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CHEMICAL STUDIES ON POLLEN AND POLLEN EXTRACTS I. DISTRIBUTION OF NITROGEN EXTRACTED BY VARIOUS SOLVENTS

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Whether one is inclined to accept or to reject the theory that pollen sensitization or allergy is an anaphylactic phenomenon due to one or more specific proteins of the pollen, it is evident that a careful chemical study of different pollens is desirable as a basis for further research which may lead to the isolation of the actual causative agent or agents.

The literature yields only very limited information on the subject of pollen composition. As proteins are usually considered to be the offending agents in cases of allergy, it seemed desirable to begin such a study by determining the nitrogen extractable by various solvents.

Heyl,[1,2] Caulfeild, Cohen and Eadie,[3] Koessler[4] and Csonka, Bernton and Jones[5] have contributed valuable information on this subject. However, because of the varying methods of attack the results of the different investigators do not agree.

Certain definitions which have been made to fit the animal proteins cannot be applied to vegetable proteins. This fact must be kept in mind by anyone who works with the proteins of pollen. In this work the purpose was to extract the pollen with different solvents in such a manner as to cause the least possible denaturation. No heat or concentrated solutions of electrolytes were used. The development by Koch and McMeekin[6] of a satisfactory direct colorimetric micro-analysis for nitrogen has made it possible to determine nitrogen in pollen and pollen fractions with greater ease and accuracy than could be secured by any macro method.

For this work four pollens have been chosen; two representative of the type causing spring hay fever, timothy (*Phleum pratense L.*) and orchard grass (*Dactylis glomerata L.*) and two of the type causing autumnal hay fever, short ragweed (*Ambrosia elatior L.*) and giant ragweed (*Ambrosia trifida L.*).

[1] F. W. Heyl, "Analysis of Ragweed Pollen," THIS JOURNAL, **39**, 1470–1476 (1917).

[2] F. W. Heyl, "Analysis of Ragweed Pollen," *ibid.*, **41**, 670–685 (1919).

[3] A. H. W. Caulfeild, C. Cohen and G. S. Eadie, "The Antigenic Properties of Pollen Fractions," *J. Immunology*, **12**, 153–175 (1926).

[4] J. H. Koessler, "Studies on Pollen and Pollen Diseases. I. The Chemical Composition of Ragweed Pollen," *J. Biol. Chem.*, **35**, 415–434 (1918).

[5] F. A. Csonka, H. S. Bernton and D. B. Jones, "Proteins of Timothy and Orchard Grass Pollen and their Relation to Vernal Hay Fever," *Proc. Soc. Exptl. Biol. Med.*, **23**, 14–16 (1925).

[6] F. C. Koch and T. L. McMeekin, "A New Direct Nesslerization Micro-Kjeldahl Method and a Modification of the Nessler-Folin Reagent for Ammonia," THIS JOURNAL, **46**, 2066–2069 (1924).

Small samples (*i. e.*, two to four grams) of pollen were used. This obviated the necessity of handling large volumes of solutions when exhaustive extraction was attempted. The nitrogen determination by the modified micro-Kjeldahl method of Koch and McMeekin[6] permitted accurate estimation of the nitrogen in these solutions.

Extractions were made of at least four samples of each pollen studied. Preliminary work had shown the amount of solvent necessary for practically complete extraction with each extracting menstruum.

All water used was redistilled from alkaline permanganate and tested for ammonia.

Method

An accurately weighed sample of dry pollen, usually two or four grams, was placed in a dry beaker. A suitable portion of ether was then added and mixed with the pollen sample. The mixture was filtered by suction through a hardened paper in a 4.5-cm. Büchner funnel. The residue was washed with a little ether and again carefully transferred to the beaker to be re-extracted. Previous work had shown that it was possible to determine the completeness of extraction by the color imparted by the residue to the fresh ether. The ragweed pollens required a greater number of ether extractions than did the grass pollens, the total volume of ether solution being for the ragweeds 100 cc. and for the grasses 50 cc.

The same method was used in carrying out the other extractions upon the ether-extracted samples, the residue from filtration being transferred back to the beaker for mixing with fresh portions of the solvent. The volumes of each extract obtained, using four-gram samples, were: water, 250 cc.; salt solution (10% sodium chloride at 60°), usually 100 cc.; alcohol solution (75%), 100 cc.; alkaline solution (0.2% sodium hydroxide), usually 100 cc.

Nitrogen was determined on 5- or 10-cc. aliquots of each of these extracts and on weighed samples (preferably 20 to 30 mg.) of the original dry pollen and of the residue which had been thoroughly washed with water to remove alkali and dried in air and over sulfuric acid. In the case of the grass pollens, enormous swelling took place in the alkaline solution and the residue finally obtained, even after repeated washings with

TABLE I

NITROGEN DISTRIBUTION

	Fraction	Giant ragweed	Short ragweed	Orchard grass	Timothy
	Whole pollen	4.36	4.26	4.37	4.92
(A)	Ether-soluble	0.079	0.053	0.005	0.003
(B)	Water-soluble	1.31	1.315	1.38	1.49
	(1) Rptd. by dialysis	0.074	0.125	0.021	0.173
	(2) In filt. from dialysis	.053	.094	.031	.19
(C)	Salt-soluble (10% NaCl)	.48	.354	.23	.705
	(1) Rptd. by dialysis	.013	.013	.016	.064
	(2) In filt. from dialysis	.02	.042	.021	.05
(D)	Alcohol-soluble (75% EtOH)	.345	.30	.029	.035
(E)	Alkali-soluble (0.2% NaOH)	.118	.135	.26	.50
	(1) Rpt. by acid	.065	.05	.053	.19
(F)	Residual N	1.98	2.18	2.48	1.89
	Total	4.31	4.34	4.38	4.62

water, formed a hard cake on drying, probably due to incomplete removal of the alkali. The ragweed pollens gave residues which were easily powdered.

Aliquots of 100 and 50 cc., respectively, of the water and of the salt extracts were used for dialysis. They were electrodialyzed under toluene in gold beaters' sacs at about 2° for approximately a week. The distilled water outside the sacs was frequently changed. Carbon electrodes with a potential difference of 110 volts were used. In the case of the salt extract a preliminary dialysis was necessary in order to remove the excess sodium chloride before electrodialysis.

Precipitates were formed by electrodialysis of both water and salt extracts. These were separated from the solutions by centrifugation. Nitrogen was determined on these precipitates and on aliquots of the solutions.

Table I and the figures give the results obtained. The values for nitrogen are all calculated on the basis of the original dry pollen.

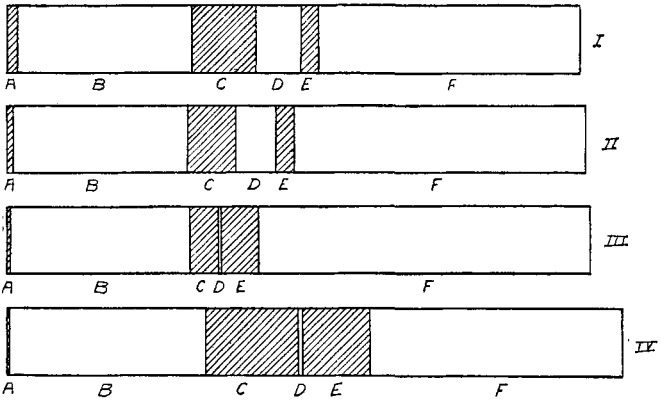

Fig. 1.—Nitrogen extracted. I, Giant ragweed; II, short ragweed; III, orchard grass; IV, timothy. A, by ether; B, by water; C, by sodium chloride solution; D, by ethyl alcohol solution; E, by sodium hydroxide solution; F, in residue.

Discussion

Only a very small amount of nitrogen was extracted by ether, and this is probably non-protein in nature. However, recent work by Milford[7] indicates that this fraction may be of allergic importance. Although by far the largest part of the nitrogen extractable by the solvents used appeared in the water solution, only a small percentage of this was non-dialyzable under the conditions of these experiments. This may be taken to mean that most of the nitrogen thus extracted is not protein nitrogen. It has been suggested that much of the nitrogen present in the pollen may occur in the form of water-soluble diffusible bases such as betaines. It is also conceivable that enzymes contained in the pollen may have been able to hydrolyze the proteins and change their nitrogen into a dialyzable form

[7] Edgar L. Milford, "Specific Studies in Allergy. I. The Specific Activity of Pollen Oil," *J. Allergy*, **1**, 331–333 (1930).

during the prolonged dialysis. The former theory is supported by the results of Heyl,[2] who found the total coagulable protein plus material precipitated by saturation with zinc sulfate to represent only a small portion of the nitrogen extractable by water. It might be assumed that the action of enzymes would be greatly retarded by the low temperature and the toluene used in the dialysis. However, because of the large number of enzymes found in pollen (Paton[8]) and because of the small amount of investigation that has been done on them, it would seem unwise to make any assumptions as to their action.

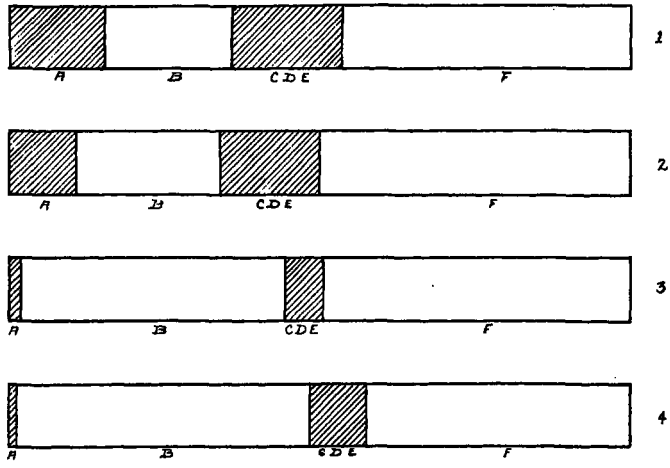

Fig. 2.—Total solids extracted. 1, Giant ragweed; 2, short ragweed; 3, orchard grass; 4, timothy. A, By ether; B, by water; CDE, by sodium chloride, ethyl alcohol and sodium hydroxide; F, in residue.

It is of interest to note that in every case about one-half of the nondialyzable nitrogen of the water extract is precipitated by dialysis. This behavior would not be expected if the water extract contains only albumin and proteose, as is generally assumed. On the basis of Osborne's[9] definition, this precipitable material should be classified as a globulin. It might be postulated that the albumin has been denatured by continued contact with pure water, but it seems more logical in the present state of our knowledge to assume that most of the globulin present can be extracted by redistilled water with the aid of salts present in the pollen itself.

The nitrogen extracted by salt solution (10% NaCl) is small in amount and a considerable portion of it is dialyzable. The same argument as to

[8] Julia B. Paton, "Enzymes of Pollen," *Proc. Soc. Exptl. Biol. Med.*, **17**, 60–61 (1919).

[9] Thos. B. Osborne, "The Vegetable Proteins," Longmans, Green and Company, New York, **1924**, p. 18.

whether or not this is due to enzyme action applies here as in the water extract. The precipitate may be safely classified as globulin. The fractions soluble in water and diluted salt solutions are generally conceded to be the most important ones as causative agents in allergic conditions.

The alcohol solution (75% EtOH) extracts a small amount of nitrogen. No further work was done on this fraction to determine whether this nitrogen is protein in nature. Clinical studies by various investigators have shown some allergic activity in the alcohol-soluble material from pollens.

A further small amount of nitrogen is extracted by dilute alkali (0.2% NaOH) of which approximately half is precipitated by acidification of the extract. This precipitated material is probably a glutelin, and its importance as a factor in allergy is doubtful.

Approximately half of the nitrogen originally present in the pollen remains in the residue after extraction with all the above solvents. Its nature is not known but as it appears highly probable that the toxic agent in allergic conditions is one easily extracted by body fluids, it may be assumed that this nitrogen is not of clinical importance.

The allergic properties of the various fractions are being studied. No significant difference was found in the amount of nitrogen extracted from different pollens by the solvents used.

A difference between the ragweed and grass pollens is shown in the quantities of material extracted by ether and by water. Table II shows the total solids extracted by ether and by water, and the weight of material left after extraction by all of the solvents mentioned above. These percentages are calculated on the basis of the original dry pollen.

TABLE II

TOTAL SOLIDS

	Giant ragweed	Short ragweed	Orchard grass	Timothy
Ether-soluble	15.4	11.1	1.9	1.6
Water-soluble	20.7	22.9	42.5	47.0
Residual	46.3	49.8	49.6	42.7
Soluble in 10% NaCl, 75% EtOH and 0.2% NaOH (by difference)	17.6	16.2	6.0	8.7

Summary

Pollens of giant ragweed (*Ambrosia trifida L.*), short ragweed (*Ambrosia elatior L.*), timothy (*Phleum pratense L.*) and orchard grass (*Dactylis glomerata L.*) were exhaustively extracted with ether, water, salt solution, alcohol and alkali in the order named. Nitrogen was determined on each of these extracts and on the original pollen and the residue.

The water and salt solution extracts were dialyzed. In both dialyses a precipitate was thrown down. Nitrogen was determined in the precipitate and in the filtrate which remained in the dialyzing sac.

The total materials extracted by the ether and by the water were determined as well as the amount of residue remaining. The quantity of material extracted by the combined salt, alcohol and alkali extractions was calculated by difference.

The similarity between the two grass pollens and between the two ragweed pollens is very marked. The grass pollens swelled greatly in alkali while the ragweed pollens did not. The ragweed pollens contain much more ether-soluble material, but only approximately half as much material extractable by water.

The differences in nitrogen content in the similar fractions of the different pollens do not appear to be significant. About half of the nitrogen was not extractable by the solvents used.

The results on the water extracts indicate that most of the nitrogen extracted by this solvent is non-protein. It also appears that the water extract contains globulin.

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THE EFFECT OF SUBSTITUENTS UPON THE REARRANGEMENT OF BENZOPINACOL

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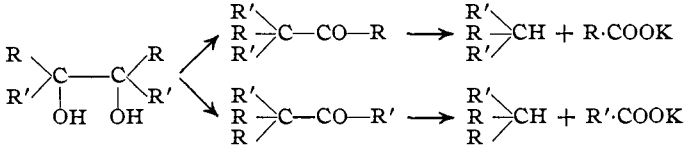
The relative migratory tendencies of organic radicals have often been determined by means of molecular rearrangements. Any rearrangement in which two groups occupy identical positions and are equally free to move might theoretically be used for this purpose, but if the two groups are nearly equal in migratory tendency, each will migrate to some extent, and a mixture of two isomeric products will be formed. The separation of these, or a determination of the relative amounts in which they are present, is often difficult. In the case of the pinacol rearrangement, however, each of the two pinacolones which are formed may be split by the action of alcoholic potassium hydroxide into a hydrocarbon and the potassium salt of an acid. These acids are often readily separated.



Thus Montagne has determined the relative migratory tendencies of the *p*-chlorophenyl and phenyl groups[1] and the *p*-bromophenyl and

[1] Montagne, *Rec. trav. chim.*, **26**, 253 (1907).