

THE PREPARATION OF HIGHLY CONCENTRATED ALLERGENIC EXTRACTS BY INCREASING THE CONCENTRATION OF EXTRACTION

I. TIMOTHY AND LOW RAGWEED POLLENS

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EARLIER impressions concerning pollen extraction¹⁻¹⁶ implied that it was not advantageous to extract pollen at concentrations greater than 3 per cent to 8 per cent weight by volume. This opinion was based on the observation that the relative activity of such extracts apparently failed to increase in proportion to the increase in extracting concentration.

Brown, in 1932,¹ reported on yields of extraction up to 20 per cent weight by volume ratio. The more concentrated extracts, as a group, were more active by direct skin tests than the less concentrated ones. The total nitrogen values increased in regular proportion up to 12.5 per cent, but at 17.5 per cent and 20 per cent they were not proportionately stronger. Stull and his associates,¹² in 1933, briefly reported studies using a maximum weight by volume ratio of 9 per cent. The values of total and phosphotungstic acid-precipitable nitrogen of extracts, ranging between 1 per cent and 9 per cent concentrations, were linear even after the volume of the filtrates was adjusted to the original volume of fluid added. Sullivan and Vaughan¹⁶ found that a 10 per cent extract was five times more active than a 2 per cent extract. Rappaport¹⁶ prepared an extract which was four and one-half times stronger than the original extract by drying at 40° C. and restoring to the desired volume. There are reports of the use of 10 per cent to 20 per cent extracts for clinical and experimental purposes.^{6, 10, 21, 30, 35, 37, 17} Malkiel²⁷ first informed this laboratory of his findings that the yields of nitrogen increased beyond that to be expected from an examination of the early reports on this subject.

Considerable advantage could result from the preparation of pollen extracts of higher concentration, since those prepared in the proportion of 3 to 5 Gm. per 100 ml. extracting fluid, have been found to be relatively poor antigens for lower animal experimentation. It was felt that greater concentrations of extracts would offer more desirable antigens, not only for sensitization of the lower animal, but also for clinical use.

Furthermore, since it has been shown that glycerin in the strength of 50 per cent preserves the activity of pollen extracts²⁰ and since the injection of

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such high concentrations of glycerin may be painful, it seemed desirable to prepare stronger extracts, so that when dilutions of such extracts are prepared for clinical use the glycerin content would be of weaker, more practical strengths.

The present investigation was designed to determine the effect of increasing the weight by volume ratios on the concentration of allergenic extracts.

METHODS

Preparation of Extracts.—Extracts of low ragweed (*Ambrosia elatior*) and timothy (*Phleum pratense*) pollens were prepared by adding weighed aliquots (0.5 to 25 grams) of a single lot of ether-defatted (Soxhlet) pollen to 50 ml. aliquots of extracting fluid. Evans-buffered saline¹⁷ was designated as "plain" extracting fluid and Evans-buffered saline containing glycerin in the concentration of 50 per cent was designated as "glycerinated" extracting fluid. Fifty per cent glycerin was used, since this concentration of glycerin had been shown to maintain stability of ragweed pollen extracts.²⁰

Direct extraction was carried out with 50 per cent glycerinated Evans fluid. It was evident that this procedure would preclude the dilution that necessarily results when glycerin is added to stock solutions after extraction. There are many reports of the use of glycerin as an extractant.^{1-3, 5, 7, 40, 46}

The weight by volume ratios of pollen to extracting fluid used in this study were 5, 10, 20, 30, 40, and 50 grams per 100 ml. These extracts were designated as 5, 10, 20, 30, 40, and 50 per cent extracts, respectively. All extracts were processed in an identical manner. Thorough mixing and wetting were accomplished by manual agitation. The pollen suspensions were then placed in an automatic shaker for one hour at 4° C. and refrigerated overnight. The insoluble residue was removed by Buchner filtration through a single sheet of Whatman #1 filter paper and the volume of each filtrate measured. Discrepancies due to the effect of Seitz filtration were minimized by passing a standard volume (20 ml.) through the sterilizing filter pad (St-1, 37 mm.). The Seitz filtrates were dispensed into sterile containers, cultured, and stored at 4° C.

Analytic Procedures.—The analytic techniques which were used in these studies were essentially as follows:

Nitrogen content was determined by micro-Kjeldahl methods described by Conway,²¹ Ma and Zuazaga,²² and Miller and Houghton.²³

Phosphotungstic acid-precipitable nitrogen was determined according to the method of Cooke and Stull.¹⁰ Hereafter this value will be referred to as PTA nitrogen. One protein nitrogen unit (PNU) is equivalent to 0.00001 mg. phosphotungstic acid-precipitable nitrogen.

Reducing substances, hexoses, and pentoses, were quantitatively estimated by the methods of Somogyi,²⁴ Nelson,²⁵ Meybaum,²⁶ and Lusena.²⁵ Hydrolysis was carried out with 5 N sulfuric acid for three hours at approximately 100° C., followed by neutralization with sodium hydroxide. All values of concen-

trations of reducing substances were reported as glucose in milligram per milliliter. The values of bound carbohydrate were obtained by subtracting the values of free reducing substances in unhydrolyzed samples from their corresponding values of total reducing substances (after hydrolysis).

Qualitative chromatograms for identification of amino acid and carbohydrate components of the substances analyzed were run according to the methods of Consden and co-workers,⁸ Dent,¹⁵ and Partridge.^{33, 34} Hydrolysis for amino acid chromatograms was accomplished with 6 N hydrochloric acid in sealed tube at 110° C. for eighteen hours followed by drying and aeration over sodium hydroxide. Hydrolysis for carbohydrate chromatograms was accomplished with 1 N sulfuric acid for one hour at 100° C. followed by neutralization with barium hydroxide.

Determinations of pH were performed with a Beckmann research G apparatus.

The allergenic activity of each extract was estimated by the quantitative determination of its capacity to neutralize skin-sensitizing antibody (reagin), according to the method of Cooke and co-workers,^{11, 12} using pooled high-titer serum obtained from patients with ragweed and timothy pollinosis. Briefly, the technique was as follows: Equal volumes of a single dilution of serum capable of passively sensitizing normal human skin to ragweed and timothy pollens were mixed with the same volume of a series of dilutions of each extract. Two to six hours after in vitro mixture, 0.1 ml. of each mixture was injected intradermally into the backs of nonreactive human subjects. Ninety-six hours later, each site was tested with approximately 0.02 ml. of a known active extract containing 1,000 phosphotungstic acid-precipitable nitrogen units (PNU) per milliliter. The immediate wheal reactions were evaluated after about thirty minutes. The lowest concentration of extract present in the serum-extract mixture which inhibited a positive reaction on subsequent retest was designated as the end point of neutralization.

RESULTS

Physical Characteristics of Extracts.—Freshly prepared extracts showed certain differences as the concentration of extracting mixtures was increased from 5 to 50 per cent weight by volume.

The volume of the Buchner filtrate decreased from 96 to 44 per cent of the original volume of fluid added for extraction. The time required for Seitz filtration increased approximately sixfold. The pH of the filtrates decreased from 6.0 to 5.4. The clarity of the Buchner filtrates decreased. The color intensity increased.

Several changes in appearance of the extracts were noted after varying periods of storage at refrigerator temperature.

Sedimentation occurred in all unglycerinated extracts of both timothy and ragweed pollens. There was increased sedimentation with increasing per cent extraction. Crystals appeared in certain extracts of timothy. Observations on the nature of these deposits are described in detail in the *Sedimentation* section of this article.

The pH of undiluted, one-year-old extracts showed a drop from their original values (Table I). The plain extracts, as a group, tended to show greater fall than the glycerinated extracts. The greatest change appeared in the plain 40 per cent weight by volume ragweed extract, which showed a drop from 5.62 to 4.93. The range of pH of one-year-old extracts was 5.95 (5 per cent) to 4.9 (40 per cent), compared with the original range of 6.0 to 5.4.

TABLE I. DETERMINATION OF pH OF UNDILUTED EXTRACTS

	PER CENT EXTRACTION (WEIGHT BY VOLUME)	ORIGINAL	pH 1 YEAR AT 4° C.	CHANGE
<i>Ragweed Pollen</i>				
Plain	5	6.00	5.95	-0.05
	10	5.82	5.50	-0.32
	20	5.70	5.18	-0.52
	40	5.62	4.93	-0.69
50% Glycerinated	5	5.92	5.80	-0.12
	10	5.70	5.75	+0.05
	20	5.63	5.20	-0.33
	30	5.59	5.25	-0.24
	40	5.58	5.20	-0.38
<i>Timothy Pollen</i>				
Plain	5	6.00	5.60	-0.40
	10	5.70	5.28	-0.42
	20	5.50	5.20	-0.30
	40	5.40	5.12	-0.28
50% Glycerinated	5	6.00	5.85	-0.15
	10	5.82	5.56	-0.26
	20	5.62	5.51	-0.11
	30	5.60	5.51	-0.09
	40	5.60	5.43	-0.17
	50	5.60	5.42	-0.18
<i>Extracting Fluids</i>				
Plain and 50% Glycerinated		7.00	7.00	0.00

Dilution of the extracts with buffered saline to concentrations of 0.10 to 0.001 mg. PTA nitrogen per milliliter, in some instances, resulted in a slight increase in pH after storage for one year. Crip and associates¹³ noted a similar change in pH with aging of pollen extracts that had been diluted with buffered saline to concentrations of 0.01 mg. nitrogen per milliliter.

The color of most of the extracts became darker with age, particularly the plain extracts of high weight by volume concentration.

Chemical Analyses.—

A. Total and phosphotungstic acid-precipitable nitrogen: There was a linear, proportional increase in the total and PTA nitrogen content of both plain and glycerinated ragweed and timothy pollen extracts as the weight by volume ratio of extraction was increased from 5 to 50 per cent (Table II). These results have been confirmed by the analysis of extracts, prepared in a similar manner, of four other lots of timothy and ragweed pollens.

The total and PTA nitrogen concentrations of the fresh extracts, when plotted against concentrations of extraction, yielded straight lines (Figs. 1 and 2). No plateau was encountered with the extraction concentrations used in this study with either timothy or ragweed pollen.

TABLE II. ONE-DAY-OLD POLLEN EXTRACTS*: ANALYTICAL DATA

		WEIGHT BY VOLUME RATIO							K-VALUE** (RANGE)
		5 PER CENT	10 PER CENT	20 PER CENT	30 PER CENT	40 PER CENT	50 PER CENT	AVER- AGE	
<i>Ragweed</i>									
Plain	PTA-N	0.18	0.42	0.79	----	1.92	----	0.041	(0.036-0.048)
	TOTAL-N	0.54	1.00	2.04	----	4.33	----	0.105	(0.100-0.108)
	BOUND-CHO	2.30	4.60	9.00	----	18.50	----	0.460	(0.450-0.460)
	TOTAL-CHO	4.70	9.06	18.00	----	36.00	----	0.911	(0.900-0.911)
50% Glycerinated	PTA-N	0.16	0.36	0.75	1.20	1.74	----	0.038	(0.032-0.044)
	TOTAL-N	0.41	0.83	1.62	2.60	3.59	----	0.985	(0.081-0.090)
	BOUND-CHO	1.85	4.26	8.45	12.60	16.90	----	0.412	(0.370-0.430)
	TOTAL-CHO	3.70	8.50	17.00	25.00	34.40	----	0.826	(0.740-0.860)
<i>Timothy</i>									
Plain	PTA-N	0.27	0.52	1.14	----	2.45	----	0.055	(0.052-0.061)
	TOTAL-N	0.46	0.83	1.95	----	4.15	----	0.094	(0.083-0.104)
	BOUND-CHO	***	---	---	---	---	---	---	---
	TOTAL-CHO	13.00	25.00	48.00	----	95.00	----	2.470	(2.400-2.600)
50% Glycerinated	PTA-N	0.23	0.48	1.06	1.58	2.16	2.65	0.051	(0.046-0.054)
	TOTAL-N	0.35	0.70	1.55	2.55	3.19	3.65	0.075	(0.070-0.085)
	BOUND-CHO	***	---	---	---	---	---	---	---
	TOTAL-CHO	12.00	23.50	46.00	65.00	90.00	110.00	2.280	(2.190-2.400)

PTA-N = Phosphotungstic acid-precipitable nitrogen (milligram per milliliter).

TOTAL-N = Total nitrogen (milligram per milliliter).

BOUND-CHO = Total reducing substances minus free reducing substances (as glucose; milligram per milliliter).

TOTAL-CHO = Total reducing substances (hydrolyzed samples) (as glucose; milligram per milliliter).

*Seitz-filtered extracts.

**K-value = Ratio of concentration in milligram per milliliter to per cent extraction.

***Timothy extracts showed no increase, often a slight decrease, in CHO upon hydrolysis.

The total recovery of PTA nitrogen was progressively less, even though the concentration of PTA nitrogen increased with increasing concentration of extraction. Table III tabulates the relative recoveries, obtained with ragweed extracts in the upper half and timothy extracts in the lower half. The extraction ratio for both glycerinated and plain extracts is shown in the first column. There was progressive reduction in volume recovered as the concentration of extraction increased, until with glycerinated 40 per cent ragweed extract, for example, only 58 per cent of the added volume of fluid was recovered as extract, as recorded in the second column. Column 3 shows the linear increase of PTA nitrogen per milliliter of extract as the concentration increased. Column 4 expresses this increased protein content as per cent of the content of the 5 per cent weight by volume extract. Thus, the 40 per cent ragweed extract was 910 per cent stronger than the 5 per cent extract. Column 5 shows that the total PTA nitrogen recovered per 100 Gm. of extracted pollen decreased progressively as the concentration of extraction increased. This is

expressed in Column 6 as diminishing per cent of recovery. Therefore, the total yield of PTA nitrogen from glycerinated 40 per cent ragweed extract was only 72 per cent of that recoverable from the 5 per cent extract.

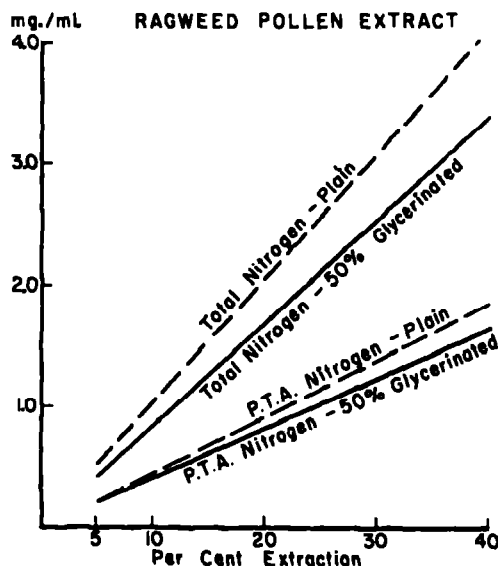


Fig. 1.

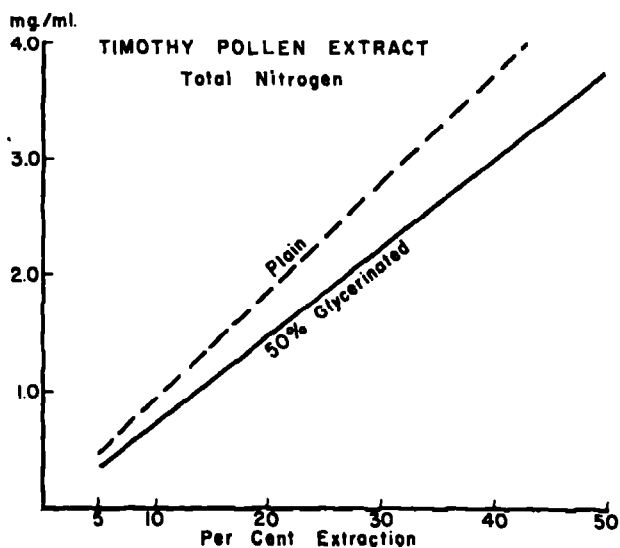


Fig. 2.

The same pattern of recovery was encountered for all the other extracts as recorded in Table III.

The total and PTA nitrogen values of glycerinated extracts of both timothy and ragweed pollen were 10 to 20 per cent lower than those of plain extracts (Tables II and III and Figs. 1, 2, and 3). Even though glycerin thus

TABLE III. RELATIVE RECOVERIES COMPARED WITH 5 PER CENT POLLEN EXTRACTS*

	PER CENT EXTRACTION (W/V)	PER CENT ORIGINAL VOLUME (50 ML.) RECOVERED	PTA NITROGEN (MG. PER ML.)	PER CENT OF 5% EXTRACT	TOTAL PTA- NITROGEN RECOVERED PER 100 GM. OF POLLEN (MG.)	PER CENT OF 5% EXTRACT
	(1)	(2)	(3)	(4)	(5)	(6)
<i>Ragweed</i>	5	92	0.20	100	368	100
	10	83	0.42	208	348	95
50% Glycerinated	20	68	0.84	420	286	78
	30	68	1.23	615	279	76
	40	58	1.82	910	264	72
	5	96	0.26	100	516	100
	10	92	0.48	178	440	85
Plain	20	82	0.95	356	391	76
	40	70	2.26	843	290	76
<i>Timothy</i>	5	94	0.28	100	533	100
	10	88	0.59	206	514	97
50% Glycerinated	20	74	1.18	416	436	82
	30	58	1.88	663	363	68
	40	46	2.59	913	298	56
	50	50	3.65	1,285	350	65
	5	90	0.35	100	624	100
	10	86	0.66	192	570	92
Plain	20	68	1.47	424	500	80
	40	44	3.13	905	345	55

*Fresh extracts, not Seitz-filtered.

TABLE IV. PHOSPHOTUNGSTIC ACID-PRECIPITABLE NITROGEN

	PER CENT EXTRACTION (W/V)	ORIGINAL	PTA-N. (MG. PER ML.) ONE YEAR AT 4° C.	PER CENT LOSS
<i>Ragweed Pollen</i>	5	0.18	0.15	17
Plain	10	0.42	0.30	29
	20	0.79	0.62	22
	40	1.92	1.68	13
	5	0.16	0.14	13
50% Glycerinated	10	0.36	0.33	8
	20	0.75	0.73	3
	30	1.20	1.12	7
	40	1.74	1.68	4
<i>Timothy Pollen</i>	5	0.27	0.16	41
Plain	10	0.52	0.30	43
	20	1.14	0.64	39
	40	2.45	1.38	44
	5	0.23	0.20	12
50% Glycerinated	10	0.48	0.45	6
	20	1.06	0.96	10
	30	1.58	1.48	6
	40	2.16	2.10	3
	50	2.65	2.58	3

reduced the yield of nitrogen, its use had the advantage of providing clear extracts in contrast to the extremely turbid and opalescent extracts obtained with plain extracting fluids, as described later in this article under *Sedimentation*.

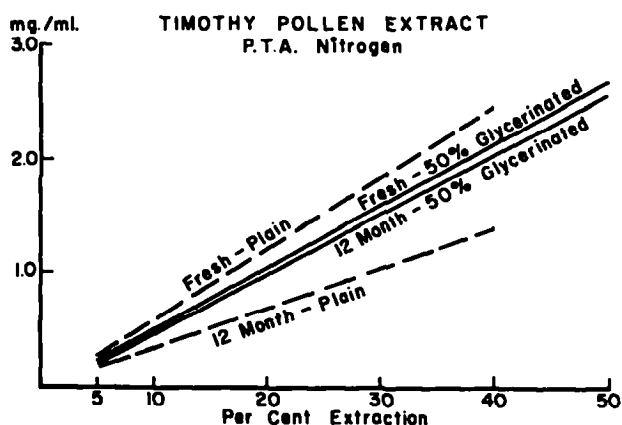


Fig. 3.

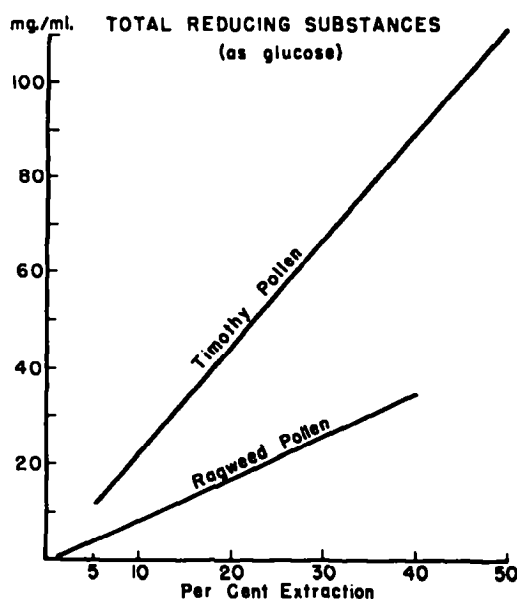


Fig. 4.

All extracts showed some loss of PTA nitrogen, varying from 2.5 to 44 per cent after storage for one year at 4° C. (Table IV and Fig. 3). The glycerinated extracts showed smaller losses than the unglycerinated extracts. This preservative effect of glycerin against loss of PTA nitrogen has been noted in other studies from this laboratory.²⁰

B. Reducing substances: There was distinct linearity between concentration of extraction and content of reducing substances in extracts of both pollens (Table II and Fig. 4).

1. Ragweed Pollen Extracts:

All fresh extracts of ragweed pollen showed an increase of approximately 100 per cent in reducing substances after hydrolysis as shown by the values of bound and total carbohydrate in Table II.

Bound carbohydrate could no longer be detected in the plain ragweed extracts after storage for one year at 4° C., whereas the glycerinated extracts showed only very slight losses (Table V).

TABLE V. RAGWEED POLLEN EXTRACTS; BOUND REDUCING SUBSTANCES

PER CENT EXTRACTION (W/V)		ORIGINAL	MG. PER ML. (AS GLUCOSE) ONE YEAR AT 4° C.	PER CENT LOSS
Plain	5	2.30	0.30	87
	10	4.60	0.70	85
	20	9.00	0.80	91
	40	18.50	2.00	90
50% Glycerinated	5	1.85	1.80	3
	10	4.26	3.90	9
	20	8.45	8.10	4
	30	12.60	11.00	12
	40	16.90	14.00	17

Chromatography studies indicated that glucose and fructose were present before hydrolysis and glucose and arabinose after hydrolysis. These results are in agreement with those on ragweed extracts reported by Dankner and his co-workers¹⁴ and Frankel and associates.¹⁸

2. Timothy Pollen Extracts:

The concentration of reducing substances in timothy pollen extracts showed no change, or only very slight losses, upon hydrolysis. Stull and his co-workers^{41, 43} reported similar findings in studies with extracts of cattail and timothy pollens.

Aging did not result in any quantitative change in carbohydrate content, since all the measurable reducing substances in the timothy extracts were in the free form in contrast to those of the ragweed extracts.

Chromatographic studies of timothy extracts, both before and after hydrolysis, revealed the presence of glucose and fructose. Gough,¹⁹ using methods other than chromatography, found 1- arabinose and galactose after hydrolysis of sodium hydroxide extracts of timothy grass pollen. This apparently resulted in extraction of bound reducing substances and destruction of free reducing substances. This finding is in contrast to the studies on aqueous and buffered saline extracts of timothy in this study and that by Stull and associates^{41, 43} in which quantitative determinations revealed no bound carbohydrate.

Calculation of the total recovery of reducing substances in relation to concentration of extraction yielded the same differences already described for the yields of PTA nitrogen.

Reagin Neutralization Studies.—The final criterion of allergenic yield should be evaluated in terms of biologic activity of the extracts, rather than any single chemical determination. In these studies, the allergenic activity of each extract was estimated by the quantitative determination of its capacity to neutralize skin-sensitizing antibody (reagin).

TABLE VI. REAGIN NEUTRALIZATION ACTIVITY OF NINETEEN-WEEK-OLD RAGWEED POLLEN EXTRACTS

CONCENTRATION OF EXTRACT MIXED WITH SERUM DILUTION (IN EQUAL VOLUMES) PNU* PER ML.	WEIGHT BY VOLUME EXTRACTION RATIO (PER CENT)							
	50% GLYCERINATED				PLAIN			
	5	10	20	40	5	10	20	40
5	+++	+++	++	+	+++	+++	+++	++
10	++	+	+	0	++	+++	+++	+
25	+	+	+	0	+	++	+	+
50	0	0	0	0	±	±	±	±
100	0	0	0	0	0	0	0	0
200	0	0	0	0	0	0	0	0
400	0	0	0	0	0	0	0	0
800	0	0	0	0	0	0	0	0
Diluent	++++	++++	++++	++++	++++	++++	++++	++++

*PNU = Phosphotungstic acid-precipitable nitrogen units.

A. Ragweed pollen extracts: Six neutralization experiments were performed with relatively fresh ragweed extract. Five, 10, 20, and 40 per cent extracts, studied as described under *Methods*, after six, ten, and nineteen weeks' storage showed clear-cut linearity in that the neutralization points for each extract, when diluted to the same PTA nitrogen concentration, were essentially of the same values (Table VI). Earlier studies, when the extracts were three and four weeks old, showed an unexplained trend toward increasing activity with increasing per cent extraction (Table VII), which was encountered in three separate experiments.

TABLE VII. REAGIN NEUTRALIZATION ACTIVITY OF FOUR-WEEK-OLD RAGWEED POLLEN EXTRACTS

CONCENTRATION OF EXTRACT MIXED WITH SERUM DILUTION (IN EQUAL VOLUMES) PNU* PER ML.	WEIGHT BY VOLUME EXTRACTION RATIO (PER CENT)							
	50% GLYCERINATED				PLAIN			
	5	10	20	40	5	10	20	40
10	+++	+++	+++	++	+++	+++	+++	++
25	++	++	+	±	++	++	+	±
50	++	+	±	0	++	++	±	0
75	++	+	0	0	++	++	0	0
100	++	0	0	0	++	++	0	0
200	++	0	0	0	++	++	0	0
400	±	0	0	0	++	++	0	0
800	0	0	0	0	+	0	0	0
Diluent	++++	++++	++++	++++	++++	++++	++++	++++

*PNU = Phosphotungstic acid-precipitable nitrogen units.

Furthermore, each extract, after storage for one year at 4° C., neutralized human ragweed-sensitive serum in the same linear range (Table VIII). Incidentally, plain extracts, after storage for one year, showed a loss in potency in comparison with glycerinated extracts in that only 50 to 100 units of glycerinated, but as much as 100 to 400 units of the plain, extracts were required for neutralization of the reaginic serum used in this study (Table VIII). These findings are similar to those previously reported from this laboratory.²⁰ The results with plain extracts agree with those of Stull and Sherman⁴⁵ who noted a loss of PTA nitrogen and neutralization capacity of extracts after storage for ten months at 7° C.

TABLE VIII. REAGIN NEUTRALIZATION ACTIVITY OF ONE-YEAR-OLD RAGWEED POLLEN EXTRACTS

CONCENTRATION OF EXTRACT MIXED WITH SERUM DILUTION (IN EQUAL VOL- UMES) (PNU* PER ML.)	WEIGHT BY VOLUME EXTRACTION RATIO (PER CENT)									
	50% GLYCERINATED					PLAIN				
	5	10	20	30	40	5	10	20	40	
5	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
10	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
25	++	++	++	++	+	+++	+++	+++	+++	+++
50	0	+	±	+	0	+++	+++	+++	+++	+++
100	0	0	0	0	0	+	+	+	+	+
200	0	0	0	0	0	+	0	0	0	±
400	0	0	0	0	0	0	0	0	0	0
800	0	0	0	0	0	0	0	0	0	0
Diluent	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

*PNU = Phosphotungstic acid-precipitable nitrogen units.

B. Timothy pollen extracts: Three neutralization experiments, performed with four- and five-week-old timothy extracts revealed distinct uniformity of neutralization activity of all concentrations from 5 to 50 per cent weight by volume (Table IX). The plain extracts showed some loss in activity, after storage for one year, when compared with the glycerinated extracts (Table X), indicating the preservative effect of glycerin, as was encountered with ragweed extracts previously noted.

TABLE IX. REAGIN NEUTRALIZATION ACTIVITY OF FOUR-WEEK-OLD TIMOTHY POLLEN EXTRACTS

CONCENTRATION OF EXTRACT MIXED WITH SERUM DILUTION (IN EQUAL VOL- UMES) (PNU* PER ML.)	WEIGHT BY VOLUME EXTRACTION RATIO (PER CENT)									
	50% GLYCERINATED					PLAIN				
	5	10	20	40	50	5	10	20	40	
10	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
50	±	±	0	±	±	±	±	0	0	0
100	0	0	0	0	0	0	0	0	0	0
150	0	0	0	0	0	0	0	0	0	0
200	0	0	0	0	0	0	0	0	0	0
400	0	0	0	0	0	0	0	0	0	0
Diluent	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

*PNU = Phosphotungstic acid-precipitable nitrogen units.

TABLE X. REAGIN NEUTRALIZATION ACTIVITY OF ONE-YEAR-OLD TIMOTHY POLLEN EXTRACTS

CONCENTRATION OF EXTRACT MIXED WITH SERUM DILUTION (IN EQUAL VOLUMES) (PNU* PER ML.)	WEIGHT BY VOLUME EXTRACTION RATIO (PER CENT)									
	50% GLYCERINATED						PLAIN			
	5	10	20	30	40	50	5	10	20	40
5	+	+	+	+	+	+	+	+	+	++
10	0	0	0	0	0	0	+	±	+	+
25	0	0	0	0	0	0	0	0	±	+
50	0	0	0	0	0	0	0	0	0	+
100	0	0	0	0	0	0	0	0	0	+
200	0	0	0	0	0	0	0	0	0	0
400	0	0	0	0	0	0	0	0	0	0
Diluent	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++

*PNU = Phosphotungstic acid-precipitable nitrogen units.

Table XI shows that, since the neutralization activity of each extract was linear, dilution of each fresh timothy extract to the same PTA nitrogen content resulted in approximately equal values when expressed in terms of total nitrogen content or of weight by volume dilutions.

TABLE XI. TIMOTHY POLLEN EXTRACTS; COMPARATIVE VALUES

	PER CENT EXTRACTION	PTA NITROGEN (PNU PER ML.)	WEIGHT BY VOL- UME DILUTION	TOTAL NITROGEN (UNITS PER ML.)
50% Glycerinated	5	10	1-45,000	15.6
	10	10	1-48,400	14.4
	20	10	1-52,500	14.7
	30	10	1-47,400	16.1
	40	10	1-54,000	14.3
	50	10	1-52,800	12.2
Plain	5	10	1-54,500	17.0
	10	10	1-51,600	16.0
	20	10	1-57,000	17.7
	40	10	1-49,800	16.7

Sedimentation.—There was progressive sedimentation during aging, with increasing per cent extraction, in all plain extracts of timothy and ragweed pollens (Table XII). The protective effect of glycerin in the concentration of 50 per cent against the formation of this amorphous sediment was marked.

TABLE XII. SEDIMENTATION IN UNDILUTED POLLEN EXTRACTS

Per Cent Extraction	RAGWEED										TIMOTHY									
	PLAIN					50% GLYCERINATED					PLAIN					50% GLYCERINATED				
	5	10	20	40	5	10	20	30	40	5	10	20	40	5	10	20	30	40	50	
Original	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
2 Weeks	+	++	+++	++	0	0	0	0	0	0	+	++	+	0	0	0	0	0	0	
4 Weeks	++	+++	++++	++++	0	0	0	0	0	+	++	++	++	0	0	0	0	0	0	
6 Weeks	+++	+++	++++	++++	0	0	0	0	0	+	++	++	++	0	0	0	0	0	0	
1 Year	+++	+++	++++	++++	0	0	0	0	0	+	++	++	+++	0	0	0	0	0	0	

Other observations in this laboratory indicate that glycerin in a concentration of 30 per cent or more is needed to prevent formation of such sediment in undiluted 20 per cent weight by volume ragweed pollen extracts.²² Sedimentation did not occur in the undiluted 50 per cent glycerinated extracts. However, dilution with unglycerinated buffered saline of fresh or one-year-old 20 per cent weight by volume, glycerinated extracts from 100,000 to 20,000 and 10,000 PNU per milliliter, in some instances, resulted in the formation of a small amount of sediment within a few weeks. No such sedimentation occurred in dilutions of 1,000 PNU per milliliter or weaker concentrations.

A. Ragweed pollen extracts: All glycerinated ragweed extracts remained clear during the one-year period of observation. An amorphous, yellow sediment did appear after two weeks' storage in the plain ragweed extracts and increased in quantity to an apparent maximum at six weeks (Table XII). The original change in appearance was a homogeneous opalescence and turbidity which later separated into layers of yellow, amorphous sediment and clear supernate. The amount of sediment increased as the per cent extraction increased. There were copious quantities in the 20 and 40 per cent weight by volume extracts.

A few studies were made with the amorphous sediment of the 40 per cent weight by volume plain extract after six weeks' storage. The loss in total and PTA nitrogen (0.15 mg. per milliliter), after removal of the sediment from this extract by centrifugation, was accounted for entirely by the nitrogen of the sediment.

Reagin neutralization studies were performed with portions of such sediment after it had been washed five times with cold-buffered saline and redissolved. The whole extract, supernate, and sediment solution were diluted to equal PTA nitrogen concentration. The sediment neutralized against itself, but not against the whole extract. Whole extract neutralized against the sediment in the same range as whole extract against itself.

The whole extract and the supernate had the same end point of neutralization. Thus, there was no apparent loss in activity, even though the isolated sediment had marked neutralizing activity when diluted on an equal PTA nitrogen basis.

Stull and associates^{42, 44} found sediment in 9 per cent weight by volume extracts and reported it as probably nonnitrogenous. The present analysis was on a 40 per cent weight by volume extract which may have resulted in 4.5 times more sediment than Stull's extract. The quantities thus involved probably account for their detectability by difference in nitrogen content before and after removal of the sediment from the extract by centrifugation.

Moore and co-workers³⁰ noted slow, spontaneous, noncrystalline sedimentation in aqueous ragweed extracts and reported the precipitate to be soluble in hot water and to be skin reactive. It was suggested that the reactivity may have been due to contamination with active components from the whole extract.

Chromatographic studies of an unhydrolyzed portion of sediment from two-week-old, plain, 40 per cent weight by volume ragweed extract yielded

no ninhydrin-reacting areas. After hydrolysis, however, there was an amino acid pattern such as that found by Dankner and co-workers¹⁴ and Frankel and associates¹⁸ in their chromatographic analysis of dialyzed residue of plain extract. The change after hydrolysis indicated that this particular sediment contained substances which were protein or polypeptide in nature. The presence of bound carbohydrate was also established for this sediment and this was identified as arabinose by chromatography.

In the present study, no attempt was made to establish activity or to characterize the sediment in the extracts other than that of 40 per cent weight by volume plain ragweed extract, or to identify the possible flavonoid components of the sediments as studied by Heyl,²¹ Stevens,³⁹ Moore and co-workers^{30, 31} and Stull and associates.^{42, 44}

B. Timothy pollen extracts: The changes in appearance of timothy extracts during storage at refrigerator temperature were somewhat different from those noted in ragweed extracts. An amorphous sediment appeared in the plain extracts after standing two weeks, but only in small amounts, though increasing with increasing concentration of extraction. Sedimentation of amorphous particles did not appear in the glycerinated timothy extracts. However, moderate quantities of a fine, feathery, light yellow, crystalline precipitate appeared in some of the extracts. Since aliquots of the same extract distributed in separate bottles yielded very different amounts of these crystals, it is possible that the surface of the container itself may have influenced the deposition of crystals. These crystals usually appeared after two days' storage at 4° C. and dissolved readily when the extract was warmed to room temperature.

It is believed that these crystals are identical to those described by Moore and Moore³¹ as a glucoside, "dactylin," appearing in extracts of orchard grass and timothy pollen. Stull and associates⁴³ reported such crystals and confirmed Moore and Moore's observations.³¹ Studies on the identification and allergenic activity of these crystals are in progress.²³

COMMENTS

Highly concentrated, potent pollen extracts were obtained by simple, direct extraction methods, without resorting to subsequent concentration procedures. It might be possible to obtain even higher than 50 Gm. per 100 ml. ratio, since, in this study, no plateau or any other evidence of saturation was noted.

The filtrates of the high weight by volume suspensions showed losses up to 56 per cent of the volume of fluid added (Table III). These losses apparently were due to the greater absorption of fluid by the larger amount of dry pollen per unit of fluid added originally for extraction. No attempt was made to recover the absorbed fluid by re-extraction. It was not the purpose of this study to extract the maximum from a particular weight of pollen, but to demonstrate that when increasing quantities of pollen were added to the same volume of fluid, proportionately stronger extracts could be obtained. Furthermore, re-extraction may yield extracts of different qualitative composition. Therefore, for the sake of uniformity, the only variable in these experiments was the weight of pollen per unit of extracting fluid.

Fifty per cent glycerin protected against sedimentation. Plain extracts of high weight by volume ratios became markedly turbid, while glycerinated extracts of the same respective concentrations and pH remained clear. It has been shown previously that, when glycerin was added to freshly prepared pollen extracts to a final concentration of 50 per cent, no differences in reagin neutralization activity of the plain extracts and such glycerinated extracts could be detected.²⁰

In this study, the solubility of pollen antigen in 50 per cent glycerinated buffered saline was about 90 per cent of its solubility in buffered saline alone. Equal reagin neutralization activity was observed, when both plain and glycerinated fresh extracts were diluted to the same PTA nitrogen content. This would seem to indicate that the same antigens were extracted by both plain and glycerinated buffered saline, at least within the limitations of reagin neutralization methods used in this study. Multiple antigens and antibodies, and their possible variabilities in reactivity of reaginic sera must be considered in evaluating the results of any experimental studies such as herein presented. The fact remains, however, that potent, highly concentrated pollen extracts can be prepared by direct extraction with either plain or glycerinated fluids, the latter being more practical.

It was not the purpose of this study to infer that there is necessarily complete correlation between allergenic activity and concentration of total and PTA nitrogen and reducing substances, since, in another study,²² ragweed pollen extract, heated to 56° C. for forty-five minutes, showed loss of reagin neutralizing activity, without any loss of PTA nitrogen or bound carbohydrate. Using freshly prepared, unmodified extracts, however, the quantitative relationship between concentration of these components and reagin neutralization activity in relation to weight by volume extraction ratio was distinctly proportional.

There were certain observations in these experiments that remain unexplained.

Four-week-old ragweed extracts, both plain and glycerinated, showed an increasing neutralizing capacity as the concentration of extraction increased (Table VII). This was less evident at nineteen weeks (Table VI) and even less demonstrable after one year (Table VIII).

Timothy pollen extracts, both plain and glycerinated, showed no increased neutralizing capacity at four weeks, as was the case with ragweed. One-year-old plain timothy extract actually showed a decreasing neutralizing capacity as the concentration increased, although there was a distinct proportional linearity of all glycerinated extracts after one year.

The possibility that unequal dispersion of sediment particles during dilution of the timothy extract might account for this observation is doubtful, since the plain ragweed extracts containing even greater amounts of sediment did not show this phenomena. Further study in the comparison of the chemical and biologic properties of the timothy and ragweed sediments might clarify these observations.

The limitations of end point evaluation in reagin neutralization, however, may not permit definitive end point determinations in a narrow range of 25 protein nitrogen units or less.

The fact remains, however, that more active ragweed and timothy pollen extracts were prepared by increasing the weight by volume concentration of extraction.

SUMMARY

1. Highly concentrated timothy and low ragweed pollen extracts have been prepared by direct extraction.

2. Extracts of timothy and low ragweed pollens, prepared in increasing weight by volume ratios, from 5 to 50 per cent, showed a distinct proportionality, indicating extraction in linear ratios, as measured by total and phosphotungstic acid-precipitable nitrogen content, concentration of reducing substances, and reagin neutralization activity.

3. The use of glycerinated extracting fluids was necessary in the preparation of such highly concentrated and potent extracts to avoid sedimentation and to preserve activity.

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