

The international collaborative study establishing the first international standard for timothy (*Phleum pratense*) grass pollen allergenic extract

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A selected candidate international reference preparation of timothy grass (Phleum pratense)-pollen extract was studied together with two other freeze-dried timothy pollen allergenic extracts in a multinational study. The collaborators used RAST inhibition, histamine release, quantitative immunoelectrophoresis (crossed immunoelectrophoresis/crossed radioimmunoelectrophoresis and rockets), isoelectric focusing, and other methods. The total allergenic potencies measured in RAST inhibition were evaluated for validity of linearity and parallel-line response. The relative concentrations of some important individual allergenic components were measured. The relative potencies for the total allergenic activity and the timothy components studied in each preparation were expressed relative to the selected candidate. This preparation was established in 1983 by the World Health Organization expert committee on biologic standardization as the international standard for timothy grass-pollen extracts with assigned units of 100,000 IU per ampule. (J ALLERGY CLIN IMMUNOL 75:258-67, 1985)

Standardization of allergenic extracts has increasingly been required from clinical allergologists in order to increase the precision of diagnosis and the safety of specific immunotherapy. The allergenic extracts consist of complex mixtures of proteins with variable allergenic activities among which only very few have been purified. It is therefore difficult to express the

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Abbreviations used

CIE:	Crossed immunoelectrophoresis
CRIE:	Crossed radioimmunoelectrophoresis
GCV:	Geometric coefficient of variation
HPLC:	High performance liquid chromatography
IEF:	Isoelectric focusing
IS:	International standard
IUIS:	International Union of Immunological Societies
NIBSC:	National Institute for Biological Standards and Control, London, England
QIE:	Quantitative immunoelectrophoresis
RIE:	Rocket immunoelectrophoresis
SDS-PAGE:	Sodium dodecylsulphate polyacrylamide gel electrophoresis
WHO:	World Health Organization

concentration of a specific component or the allergenic activity of the total mixture in absolute units. It is more appropriate to express the composition of an allergenic extract relative to a standard.

The IUIS organized an Allergen Standardization Subcommittee with representation of allergologists

from various professional societies, allergen manufacturers, national health authorities, and allergen scientists. At a meeting in Geneva in 1981,* the subcommittee decided to prepare candidate IS for some of the most common allergen sources, the first three being timothy grass (*Phleum pratense*), short ragweed (*Ambrosia elatior*), and house dust mite (*Dermatophagoides pteronyssinus*).

A small working group on timothy grass-pollen extracts consisting of L. Jäger, D. G. Marsh, and H. Løwenstein specified the requirements for IS candidates and verified the fulfillment of these among the three candidates supplied. On the basis of this preliminary study, one preparation was chosen as the proposed IS.

The IUIS Allergen Standardization Subcommittee subsequently organized a collaborative study.† The aim of the study was to determine the suitability of the material to serve as the IS for timothy grass-pollen extracts, both for the measurement of total allergenic potency and for the measurement of important individual allergenic components. The study was designed taking into account the WHO guidelines.¹

Experimental data are presented here concerning: (1) the relative potencies as measured in valid parallel-line assays, (2) the relative allergenic activity of the important and analytically suitable components, (3) the thermal stability of the total allergenic potency and of the activity of some allergens of the proposed

IS, (4) the recommendation of the preparation to the WHO expert committee on biologic standardization as the IS for calibrating the potency and concentration of the components from timothy pollen allergenic extracts, and (5) the assignment of 100,000 IU to the preparation when it was used to measure the total allergenic potency or the allergenic components in other timothy preparations that are suitable for analysis.

MATERIAL AND METHODS

Extracts. Three extracts were prepared for the study. One of these was included twice. The four samples were coded A, B, C, and D.

IS extract A. Timothy pollen from two different lots, not contaminated with foreign pollen or plant debris, was extracted with 0.0125M ammonium bicarbonate at 1:10 w/v. The extraction was performed at a temperature of 2° to 8° C with constant agitation for 24 hr. The extract was filtered through a Whatman no. 4 filter. The pollen cake was reextracted at 1:5 w/v in the same manner for 1 hr. The extract was again filtered through a Whatman no. 4 filter. The supernatants from the two extractions were combined and sterile filtered, ending with an 0.2 µm membrane filter.

The finished extract (4.5 L) was sent at 0° C by air to NIBSC where it was sterile filtered again, and glass ampules were filled, 1.0 ml in each, freeze-dried, sealed under nitrogen,² and coded 82/520 (the mean content of 68 ampules was 1.0021 gm, range ± 0.49%). The lyophilized material had a water content <1% as measured at NIBSC, and it was extremely hygroscopic. Some ampules were frozen and kept at -20° C without freeze-drying.

Extracts B and D. Timothy pollen was extracted (1:10 w/v) with distilled water at pH 7.0 for 18 hr at 2° to 8° C with constant stirring. The extract was filtered through a nylon cloth. The pollen cake was washed with distilled water (1:2). Mannitol (5% w/v) was added to the combined filtrates. The extract was sterile filtered. The finished extract was shipped in bulk as an aqueous extract to NIBSC where it was lyophilized in bulk. Some of the material was redissolved in distilled water, sterile filtered, and ampules were filled as above.

Extract C. This was standard E5-Ti of the Bureau of Biologics prepared in June 1980, kindly supplied by Dr. Harold Baer. Defatted timothy pollen was extracted 1:10 w/v with twice-distilled water at 4° C for 20 hr with gentle stirring. The mixture was centrifuged at 10,000 × g at 4° C for 30 min. The supernatant was filtered through a 0.22 µm membrane filter and lyophilized in ampules, 0.5 ml per ampule. The ampules were filled with nitrogen and glass sealed.

RAST inhibition. This was performed by modifications of the methods of Ceska et al.³ and Gleich et al.⁴ Eight laboratories used paper discs as the solid phase, whereas four laboratories used acrylic beads (lab 1), polystyrene beads (lab 9), cellulose (lab 12), or Sepharose (lab 13) as the solid phase. Four laboratories used the Phadebas RAST kit (Pharmacia, Uppsala, Sweden) (labs 2, 6, 8, and 14).

*Geneva meeting documents are available in Europe from H. Løwenstein, The Protein Laboratory, Sigurdsgade 34, DK-2200, Copenhagen, Denmark; documents are available outside Europe from R. Goldstein, NIAID, National Institutes of Health, Westwood 755, Bethesda, MD 20205.

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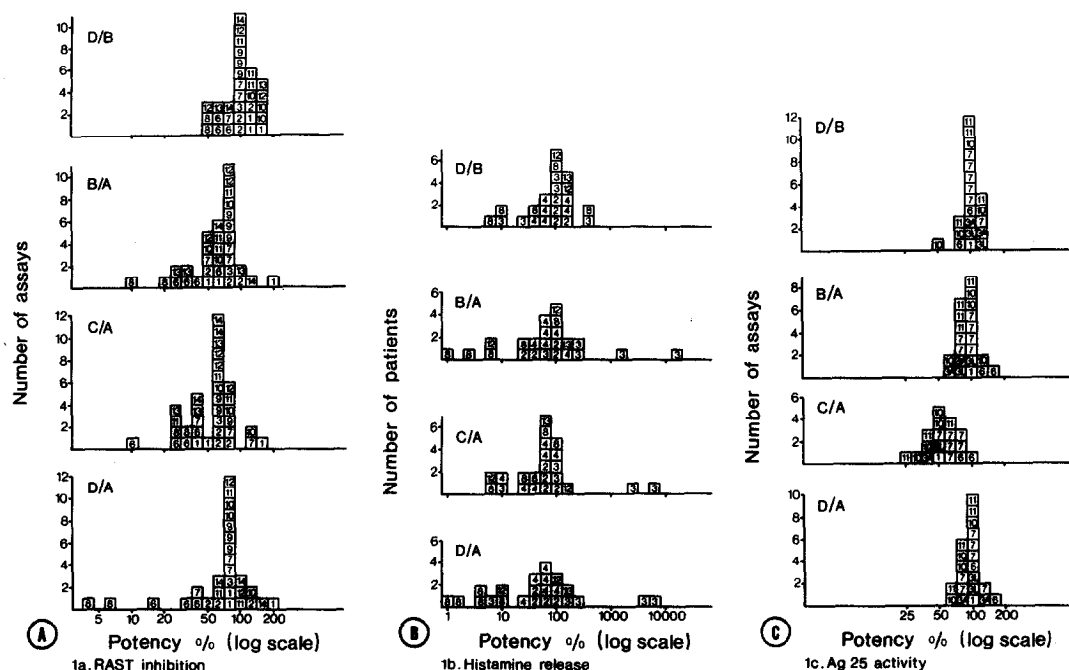


FIG. 1. Frequency distributions of potency estimates. Each box denotes the estimate from one test. The numbers in the boxes refer to the code numbers of the laboratories. **A**, RAST inhibition. **B**, In vitro histamine release. **C**, Ag 25 activity by QIE. D/B: extract D relative to B; B/A, C/A, D/A: extracts B, C, and D relative to A.

Labs 7, 9, and 10 used extract A coupled to the solid phase, whereas the rest of the laboratories used their own timothy extracts on the solid phase. All the laboratories used different pools of patient sera. Eight laboratories used Pharmacia radiolabeled anti-IgE (Pharmacia), whereas the other laboratories (labs 1, 9, 12, and 13) obtained the labeled antibody from other sources.

Histamine release in vitro. Washed leukocytes (or whole blood in the case of lab 4) from timothy-allergic patients were used as reagents for the study of allergen-induced release of histamine from basophil granulocytes as described by Lichtenstein and Osler,⁵ May et al.,⁶ and Siraganian and Hook.⁷ Labs 2 and 4 used an automated method, whereas labs 3, 8, 12, and 13 used a manual method. Labs 4 and 13 diluted in steps 1:3, whereas the other laboratories used 1:10 steps. All laboratories used single determinations at each concentration.

IEF. Nine laboratories used the LKB PAG plates (LKB, Bromma, Sweden), pH range 3.5 to 9.5, following the manufacturer's instructions with small modifications. Two laboratories (labs 2 and 12) produced their own polyacrylamide gels. Eight of the laboratories demonstrated a pH gradient indication measured either by means of surface electrodes, by eluting the ampholytes with water and measuring the resultant pH, or by the position of marker proteins. Two laboratories (labs 5 and 10) measured the band densities by scanning the plates after staining with Coomassie brilliant blue.

QIE. CIE, CRIE, and RIE were performed essentially as described by Clarke and Freeman⁸ and Løwenstein.⁹ Labs 3, 6, 7, 10, 11, and 12 used ALK (ALK, Copenhagen, Denmark) anti-timothy rabbit antibody for CIE, whereas

labs 1 and 2 used their own antibodies. Lab 7 used two mono- or oligospecific antibodies in RIE. Lab 6 performed crossed enzyme immunoelectrophoresis by use of the same antibody as in CIE and enzyme-labeled anti-IgE from Pharmacia.

Other methods. The following methods were used by only one or two laboratories: protein determination by means of Lowry et al.¹⁰ in lab 7 or by the Kjeldahl method¹¹ in lab 1, SDS-PAGE¹² in lab 6, HPLC¹³ and ELISA-inhibition¹⁴ in lab 11, and complement inactivation¹⁵ in lab 10.

Changes caused by lyophilization. The content of some ampules of the proposed IS was stored at -20°C without lyophilization. This material was compared with the freeze-dried material in RAST inhibition and CIE.

Stability. The shelf life of the proposed IS was studied by comparing ampules stored at different temperatures for different periods of time with ampules kept at -80°C . The storage temperatures were -20°C (recommended storage), 5°C , room temperature (approximately 22°C), 33°C to 35°C , and 55°C . The ampules were compared after 2 and 6 mo of storage by RAST inhibition, QIE and IEF.

Design of study. Each laboratory received a number of coded ampules in August 1982. The approximate amount of protein per ampule was stated. Each collaborator was free to choose any version of methods but for some methods was asked to perform a minimum number of assays, i.e., RAST inhibition, three independent assays; histamine release, at least four patients; CIE and IEF, two independent assays. There was no limitation on type of reagents. The participants were instructed to submit raw data and their own interpretation of the data expressed relative to extract A per ampule.

TABLE I. Geometric means of potency estimates from replicate assays of RAST inhibition for each laboratory

Lab	No. of assays	Potency (%) D/B	Potency relative to extract A (%)		
			B	C	D
1	3	140 (12)	91 (100)	70 (94)	100 (61)
2	3	110 (26)	70 (40)	71 (15)	75 (61)
3	1	110 (—)	82 (—)	65 (—)	79 (—)
6	4	64* (21)	38 (49)	26 (93)	28* (52)
7	3	93 (10)	72 (30)	71 (64)	65 (37)
8	2	47 (0)	14 (66)	29 (13)	5.3 (45)
9	3	100 (10)	81 (5)	70 (11)	81 (7)
10	3	150 (13)	62 (25)	91 (31)	94 (25)
11	3	110 (10)	69 (19)	54 (80)	78 (29)
12	3	94 (76)	74 (33)	69 (2)	87* (14)
13	3	97* (89)	41 (100)	38 (62)	—* (—)
14	3	93* (26)	82* (68)	57 (24)	95 (53)
Geometric means of 11 labs (ex. lab 8)		102 (25)	67 (32)	59 (43)	75 (45)

The figures in parentheses are the GCV of the potency estimates.

*Incomplete data, nonparallel line in assay (t test, $p < 0.05$); therefore no potency estimate.

Participants. Fourteen laboratories from 10 countries participated in the collaborative study, and they were identified only by a code number that bore no relation to the order in which they were listed (see footnote page 259).

Statistical analysis. All calculations were performed at the Protein Laboratory and at NIBSC except where a method was used by one laboratory only, in which case the calculations made at that laboratory were used.

For each assay potencies of the extracts were expressed relative to extract A, the proposed IS. Since extracts B and D were coded samples of the same extract, the potency of D was also expressed relative to extract B. This was done in order to determine the extent to which variations occurred in a comparison of like with like.

Assay data for RAST inhibition were analyzed as parallel-line assays^{16, 17} relating percentage inhibition to log dose. All raw data were plotted in order to select the range in which the log dose-response relationships were linear; only data in this range were included in the analysis. The analysis included statistical tests for deviations of the relationships from linearity and from parallelism.

The relative potency of extract B from the in vitro histamine release data was defined as

$$\frac{C_A}{C_B} \times 100$$

where C_A and C_B were the concentrations of the extracts producing 30% histamine release from the respective graphs of histamine release versus log concentration of extracts A and B. The relative potencies of extracts C and D were obtained similarly.

From the QIE plates the content of individual allergens was estimated from the area under the corresponding pre-

cipitation arcs. This was done by (1) drawing the shape of the enlarged precipitation arcs, cutting out the area under the arc, and weighing the paper or (2) planimetry by use of an area integrator.⁹ The results were expressed relative to extract A.

The significance of the differences between potency estimates obtained by the laboratories was assessed by one-way analysis of variance of the log potency estimates. Potency estimates were combined by taking their geometric mean. The reproducibility of the potency estimates was calculated as the GCV.¹⁸

RESULTS AND DISCUSSIONS

RAST inhibition

Twelve laboratories performed 34 independent RAST-inhibition assays. All laboratories produced statistically valid results except for the curves of extract D obtained in one laboratory where the slopes deviated from those of extract A (t test, $p < 0.05$); no potency could therefore be estimated.

The frequency distributions of the potency estimates from independent assays are demonstrated in Fig. 1, A. For extract D relative to its duplicate, extract B, most of the potency estimates were within a factor of 2 of the expected value of 100%. The ranges for the potencies of B, C, and D relative to A were somewhat wider. For lab 8 the potency estimates of the duplicate extracts relative to extract A were considerably lower than those of the other laboratories.

The geometric means of the potency estimates for each laboratory are demonstrated in Table I. This table

TABLE II. Geometric means of potency estimates from histamine release tests for each laboratory

Lab	No. of assays	Potency (%) D/B	Potency relative to extract A (%)		
			B	C	D
2	4	99 (19)	52 (81)	64 (12)	51 (65)
3	5	62 (340)	550 (780)	250 (1200)	340 (1500)
4	6	74 (68)	67 (57)	35 (100)	49 (75)
8	5	37 (460)	6.9 (520)	26 (190)	2.5 (130)
12	2	110 (64)	21 (750)	29 (900)	23 (410)
13	1	120 (—)	120 (—)	58 (—)	150 (—)
Geometric means of 6 labs		78 (57)	57 (350)	54 (130)	44 (450)

The figures in parentheses are the GCV of the potency estimates.

also illustrates the reproducibility of the assays within the laboratories as measured by the GCV; these values are illustrated in parentheses after the potency estimates. Analysis of variance demonstrated no significant differences between the laboratories in their estimation of potency of extract D relative to its coded duplicate or of extract C relative to extract A. However, significant differences were found between the estimates of the laboratories for both extract B and extract D relative to extract A, most of the variation being attributable to the low estimates of lab 8. When these values were excluded, the differences between the potency estimates of the laboratories were no longer statistically significant. The laboratory-to-laboratory variations are demonstrated in Table I as the GCV in parentheses after the geometric means of all the laboratories excluding lab 8.

Since four laboratories (labs 2, 6, 8, and 14) used the Phadebas RAST kit, it might be expected that closer agreement would be found between their potency estimates than among those of the other laboratories where different experimental conditions and assay reagents were used. However, this was not so. In fact, the ranges in values for the estimates of these laboratories were generally wider than the ranges of estimates among the other laboratories (Fig. 1).

Eight laboratories (labs 2, 3, 6, 7, 8, 10, 11, and 14) used paper discs in their assays. Comparison of the potency estimates obtained by these laboratories with those of the other laboratories demonstrated no evidence of differences between the means of the two groups, and they also had similar ranges of assay-to-assay variation (Table I).

Three laboratories included four local standards in their assays; they all revealed log dose-response relationships with lines parallel to the proposed standard.

Histamine release in vitro

Six laboratories performed in vitro histamine release by use of leukocytes from 23 patients. Two laboratories (labs 2 and 4) used an automatic analyzer, whereas the others used manual methods. The extracts were tested only at a few concentrations and not in duplicate. In most cases the dose-response relationship had a peak in the percent histamine release with decreasing values at higher concentrations. This is a common finding for the histamine-release assays. About 25% of the assays did not have enough data points to fulfill the strict criteria for linearity and/or parallelism when the assays were evaluated by dose-response parallel-line statistics. The frequency distributions of the graphically estimated potencies from all the assays are demonstrated in Fig. 1, B. There were considerable differences between the potency estimates, even for the comparison of the duplicate samples, where the values ranged from 5% to 440% (the expected value was 100%).

The geometric means and GCVs of the potency estimates of the individual laboratories are demonstrated in Table II. The large variation between tests (each one involving the cells from a single patient) is illustrated by the high values of the GCVs, particularly for labs 3, 8, and 12; labs 2 and 4 (which used automatic analyzers) obtained better reproducibility between tests with GCVs of 100% or less. These values are illustrated in parentheses after the potency estimates. Analysis of variance revealed that there were no significant differences between potency estimates of the laboratories for extract D relative to B (its coded duplicate) or for extract C relative to A. However, there were significant interlaboratory differences ($p < 0.01$) for both B and D relative to A: the estimates from lab 8 were consistently lower, and estimates for

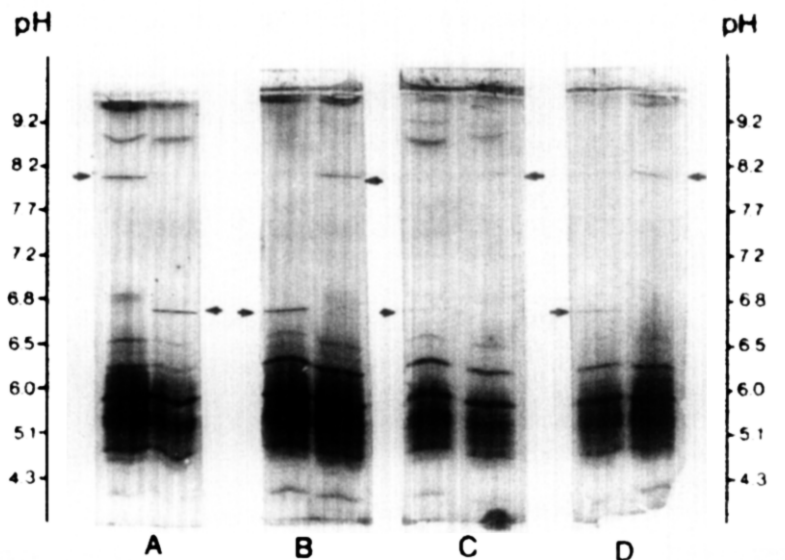


FIG. 2. A characteristic IEF with pH gradient (lab 10). Approximately 200 μ g protein applied. IEF on LKB PAG plate run according to manufacturer's instructions with small modifications; pH measurements were obtained from gel pieces (0.5×1 cm) extracted with 5 ml of distilled water for 3 hr. Arrows demonstrate points of application.

lab 3 were consistently higher. The laboratory-to-laboratory variation is demonstrated in Table II as the GCV in parentheses after the geometric means of the six laboratories.

IEF

Eleven laboratories performed a total of 19 IEF experiments. Nine of the laboratories used LKB PAG plates following the manufacturer's instructions with small modifications. Eight of the laboratories indicated a pH gradient. The IEFs demonstrated very similar patterns both among the extracts A, B, C, and D and between the laboratories. Nearly all had (1) heavy characteristic bands at pHs of 5.8, 6.2, and 6.5, (2) most proteins in the extracts had pIs in the range 4.5 to 6.5, and (3) extracts A and C had more basic bands than the others, although all extracts had some basic bands.

No laboratory attempted to identify a particular band with a specific antigen. Three laboratories measured the relative band density by scanning. Fig. 2 demonstrates a characteristic IEF.

Eleven of the fourteen laboratories performed at least one IEF. Comparison of extracts by this method allows evaluation of particularly characteristic bands; we are not, however, able to say whether absence of a band means that an important allergen is missing or whether it means that the extract is without a superfluous inactive protein.

QIE

Eight laboratories performed CIE experiments. Six laboratories used ALK rabbit antibodies. Three other antibodies were used for at least two independent determinations each.

Two mono- or oligospecific antibodies were used in RIE.

Allergen identification¹⁹

Six laboratories contributed data from a total of 21 QIE tests in which the Ag 25 content could be determined (closed, bell-shaped precipitates). Two laboratories performed the CIE under conditions in which Ag 25 could not be measured.

The frequency distributions of all the assays are demonstrated in Fig. 1, C. Most of the potency estimates for extract D relative to extract B were in the range 75 to 130, close to the expected value of 100. In addition, the potency estimates for each of the other comparisons were in close agreement.

The geometric means and GCV of the potency estimates for each laboratory are demonstrated in Table III. Analysis of variance demonstrated no significant differences between the potency estimates of the laboratories for any of the comparisons.

Labs 1 and 2 used their own antisera. Lab 3 carried out some assays by use of its own antiserum (3L) as well as ALK antiserum (3A). However, the use of different antisera did not appear to make any differ-

TABLE III. Geometric means of potency estimates of Ag 25 from QIE tests for each laboratory

Lab	No. of assays	Potency (%) D/B	Potency relative to extract A (%)		
			B	C	D
1	1	100 (—)	110 (—)	46 (—)	110 (—)
3L	1 for C and	120 (13)	93 (13)	53 (—)	110 (0)
3A	2 for A,B,D	120 (17)	75 (18)	41 (—)	91 (38)
6	2	88 (26)	140 (9)	84 (19)	120 (38)
7	6	100 (13)	93 (10)	65 (13)	95 (17)
10	4	86 (52)	94 (41)	42 (27)	81 (28)
11	2 for C	98 (21)	82 (11)	39 (58)	81 (19)
	4 for A,B,D				
Geometric means of 6 labs		101 (14)	96 (22)	51 (33)	97 (17)

The figures in the parentheses are the GCV of the potency estimates.

TABLE IV. Results of various tests as measured and calculated in different laboratories

Extracts	Protein determination (mg/ampule)		Ag 15 by CRIE (relative activity) Lab 7	Ag 19 by RIE (relative activity) Lab 7	ELISA inhibition (relative potency) Lab 11	Complement in activation (relative potency) Lab 10
	Kjeldahl Lab 1	Lowry Lab 7				
A	6.7	5.7	100	100	100	100
B	7.1	6.3	126	78	104	111
C	3.4	3.8	76	65	56	46
D	6.8	as B	102	76	164	114

All results are expressed per ampule relative to the respective values for extract A (100%).

ence to the potency estimates obtained in accordance with the theoretic expectation.

Seven laboratories performed CRIE. The labeling of the immunoprecipitates with binding to IgE antibody and radiolabeled anti-IgE clearly demonstrated Ag 19 and Ag 25 on all CRIE. In addition, other allergens could be observed but not easily identified. Ag 15²⁰ demonstrated a good radiostaining precipitate when it was allowed to migrate into a gel containing a specific antibody (not commercially available).

Table IV demonstrates the relative content of Ag 15 and Ag 19 in the various extracts as measured by lab 7. Lab 6 performed crossed enzyme immunoelectrophoresis that demonstrated the same allergens as found by CRIE. Fig. 3, A is a drawing of the precipitates observed in the CIE (Fig. 3, B) and CRIE (Fig. 3, C) of the proposed IS run under defined conditions. Only the easily recognized precipitates are labeled.¹⁹

Antigen 25 is the most important allergen in timothy grass.¹⁹ It reveals well-defined, bell-shaped precipitates under most of the conditions used in this study. Most other allergens in timothy grass are not easily

identified and quantitated without specific antibodies (not commercially available). The relative contents of Ag 15 and Ag 19 were measured by only one laboratory.

All the CRIE experiments demonstrated heavy radiostaining of Ag 25, but all the pictures also demonstrate Ag 19 and the other allergens. The total number of radiostained allergens depended on the conditions of the assay.

Other methods

The following methods were applied by one or two laboratories only: SDS-PAGE, HPLC, ELISA inhibition, and complement inactivation. The results as determined by each laboratory are described in Table IV where possible (not in the case of SDS-PAGE and HPLC).

Protein determination

Two laboratories measured the protein content (Table IV). Lab 1 used the Kjeldahl method to measure protein nitrogen, and the total protein was calculated

by multiplication with 6.25. Lab 7 used the Lowry method by use of bovine serum albumin as the reference. The two determinations do not differ much. Extract C contains less protein per ampule than the other extracts. This might be related to the general tendency that the measured potency per ampule was smaller in C than in the other extracts. In most cases this difference was not statistically significant.

SDS-PAGE and HPLC

Lab 6 estimated the apparent molecular weight of individual protein compounds by SDS-PAGE, whereas lab 11 performed HPLC for the same estimate. Both laboratories demonstrated that the components of all the extracts have similar molecular weight distributions with molecules mainly in the range 36,000 to 94,000 daltons and prominent bands at 19,000 and about 14,000 daltons (lab 6) or with some molecules throughout the range 10,000 to 150,000 daltons (lab 11).

ELISA inhibition

Lab 11 measured the inhibitory potency of the extracts in the reaction between solid-phase bound timothy grass extract and ALK rabbit antibody (Table IV). On an ampule-to-ampule basis, extract C was less potent than the others.

Complement inactivation

This is a method without a well-defined theory, but it has been suggested that it may be used to measure the relative allergen potency.¹⁴ Lab 10 measured the relative potencies by this method (Table IV) and found extracts A, B, and D to be very similar, whereas extract C was weaker per ampule.

Comparison of methods and reproducibility

The GCVs of the potency estimates of extract D of the laboratories relative to B demonstrate the extent to which random variation occurred when extracts are compared like with like. This was less than the variation that was found when the different extracts were compared, which presumably reflects differences in the specificity of the assay systems used. When the GCVs for RAST inhibition were compared with those for histamine release (both methods for measuring general allergenic activity), it was demonstrated that histamine release had much greater variation between laboratories. Some of this variation is expected since the RAST-inhibition experiments were performed by use of serum pools, whereas histamine release was measured on individual patients' leukocytes. QIE, for the measurement of the specific allergen Ag 25, demonstrated the closest agreement between laboratories.

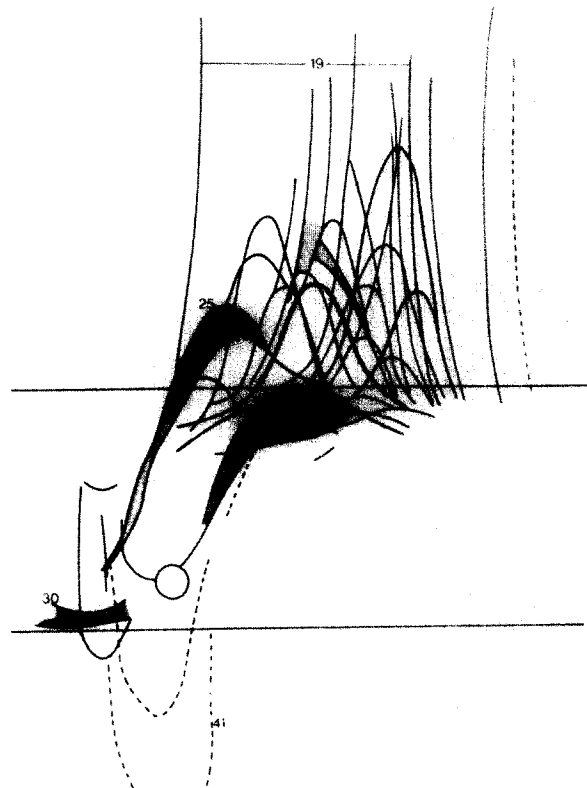


FIG. 3A. Identification of components of extract A; a drawing of the precipitates observed on the CIE of Fig. 3, B, and the CRIE of Fig. 3, C.

Lyophilization

Some of the ampules of the proposed IS were not lyophilized but were stored at -20°C . They were compared with the freeze-dried ampules after storage for 1 to 3 mo. The total allergenic activity measured by RAST inhibition and the content of individual allergens (Ag 15, 19, and 25) in the lyophilized ampules varied between 77% and 123% of the nonlyophilized samples, which was within the expected variation of the methods. The aim was to produce a timothy grass-pollen extract that did not lose more than 10% of its activity in the freeze-drying process. It was not feasible to compare the nonlyophilized ampules without storage and thus freezing and thawing. Within the accuracy of the methods, it was not possible to estimate whether any loss occurred during the freeze-drying process or losses caused by freezing and storage for 1 to 3 mo followed by thawing were comparable to losses caused by the process of freeze-drying.

Thermal stability

Some ampules of extract A were stored at the following temperatures: -80°C , -20°C , 3° to 5°C ;

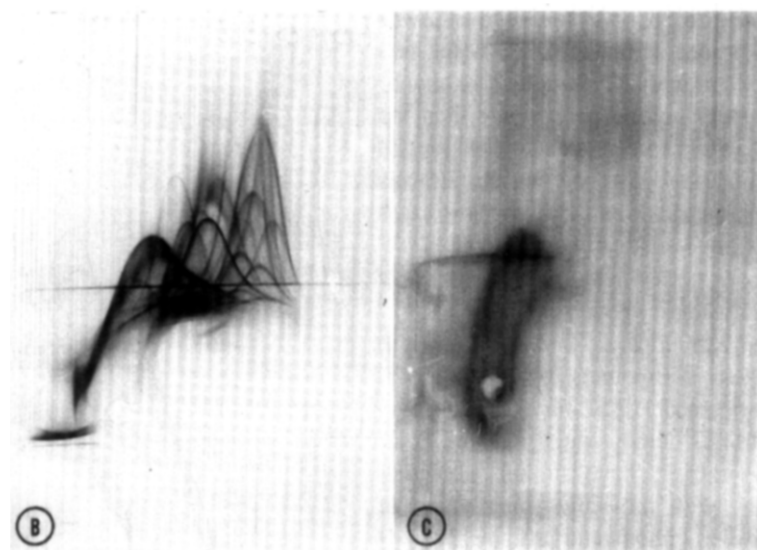


FIG. 3, B and C. CIE and CRIE. One ampule of extract A was dissolved in 100 μ l 50% (v/v) glycerol/saline and 7 μ l was applied. Agarose (HSA, Litex), 1% (w/v) solution, 1.5 mm thick (0.073M TRIS, 0.024M barbital, 0.0006M calcium lactate, pH 8.6) at 15° C. First dimension electrophoresis was performed at 10 V/cm for 25 min. Second dimension used a 1.3 mm thick gel containing ALK anti-timothy antibody (13 μ l per cm). The gel was run at 1.5 V/cm for 18 hr. The plate was pressed, washed in 0.9% saline solution, cold-air dried, and incubated with a timothy-allergic patient serum (diluted 1:15) for 22 hr. After washing, the plate was incubated with Pharmacia radioiodine-labeled anti-IgE, approximately 0.25 μ Ci for 24 hr. After washing, the plate was hot-air dried and radioautographed for 7 days at -20° C on Kodak XL 1 x-ray film in a cassette fitted with an intensifier screen. The plate was washed in distilled water for 1 hr, hot-air dried and stained in Coomassie brilliant blue. According to the IUIS nomenclature proposal for isolated and/or well characterized allergens, the new names will be: Ag 25 = Phl.p.I; Ag 19 = Phl.p.II; Ag 30 = Phl.p.III. Ag 25 makes up approximately 11% of the total protein or about 0.7 mg purified Ag 25 is equal to 100,000 IU. (Gjesing B, Løwenstein, H: Personal communication.)

room temperature, 33° to 34° C and 55° to 56° C. The temperatures were recorded 5 days each week. The room temperature was mainly in the range of 21° to 24° C; on a few days it was as high as 31° C. The other temperatures were very stable. After 2 mo and after 6 mo, three ampules from -80° C and two ampules from each of the other temperatures were tested. The relative potencies were measured by RAST inhibition, and the activities of Ag 25 and Ag 19 were measured by QIE. IEF was used for a qualitative evaluation. Storage at 56° C completely denatured the freeze-dried material within 2 mo. No precipitation bands were visible on the CIE/CRIE, the IEF band pattern was unrecognizable, and the potency in RAST inhibition was very low. The data revealed a slope different from the material kept at the other temperatures. The ampules kept at other temperatures up to 33° C were unchanged for 2 mo. After 6 mo the material kept at 33° C began to demonstrate signs of degradation. Half the ampules had material with brown edges. No significant loss in activity for the material stored at 33° C was detected by RAST inhibition. The Ag 25 activity increased by 16% indi-

cating changes of antigenic determinants,²¹ whereas the Ag 19 activity remained constant. On the IEF, one basic band disappeared, whereas the rest of the pattern was very similar to the other results. The ampules kept at room temperature (21° to 31° C) demonstrated no measurable changes, although the basic band on the IEF that had disappeared at 33° C was less stained compared with ampules kept at lower temperatures. Data from NIBSC demonstrated that storage at 4° C and 20° C for 20 mo did not change the RAST-inhibition activity.

GENERAL DISCUSSION

The potency estimates by RAST inhibition and histamine release, both assays of general allergenic activity, were in reasonable agreement, although the estimates for histamine release demonstrated large variations between the laboratories. For statistical reasons it was decided not to include the RAST-inhibition data from lab 8 in the overall mean. In view of the thermal stability of the materials, it is unlikely that the low values of lab 8 were caused by deterioration during shipment. We have no explanation for

the low values, and the overall means would not have been changed much by including them.

In the QIE system it is possible to quantitate any identifiable antigen. On a QIE plate, Ag 25 is easily recognizable by its strong radiostaining in CRIE. Different conditions during electrophoresis will allow identification of other allergens and antigens and thus make them quantifiable relative to the reference. Among the quantitative methods used in this study, QIE demonstrated the clearest agreement between the laboratories.

The IEFs from the various laboratories revealed very similar patterns, but as the individual bands have not yet been defined, presence or absence of important allergens and the antigenic relationship between the bands cannot be checked. It is therefore not possible at present to make use of the reference to quantitate components of a timothy extract by the IEF method.

CONCLUSIONS

Results from this study have demonstrated that the material (coded 82/520) is suited to serve as an IS. In the assay system of RAST inhibition, the dose-response relationships of the material 82/520 were comparable with those of the other two timothy grass extracts included in this study. The material 82/520 was also suited to function as a standard in the other tests reported here. Accelerated degradation studies have demonstrated that the material has adequate stability. With the approval of the participants, the material coded 82/520 was established in 1983 at the thirty-fourth meeting of the WHO Expert Committee on Biological Standardization as the IS* for timothy grass (*Phleum pratense*)—pollen extract and assigned arbitrary units of 100,000 IU per ampule. The individual allergen components that it contained were also assigned units of 100,000 IU. The value of 100,000 was chosen so that doses used in assay systems would have concentrations in whole numbers of IU.

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*The IS is available on request from the Director, National Institute for Biological Standards and Control, Holly Hill, Hampstead, London, NW3 6RB, U.K.

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