[CONTRIBUTION FROM THE CHEMICAL RESEARCH LABORATORY, THE UPJOHN COMPANY.]

ANALYSIS OF RAGWEED POLLEN.

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The work of Blackley[1] established the theory that pollen is the etiological agent in hay fever. Dunbar[2] came to the same conclusion. Dunbar considers that pollen grains contain a toxin,[3] *i. e.*, a poisonous substance, which has the property of stimulating antitoxin production in the animal body. Kamman[4] classifies it as a water-soluble toxalbumin. The work of Dunbar proceeded to the preparation of an antitoxic serum ("pollantin") for passive immunization against hay fever; but immunologists are not yet agreed as to whether or not the pollen albumin is accompanied by a toxin or whether it itself becomes by degradation an anaphylatoxin. The work of Noon and Freeman[5] reports the results obtained by active immunization, using timothy pollen extracts. All the work mentioned thus far has been carried out almost entirely with pollen of the grasses, particularly rye pollen.

In this country, the pollen of the ragweed (*Ambrosia*) is undoubtedly the chief causative agent in autumnal catarrh, and a lively interest has developed in studying immunization by using various preparations made from it.[6] The term "pollen unit" has come into requisition for the purpose of calculating dosages. The expression as used by Noon designates 0.0_51 g. timothy pollen. Koessler uses it to designate 0.0_725 g. ragweed pollen, and assuming the presence of 40% protein he speaks of this being equivalent to 0.0_71 g. ragweed pollen protein.

Last year we were able to gather a considerable quantity of ragweed pollen in the immediate vicinity of Kalamazoo, and considering the importance which is being attached to this substance, it was considered of interest to subject our sample to a chemical investigation. Although the several pollens mentioned have been used in numerous immunological studies, the writer can find nothing whatever as to the composition of the pollen of *Ambrosia*, and our information as to rye pollen is confined to a very brief analysis. Rye pollen has the following composition:[7] Water, 10.2%; ash, 3.4%; fat and wax, 3.0%; starch, 25%; non-protein nitrogenous substances, 18%; and albuminous substance, 40%.

[1] "Experimental Researches on the Cause and Nature of Hay Fever," London (1873); *Lancet*, **1881**, 371; *Brit. Med. J.*, 1, 867 (1898).

[2] "Zur Ursache und specifischen Heilung des Heufiebers," München (1903); *Deut. Med. Wochschr.*, Nov., 1911.

[3] *Berl. klin. Wochschr.*, **41**, 537, 567, 596 (1903).

[4] *Biochem. Z.*, **46**, 151 (1912).

[5] *Lancet*, **1914**, 1179.

[6] K. K. Koessler, "Forscheimer's Therapeusis of Internal Diseases," p. 671 (1914).

[7] Report of Schimmel & Co., April, 1914.

Various nontoxic pollens, which are more readily accessible, have already been examined. The pollen of *Pinus sylvestris* contains[1] 11.24% sucrose and 7.06% starch. This work was repeated and confirmed by Kresling.[2] In the pollen of *Coryllus avellana* was found[3] 14.7% sucrose and 5.26% starch. Stift[4] found only a trace of sucrose and 0.8% of starch and dextrin in sugar-beet pollen. Planta[5] found 4.2% fat in hazel pollen and 10.6% fat in fir pollen. Kressling[5] analyzed fir pollen and found triolein, tripalmitin, myricyl alcohol, and cerotic acid. Our knowledge of the proteins in these non-toxic pollens is limited to some analytical data; thus in the pollen of *Pinus sylvestris*, Planta found 16.6% protein, while Kresling found 15.9%. *Corylus avellana* pollen contained 30.1% protein, according to Planta, and that of *Beta vulgaris* yields 16.9% according to Stift.

In studying the nitrogenous constituents, Planta[6] and E. Schulze and Planta[7] found in all cases nuclein bases. Planta obtained from the pollen of *Corylus* 0.15%, from *Pinus* pollen 0.04%, hypoxanthine and guanin. Kresling found in pine pollen, 0.015% xanthine, 0.02% guanin and 0.09% hypoxanthine. The most interesting nitrogenous glucoside, vernin, was found in *Corylus* and pine pollen. This was later shown[8] to yield guanin and glucose and have the composition $C_{10}H_{13}O_5N_5$.

It is generally stated[9] that pollen contains a considerable quantity of lecithins.

In connection with the toxic pollens it is important to observe the presence of enzymes,[10] especially those of proteolytic nature, since they might naturally tend to disturb the original composition of various pharmaceutical preparations that might be prepared from pollen. Kamman's work indicates the presence of a protease, diastase, oxydase, and a lipase in rye pollen. A cystase and an invertase have been proved to be present in several nontoxic pollens.

The present paper reports the results of the proximate analysis of ragweed pollen (*Ambrosia artemisifolia* L.) along with other interesting data that have accumulated in this laboratory. We shall later report upon several of the individual constituents with special reference to the nitrogenous constituents.

[1] Schulze and Planta, *Z. physiol. Chem.*, **10**, 326 (1886).
[2] *Arch. Pharm.*, **229**, 389 (1891).
[3] Planta, *Landw. Versuchsstat.*, **31**, 97 (1884); **32**, 215 (1885).
[4] *Just. Jahresber.*, 1, 304 (1895); *Chem. Zentr.*, **1**, 45 (1896); **1**, 903 (1901).
[5] *Loc. cit.*
[6] A. v. Planta, *Landw. Versuchsstat.*, **31**, 97 (1884).
[7] E. Schulze and Planta, *Z. physiol. Chem.*, 10, 326 (1886).
[8] *Ibid.*, **41**, 457 (1909).
[9] *Biochem. Handlexicon*, **3**, 224 (1911).
[10] See Czapek, *Biochem. Pflanzen*, 1, 490 (1913).

Experimental.

Preparation of Material.—Our sample was collected between Aug. 21 and Sept. 2, 1916. The selected plants were pulled up by the roots and brought indoors. Glazed, transparent paper in the form of a bag was tied loosely over the flowering tops and the roots were then immersed under water in specially made troughs, so that the plants inclined over the sides. After being kept in this manner, the pollen grains were collected for several days when the bag was taken off and the yield passed through a very fine sieve. The material collected in this manner was almost pure and had a bright yellow appearance. A further collection of somewhat dirtier pollen could be obtained by placing the plants in a larger paper bag and shaking the pollen off. The pure material after drying *in vacuo*, over sulfuric acid for several months, lost 8.2% of water. This material forms the basis for the work reported.

Microscopic examination showed that the pollen was in the three-nuclear stage. It failed to respond to an iodine test for starch.

The ragweed pollen grain is a small spherical structure, measuring from 0.0095 mm. to 0.02 mm. in diameter and has an average volume of 0.069 cu. mm. The cell wall forms a very conspicuous portion of the cell and amounts to practically 65% of the structure. The cell wall is thrown into numerous projections averaging about 0.035 mm. in length. The protoplasmic substance therefore is not so important here, as in the rye pollen, which is rich in starch and protein. The nuclear volume is highly variable, due mainly to the divergence in size of the three separate nuclei. The nuclear volume is estimated to occupy about 20% of the protoplasmic substance. The structure of cell is certainly well adapted to its function to serve as a wind-borne pollen.

A suspension of 1–500 was counted in a haemocytometer and it was found that it required about 610 million cells to yield 1 g. of pollen.

The work was begun by extracting with various volatile solvents. By exhaustively macerating with anhydrous ether 15.3 g. yielded an ether extract weighing 1.811 g., equivalent to 11.83%. The ether-extracted residue weighed 13.211 g., equivalent to 86.1%.

4.0 g. pollen extracted in a Soxhlet yielded 0.5305 g. ether-soluble material, equivalent to 13.26%.[1]

An ether extract (Soxhlet) made upon a sample of pollen dried at 100° in a stream of hydrogen, gave 12.55%. A similar estimation of ligroin-soluble material gave 10.82%.

0.8643 g. ether-extracted pollen (equivalent to 1.0 g. undried pollen) was repeatedly extracted with hot absolute alcohol, and the alcoholic extract was made up to 200 cc. An aliquot evaporated to dryness showed the presence of 20.9% alcohol-soluble material.

[1] Material collected the preceding year yielded 15.7%.

1.0 g. undried pollen, exhausted with hot 95% alcohol, yielded 42.9% soluble material.

The proximate analysis yielded the following results:

	Per cent.		Per cent.
Moisture	5.28; 5.12	Total nitrogen	4.99
Starch (diastase)	None	Nitrogen in alcohol extract	1.08
Crude fiber	12.2	Ash	5.39
Pentosans	7.26	Dextrin	2.1

The quantitative examination of the alcohol-soluble carbohydrates resulted as follows:

A quantity of fat-free pollen equivalent to 10 g. pollen, was thoroughly extracted with boiling neutral 95% alcohol. The alcoholic solution was concentrated to a small volume and the residue was taken up with water. The resin that separated was filtered off and amounted to 17.4% of the pollen. The filtrate from the resin was treated with a slight excess of basic lead acetate solution, which yielded a yellow precipitate, and then made up to a volume of 50 cc. The direct and invert readings at 24° in a 2-dcm. tube are —1.6° V., and —2.0° V., respectively. The invert reading at 86° in a 2-dcm. tube was —2.0° V. Hence, sucrose = 0.4%, and there is no evidence of levulose. 25 cc. of the above solution were freed from lead with anhydrous sodium carbonate, and 5 cc., equivalent to 1.0 g. pollen when examined by the Walker-Munson process, gave 0.0375 g. Cu_2O. This corresponds to 1.58% glucose.

Estimation of phosphorus showed that the entire pollen contained 0.37%; that the ether extract was phosphorus-free, and that the alcoholic extract contained a minute quantity equivalent to about 0.03%. Most of the phosphorus is in the ash.

On entire pollen: 0.090 g. pollen yielded by Raper's[1] process 48.5 mg. $PbMoO_4$, equivalent to 0.335 mg. P. Found, 0.37% P, or 0.86% P_2O_5.

On ether extract: The quantity of phosphorus present was so slight that it was necessary to add 5 cc. of standard P_2O_5 solution to insure the complete separation of the phosphomolybdate precipitate. Triplicate blanks on the 5 cc. standard gave 0.0824, 0.0817, and 0.0843 g. $PbMoO_4$. A quantity of ether extract, equivalent to 0.32 g. and a duplicate of 0.8 g. pollen, gave 0.0816 and 0.824 g. Hence, no lecithins can be present in the ether extract.

On the alcoholic extract of the ether-extracted pollen: The precipitate of phosphomolybdate was so slight that it was again necessary to add a standard solution of P_2O_5. A quantity of the alcoholic extract, equivalent to 0.5 g. of the original pollen, yielded (after subtracting blank) 0.0217 g. $PbMoO_4$, equivalent to 0.345 mg. P_2O_5 or 0.069%. A duplicate gave 0.063% P_2O_5. This is equivalent to 0.75% lecithin (distearyllecithin).

On ash: 0.1781 g. pollen gave 0.0096 g. ash and this gave 0.1134 g. $PbMoO_4$. P_2O_5 in ash 18.75%, equivalent to 1.01% P_2O_5 or 0.44% P in pollen itself.

Distribution of the Nitrogen and Protein Extractions.

Quantitative nitrogen estimations showed that the pollen contained 4.99% N, of which 1.08% was alcohol-soluble. Saline extractions remove about 2.6% but this represents both protein (0.6%–0.8%) and a very considerable quantity of basic substances (1.9%). After complete saline extraction, 0.2% alkali extracted a large quantity of nitrogenous material

[1] H. S. Raper, *Biochem. J.*, 8, 649 (1915).

(0.8%–1.1% N). After extracting with dilute sodium chloride (10%) and 0.2% KOH solution, 1% sulfuric acid was found to remove only a small quantity of nitrogen, 0.12%; the residue contained 1.2% nitrogen.

A few brief quantitative estimations on protein extractions were made on the material collected in 1915. The material containing 5.06% water and 4.91% nitrogen was triturated with 8.5% salt solution for one hour, and then shaken mechanically for two hours. The exhausted residue was filtered off on a gooch. The nitrogen soluble in dilute salt solution equals 2.52%. A duplicate made in the same way, except that the mixture was incubated for sixteen hours after having been mechanically agitated, gave 2.6%.

The nitrogen in the saline extract was not entirely of a protein nature. By precipitating the saline extract (slightly acid reaction) with ten volumes of 95% alcohol and filtering off the flocculent precipitate, it was found that the filtrate contained 1.93% nitrogen. Hence, only 0.63% proved to be protein nitrogen. This would be approximately only 3.9% protein. The protein weighed, amounted to about 6.0%.

These figures are interesting because they indicate a great difference between the composition of the ragweed pollen, and the rye pollen, which is stated to contain 40% protein. There has existed the tendency to ascribe to ragweed pollen a similar composition as far as protein is concerned, and this has led Koessler into formulating a so-called pollen unit using ragweed pollen protein as a unit, and assuming this quantity to represent 40% of the pollen involved. The non-protein constituents of the alcoholic filtrate gave a heavy precipitate with phosphotungstic acid.

The pollen collected this year contained 5.28% water and 4.99% N. In order to see if we could increase the yield of protein extracted with salt solution we first extracted a sample with ether, and then extracted with 10% salt solution. (Total N = 2.7%.) Upon precipitating this extract with alcohol the crude protein that separated amounted to 4.9% (0.78 × 6.25). The filtrate in this case again showed the presence of considerable nitrogen—1.9%.

The residue from the above saline extract was now extracted with 0.2% KOH, and duplicate determinations showed the presence of 0.8% and 1.07% nitrogen. In one analysis the residue left after these extractions showed the presence of 1.45% N. In another the residue was first extracted with 1% H_2SO_4 which dissolved 0.12% N; whereafter the residue contained 1.21% nitrogen. These determinations were made by shaking the pollen with several portions of the extracting solution mechanically for about an hour and centrifuging to a good separation.

As is well known, the protein precipitated from saline extract by the addition of alcohol is the basis for the several vaccines now extensively used in actively immunizing against hay fever. Its activity may be graded by the power it has to set up a local irritation of the eye, thus producing in a way an experimental hay fever. It is also generally considered that this toxic property of pollens resides entirely in the proteins thus prepared, or perhaps with a toxin that is associated with the protein precipitated by alcohol.

In order to guide us in some work with ragweed pollen proteins, which we have in progress, we endeavored in the first place to establish the sensitiveness of several hay fever subjects. They were subjected to the ophthalmic tests with two preparations made as follows:

I. *Galenical extract:* A quantity of fat-free ragweed pollen, equivalent to 0.25 g. pollen (5.28% moisture), was triturated with 10 cc. 8.5% sterile salt solution containing 0.5% phenol added drop by drop and the mixture was then shaken mechanically for two hours. After standing over night it was again shaken and then centrifuged. A quantity of the clear supernatant fluid, equivalent to 0.125 g. pollen, was pipetted off and was diluted to a volume of 125 cc. (45 cc. water + 75 cc. 0.85% saline soln. + 0.5% phenol). 1 cc. = $^1/_{1000}$ g. pollen.

This solution was the basis for serial dilutions used in eye-testing with galenical pollen preparations.

II. *Protein extract:* The fat-free pollen was extracted as described above, and 5 cc., after withdrawal, were precipitated with the addition of 50 cc. 95% alcohol. The protein was separated by centrifuging. This precipitate was dissolved in 125 cc. physiological salt solution containing 0.5% phenol. 1 cc. = protein of $^1/_{1000}$ g. pollen.

This solution was the basis for serial dilutions used in eye-testing with the protein preparation.

"A" was tested with Preparation I; quantities amounting accurately to 0.0_4125,[1] 0.0_41, and 0.0_562 g. being dropped in the eye and the reactions noted were, respectively, deep ($^1/_2$ hr.), marked, and very slight. When the same subject was treated with Preparation II, in quantities amounting to 0.0_4125, 0.0_41, 0.0_583, and 0.0_562 g., reactions were, respectively, marked, distinct, slight, and negative.

"B" was somewhat more resistant. Using Preparation I, we found with 0.0_4125 the reaction was slight, with 0.0_4167 distinct, and with 0.0_4250 marked, lasting an hour. With the protein preparation he gave a distinct but transitory test with 0.0_425 and practically no test with 0.0_4167 g. The reactions with protein preparations in both cases did not last as long subjectively, even when positive.

In order to find out whether or not the decanted alcoholic supernatant liquid (1.9% nitrogen) exerted any influence in setting up this ophthalmic reaction, the alcoholic liquid decanted from the precipitated proteins was freed from alcohol under diminished pressure, and the liquid was diluted to 50 cc. (45 cc. water + 0.5% phenol). 1 cc. = 2.5 mg. pollen saline extract minus 4.9% protein. When the ophthalmic test was made with this solution "B" failed to respond even with a quantity equivalent to 0.0_3125 g. or ten times the quantity above mentioned. With "A," on the other hand, a marked reaction could be set up with protein-free solutions. In fact, a distinct reaction was obtained with 0.0_425 g.

"A" may be said to be sensitive to about 0.0_41 g. pollen and somewhat less so to $0.0_41 \times 0.05 = 0.0_55$ g. ragweed pollen protein. "B" reacts to 0.0_4167 g. pollen and to $0.0_425 \times 0.05 = 0.0_512$ g. protein. "B" represents a less sensitive type than "A." Dunbar reported that for rye pollen 1 to 2 drops of a 1 : 40,000 dilution of the protein was the average minimum limit of activity. This amounts to about 0.0_525 g. rye pollen proteins,

[1] 0.05 cc. of solution containing 0.25 mg. pollen.

a quantity of about the same dimensions as we have found for ragweed pollen protein.

Summary.

1. Ragweed pollen has the following composition: Alcohol soluble, 42.9%; moisture, 5.3%; crude fiber, 12.2%; pentosans, 7.3%; ash, 5.4%; dextrin, 2.1%; protein, 24.4%. Of the protein, about 7.5% could not be extracted, while 6.75% was extracted with dilute alkali, and only about 5% with 10% salt solution. The albumin and globulin fraction is therefore quite small. The analytical figures indicate the presence of proteoses. The nitrogen in the alcoholic extract (1.08%) is probably a base, and the nitrogen in the saline extract after alcohol had precipitated the proteins (1.9%) probably contains this base, and also some proteose.

The alcoholic extract (42.9%) contains fat, 10.8%; lecithin, 0.75%; ether soluble but not ligroin soluble, 1.75%; sucrose, 0.4%; glucose, 1.6%; resin, 17.4%; and a nitrogenous base.

2. A characteristic ophthalmic test could be obtained in the case of two average hay fever subjects with quantities of ragweed pollen protein amounting to $0.0_{5}1$ to $0.0_{5}5$ g.

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INFLUENCE OF CERTAIN ELECTROLYTES UPON THE COURSE OF THE HYDROLYSIS OF STARCH BY MALT AMYLASE.

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The influence of electrolytes and of hydrogen-ion concentration upon enzyme action is now so well recognized as to require no elaboration of the statement that experiments conducted with malt extract or commercial "diastase" containing unknown kinds and amounts of organic and inorganic material cannot be regarded as conclusive.

Osborne's highly purified malt amylase preparations[1] were devoted to determinations of diastatic power and especially to the study of the chemical nature of the enzyme. Recent investigations in this laboratory upon methods of purification[2] and the influence of various electrolytes upon the activity of the purified enzyme[3] have made possible the present study of the general course of the hydrolysis of starch by malt amylase

[1] THIS JOURNAL, **17**, 587 (1895); **18**, 536 (1896).

[2] Sherman and Schlesinger, *Ibid.*, **35**, 1617 (1913); **37**, 643 (1915).

[3] Sherman and Thomas, *Ibid.*, **37**, 623 (1915).