

Antigenic and allergenic changes during storage of a pollen extract

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The changes that occur in the antigens and allergens of a timothy grass pollen extract during storage were measured by radioallergosorbent test (RAST) inhibition, isoelectric focusing, (IEF), crossed immunoelectrophoresis (CIE) and crossed radioimmunoelectrophoresis (CRIE) and a dye-binding protein assay. Storage intervals varied from 7 to 92 days at temperatures of 4°, 22°, and 35° C and the freeze-dried extract was rehydrated and stored in either phosphate-buffered saline with 50% glycerol (PBSG) or Coca's solution (CS). For extracts stored in CS at 35° C, allergenic activity as measured by RAST inhibition dropped by almost one half at 7 days and certain of the IEF, CIE, and CRIE bands were no longer visible, showing that these properties correlated with allergenic activity. After 65 days, no IEF bands were visible but three CIE bands could be visualized. At 22° the changes were slower and at 4° C no changes were observed for the 92-day storage time. Extracts stored in PBSG showed some of the same changes but were much more stable at all temperatures. During storage, the protein content as measured by dye binding was also reduced, showing that proteins were fragmenting. Because some of the allergenic proteins disappeared more rapidly than others, there was not only a loss in total allergenic activity but also a change in specificity. This raises serious questions concerning the use of such extracts, especially for diagnosis. (J ALLERGY CLIN IMMUNOL 69:3, 1982.)

The stability of pollen extracts is important in their successful use for the diagnosis and immunotherapy of allergic disease. Changes in allergenic activity during storage have been observed but are not well understood; therefore this study was initiated to examine the changes that develop in a concentrated timothy pollen extract under various storage conditions.

Since allergenic extracts contain many allergens of different molecular weights and properties, these components probably differ in stability, and changes that occur may be exceedingly complex. RAST inhibition, CIE, and CRIE were used to detect and visualize changes in allergenic and antigenic activity,

and biochemical changes were investigated by IEF and a dye-binding protein assay. Enzymatic activities of the extract in the presence of different diluents were examined to determine the relationship between stability and active enzymatic composition.

METHODS

Extraction procedure

Timothy grass pollen (*Phleum pratense*) (International Biologicals, Bethany, Okla.; Lot 6680ADD, vacuum collected in 1977) was defatted with petroleum ether and extracted with gentle stirring at 4° C for 20 hr in 0.05M ammonium bicarbonate containing 1% diethyl ether as a preservative, at a ratio of one part pollen to 10 parts extracting fluid (1:10 w/v). The pollen mixture was centrifuged at $4600 \times g$ for 30 min at 4° C, and the supernatant was filtered through 0.45- and 0.22- μ m membrane filters and immediately freeze-dried in 1-ml aliquots. After drying for 72 hr, with the last 12 hr performed with a shelf temperature of 22° C, the vials were sealed under dry nitrogen and stored in a refrigerator at 2° to 8° C until rehydrated.

From the Bureau of Biologics, Food and Drug Administration, Bethesda, Md.

Received for publication March 31, 1981.

Accepted for publication Aug. 26, 1981.

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Abbreviations used

PBSG:	Phosphate buffered saline containing 50% glycerol by volume
CS:	Coca's solution
RAST:	Radioallergosorbent test
IEF:	Isoelectric focusing
CIE:	Crossed immunoelectrophoresis
CRIE:	Crossed radioimmunoelectrophoresis

Storage conditions

Extracts were rehydrated with either PBSG (0.85% NaCl in 0.1M phosphate buffer, pH 7.5), with 50% glycerol by volume or CS (0.5% sodium chloride, 0.275% sodium bicarbonate, and 0.4% phenol) to produce a 1:10 w/v extract. Vials of freeze-dried extract were rehydrated on days 0, 27, 56, 70, and 85 of the study, so that continuous storage of the rehydrated vials at the specified temperature was maintained for 0, 7, 22, 36, 65, and 92 days. In this manner, all analyses could be conducted simultaneously, using a freshly rehydrated sample as a control.

Testing procedures

Dye-binding assay. Extracts were tested for protein content by their ability to bind Coomassie Brilliant Blue G-250 in phosphoric acid (Bio-Rad Laboratories, Richmond, Calif.). This protein assay is free of interference from phenol and ammonium ion; polypeptides having molecular weights under 300 daltons, as well as some oligopeptides, do not bind the dye.^{1,2} A standard curve was developed by means of a freshly rehydrated vial of the freeze-dried timothy pollen extract. The protein content, 9.69 mg/ml, was determined by multiplying the Kjeldahl nitrogen value, 1.55 mg/ml, by the factor 6.25. Changes in the dye-binding affinity of the extract proteins due to storage conditions were detected with this procedure.

RAST inhibition. The details of this procedure have been described.^{3,4} A microparticulate support was prepared from a fresh timothy pollen extract (1:10), which was extracted in the same manner as the freeze-dried extract and was shown to be similar by IEF. Thirty milliliters of the freshly prepared extract were added to 0.5 gm (dry weight) of activated acrylic beads suspended in 7.5 ml of dioxane (Matrex; Amicon Corp., Lexington, Mass.), resulting in the covalent binding of proteins to this support. The mixture was rotated at 4° C overnight, washed six times with borate buffer, pH 8.0, and a final wash with RAST A buffer (0.1M phosphate buffer, pH 7.5, containing 1% bovine serum albumin, 0.1% Tween 20, and 0.1% sodium azide). The sensitized particles were resuspended in 500 ml of RAST A buffer and stored at 4° C.

Samples of the fresh extract were diluted to a concentration that produced between 80% and 90% inhibition. Stored extracts were diluted similarly and their ability to inhibit was determined. Analyses were performed in tripli-

cate. The mean percent inhibition was the ratio of total counts bound by a positive control sample and those bound by the stored sample.

Extracts and control samples were mixed with RAST A buffer and 500 μ l of sensitized support and then rotated at 22° to 24° C for 18 to 24 hr. The mixture was centrifuged, and the precipitate washed twice with RAST B buffer (0.1M phosphate buffer, pH 7.5 containing 1% Tween 20). Affinity-purified goat anti-human IgE (Atlantic Antibodies, Scarborough, Me.) radiolabeled with ¹²⁵I by the method of Hunter and Greenwood⁵ was diluted with RAST C buffer (0.1M phosphate buffer, pH 7.5, containing 0.4% calf serum, 0.1% Tween 20, and 1% sodium azide) to contain approximately 20 ng of antibody and approximately 25,000 cpm per assay. The samples were rotated at 22° to 24° C overnight, centrifuged, and washed three times with RAST B buffer and counted in a gamma counter. The percent inhibition was calculated by subtracting the average cpm of the sample from a noninhibited positive control and dividing this figure by the difference between the positive and negative (5% human serum albumin) control values. The freshly rehydrated control sample was considered to produce the maximal inhibition value; therefore all samples were expressed as the percent of the time-zero inhibition value.

IEF.⁶⁻⁸ Samples were focused in a pH gradient 3.5 to 9.5, using commercially available polyacrylamide gels (PAG plates; LKB Instruments, Rockville, Md.). Fifteen microliters of each sample was applied to 5 by 10 mm filter papers placed directly on the gel, and an electric current not exceeding 50 mAmp was applied to the acrylamide gels measuring 12 by 24 cm; the voltage was not permitted to exceed 300 V. At the conclusion of the run, the gels were fixed for 30 min in a solution of 11.4% trichloroacetic acid containing 34 gm/L of sulfosalicylic acid. After two rinses in destaining solution (25% ethanol containing 8% glacial acetic acid), the gels were stained overnight with Coomassie Brilliant Blue R-250 (0.9% stain in destaining solution). Destaining was accomplished with repeated rinses of destaining solution and removal of stain from the gel surface by gently rubbing with gauze. For each electrophoresis, the pH gradient was determined by extracting 5-mm sections of gel with deionized water and measuring the pH with a conventional electrode.

CIE and CRIE. The methods for CIE and CRIE have been described.⁹⁻¹¹ Briefly, 2 μ l of the stored pollen extracts underwent electrophoresis at 10 V/cm for 25 min at 10° C in 2-mm thick, 1% agarose gels (Litex agarose, type HSA, batch 523; distributed by Accurate Chemical and Scientific Co., Hicksville, N. Y.), with Svendsen's buffer (2.24% diethyl barbituric acid, 4.4% Tris 7-9, 0.05% calcium lactate, 0.1% sodium azide, pH 8.7, ionic strength 0.1, diluted 1:5 for use) as the running buffer. After the first electrophoresis, the gel strips containing the electrophoresed pollen extracts were placed on 5 by 7 cm glass slides and the same agarose now containing sheep antibody globulin preparation to timothy pollen extract was poured above and below the gel strip. These second, antibody-containing gels were 1.8 mm in thickness and contained 200

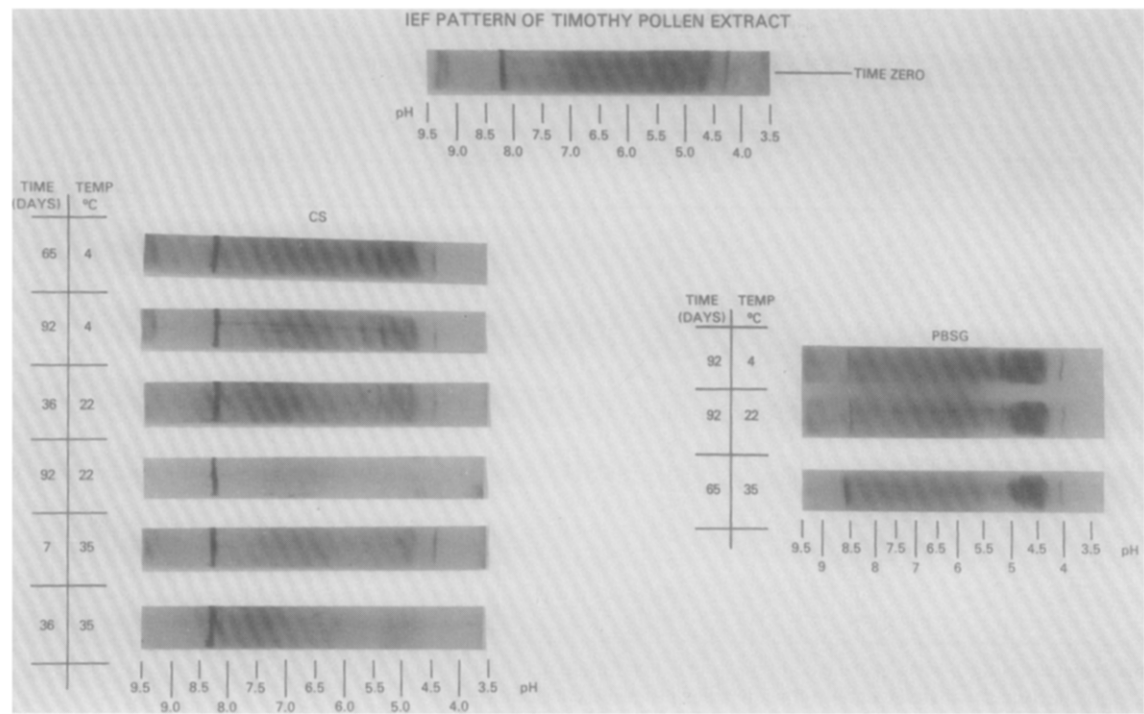


FIG. 1. IEF patterns of timothy pollen extract stored at various times and temperatures in CS and PBSG.

μ l of globulin preparation in the upper gel and 150 μ l in the lower gel. A second electrophoresis was then run for 18 to 20 hr at 10° C and 1.5 V/cm at right angles to the direction of the first electrophoresis; the plates were then pressed and dried.

For CRIE, the dried plates containing the immobilized antigen were covered with serum from a pool of untreated grass-allergic individuals, incubated overnight at room temperature with gentle rotary shaking, washed with normal saline, covered with radiolabeled anti-human IgE (4 μ g antibody, 100,000 cpm), and incubated overnight with gentle shaking. The human serum pool and the anti-human IgE were also used in the RAST studies. The plates were washed thoroughly with normal saline, then loaded into x-ray film cassettes with Kodak Royal Blue x-ray film (Eastman Kodak Co., Rochester, N. Y.). After 4 wk, the films were developed for the CRIE images. The CIE pattern was revealed by staining the dry plates with Coomassie Brilliant Blue R-250. The 4-wk development period gave maximal line resolution for our test extracts. Controls consisting of sera from two nonallergic individuals, 5% human serum albumin, and normal sheep serum produced no film images when exposed for the same time period.

Antiserum for globulin preparation for CIE and CRIE studies was produced in a sheep that was immunized with a freeze-dried timothy pollen extract, similar to the freeze-dried extract in this study by IEF and had 9.14 mg of protein/ml. The animal was immunized with 1 ml of extract in 2 ml of complete Freund's adjuvant, and a booster immunization was given 9 days later. Another booster was

given 2 wk after the second injection. Antibody was demonstrated by the Ouchterlony gel diffusion method in a serum sample taken 1 wk after the last injection. The sheep underwent plasmapheresis, and approximately 2 L of plasma were collected during the next 4 wk. The plasma was pooled and defibrinated by adding 30 gm of barium chloride to the pooled material. The mixture was centrifuged (relative centrifugal force 9150, 30 min, 4° C) and 19 gm of ammonium sulfate was added to the supernate. After a second centrifugation, sufficient ammonium sulfate was added to the supernate to produce a final concentration of 25% ammonium sulfate. This was mixed well and allowed to stand at room temperature (22° to 24° C) overnight. The mixture was again centrifuged and the precipitate was resuspended in 200 ml of deionized water, and dialyzed against four changes of 7 L of deionized water at 4° C for 48 hr. Dialysis was continued against 0.1M sodium acetate (7 L, four changes, 4° C, 48 hr). The globulin was then centrifuged, dialyzed against 0.85% sodium chloride for 48 hr, two changes at 4° C, filtered through membrane filter of 0.45- μ m porosity, and stored at -20° C.

Enzyme analysis. Examination of the pollen extracts for the presence of 19 enzymatic activities was performed with API-ZYM kits (Analytabs Products, Plainview, N. J.). Extract rehydrated in PBSG or CS without phenol was further diluted 1:20 with normal saline, and 50 μ l of solution were added to each specific substrate well. After incubation at 35° C for 4 hr, the presence of the enzyme activity was revealed by color reagents provided with the kit. Color inten-

TABLE I. Percent activity remaining after storage

Storage		Diluent*			
Temperature (°C)	Time (days)	CS		PBSG	
		Dye binding (%)	RAST† (%)	Dye binding (%)	RAST† (%)
4	7	91	98	91	96
4	22	91	107	103	102
4	36	74	102	81	98
4	65	78	101	109	101
4	92	104	97	106	103
22	7	89	102	89	99
22	22	68	107	86	98
22	36	57	91	90	98
22	65	61	84	87	99
22	92	48	67	101	99
35	7	88	57	109	100
35	22	63	27	99	96
35	36	42	33	83	90
35	65	40	15	55	82

*Values expressed as the percent of the ratio of the sample to a freshly rehydrated sample in the same diluent.

†RAST % is the percent inhibition of a fresh or stored extract as described in Methods.

sity was graded on a scale of 0 to 5, using the color scale provided.

RESULTS

The effects of temperature, diluent, and time on the dye-binding and RAST-inhibition capacities of the timothy grass pollen extract are presented in Table I.

Storage at 4° C. Extracts stored in either CS or PBSG showed no changes in dye-binding or RAST-inhibition capacity during the 92-day storage period.

Storage at 22° C. Extracts stored in PBSG showed no loss in dye-binding or RAST activity after 92 days. Extracts stored in CS showed a slight loss in dye-binding capacity after 22 days, with a continued decline over the 92-day test period, and the RAST dropped to 67% of the original activity.

Storage at 35° C. Storage of the extract in PBSG caused a clear reduction in dye-binding capacity after 65 days but no loss of RAST-inhibition activity. Extract stored in CS showed a rapid loss of RAST inhibition to a low of 15%, and dye-binding capacity decreased to 40% of the original activity.

The effect of storage on the IEF patterns of timothy pollen extract is shown in Fig. 1. The pattern of a freshly rehydrated sample is shown at the top of the figure, along with the corresponding pH gradient.

Storage at 4° C. Extract stored in PBSG lost bands in the pH areas of 5 to 6.5 after 92 days of storage. In CS, extracts displayed less distinct binding patterns in the pH 9 to 9.5 region after 65 days, and no additional changes were apparent after 92 days.

Storage at 22° C. Bands in the pH regions of 5.5 to 6.5 were lost after 92 days in PBSG, and bands in the pH areas of 9 to 9.5 were less distinct. Extracts stored in CS displayed changes in the IEF patterns after 36 days when bands at pH 5.5 to 6.5 and 9 to 9.5 were less distinct. Very few bands were discernable in extract stored in CS for 92 days at 22° C.

Storage at 35° C. Those extracts stored in PBSG lost bands at pH 5.5 to 6.5 and displayed blurred bands at pH 9 to 9.5 after 22 days (not shown). After 65 days of storage, no bands were visible in pH regions above 5.5 in PBSG extract. CS-stored extracts showed a marked loss of bands after 7 days, and after 36 days of storage IEF patterns were no longer visible.

The CIE and the CRIE patterns of the freshly rehydrated timothy pollen extract are shown in Fig. 2 with schematic tracings of the patterns to illustrate the 18 CIE antigen bands and the seven CRIE allergen bands. CRIE, which uses serum from untreated grass pollen-allergic individuals, indicates those bands that react with the radiolabeled anti-human IgE and are therefore considered to be allergens. Bands 2 and 10, which are allergens as determined by this serum pool, were not intensely radiolabeled and are difficult to discern in the autoradiograph.

During storage under the various conditions of this study, a number of changes occurred in the CIE patterns of these extracts. Because of the complexity of the patterns, only those changes that are obvious will be discussed (Fig. 3).

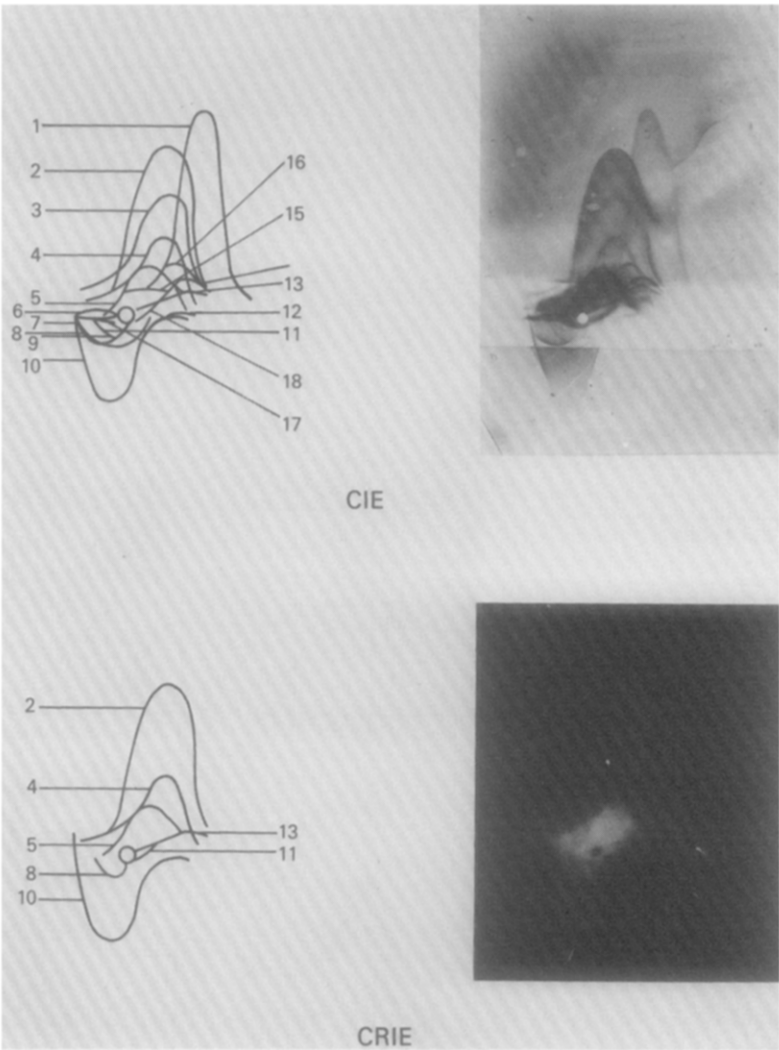


FIG. 2. CIE and CRIE patterns of timothy pollen extract showing the antigens and allergens of a freshly rehydrated sample. The schematic drawings provide a simple numbering system to identify and trace certain components.

Storage at 4° C. Extracts stored in PBSG for 92 days were found to be unchanged. Extracts stored in CS for 92 days displayed a reduction in the height of band 1 and the loss of band 13. Other minor changes were also apparent.

Storage at 22° C. Bands that migrate to the cathode in PBSG extracts were reduced in height after storage for 92 days, and the change in band 10 was most noticeable. After 36 days of storage, CS extracts displayed different CIE patterns, and continued storage resulted in the almost complete loss of band 1 and a substantial reduction in the height of band 2.

Storage at 35° C. PBSG extracts stored for 65 days displayed marked changes in patterns, showing a significant reduction in band 1 and changes in other anodic-migrating bands, with disappearance of the prominent bands that focus at the basic end of the gel.

In the CS-rehydrated extract, bands were no longer identifiable after only 7 days of storage. After 65 days, three bands were produced in CIE, but these did not resemble freshly rehydrated extract.

The enzyme profiles of freshly rehydrated timothy extract in PBSG and normal saline are presented in Table II. Extract rehydrated in diluent containing glycerol had a diminished activity with sugar-splitting enzymes.

DISCUSSION

Many publications have reported that allergenic extracts lose potency during storage under various conditions.¹²⁻¹⁹ Heat is known to be detrimental to extracts, and conversely, refrigerated storage is an aid to maintaining potency; very little data exist on the effects of freezing, although one study indicated that

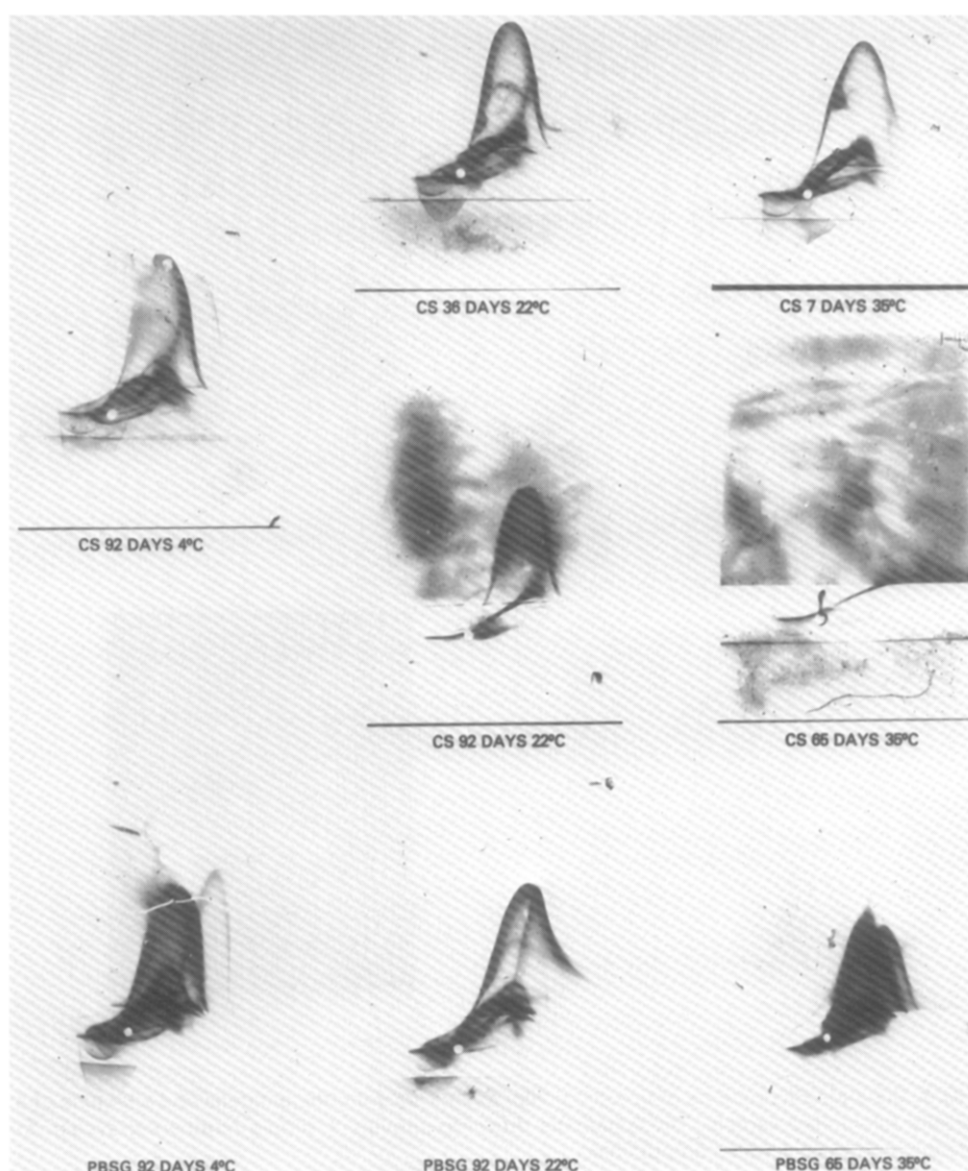


FIG. 3. CIE patterns of a timothy pollen extract stored at various times and temperatures in CS and PBSG.

this results in a loss of potency in the presence of the preservative phenol.¹⁵ High concentrations of glycerol are known to stabilize extracts, and freeze drying of pollen extracts is considered to be the best method of ensuring stability.

Since all allergenic extracts studied thus far are a complex mixture of allergenic and nonallergenic proteins, loss of potency is probably not the result of the loss of a single substance.¹²⁻¹⁴ The various components appear to degrade at different rates, since it is known that there are heat-stable and heat-labile materials in extracts, and some allergenically active proteins will survive boiling.¹⁴

The timothy grass pollen extract employed differed

from other well-characterized timothy grass pollen extracts previously described. One extract was prepared from nondefatted pollen, contained 1.67 mg/ml protein in a 1:10 extract, was stored at -20°C , and showed no cathodic-migrating proteins.¹⁸ Timothy pollen extracts prepared in a different manner had only one basic band called antigen 30.²⁰⁻²² The extract used in this study was prepared from defatted pollen, contained 7.37 mg/ml of protein, was not dialyzed, was freeze-dried, and contained at least three proteins that migrated to the cathode at pH 8.7; two of these antigens are allergens with our human serum pool.

The present study was undertaken in an attempt to

demonstrate the nature of the changes that occur during storage and makes the assumption that no changes occurred in the freeze-dried extract during the 92-day storage period.

The results confirm previous findings that maintaining an extract at 4° C or storage in 50% glycerol prevents deterioration of antigens and allergens. As the temperature increases, there is a degradation of some proteins, demonstrating that timothy grass pollen extract is a mixture of heat-stable and heat-labile components, and in this respect it is similar to other pollen extracts.^{14, 17} Since some of the allergenically active proteins are stable, total inactivation of bioactivity is not observed, but the stored extract now possesses an altered specificity. It must be assumed that a degraded timothy pollen extract would show a different pattern of skin reactivity, as previously demonstrated with short ragweed pollen extract.¹⁴ If such an extract is used in diagnosis or immunotherapy, results would be questionable. It is also interesting to note that many commercial extracts produce IEF patterns that resemble those of the degraded extracts in this study.⁶ Thus an extract that fails to produce well-defined protein bands or lacks bands is almost certainly degraded and will most likely have low allergenic activity.

The destruction of proteins in the extract is undoubtedly caused by several processes. Some of the proteins are probably destroyed by thermal denaturation; to establish this would require studies of purified extract proteins. It has been shown that solutions of pure antigen E of ragweed pollen degrade in a manner suggestive of thermal denaturation,²² whereas the antigen E in the whole extract degrades in a more complex manner. The discovery of many enzymes in timothy pollen extract, including peptidases, indicates that some breakdown of proteins is probably the result of enzymatic activity. The enzymes found in timothy pollen extract in this study are the same as those reported earlier.²³ The API-ZYM kit uses a 35° C incubation temperature; therefore the more rapid breakdown of the extract proteins (many of which are the allergens) as temperature increases from 4° to 35° C could partly be the result of increased enzyme activity. It is interesting that the presence of 50% glycerol seems to markedly inhibit only the sugar-splitting enzymes. One possible explanation for this is that glycerol is acting as a competitive inhibitor. This would further seem to suggest that the stabilizing effect of glycerol is not a general effect on enzymatic activity, since the proteolytic enzymes still seem reactive (possibly slightly reduced in activity) in this medium.

In conclusion, prolonged storage under certain

TABLE II. Enzyme activities of timothy pollen extracts*

Enzyme activity	CS (without phenol)	PBSG
Alkaline phosphatase	5	5
Esterase (C4)	0	0
Esterase lipase (C8)	5	4
Lipase (C14)	0	0
Leucine aminopeptidase	5	5
Valine aminopeptidase	2	1
Cystine aminopeptidase	0	0
Trypsin	1	1
Chymotrypsin	0	0
Acid phosphatase	5	4
Phosphoamidase	5	3
Alpha galactosidase	3	0
Beta galactosidase	5	0
Beta glucuronidase	0	0
Alpha glucosidase	0	0
Beta glucosidase	0	0
N-Acetyl glucosaminidase	3	0
Alpha mannosidase	0	0
Alpha fucosidase	0	0

*All extracts were tested at 2.5 w/v in the indicated diluent. CS was used without phenol because of interference in the control wells. Data expressed as arbitrary grades of reactivity from 0-5 based on a color chart provided by the manufacturer of the enzyme kit.

conditions results in an extract with low allergenic activity, a process that is accelerated by increased storage temperature and the absence of a stabilizing agent. The profound changes in the proteins and, consequently, the antigens and allergens indicates that this low potency cannot be improved by using extracts at increased protein levels, since the allergenic composition of these materials is not the same as that of a freshly prepared pollen extract.

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