

The lability of dilutions of skin-sensitizing antibody and antigen (ragweed) on freezing

Alan R. Feinberg, M.D., Samuel M. Feinberg, M.D., and
Fumiko Lee, B.A., Evanston, Ill.

In the course of investigations on other aspects of antigen-antibody reactions, we became suspicious that the potency of both diluted reaginic serum and antigen may be impaired by freezing. The following protocol was carried out. Ragweed reaginic serum (KA), which had been kept frozen for 2 years at -20° C., was diluted in threefold serial dilutions from 1:9 to 1:2,187. One set was kept frozen for 4 days, another was refrigerated for a similar period, and a third was prepared and used immediately. Three nonatopic subjects were passively sensitized with these 3 series of dilutions at one sitting and the sites were challenged 48 hours later with ragweed extract, 1:300. In one subject the procedure was repeated a second time. The preparations which had been frozen required 9 to 27 times the concentration of the fresh and refrigerated serum dilutions to produce an end point. The lability of the diluted ragweed extract upon freezing was similarly tested. Several sets of threefold serial dilutions were prepared from stock refrigerated extract and treated as follows: (1) used immediately, (2) refrigerated for 1 day to 4 weeks, (3) frozen for 1 day to 4 weeks in glass vials, and (4) frozen in siliconized vials. Titrations were made on 4 ragweed-sensitive subjects and on 2 nonatopic individuals sensitized with reaginic serum diluted 1:9. The results indicate a substantial loss of skin-reacting potency from freezing. In addition, other aspects of antigen lability were measured.

In the course of investigations involving passive local sensitization of the human skin with serially diluted, reaginic sera which, for expediency, had been frozen after dilution several days earlier, disturbing results on challenge with antigen made us suspicious that the diluted reagins might have been damaged by freezing. The impression prevails that the reaginic whole serum maintains its potency after being frozen for years. Perlman and Layton¹ have reported that freezing at -20° C. has very little effect on reaginic serum; our experience has confirmed these conclusions. However, our preliminary experience with the

From the Allergy Research Laboratory, Evanston Hospital, and the Department of Medicine, Northwestern University Medical School.

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sera frozen in high dilutions made us suspicious that the antibodies might not be stable under those conditions. We, therefore, undertook a study to investigate the lability of diluted reaginic sera on freezing and extended our investigations to include similar treatment of dilute antigen solutions at -20°C . and -70°C .

MATERIALS AND SUBJECTS

Ragweed reaginic serum (KA) drawn 30 months earlier and kept at -20°C . was diluted with buffered saline in threefold serial dilutions ranging from 1:9 to 1:2,187. One set was prepared immediately before passive transfer; while another set was prepared 4 days earlier, frozen at -20°C ., and thawed immediately before use. A third set was diluted and refrigerated at $+5^{\circ}\text{C}$. for 4 days. Passive transfer tests were done on 4 nonatopic male subjects and the sites were tested with a 1:300 mixed ragweed extract.

A 3 per cent extract, prepared from equal parts of short and giant ragweed pollen several months earlier and stored at refrigerator temperature, was the source of all antigen preparations used in this study. Phenolized buffered saline was the diluting fluid. The following variations were used in preparing sets of threefold serial dilutions ranging from 1:1,000 to 1:59,049,000:

1. Freshly diluted immediately before use.
2. Diluted and allowed to stay in refrigerator 1 to 4 weeks.
3. Diluted and frozen at -20°C . for 1 day to several weeks.
4. Diluted and frozen at -70°C . for 1 day.
5. Diluted and frozen at -20°C . in siliconized vial, followed by refrigeration for 1 week.
6. Stock solution frozen at -20°C ., then diluted.
7. Stock solution frozen at -70°C ., then diluted.

Five milliliter quantities of all solutions were kept in the same size vials. Six subjects, with varying degrees of clinical and skin sensitivity to ragweed, were tested intradermally with ragweed dilutions of 1:81,000 to 1:59,049,000. Two nonatopic subjects were used to test the ragweed dilutions on passively sensitized sites.

Immunodiffusion in agar was done with dilutions of ragweed, frozen and refrigerated, against potent rabbit antiragweed serum. Histamine release from the leukocytes of a treated, ragweed-sensitive subject was determined with the use of ragweed dilutions which were refrigerated and then frozen at -20°C . and -70°C ., according to the technique of Lichtenstein and Osler.²

RESULTS

Table I shows the results of challenge with 1:300 ragweed on passively sensitized subjects with serial dilutions of serum. No significant difference was shown on sites prepared with the 1:9 serum dilution. However, frozen dilutions of 1:27 and more showed a marked reduction or absence of reaction on challenge. Dilutions not frozen, but refrigerated for the same length of time as the frozen dilutions, showed no difference in reaction as compared with the fresh, diluted sera.

Table II shows the results of passive transfer of a 1:9 dilution of serum chal-

Table I. *Passive transfer of ragweed reaginic serum, freshly diluted, refrigerated at +5° C., and frozen at -20° C.*

Serum dilutions	Subject 1		Subject 2		Subject 3			Subject 4		
	Fresh	-20° C. 4 days	Fresh	-20° C. 4 days	Fresh	+5° C. 4 days	-20° C. 4 days	Fresh	+5° C. 4 days	-20° C. 4 days
1:2,187	0	0	0	0	0	0	0	0	0	0
1:729	0	0	±	0	+	+	+	0	0	0
1:243	+	0	++	0	++	++	±	++	+	0
1:81	++	0	+++	0	++	++	±	++	++	0
1:27	+++	0	+++	0	+++	+++	±	+++	+++	0
1:9	++++	++++	++++	++++	+++	+++	+++	++++	++++	+++

Reactions on challenge with 1:300 ragweed extract.

Table II. *Passive transfer of ragweed reaginic serum diluted 1:9 and reactions on challenge with dilutions of ragweed freshly diluted, dilutions refrigerated at +5° C., and dilutions frozen at -20° C.*

Ragweed dilutions	Subject 5					Subject 6				
	Fresh	+5° C. 1 week	+5° C. 4 weeks	-20° C. 1 week	-20° C. 4 weeks	Fresh	+5° C. 1 week	+5° C. 4 weeks	-20° C. 1 week	-20° C. 4 weeks
1:6,561,000	0	0	+	0	±	0	±	±	0	0
1:2,187,000	0	0	0	0	0	±	+	0	0	0
1:729,000	±	±	+	0	0	±	0	+	0	0
1:243,000	++	++	++	0	0	+	0	+	0	0
1:81,000	+++	+++	++	0	0	++	+	++	0	0
1:27,000	+++	+++	+++	0	0	+++	+++	+++	0	0

Table III. *Challenge of clinically sensitive ragweed subjects with fresh dilutions of ragweed, dilutions refrigerated at +5° C. and dilutions frozen at -20° C.*

Ragweed dilutions	Subject 7					Subject 8				
	Fresh	+5° C. 1 week	+5° C. 4 weeks	-20° C. 1 week	-20° C. 4 weeks	Fresh	+5° C. 1 week	+5° C. 4 weeks	-20° C. 1 week	-20° C. 4 weeks
1:19,683,000						0	0	0	0	0
1:6,561,000	+	0	+++	+	0	0	+	0	+	0
1:2,187,000	++	++	++	0	0	+	+	++	0	0
1:729,000	+++	++	+++	0	±	±	++	+	0	+
1:243,000	+++	+++	+++	±	+	++++	+++	+++	+	++
1:81,000	++++	++++	+++	+	++	+++	+++	+++	+	+

lenged at all sites with dilutions of ragweed, fresh, refrigerated, and frozen. No reaction was seen on challenge with the frozen ragweed dilutions; whereas, the freshly diluted and refrigerated dilutions reacted similarly.

Table III shows the reactions on skin testing 2 atopic subjects with fresh, refrigerated, and frozen dilutions of ragweed. There was marked diminution of reactions with the frozen dilutions; while very little change was evident in the refrigerated, as compared with the fresh dilutions. Table IV shows tests in 2 additional atopic subjects. Dilutions frozen at both -20° C. and -70° C. showed loss of reactivity. When the concentrates were frozen at the same temperatures

Table IV. *Challenge of clinically sensitive ragweed subjects with fresh dilutions of ragweed, dilutions frozen at -20° C., then refrigerated, dilutions frozen at -70° C., and dilutions prepared from stock 1:33 ragweed frozen at -20° C. and -70° C.*

<i>Ragweed dilutions</i>	<i>Subject 9</i>					<i>Subject 10</i>				
	<i>Dilutions frozen</i>			<i>1:33 frozen</i>		<i>Dilutions frozen</i>			<i>1:33 frozen</i>	
	<i>Fresh</i>	<i>-20° C. 1 week, +5° C.</i>	<i>-70° C.</i>	<i>-20° C. 6 days, then diluted</i>	<i>-70° C. 4 days, then diluted</i>	<i>Fresh</i>	<i>-20° C. 1 week, +5° C.</i>	<i>-70° C.</i>	<i>-20° C. 6 days, then diluted</i>	<i>-70° C. 4 days, then diluted</i>
		<i>1 week</i>	<i>1 day</i>				<i>1 week</i>	<i>1 day</i>		
1:59,049,000	0	0	0	0	0				±	0
1:19,683,000	+	0	0	+	+	0	0	0	±	0
1:6,561,000	++	0	0	++	++	±	0	0	±	±
1:2,187,000	++	0	+	++	++	+	0	0	+	+
1:729,000	+++	0	++	+++	+++	+	0	0	+	++
1:243,000						++	±	+	++	++

Table V. *Challenge of clinically sensitive ragweed subjects with dilutions of ragweed freshly diluted, refrigerated at $+5^{\circ}$ C., frozen at -20° C., and frozen at -20° C. in siliconized and nonsiliconized vials and then refrigerated at $+5^{\circ}$ C.*

Ragweed dilutions	Fresh	+5° C. 1 week	+5° C. 2 weeks	+5° C. 4 weeks	-20° C. 1 week	A*	B†	C‡	D§
						-20° C. 1 week, +5° C. 1 week	-20° C. 1 week, +5° C. 1 week	-20° C. 1 week, +5° C. 1 week	-20° C. 1 week, +5° C. 1 week
						1 week	1 week	1 week	1 week
Subject 11									
1:19,683,000	0	+	0	0	0	0	+	0	0
1:6,561,000	++	++	+	++	0	0	0	0	0
1:2,187,000	++	++	++	++	0	0	0	±	0
1:729,000	+++	+++	+++	+++	++	+	0	0	0
1:243,000					++	++	+	+	0
1:81,000					++	+++	++	++	++
Subject 12									
1:6,561,000	±	±	±	±	0	0	0	0	0
1:2,187,000	+	±	+	+	0	0	0	0	0
1:729,000	++	+	+	+	0	0	0	0	0
1:243,000	++	++	++	++	+	0	0	0	0
1:81,000	++	++	++	++	+	+	++	0	+

*A = Dilutions in ordinary vials.
†B = Dilutions in siliconized vials.
‡C = Portion removed from A and placed in another ordinary vial after thawing.
§D = Portion removed from B and placed in another siliconized vial after thawing.

and then diluted, no significant difference was observed in comparison with fresh dilutions (Fig. 1).

Table V shows the results of tests with intradermal ragweed dilutions in 2 atopic subjects, comparing the fresh, refrigerated, and frozen dilutions. The results were similar to those in the preceding experiments. In addition, the following preparations were used: dilutions frozen in siliconized vials (B), a portion

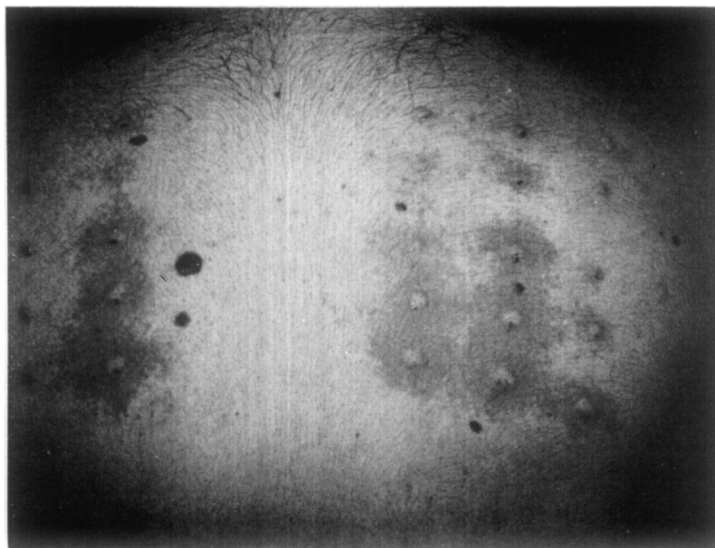


Fig. 1

Skin reactions of Subject 9. Threefold serial dilutions of ragweed extract in a clinically sensitive subject. Dilutions range from 1:729,000 to 1:59,049,000: first column, dilutions frozen -20° C. one week, refrigerated one week; second column, stock frozen -20° C. 4 days, then diluted; third column, fresh dilutions; fourth column, stock frozen -20° C. for 6 days, then diluted; fifth column, dilutions frozen -70° C. for one day.

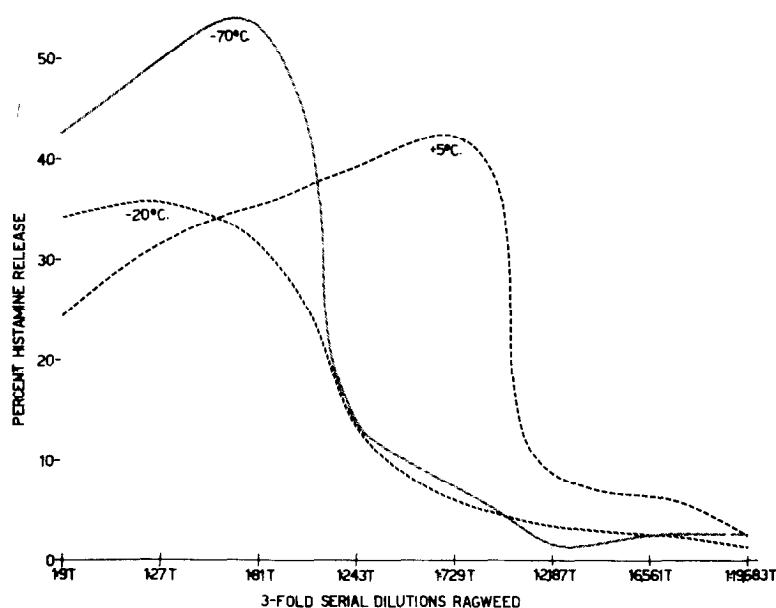
removed from B and placed in another siliconized vial after thawing (D), and a portion removed from dilutions frozen in ordinary vials and placed in another ordinary vial after thawing (C). After thawing, all dilutions were refrigerated for 1 week. None of the variations modified the diminution of potency from freezing.

Immunodiffusion tests were not satisfactory because the rabbit antiragweed serum did not show precipitin lines with antigen dilutions weaker than 1:100, and no difference was noted at the 1:100 strength.

Histamine release from leukocytes is shown in Fig. 2. It may be seen that the peak histamine release was achieved with a weaker strength of ragweed with the refrigerated than with the frozen dilutions. The dilutions represented in this graph were from refrigerated and frozen ragweed 9 times the indicated strength, with the final dilutions done just before testing. If human serum albumin (0.3 per cent) was added to the diluting fluid, it was observed that no diminution of activity of the ragweed solution took place. The pH of the various solutions used in these studies was tested, and it was found that the freezing caused no changes.

DISCUSSION

Our experiments demonstrate that the freezing of highly diluted reaginic serum and ragweed antigen at -20° C. and at -70° C. results in reduction of

**Fig. 2**

Histamine release from leukocytes of a ragweed-sensitive donor on challenge with ragweed dilutions that had been refrigerated at -5°C ., frozen at -20°C . and -70°C . ($T = \text{thousand}$).

potency, as demonstrated by the skin test. Refrigeration over a period of 4 weeks causes no significant change. The findings also indicate that the diminution of potency with freezing is not due to sticking of protein to the glass of the vial. Temporary polymerization or other temporary physical change from freezing does not explain the potency decrease, since refrigeration for a week after freezing did not restore potency.

Apparently, the amount of protein in the solution is an important factor that affects stability. Stanworth and Kuhns,³ in their paper on factors affecting the Prausnitz-Küstner test, mention that the practice of diluting reaginic serum with a protein solution enhances the stability of isolated reagin fractions. Augustin⁴ also notes that deterioration may be reduced by adding serum albumin or normal serum. She prepares high dilutions just before testing, since low-protein solutions deteriorate upon storage. Reid and Minden⁵ observed loss of reaginic activity of isolated fractions of serum after repeated freezing and thawing, but they did not feel that this was dependent on protein concentration alone. McKaba and Sussdorf⁶ tested serial dilutions of ragweed prepared by various extracting methods and found that the preparation containing the least protein concentration lost the most potency after freezing.

The cause of loss of potency has not been demonstrated. For example, it may be an irreversible polymerization or denaturation of the protein. At any rate, it must be borne in mind that in any experimental work utilizing dilution of antigens or antibodies, the freezing of such dilutions may seriously damage their reactivity.

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