†

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THERE has been considerable speculation as to the molecular size of pollen excitants. Abundant confirmation was found for the early claim of Grove and Coca⁶ that the activity did not diffuse to any important degree through cellophane. Of the opposite opinion were Unger, Cromwell and Moore,²¹ Johnson and Rappaport,⁷ Spain and Newell,¹⁷ Benjamins, Dishoeck, and German,³ Long and Teller,⁸ and, more recently, Robbins, Samuels, and Mosko,¹⁴ Stone, Harkavy, and Brooks,¹⁹ Cohen and Friedman,⁴ and Stevens and Moore.¹⁸ Stull, Sherman, and Wing²⁰ found activity in both diffusible and nondiffusible fractions prepared by salting-out, a situation also encountered by the writer during ultrafiltration studies.¹¹ From the latter 2 laboratories came the additional observation that the susceptibility for smaller substances varied with the patient, some failing to respond altogether, whereas the larger molecules were allergenic without exception. That this selectivity rested on specificity differences was demonstrated by serologic cross-tests.

Abramson, Moore, and Gettner¹ concluded from ultracentrifugal and diffusion studies that the major allergen of undialyzed ragweed extract had a molecular weight of 5,000, was unpigmented and essentially immobile in the electric field (electrophoresis). Rockwell,¹⁵ using chemical methods instead of electrophoresis for his separations, agreed with Abramson and colleagues as to molecular size. Sanigar,¹⁶ however, noted marked heterogeneity in the ultracentrifuge and could only conclude that most of the pollen material was below 17,000 in molecular weight. Newell,¹³ attempting to rid the extract of the low-molecular excitant of Abramson, subjected it to 20 hour dialysis through cellophane before analyzing it electrophoretically. The pattern, nevertheless, resembled that of Abramson's undialyzed preparation. When Newell examined in this apparatus the 3 fractions salted out by Stull, one of which ("Fraction 2") was dialyzable while the others were not, he obtained for each the familiar pattern—a major, immobile, colorless constituent, and a minor, mobile, pigmented group. Thus, a dilemma arose as to the nature of Abramson's major component, since he reported it to be dialyzable, whereas Newell was unable to eliminate it through 20 hour dialysis and found a similar component in all of Stull's fractions.

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Differences in the immunizing potentialities of the diffusible and non-diffusible portions of low ragweed pollen have been examined by the Roosevelt Hospital group²⁰ and the writer.¹¹ In both studies it was found that the non-diffusible material was far more efficient in producing blocking antibodies and clinical resistance than was the dialyzable material. Finally, chromatographic separations have revealed¹¹ that the ultrafiltrate contains several active pigments of different specificity.

The situation to date could be summarized as follows: Small dialyzable constituents in ragweed pollen differ in specificity from nondiffusible components. They can provoke clinical allergy in most, but not all, patients with a history of autumnal hay fever who are reactive to whole extract. They are ineffectual in developing clinical resistance and thermostable (blocking) antibodies, behaving like haptens. To date, no evidence has been published to show that the diffusible and nondiffusible portions of the extract differ from each other electrophoretically. Such evidence will be presented in this article. From the chemical viewpoint, Abramson considered his major fraction to be protein-like although it contained carbohydrate. Likewise, Stull decided that the nitrogenous portion of his dialyzable fraction 2 was the important immunologic substance, albeit some samples gave a positive Benedict's test. The author found both precipitable nitrogen and Molisch-positive constituents in her ultrafiltrates.

The relationships between molecular size and the chemical, electrophoretic, and allergenic nature of ragweed pollen antigens are, therefore, far from clear. This is due to the complexity of the problem, which has induced many workers to concentrate on the separatory phase at the expense of immunologic and other tests for purity. It was the purpose of the present investigation to converge the 2 aspects. Pollen dialysate, for example, has been visualized for the first time in the Tiselius apparatus. Preliminary electrophoretic comparisons have been made between the diffusate, the dialyzed sac contents, and whole extract. In addition, the dialysate has been separated into fractions by means of electrophoresis as well as by chromatography, and specificity differences have been shown to exist between them by means of immunologic cross tests. Not only has the significance of the small molecular material for the patient been extensively appraised by direct sensitivity tests, but its immunizing potentialities have been re-explored by therapeutic injections given man. Its stability has been observed under freeze-drying and under heat. The nature and degree of the loss of material and activity from the extract during dialysis have been investigated under controlled conditions for the first time.

MATERIAL AND METHODS

All extraction and separatory steps were carried out in the 4° C. coldroom with precautions against contamination by molds and bacteria. Periodic tests for sterility allowed the prompt interruption by filtration of any contamination.

Allergenic Solutions.—With 2 exceptions all pollen had been collected under the "gold seal" precautions of Hodgson during the autumns of 1947 and 1948. Whole extracts were made by exposing approximately 20 Gm. of ether-defatted, low ragweed pollen (*Ambrosia elatior* or *A. artemisiifolia*)

to 100 ml. of phosphate buffer of .06 ionic strength at pH 7.4 for 2 hours with mechanical stirring, or for 42 hours without stirring. In 1 large set of conjunctival comparisons between dialysate and our "standard" whole extract, the preparation differed from other whole extracts only in that alkaline-saline solution had been used as extractant. Each extract was sterilized by passage through bacterial filters of the Seitz or sintered glass variety, and labelled according to the date of its analysis and its content of phosphotungstic-acid precipitable nitrogen, 0.1 mg. representing 10,000 PTA-N units.

Dialysates and filtrates were prepared from whole extracts in 2 ways. The first was the conventional procedure of placing a measure of extract inside a Visking cellophane (sausage casing) sac against 1 to 3 parts of either distilled water or phosphate buffer for a period ranging from 16 to 87 hours. The other was the application of gentle suction with a water filter pump under aseptic conditions to a cellophane sac placed within a dry sterile suction flask, until most of the fluid had been drawn out of the sac during 17 days. The product was labelled "vacuum dialysate" (or "filtrate"). In 1 instance a secondary dialysate was made by re-exposing the sac contents to a different buffer; namely, whole extract of Jan. 31, 1949, was first exposed to 2 volumes of phosphate buffer for 18 hours and subsequently to 3½ volumes of barbital buffer, ionic strength 0.1, pH 7.9, for 46 hours.

In most of the earlier studies, once an initial diffusate had been prepared for testing, the balance of the voluminous dialysates was discarded. In an effort to rid the sac contents of all diffusible molecules, running water had been employed for about 5 days and electro dialysis for 72 hours. So striking were the losses of material and activity from the sac contents during these prolonged dialyses that it was decided to modify one set of experiments by saving aliquots of dialysate at each stage in order to see whether these losses were referable to a mere shift of materials or to degradation processes. Thus, in Experiment XVII 4 identical preparations of extract were exposed to 1 or 1½ parts of distilled water for 2½ days, to provide initial dialysate. Next, 36 L. of distilled water were allowed slowly to pass the sac under a hydrostatic head of about 2 feet of water which maintained continuous negative pressure outside the sac. (This secondary diffusate was referred to as "hydrostatic" dialysate.) Finally, electro dialysis was carried out on the sac contents by surrounding the sac with 16 L. of continuously circulating distilled water which was changed 3 times during 96 hours. Meantime, a current of 27 Ma., which gradually fell to 6 Ma., was used at 105 volts. Samples were removed from the various diffusates and sac contents at each stage so that activity and chemical studies could be done. Aliquots of the highly diluted dialysates were dried in the frozen state so that they could be reconstituted later in small volumes.

Stability of whole extract XIV, whole standard extract, and vacuum dialysate was achieved by addition of an equal volume of glycerine, whereas the sac contents of Experiment XVII, like the dialysates, were kept in the lyophilized state. Whole extract XII and sac contents XI, like the first and second dialysates of Jan. 31, 1949, were unpreserved but were tested within a few months of their preparation.

Chemistry.—All fractions, with the exception of the small yields from the electrophoretic apparatus, were analyzed for PTA-precipitable nitrogen and nonprecipitable nitrogen by the micro-Kjeldahl method, as well as for combined and free reducing sugars by a slightly modified Folin-Malmros procedure.⁵ Assays on secondary dialysates were considered less reliable than on the other solutions, due to their small content of solids. The aim of the semiquantitative analyses was to establish roughly the chemical nature of our incompletely purified fractions and to determine whether simple diffusion or enzymatic breakdown was responsible for the loss of activity from the dialyzing sac.

Activity Tests.—Potency was judged by means of serologic, conjunctival, and intracutaneous tests carried out in a controlled manner.⁹ Although our original intention was only to determine, by any of these methods, the approximate dose of our several derivatives which would provoke comparable responses in a given patient, we subsequently attempted to gain an over-all impression of each fraction by grouping the combined results of the 3 techniques under 5 headings, namely, +++, ++, +, ±, and 0.

In preliminary Prausnitz-Küstner experiments, we found that +++ responses could be elicited with one-third of the serum specimens when 40 unit unglycerinated whole extract or sac contents was selected for the test. To procure this proportion of marked reactions with dialysate, however, it was necessary to use 160 unit material. The serum of each patient was accordingly examined with 0.03 ml. of these 2 strengths, and the potency of the 3 unglycerinated preparations was judged by the relative number of ++, +, and negative responses each provoked.

Whereas a single test with 0.03 ml. of 160 unit unglycerinated dialysate served for the crude classification of patients during several months by intracutaneous test, more careful examinations were carried out with glycerinated whole extract by means of serial strengths which located the minimal reactive dose. In order to include these results in the composite analysis, the threshold values were later assorted into 5 classes, based on the response to 0.03 ml. of 1 unit whole extract, a yardstick which placed a little over one-third of the patients in the +++ class. The need to select so dilute a test material confirms the observations that the intracutaneous test is more sensitive than is the Prausnitz-Küstner method and that glycerine preserves pollen activity.

The conjunctival test was also carried out in such a manner as to indicate the thresholds of susceptibility for unfractionated and derivative antigens. The results were again sorted roughly into 5 groups according to the thresholds. As in the case of the Prausnitz-Küstner results, a fourfold handicap had to be allowed dialysate.

In addition to estimating the capacity of dialysate to combine with preformed reagents by the preceding means, we also recorded any systemic manifestations which appeared following the therapeutic use of vacuum dialysate in 8 patients.

Dialysate and sac contents were heated in a water bath at 60° C. and 100° C. for 1 hour and then examined serologically for potency. The effect of freeze-drying was likewise examined by this technique. Specificity differences in the

various dialysates, sac contents, and starting materials were examined by means of cross-neutralization studies carried out in normal test subjects. Twenty-four hours after a series of intracutaneous injections had been made with sensitizing serum to which a pollen fraction had been added in excess, the sites were tested with the homologous allergen and with other fractions. Whereas in vitro neutralization of the homologous allergen had usually occurred, as evidenced by the lack of response to its reintroduction in high concentration on the following day, fractions which differed from it in specificity could still elicit a wheal-and-flare response in high dilution. The amount of PTA-nitrogen required to provoke this cross-reaction was then compared with the amount needed to reproduce the urticarial result in a site which had received only serum and saline solution. The nonirritating quality of the antigen was established by its concurrent use in high concentration in areas of normal skin. By comparing these 3 reactions, it was possible to classify the specific cross response as being maximal, half-maximal, $\pm$, or negative.

Therapeutic Procedure.—Eight dialysate-allergic patients were given a single therapeutic injection of vacuum dialysate XIV in a dose ranging from 1,600 PTA units to 3,000 units. Just before use, the undiluted filtrate containing .77 mg. PTA-nitrogen per c.c. was mixed with 6.7 parts of the patient's own serum, in the hope that specific spontaneous conjugation would take place and render this haptenlike preparation more antigenic as far as immunization was concerned. In 2 control cases saline solution was substituted for the patient's serum. The dialysate was then stirred with mineral oil and Falba to make a stable, water-in-oil emulsion which was then placed in a subcutaneous depot, an abbreviated immunization procedure which the author has found effective for whole pollen extract.¹⁰ All but 2 of the subjects also received whole extract or sac contents in another such depot. The immunologic response to this complementary therapy was followed by monthly eye tests, and such clinical resistance as developed was compared with that of past seasons when depot therapy with whole ragweed extract alone had been involved.

The antibody response to whole extract, with its dialyzable constituents intact, was judged by monthly eye tests with both vacuum dialysate and whole extract on 84 patients who had been treated with unfractionated extract in emulsified state.

Electrophoretic Studies.—Patterns were obtained on samples of whole extract XII, electrodialed sac contents XI, and first dialysate. Heretofore, characterization of dialysates of pollen has not been accomplished, presumably because of their ready dialyzability, which complicates the interpretation and leads to marked loss of material from the sample during its equilibration against a large volume of the buffer to be used in the run. This loss was discouraged by the use of 7 thicknesses of cellophane and by restricting the period of equilibration to 3 hours. The result was only partially satisfactory, since the sample was brought to a resistance of 300 ohms, whereas the buffer registered 467 ohms. The possible influence of this disequilibration on the pattern will be discussed. Whole extract and sac contents were equilibrated by exposure to 250 volumes of phosphate buffer for 19 and 67 hours, respectively.

Whereas the unfractionated extract and electrodialed material were analyzed in their original concentrations, first dialysate had been reduced to dryness in the frozen state and taken up in one-fifth its original volume of distilled water so as to increase the concentration of its solids. Preceding its exposure to the Tiselius buffer, this dialysate was protected by 3 thicknesses of cellophane and suspended in 9 volumes of distilled water for 30 minutes in order to lower its phosphate salt content.

In all electrophoretic runs phosphate buffer at pH 7.5 and ionic strength 0.1 was employed with a current of 13.5 Ma. at 175 to 200 volts. The experiment was interrupted after 135 minutes in the case of whole extract and after 153 minutes in the case of sac contents, whereas first dialysate was observed for 357 minutes to see whether its major stationary component would move. Mobilities were not calculated due to the low peaks of the pattern of the unconcentrated samples and the disequilibrium of the dialysate, but the relative areas of mobile and immobile components were estimated for each sample.

At the conclusion of the runs on sac contents and dialysate, a small sample of the fastest- and of the slowest-moving material was removed from the cell for immunologic cross-testing.

Chromatography.—Several hundred c.c. of the distilled water dialysates, XVII No. 7, No. 8 and No. 9, were adjusted to pH 5 as in earlier absorptions,¹¹ and each was allowed to seep through 3 to 5 Gooch crucible holders which had been packed with mixtures of alumina and Supercel and connected in vertical series. The 3 yellow bands of adsorbed material were dug out and eluted in phosphate buffer at pH 8. Only a small portion of the adsorbed pigment was removable. Eluates were nevertheless analyzed for specificity differences and potency by the serologic technique.

FINDINGS

Chemistry.—The nitrogen and carbohydrate contents of the extract dwindled as dialysis proceeded. The dialysates procured by allowing 1 or 1½ volumes of distilled water to stand against whole extract for 2 to 3 days robbed the cellophane sac of two-thirds of its nonprecipitable nitrogen, one-half its reducing sugars, one-fourth of its PTA-nitrogen, and 10 per cent of its combined sugars. These averages based on 4 similar preparations are shown in the lower lines of Table I. In estimating the material balance between the sacs and their initial dialysates, it was found that all but 10 per cent of the original precipitable nitrogen had been recovered but that 30 per cent of the combined sugars had been lost from the system. At the same time, there appeared to be a compensatory gain in the smaller nitrogenous and reducing substances, indicating that larger molecules had been converted into smaller ones.

After "hydrostatic" dialysis against 30 to 35 L. of distilled water had been allowed to proceed for an additional 2 or 3 days, the losses through the cellophane had mounted; after a final exposure of the extracts to electro dialysis for 96 hours, only 22 to 31 per cent of the original combined sugars remained in the sacs of XVII No. 2, No. 6, No. 7, and No. 8 (Table II). The precipitable nitrogen dropped to between 13 and 21 per cent, the reducing substances to

TABLE I. DISTRIBUTION OF NITROGENOUS AND CARBOHYDRATE CONSTITUENTS OF WHOLE RAGWEED EXTRACT BETWEEN INITIAL DIFFUSATE AND CELLOPHANE SAC CONTENTS
(AFTER 2 TO 3 DAYS OF DIALYSIS AGAINST 1 OR 1½ VOLUMES OF DISTILLED WATER)

STARTING MATERIAL	RELATIVE VOLUME OF DIALYSANT	TOTAL RECOVERY OF MATERIAL FROM DIALYSATE AND SAC CONTENTS (EXPRESSED AS PER CENT)							
		COMBINED SUGARS (EXPRESSED AS GLUCOSE)		PTA-PRECIPTABLE NITROGEN		REDUCING SUGARS (EXPRESSED AS GLUCOSE)		NONPRECIPITABLE NITROGEN	
		DIALYSATE	SAC CONTENTS	DIALYSATE	SAC CONTENTS	DIALYSATE	SAC CONTENTS	DIALYSATE	SAC CONTENTS
Whole XVII No. 2	1	7	47	21	61	35	80	48	53
Whole XVII No. 7	1½	13	66	33	60	64	72	57	65
Whole XVII No. 8	1½	13		29		53		80	
Whole XVII No. 9	1½	5		29		53		85	
Average dialysate		9½		28		52		68	
Average sac contents			56		60		76		59
Average recovery from dialysate + sac contents		66		88		128		127	

between 7 and 9 per cent, and the nonprecipitable nitrogen was present only in traces (1 to 4 per cent). Although the missing materials were roughly accounted for in the accumulated dialysates, the figures for all but the initial preparations were considered doubtful due to the small amount of solids in the samples and the large factors by which the results had to be multiplied.*

TABLE II. PER CENT OF ORIGINAL EXTRACT REMAINING IN SAC CONTENTS AFTER ELECTRODIALYSIS OF RAGWEED EXTRACTS XVII

EXTRACT NUMBER	COMBINED SUGARS (GLUCOSE) (PER CENT)	PTA-N (PER CENT)	REDUCING SUGARS (GLUCOSE) (PER CENT)	NONPRECIPITABLE NITROGEN (PER CENT)
2	22	13	7	4
6	25	18	7	4
7	30	16	9	2
8	31	21	8	1
Range	22-31	13-21	7-9	1-4

Since, for reasons to be given later, the PTA-N and combined sugars (CS) would theoretically be better guides to potency than would the smaller molecular constituents (nonprecipitable nitrogen and reducing sugars), it will be of interest to know the relationships between the 2 in various fractions. The PN:CS ratio in whole extract XVII was about 1:5. More PTA-N than CS escaped into the initial dialysate, bringing its ratio to 1:1½. The ratio was overcorrected in the hydrostatic dialysate, being found at 1:7. At the conclusion of electro-dialysis, the final product showed a marked reduction in PTA-N, while the CS had been lost at about the same rate as in the preceding, hydrostatic sample, the PN:CS ratio now being 1:18 in the electro-dialysate.

The technique followed in preparing an initial dialysate appeared to influence its chemical make-up. Phosphate buffer, for example, withdrew as much nonprecipitable nitrogen as did distilled water but was less efficient in extracting PTA-nitrogen and particularly in bringing out carbohydrates, as shown by comparing first dialysate with the XVII dialysate in Table III. Secondary exposure of the sac contents used for first dialysate to barbital buffer at pH 7.9 yielded a diffusate with equal content of combined sugars but with only one-sixth as much precipitable nitrogen per cubic centimeter as its predecessor. The most striking differences were encountered when simple suction was used to procure filtrates for whole extract. Since no dilution was involved, naturally all constituents were present in higher concentration than when a dialysant was used. The ratio, however, between precipitable nitrogen and combined sugars was nearly 1:5, whereas in the initial dialysates of XVII, it did not exceed 1:2. About 40 per cent of each of these 2 components had apparently been pulled out of whole extract XIV without disturbing their original relationship. It will be recalled that only 10 per cent of the combined sugars initially escaped during conventional dialysis (Table I), whereas the loss of PTA-N was nearly 30 per cent. Whole extracts XIV and XVII, like their electro-dialyzed sac contents, did not differ significantly, showing that the manner of preparation of the initial diffusates was responsible for the chemical differences rather than some variable in the pollens used.

*Detailed analytical results will be supplied on request.

TABLE III. CHEMICAL COMPOSITION OF INITIAL, SECONDARY, AND VACUUM-PREPARED DIFFUSATES OF LOW RAGWEED EXTRACT

PREPARATION	RELATIVE VOLUME OF DIALYSANT	DIALYSANT	DURATION (HOURS)	CHEMICAL COMPOSITION OF DIALYSATE			
				COMBINED SUGARS (MG. GLUCOSE/C.C.)	PRECIPITABLE NITROGEN (MG./C.C.)	REDUCING SUGARS (MG. GLUCOSE/C.C.)	NONPRECIPITABLE NITROGEN (MG./C.C.)
XVII No. 7, First dialysate	1½	Aqua destillata	87	0.5	0.24	2.1	0.25
First dialysate of 1/31/49	2	Phosphate buffer, pH 7.5	18	0.2	0.15	0.9	0.26
Second dialysate of 1/31/49	3.2	Barbital buffer, pH 7.97, ionic .1	46	0.2	0.025	0.3	0.04
Vacuum dialysate XIV	suction	none	17 days	3.6	0.77	4.5	0.58

Activity Tests.—As a preliminary to comparisons between sac contents and dialysates, experiments were set up to determine whether the chemical differences noted previously between vacuum dialysate XIV and first dialysate in phosphate buffer influenced their potencies. Among 22 patients concurrently tested with the 2 by conjunctival test, only 6 individuals showed any difference in the 2 threshold requirements. From 4 to 6 times more PTA-nitrogen was demanded of first dialysate by these exceptional cases. Since, as will be shown later, certain active substances were present in vacuum dialysate which were lacking in the first dialysate, the occasional difference noted in potency may have been the outcome of selective susceptibility to the extra antigens.

More extensive comparisons of potency were now drawn between one or other of the dialysates and whole extracts by means of serologic, conjunctival, and intracutaneous tests done on members of an experimental group of 125 ragweed-allergic patients. As shown in Table IV, Prausnitz-Küstner experiments on the pretreatment sera of Group A revealed that one-third of them could be made to give +++ responses to original extract if this allergen were injected in a strength of 40 units. First dialysate, on the other hand, had to

TABLE IV. SHOWING THAT LESS PTA-N IS REQUIRED FOR SKIN, EYE, AND PRAUSNITZ-KÜSTNER RESPONSES TO WHOLE RAGWEED EXTRACT OR ELECTRODIALYZED SAC CONTENTS THAN TO DIALYSATE

	PTA-N UNITS PER C.C.	CASES	SENSITIVITY CLASSES (PER CENT)				
			+++	++	+	±	NEGATIVE
<i>Serologic (Prausnitz-Küstner) Tests in Group A</i>							
Whole XII	40	15	27	40	33		
First dialysate of 1/31/49	160	35	31	26	20		22
Sac contents XI	40	15	40	47	13		
<i>Intracutaneous Tests in Group B</i>							
First dialysate	160	57	32	14	17	17	20
Whole "standard," glycerinated	1	23	43	17	17	22	
<i>Conjunctival* Tests in Group C</i>							
First dialysate	160	31	6	26	19	23	26
Whole XIV, glycerinated	40	31	16	45	26	13	
<i>Conjunctival* Tests in Group B</i>							
Whole XIV, glycerinated	40	14	28	43	29		
Sac contents XIV, glycerinated	40	14	51	43	7		
<i>Conjunctival* Tests in Entire Membership</i>							
Vacuum filtrate XIV, glycerinated	160	125	31	26	16	18	8
Whole "standard," glycerinated	40	125	26	43	22	9	

*Basis of classification: strength of solution giving rise to threshold reaction in eye.

	Class +++	Class ++	Class +	Class ±	Negative
Whole:	40 U or less	80 to 160	320 to 640	1280 to 10,240	
Dialysates:	160 U or less	320 to 640	1280 to 2560	5120 to 20,480	40,480

be employed in 160 unit strength to provide a corresponding number of marked reactions. The adoption of these 2 strengths as the standard test agents provided positive results with the mother solution in all instances, but negative reactions in 22 per cent of trials with dialysate. When sac contents were used in 40 unit concentration, a somewhat higher proportion of sera gave +++ reactions than with whole extract, indicating that the PTA-nitrogen left in the sac after electro dialysis had greater potency than that in the original extract.

Fifty-seven individuals were examined by direct intracutaneous tests. As in the case of the serologic studies, 160 unit dialysate served to classify patients, since it provided 32 per cent of +++ responses. There were 20 per cent of negative reactions. However, when 1 unit strength of our standard glycerinated whole extract was chosen as a basis for segregation, more cases fell into the +++ category than with 160 unit dialysate, and there were no negative results.

The data accumulated during conjunctival tests of the threshold variety on 31 individuals were handled somewhat differently. When the patients were listed in the order of their susceptibility, it was found that one-third of them reacted to glycerinated whole extract XIV in a strength of 40 units or less, whereas the test with first dialysate had to be carried to the 160 unit preparation before one-third of the group showed the standard reaction. The basis of distribution for the rest of the group, who were less highly allergic, is stipulated in Table IV. It also shows that no responses were encountered in 26 per cent of the group when dialysate was employed, whereas whole extract was active in all subjects without exception. Fourteen subjects evaluated with sac contents proved more reactive to this fraction than to the mother material in equal strength.

The entire membership of 125 was included in the final comparisons between standard whole extract and vacuum filtrate, both of which had been stabilized by glycerination. Using the same system of classification as that just described for conjunctival tests, it was found that at least 4 times more PTA-nitrogen usually had to be allowed for dialysate than for whole extract to elicit a comparable response. Whereas about one-third of the patients were classified as having marked allergy for dialysate, nearly 10 per cent of the members proved to be insusceptible to this fraction; all were affected by whole extract of 10,240 unit strength or less. Patients showing no conjunctival erythema until 40,960 unit vacuum filtrate had been instilled were considered unresponsive. Table IV shows that a lower incidence of negative reactions was obtained for vacuum filtrate than for conventional dialysate, a difference which might be referable to the more complex chemical and antigenic structure of the former, and to its stabilization with glycerine.

These relationships between the threshold doses of whole extract and of vacuum dialysate have been presented in the form of frequency curves (Fig. 1) and cumulative frequency curves (Fig. 2). Both show that more nitrogen is in general required of dialysate than of the mother solution, the distribution curves for the fraction being shifted to the right. No threshold response was encountered, for example, to a solution containing less than 40 units of dialysate per c.c., whereas 10 unit material was effective in a small proportion of the tests with

whole extract. At the other extreme, it was found that only 2 patients required antigen as strong as 10,240 units for their responses to whole extract, whereas 25 members needed from 10,240 to 40,960 for the comparable response to dialysate.

It will be seen in Fig. 2 that 90 per cent of the 125 patients responded to whole extract by the time 640 unit dilution had been instilled, whereas 20,480 unit dialysate had to be reached before 90 per cent of them reacted to this derivative. Since the end points in the conjunctival test can be reproduced within 1 tube and often within half a dilution step, this difference of 5 dilution tubes has definite statistical significance.

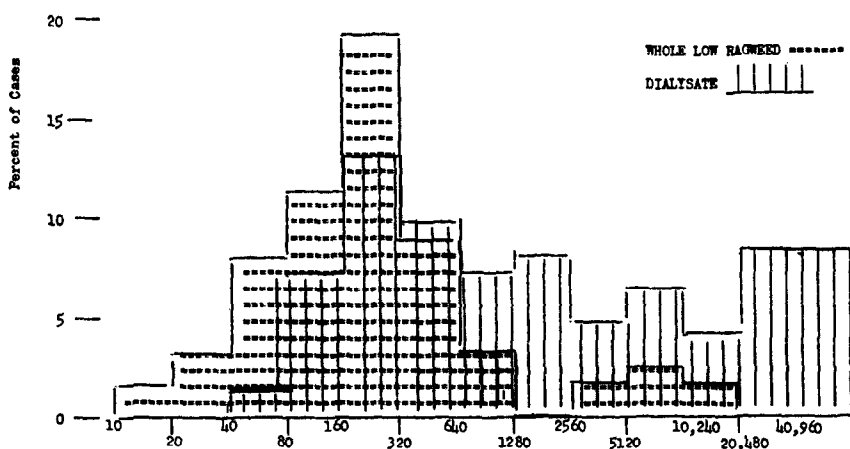


Fig. 1.—Threshold of conjunctival reaction to dialysate and to whole low ragweed extract in 125 patients prior to booster therapy.

Although a change in activity has obviously been brought about by fractionation, specificity differences must also be involved. The basis for this claim is that the over-all shapes of the curves for the two test solutions are different. It is therefore not tenable to assume that they differ merely in the potency of their PTA-N, that inert nitrogen from the mother solution has diluted the active nitrogen of the dialysate. The reason for the altered shape of the diffusate curve became evident when the individual threshold doses determined for the 2 materials were examined for successive members of the group. For 56 patients with marked susceptibility to filtrates, the dialysate: whole extract ratios ranged from $\frac{1}{8}$ to 40, with a median value of 3. At the other extreme were 10 patients who showed little or no response when tested with dialysate 40,960. Their dialysate: whole extract ratios ranged all the way from 4 to 500, with a median value of 85. Meanwhile the absolute values for whole extract did not exceed 320 among the first group, but were never less than 1,280 for the latter group. Thus, there was a general trend for the susceptibility to increase for 1 solution as it did for the other, but the dialysate: whole extract relationship varied widely with the individual.

The difference in shape of the 2 curves would presumably have been more pronounced had conventional dialysate rather than the more complex vacuum preparation been used for these comparisons.

A single antigenic mixture in the 2 solutions could not give rise to these wide variations. If the dialysate antigens were identical with those of whole extract but had been diluted with inert nitrogenous material which escaped during dialysis, then the shapes of the 2 distribution curves should be alike, and the dialysate data should have been merely shifted to the right by its lower potency. If, on the other hand, individual specificities were involved in the dialysate and in the sac contents, the observed difference in shape between mother extract and fraction would be expected.

Fig. 2 makes it clear that the median requirement was 160 for whole extract, 640 for dialysate, whereas the most probable requirement for either solution was 160 unit strength (Fig. 1).

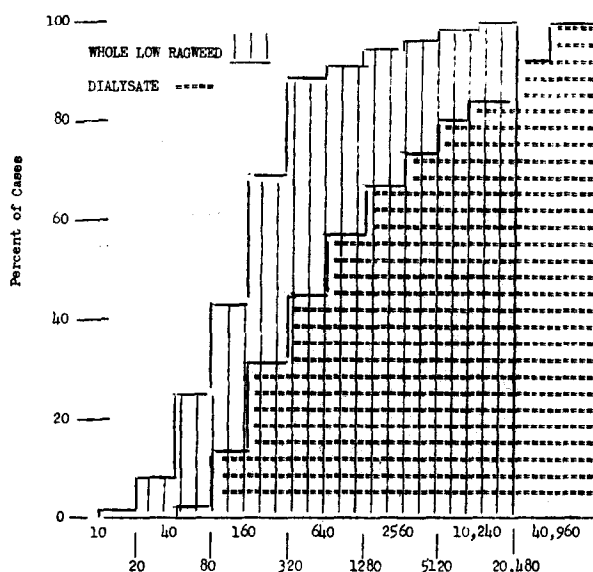


Fig. 2.—Threshold of conjunctival reaction to dialysate and to whole ragweed extract in 125 patients prior to booster therapy (cumulative).

Serologic and conjunctival comparisons for potency were carried out with the yields of conventional dialysis at its several stages. The initial dialysate of XVII possessed about twice the potency per PTA-N unit as the hydrostatic sample, and from 3 to 6 times the activity of the vacuum dialysate of XIV when both were examined in several Prausnitz-Küstner experiments as well as in 9 sets of eye tests. It was also stronger than the electro-dialysate, from $1\frac{1}{2}$ to 32 times less PTA-N being required of it for conjunctival responses in 10 patients, the median value being 4. Its potency in Prausnitz-Küstner studies appeared about 25 times greater than that of the final dialysate.

A puzzling situation arose when such comparisons were made between the first and second dialysates of the Extract of Jan. 31, 1949. Volume for volume, the activity of the secondary yield (in barbital buffer) was distinctly greater (tenfold) than its predecessor in phosphate buffer, in spite of the fact that it contained only one-sixth as much PTA-N. Furthermore, during neutralization

experiments it appeared that 120 units of second dialysate sufficed to exhaust the sensitizing power of a ragweed-sensitive serum (RO), whereas 1,280 units were required of first dialysate. No explanation was found for this reversal of the trend noted previously with the distilled water dialysates to become less potent per PTA-N unit as dialysis continued. It might be assumed that the 2 buffers played some selective role.

Heating of initial dialysates of XVII in a water bath at 60° C. and 100° C. had no observable effect on their potency, as judged by the minimal dose required for Prausnitz-Küstner responses. Electrodialyzed sac contents XVII No. 3, on the other hand, showed a loss of perhaps one-half its activity following exposure to 60° C. and further damage after 100° C.

Freeze-drying apparently did not reduce the potency either. The allergenic activity of the initial 1:2 dialysate of XVII No. 2 was as great after being lyophilized 1 or 4 times as it was in the original state. The same situation obtained for the initial and the hydrostatic samples of XVII No. 7 when the once-dried samples were compared with the 4 times lyophilized samples. Furthermore, no impressive differences in potency were noted between Prausnitz-Küstner reactions of 35 sera to fresh first dialysate (Table IV) and the responses of several sera to the lyophilized samples of XVII dialysates at a later date.

In addition to the foregoing evidence for the capacity of dialysates to combine with preformed sensitizing antibodies in the serum, skin, and eyes of certain pollen-allergic patients, confirmation was given by 2 subjects who developed prompt systemic reactions following slight overdosage with therapeutic injections of diffusate.

Cross Tests for Immunologic Specificity (First and Second Dialysates).—In spite of their chemical and potency differences, neither the first and second dialysates nor the initial, hydrostatic, and electrodialysates of XVII could be differentiated from each other in cross tests. Table V shows that a site of normal skin, previously injected with a mixture of serum and excess dialysate, reacted negatively to test with either the homologous or the heterologous dialysate in high concentrations. The same relationships obtained for the successive diffusates of XVII. By contrast, any such site would readily respond to retest with dilute sac contents or whole extract, indicating the satisfactory state of the area and showing that the dialysates were simplified fractions. Sites which had been injected with serum-saline mixtures responded to very small amounts of precipitable nitrogen when second dialysate, sac contents, or whole extract were injected, whereas many more gamma of precipitable nitrogen per cubic centimeter had to be used for equivalent results with first dialysate. None of the testing solutions was nonspecifically irritating, as control tests in normal areas revealed.

Cross tests between heated and unheated samples of XVII dialysates showed that the heat had failed to reduce their antigenic complexity, the diffusates heated to 100° C. exhausting a sensitized site completely toward retest with either the 60° C. heated specimen or the unheated solution. Similar experiments with heated sac contents XVII suggested that certain of the nondiffusible

antigens were more effected by heating than others. A similar situation was encountered in the case of XVII dialysates following their exposure to freeze-drying, especially when it was repeated as often as 4 times. This destruction of some of the antigenic components was not sufficiently widespread to be reflected in the less delicate potency tests referred to previously.

TABLE V. SEROLOGIC CROSS TESTS SHOWING ANTIGENIC SIMILARITY OF FIRST AND SECOND DIALYSATES AND THEIR SIMPLIFICATION OVER SAC CONTENTS AND WHOLE EXTRACT (TEST SUBJECT SW; REACTION TO 24 HOUR TEST WITH ANTIGEN)

		1st Dialy- sate (mg Pta-N/cc)	2d Dialy- sate (mg Pta-N/cc)	SacContents XI (mg Pta-N/cc)	Whole Ex- tract XII (mg Pta-N/cc)
Antigen mixed with RO Serum	1st Dial- ysate	0.15 0	0.02 0	0.0004 1½	0.00015 3-
	2nd Dial- ysate	0	0	2	3-
	Serum- saline	.015= 2½ .150= 3	.0001= 2½ .0030= 3	.0001= 3 .	.00015= 3
	Normal skin con- trol	.150= ±	.0300= 0	.0004= 0	.00150= 0

Sac contents, dialysate and whole extract: Table VI covers the cross test results of first dialysate, electrolyzed sac contents XIV, and whole extract XII, all of which were prepared within a few weeks' time. As would be expected, a serum-antigen mixture containing an excess of the starting material failed to respond next day to test with either of the derivatives, sac contents and a diffusate of either the conventional or vacuum type. On the other hand, sites neutral to sac contents still responded partially to whole extract or either of the filtrates. In the reverse test, a vacuum filtrate site was one-half reactive to whole or sac contents but not to first dialysate. The vacuum diffusate therefore must have lacked activities present in whole extract and in sac contents, but contained all the antigens which made up the initial dialysate. When the 2 dialysates were used in the opposite order, a half-maximal wheal developed after vacuum filtrate was introduced. This indicated that it was less simple than first dialysate, confirmation for which was found in the nearly maximal results obtained with whole extract and with sac contents in these first dialysate sites,

whereas only partial responses had been detected in the vacuum dialysate sites. To all appearances, suction is a less desirable means of preparing diffusates than is conventional dialysis.

Three sera bearing no sensitizing power for dialysates were mixed with large amounts of first dialysate and were subsequently found to be maximally reactive to cross test with sac contents and with whole extract, showing again that the nondiffusible specificities differed importantly from the diffusible.

TABLE VI. DIFFERENCE IN SPECIFICITY OF DIALYSATE, SAC CONTENTS, AND UNFRACTIONATED EXTRACT OF LOW RAGWEED POLLEN (TEST SUBJECT HEL; RESPONSE TO 24 HOUR TEST)

		Whole Extract	SacContents	Vac.Filtrate	1stDialysate
Antigen mixed with BAU Serum in vitro	Whole Extract XII	0	0	0	0
	Sac Contents XIV	1/2—	0	1/2—	1/2—
	Vacuum Filtrate XIV	1/2	1/2—	0	0
	1st Dialysate	max—	max—	1/2	0
		BAU (mg Pta-N/cc)	BAU (mg Pta-N/cc)	BAU (mg Pta-N/cc)	BAU (mg Pta-N/cc)
		Serum-salt	Serum-salt	Serum-salt	Serum-salt
		.00014 = 3½	.00008 = 2, .0008 = 2½	.0008 = 2, .0016 = 2½	.01280 = 0, .15400 = 2½
		Normal skin	Normal skin	Normal skin	Normal skin
		.10000 = 0	.01800 = 0	.01280 = 0	.01280 = 0, .15400 = ±

After obtaining the same cross results among whole extract, sac contents, and filtrates with 6 different dialysate-allergic sera, it was concluded that common as well as individual activities were possessed by each derivative. In other words, although diffusible had been separated from nondiffusible antigens during dialysis, the process had not been carried quite to completion, residual specificities of the small molecular type still being detectable in the sac contents after electro-dialysis. At the same time, certain large molecular substances remained exclusively behind in the sac contents. Sera neutralized with either of the simplified derivatives could still respond to the starting extract by virtue of

its more complex makeup; but the reverse test was always negative. Thus, dialysis effected separation into 2 composite fractions with some overlapping between the 2.

When sac contents and filtrate were both added to the mixture, there was no response to later tests with whole extract, indicating that original specificities had been retained throughout fractionation.

Immunizing Power of Dialyzable Components.—Eight patients with ++ or +++ dialysate-allergy were given vacuum filtrate therapeutically in a large single dose (1,600 to 3,000 PTA units, median 2,000) which was mixed with the individual's own serum in 6 instances and with saline solution in 2 control cases. Each mixture was incorporated into a water-in-oil emulsion before being injected into a subcutaneous depot. One patient in each group developed a mild, classical, systemic reaction of overdosage. One subject in each category received no therapy in addition to the depot, whereas the rest of the patients were also given a later injection with a water-in-oil preparation of whole extract. Judged by periodic conjunctival tests, the antibody response to dialysate during the next several months were neither negative nor consistently positive. The maximal increase in the threshold requirement of vacuum dialysate noted for the 2 controls given filtrate-saline depots amounted to 2 and 6, respectively. Three subjects given serum-filtrate mixture showed ratios no higher than 2, which we considered of no significance; 1 showed a change of threefold, and another of 12 fold. One patient appeared to have increased his sensitivity, for his threshold dose dropped threefold. The ratios for the entire group ranged from $\frac{1}{3}$ to 12, median $2\frac{1}{2}$. The 6 subjects meanwhile were responding rather better to their depots of whole extract or sac contents, maximal ratios for the whole extract test solution ranging from 2 to 32, with a median value of 4. Thus, by inference, blocking antibodies were formed somewhat more efficiently for the mother solution than for the fraction following the use of a large, complementary dose of vacuum dialysate in addition to whole extract or sac contents.

These ratios were compared with others concurrently set up for 39 ++ and +++ dialysate-allergic patients who took only whole extract in their single depot injections. Vacuum filtrate was used in conjunctival tests to determine their threshold doses during the subsequent few months and revealed that 61 per cent of the group remained unchanged, with ratios of 1 or 2, whereas for 29 per cent these ratios ranged between 3 and 12, and for 10 per cent reached 16 to 64. Thus, although the experimental group of 8 was too small to permit final conclusions, it appeared that the serum-dialysate mixture had been somewhat less efficient than whole extract in setting up immune responses toward the diffusible antigens of ragweed pollen.

It was our impression, also, that the patients tolerated larger doses of filtrate when it was mixed with reaginic serum than with saline solution. We had predetermined for each serum-dialysate mixture approximately how much "free" dialysate was available per cubic centimeter by evoking comparable conjunctival responses with it and with plain dialysate in the patient who was to receive the mixture. At least 80 per cent of the activity appeared to have

been bound by the 6 sera. The author has shown that pollen reagin (admixed with extract) can prevent the allergen from eliciting responses in the allergic eye,¹² presumably by binding the antigen in vitro.

It was incidentally observed, among a larger group of 84 whole extract-treated patients with various reactivities to filtrate, that 8 developed an increase in susceptibility for diffusate by eye test, the degree ranging from 3 to 30 fold. The ratios of increased resistance for whole, meantime, ranged from 1 to 170 in this large group, with a median value of 8, showing that unfractionated extract was a more effective immunizing agent than dialyzable antigens whether or not they had been admixed with reaginic serum (median ratio, $2\frac{1}{2}$).

The clinical immunity of the filtrate-inoculated patients did not appear changed significantly from earlier years when depots of whole extract had been administered in equal or smaller doses. If these preliminary findings with injected serum-filtrate mixtures and with filtrate-containing whole extract can be confirmed, some explanation must be sought for the failure of diffusible antigens admixed with reaginic serum to enhance clinical immunity, in the face of their demonstrated capacity to develop conjunctival resistance in a sizable minority of patients given the mother solution without serum. It is, of course, possible that our reaginic serum covered up the active groupings of these haptens and thereby discouraged antibody responses to the serum-filtrate mixtures. One might anticipate that such a mechanism would, however, also have operated in vivo during treatments with whole extract, thereby preventing the observed increases in conjunctival tolerance. Further studies are indicated.

Electrophoretic Patterns.—In Fig. 3, comparison can be made between the electrophoretic patterns of undialyzed whole extract and of a concentrated sample of first dialysate. The dialysate is less complex in constitution, containing only 2 well-defined peaks, whereas whole extract contains a broadly distributed spectrum of mobile pigmented components in addition to the large immobile peak.

The marked lack of symmetry in the ascending and descending patterns, as well as in the pigmented peak, may be in part due to imperfect equilibration of the fraction with the buffer. It will be recalled that dialysis against the Tiselius buffer was curtailed in duration and modified by the use of 7 thicknesses of cellophane in order to discourage loss of the readily diffusible material while the salt concentrations were becoming equalized. Because the resistance of the buffer was found to differ from that of the sample, reliable absolute value of mobilities could not be determined. This may also have given a false impression of the area of the immobile component, since it is superimposed on the anomaly and the latter may be abnormally large due to imperfect equilibration. In order to decide the question of heterogeneity of either peak, reversible boundary spreading experiments (Alberty²) would have to be done.

Although the use of averages for estimating the concentrations of components is somewhat doubtful, for reasons just given, when such estimates are made, 31 per cent of the constituents of whole extract appear to be mobile and 69 per cent immobile at pH 7.5, whereas the corresponding areas of dialysate are 5 per cent and 95 per cent, respectively. The patterns obtained with sac

contents were so low, due to loss of substance during the prolonged dialysis of fractionation, that the photographs will not be presented. Direct visualization of the magnified peaks during the 2½-hour run at pH 7.5 showed a major, very slowly moving, colorless component and a mobile, colored constituent which was heterogeneous. Although it appeared to be less complex than the pattern for undialyzed whole extract, definite conclusions must await the analysis of a concentrated sample of both preparations. However, it was estimated that dialysis had effected a reduction in the immobile components to about 47 per cent of the total area while a proportionate increase had taken place in the mobile constituents until they comprised 53 per cent of the area.

These preliminary analyses show qualitatively that the shift of material through cellophane into the dialysate had been selective, involving largely those substances which were electrophoretically immobile at pH 7.5.

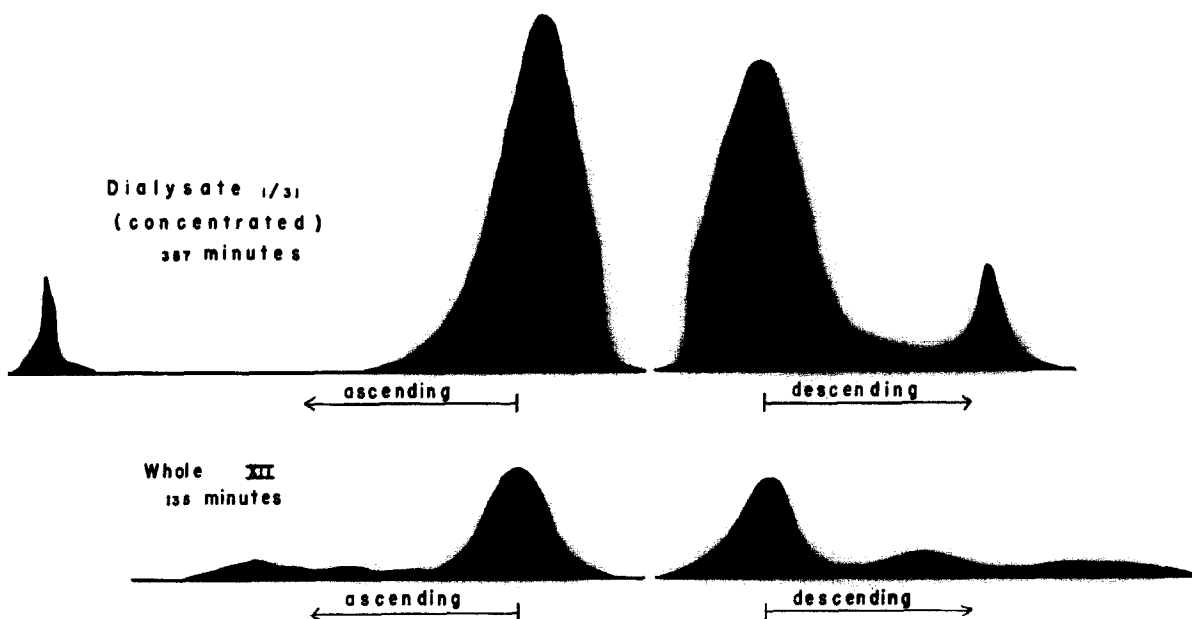


Fig. 3.—Tiselius electrophoretic patterns of low ragweed extracts (phosphate buffer of ionic strength 0.10, pH 7.50).

The sample of dialysate used for the Tiselius run contained 50.5 mg. of solids per milliliter (after excluding the weight of its buffer salts), of which 8 per cent was reducing sugars, 2.4 per cent was nonprecipitable nitrogen, 1.7 per cent was combined sugars expressed as glucose, and 1.3 per cent was PTA-N. Since the initial dialysates contained a much higher proportion of PTA-N to combined sugars than did the mother material (Experiment XVII), their PN:CS ratios being 1:1½ and 1:5, respectively, it might be deduced that the outward shift of immobile material into the dialysate was concerned more importantly with precipitable nitrogen than with the combined sugars. Polypeptides and smaller forms of nitrogen down to amino acids would be brought down by phosphotungstic acid and might be assumed to be the types involved. It is, of course, not possible to exclude the reducing sugars and nonprecipitable nitrogen

as factors contributing to the increase in the immobile components of the dialysate, since these substances diffused out quickly and freely through the cellophane and could quite possibly have been visualized by the electrophoretic technique.

Cross tests of electrophoretic fractions: Samples of the fastest- and slowest-moving materials were withdrawn from the Tiselius apparatus at the conclusion of the runs on first dialysate and sac contents so that their relative specificities could be appraised by means of serologic cross tests. Sites which had been injected with a serum-antigen mixture containing an excess of the fastest-moving, dialyzable fraction (pigmented) still responded to cross test with its slowest fraction (uncolored), and vice versa. These reactions were read as $1\frac{1}{2}$ plus ($+\pm$) and probably were about half-maximal, although sufficient material was not available to establish this definitely in serum-saline sites. Either site reacted at least half-maximally when tested with the fastest component of sac contents, showing that mobile activities had been retained by the cellophane which differed in specificity from both the immobile and mobile substances that diffused out. In the converse experiment, a site exhausted with the fast material from sac contents responded $++$ to retest with the fast fraction of first dialysate. Since these 2 way tests with nonirritating solutions provided mutually positive results, it could be concluded that each electrophoretic fraction possessed at least one specificity not present in the other. The supply was insufficient to determine whether they also contained mutual antigens, but this situation had been encountered in cross-tests between the unfractionated sac contents and dialysate (Table VI).

From these limited immunologic comparisons, evidence is at hand that low ragweed pollen contains at least 3 different specificities separable by cellophane and electrophoresis: 2 in the dialysate which differ from a third, mobile component of the nondiffusible portion of the extract.

Chromatography.—Cross tests among the eluates taken from the several crucible holders revealed that 3, and probably 4, different antigens were present in the initial dialysate of XVII. When, for example, normal skin was injected with a mixture of standard sensitizing serum and the pale yellow filtrate which had emerged from the lowermost crucible holder, it proved unresponsive next day when tested with this filtrate in undiluted form (PTA-N 0.17 and CS 0.6 mg. per ml.). An identical site reacted half maximally, however, to test with a yellow eluate taken from the upper portion of the second holder, fraction No. 2-a, which contained only 0.01 mg. PTA-N per ml. In the reverse experiment, No. 2-a inactivated sites toward cross test with undiluted filtrate. Thus, No. 2-a must have contained all the allergens of the filtrate, plus at least one additional activity. In spite of its complexity, the eluate was found to be simpler than another pigmented fraction eluted from the lower half of the top holder, fraction No. 1-b, which had a PTA-N value of only 0.02 and a CS content of around 0.2 mg. per ml. and which contained all activities present in No. 2-a and the filtrate, as well as a distinctive antigen. This showed that no fewer than 3 entities had been originally introduced into the adsorption column. Since

each of the foregoing derivatives failed to inactivate its sites for the unfractionated dialysate, one could deduce that at least 4 activities were involved in the starting material, initial dialysate XVII. Serum-saline control tests indicated that the faintly tinted filtrate was less potent than an equal volume of the definitely colored eluates, in spite of its higher content of both PTA-nitrogen and CS.

SUMMARY AND DISCUSSION

Phosphate buffer extracts of low ragweed pollen have been divided into 2 portions by means of suction or dialysis through commercial cellophane membrane. The diffusates differ from the sac contents in their carbohydrate: nitrogen ratios, in antigenic potency as well as specificity, and in electrophoretic constitution. Although both derivatives were simplified over the starting material, whole extract, neither was single as judged by electrophoretic, immunologic, and chromatographic analysis.

Confirmation was found for the claims of others^{3, 4, 7, 8, 11, 13, 16-18, 20} that pollen materials and activity diffuse out through cellophane. The extent of these losses has been approximated for the first time by the expedient of saving all yields of prolonged dialysis for a number of identical experiments. During initial dialysis against 1 or 1½ volumes of distilled water, the several phosphate buffer extracts of XVII lost about two-fifths of their nonprecipitable nitrogen, one-fourth their reducing sugars, two-fifths of their phosphotungstic acid-precipitable nitrogen (PTA-N), and 45 per cent of their combined sugars (CS). Meantime, some 30 per cent of the combined sugars and 10 per cent of the PTA-N had been converted to reducing sugars and nonprecipitable nitrogen, respectively. Although the extent of these gains in smaller molecular material at the expense of larger substances was not great, the shifts were suggestive of a possible breakdown of enzymatic or other nature taking place in the extract at 4° C. and under sterile conditions.

By the conclusion of 5 or 6 days of additional exposure of the sac contents to hydrostatic dialysis and to electrodialysis, the losses into the diffusate had mounted. Indeed, the residual extract possessed only 25 per cent of its original combined sugars, 15 per cent of its PTA-N, 8 per cent of its reducing sugars, and a trace of its nonprecipitable nitrogen. Meanwhile, an attempt to establish a crude material balance revealed that approximately one-fourth of the original PTA-N had been lost from the system while a compensatory gain of over 10 per cent had taken place in nonprecipitable nitrogen. An excess of free reducing sugars was also recovered.

The potency per PTA-N unit was reduced in the initial dialysate as compared to whole extract, all but a few of the 125 ragweed-allergic subjects tested by the conjunctival method requiring decidedly more nitrogen of the diffusate than of the whole extract. In patients showing minimal susceptibility to dialysate, the requirement was 500 times that of the unfractionated extract. These findings show not only that the diffusible nitrogen possesses a generally lower potency but also that the susceptibility for sac contents and for diffusate varies independently. Hence, different specificities must be involved. As dialysis

proceeded, the yields showed an even greater reduction in their potency as gauged by conjunctival and Prausnitz-Küstner tests, the hydrostatic product possessing about one-half the activity of its predecessor, the initial dialysate, and the electro-dialysate having only a fraction of their potency per PTA-N unit.

An effort was made to correlate these shifts in potency with shifts in the 4 chemical constituents of the fractions. Since over 90 per cent of the reducing sugars and nonprecipitable nitrogen were removed from the extract during dialysis and could be roughly accounted for in the accumulated diffusates, and since the dialysates proved in general far less potent per unit of carbohydrate or of nitrogen than did the sac contents, one could conclude that these highly diffusible, small substances played no significant role in activity. This leaves the better retained PTA-N and combined sugars for consideration. The PN:CS ratio changed as follows: from 1:5 in whole extract to 1:1½ in the initial dialysates, to 1:7 in hydrostatic dialysates, and to 1:18 in the final electro-dialysates. Although the concentration of PTA-N and the potency per nitrogen unit progressively fell in successive diffusates, rendering this assay method an unsatisfactory index to activity, the carbohydrate content was even less reliable inasmuch as the PN:CS ratio rose as the concentration and potency of the PTA-N steadily decreased. If the precipitable nitrogen was a determinant of activity, it must have been accompanied by considerable and increasing proportions of inert PTA-N as it found its way out of the sac. Spain and Newell¹⁷ found that 20 to 30 per cent of the PTA-N of ragweed extract diffused through heavy cellophane and was inactive. Meantime, our sac contents was enhancing somewhat its activity per PTA-N unit and its PN:CS ratio, the latter reaching 1:8 in the final, electro-dialyzed sac contents. Either assay method therefore could have served as a reasonably satisfactory means of reference to activity in successive sac contents. For the whole extract, no single chemical procedure was ideal due to the selective susceptibility of patients to dialysate, but the PTA-N content appears to follow activity more closely on the average than does CS.

A few speculations are permissible regarding the relationship of these chemical and activity characteristics to the electrophoretic results. The striking difference between the whole extract and its derivatives was a change in the relative proportions of the mobile and the immobile constituents, the latter increasing from 69 per cent in whole extract to 95 per cent of the total area in dialysate and being reduced by a like amount in the sac contents. Inasmuch as the immobile component of the diffusate was found to be serologically active, and it has been reasoned that the PTA-N is the most likely chemical category to account for the allergenicity of diffusates, it might be inferred that the PTA-N contributed activity and area to the immobile component of the dialysate. By way of contrast, the slowest-moving unpigmented material (SU) remaining behind in the final electro-dialyzed extract at pH 7.5 appears to possess little activity, as will be shown in a later publication. Furthermore, this retained SU material contains considerable carbohydrate and some PTA-N, if the limited assays done to date can be reproduced. Since the filtrate delivered from the chromatographic columns still possessed considerable CS and PTA-N, some

color, and only moderate potency per PTA-nitrogen unit, one might conclude tentatively that at least part of the PTA-nitrogen in our conventional diffusates was relatively inert. One might speculate that the small amount of activity possessed by this filtrate from the adsorption column might depend on its trace of pigment. The latter differed from other pigmented allergens in the dialysate by its inability to be absorbed on alumina at pH 5.

It would be profitable to establish the relationship of our findings to those of others in the literature. Abramson, Moore and Gettner, like Rockwell, attribute the allergenicity of ragweed pollen chiefly to its diffusible component of 5,000 molecular weight. Nevertheless, only 9 of their 15 patients reacted when this SU fraction was sprayed into their nostrils. This places it in the category of Stull's dialyzable fraction 2 and of our dialysates, which elicited responses in only a portion of the ragweed-allergic population. In view of the fact that undialyzed phosphate buffer extract had been employed for Abramson's electrophoretic separations, one is perplexed that no mention is made of any high molecular weight constituents. Both Stull and the writer have reported non-diffusible allergens in this pollen which are more regularly and more potently active than are the diffusible materials. Newell also found activity and an SU component in his electrophoretic analyses of whole extract which had been dialyzed for 20 hours, and in all 3 of Stull's fractions, only 1 of which (Fraction 2) is diffusible.

Rockwell further held that maximal protection from hay fever has been procured by patients given his 4,500 molecular weight fraction as an immunizing agent. This stands in contrast to the findings of Stull and associates with fraction 2 and of the writer who employed both ultrafiltrates¹¹ and suction-diffusates therapeutically. Since diffusible antigens are only selectively active, have lower potency, and are far less efficient in immunization, one would assume that they are of less over-all importance to patients with hay fever than are nondiffusible constituents in the pollen.

The incompatible results of Abramson and associates and of Newell with reference to the diffusibility of the slow, unpigmented components have been reconciled by our finding of a similar major electrophoretic constituent in both the dialysate and the electro-dialyzed sac contents. Although the 2 could readily be confused if one examined but 1 of them in a given electrophoretic experiment, they can be distinguished when both halves of the extract are analyzed concurrently. The diffusible type remained stationary throughout a 6 hour period of observation at pH 7.5, whereas the nondiffusible SU moved very slowly during a shorter run. The former was active, the latter questionably so. Since the 20 hour dialysis of Newell¹³ failed to rid the extract of its SU and our prolonged electro-dialysis removed only a portion of this major component from the sac, Abramson's undialyzed fraction must have contained both the diffusible and the nondiffusible varieties of colorless material.

Although many attempts have been made during the past half century to isolate an allergen from pollen, no one prior to the present report has come

closer to this goal than Abramson's group. Their electrophoretic separation would have been an outstanding achievement if it had been associated with proof as to the fate of the nondiffusible components of the pollen as well as immunologic evidence of purity.

The significance and implications of our own findings would appear to be as follows: Although pollen is a highly complex mixture of chemical, electrophoretic, and immunologic entities, an almost complete separation of diffusible from nondiffusible activities has been effected by means of dialysis through Cellophane membrane. The majority of the material to escape was electrophoretically immobile at pH 7.4. Attempts to refractionate the diffusate by electrophoretic and chromatographic means yielded at least 3 different excitants. The major electrophoretic component was colorless and contained at least 1 antigen which was serologically different from its colorless, major, very slowly-moving counterpart left inside the sac. Since patients show a marked degree of difference in their relative susceptibility to the diffusate and to the sac contents, it is to be expected that they may also show selectivity in their responsiveness to the various constituents of either of these complex fractions. In order to understand the exact relationships between clinical allergy for pollens and the presence of sensitizing antibodies in the patient, it will therefore be indicated in the future to isolate each of the numerous excitants in pollen and to utilize these to determine the titer of each related reagin. This means that the antigen must appear homogeneous by several criteria: namely, its behavior in the ultracentrifuge, in diffusion studies, in the electrophoretic field, as well as in its chemical composition and its immunologic specificity. One or more of these criteria may then be found to serve as a truly reliable means of reference, to replace the present unsatisfactory chemical and "weight-by-volume" indices of potency.

The next step toward the isolation of excitants from pollen will be the refractionation of our 2 composite fractions, diffusate and sac contents. Gentle physical methods which will not risk denaturation would seem preferable to older, chemical methods. This laboratory will soon publish its pioneering experiments with electrophoresis-convection in the case of the nondiffusible portion of the extract. It will be of particular interest to re-examine in greater detail the chemistry, specificity, and potency of the 2 major components (SU) of the sac contents and diffusate, so as to establish clearly their similarities and differences. Some means should be sought for ridding the fractions of this ubiquitous major unpigmented material, so that other antigens may be more easily obtained in pure form. The several pigments inside and outside the dialyzing sac must also be separated from each other in quantities sufficient for characterization.

When one or more isolated antigens have been procured, it will be possible to explore the relationships of the spectrum of sensitizing antibodies to clinical allergy and to evaluate the connection between the related thermostable, or blocking, antibodies and clinical resistance. Eventually, it should be possible to contribute further evidence for the theory that allergic man is capable of

forming 2 competitive types of antibody for the same single antigen, one being concerned with hypersensitiveness whereas the other is capable of increasing clinical tolerance.

It is urgent that all fractionations and characterizations be carried out under rigidly controlled conditions, since the outcome may be importantly influenced by such factors as aging, heat, freeze-drying, and contamination by molds or bacteria. Furthermore, the nature of the diffusate varies with the use of suction, certain buffer salts, and especially with the susceptibility of the human subject selected for the potency tests.

CONCLUSIONS

1. An appreciable proportion of the large losses of nitrogen and carbohydrate from low ragweed extract during extensive, conventional dialysis through cellophane might be due to the breakdown of larger into smaller filtrable forms. The extent of this conversion has been approximated for the first time by establishment of a material balance. It amounts to 10 per cent for the nitrogenous materials and to about 30 per cent for the carbohydrates. It occurs at 4° C. and under aseptic conditions.

2. The total losses from the sac during dialysis and electrodialysis amounted to approximately 98 per cent of the nonprecipitable nitrogen, 92 per cent of the reducing sugars, 85 per cent of the phosphotungstic acid-precipitable nitrogen, and 75 per cent of the combined sugars.

3. Whereas all ragweed-allergic patients reacted to the nondiffusible constituents, at least 20 per cent of them were unaffected by diffusates in extensive tests of the skin, eye, and serum.

4. Diffusates differed from nondiffusible constituents in potency per PTA-N unit as gauged by intracutaneous, conjunctival, and serologic tests. Their specificities were also different as shown by serologic cross tests, as well as by concurrent eye tests with whole extract and diffusate in 125 allergic subjects whose susceptibilities to the 2 fractions varied independently.

5. Electrophoretically, the initial dialysate differed from whole extract in its greater proportion of immobile component at pH 7.4. The sac contents showed a corresponding reduction of this area.

6. Successive diffusates showed a loss of potency per PTA-nitrogen unit as dialysis proceeded and a progressive increase in the precipitable nitrogen: combined sugar ratio. The sac contents showed a slight increase both in potency per PTA-N unit and in PN:CS ratio.

7. Cellophane dialysates, although simplified over whole extract, contained 3 and possibly 4 chromatographic antigens, at least 2 electrophoretic entities. Not less than 1 additional antigen is possessed by sac contents.

8. Confirmation was found for the claim¹ that pigmented, mobile material as well as unpigmented, slow-moving substances of low molecular weight in low ragweed pollen have allergenic activity and that both nitrogenous and carbohydrate materials diffuse out through cellophane.

9. Diffusates were not only less potently and less frequently allergenic than whole extract and sac contents, but were less efficient as immunizing agents in

the production of thermostable antibodies and of clinical resistance. This confirms the opinion of Stull and his associates but is in disagreement with that of Abramson and Rockwell, who hold that molecules of about 5,000 molecular weight are the chief allergens of ragweed pollen.

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