

Comparison of the stability of a mite and a pollen extract stored in normal conditions of use

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Summary

Pollen extracts are stable in solutions containing 50% glycerol. The stability of *Dermatophagoides pteronyssinus* (*Dp*) extracts have not been determined. Two lyophilised extracts (*Dp* and cocksfoot pollen) were reconstituted in 50% glycerol and stored for up to 6 months at +4°C. Every day the extracts were placed at room temperature for 1 hr to mimic the natural use of allergen extracts by allergists. Every 2 months the extracts initially prepared were compared to freshly reconstituted extracts by means of prick skin tests in fifteen allergic patients. It was observed that cocksfoot pollen extract is stable but there appears a slight but significant ($P < 0.05$) decay in activity when the extract stored for up to 6 months was compared with a freshly prepared extract. On the other hand there is a significant ($P < 0.01$) decay in activity of the *Dp* extract after 2 months of storage. *Dermatophagoides pteronyssinus* and cocksfoot pollen extracts behave differently when stored. These findings have particular importance in the diagnosis and treatment of allergic disorders.

Introduction

Allergen extracts are composed of very heterogeneous mixtures of biological substances which may contribute to their rapid degradation. For pollens, saline extracts are rapidly degraded when preserved at room temperature (Baer, 1980; Center Schuller & Zelzanick, 1974; Hjorth, 1958; Norman & Marsh, 1978). Addition of human serum albumin (Norman & Marsh, 1978) or Tween 80 (Hjorth, 1958; Norman & Marsh, 1978) could delay the degradation of diluted extracts. Parallel experiments made with 50% glycerol, several concentrations of phenol, paraben or thiomersal showed clearly that thermal decay occurred with all preservatives except glycerol (Hiatt, Baer & Hooten, 1977). Such protective activity of 50% glycerol solutions has been reported for different pollen species (Nelson, 1979; Bousquet *et al.*, 1983a).

Dermatophagoides pteronyssinus (*Dp*) is the major mite allergen of housedust in Europe (Voorhorst, Spieksma-Boezeman & Spieksma, 1964; Cunningham, 1967;

Maunsell, Hughes & Wraith, 1970) and probably represents one of the most prevalent stimuli to allergic sensitization in the world. However, the stability of *Dp* extracts has never been established.

Most studies of the shelf life of allergenic extracts are made at one of two different temperatures. Either the extract is carefully preserved at +4°C, or the extract is placed at a given temperature which is maintained constant throughout the study. However these conditions rarely apply to the preservation of allergen extracts in the clinics. Usually the extracts are stored at +4°C and stay on the bench while the allergist is working.

The stability of allergen extracts may be studied by *in vivo* or *in vitro* methods (Baer, 1978; Panel on Review of Allergenic Extracts, 1981; Bousquet *et al.*, 1983a). Direct skin-test titration appears to be a satisfactory method to obtain the relative potency of allergen extracts (Brighton, 1980) especially when the potency of the extract is related to the reaction elicited by a positive control solution (Aas & Belin, 1972). Moreover, it was shown that the potency of some pollen extracts as determined by skin-test end-point correlated significantly with *in vitro* studies (Baer *et al.*, 1974; Gleich *et al.*, 1974; Pepys *et al.*, 1975). When skin-test titration is used for standardization purposes the performance of the test should follow precise guidelines (Stevens, 1980), and, due to the considerable variability in sensitization from patient to patient, a sufficient number of allergic subjects should be treated (Grimmer *et al.*, 1979; Brighton, 1980).

In 1981, standardized and lyophilized extracts were available and it was therefore possible to assess the stability of reconstituted extracts. *Dermatophagoides pteronyssinus* (*Dp*) and cocksfoot (*Dactylis glomerata*) pollen extracts reconstituted in 50% glycerol were stored for up to 6 months and their stability was examined every 2 months by means of skin-test titration. The extracts were preserved at +4°C throughout the study, but were removed from the refrigerator for 1 hr every day.

Materials and methods

Preparation of extracts

Dermatophagoides pteronyssinus (*Dp*) extracts were grown on medium containing equal parts of *Saccharomyces cerevisiae* and human dander. After 3 weeks of culture, mites were carefully isolated from the culture medium. Somatic and metabolic antigens were extracted by ammonium bicarbonate (0.125 M) at pH 8.0. The extracting procedure with continued stirring at +4°C was performed for 24 hr. The extracting liquid is then passed through a sterilizing grade filter, an aliquot was removed for control testing and the bulk of the extracting solution was frozen.

Cocksfoot pollen extract was prepared from pure pollens harvested from cultivated plants over a period of 3 years to avoid possible annual variations. Before extraction, pollens were analysed for purity. They were free from other vegetable matter and other pollens. Unde-fatted pollens were extracted as previously indicated for mite antigens.

Control and lyophilisation of the extracts

A control of the potency of an aliquot was performed by RAST inhibition (Gleich *et al.*, 1974), iso-electric focussing (Brighton, 1975) and compared with the internal standard which was controlled by RAST inhibition, iso-electric focussing, cross-immuno-electrophoresis (Løwenstein, 1978), and skin-test titration. A given amount of the extracting solution was then poured into vials which were subsequently lyophilized.

Table 1. Serial dilutions of extracts tested

Log ₃ allergen dose	Allergen	
	<i>D. pteronyssinus</i>	Cocksfoot pollen
1	1/100	1/20
2	1/300	1/60
3	1/900	1/180
4	1/2700	1/540
5	1/8100	1/1620
6	1/24 300	1/4860
7	1/72 900	1/14 580
8		1/43 740
9		1/131 220
10		1/393 660

A control of the final product by RAST inhibition and iso-electric focussing was finally performed. The freeze-dried extracts were stored at +4°C.

The same batch of extract was used throughout the study.

Test procedures

Allergen extracts were reconstituted in 50% glycerol and stored for periods up to 6 months. The extracts were kept at +4°C and placed at room temperature for 1 hr every day to better mimic natural conditions of usage of allergen extracts.

Every 2 months another vial was reconstituted and 3-fold dilutions were made in 50% glycerol. For *Dp* extracts the dilutions were tested from 1/100 to 1/72 900. For cocksfoot pollen extracts the dilutions ranged from 1/20 to 1/393 660. The highly diluted extracts were chosen in order to find out whether anomalous effects of allergen concentration might be observed (Huggins & Brostoff, 1979) (Table 1).

Prick tests were performed by the Stallerpointe® derived from the Morrow-Brown standardized needle (Morrow-Brown, Su & Thantrey, 1981). They were done on the volar surface of the right arm. Negative and positive controls and allergens were tested in random order. A 5% codein-phosphate solution in 50% glycerol was used as a positive control and a 50% glycerol solution as a negative control. To avoid possible circadian differences in skin-test reactivity, tests were done between 0900 and 1100 hr. None of the subjects had received any antihistamine, systemic corticosteroids or any form of theophylline for at least 72 hr before the tests and ketotifen or imipramine for at least 2 weeks before the tests since these drugs might affect the formation of the weal and flare reaction (Menardo, Bousquet & Michel, 1981, 1982).

All tests were done by the same person.

The weals induced by the extracts were recorded 15 min after the tests for allergens and 10 min after the tests for codein phosphate by the scotch-tape method. The mean weal diameter was subsequently measured. The strength of an extract which produced a weal size of over 75% of the positive control solution was arbitrarily defined as the threshold strength of that extract according to Shultze-Werninghaus (1980) and Menardo *et al.* (1982). Moreover the size of the greatest test result was also analysed.

Skin tests were performed on very sensitive subjects who never had immunotherapy. Their sensitivity was assessed by positive skin tests to the relevant allergen and the

presence of increased titres of serum-specific IgE antibodies in the RAST class 3–4 of the Pharmacia scoring system.

All freshly prepared extracts were tested in ten subjects within 1 week after reconstitution to assay the strength of extracts.

After storage, fresh extracts and those prepared 2, 4 and 6 months before were tested in the same patient within 1 week after the reconstitution of the freshly prepared extract. Fifteen patients were tested in the study.

Statistical analysis of the data

The comparison of skin-test results was made by non-parametric tests for paired samples using the Wilcoxon *W* test.

Results

No bleeding at the skin-test site was noted and the test results were therefore considered valid. No anomalous effects of weak concentrations were found.

The end-point prick-test titration and the mean size of the greatest prick test was similar for freshly prepared extracts throughout the study (Tables 2 and 3).

Table 2. Mean results of prick tests induced by cocksfoot pollen extracts freshly prepared

		Time of reconstitution (months)			
		0	2	4	6
Mean end-point	$\bar{x}$	4960 ⁻¹	5020 ⁻¹	4776 ⁻¹	3325 ⁻¹
	s.d.	1090	1256	1845	1652
Mean size of greatest test	$\bar{x}$	8.1	8.4	7.7	8.0
	s.d.	3.9	4.1	3.7	3.8

Table 3. Mean results of prick tests induced by *D. pteronyssinus* extracts freshly prepared

		Time of reconstitution (months)			
		0	2	4	6
Mean end-point	$\bar{x}$	7020 ⁻¹	6503 ⁻¹	7345 ⁻¹	7210 ⁻¹
	s.d.	2216	2456	3215	2310
Mean size of greatest test	$\bar{x}$	6.1	5.8	6.3	6.1
	s.d.	3.2	3.4	2.9	3.1

In tests on the activity of cocksfoot pollen extracts stored for up to 6 months, the end-point titre was significantly ($P < 0.05$) decreased when the initial extracts were compared to the extracts prepared 6 months later. However, the mean size of the greatest weal was unchanged after 6 months storage.

In contrast, the decay of activity of the *Dp* extract is significant ($P < 0.01$) after 2

months of storage when the activity of the extract is either analysed by the prick-test end-point titre or the size of the greatest weal. The activity of the *Dp* extract continuously decreased when the extract was stored over a longer period of time.

Discussion

Mite and pollen extracts were shown to behave differently when lyophilized materials were reconstituted with the same diluent which was chosen as it was found to be appropriate for the preservation of pollen extracts (Baer, 1980; Center *et al.*, 1974; Hiatt *et al.*, 1977; Bousquet *et al.*, 1983a; Nelson, 1979).

Skin-test titration, when carried out properly, is a reliable procedure for obtaining the relative potency of allergen extracts (Baer, 1978; Bousquet *et al.*, 1983a; Brighton, 1980; Aas & Belin, 1972; Gleich *et al.*, 1974), and is consistent with the results of our tests since all lyophilized freshly reconstituted extracts induce a similar mean prick response (Table 1). Only fifteen allergic patients were tested although it is estimated that twenty (Grimmer *et al.*, 1979) to thirty patients (Brighton, 1980) should be included in the same group. However, it was found more desirable to assay the extracts within 1 week and it was not practical to test more than fifteen patients. The analysis of skin tests may be performed using the end-point method (Aas & Belin, 1972) or by plotting the geometric mean weal size of 10-fold diluted extracts (Brighton, Topping & Henocq, 1977). This method could not be used because it requires thirty patients. The end-point titration was therefore performed using codein phosphate instead of histamine as a positive control because it was found to be more stable. In order to avoid a possible anomalous effect of allergen concentration on weal size (Huggins & Brostoff, 1979), we used highly diluted extracts in the serial dilutions. End-points could be clearly identified. By means of the Stallerpointe, no bleeding occurred. This is critical when extracts preserved in 50% glycerol are tested by the prick method (Menardo *et al.*, 1983).

The results observed for pollen extracts did not differ from those already published. In 50% glycerol, the stability of various species of pollen extracts is remarkable (Baer, 1980; Center *et al.*, 1974; Nelson, 1979, 1981; Bousquet *et al.*, 1983a,b). In the present study it was found that after 6 months of storage there was a slight but significant decay in activity of cocksfoot pollen extracts when the end-point titration was taken into consideration but the size of the greatest test response did not vary. This latter finding can be attributed to the relatively high concentrations of the first extracts (1/20 and 1/60) since it is commonly observed that the size of the tests increases with the strength of the extracts then reaches a plateau and in some instances subsequently decreases. These experiments suggest that a cocksfoot pollen extract can be used for 6 months after reconstitution with 50% glycerol.

In contrast *Dp* lyophilized extracts reconstituted in 50% glycerol very rapidly lose their activity and after reconstitution they should not be kept for more than 1 month. However, since the plateau of the size of skin tests was not reached, more concentrated extracts may be more stable and kept for a longer period of time. Glycerol extracts of *Dp* should be more stable at 4°C. When stored at +4°C in saline containing human serum albumin, *Dp* extracts were found to be quickly degraded and solutions used for immunotherapy had to be removed after 1 month of storage.

The results found in the present study conflict with recent data published by Naerdal and Vilsvik (1983) who observed that a *D. farinae* extract preserved in saline was fairly unstable, whereas preserved in saline containing 0.03% of human serum

albumin the extract was quite stable over a period of 8 months. However, in this study it appears that HSA-containing preparations may lose up to 50% of their activity within 1 week. Moreover, in some patients it was found that after 6 weeks the estimated allergenic activity was sharply decreased.

The reasons for the differences observed between pollen and mite extracts are unknown. Glycerol was found to be a very potent preservative for pollen extracts and its role was eventually related to its anti-enzyme activity (Anderson & Baer, 1982). Pollen extracts contain large amounts of several enzymes (Bousquet *et al.*, 1980a) not found in extracts of mites (Bousquet *et al.*, 1980b). Moreover, mite allergens are less characterized than pollens and the complexity of mite extracts may be greater than that of pollens.

These observations indicate that the shelf life for different allergen extracts should be determined and that similar allergens made by different manufacturers should be evaluated. Finally, at least for mite extracts, an adequate preservative must be found.

References

- AAS, K. & BELIN, L. (1972) Standardization of diagnostic work in allergy. *Acta Allergologica*, **27**, 439.
- ANDERSON, M.C. & BAER, H. (1982) Antigenic and allergenic changes during storage of a pollen extract. *Journal of Allergy and Clinical Immunology*, **69**, 3.
- BAER, H. (1978) Allergenic extracts. In: *Allergy, Principles and Practice* (eds E. Middleton Jr, C.E. Reed and E.F. Ellis), p. 226. Mosby Co., St Louis.
- BAER, H. (1980) Stability of allergen extracts. In: *Regulatory Control and Standardization of Allergenic Extracts* (eds H.D. Brede and H. Going), p. 175. Gustav Fisher, Stuttgart.
- BAER, H., MALONEY, C.J., NORMAN, P.S. & MARSH, D.G. (1974) The potency and Group I antigen content of six commercially prepared grass pollen extracts. *Journal of Allergy and Clinical Immunology*, **54**, 157.
- BOUSQUET, J., GUERIN, B., FURIC, R. & MICHEL, F.B. (1983a) Standardization of allergen extracts. In *Provocative Testing* (ed. S. Spector). CRC Press, Boca Raton, Florida. p. 45.
- BOUSQUET, J., GUERIN, B., HEWITT, B. & MICHEL, F.B. (1980a) Analysis of commercial pollen extracts by enzyme determination. I. Comparison of RAST-inhibition assay and enzyme titration for orchard grass, rye grass and short ragweed pollen extracts. *Annals of Allergy*, **45**, 310.
- BOUSQUET, J., GUERIN, B. & MICHEL, F.B. (1984) Etude de la stabilité des extraits de pollens en milieu aqueux. *Revue française d'Allergologie*, (in press).
- BOUSQUET, J., HALE, R., GUERIN, B. & MICHEL, F.B. (1980b) Enzymatic activities of house dust extracts. *Annals of Allergy*, **45**, 304.
- BRIGHTON, W.D. (1975) Profiles of allergenic extract components by iso electric focusing and radio immunoassay. *Developments in Biologic Standardization*, **29**, 367.
- BRIGHTON, W.D. (1980) Introduction on possibilities and limitations of potency determination. In: *Regulatory Control and Standardization of Allergen Extracts* (eds H.D. Brede and H. Going), p. 111. Gustav Fisher, Stuttgart.
- BRIGHTON, W.D., TOPPING, M.D. & HENOCQ, E. (1977) Activity units for allergen extracts. *Clinical Allergy*, **9**, 591.
- CENTER, J.C., SCHULLER, N. & ZELZANICK, L.D. (1974) Stability of antigen E in commercially prepared ragweed pollen extract. *Journal of Allergy and Clinical Immunology*, **54**, 305.
- CUNNINGTON, A.M. (1967) The mite flora of house dust. *Acta Allergologica*, **22**, 415.
- GLEICH, G.J., LARSON, J.B., JONES, R.T. & BAER, H. (1974) Measurement of the potency of allergy extracts by their inhibitory capacities in the radioallergosorbent test. *Journal of Allergy and Clinical Immunology*, **53**, 158.
- GRIMMER, O., FROSTAD, A.B., SANDVIK, L. & AAS, K. (1979) A practical and statistical method for the precise biological standardization of allergen extracts. *Journal of Allergy and Clinical Immunology*, **68**, 26.
- HIATT, C.W., BAER, H. & HOOTEN, M.L. (1977) Kinetics of thermal decay of antigen E from short ragweed pollen. *Journal of Biological Standardization*, **5**, 39.
- HJORTH, N. (1958) Instability of grass pollen extracts. *Acta Allergologica*, **12**, 316.

- HUGGINS, K.G. & BROSTOFF, J. (1979) An anomalous effect of allergen concentration on weal size elicited by skin testing for immediate hypersensitivity. *Clinical Allergy*, **9**, 37.
- LÖWENSTEIN, H. (1978) Quantitative immunoelectrophoretic methods as a tool for the analysis and isolation of allergens. *Progress in Allergy*, **25**, 1.
- MAUNSELL, K., HUGHES, A.M. & WRAITH, D.G. (1970) Mite asthma: cause and management. *Practitioner*, **205**, 779.
- MENARDO, J.L., BOUSQUET, J., BATAILLE, A., RESTAGNY, G. & MICHEL, F.B. (1983) Effect of diluents on skin tests. *Annals of Allergy*, **51**, 315.
- MENARDO, J.L., BOUSQUET, J. & MICHEL, F.B. (1981) Standardization of skin tests. In: *Proceedings of the Annual Meeting of the European Academy of Allergy and Clinical Immunology, Clermont Ferrand, September 1981* (ed. C. Molina), p. 435.
- MENARDO, J.L., BOUSQUET, J., MICHEL, F.B. (1982) Comparison of three prick test methods with the intradermal test and with the RAST in the diagnosis of mite allergy. *Annals of Allergy*, **48**, 235.
- MORROW-BROWN, H., SU, S. & THANTREY, N. (1981) Prick test standardization using a precision needle. *Clinical Allergy*, **11**, 95.
- NAERDAL, Å. & VILSVIK, J.S. (1983) Stabilisation of a diluted aqueous mite allergen preparation by addition of human serum albumin. An intracutaneous test study. *Clinical Allergy*, **13**, 149.
- NELSON, H.S. (1979) The effects of preservatives and dilution on the deterioration of Russian thistle (*Salsola pestifera*). A pollen extract. *Journal of Allergy and Clinical Immunology*, **63**, 417.
- NELSON, H.S. (1981) Effects of preservatives and conditions of storage on the potency of allergy extracts. *Journal of Allergy and Clinical Immunology*, **67**, 64.
- NORMAN, P.S. & MARSH, D.G. (1978) Human serum albumin and tween 80 as stabilizers for allergen solutions. *Journal of Allergy and Clinical Immunology*, **62**, 314.
- PANEL ON REVIEW OF ALLERGENIC EXTRACTS (1981) *Final Report, March 13*. Bureau of Biologics, Food and Drug Administration, Bethesda.
- PEPYS, J., TANGEN, O., PERKINS, F.T., TATE, H. & BRIGHTON, W.D. (1975) Examination of cocksfoot pollen (*Dactylis glomerata*) extracts by skin prick-tests in man, RAST-based allergen assay and protein content. *Developments in Biological Standardization*, **29**, 284.
- SCHULTZE-WERNINGHAUS, G. (1980) Comparison of standardized skin and provocation tests with RAST and histamine release from leukocytes in bronchial asthma. *Allergy*, **35**, 259.
- STEVENS, E. (1980) Cutaneous tests. In: *Regulatory Control and Standardization of Allergenic Extracts* (ed. H.D. Brede and H. Goings), p. 133. Gustav Fisher, Stuttgart.
- VOORHORST, R., SPIEKMA-BOEZEMAN, M.I.A. & SPIEKMA, F.TH.M. (1964) Is a mite (*Dermatophagoides* spp.) the producer of the house dust allergen? *Allergie und Asthma*, **10**, 329.

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