

FRACTIONATION OF POLLEN EXTRACTS

II. CHEMICAL AND PAPER CHROMATOGRAPHIC INVESTIGATION OF RAGWEED POLLEN EXTRACTS

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A PREVIOUS report from this laboratory recorded observations on the separation of the dialyzable components of ragweed extract dialysate by the method of partition paper chromatography.¹ The data incorporated in that report were concerned, in part, with determinations of the biologic activity and the amino acid content of some of the fractions obtained by one dimensional paper chromatography with water saturated lutidine (2, 4-dimethylpyridine). Later studies with the resulting components of dialysate revealed that lutidine alone was capable of provoking positive skin reactions in ragweed-sensitive subjects. The fractionation of ragweed extract dialysate was, therefore, repeated, using a nonskin reacting solvent, methyl cellosolve (ethylene glycol monomethyl ether). The present report is concerned with this separation and with the analysis of the resulting fractions. When it was found that further manipulation of the fractions resulted in successive breakdown and loss of material, a preliminary study was made of the nondialyzable residue, certain data of which are included in this report.

METHODS AND RESULTS

Low ragweed pollen was extracted and separated into its dialyzable and nondialyzable components by methods described in the earlier publication.¹ The present procedure differed only in that water was used throughout instead of buffer for the initial extraction of the pollen, and the whole extract was concentrated twentyfold by vacuum distillation at a temperature which never exceeded 35° C.

A. *Dialysate Fractions.*—

I. Lutidine: Paper chromatographic fractions of the dialyzable portion of ragweed extract were prepared in large quantities by the Nepera Chemical Co., Inc. of Yonkers, New York.

Dialysate of ragweed extract was stripped across a short edge of a large sheet of filter paper and developed one dimensionally using lutidine. Spraying

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a guide strip of the dried paper with ninhydrin revealed four polypeptide bands (called A, B, C, D) while spraying another guide strip with aniline hydrogen oxalate revealed the presence of one carbohydrate containing band (called CHO). After lyophilization, the eluted fractions A, B, C, and D were obtained as brown powders and fraction CHO as a dark brown syrup. All fractions were skin reactive upon intradermal test in ragweed-sensitive subjects, and their total nitrogen and amino acid contents were similar to those of the fractions studied previously.

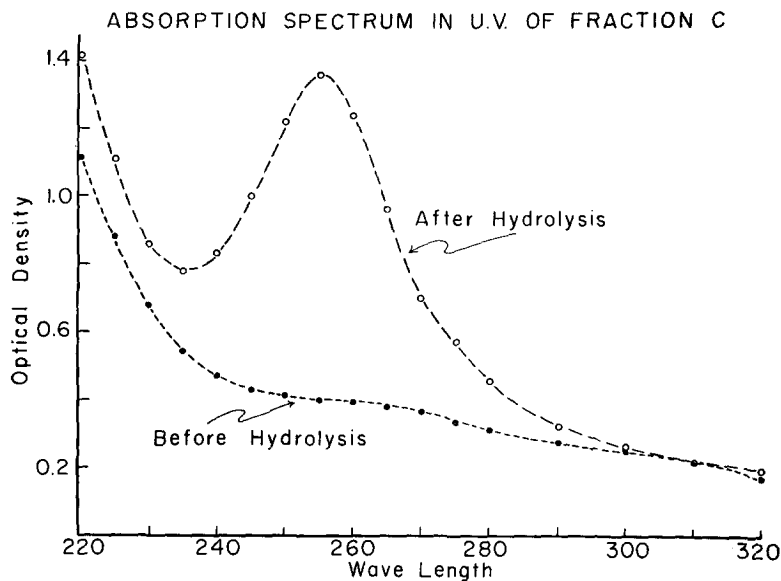


Fig. 1.

A portion of each fraction was placed in sealed tubes and hydrolyzed with 6 N hydrochloric acid at 100° C. for eighteen hours. Despite such drastic treatment, fractions A, C, and CHO retained skin reactivity, while fractions B and D became inactive. Ultraviolet absorption spectra (Model DU Beckman) obtained before and after hydrolysis revealed the appearance of an absorption band at 260 mμ in only those hydrolyzed fractions which retained skin reactivity. Fig. 1 shows curves of fraction C before and after hydrolysis, and is typical of the behavior of all three fractions.

These unexpected results prompted an investigation of lutidine alone, and it was found that the absorption spectrum of a very dilute solution of this material was very similar to those obtained with the hydrolyzed fractions. Furthermore, positive skin reactions were obtained when lutidine alone was tested intradermally in ragweed-sensitive subjects. This may well be correlated with the findings of Winkenwerder, Buell, and Howard² who showed that nitrogenous materials derived from nucleic acid exhibited skin reactivity in ragweed-sensitive subjects. It seemed possible that lutidine had combined chemically with the fractions during separation and was itself responsible for

the biologic activity previously attributed to the fractions. For this reason the fractionation of dialyzable ragweed extract was repeated with the aid of a nonskin reacting solvent. Methyl cellosolve, which had been reported as giving Rf values for amino acids similar to those with lutidine,³ was chosen for this fractionation after preliminary study revealed it was not itself skin reactive.

II. Methyl cellosolve: The cellosolve separation was accomplished by processing 150 sheets of water-washed filter paper each of which had been streaked with 110 to 125 mg. of concentrated dialysate. These sheets were then developed, one dimensionally with 90 per cent aqueous methyl cellosolve in the same manner previously employed with lutidine. Although lutidine had separated four ninhydrin reacting bands and one carbohydrate containing band, methyl cellosolve separated eight ninhydrin reacting bands and one carbohydrate containing band.

TABLE I. NITROGEN AND CARBOHYDRATE ANALYSES OF CELLOSOLVE FRACTIONS

SAMPLE	Rf* (CELLOSOLVE)	% TOTAL NITROGEN	% BOUND CARBOHYDRATE†
0	0.00	6.1	0.0
1	0.10	--	--
2	0.30	6.4	0.0
2A	0.42	6.3	2.3
3	0.49	3.4	0.0
3A	0.58	4.1	7.0
4	0.70	6.3	0.0
5	0.87	7.5	9.2
CHO	0.93	1.6	21.0 (free)

*Rf represents the fraction of the distance traveled based on the distance of the solvent which is taken as 1.

†The bound carbohydrate present in the fractions was arabinose, while the free carbohydrate in fraction CHO was a mixture of glucose and fructose.

Table I shows the results of nitrogen and carbohydrate analyses performed on the cellosolve fractions. The nitrogen determinations were done by semi-micro Kjeldahl, the carbohydrates by the method of Nelson.⁴ The separation between some of the bands (2A and 3 and 5 and CHO) was not great as indicated by their Rf values. Nevertheless, elution of the fraction was carried out only when a discrete white area was present between the extremes of the blue bands made by ninhydrin reaction with the separate fractions. No blue color appeared in the area called CHO, but it yielded a typical brown band for carbohydrate after treatment with aniline hydrogen phthalate. The nitrogen values of the ninhydrin reactive bands ranged from 3.4 to 7.5 per cent while the carbohydrate content of these fractions ranged from 0 to 9.2 per cent and, where present, was exclusively bound arabinose. The 21 per cent carbohydrate content of the CHO fraction was entirely in the free form and was identified by paper chromatography as glucose and fructose.

Each of the eight ninhydrin reacting bands after removal from the paper proved to be skin reactive. Rechromatography of these bands, however, with 80 per cent aqueous isopropanol, for further purification, resulted in separation

of each band into several smaller bands. This successive breakdown into smaller units is indicative of considerable instability of the isolated fractions. This was further confirmed by the fact that several amino acids appeared on two dimensional chromatograms of all the fractions even before they were subjected to acid hydrolysis. The major portion of each fraction obtained by isopropanol rechromatography was nevertheless analyzed for amino acid composition before and after acid hydrolysis.

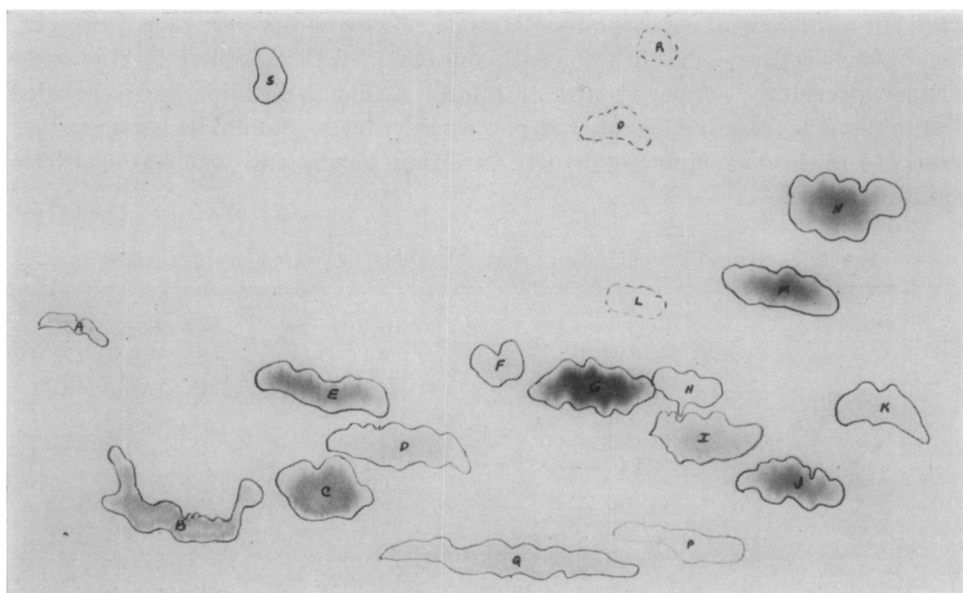


Fig. 2.—Dialysate of ragweed hydrolyzed 3 mg.

Key to Symbols: A, Cystine; B, Aspartic Acid; C, Glutamic Acid; D, Glycine; E, Serine; F, Threonine; G, Alanine; H, Hydroxyproline; I, Histidine; J, Gamma-aminobutyric Acid; K, Proline; L, Methionine; M, Valine; N, Leucine; O, Tyrosine; P, Arginine; Q, Lysine; R, Over Tyrosine; S, Unknown; W, Beta-alanine.

Two dimensional chromatograms of these partially purified fractions revealed similar but not identical patterns for all the fractions each of which contained fewer amino acids than had the unfractionated dialysate (Fig. 2). Common to all fractions was the presence of aspartic and glutamic acids, glycine, serine, alanine, and a trace of cystine. The rechromatographed cellosolve fraction 3, after acid hydrolysis, contained a rather large quantity of gamma-aminobutyric acid and a trace of hydroxyproline (Fig. 3). Cellosolve fraction 4, however, showed an undetectable amount of these amino acids but a large amount of valine and a trace of leucine (Fig. 4). Histidine, which was very evident in the unfractionated dialysate, did not appear in either of these fractions. In several of the unhydrolyzed fractions glutamine was detected and in two of them asparagine was present.

The free carbohydrates present in the unfractionated dialysate were shown by paper chromatography to be glucose and fructose. This was further confirmed by the preparation of the glucosazone and the use of spraying

reagents specific for ketoses. Brown and Benotti⁵ prepared a glucosazone from Norite A decolorized ragweed pollen extract, which is not capable, however, of distinguishing fructose from glucose. The fact that glucose and fructose migrate at the same rate in the developing solvents used in the present study, accounts for the fact that both sugars appeared in the single band labeled CHO.

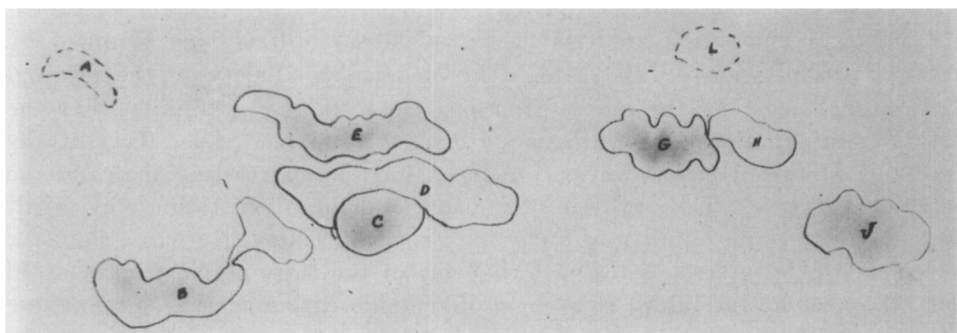


Fig. 3.—Cellosolve fraction 3 rechromatographed 80 per cent isopropanol hydrolyzed 3.6 mg. (For Key to Symbols, see opposite page.)

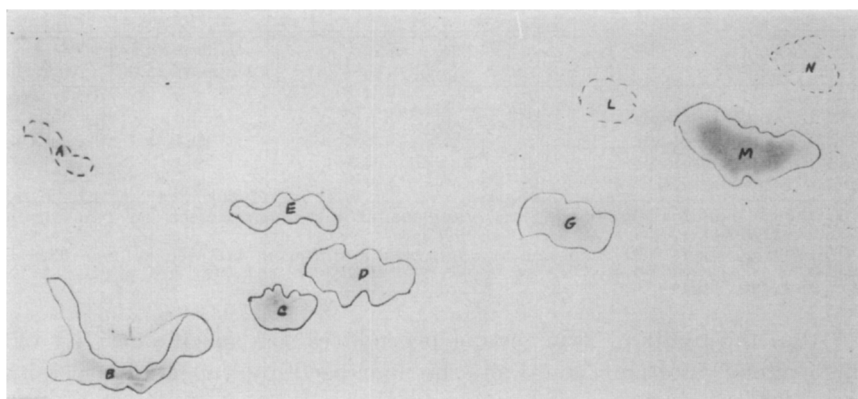


Fig. 4.—Cellosolve fraction 4 rechromatographed 80 per cent isopropanol hydrolyzed 3 mg. (For Key to Symbols, see opposite page.)

III. Alcohol and acetone: Several investigators^{10, 16-19} have succeeded in recovering active fractions from whole ragweed by precipitation with alcohol and/or acetone. Advantage was taken of this observation in an attempt to fractionate whole dialysate in order to obtain a less complex mixture for subsequent paper chromatographic separation. It was found that nearly all of the skin reactivity present could be precipitated at 60 per cent concentration of either solvent. Upon re-solution of the precipitate in water there remained a white, insoluble residue which proved to be calcium phosphate. Moreover Brown's observation that all phosphorus present in ragweed extract was inor-

ganic⁵ was confirmed. Nitrogen, carbohydrate, and amino acid analyses, as well as skin reactivity determinations were done on several fractions obtained by serial precipitations with increasing concentrations of either alcohol or acetone. The principal amino acids appearing on two dimensional paper chromatograms of these isolated precipitates were essentially the same as those listed for the cellosolve fractions with the exceptions that large amounts of histidine and only very faint traces of hydroxyproline and proline were present.

Table II shows the analysis of a typical set of fractions obtained by acetone precipitation of dialysate. The chemical similarity to the original dialysate of most of the active fractions suggests the possibility that the precipitated fractions are the same except for polymer size. Two of the fractions produced larger wheals on direct intradermal testing than did the original dialysate. The presence of unusual amounts of histidine may result from its relative insolubility in fairly high concentrations of organic solvents. Of considerable interest is the fact that one of the more active fractions (40 per cent acetone insoluble) showed no detectable carbohydrate. From these data, it would appear that the allergenic activity of the various fractions may not be proportional to the carbohydrate content as was claimed for the hydrochloride fractions obtained by Rockwell.⁹

TABLE II. ACETONE FRACTIONS OBTAINED FROM DIALYSATE

SAMPLE	HISTIDINE*	% TOTAL NITROGEN	% BOUND CARBOHYDRATE	ACTIVITY†
Dialysate	L	6.5	7.6	+++
40% Acetone insoluble	L	5.5	0.0	++++
60% Acetone insoluble	L	6.3	10.4	++++
70% Acetone insoluble	L	6.6	9.4	++
100% Acetone soluble	0	2.7	8.6	0

*L under histidine signifies a large amount of histidine as seen on two dimensional paper chromatograms.

†Intradermal tests with solutions of 5 mg. per milliliter in buffered saline. The number of plus signs represents an average of many determinations and they are relative to unfractionated dialysate at +++.

Within the limits of skin testing procedures, the specific activity of each of the acetone fractions could not be increased by repeated precipitation. For this reason and because there was progressive loss of material with each succeeding purifying procedure this approach to isolation of an active fraction was discarded, and the next phase of study was concerned with an analysis of nondialyzable fractions.

B. Residue Fractions (Nondialyzable).—The nondialyzable portion of the ragweed extract retained by far most of the skin reactivity. Neither ninhydrin nor aniline hydrogen phthalate reacting components appeared on chromatograms of unhydrolyzed residue. Both types of constituents, however, appeared in large amounts after acid hydrolysis. Paper chromatographic analysis of 1 N acid hydrolysates revealed the presence of very large amounts of arabinose in agreement with the findings of Rockwell⁷ and Stull, and co-workers.⁸ Fig. 5 shows that the amino acids appearing after 6 N acid hydrolysis were cystine, aspartic

and glutamic acids, glycine, serine, threonine, alanine, valine, leucine, arginine, lysine, beta-alanine, gamma-aminobutyric acid, methionine, traces of tyrosine and an unknown material appearing above tyrosine in the approximate position of one of the iodotyrosines. Extremely large amounts of hydroxyproline and proline were present in all preparations and in all subsequent fractions obtained from them although the yellow color characteristic of these compounds is not clearly evident in the black and white reproduction.

The exhaustively dialyzed residue contained, by weight, 4.5 per cent total nitrogen which was entirely precipitable by phosphotungstic acid, and 40.8 per cent bound carbohydrate determined by the method of Nelson and by the orcinol method for pentoses. Qualitative analysis of the sugar component by paper chromatography showed the presence of arabinose alone.

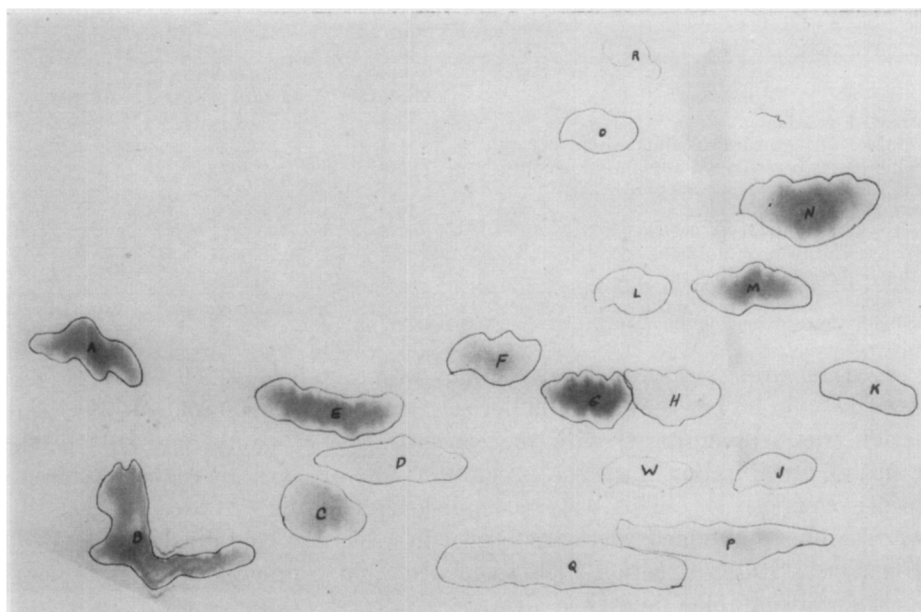


Fig. 5.—Nondialyzable residue of ragweed hydrolyzed 3 mg. (For Key to Symbols, see page 36.)

Several fractionation procedures were used in order to determine whether the biologic activity was due to a polypeptide, a carbohydrate, or possibly to an intact polypeptide-carbohydrate complex. The limit of chemical manipulation tolerable to the active components was evaluated in the process of attempting to isolate the purest possible active material. Several investigators have attempted this sort of fractionating procedure but without much success. The carbohydrate fractions isolated by Black¹⁰ and Caulfeild¹¹ each contained appreciable amounts of nitrogen. Rockwell⁹ isolated five fractions each consisting of a flavonol-polypeptide-carbohydrate complex and each exhibited biologic activity, but it was not proved that the activity was dependent on the entire complex or any part of it. Stull¹² obtained by ammonium sulfate precipitation a carbohydrate-free fraction which retained activity, but this was not confirmed in the present

study. Rockwell¹³ reports that the pollen antigen was destroyed by alkalies, yet in this laboratory it was possible to prepare a fraction by alkaline hydrolysis at 37° C. which contained 74 per cent bound carbohydrate and only 1.5 per cent nitrogen, and which retained about half of the activity exhibited by the original residue (Table III). It was found that activity could be preserved even after treatment with 1 N acid or 1 N alkali if the temperature was maintained below about 40° C. A few fractions were obtained free of carbohydrate, but the treatment required hydrolysis with 1 N hydrochloric acid in boiling water for one hour which destroyed the skin reactivity. In no case was it possible to obtain from residue an active fraction which was completely free of either nitrogen or carbohydrate.

TABLE III. ANALYSES OF FRACTIONS FROM THE NONDIALYZABLE PORTION OF RAGWEED POLLEN EXTRACT

SAMPLE	% TOTAL NITROGEN	% BOUND CARBOHYDRATE	ACTIVITY*
Untreated residue	4.5	40.8	++++
Insoluble 50% saturated ammonium sulfate	7.7	22.7	+++
Insoluble 100% saturated ammonium sulfate	5.9	44.9	+++
Insoluble 69% aqueous acetone	7.7	20.8	++++
After HCl hydrolysis (hot)	10.4	00.0	0
After NaOH hydrolysis (warm)	1.5	74.4	++
Lead precipitation (Caulfeild)	3.4	55.9	++
Phenol extraction (Morgan)	2.1	72.4	++

*Each solution was adjusted to 100 PNU per milliliter on the basis of the total nitrogen which, in these experiments, was the same as the PTA nitrogen.

I. Ammonium sulfate: Two fractions were obtained which were insoluble, respectively, in 50 per cent and 100 per cent saturated ammonium sulfate. Each fraction was reprecipitated with the concentration of ammonium sulfate used for initial precipitation until the precipitate was of constant carbohydrate and nitrogen composition. This was accomplished after three reprecipitations, but each fraction so obtained was dark brown in color and contained carbohydrate. By contrast, Stull's Fraction I obtained after ten reprecipitations, was nearly colorless and contained no carbohydrate.¹²

The 50 per cent and 100 per cent ammonium sulfate insoluble fractions obtained in this study were of approximately equal skin reactivity on direct intradermal testing. The 50 per cent insoluble fraction, however, contained more nitrogen and less carbohydrate than did the 100 per cent fraction. Each fraction was probably a complex mixture as evidenced by its electrophoretic inhomogeneity in phosphate buffer at pH 6.7. The fraction insoluble at 50 per cent salt saturation contained 7.7 per cent total nitrogen and 22.7 per cent bound arabinose, while the fraction insoluble at 100 per cent salt saturation contained 5.9 per cent total nitrogen and 44.9 per cent bound arabinose.

II. Acetone precipitation: A light tan powder was obtained by bringing the aqueous solution of residue to 90 per cent acetone concentration. This powder exhibited the same activity as the original solution. Further fractionation of the powder with 60 per cent, 70 per cent, 80 per cent, and 89 per cent acetone produced materials of similar skin reactivity and chemical composition

with the exception of the 80 per cent soluble, 89 per cent insoluble fraction which had a bound carbohydrate higher than the others. The nitrogen values ranged from 7.2 per cent to 7.9 per cent and the bound arabinose from 19.3 per cent to 21.7 per cent, the 89 per cent fraction having 31.3 per cent bound carbohydrate.

III. Acid hydrolysis: It had been repeatedly demonstrated in the course of this study that the bound carbohydrate of ragweed pollen is completely hydrolyzed by 1 N hydrochloric acid in boiling water for one hour. Under these conditions, very little hydrolysis of protein occurs. Accordingly this procedure was attempted in order to obtain a carbohydrate-free fraction. It was indeed possible to obtain such a fraction, but this was accompanied by an expected destruction of skin reactivity. The faintly colored material which precipitated during the hydrolysis contained 10.4 per cent nitrogen and no carbohydrate. Milder methods of hydrolysis, employing weaker acid and lower temperatures failed to produce a fraction which was entirely free of carbohydrate although it was possible to retain about one-half of the original skin reactivity.

IV. Alkaline hydrolysis: It seemed possible that the bound arabinose in ragweed extract was present as a polysaccharide. If present as such a polymer, the carbohydrate should resist hydrolysis with alkali while any polypeptides present could be expected to be degraded under these conditions. Therefore, the aqueous residue was made 1 N with sodium hydroxide and allowed to stand in a warm room at 37° C. for twenty-four hours. The hydrolysate was neutralized and reduced in volume in vacuo. Acetone was added to 90 per cent, the precipitate removed, washed with 90 per cent acetone, 100 per cent acetone, and dried under vacuum. There was present 1.5 per cent nitrogen, 74.4 per cent bound arabinose and approximately half of the original skin reactivity representing the best separation with retention of activity in the entire series. Since the carbohydrate component was in the bound state even though there was such a small amount of nitrogen present, it is evident that the arabinose is present as a polysaccharide rather than in the monomolecular form as pictured by Rockwell.⁷ The small amount of nitrogen was shown by paper chromatography to be in the form of amino acids rather than nitrogen-containing carbohydrates.

V. Miscellaneous: Other methods were employed in order to obtain a carbohydrate fraction, but none produced a totally nitrogen-free fraction. The method of Caulfeild¹¹ produced an active fraction containing 3.4 per cent nitrogen and only 55.9 per cent bound arabinose. Some of the methods used by Morgan¹⁴ in his fractionation of a polypeptide-polysaccharide antigen were attempted. One procedure called for extraction with phenol which, when applied to residue, produced an active fraction containing 2.1 per cent nitrogen and 72.4 per cent arabinose. Again it was shown that the nitrogen was present as amino acids rather than amino sugars, and that the only carbohydrate detectable was arabinose.

CONCLUSION

All of the fractions obtained from residue by the various methods used agree in general with the results obtained by Rockwell,⁹ namely, that these are

polypeptide-carbohydrate-pigment complexes, with the exception that the carbohydrate component appears to be an araban rather than arabinose. Only in the dialyzable portion, however, was it possible to obtain an active fraction which was completely free of carbohydrate. Regardless of the fractionation procedures employed, both paper chromatographic and chemical, there was never obtained from residue an active fraction which was entirely free of either nitrogen or carbohydrate. This does not necessarily imply that the combination of polypeptide and polysaccharide is essential for allergenic activity, but rather that suitable methods for a complete separation result in a destruction of biologic activity.

Augustin¹⁵ has stated that the active components of the grass pollens are nondialyzable. If the active materials in dialysate are truly degradation products from residue and since CHO free fractions were obtained from dialysate, then the separation of the components of the active fractions of residue should be possible.

It would seem that paper chromatography and the chemical procedures used in this study are unable to define the chemical nature of ragweed activity without the addition of such other methods as resin column extractions and electrophoresis by any of the varied techniques available.

SUMMARY

1. The solvent for paper chromatographic separations of skin reactive allergens must be chosen with great care. Lutidine can combine chemically with certain compounds and is in itself capable of producing skin reactions.

2. Fractions very similar to those previously obtained with lutidine were separated by use of a nonskin reactive solvent, methyl cellosolve. Such fractions exhibited skin reactivity. From this it has been concluded that the originally separated lutidine fractions were actually active components of dialysate.

3. Activity in the dialysate of ragweed extracts appears to be due to breakdown of originally larger particles, as shown by the extreme lability of the fractions isolated by paper chromatography.

4. Nearly all of the skin reactivity present in dialysate was precipitable by making an aqueous solution 60 per cent with respect to acetone.

5. Chemical fractionation of the nondialyzable portion of ragweed extract failed to produce an active fraction which was completely free of either protein or carbohydrate, respectively. Warm alkaline hydrolysis and phenol extraction came closest to producing such a fraction although the specific activity was reduced in both cases. Dialysate did, however, produce a few skin reactive carbohydrate-free fractions.

6. The active components appear to consist of a protein-carbohydrate-pigment complex, the carbohydrate component being a polysaccharide of arabinose.

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