

Human serum albumin and Tween 80 as stabilizers of allergen solutions*

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Intradermal skin testing may often give inaccurate results because of poor stability of allergens and loss of protein by adsorption to the walls of containers and syringes during the process of making the extreme dilutions required with potent extracts. To test the ability of stabilizers to prevent such losses of allergenic activity, three diluents for allergens were compared: standard phosphate-buffered saline (PBS), pH 7.4, containing 0.4% phenol and the same buffer containing either 0.03% human serum albumin (HSA) or 0.005% Tween 80. Tenfold dilution series of ragweed, grass, Alternaria, and dust allergens were tested by the intradermal threshold dilution technique in the same group of patients five times over six months, comparing stored dilutions with dilutions freshly made from the same batches of lyophilized extracts. Results with Tween 80 and HSA buffers were identical and highly reproducible; however, each new set of dilutions in standard buffers frequently showed within 24 to 48 hr after preparation a lower skin test potency which varied unpredictably between ten and one thousandfold. Furthermore, upon prolonged storage at 4° C, dilutions in standard buffer lost further activity. Storage of radiolabeled antigen E (AgE) in ordinary glass tubes for 24 hr showed adsorption of about 5% of the labeled protein to glass in the absence of stabilizers but only 0.5% in the presence of stabilizers. We conclude that stabilizing agents should be added to diluting fluids in preparing allergens for skin testing or immunotherapy.

Several reports indicate that allergen extracts may lose activity very rapidly upon dilution to the very low concentrations required for intradermal skin testing.¹⁻⁵ A similar phenomenon (termed the "volume effect")⁶ noted with tuberculin could be removed by the simple addition of proteins or detergents to the solution.⁷ Indeed, it is only by the introduction of some such agent that the volume effect can be assessed in ordinary diluting fluids.

The two explanations usually invoked to explain this effect are adsorption of protein to glass and de-

naturation of protein at the liquid-to-air interface. As either of these phenomena would remove or change only minute amounts of protein, their effects would become evident only with very dilute solutions of protein where a very sensitive test of activity is available. Dilutions of pollen extracts for intradermal tests are often to concentrations below 0.1 PNU/ml so that these conditions are often fulfilled in allergy practice.

The present study reexamines the phenomenon with extracts of four different allergens and demonstrates that in some patients some extracts diluted in the usual buffer can appear as much as ten thousandfold less active than they do in a buffer containing a stabilizer. Addition of either 0.03% HSA or 0.005% Tween 80 abolishes the volume effect and results in highly reproducible skin test titrations.

MATERIALS AND METHODS

Extracts for skin test

In order that fresh dilutions could be made from lyophilized or concentrated materials on a number of occasions, several vials of each of four allergenic extracts were purchased from Center Laboratories, Port Washington,

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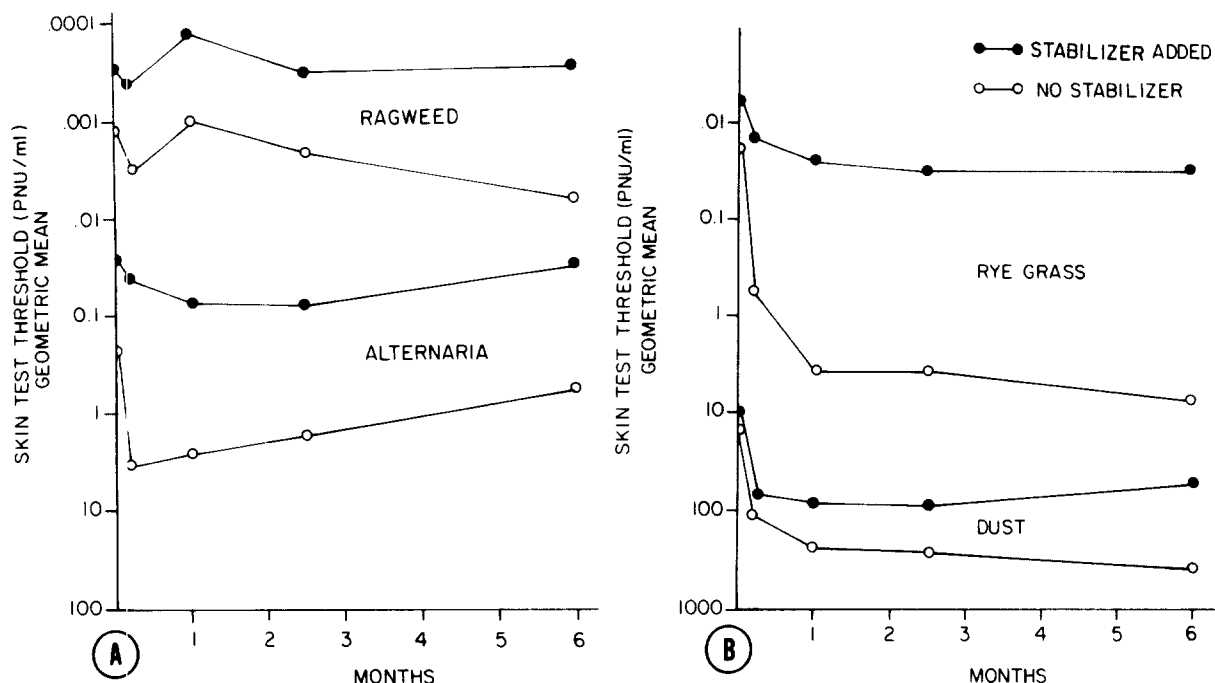


FIG. 1. Geometric mean titers of intradermal skin tests in 4 patients on a set of dilutions held for 6 months at 4° C determined by the intradermal route with the threshold dilution technique. Although two stabilizers (0.005% Tween 80 and 0.03% HSA) were tested, results are shown as single points since identical results were obtained. *A*, Ragweed and *Alternaria*. *B*, Rye grass and dust.

N. Y.: lyophilized ragweed extract (500,000 PNU/vial) (antigen E (AgE) content reported to be 560 μ g/vial by double diffusion in gel); aqueous rye grass extract (10,000 PNU/ml); lyophilized dust extract (500,000 PNU/vial); and lyophilized *Alternaria* extract (100,000 PNU/vial).

Diluents for skin testing

Three sets of diluents in 5.0-ml vials containing 4.5 ml of diluent were prepared by The Johns Hopkins Hospital Pharmacy. Standard phosphate-buffered saline (PBS) was prepared according to formula regularly furnished commercially (0.036% w/v KH_2PO_4 , 0.107% w/v $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ 0.5% w/v NaCl and 0.4% $\text{C}_6\text{H}_5\text{OH}$ [phenol] in distilled pyrogen-free water). The buffer was divided into three batches with the following additives: (1) none; (2) 0.03% (w/v) HSA (Cutter Laboratories); (3) 0.005% (v/v) Tween 80. The filled vials passed the standard FDA tests for pyrogenicity in rabbits and for sterility by culture.

Patients

Four patients with spring and fall seasonal allergic rhinitis but no asthma and known to have positive skin tests to multiple allergens, including the four to be tested, agreed to participate in a six-month study retesting the four extracts in various dilutions at intervals.

Skin tests

Testing was performed intradermally on the skin of the back according to the threshold dilution technique described

in detail elsewhere.¹⁰ Briefly, serial tenfold solutions were tested by injecting 0.05 ml intradermally. The end point was the least dilution which gave a 2+ reaction (10 mm wheal, 20-30 mm erythema, without pseudopods). If one dilution gave a large reaction, 3+ or greater, and the next, a negative or trace reaction (<10 mm wheal and erythema), an interpolated value halfway (on a $\log_{10}$ scale) was considered the end point.

Materials for in vitro tests

Ragweed antigen E was obtained from the National Institutes of Health Research Resources Branch. The glycerinated solution of antigen E was dialyzed against aqueous buffer and further purified by chromatography on a Sephadex G-75 column (180 cm $\times$ 1.5 cm diameter, equilibrated in 0.2 M NH_4HCO_3). The preparation was judged to be relatively homogenous by immunodiffusion, immunoelectrophoresis, and agarose gel electrophoresis, although subsequent more rigorous analysis of a similar fraction by crossed immunoelectrophoresis has revealed the presence of small quantities of several impurities.⁸ A different lot of lyophilized whole ragweed extract than that used for skin testing was purchased from Center Laboratories. It was found to contain 27 μ g antigen E per 10,000 PNU, as measured by radial immunodiffusion analysis using anti-AgE prepared in rabbits against NIH antigen E. The procedure was similar to that previously described for the determination of Group I content of grass pollen extracts.⁹ The Sephadex-purified preparation of antigen E served as a reference standard.

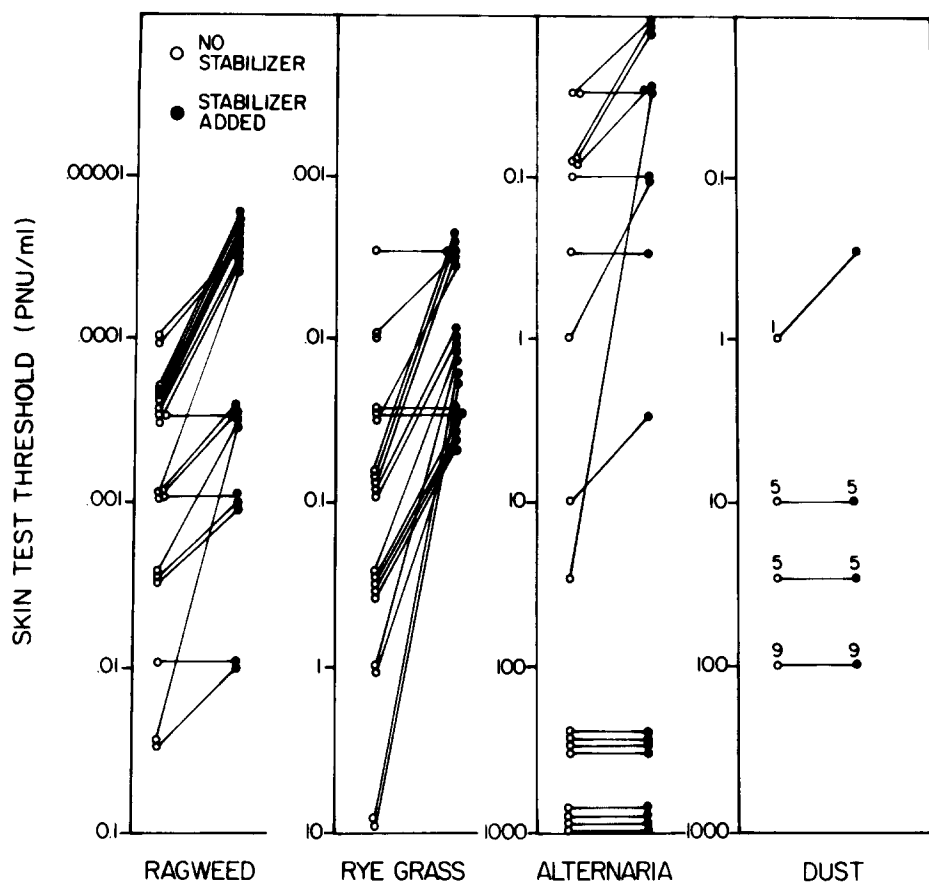


FIG. 2. Skin test results in 4 patients with new sets of dilutions prepared from stored concentrated solutions or lyophilized extracts on five occasions over six months. Each skin test pair (with and without stabilizer) is shown (connected by a line) for each patient.

Buffers for in vitro testing

Phosphate-buffered saline (PBS) was prepared according to the formula used for the skin test diluent. Tween 80 (J. T. Baker Chemical Co., Phillipsburg, N. J.) was added in concentrations varying from 0.001% to 0.1% (v/v). Tris-HSA buffer (pH 7.5) was prepared as follows: 7.015 gm NaCl; 3.728 gm KCl; 3.725 gm Trizma (Sigma Chemical Company); HSA solution sufficient to make 0.03% (w/v), distilled water to 1 l.

Glass tubes

Tubes of conventional soda glass were purchased from General Medical Scientific Company. The tubes measured 75 mm $\times$ 12 mm OD (10 mm ID).

Labeling of antigen E

The antigen E preparation [108 μ g] was radiolabeled with ^{125} I (5 mCi) using the chloramine-T method⁹ with modifications. The reaction was allowed to proceed in 0.25 M borate buffer, pH 9.5, containing 1 M urea for 70 sec at room temperature, using chloramine-T (40 μ g) at 230 μ g/ml and antigen E at 620 μ g/ml. The reaction was terminated by adding a slight excess of $\text{Na}_2\text{S}_2\text{O}_5$ (10 μ l of solution at 4 mg/ml) over that needed to neutralize the chloramine-T. In order to minimize denaturation of antigen

E, the pH of the solution was rapidly adjusted to 7.5 by addition of 217 μ l 0.4 M phosphate-citrate buffer at pH 5.9. Excess reagents were removed by exhaustive dialysis against physiologic borate-saline buffer, pH 7.5. The labeled antigen E was adjusted to 10 μ g/ml and ultracentrifuged to remove any microaggregates. It was found to possess a specific radioactivity of 3.5×10^6 cpm/ μ g.

Adsorption to glass

The labeled antigen E was incubated in glass tubes for one or seven days in buffers with and without stabilizers. All analyses were performed in triplicate. Radiolabeled antigen E was used in concentrations of 3.3×10^{-4} μ g/ml, 3.3×10^{-3} μ g/ml, and 3.3×10^{-2} μ g/ml. In certain experiments, short ragweed extract at 10,000 PNU/ml [28 μ g AgE/ml] was added to the buffers. The tubes used for the adsorption tests contained 3 ml of antigen E solution. We estimate that the solutions were in contact with 12 sq cm of glass during the majority of the incubation period, although handling might cause wetting of a somewhat greater area. Tubes were handled in a similar fashion to standardize these unknown variables as much as possible. At the end of the incubation period the tubes plus contents were counted in a gamma counter. A 0.5-ml aliquot was removed and counted separately. The incubation tubes were emptied, washed

three times with 3.0 ml of buffer, and counted. The percentage adsorption of ^{125}I -AgE to the glass was calculated from these counts.

RESULTS

Serial tenfold solutions were made in each of the three diluting fluids and tested intracutaneously on the skin of the back of the four subjects within 48 hr after the dilutions were made. The original set of dilutions along with unopened vials of lyophilized or concentrated aqueous extract were stored at 4° C. Retesting was performed at one week, one month, two and one-half months, and six months. Before each retesting, fresh unopened vials of the extracts were reconstituted and rediluted and the new set of dilutions compared with the original set of dilutions. It was noted that the vials of lyophilized ragweed extract showed evidence of a vacuum while the vials of *Alternaria* and dust extracts did not.

The Tween 80 and HSA diluents gave indistinguishable results throughout the experiment. This being so, results with the two diluents have been illustrated as single points on the graphs. Fig. 1 shows geometric mean titers for the skin test threshold for the original set of dilutions. With each extract, the first tests (performed within 48 hr after dilution) show that the extract in buffer without stabilizer was considerably less active than the extract diluted with Tween 80 or HSA present. Without stabilizer, *Alternaria*, rye grass, and dust extracts lost additional activity in the first month; thereafter the activity remained relatively constant. Ragweed extract seemed more stable at first but showed progressive dissipation of activity. In the presence of stabilizer loss of activity was reduced with all extracts.

Inspection of the data (Fig. 1) suggests that the patients varied somewhat in their skin sensitivity or reactivity from one sitting to the next. As a result, the ragweed extract actually appeared slightly more active at the end of six months than at the beginning (Fig. 1, A). Despite this variability, it was evident that the dust (Fig. 1, B) and *Alternaria* (Fig. 1, A) extracts lost activity in the lyophilized state. This was particularly evident in the case of *Alternaria* because the "controls" lost activity more rapidly than the original set of dilutions. We were subsequently informed that lyophilized extracts manufactured during the period at times had an excessive moisture content, a problem which has since been corrected. The maintenance of a vacuum in the ragweed vials while there was none in the *Alternaria* and dust vials may account for the difference in stability.

The difference between extracts diluted with and without stabilizer was observed each time a new set of dilutions was made. Fig. 2 shows the skin test titers

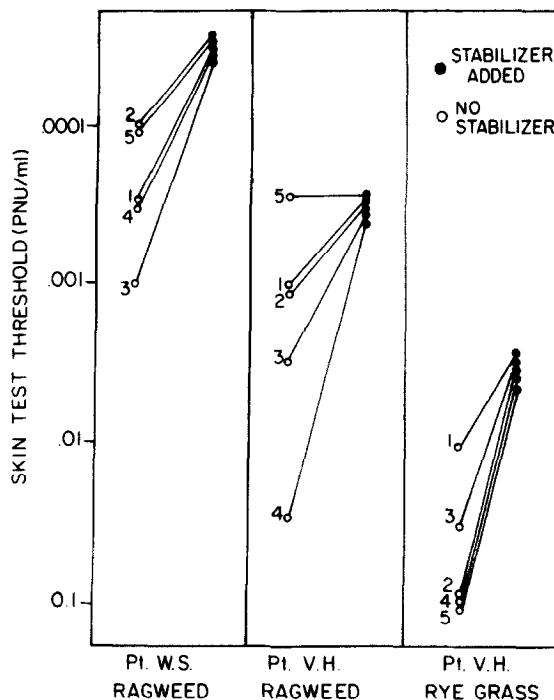


FIG. 3. Skin test results in representative individual patients with new sets of dilutions prepared over six months. Each dilution pair (with and without stabilizer) is numbered (1 = 0 time, 2 = 1 week, 3 = one month, 4 = 2½ months, 5 = six months).

TABLE I. Adsorption of ^{125}I -AgE ($3.3 \times 10^{-2} \mu\text{g/ml}$) in phosphate-saline phenol buffer to glass after 24 hr at room temperature

Conc. of Tween 80 (v/v)	% AgE adsorbed to glass tube
0	5.30%
0.0001%	1.27
0.0005	2.10
0.001	0.69
0.002	0.37
0.005	0.36
0.01	0.13
0.1	0.23

with and without stabilizer for the four patients for each extract on each of the five occasions a new set of dilutions was tested. The apparent loss of activity without stabilizers shows best with the highly active ragweed and rye grass extracts with which positive skin reactions develop using extremely dilute solutions. *Alternaria* and dust extracts, on the other hand, require higher concentrations of protein to elicit positive reactions and show less immediate difference due to stabilizers.

The activity found upon dilution without stabilizer varied considerably from one time to the next. Fig. 3

TABLE II. Percent ^{125}I bound to glass

	Final concentration of ^{125}I -AgE					
	3.3×10^{-4} $\mu\text{g/ml}$		3.3×10^{-3} $\mu\text{g/ml}$		3.3×10^{-2} $\mu\text{g/ml}$	
	Incubation period					
	24 hr	7 days	24 hr	7 days	24 hr	7 days
Phosphate-saline phenol buffer alone	4.89	4.47	5.20	5.95	4.23	4.20
Phosphate-saline phenol buffer with 0.1% Tween 80	0.76	2.10	0.17	0.39	0.23	0.41
Phosphate-saline phenol buffer with 0.01% Tween 80	0.54	2.3	0.22	0.41	0.28	0.54
Phosphate-saline phenol buffer with 0.03% HSA	0.77	1.56	0.71	4.80	0.58	0.90
Tris buffer with 0.03% HSA	1.87	1.30	0.49	1.48	0.68	1.95

illustrates this point by showing skin test titers on all five sets of dilutions with ragweed extract in two patients and rye grass extract in one patient. In each case the titer in the presence of stabilizers is constant whereas it is highly variable but nearly always lower in the absence of stabilizer.

To test whether adsorption to glass could account for the early loss of activity in unstabilized diluting fluids, antigen E was radiolabeled with ^{125}I . Table I shows that approximately 5% of ^{125}I -AgE was adsorbed to glass after 24 hr of contact in standard phosphate-saline phenol buffer. Addition of as little as 0.001% (v/v) Tween 80 inhibits adsorption significantly and concentrations of 0.002% and above inhibit more than 90% of the adsorption.

Another experiment is shown in Table II which compares HSA in standard and Tris buffers with Tween 80 at 0.1% and 0.01% after 24 hr and 7 days of incubation. No advantage for the higher concentration of Tween is revealed and relatively little for Tween over HSA. (The value of 4.8% for 7 days' incubation of $3.3 \times 10^{-3} \mu\text{g/ml}$ ^{125}I -AgE in standard phosphate-saline-phenol buffer plus HSA is probably an experimental error.) Tris buffer seems to offer no advantage over the standard buffer in the presence of HSA. Although the percent ^{125}I -AgE adsorbed is constant in the absence of stabilizer in the range of concentrations tried, skin test data suggest that a slightly higher percentage is adsorbed from more dilute solutions when no stabilizer is present.

Table III shows the effect of adding whole ragweed extract in a concentration (10,000 PNU/ml) com-

monly furnished to allergists by suppliers. At this concentration the proportion of labeled antigen adsorbed to glass is negligible.

DISCUSSION

These experiments confirm that preparing serial dilutions of allergens in buffers without stabilizers introduces a major source of error in the interpretation of skin tests. Such an error would be manifested as an unexplained variability in potency until some technique for eliminating it is employed. The addition of stabilizing additives which prevent adsorption of protein to glass is one way of eliminating much of this error. From the skin test data the error appears to be larger in extracts diluted to 0.1 PNU/ml or further. In the experiments with ^{125}I -AgE, the likely errors arising from adsorption to glass could not be completely ascertained because the radiolabel was not active enough to test the extreme dilutions required for intradermal skin tests. Such dilutions may extend to $10^{-6} \mu\text{g AgE/ml}$ in the case of ragweed, where a larger proportion of the antigen than 5% may be adsorbed. Furthermore, preparation of a long series of dilutions compounds the error. If 5% of the antigen is adsorbed in each vial, the dilution series for ragweed to 0.00001 PNU from 10,000 PNU would involve nine vials and result in 63% of the expected strength of the antigen in the ninth vial ($0.95^9 = 0.63$). The error found is often much greater so that either larger proportions of antigen are adsorbed from very dilute solutions or other sources of protein loss, such as adsorption to the syringes employed and possibly sur-

TABLE III. Effect of adding short ragweed extract (10,000 PNU/ml) on the adsorption of ^{125}I -AgE to glass

	Percent ^{125}I -AgE bound			
	Final concentration of ^{125}I -AgE			
	$3.3 \times 10^{-3} \mu\text{g/ml}$		$3.3 \times 10^{-2} \mu\text{g/ml}$	
	Incubation period			
	24 hr	7 days	24 hr	7 days
Phosphate-saline phenol alone	5.20	5.95	4.23	4.20
Phosphate-saline phenol + ragweed	0.28	0.23	1.60	1.85
Phosphate-saline phenol + 0.1% Tween 80	0.17	0.39	0.23	0.41
Phosphate-saline phenol + 0.1% Tween 80 + ragweed	0.12	0.19	0.19	0.28
Tris buffer + 0.03% HSA	0.49	1.48	0.60	1.95
Tris buffer + 0.03% HSA + ragweed	0.17	0.35	0.24	0.18

face denaturation from foaming, must be involved. Such errors are likely to vary considerably from one dilution series to the next and probably largely account for the highly variable and unpredictable magnitude of the error.

In any event, the addition of either HSA or Tween 80 effectively eliminates most of the error. Whether other agents would act as well was not explored. It is also possible that silicone coating of glassware might be helpful.⁴

For use in the allergist's office a simple additive to diluting fluids seems highly practical. The use of Tween 80 for tuberculin is now universally adopted. In our clinic extracts prepared for both skin testing and treatment of patients on research protocols have been diluted in both Tris and phosphate buffers containing 0.03% HSA without evidence of sensitization as indicated by buffer control skin tests.^{11, 12} Tuberculin in Tween 80 diluents has been used on many more patients but not so frequently in individual patients as allergen solutions in HSA. Although we are not recommending Tris buffers over phosphate buffers, it should be noted that Tris (Tris [hydroxymethyl] aminomethane) used as a buffer compound is a different chemical from the Tris used as a fire-resisting agent in clothing and suspected as a carcinogen.

The physician who employs extracts stabilized with one of these materials is likely to find that his extracts appear to be much more potent on skin testing by the intradermal route and that he must prepare higher dilutions to avoid excessively large reactions. More concentrated materials used for scratch tests are less likely to appear stronger as the higher concentration

of protein in these materials makes the loss by adsorption negligible. The same applies to the more concentrated materials used for treatment.

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