

Studies on the stability of diluted allergen extracts using the radioallergosorbent test (RAST)

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Summary

Allergen extracts from birch pollen, timothy pollen and horse dandruff were investigated. The influence of storage time, volume of extract in the vial and various additives on the potency of diluted extracts was tested with the radioallergosorbent test (RAST). The potency of diluted pollen extracts was found to decrease more rapidly than the potency of diluted dandruff extracts. The differences in allergen activity seen in RAST between freshly diluted extracts and stored extract dilutions were roughly reproducible in skin tests and histamine release tests from passively sensitized chopped human lung. The higher allergen stability of the dandruff extracts could, at least in part, be explained by the higher protein content of these extracts. An adsorption of allergen to the glass wall was shown to be the most probable cause to the more rapid decrease of allergen activity seen in pollen extracts stored in vials only partly filled. With addition of 0.2% Tween 20 after storage the potency of the extracts became almost equivalent to that of corresponding dilutions stored in filled vials.

Introduction

Allergens in concentrated solution have been reported to be stable for one to several years (Augustin & Hayward, 1962; Derrick & Trinca, 1964). Diluted extracts, on the other hand, are more labile and deteriorate faster the more diluted they are (Hjorth, 1957; Malley, Reed & Lietze, 1962). For the allergologist it is important to know not only the potency of an allergen extract but also the stability at various dilutions. Such information is scarce, however, probably due to a lack of simple and reliable test-methods.

The radioallergosorbent test (RAST) (Wide, Bennich & Johansson, 1967) was primarily designed for the determination of IgE-antibodies to various allergens and the test has been shown to be of great value in the diagnosis of human reaginic allergy (Berg, Bennich & Johansson, 1971; Aas & Johansson, 1971). Recently, RAST has been shown to be of value also for the characterization of allergen extracts (Ceska,

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Eriksson & Varga, 1972; Foucard *et al.*, 1972) and in the present study we have used RAST to study the effect of storage on the stability of diluted allergen extracts.

Material and methods

Serum samples were obtained from patients undergoing allergological investigations at the University Hospital, Uppsala. The sera were screened with RAST for IgE antibodies to relevant allergens and sera with high titres were chosen for the present study.

Allergens. Commercial water-soluble stock extracts of birch pollen, timothy pollen and horse dandruff from the following manufacturers were used: Vitrum Allergen Laboratory (V), Stockholm, Sweden; Dome Laboratories (D), New York, distributed by MEDA Ltd, Gothenburg, Sweden; Allergological Laboratory (C), Copenhagen, Denmark. The potency of the stock extracts was noted as 1/10 w/v (V), 1/100 w/v (C) and 10,000 PNU (D). The same batches were used throughout the study. Furthermore, an extract of timothy pollen allergen was prepared essentially as described by Augustin & Hayward (1962) and by Johnson & Marsh (1965) and partially purified by gel filtration. The procedure is summarized as follows: 100 g of *Phleum pratense* pollen (AB Cernelle, Sweden) were defatted three times with 300 ml acetone at room temperature. The defatted product was extracted twice with 300 ml 10 mM ammonium bicarbonate, pH 8, by gentle stirring for 16 hr at +4°C. Insoluble matter was removed by centrifugation and the supernatant was dialysed (Visking tubing, 23/32 in.) at +4°C against bicarbonate buffer (see above) and finally freeze-dried. The extract was dissolved in 15 ml 50 mM ammonium bicarbonate, pH 8, and an aliquot was taken for determination of allergen activity by RAST (Foucard *et al.*, 1972). The remaining solution was applied on a 700 ml column of Sephadex G-75 equilibrated with the 50 mM ammonium bicarbonate. The effluent was measured for absorbancy at 280 and 260 nm and for allergen activity. Active fractions were pooled, concentrated by freeze-drying, redissolved in 15 ml 50 mM ammonium bicarbonate and finally applied on a 700 ml column of Sephadex G-50. Allergen activity was recovered in the effluent between 0.47 and 0.58 of the total bed volume. After freeze-drying, a stock solution of partially purified timothy allergen was made in 10 mM ammonium bicarbonate, to be used within a month from the day of preparation.

All allergen extracts were diluted in Coca's solution and stored in 14 ml rubber-capped soda glass vials (Apotekarnas Droghandel, Gothenburg, Sweden).

Skin tests with birch and timothy pollen allergens were performed as intradermal tests on the backs of allergic children. One-tenth ml of the allergen dilutions 10^{-3} – 10^{-5} w/v (V) was injected. 20 min later the mean diameter of the separate weals was calculated from two perpendicular diameters. The horse dandruff extracts were titrated using prick tests on the volar surface of the forearms of allergic children at allergen dilutions 10^{-1} – 10^{-3} w/v (V). The weals formed after 10–15 min were recorded in the same way as for the intradermal tests. The allergen extracts used for all skin titrations were coded and the vials were recoded for each test occasion.

The histamine release tests (CHLT) were performed on passively sensitized chopped human lung (Sheard, Killingback & Blair, 1967) as previously described (Foucard, 1972). In brief, 100 mg samples of chopped and washed human lung were incubated for 18 hr at room temperature with a suitable dilution of a reaginic serum. After washings, dialysed allergen extracts in serial tenfold dilutions were added. Tyrode was used for all dilutions and washings. The samples were incubated at +37°C for 20 min, cooled,

centrifuged, and the supernatants were analysed for histamine using an atropinized guinea-pig ileum. The histamine remaining in the lung samples was determined after boiling of the pellets. The release of histamine was calculated as a percentage of the total histamine.

The radioallergosorbent test (RAST) (Wide *et al.*, 1967) was applied for allergen quantitation as described (Foucard *et al.*, 1972). Serially diluted allergen extracts were coupled to CNBr-activated filter paper discs (Ceska *et al.*, 1972), 0.2 ml allergen dilution per paper disc. Each allergen-polymer complex (APC) was tested with two reaginic sera as follows: 100 μ l of reaginic serum was incubated with one APC disc for 3–18 hr at room temperature. After six washings in saline containing 0.1% Tween 20, 100 μ l (= 80,000 cpm) of ^{125} I-labelled immunosorbent purified rabbit anti-Fc $_{\epsilon}$ (ND) was added. 20 hr later the APC disc was washed six times and the radioactivity was measured in a gamma scintillation counter.

The protein content of the allergen extracts was kindly analysed by the Department of Biochemistry, Pharmacia Ltd, Uppsala, using a micro-Kjeldahl technique. The analyses were performed on 0.5 ml of the stock extracts dialysed for 24 hr against 2×1500 ml distilled water (Visking tubing, 8/32 in.).

Results

Preliminary experiments using RAST indicated that the potency of diluted pollen extracts decreases during storage in comparison with dilutions made fresh from stock extracts. To exclude that the observed difference in potency reflected only a decreased ability of the diluted and stored allergens to bind to activated cellulose discs, the reaginic activity had to be assessed along with the RAST studies. Separate dilutions of Vitrum extracts (5 ml in 14 ml glass vials) from birch pollen, timothy pollen and horse dandruff were therefore stored for 4 months at $+4^{\circ}\text{C}$ prior to analyses with RAST, skin tests and CHLT. As seen from Figs. 1 and 2 the potency of the pollen extracts stored at a concentration of 10^{-3} w/v was significantly lower as compared with the corresponding dilution freshly prepared from 10^{-1} w/v. A decrease in potency was seen in all three test systems but to a somewhat varying degree. In contrast, the diluted extract of horse dandruff appeared to be very stable; the slight difference in allergen

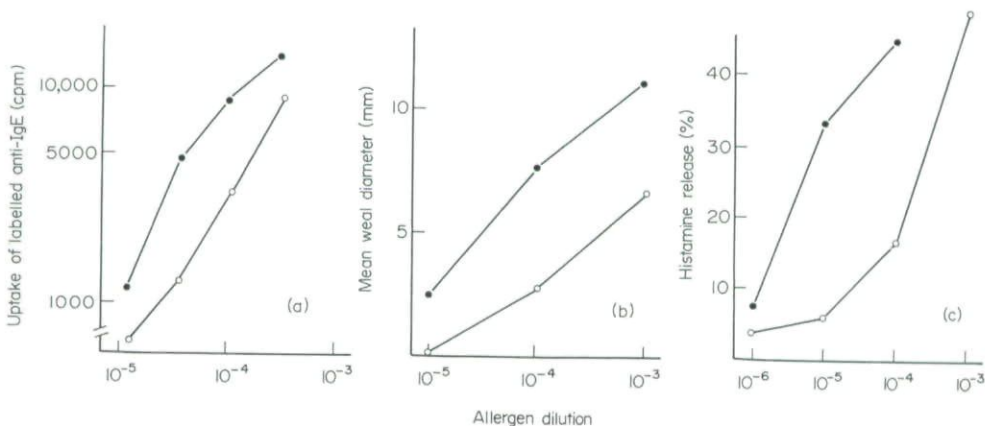


Fig. 1. The potency of a birch pollen extract stored as 10^{-3} w/v (○) for 4 months at $+4^{\circ}\text{C}$ in relation to the same dilution made fresh from the stock extract, 10^{-1} w/v (●). (a) RAST; (b) skin titrations, mean weal diameter in eight patients; (c) CHLT.

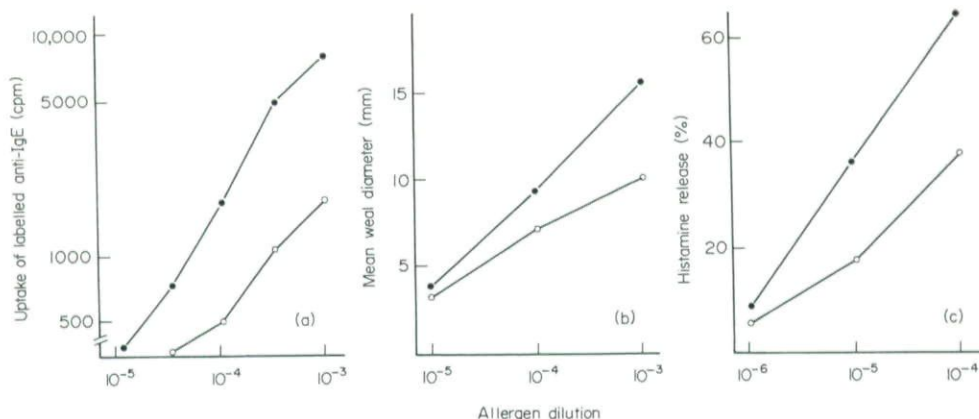


Fig. 2. The potency of a timothy pollen extract stored as 10^{-3} w/v (○) for 4 months at $+4^{\circ}\text{C}$ in relation to the same dilution made fresh from the stock extract, 10^{-1} w/v (●). (a) RAST; (b) skin titrations, mean weal diameter in eight patients; (c) CHLT.

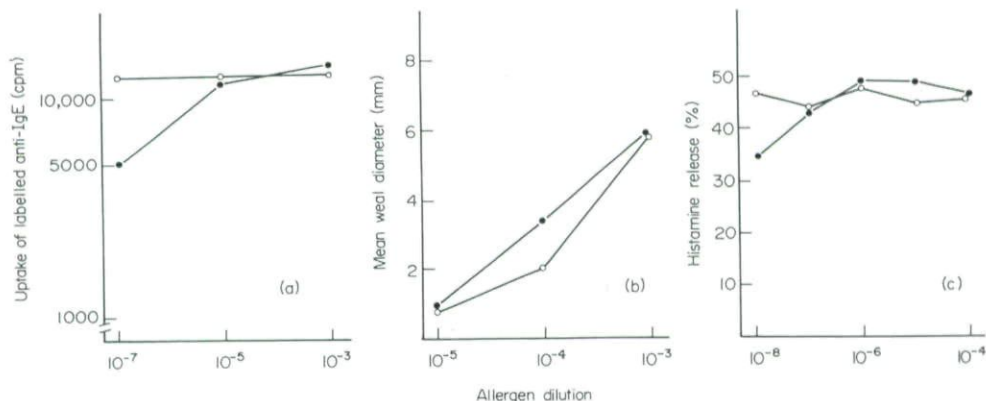


Fig. 3. The potency of a horse dandruff extract stored as 10^{-3} w/v (○) for 4 months at $+4^{\circ}\text{C}$ in relation to the same dilution made fresh from the stock extract, 10^{-1} w/v (●). (a) RAST; (b) skin titrations, mean weal diameter in seven patients; (c) CHLT.

activity noted in RAST and CHLT being actually in favour of the diluted extract (Fig. 3).

For further studies on the effect of storage time on the potency of dilute allergen extracts the following experiments were designed: 5 ml of extracts from different manufacturers were stored in 14 ml vials at $+4^{\circ}\text{C}$ and analysed at separate time intervals. Two or three complete sets of extracts were prepared, one was used for the analyses during the first week, another after 6 weeks and the third after 4–5 months. As the slopes for freshly diluted extracts and stored extract dilutions in most cases were parallel in RAST, the decrease of the allergen activity was easy to calculate. For the pollen extracts, the sensitivity of RAST did not allow, except in a few cases, any measurement to be made below 10^{-4} w/v. To calculate the decrease of the potency of a pollen extract stored as 10^{-4} w/v compared with the same dilution made fresh, it was therefore assumed that the RAST value obtained for the extract stored as 10^{-4} w/v represented a point on a slope parallel with the slopes obtained with the more concentrated solutions (Fig. 4).

As seen in Table 1, a decrease in allergen activity was obtained mainly with diluted pollen extracts and both the rate and the degree of deterioration increased with the dilution. No difference in allergen stability was found between extracts from different manufacturers or for our own timothy allergen preparation. Extracts of horse dandruff had to be diluted to at least 10^{-6} w/v before any potency decrease was noted during the storage. The difference in allergen stability seen between the pollen extracts and the horse dandruff extracts was suspected to be caused, at least partly, by a difference in

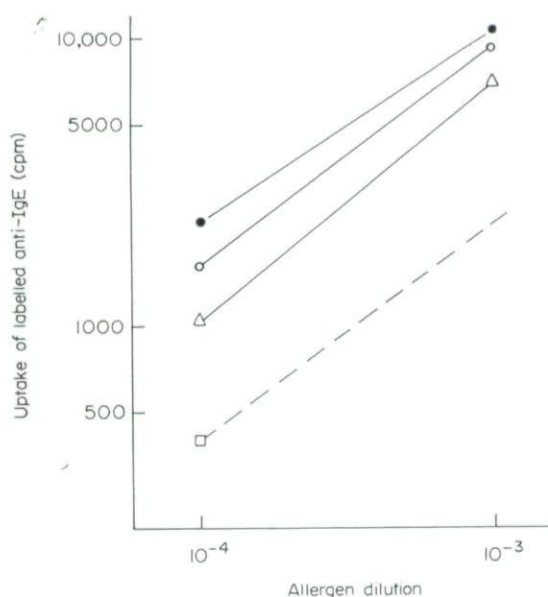


Fig. 4. A comparison of the potency of a birch pollen extract stored as 10^{-1} – 10^{-4} w/v for 6 weeks at $+4^{\circ}\text{C}$. Dilutions were after storage made fresh from 10^{-1} (●), 10^{-2} (○) and 10^{-3} (△) w/v. No dilution was made from 10^{-4} w/v (□) but for the calculations parallelism was supposed to exist with the slopes for the other dilutions.

protein concentration. Table 2 shows that the protein concentration of the horse dandruff extracts were 5–75 times higher than that of the commercial pollen extracts. Our own timothy allergen preparation, however, had a protein concentration very similar to one of the horse dandruff extracts.

Effect of the extract volume in the vial on allergen stability

From 10^{-1} , 10^{-3} and 10^{-5} w/v of 'full' vials of Vitrum allergen extracts (10 ml) 9 ml was transferred to one empty vial and 1 ml to another. These vials were stored for 6 weeks at $+4^{\circ}\text{C}$ and the allergen content was then tested with RAST. As seen from the timothy pollen experiment (Fig. 5) there was no difference in potency between dilutions made fresh from the '9 ml' and the '1 ml' vial. When the freshly prepared dilution 10^{-3} w/v was compared with the corresponding dilution stored for 6 weeks, a marked decrease of the uptake of radioactivity was found with the latter. A decrease was seen both in the '9 ml' and in the '1 ml' volume but it was more pronounced in the smaller volume. The potency of the '1 ml' extract seemed to be less than 1/10 of that of the '9 ml' extract. The results were almost identical for the extracts from birch and timothy pollen. As in the storage experiments, the horse dandruff extract was very stable and

Table 1. The relative deterioration of diluted allergens during storage at +4°C. The potency is expressed in per cent of that of a dilution made fresh from the stock extract

	Allergen dilution			w/v	Storage time
	10 ⁻¹	10 ⁻³	10 ⁻⁴		
Birch V89205	100	100	100		48 hours
	100	100	55		1 week
	100	50	≤ 15		6 weeks
	100	30	≤ 10		4 months
	100	30	NT		5 months
Birch D816058	10000	1000	100	PNU	Storage time
	100	85	35		6 weeks
	100	75	20		5 months
Timothy D808058	100	80	60		6 weeks
	100	45	20		5 months
Timothy V89185	10 ⁻¹	10 ⁻³	10 ⁻⁴	w/v	Storage time
	100	70	70		48 hours
	100	50	35		1 week
	100	50	≤ 10		6 weeks
	100	25	≤ 10		4 months
	100	25	NT		5 months
Timothy (own preparation)	10 ⁻¹	10 ⁻²	10 ⁻³	w/v	Storage time
	100	100	100		1 week
	100	100	35		6 weeks
	100	50	≤ 10		4 months
Horse dandruff V89195	10 ⁻¹	10 ⁻³	10 ⁻⁵	w/v	Storage time
	100	100	100		6 weeks
	100	100	100		5 months
C 125/012132	10 ⁻²	10 ⁻⁴	10 ⁻⁶	w/v	Storage time
	100	100	85		6 weeks
	100	100	85		4 months

NT = not tested.

the volume of the extract in the vial did not seem to be of any importance for the 10⁻³ w/v dilution. In the dilution 10⁻⁵ w/v, however, a decrease of the allergen activity was seen in the smaller volume (Fig. 6). The volume effect was further studied with our own preparation of timothy pollen extract. Vials with 10 ml, 5 ml and 1 ml of the extract were stored for 5 weeks at +4°C at the concentrations 10⁻¹–10⁻³ w/v. The RAST analyses showed that the potency of the most concentrated solutions were the same at the 10 ml and the 5 ml volumes but at the 1 ml volume a decrease was seen

(Table 3). For the more diluted extracts a decrease of the potency was seen already in 'half-filled' and 'filled' vials, but again the loss was more pronounced in the smallest volume. In vials with 1 ml allergen a more pronounced decrease in potency was seen when the vials had been vertically rotated for a few minutes once a week in comparison with vials not rotated. A very good agreement was seen between the results obtained with the two reaginic sera. No major difference in allergen activity was seen after 3 days storage between a 1 ml volume stored in a previously empty vial and the last 1 ml volume taken from a full vial from which 9 ml had been removed before the storage (Table 4).

Table 2. The protein concentration of allergen extracts as measured by a micro-Kjeldahl technique

Allergen extract		Nitrogen (mg/ml)		
		Test 1	Test 2	Mean
Birch	V 89205	0.047	0.040	0.044
	D 816058	0.068	0.061	0.065
Timothy	V 89185	0.076	0.076	0.076
	D 808058	0.040	0.040	0.040
	Own preparation	0.315	0.298	0.307
Horse dandruff	V 89195	2.996	3.031	3.014
	C 125/012132	0.259	0.266	0.263

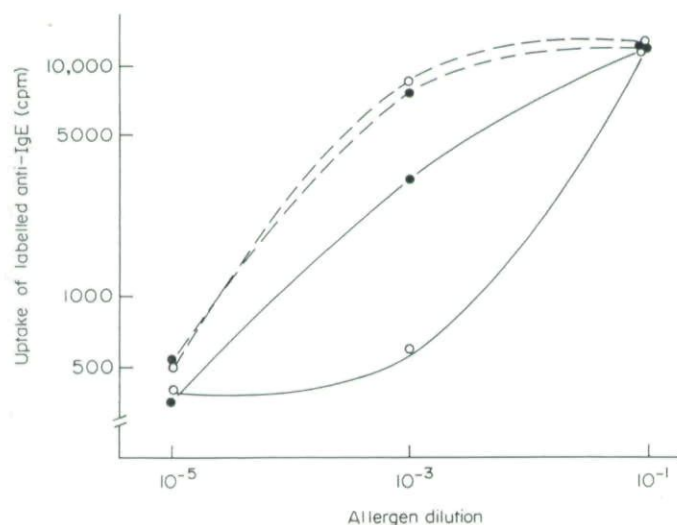


Fig. 5. The potency of a timothy pollen extract stored in filled (●) and partly filled (○) vials for 6 weeks at +4°C. Dilutions made fresh from 10⁻¹ w/v (---) and extracts stored at respective dilution (—).

Effect of additives on the 'volume effect'

To study whether it was possible to eliminate or markedly reduce the decrease of the allergen potency obtained with diluted extracts upon storage at small volumes, several additives were investigated. As seen in Fig. 7, Tween 20 was the only additive that

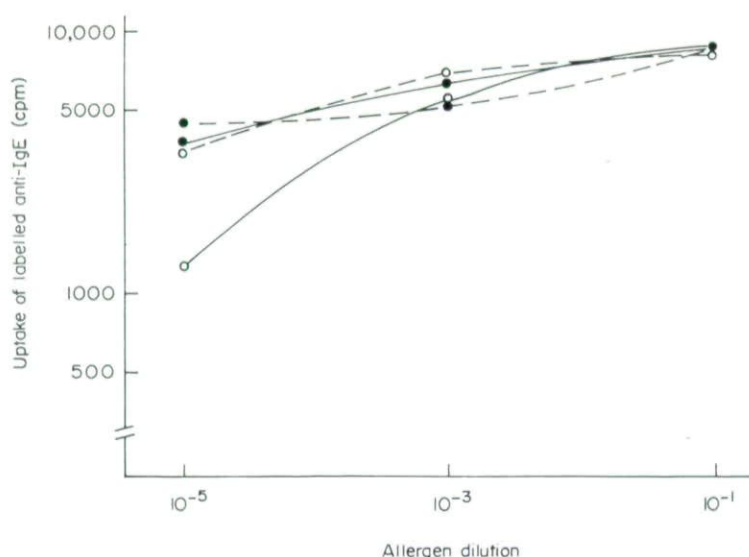


Fig. 6. The potency of a horse dandruff extract stored in filled (●) and partly filled (○) vials for 6 weeks at +4°C. Dilutions made fresh from 10^{-1} w/v (---) and extracts stored at respective dilutions (—).

Table 3. The effect of the volume in the vial on the deterioration of a timothy pollen extract (own preparation) stored for 5 weeks at +4°C. The figures are given in per cent of that of the corresponding dilutions made fresh from a full vial 10^{-1} w/v. The paired results represent those obtained with two different test-sera

Allergen dilution	10 ml	5 ml	1 ml	1 ml*
10^{-1} w/v	100–100	85–100	70–70	40–50
10^{-2} w/v	100–100	100–100	20–25	5–5
10^{-3} w/v	50–70	15–15	10–10	<5–<5

* Vertically rotated for a few minutes once every week.

Table 4. The 'volume effect' in (A) previously empty vials; (B) previously full vials from which 9 ml were removed 3 days before the analyses. 1 ml test volumes were stored in the vials for 3 days at +4°C. The same timothy pollen extract as in Table 3 was used. The figures are given as per cent of that of the corresponding dilutions made fresh from a full vial 10^{-1} w/v. The paired results represent those obtained with two different test-sera

Allergen dilution	A	B
10^{-1} w/v	100–100	100–100
10^{-2} w/v	70–65	65–65
10^{-3} w/v	<10–<10	20–10

seemed to have the expected effect and it did not matter whether Tween was present in the vial during the whole storage time (1 month) or was added a few hours before the analysis. For a timothy extract, Tween seemed to be of less benefit as a preservative of the allergen activity when it was present in the vial during the storage and full effect was seen only when Tween was added after the storage period. Tween itself did not facilitate the coupling of the allergen to the activated cellulose discs. On the contrary, a decreasing uptake of labelled anti-IgE was observed with increasing concentration of

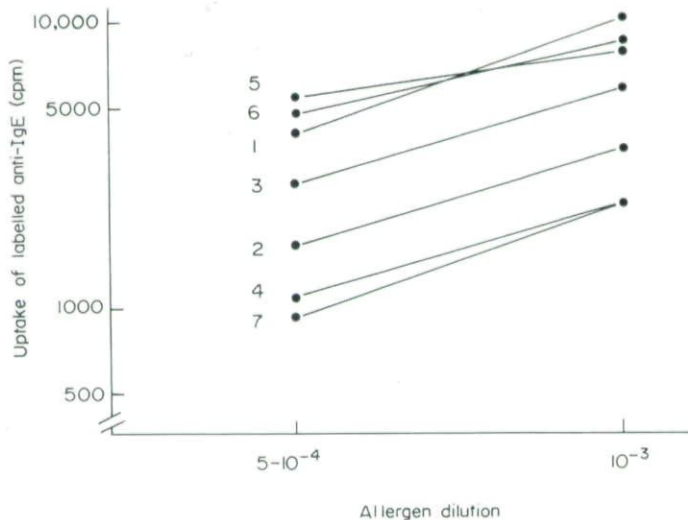


Fig. 7. The effect of additives on the 'volume effect' in dilutions of a birch pollen extract. (1) 10 ml without additive; (2) 1 ml, siliconized vial; (3) 1 ml + HSA 0.1%; (4) 1 ml + HSA 1%; (5) 1 ml + Tween 0.2%; (6) 1 ml without additive, Tween 0.2% added before analysis; (7) 1 ml without additive.

Tween added immediately before the coupling procedure. Besides the additives shown in Fig. 7, the following chemicals have been tested and found unable to substantially reduce the volume effect: dithiothreitol (Nutritional Biochemicals Corporation) 0.02%, tranexamic acid (Cyclokapron®, KABI Ltd) 2% and helium gas.

Discussion

Comparative studies of the potency of allergen extracts are usually performed with skin titrations. This technique may be difficult to use with less common allergens as patients with such allergies are rare. Furthermore, the accuracy of skin titrations is open to discussion. Some authors claim that a difference in potency of more than 50% is needed for detection (Augustin, 1959) and others have reported that a tenfold or higher difference may be overlooked (Stull, Cooke & Tennant, 1933; Arbesman & Eagle, 1939).

In vitro techniques based on histamine release from passively sensitized chopped human lung (Foucard *et al.*, 1972) or leucocytes (Baer *et al.*, 1970) by serial dilutions of allergen are easier to standardize than the skin tests but both methods are laborious. In the present study, RAST, skin titrations and titration on passively sensitized chopped human lung did all give results showing a potency decrease of pollen extracts stored for 4 months at +4°C at a concentration of 10⁻³ w/v in comparison with the same dilution freshly prepared from the stock extract. In contrast the horse dandruff

extract exhibited no decrease in potency during storage. For the birch extract analyses by RAST showed a smaller decrease of the potency than did the biological methods. This may reflect a tendency of RAST to underestimate differences in allergen activity but this hypothesis is not supported by the agreeing results obtained in a previously reported study of birch allergen extracts (Foucard *et al.*, 1972) and in the present study with the timothy and horse dandruff allergens. The most discordant results in the testing of the birch extract were obtained in RAST and the lung test. Possibly, a better agreement would have been obtained if there had been allergen enough for double or triple estimations in the lung test.

On the whole, the results indicated that the decrease of allergen activity seen with RAST corresponded to the loss of the biological activity of the extracts.

The stability of allergens is dependent on several factors such as the chemical composition of the allergens, the allergen concentration, the concentration of enzymes and non-allergenic proteins, pH of the extract and the storage temperature (Berrens, 1971). The great difference in stability seen in this study between the pollen and the horse dandruff extracts is therefore not surprising and it can partly be explained by the higher protein concentration in the latter extracts. These proteins are probably both allergenic and non-allergenic. One of the horse dandruff extracts and our own timothy pollen extract had a very similar protein concentration but, nevertheless, the diluted extracts showed great differences in stability upon storage. This may depend on a high proportion of non-allergenic protein in the timothy extract. Pollen extracts seem to contain large amounts of non-allergenic protein. King, Norman & Connell (1964) found that the predominant allergen from ragweed pollen only constituted about 6% of the protein moiety in the pollen extract and similar experiences have been reported by other workers (Baer *et al.*, 1970; Varga & Ceska, 1972). The fact that the horse dandruff extracts could be highly diluted and still give a high uptake of labelled anti-IgE indicates that the high protein concentration in these extracts reflects a high concentration of allergen. Furthermore, the allergens in the dandruff extract may be more stable than the pollen allergens which also would contribute to the differences obtained. The diluted extracts lost their activity more rapidly than the more concentrated solutions which is consistent with the findings of other workers (Frankland, 1955; Augustin & Hayward, 1962; McKaba & Sussdorf, 1967). This instability of diluted allergen is probably dependent on the lower concentration of both allergenic and non-allergenic protein.

The greater instability of diluted allergens when stored in vials filled to various levels compared with full vials is called the 'volume effect' and has been previously described (Hjorth, 1957; Derrick & Trinca, 1964; Osmundsen & Aastrup, 1967a, b). The phenomenon has been found in diluted extracts of grass pollen and to a less degree in extracts of house dust and horse dandruff. The 'volume effect' was seen in the present study with all allergens but the horse dandruff extract had to be diluted a hundred times more than the pollen extracts before any 'volume effect' could be demonstrated. Again, this higher stability of the dandruff extract may probably be explained by the high concentration of allergen and non-allergenic protein in the dandruff extract. The 'volume effect' is believed to be caused by adsorption of protein to the glass wall and addition of 0.01% Tween 80 eliminated this effect in grass extracts (Hjorth, 1958; Derrick & Trinca, 1964) but not in extracts of house dust and horse dandruff (Osmundsen & Aastrup, 1967a, b). According to Malley *et al.* (1962) the adsorption of pollen proteins to the glass wall may be prevented by using siliconized

vials. In our study Tween 20 was the only additive that reduced the 'volume effect' substantially. The fact that addition of Tween 3 hr before the allergen analysis had at least as good effect as when Tween was mixed with the extract from the beginning is consistent with the conception that allergens adsorb to the glass wall. The observation that the 'volume effect' was enhanced by rotation of the vial during storage points in the same direction. A similar enhancing effect was noted in a tuberculin solution when glass particles were added to the vial (Magnus *et al.*, 1955).

In contrast to the findings of Malley *et al.* (1962) siliconized glass reduced but did not prevent the 'volume effect' in our study. No effect of siliconizing was observed by Magnus *et al.* (1955). The 'volume effect' was not influenced by a reducing agent, an enzyme inhibitor or by keeping the samples under helium gas which speaks against oxidation or enzymic degradation as being the cause of the 'volume effect'. Addition of serum albumin did not seem to stabilize the activity, probably because the albumin competes during coupling with the allergens for the active sites on the filter paper discs. Thus a lower uptake of radioactivity is obtained the higher the concentration of albumin in an extract. Similar results were obtained when, prior to the coupling, albumin was added in increasing amounts to a freshly diluted allergen extract. RAST is therefore not suitable for evaluation of the possible protective effect of added proteins on the allergen stability.

The great variation in the stability of diluted allergen extracts in addition to the great difference in potency of commercial allergen extracts reported elsewhere (Aas 1966; Baer *et al.*, 1970; Foucard *et al.*, 1972) is of considerable annoyance to the allergologist. Standardization of commercial allergen extracts is an urgent need and should be accompanied by a statement of the stability of the diluted extracts.

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