

Stabilizing effect of epsilon-aminocaproic acid on allergenic extracts

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The effect of ϵ -aminocaproic acid (EACA) on the degradation of an aqueous Lolium perenne extract was studied by intracutaneous tests and by RAST inhibition. Extracts for skin testing stored at 4° C for 12 months and at 37° C for 6 weeks were significantly protected from degradation by addition of 0.1 mol/L of EACA before storage. Extracts were stored for RAST inhibition at 4° C for 6 months and at 37° C for 7 days. Shelf life was twofold to threefold increased when 0.1 mol/L of EACA was added to the dilution medium. EACA also protected the extracts from the effect of freezing and thawing. Comparison with the effect of human serum albumin indicated a rather short activity of human serum albumin, whereas the effect of EACA lasted longer. It is suggested that EACA can be used to increase the stability of aqueous allergen extracts for skin testing and for hyposensitization therapy. (J ALLERGY CLIN IMMUNOL 76:543-50, 1985.)

“Stability” means the duration of allergenic activity of an allergen or allergenic product under various conditions of storage such as temperature, light, solvent, etc., including the effect of incorporated preservatives and adjuvants on this activity.¹ Several authors have demonstrated the degrading effect of temperature,²⁻¹⁰ dilution,^{2, 9, 11} diluent,⁹ type of allergens^{11, 12} mixture effect,^{10, 12} volume effect,^{3, 10-12} and preservatives^{2, 9} on allergenic extracts. Other authors have proposed remedies against the instability of allergens such as the addition of glycerin,^{2, 9, 10} HSA,^{6, 9-13} Tween 80,^{2, 3, 6, 11} freeze drying,² and adsorption to insoluble supports² to the extracts. The potency of allergenic extracts has been measured in these studies by skin testing,^{3, 5, 12, 13} antigen E determination,^{3, 14} RAST inhibition,^{4, 5, 9, 10} and Coomassie blue binding.⁵

Pollen extracts are known to contain numerous enzyme activities.^{4, 15} It appears possible that the breakdown of proteins in the extracts is partly the result of enzymatic activity. Therefore, we studied the effect of EACA, which is a potent enzyme inhibitor,¹⁶⁻¹⁹ on degradation of aqueous allergenic extracts. The potency of the extracts was evaluated by RAST-inhibition tests and skin testing.

Abbreviations used

NU:	Noon unit
PBS:	Phosphate-buffered saline
EACA:	ϵ -aminocaproic acid
HSA:	Human serum albumin

MATERIAL AND METHODS

Allergens

Different extracts of *Lolium perenne* pollen were used. The extract concentration is expressed in Noon units. The material extracted from 1 gm of pollen is arbitrarily stated as 1 million NU. This quantity is roughly equivalent to 360,000 PNU.²⁰

A *Lolium perenne* extract (provided by HAL Laboratories, The Netherlands) of 50,000 NU was stored in 50% glycerol at -20° C for various lengths of time up to 2 years. For RAST inhibition this material was diluted to 2000 NU in PBS, pH 7.4, 0.15 mol/L with or without 0.1 mol/L of EACA. The *Lolium perenne* extract for skin tests was diluted to 10 NU in Coca's solution and in Coca's solution containing 0.1 mol/L of EACA.

Fresh extracts were used for the experiment comparing the stabilizing effect of HSA and EACA. An aqueous extract of 0.5 gm of *Lolium perenne* pollen (provided by HAL Laboratories) in 5 ml of PBS containing 100,000 NU/ml was prepared and immediately diluted to 10,000 NU/ml in PBS and PBS containing 0.03% crystalline HSA or EACA at a concentration of 0.1 mol/L.

All extracts contained 0.5% phenol.

RAST-inhibition tests

A pool of 50 sera containing high titers of IgE antibody against *Lolium perenne* was used as the source of antigen-

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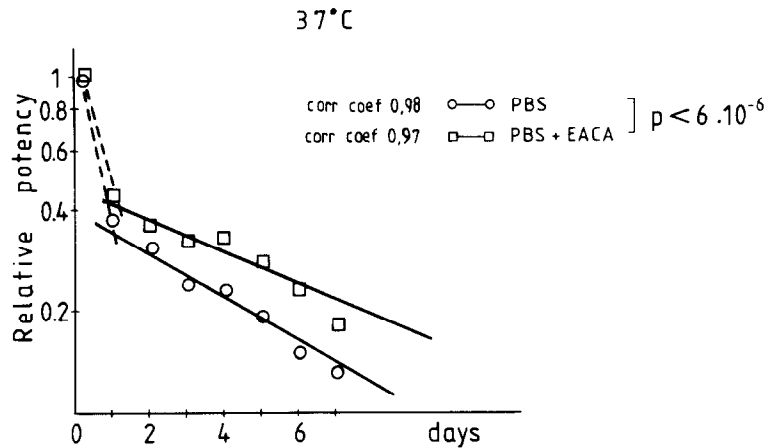


FIG. 1. *Lolium perenne* extracts (2000 NU), diluted in PBS or in PBS plus 0.1 mol/L of EACA, are incubated for 1, 2, 3, 4, 5, 6, and 7 days at 37° C. Results of RAST inhibitions for each incubation time at 37° C are expressed as relative potencies compared to a sample kept at 4° C for 8 days. Statistical evaluation by two-way variance analysis (mean of three experiments).

TABLE I. Relative potencies of non-EACA and EACA extracts after freezing and thawing compared to an extract stored at 4° C for 1 hour

Experiment No.	Non-EACA extract	EACA extract
1	0.82	1.27
2	0.77	1.08
3	0.65	1.06
4	0.42	0.59
5	0.51	0.86
6	0.51	0.78
7	0.57	0.76
8	0.39	0.81
9	0.36	0.70
10	0.38	0.82
Mean	0.54	0.87
SD	0.16	0.20

T = 10.567; $p < 2 \times 10^{-4}$

specific IgE. The 50 sera were selected from sera received by the laboratory for routine RAST determination. They all demonstrated a RAST score of + + + (five to 11 times the negative control) or + + + + (more than 11 times the negative control). *Lolium perenne*-coupled Pharmacia discs (Pharmacia Belga, Brussels, Belgium) were used for routine testing. For a negative control of about 200 cpm, the serum pool scored 40 to 50 times higher.

RAST-inhibition tests were carried out as follows: Fifty microliters of the allergenic extract was incubated with 50 µl of the serum pool. After 3 hours *Lolium perenne*-coupled discs were added to the tubes. For this purpose *Lolium perenne* extract (10 mg dry weight) was coupled to 1 gm

of CNBr-activated Whatman cellulose paper discs according to the method of Ceska et al.²¹ After 6 hours of incubation, the discs were washed three times and incubated with 50 µl of radioiodinated anti-IgE for at least 6 hours. After three washings radioactivity was measured in a gamma counter, and the percent of inhibition was calculated.

Inhibition curves were drawn with seven twofold dilutions of each sample. The inhibitory activity of the extract is expressed as relative potency compared to the control. Relative potencies were calculated according to the method of Anderson and Baer.²²

The coefficient of variation of four day-to-day measurements on two samples with this system was 0.08 and 0.10, respectively, which is in agreement with data from the literature.²³ In order to correct for this variability, all experiments were carried out threefold to tenfold, except for the experiment on storage at 4° C that was carried out only once. Statistical evaluation was done by two-way variance analysis of relative potencies determined after different storage periods. The logarithms of relative potencies were plotted against storage time, and the linear regression was calculated for the linear part of the curve. For freezing and thawing experiments, statistical analysis was done by t test on paired variates.

Skin tests

Patients were randomly selected from our outpatients' allergy clinic. None of them received antihistamines at the time of testing. Two selections were made.

For a short term experiment with EACA- and non-EACA-containing extracts stored at 37° C during 6 weeks, three patients were selected and tested at the start and after 1, 3, and 6 weeks.

In order to demonstrate differences between EACA- and non-EACA-treated extracts over a 1-year storage period at 4° C, different groups of 17, 7, 10, 8, and 6 patients were

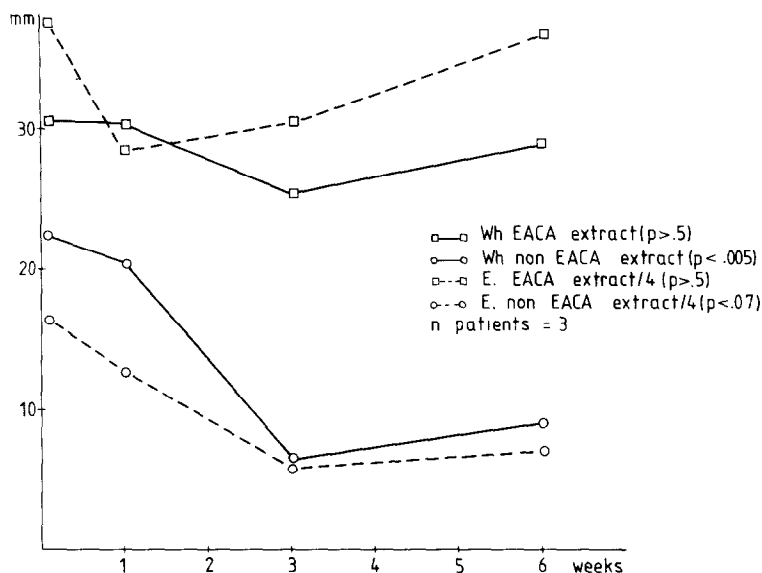


FIG. 2. *Lolium perenne* extracts (10 NU), diluted in Coca's solution or in Coca's solution plus 0.1 mol/L of EACA, are stored for 1, 3, and 6 weeks at 37° C. The skin test results (sum orthogonal diameters) of three patients tested repetitively are expressed as mean values of wheal-and-flare reactions. Statistical evaluation by two-way variance analysis.

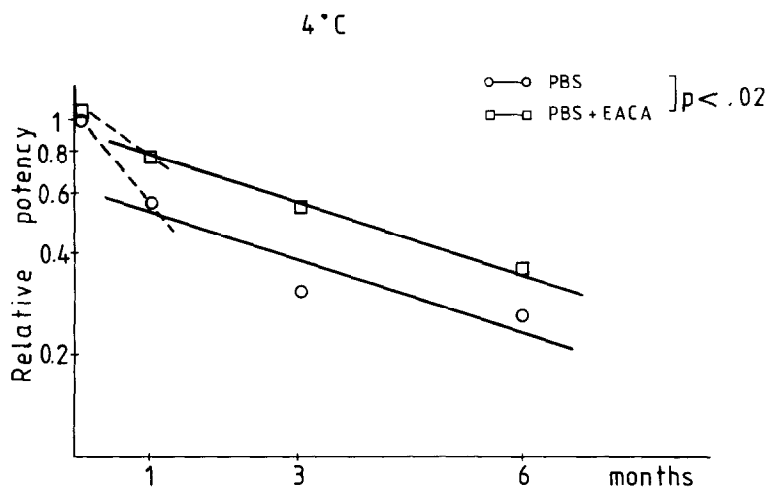


FIG. 3. *Lolium perenne* extracts (2000 NU), diluted in PBS or in PBS plus 0.1 mol/L of EACA, are incubated for 1, 3, and 6 months at 4° C. Extracts were then frozen at -70° C until they were tested. Results of RAST inhibitions for each incubation time are expressed as relative potencies compared to a sample immediately frozen at -70° C. Statistical evaluation by two-way variance analysis.

selected at the start of the experiment and after 3, 6, 9, and 12 months, respectively.

Hyposensitization during more than 1 year was used as a rejection criterion because after 1 year, our hyposensitized patients are regularly skin test negative. The groups were limited by the number of patients we were able to collect during 1 week owing to these criteria.

EACA- and non-EACA-containing extracts were compared by single intradermal injections.⁵ Intradermal injections (0.05 ml) were administered with a 26-gauge needle

at the volar-ulnar side of both forearms; 10 NU were injected at 5 cm from the wrist. The normal extracts were injected on the right, and EACA-containing extracts were injected on the left forearm. Skin tests were read after 15 minutes. The wheal-and-flare reactions were delineated on a transparent sheet, and the sum of the longest and midpoint orthogonal diameter was recorded. Injections and readings were double-blind. Statistical analysis was done by variance analysis. The overall coefficient of variation of our skin test technique calculated for 98 histamine dihydrochloride and

TABLE II. Wheal-and-flare reactions to non-EACA- and EACA-treated extracts stored at 37° C in three patients tested repetitively during a 6-week period

Patient	Start				1 wk at 37° C				3 wk at 37° C			
	Non-EACA		EACA		Non-EACA		EACA		Non-EACA		EACA	
	ΣWh	ΣE	ΣWh	ΣE	ΣWh	ΣE	ΣWh	ΣE	ΣWh	ΣE	ΣWh	ΣE
L. M.	25	111	35	103	29	79	44	92	19	71	38	92
V. V. F.	20	51	24	56	11	24	21	37	0	0	20	43
G. L.	22	35	33	66	23	48	26	42	0	0	18	48

Statistical analysis by two-way variance analysis. ΣWh = wheal; ΣE = erythema (flare). Variance analysis: non-EACA ΣWh: $p < 0.005$; ΣE: $p < 0.07$. EACA ΣWh: $p > 0.5$; ΣE: $p > 0.5$.

TABLE III. Wheal reactions to non-EACA- and EACA-treated *Lolium perenne* extracts before and after storage at 4° C for 3, 6, 9, and 12 months

Patient	Start		Patient	3 mo/4° C		Patient	6 mo/4° C		Patient	9 mo/4° C	
	Non-EACA	EACA		Non-EACA*	EACA		Non-EACA*	EACA		Non-EACA*	EACA*
H. H.	19	20	D. G.	23	22	Y. Y. K.	20	32	T. P.	0	22
W. J.	19	23	M. R.	17	21	D. S.	20	28	S. P.	18	24
M. J.	23	31	A. V.	24	40	N. D.	0	25	V. E.	19	25
V. S. R.	23	24	V. J.	0	17	C. D.	0	23	R. S.	24	29
E. D.	31	35	S. J.	20	27	D. C. L.	0	23	R. D.	20	29
V. L.	21	24	D. M.	20	23	R. J.	0	16	G. H.	17	25
V. R. E.	28	32	O. K.	0	19	B. C.	29	37	D. D.	0	19
V. R. B.	37	37				S. W.	27	37	S. C.	20	27
M. E.	35	36				S. J.	29	38			
J. D.	13	17				P. P.	0	12			
B. P.	44	50									
V. P.	24	30									
S. M.	31	41									
B. P.	36	32									
T. G.	30	33									
C. M.	22	23									
G. G.	26	22									

Statistical analysis by t test on unpaired variates and one-way variance analysis.

Variance analysis: non-EACA: $p < 0.00008$; EACA: not significant.

*Significant difference from start ($p < 0.05$).

100 48/80 injections, in the same patients ($n = 7$) on different days, was 0.14 and 0.15, respectively.

RESULTS

Freezing and thawing

After dilution from 50,000 NU to 2000 NU in PBS or in PBS plus 0.1 mol/L of EACA, the *Lolium perenne* extracts were immediately frozen at -70°C . After 1 hour they were thawed and compared by RAST inhibition to the unfrozen extract kept at 4°C . The whole experiment was carried out 10 times. Results are illustrated in Table I. The *Lolium perenne*

extract diluted in PBS plus EACA is only slightly degraded (-13%). On the contrary, the extract diluted in PBS loses 46% of its initial activity ($p < 2 \times 10^{-4}$). EACA thus significantly protects proteins from degradation by freezing and thawing.

Storage at 37° C

After dilution to 2000 NU in PBS or in PBS plus 0.1 mol/L of EACA, the extracts were partitioned in eight equal parts and stored at 4°C . Every day for 1 week, one part of each extract was transferred to an incubator at 37°C . RAST-inhibition tests were carried

6 wk at 37° C			
Non-EACA		EACA	
ΣWh	ΣE	ΣWh	ΣE
17	33	33	67
10	31	24	72
10	21	30	81

12 mo/4° C		
Patient	Non-EACA*	EACA*
Q. A.	0	20
V. A.	0	19
D. S.	0	22
S. S.	0	15
S. C.	14	23
C. J.	0	24

out on all samples on day 8. Results for each incubation time at 37° C, expressed as relative potencies compared to the sample kept at 4° C for 8 days, are illustrated in Fig. 1 (mean of three experiments). Relative potencies of the EACA-treated extracts are consistently higher with a twofold to threefold increase in shelf life. The difference between the two regression lines is highly significant ($p < 6.10^{-6}$).

After dilution to 10 NU in Coca's solution and Coca's solution plus 0.1 mol/L of EACA, each sample was partitioned in four equal parts. One part was immediately used in skin tests. The other parts were incubated for 1, 3, and 6 weeks at 37° C. Skin tests were carried out in the same three patients after each time interval. Mean values of wheal-and-flare reac-

tions for EACA- and non-EACA-treated extracts are presented in Fig. 2.

Statistical evaluation by two-way variance analysis demonstrates no significant decrease of wheal or flare reactions with EACA-treated extracts, whereas the decrease in reactivity is highly significant for the wheal reaction with non-EACA-treated extracts and approaches significance for the flare reaction. The actual sizes of wheal-and-flare reactions are illustrated in Table II.

Storage at 4° C

After dilution to 2000 NU in PBS or in PBS plus 0.1 mol/L of EACA, the extracts were partitioned in four equal parts. One part was immediately frozen at -70° C and served as the reference extract. The other parts were incubated for 1, 3, and 6 months at 4° C. Afterward they were kept at -70° C until potency determination. The results of RAST-inhibition tests are illustrated in Fig. 3. When the extracts are compared to extract that was immediately frozen, the extracts in PBS with EACA score uniformly higher relative potencies than the extracts in PBS. The difference between the two regression lines is significant ($p < 0.02$). The increase in shelf life is equivalent to the shelf life obtained in the experiment with storage at 37° C.

After dilution to 10 NU in Coca's solution and Coca's solution plus 0.1 mol/L of EACA, each sample was partitioned in five equal parts. One part was immediately used in skin tests. The other parts were incubated for 3, 6, 9, and 12 months at 4° C. Skin tests were carried out at the start and after each storage period. Results of skin tests, expressed as the difference between the wheal-and-flare reaction to EACA extracts and non-EACA extracts, are illustrated in Fig. 4. The difference in skin reactivity between EACA and non-EACA extracts increases significantly with time ($p < 2 \times 10^{-5}$, by one-way variance analysis of wheal reactions, and $p < 3 \times 10^{-4}$ for flare reactions). The actual sizes of wheal reactions and statistical differences from the start values (t test on unpaired variates) are illustrated in Table III.

Comparison of the influence of EACA and HSA on storage at 37° C

After dilution to 10,000 NU in PBS, PBS plus 0.1 mol/L of EACA, or PBS plus 0.03% of HSA, the extracts were partitioned in four equal parts and stored at 4° C. One part of each extract was transferred to an incubator at 37° C on day 1, 4, and 7. RAST-inhibition tests were carried out on all samples on day 8. Results for each incubation time at 37° C are expressed as relative potencies compared to the sample

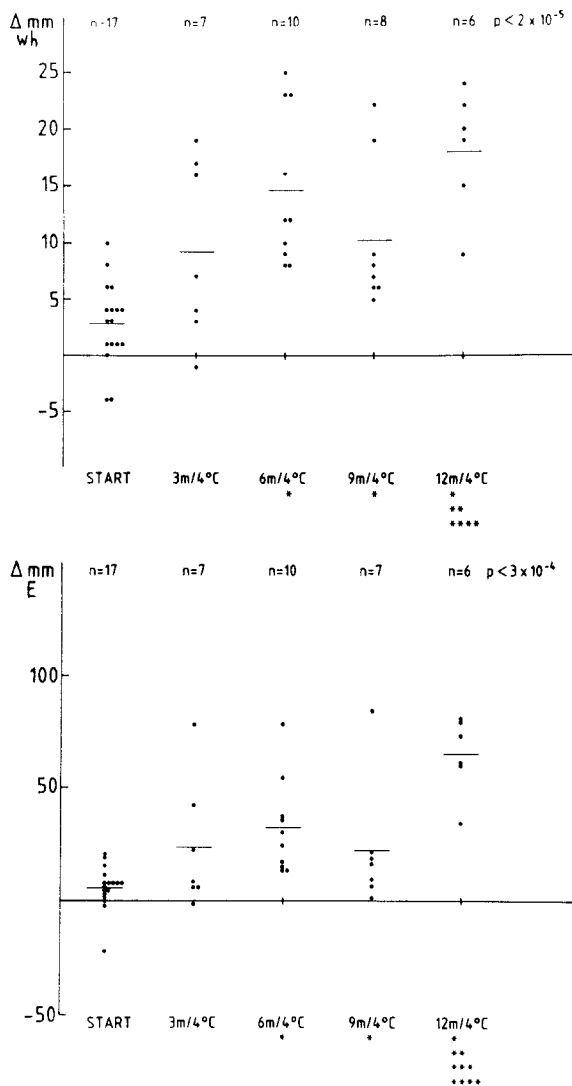


FIG. 4. *Lolium perenne* extracts (10 NU), diluted in Coca's solution or in Coca's solution plus 0.1 mol/L of EACA, are stored 3, 6, 9, and 12 months at 4°C. Results of skin tests in five groups of different patients are expressed as the difference in wheal-and-flare reactions (sum orthogonal diameters) for both extracts. Statistical evaluation by one-way variance analysis for the overall results. The significance of differences between results at each time interval were calculated by t test on unpaired variates. Significant differences from start values are indicated by *, from 3 months by **, from 6 months by ***, and from 9 months by **** ($p < 0.05$).

diluted in PBS and kept at 37°C for the same periods of time.

Fig. 5 illustrates the relative potency for EACA- and HSA-treated extracts compared to the extract in PBS (mean of three experiments). The effect of HSA is more pronounced for extracts stored at 4°C for 7 days and for extracts stored at 4°C for 6 days and at 37°C for 1 day. When the length of the storage period

at 37°C increases, the effect of EACA is superior to the effect of HSA.

DISCUSSION

The present study deals with the influence of temperature on extract stability and the inhibitory activity of EACA on temperature-dependent degradation. This activity is compared to that of HSA. Skin testing and RAST-inhibition tests are used as a tool for potency testing.

Our RAST-inhibition experiments indicate that an aqueous *Lolium perenne* extract of 2000 NU stored at 37°C loses more than 50% of its activity after 1 day and 80% after 7 days of storage. Storage at 4°C results in a potency decrease of about 80% within 6 months of conservation. These results are in agreement with the data of Foucard et al.¹¹ who found a degradation of 50% after 5 months of storage at 4°C in a timothy grass-pollen extract by use of direct RAST. EACA partly protects the allergenic extracts from this degradation during storage. In terms of half-life, this means that a *Lolium perenne* extract of 2000 NU loses 50% of its initial activity after 40 days of storage at 4°C. When the extract is stored in PBS plus 0.1 mol/L of EACA, the half-life is extended about three times to 112 days. For the experiment at 37°C, the half-life could not be determined because both extracts had lost more than 50% of their initial activity after 1 day. The same applies to HSA-stabilized extracts (data not shown).

As indicated by Norman and Marsh,¹² the preparation of extracts used for skin tests without the addition of stabilizers introduces a major source of error in the interpretation of skin tests caused by rapid degradation. Our experiments demonstrate that after 3, 6, 9, and 12 months of storage of the extract at 4°C, skin test diameters are smaller for non-EACA extracts than for EACA extracts (Fig. 4; Table III). The difference increases with the storage period. The same is true for extracts stored at 37°C and assayed repetitively in the same patients (Fig. 2; Table II). Reaction diameters are somewhat higher for EACA-containing extracts at the start of these experiments. EACA in a 0.1 mol/L concentration causes no aspecific skin reactions in double-blind experiments on 58 patients. Therefore, the small but significant difference in skin reactions at the start appears to be due to a stabilizing effect of EACA during the dilution procedure and the 6 hours of storage at room temperature before all the patients were tested. Storage at 4°C during skin test periods was deliberately omitted in order to save comparability with RAST-inhibition tests because, during the RAST-inhibition procedure, dilution of the extract and reaction of IgE with

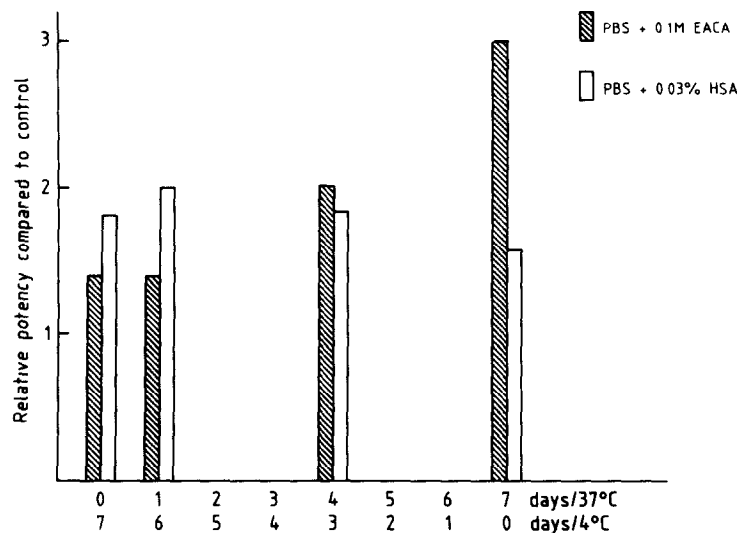


FIG. 5. Comparison of EACA- and of HSA-containing extracts to control extracts in PBS stored for the same period of time at 37° C. Results are expressed as relative potency compared to the control extract in PBS. Before incubation at 37° C, extracts were kept at 4° C. All RAST-inhibition tests were carried out on day 8 (mean of three experiments).

antigen is also separated by a period of 6 hours. Accordingly, results of RAST inhibition demonstrate the same slightly higher activity of freshly diluted EACA extracts compared to freshly diluted extracts in PBS (Fig. 5).

We have demonstrated that freezing and thawing also have a degrading effect on the activity of a diluted *Lolium perenne* extract and that EACA inhibits this degradation. The mechanism of this protective effect is unclear. EACA is known to be an enzyme inhibitor.¹⁶⁻¹⁹ However, inhibition of enzyme activity is not likely to be important for protection at low temperatures. Although results in Table I illustrate that the reproducibility of freezing and thawing experiments is rather low, statistical analysis demonstrates unequivocally the protective effect of EACA.

The time-related degradation has a biphasic course for the extract without EACA and for the extract in EACA medium as well (Figs. 1 and 3). This indicates that at least two different reactions participate in the decay of allergenic extracts. This was demonstrated already by Hiatt et al.⁸ for extracts that, such as our extracts, are not highly purified. The linear part of the degradation curve starts after 1 day of storage at 37° C (Fig. 1) and after 1 month when it is stored at 4° C (Fig. 2). For the results presented in Fig. 3, it should be emphasized that samples were frozen at -70° C for various lengths of time. They were thawed immediately before RAST-inhibition testing. The curves drawn in Fig. 3 therefore illustrate the effect of EACA on thermic degradation plus the effect on freezing and

thawing. There is, however, no appreciable difference with the results of the experiment presented in Fig. 1. (In this experiment samples were not frozen.) This similarity suggests that thermic degradation and degradation by freezing and thawing are both dependent on the same structural alteration of the antigens; otherwise, the effect of EACA would have been more pronounced when both degrading procedures were combined (Fig. 3).

A different influence of EACA on the size of the skin reaction has not been found when we compare wheal reactions of EACA- and non-EACA-containing extracts intradermally tested in the same patient (Table III), provided the patient reacts to both extracts. The same applies to flare reactions (data not shown). The table demonstrates that a subgroup of patients does not react to stored non-EACA extracts while still reacting to stored EACA extracts. Apparently, such patients react only to very labile antigens, which disappear completely from the extract when the extract is not stabilized. These findings are in agreement with those of Baer et al.⁵ and Franklin et al.³ who demonstrated that allergenic extracts are mixtures of allergens that are composed of stable and unstable components. Under influence of temperature, the extract loses its heat-labile components only.

If we compare our results with the preserving effect of 50% glycerin on diluted extracts,⁹ the protective effect of glycerin is more pronounced. However, these glycerinated extracts produce local side reactions when the extracts are injected subcutaneously. There

is some evidence for a different mechanism of action when the stabilizing properties of EACA and HSA are compared (Fig. 5). Storage for 6 and 7 days at 4° C and 1 day at 37° C results in a more pronounced effect of HSA compared to the EACA effect. However, when the extracts at 37° C are stored for a longer period, the effect of EACA becomes superior to that of HSA. Evidence has been presented that diluted extracts lose potency by adsorption of allergens to the container wall.^{9, 11} HSA reduces this adsorption of allergenic extracts to surfaces.¹² Our experiments indicate a rather short activity of HSA, whereas the effect of EACA lasts longer. This might be explained by a better protection by EACA for thermal denaturation, whereas HSA is more effective by preventing adsorption. In addition, aggregated or commercial HSA, as demonstrated by Ring²⁴ may sensitize humans. Such sensitized patients react clinically to the administration of HSA in infusions. Therefore, addition of HSA to extracts used for long-term administration is of potential danger. Indeed, HSA is present, partially in aggregated form, in plasma expanders such as human plasma and plasma protein fractions. It is conceivable that patients, sensitized by aggregated HSA in allergenic extracts, will react to HSA-containing plasma expanders. The relation between previous desensitization and systemic reactions to plasma expanders may become apparent only after a considerable period of time. EACA, on the other hand, has never been demonstrated to be allergenic, and its chemical structure certainly does not suggest allergenicity. In contrast to HSA, it is not present in frequently used life-saving drugs or preparations. EACA has no toxic effects when it is injected intravenously, even in dosages of 5 gm.²⁵ Therefore, it may represent a better alternative for stabilization of allergenic extracts.

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