

QUANTITATIVE IMMUNOLOGIC STUDIES WITH ALLERGENS* † ‡

II. DETERIORATION OF RAGWEED POLLEN EXTRACTS AS MEASURED BY PRECIPITATION, NEUTRALIZATION, AND PROTEIN NITROGEN ANALYSIS

THE PREVENTION OF DETERIORATION BY GLYCERIN

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IT IS a well-established fact that various properties of aqueous pollen extracts deteriorate with age and by heating. Clock¹ first reported deterioration of the complement fixation properties of pollen extract at 5° C. in seven weeks. He further showed that storing the extracts in 66 $\frac{2}{3}$ per cent glycerin prevented this phenomenon and suggested that the action of glycerin was in preventing molecular changes due to its hygroscopic features.² Paton³ considered the instability of pollen extracts to be due to the presence of enzymes. Later, Armstrong and Harrison^{4, 5} showed that heating low ragweed pollen extract destroyed its capacity to fix complement with rabbit antisera and reported that the complement-fixing properties bore no relation to the actual allergenic factor. Gay⁶ found that heating pollen extracts to 56° C. for one hour greatly impaired their activity in skin testing. In 1927, Stier and Hollister,⁷ and Alles and Lamson⁸ suggested the addition of glycerin to extracting fluids. Stull, Cooke, and Chobot⁹ found that ragweed extract, after heating, lost much of its skin test and reagin neutralizing activity.

Stull, Cooke, and Tennant¹⁰ reported that allergenic extracts showed 50 per cent decrease in protein nitrogen after one year's storage at 10° C., although when rediluted gave comparable reactions in skin testing. Arbesman and Eagle¹¹ reported that heating ragweed extract to 56° C. for 30 and 120 minutes destroyed 75 and 95 per cent of its reagin neutralizing capacity, respectively. They also found that extracts stored in the icebox for two, six, and ten years showed a loss of activity of 84, 95.5, and 98.2 per cent, respectively. The phosphotungstic acid precipitable nitrogen content of the extracts were not changed appreciably. Stull and Sherman¹² reported that 10-month-old low ragweed extract decreased from 40,000 protein nitrogen units per cubic centimeter to 30,000 per cubic centimeter, and that it was much less active in neutralizing human sensitive serum than fresh extract, even when diluted on the basis of 30,000 protein nitrogen units (P.N.U.) per cubic centimeter. Sullivan and Vaughan¹³ reported that the skin-reacting factor of pollen extracts did not correspond with the protein nitrogen content and that the presence of glycerin in the extracts protected against deterioration.

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Stull, Cooke, and their co-workers¹⁴ found that heating ragweed extract to 70° C. for one hour reduced the phosphotungstic acid precipitable nitrogen from 0.35 mg. per cubic centimeter to 0.29 (and 0.26) mg. per cubic centimeter although no further reduction occurred in one year. Further studies (Dale and neutralization tests) indicated that the antigenic properties of ragweed pollen extract had been altered by heating. Heated extract did not liquefy gelatin, whereas, unheated extract did. Stull and his associates^{15, 16} separated three fractions from low ragweed pollen and observed a loss of protein nitrogen after incubation of two of the three fractions. Stevens¹⁷ incubated giant ragweed pollen extract from six to nineteen days and found a smaller loss of phosphotungstic acid precipitable nitrogen than of nitrogen precipitated by half saturation and full saturation with Na_2SO_4 and suggested, as did Stull,¹⁶ that split protein products may be precipitated by phosphotungstic acid.

This is a report of a study of the deterioration of low ragweed pollen extract by heating and after storage at various temperatures as measured by precipitation of antiragweed rabbit serum and by neutralization of human sensitive serum in the passive transfer test (Prausnitz-Küstner reaction).¹⁸

METHODS

Five grams of low ragweed pollen were extracted with 100 c.c. of Coea's fluid (0.5 per cent NaCl, 0.35 per cent NaHCO_3 , and 0.4 per cent phenol) and passed through a Seitz filter. This sterile extract, after analysis by Kjeldahl for its content of phosphotungstic acid precipitable nitrogen, was divided into two portions. Equal parts of Coea's extracting fluid were added to one portion (plain extract), and equal parts of glycerin were added to the other (glycerinated extract) in a final concentration of 50 per cent glycerin. The plain and glycerinated extracts were then stored (a) at room temperature, (b) in the icebox (6° C.), (c) in the deep freeze (-25° C.), and (d) in the carbon dioxide box (-70° C.). The extracts were studied at intervals up to twelve months for their capacity to precipitate antiragweed rabbit serum and to neutralize skin sensitizing antibodies of human ragweed sensitive serum. Similar studies were made with plain and glycerinated extracts subjected to temperatures of 56° C. for thirty minutes in a water bath. Three separate extracts were used in this study, one prepared on Nov. 10, 1946 (Extract A), one on June 21, 1947 (Extract B), and one on Aug. 15, 1947 (Extract C).

A. Precipitation of Antiragweed Rabbit Serum.—The absolute amounts of precipitate resulting from union of low ragweed antigen and antiragweed rabbit serum were measured by the use of a photoelectric colorimeter, a method previously described.¹⁹

B. Neutralization of Human Ragweed Sensitive Serum.—The technique employed was that described by Cooke and his co-workers.²⁰ Two-tenths cubic centimeters of diluted human ragweed sensitive serum was added to 0.2 c.c. of plain and glycerinated low ragweed extracts in strengths ranging from 10 to 2,000 protein nitrogen units per cubic centimeter. The mixtures were shaken and placed in the icebox for one to two hours. Skin sites were prepared in

recipients, reacting negatively to skin tests with low ragweed extract, with 0.1 c.c. of the antigen human serum mixtures. These sites were tested twenty-four hours later with 0.025 c.c. of plain, refrigerated low ragweed extract, containing 1,000 protein nitrogen units per c.c.,²¹ of the same lot number as used in the neutralization studies. Reactions at the skin sites were interpreted in twenty to thirty minutes after testing as 4 plus, 3 plus, 2 plus, 1 plus, or negative. The neutralization studies were performed on the same recipients at intervals up to twelve months. Portions of the same human skin sensitizing serum, in a dilution of 1:10, were used in all experiments. This serum had been shown to be positive in passive transfer in several recipients in dilutions ranging from 1:3,000 to 1:5,000.

C. Standardization of Extracts.—All extracts were standardized on the basis of their phosphotungstic acid precipitable nitrogen, as described by Cooke and Stull.²¹ Analyses were performed in duplicate or triplicate. Dilutions of each extract were then made in protein nitrogen units per cubic centimeter for the precipitation and neutralization studies.

RESULTS

A. Storage at Room Temperature.—

1. *Precipitation:* Plain Extract A, prepared Nov. 10, 1946, stored at room temperature, showed almost complete loss of capacity to precipitate antiragweed rabbit serum in twelve months. There was marked loss even after four months. The corresponding glycerinated extract retained essentially all of its activity after four months, although there was noticeable deterioration after one year, as shown in Fig. 1.

2. *Neutralization:* Plain Extract A was first studied in neutralization after it had been stored for 6 months. At first, attempts were made to demonstrate an end point by simple passive transfer methods with the fresh extract, but sharp end points could not be demonstrated within the range desired, so that such attempts were abandoned and neutralization studies were adopted when this extract was six months of age. After six and twelve months of storage at room temperature, the plain extract failed to neutralize even in the concentration of 2,000 P.N.U. per cubic centimeters, whereas, the glycerinated extract neutralized at 400 to 500 P.N.U. per cubic centimeters at six and twelve months (Table I).

B. Storage in Icebox (6° C.).—

1. *Precipitation:* Plain Extract A, prepared Nov. 10, 1946, stored at 6° C. showed only slight loss of capacity to precipitate antiragweed rabbit serum in four months but showed some loss of precipitating capacity after twelve months. The corresponding glycerinated extract showed no loss of precipitating capacity after twelve months (Fig. 2). It was noted in this experiment, as well as others, that glycerin exerted an inhibiting effect upon precipitation, most noticeable in concentrations of 25 per cent (5,000 P.N.U. per cubic centimeter of glycerinated extract) and greater (Fig. 7). Further evidence of deterioration of the plain extract is suggested in Fig. 2 by the absence of antigen excess effect depressing precipitation, such as is encountered with fresh extracts. Plain

TABLE I. COMPARISON OF REAGIN NEUTRALIZATION ACTIVITY OF PLAIN AND GLYCERINATED RAGWEED EXTRACTS STORED AT ROOM AND REFRIGERATOR TEMPERATURES

CONCENTRATION OF ANTIGEN (P.N.U. PER C.C.)*	ROOM TEMPERATURE				REFRIGERATOR TEMPERATURE			
	PLAIN		GLYCERINATED		PLAIN		GLYCERINATED	
	6 MONTH	12 MONTH	6 MONTH	12 MONTH	6 MONTH	12 MONTH	6 MONTH	12 MONTH
10							+++	+++
25							++	++
50			+++	+++			+	+
75							0	±
100	++++	++++	++	++	++		0	0
150			+	++			0	0
200			++	+	++	+	0	0
250	++++							
300		++++	+	+	+		0	0
400			±	+	0	+	0	
500	+++	++++	±	±	0		0	
750	+++	++++	0	0	0	0		
1,000	+++	++++	0	0	0	0		
1,500	++	++++				0		
2,000	++	++++				0		
Saline	++++	++++	++++	++++	++++	++++	++++	++++

*Phosphotungstic acid precipitable nitrogen.

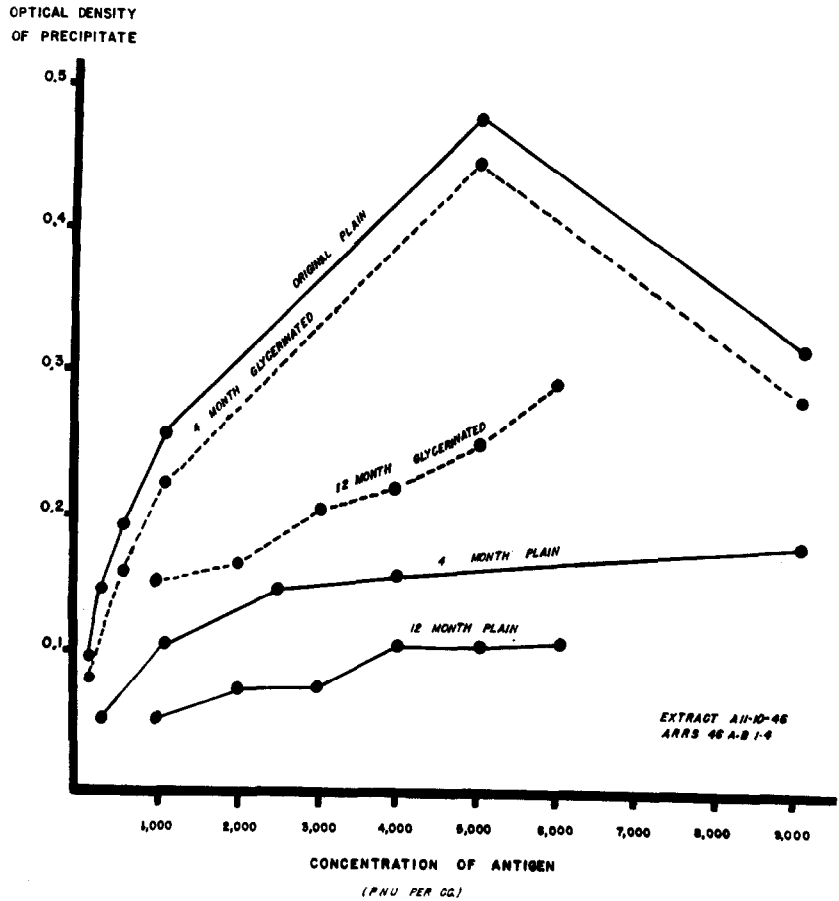


Fig. 1.—Effect of storage at room temperature of plain and glycerinated low ragweed extracts on precipitation of antiragweed rabbit serum.

Extract B, prepared June 21, 1947, showed a significant loss of capacity to precipitate after four months' storage at 6° C., whereas, the corresponding glycerinated extract showed less deterioration (Fig. 3).

2. *Neutralization*: Five hundred to 750 P.N.U. per cubic centimeters of plain Extract A, after twelve months' storage, were required to neutralize human sensitive serum, whereas, neutralization was accomplished at 75 to 100 P.N.U. per cubic centimeter of the corresponding glycerinated extract (Table I). Plain

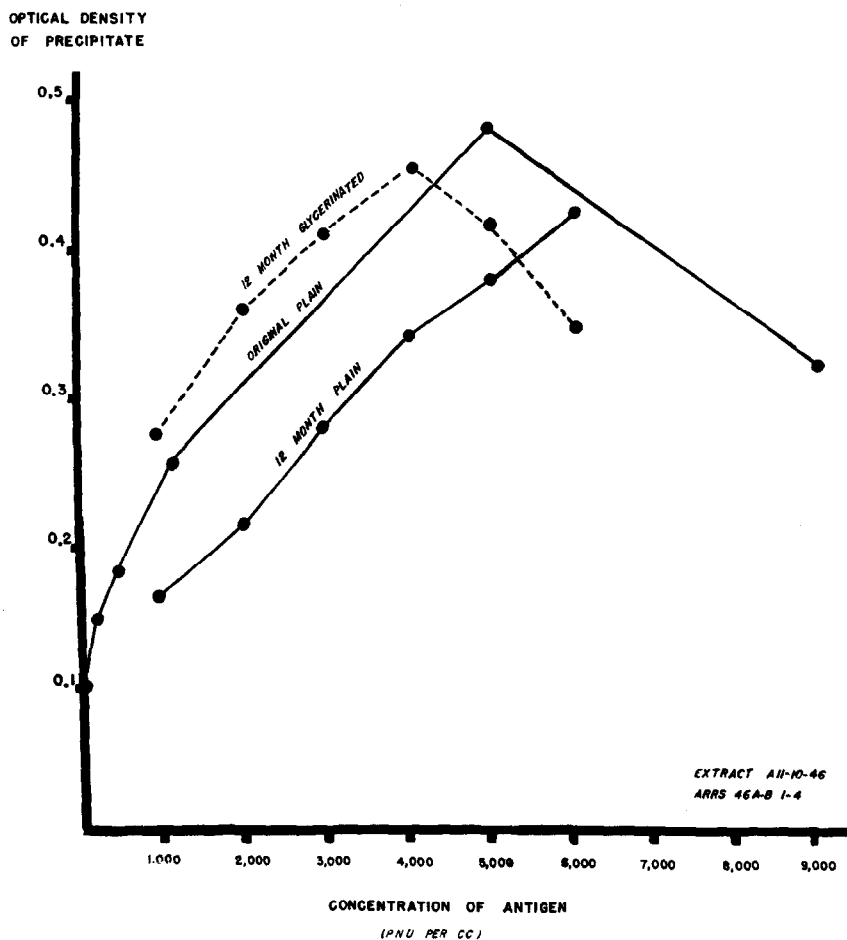


Fig. 2.—Effect of storage at refrigerator temperature of plain and glycerinated low ragweed extracts on the precipitation of antiragweed rabbit serum.

Extract B neutralized at approximately 75 P.N.U. per cubic centimeter when first prepared, although after six months' storage 200 P.N.U. per cubic centimeter of extract were required to induce complete neutralization. The corresponding glycerinated extract, however, showed essentially no loss of neutralizing qualities after six months' storage at 6° C. (Table II).

C. Storage in Deep Freeze and Carbon Dioxide Boxes.—

1. *Precipitation*: Plain Extract A, prepared Nov. 10, 1946, showed a marked loss of precipitating capacity after four months' storage in the deep

TABLE II. COMPARISON OF REAGIN NEUTRALIZATION ACTIVITY OF PLAIN AND GLYCERINATED LOW RAGWEED EXTRACTS STORED AT REFRIGERATOR TEMPERATURE

CONCENTRATION OF ANTIGEN (P.N.U. PER C.C.)*	PLAIN		GLYCERINATED	
	ORIGINAL	6 MONTH	ORIGINAL	6 MONTH
10	+++		+++	+++
25	++	++	+++	++
50	+	++	±	+
75	±		±	+
100	0	+	0	0
150	0	+	0	0
200	0	0	0	0
300		0		
400		0		
500		0		
750		0		
Saline	++++	++++	++++	++++

*Phosphotungstic acid precipitable nitrogen.

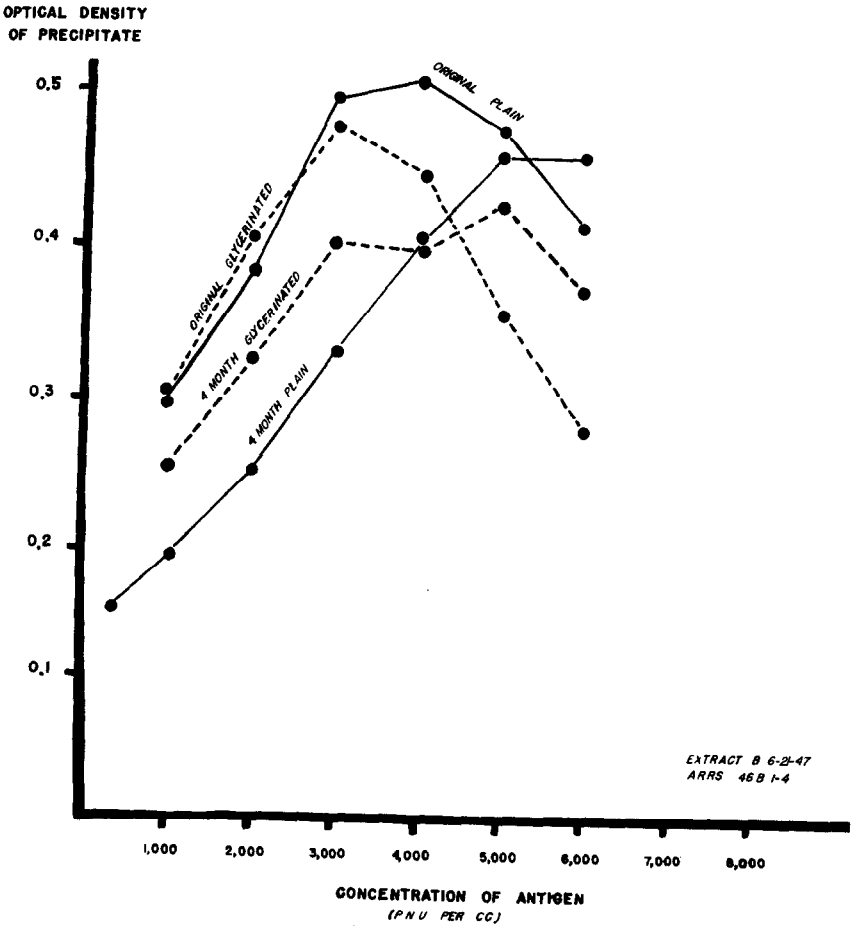


Fig. 3.—Effect of storage at refrigerator temperature of plain and glycerinated low ragweed extracts on the precipitation of antiragweed rabbit serum.

freeze box at -25°C ., and in the carbon dioxide box at -70°C . There was slightly more loss in the carbon dioxide box than the deep freeze box but there was no additional loss of activity in either instance after twelve months' storage (Fig. 4). The corresponding glycerinated extracts showed no loss of precipitating capacity, however, after four months or twelve months. The four-month precipitation curves are omitted from the graph shown in Fig. 4 since they overlapped the twelve-month curve.

OPTICAL DENSITY
OF PRECIPITATE

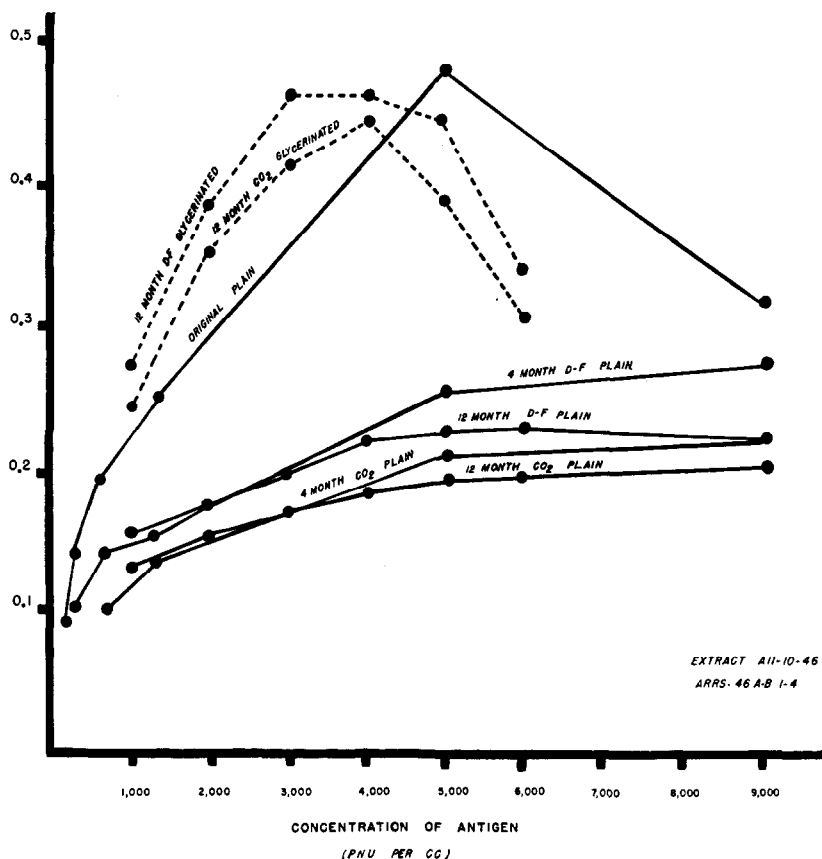


Fig. 4.—Effect of storage at deep freeze and CO_2 box temperatures of plain and glycerinated low ragweed extracts on precipitation of antiragweed rabbit serum.

2. *Neutralization*: Plain Extract A failed to neutralize human sensitive serum even in strengths of 2,000 P.N.U. per cubic centimeter upon analysis after six and twelve months' storage in the deep freeze and carbon dioxide boxes. The corresponding glycerinated extracts, however, neutralized the serum at 75 P.N.U. per cubic centimeter (Table III). Similar loss of activity was noted with Extract B after four months' storage.

D. Effect of Heat.—

1. *Precipitation*: Two freshly prepared plain extracts (Extracts B and C), heated in a water bath to 56°C . for thirty minutes, showed marked loss in

TABLE III. COMPARISON OF REAGIN NEUTRALIZATION ACTIVITY OF PLAIN AND GLYCERINATED LOW RAGWEED EXTRACTS STORED AT DEEP FREEZE AND CO₂ BOX TEMPERATURES

CONCENTRATION OF ANTIGEN (P.N.U. PER C.C.)*	DEEP FREEZE				CO ₂ BOX				REFRIGERATOR	
	6 MONTH		12 MONTH		6 MONTH		12 MONTH		12 MONTH	
	P	G	P	G	P	G	P	G	P	G
10	++++	+++		+++	++++	++++		++++		++++
25		++		+		+		++		++
50	++++	+		±	++++	+		+		+
75		0		0		±		0		0
100	++++	0		0	+++	0		0		0
150		0		0		0		0		0
200		0		0		0		0	+	0
250	+++		++++		++		++			
400				0				0	+	0
500	+++		++++		++		++			
750	+++				++				0	0
1,000	++		+++		++		++		0	0
1,500					++				0	0
2,000	++		+++		++		++		0	
Saline	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++

*Phosphotungstic acid precipitable nitrogen.

precipitating properties, whereas, the corresponding glycerinated extracts were not materially affected by heat (Fig. 5). Extract A, after six months' storage in the icebox and then heated, showed similar results.

2. *Neutralization*: Freshly prepared plain Extract B, after heating to 56° C. for thirty minutes, neutralized human sensitive serum at approximately 1,500 P.N.U. per cubic centimeters, whereas, the corresponding glycerinated extract neutralized at approximately 75 P.N.U. per cubic centimeters, the same zone of neutralization observed with the unheated fresh extract (Table IV). This same phenomenon was noted with freshly prepared Extract C, although to a lesser degree. Six-month-old refrigerated Extract A responded similarly to heat in that the heated, glycerinated extract neutralized at approximately 150 P.N.U. per cubic centimeters, whereas, approximately 1,500 P.N.U. per cubic centimeters of unheated plain extract were required to neutralize the serum.

E. *Effect of Glycerin on Precipitation*.—Precipitation studies were performed with varying concentrations of glycerin, i.e., 50, 25, 12.5, 6.25, 3.12, and 1.56 per cent, using a constant amount of antigen (4,000 P.N.U. per cubic centimeter) and antiragweed rabbit serum. Precipitation was inhibited in the presence of the higher concentrations of glycerin up to a maximum inhibition of 18 per cent with 50 per cent glycerin (Fig. 6). This accounts for the depression of the curves of the glycerinated extract at antigen concentrations of 3,000 P.N.U. per cubic centimeter and greater shown in the various figures presented.

F. *Protein Nitrogen Analyses*.—The phosphotungstic acid precipitable nitrogen of all three Extracts (A, B, and C), both plain and glycerinated, decreased after storage at the four different temperatures. The deterioration varied between 30 and 50 per cent. The three extracts did not act alike in this regard. Extract A showed no deterioration after four months' storage at the four different temperatures but approximately 30 per cent after twelve months. There was a decrease of nitrogen after four and one-half months' storage of

TABLE IV. EFFECT OF HEATING PLAIN AND GLYCERINATED LOW RAGWEED EXTRACTS ON REAGIN NEUTRALIZATION

CONCENTRATION OF ANTIGEN (P.N.U. PER C.C.)*	EXTRACT B 6-21-47				EXTRACT C 8-15-47			
	REFRIGERATED		HEATED		REFRIGERATED		HEATED	
	PLAIN	GLYCERINATED	PLAIN	GLYCERINATED	PLAIN	GLYCERINATED	PLAIN	GLYCERINATED
10	+++	+++		+++	++	++		+++
25	++	+++		+	+	++		++
50	+	+		±	+	+		+
75	±	±		±	±	0		+
100	0	0	+++	0	0	0	++	+
150	0	0		0	0	0		0
200	0	0		0	0	0		0
250								+
500			++					±
750								0
1,000			+					0
1,500			±					0
2,000			0					0
Saline	++++	++++	++++	++++	++++	++++	++++	++++

*Phosphotungstic acid precipitable nitrogen.

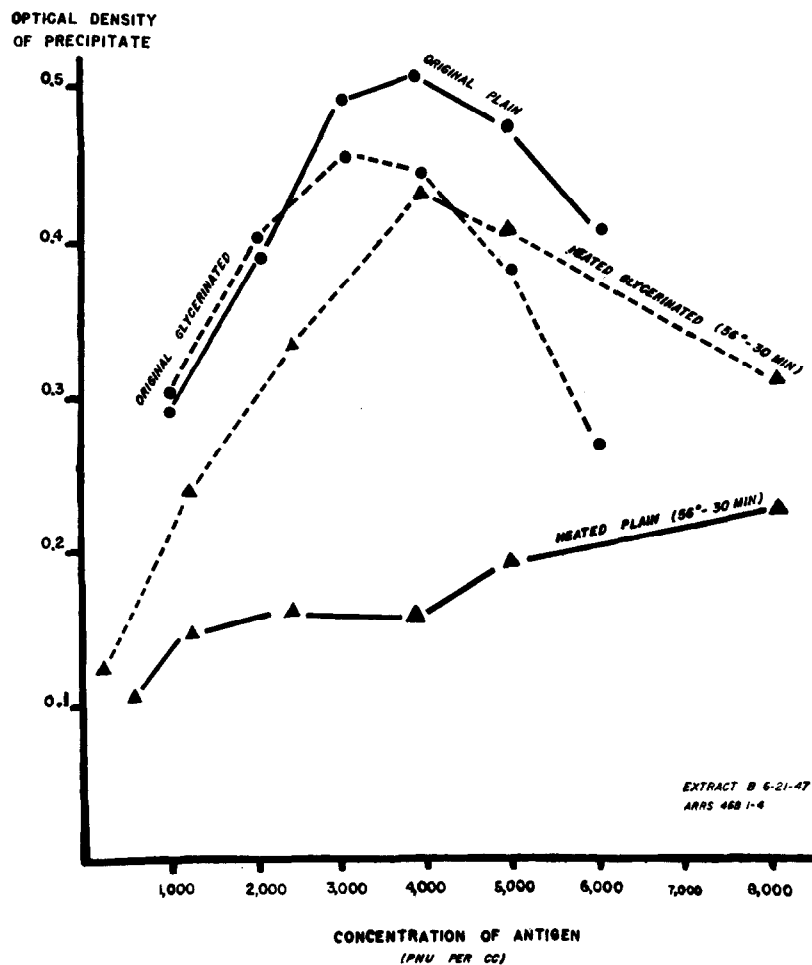


Fig. 5.—Effect of heating plain and glycerinated ragweed extracts on the precipitation of antiragweed rabbit serum.

Extract B and after two and one-half months' storage of Extract C. In general, the values of the glycerinated extracts were less than the corresponding plain extracts, suggesting that glycerin inhibited the precipitation of nitrogen by phosphotungstic acid. There was little or no decrease of nitrogen of the plain

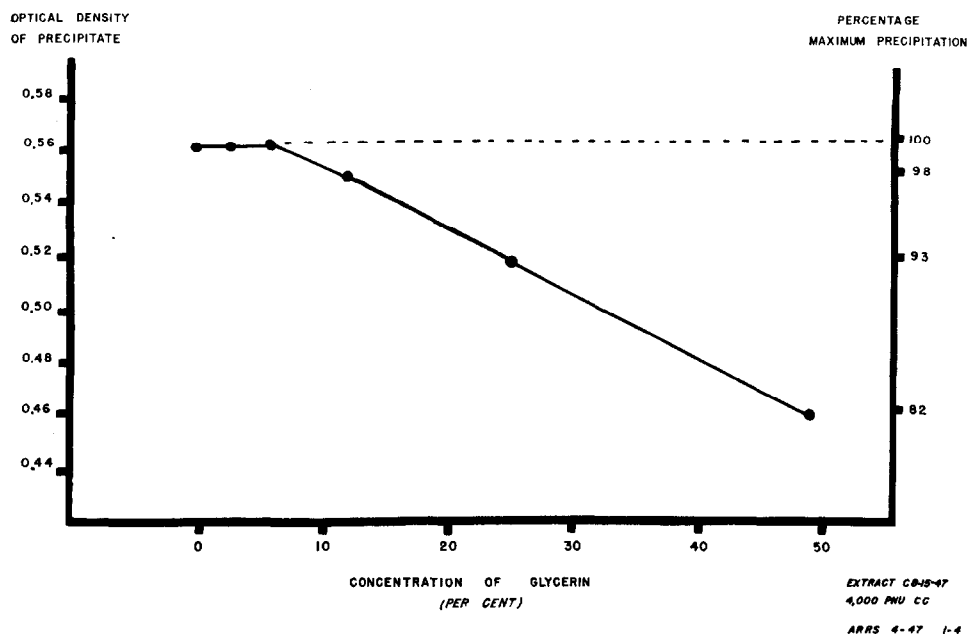


Fig. 6.—Effect of glycerin on precipitation of antiragweed rabbit serum.

TABLE V. PROTEIN NITROGEN ANALYSES* OF EXTRACTS

EXTRACT	DATE	AGE OF EXTRACT	STORAGE CONDITIONS								HEATED 56° C.	
			ROOM		6° C.		-25° C.		-70° C.			
			P	G	P	G	P	G	P	G	P	G
A	11/10/46	Fresh	.10		.10		.10		.10		.10	
	3/19/47	4 mo.	.13	.11	.12	.10	.12	.10	.11	.11	.11	.09
	11/ 3/47	12 mo.	.07	.08	.07	.05	.08	.06	.07	.06		
B	6/21/47	Fresh			.08		.08		.08		.08	
	6/25/47	4 days			.08	.08	.06	.05	.08	.08	.07	.04
	8/19/47	2 mo.					.06	.04				
	11/ 5/47	4½ mo.			.04	.03	.06	.04	.05	.03		
C	8/15/47	Fresh			.10		.10		.10		.10	
	8/19/47	4 days			.09	.07	.08	.07			.09	.06
	11/ 5/47	2½ mo.			.06	.05	.07	.03				

*Phosphotungstic Acid Precipitable Nitrogen per c.c.

P - Plain Extract.

G - Glycerinated Extract.

extracts after heating to 56° C. for thirty minutes although two of the three glycerinated extracts (Extracts B and C) showed consistently less nitrogen after heating (Table V).

DISCUSSION

The results of this study indicate that the capacity of low ragweed extracts to precipitate antiragweed rabbit serum and to neutralize skin sensitizing anti-

bodies of human ragweed sensitive serum is impaired by heating to 56° C. for one hour and by storage at room temperature, 6° C., -25° C., and -70° C. The plain unglycerinated extract deteriorated after storage at the four temperatures although there was less loss of activity in the icebox at 6° centigrade. Glycerin, in a concentration of 50 per cent in the extracts, in general, protected against such deterioration. Even glycerinated extracts deteriorated partially after storage at room temperature for one year.

It was interesting that extracts stored at -25° C. and at -70° C. showed marked loss of activity by the methods used in the study. Precipitation occurred in these extracts after freezing but the precipitates, when separated from the remainder of the extract and redissolved, showed essentially no activity by precipitation of antiragweed rabbit serum or by neutralization of human ragweed sensitive serum.

There was a definite correlation in the results of the deterioration studies as measured by the two different methods, i.e., precipitation of antiragweed rabbit serum and neutralization of human ragweed sensitive serum in passive

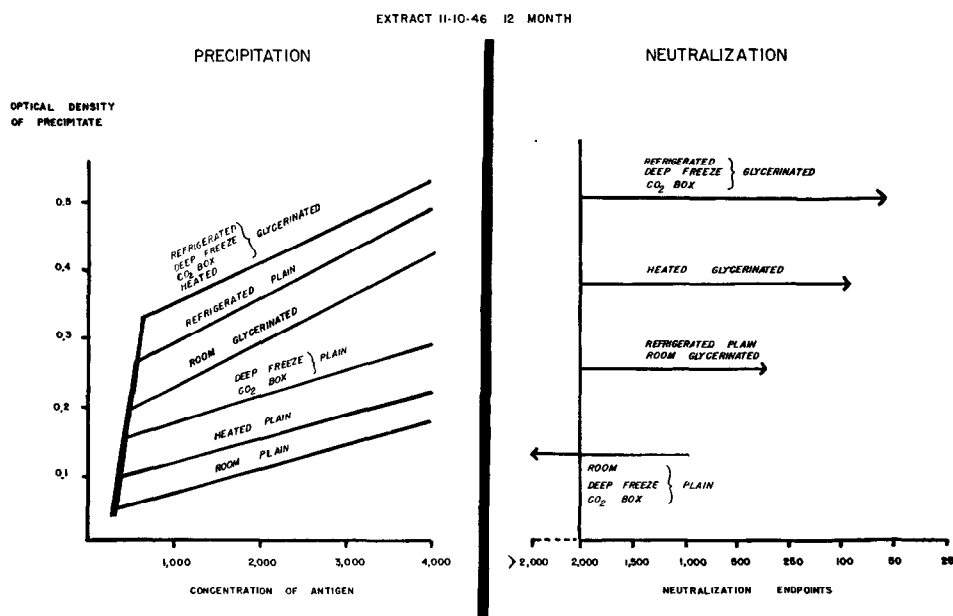


Fig. 7.—Summary of immunologic studies with low ragweed extracts.

transfer. A schematic summary of the results of the effect of heat and the various storage temperatures on deterioration of ragweed pollen extracts indicates this correlation (Fig. 7).

Clinical use of glycerinated extracts has revealed that 50 per cent glycerin irritates human skin inducing "false positive" skin tests and that it is very painful on subcutaneous injection. Even 25 per cent glycerin is painful on injection. In general, extracts containing 5 to 12 per cent glycerin are well tolerated by patients in skin testing and subcutaneous injection therapy. Therefore, 1:4 to 1:10 dilutions of concentrated extracts containing 50 per cent

glycerin are suitable for clinical use. It is possible that a concentration of less than 50 per cent glycerin would prevent deterioration of pollen extracts. Further studies in this regard are being conducted in this laboratory.

There was, in general, loss of phosphotungstic acid precipitable nitrogen in all stored extracts although this loss was not consistent in the three extracts with the same degree of aging. The protein nitrogen content of the plain extracts was not affected materially by heating, although the values were much less after addition of 50 per cent glycerin and heating. It is possible that glycerin inhibited precipitation of the extracts with phosphotungstic acid.

The protein nitrogen of the three extracts studied, as determined by precipitation with phosphotungstic acid, deteriorated 30 to 50 per cent in a year or less. This approximates the findings of Stull, Cooke, and Tennant.¹⁰ There was no correlation, however, between the decrease in nitrogen content and loss of activity of the ragweed extracts by the methods used in this study. The glycerinated extracts, in most instances, showed as much or more loss of nitrogen on storage than the plain unglycerinated extracts, even though glycerinated extracts showed no or much less deterioration as measured by precipitation of antiragweed rabbit serum and neutralization of human ragweed sensitive serum.

SUMMARY

1. Plain unglycerinated low ragweed extracts, upon heating to 56° C. for thirty minutes and upon storage at room temperature, 6° C., -25° C., and -70° C. for periods up to one year, showed loss of activity as measured by their ability to precipitate antiragweed rabbit serum and to neutralize skin sensitizing antibodies of human ragweed sensitive serum.

2. Low ragweed extracts containing 50 per cent glycerin showed no loss or less loss of activity than their corresponding plain extracts after heating or storage at the four different temperatures.

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