

Evaluation of RAST inhibition as a method for the standardization of house-dust extracts*†

P. L. B. Bruynzeel, ‡ W. Kersten §
and L. Berrens ¶||

RAST-inhibition was evaluated as a method for the *in vitro* standardization of house dust allergens, using four lyophilized house-dust preparations of different degree of purification (R_1 , R_2 , R_3 and R_4). When a serum pool of highly house-dust sensitive patients was used, a qualitatively similar potency sequence by RAST-inhibition between the different preparations could be established as with *in vivo* skin testing. When, however, individual sera were examined, striking differences were observed in both the qualitative and the quantitative potency relationships. Furthermore, large variations were noted in duplicate measurements in RAST-inhibition.

From this study it became clear that *in vitro* RAST inhibition and *in vivo* skin tests can have only statistical meaning for the standardization of house-dust allergen. In individual patients, striking differences may occur in the response to different preparations, thereby making standardization by these techniques rather unsatisfactory. By applying these techniques, we gained the impression that patients were being characterized rather than allergens.

INTRODUCTION

Although standardization of the biological activity of allergenic extracts used in

* Received for publication 9 July 1979.

† This study forms part of a multicenter project on the standardization of house dust allergens, which will be covered in full detail in future communications.

‡ Departments of Pulmonary Disease, Academic Hospital, Utrecht, The Netherlands.

§ Experimental Allergy, Academic Hospital, Utrecht, The Netherlands.

¶ Krankenhaus Bethanien, Department of Allergy, Moers, Federal Republic of Germany.

|| Correspondence to: L. Berrens, Ph.D., Department of Dermatology, Division of Experimental Allergy, University Hospital, Catharijnesingel 101, Utrecht, The Netherlands.

humans has been recognized as a problem for a good many years, the subject has recently been getting increased attention for reasons of legislation. It is now generally agreed that the methods commonly used for indicating extract potency may be very unrealistic, i.e. weight per volume, total nitrogen, or protein-nitrogen units (PNU). A long time ago, Arbesman & Eagle (1939) already demonstrated that none of these potency measurements correlated with the biological activity of allergenic extracts as determined by *in vitro* neutralization of reagins.

The development of the radioallergosorbent technique (RAST) has provided a method of measuring IgE antibody *in vitro* (Wide, Bennich & Johansson, 1967) and, recently, several investigators have employed either direct RAST or RAST-inhibition for the *in vitro* standardization of various allergens (Arbesman, Wypych & Reisman, 1977; Ceska, Eriksson & Varga, 1972; Gleich, Larson, Jones & Baer, 1974; Yman, Ponterius & Brandt, 1975). However, no attempts have been published so far to standardize the widely used house dust extracts by means of this particular technique. A major reason may be the rather poor correlations reported between the results of *in vivo* skin testing and *in vitro* RAST assay (Aas & Johansson, 1971; Hogarth Scott, McNicol, Williams & Johansson, 1973; Kersten, 1978; Pascual *et al.*, 1977; Wüthrich & Kopper, 1975).

The study of house dust allergens and their modes of action has led to the proposal that these substances may exert considerable non-immunological activity as enzyme-activating agents (Berrens, 1974). This activity is reflected in the complement-consuming capacity of purified house dust allergens, which has been suggested as a possible means of *in vitro* standardization (Berrens & Guikers, 1974).

It was the aim of the present multicenter investigations to evaluate the various now available techniques for the *in vitro* and *in vivo* standardization of house dust allergens. For this purpose, four house dust allergen preparations of different provenance and different degree of purification were obtained and submitted to Institutes in various parts of the world. The present paper documents the data on RAST inhibition in relation to skin test results with these various preparations.

MATERIALS AND METHODS

Sera

Blood samples were obtained from patients with atopic diseases attending the Out-patient Departments of Pulmonary Disease and Clinical Allergy at the Utrecht University Hospital. The samples were clotted in glass and the sera, obtained by centrifugation, were stored at -70°C until use. For serum pools, the samples of a minimum of 15 highly house dust-allergic patients were combined. Serum pools were used as standard references for the RAST inhibition studies.

Allergens

The house dust preparations used in this investigation were coded R_1 – R_4 and were obtained as follows:

R_1 was a partially purified preparation from a large lot of combined vacuum cleaner dust, purified to the stage by fraction C according to a published schedule (Berrens, 1970). The preparation was prepared and kindly donated by Haarlems Allergenen Laboratorium b.v., Haarlem, The Netherlands (batch H-772).

R_2 was obtained as a relatively crude, benzoic acid adsorbed A-fraction (Berrens, 1970) from a pool of dust obtained from an ultra-modern hotel in the Utrecht area, where absolutely no pets (cats and dogs) were allowed. From this product, a highly purified product R_4 was prepared by pursuing the schedule to fraction *E* (Berrens, 1970), followed by Sephadex G75 gel filtration and collection of the first peak off the column.

Finally, the R_3 -preparation was secured from house dust collected in Paris, France, and purified by ammonium sulphate precipitation as described (Laroche, Ky, Relyveld & Roche, 1967). It was kindly submitted by Dr E. H. Relyveld as a 25 ml solution Poussière de Maison Concentrée, lot no. 8, stated to have 0.318 mg N ml⁻¹, i.e. 31 800 units ml⁻¹.

All preparations were dialysed and dried by lyophilization. The details of analytical investigations by physicochemical means will be published elsewhere.

Allergen paper discs

For the estimation of IgE antibody and for RAST-inhibition, R_1 discs were prepared by coupling 10 mg of R_1 with cyanogen bromide to Whatman no. 1 filter paper discs (100 discs, weighing about 300 mg), according to the method of Ceska, Eriksson & Varga (1972). A similar procedure was followed to couple product R_4 . Furthermore, discs were prepared in London according to the NIBSC method, using British house dust preparations Gp1 mixture of acetone and pH 3 fractions as described (Brighton & Topping, 1977).

Radioallergosorbent tests (RAST)

The direct RAST was performed according to instructions of the manufacturer of the anti IgE reagent (Pharmacia Diagnostics AB, Uppsala, Sweden) with minor modifications. Serial dilutions of the serum pool were examined prior to RAST-inhibition experiments in order to determine the optimal dilution to be used in regular RAST-inhibition. The latter was performed essentially according to Arbesman, Wypych & Reisman (1977). Serial dilutions of each of the inhibiting preparations (R_1 , R_2 , R_3 , R_4) in 1% human serum albumin (HSA) were prepared, and 150 μ l samples were brought in duplicate rows of 11 or more polystyrene test tubes. The last tube in each row contained 1% HSA only; 50 μ l aliquots of the optimal dilution of the reference serum pool (or of individual sera) were then added to each tube. The mixture was shaken vigorously and left at room temperature overnight. A disc coupled with allergen (R_1 , R_4 or Gp₁) was then added to each tube and the tubes were left at room temperature for 20 h. After three washings with 2 ml of saline and removal of the supernatants by suction, 50 μ l of radiolabelled rabbit anti human IgE was added, and the tubes were capped, carefully shaken and left at room temperature overnight. After three additional washings with saline (2 ml), radioactivity bound to the discs was measured in a γ -counter. The results were expressed as percent inhibition, i.e.

$$100 \times \frac{\% \text{ RAST score} + \text{inhibitor}}{\% \text{ RAST score} - \text{inhibitor}}, \text{ where } \% \text{ RAST score}$$

represents the activity bound by the allergen-disc after incubation with serum as percent of the total radioactivity added to the test system. RAST scores were corrected

for aspecific binding of radioactive anti IgE to the disc in the presence of 1% HSA. The quantity of house dust preparation required for 50% RAST-inhibition was evaluated in terms of weight from logarithmic plots.

Skin tests

These were performed by the intracutaneous route in the skin of the back of house-dust sensitive patients visiting the Hospital Bethanien, Department of Allergy at Moers, using 0.05 ml of 10-fold serial dilutions of R_1 – R_4 in phosphate buffered saline. Concentrations ranged from 0.04–400 $\mu\text{g ml}^{-1}$. For endpoint titration *in vivo*, a 7 mm wheal was empirically chosen as the end point. In case the original endpoint was negative, sequentially stepped-up concentrations were used until a definite response was obtained.

RESULTS

RAST-inhibition

The four house dust preparations under investigation, R_1 – R_4 , were examined for their capacity in RAST-inhibition. An illustration of actual data obtained in a typical RAST-inhibition experiment, using R_1 paper discs, a serum pool, and the homologous R_1 product for inhibition is recorded in Table 1.

These results clearly demonstrate that large variations were obtained in duplicate measurements. This implies that the determination of the 50% inhibition point will be rather inaccurate. Although Arbesman *et al.* (1977) and Yman *et al.* (1975) consider a duplicate variance of about 15% acceptable, it is obvious that such variances prohibit the correct discrimination of extracts with only slightly different potencies. Neverthe-

TABLE 1. Typical experimental data: inhibition of R_1 house-dust RAST by the homologous house-dust preparation R_1 using a serum pool of house-dust sensitive patients

Inhibiting R_1 -concentration in μg	Net counts 4 min ⁻¹ *		Percent bound†		Mean inhibition‡
	1	2	1	2	
100	14 035	12 589	43.3	36.4	60.2
50	13 436	12 302	40.4	35.1	62.3
15	12 294	10 818	35.1	27.9	68.5
5	11 758	12 114	32.6	34.2	66.6
1.67	15 146	18 337	48.6	64.0	43.7
0.56	18 900	20 770	66.8	75.6	28.8
0.19	24 132	21 015	93.8	76.8	15.7
0.06	23 437	22 956	88.4	86.2	12.7
1% HSA	23 906	27 506	—	—	0

* These counts were corrected for background; 1 and 2 refer to duplicate samples.

† Amount of IgE antibody not neutralized by R_1 and still available to bind anti-IgE.

‡ Obtained by subtracting the mean percent bound (average of duplicates) from 100%.

less, the results do have some significance, because the duplicates show a certain reproducibility.

For the comparison of preparations R_1 , R_2 , R_3 , R_4 and the British Gp₁ product, dose-response curves for RAST-inhibition were composed, using different human sera and discs coupled to either R_1 , R_4 or Gp₁. Some typical inhibition curves are shown in Figs 1, 2, 3 and 4. Figures 1 and 2 demonstrate that the same sequence of inhibiting power $R_1/R_2/R_4$ is found in a single individual serum when discs coupled with house dust preparations of different origin and degree of purification are used. As also discussed below, this indicates that the potency sequence found in a single serum does not seem to depend on the allergen preparation coupled to the disc. However, the results may be dependent on the serum sample used, as illustrated in Fig. 3, where the same sequence of inhibition potency was found as in Fig. 1, but where the pattern of the curves was quite different owing to a change of serum.

The choice of the serum sample used may, however, also affect the potency sequence found among different preparations. This is illustrated in Fig. 4, which depicts the RAST-inhibition curves of R_1 , R_2 , R_3 and R_4 using a pool of sera of Dutch house dust

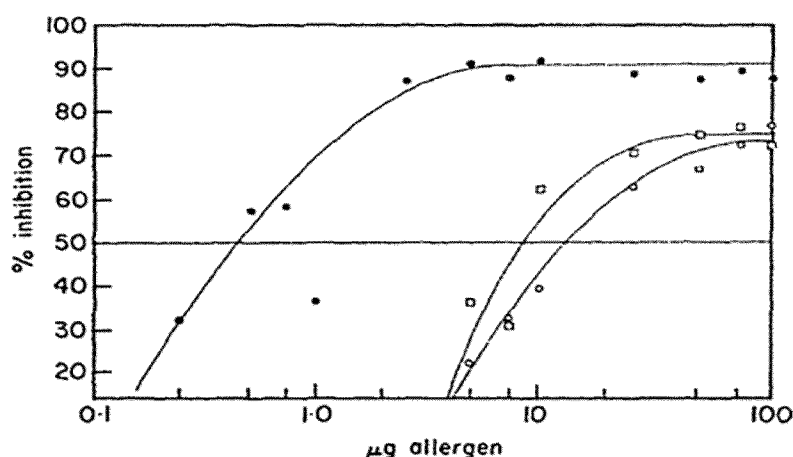


Fig. 1. Inhibition of house dust RAST by different house dust preparations. ●: R_1 , □: R_2 , ○: R_4 . Serum 77944 of a highly dust allergic patient, associated with animal dander allergy was used. Total IgE: 215 i.u. ml⁻¹, RAST (in % binding of added radio-activity): house dust R_1 : 28.1%, cat dander: 33.1%; R_1 -coupled discs were used.

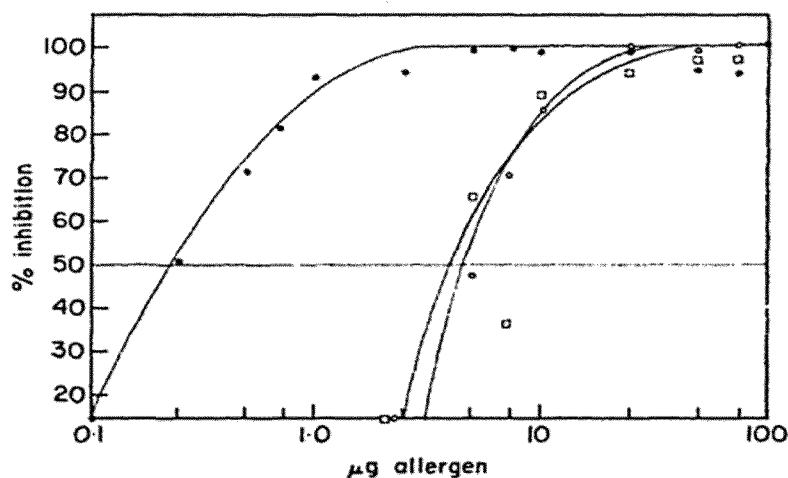


Fig. 2. Conditions as given in the legend to Fig. 1, except that R_4 -coupled discs were used.

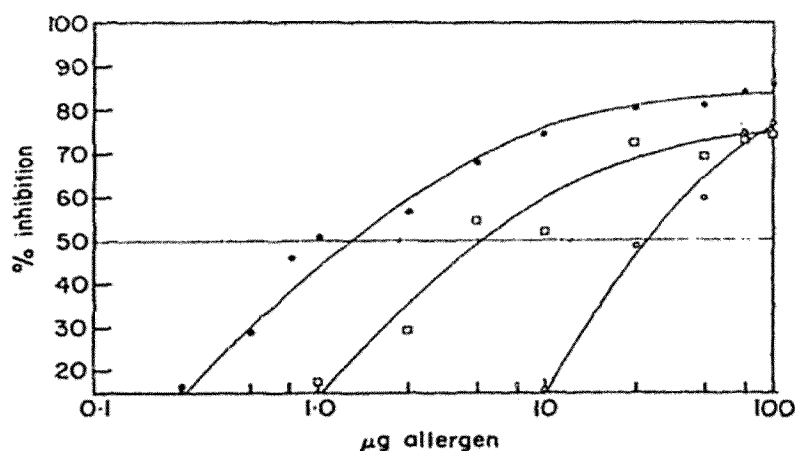


Fig. 3. Inhibition of house dust RAST with serum 77951 (atopic asthma, total IgE 1170 i.u. ml⁻¹, RAST house dust R_1 : 37.4%, cat dander: 35.4%, dog dander 3.7%, horse dander 2.9%); R_1 -coupled discs were used.

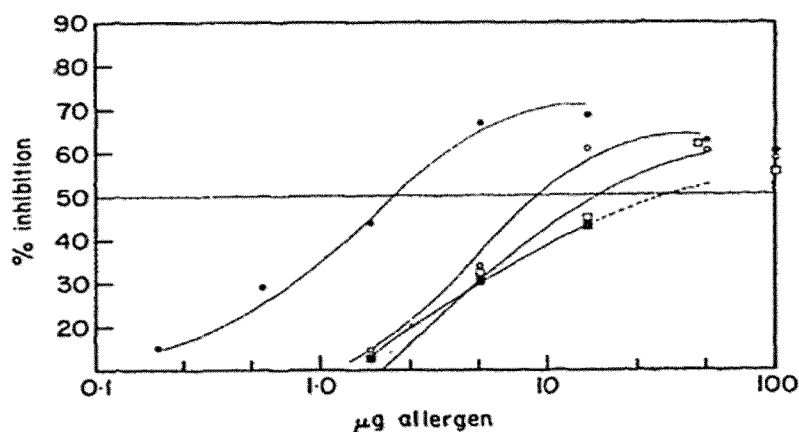


Fig. 4. Inhibition curves with a 1:8 diluted serum pool of 15 samples from highly allergic subjects (total IgE: 3800 i.u. ml⁻¹, RAST house dust R_1 : 31.9%; R_1 -coupled discs were used. ●: R_1 , □: R_2 , ■: R_3 , ○: R_4).

allergic patients. The potency sequences here was established as $R_1 > R_4 > R_2 > R_3$, using R_1 discs. Interestingly, exactly the same sequence was reported back from the London laboratory, using a British serum pool and Gp_1 discs. Working with a serum pool, we found these results highly reproducible on various occasions. In these experiments it was, moreover, again demonstrated that the results apparently did not depend on the allergen preparation coupled to the discs.

In order to evaluate the results in quantitative terms, the 50% inhibition points were established graphically. The numerical data, collectively listed in Table 2, demonstrate that, even though the potency sequences are similar, large differences are observed with different sera, even among different serum pools. If, for purposes of standardization, one allergen preparation is arbitrarily chosen for reference, the potency ratios among various products—as shown in Table 3—become very hard to interpret.

Skin test results

The results of intracutaneous tests with R_1 , R_2 , R_3 and R_4 in a randomized group of house-dust sensitive patients showed that this group could be divided into so-called

TABLE 2. Relative potencies of house dust preparations R_1 , R_2 , R_3 , R_4 , and Gp_1 as determined by RAST inhibition, in μg of product giving 50% inhibition in a standard assay

Product	77 944		77 951	Pool A	Pool B	Pool London	Serum
	R_1	R_4	R_1	R_1	R_1	Gp_1	disc
R_1	0.44	0.23	1.4	2.1	2.0	2.6	
R_2	8.2	4.0	5.4	17.2	26.0	7.2	
R_3	n.d.	n.d.	n.d.	31.0	n.d.	>100	
R_4	12.5	4.6	28.0	10.0	13.5	6.8	

TABLE 3. Potency ratios in RAST inhibition of various R -preparations, arbitrarily calculated with the R_1 -reference as unity

Product	77 944		77 951	Pool A	Pool B	Pool London	Serum
	R_1	R_4	R_1	R_1	R_1	Gp_1	disc
R_1	1.0	1.0	1.0	1.0	1.0	1.0	
R_2	0.05	0.06	0.26	0.12	0.08	0.37	
R_3	n.d.	n.d.	n.d.	0.07	n.d.	0.02	
R_4	0.04	0.05	0.05	0.21	0.15	0.38	

TABLE 4. Relative potency of house dust preparations R_1 , R_2 , R_3 and R_4 in skin testing (threshold values from end point titrations in a group of 16 patients, $\mu\text{g ml}^{-1}$) compared to relative potencies in RAST inhibition (mean of two serum pools, R_1 -discs, μg for 50% inhibition). Reference R_1 arbitrarily taken as unity

Preparation	Skin tests	RAST inhibition
R_1	1.0	1.0
R_2	0.05	0.1
R_3	0.1	0.03
R_4	1.5	0.15

strong and *weak* reactors. The strong reactors responded with wheal and flare reactions to a much lower allergen concentration ($0.04 \mu\text{g } R_1 \text{ ml}^{-1}$) than the weak reactors. Although the dose-response curves for skin titration in 10-fold serial dilutions differed among (16) individual patients, the difference in allergen reactivity among the groups of strong and weak reactors was about 100 fold for R_1 , 30 fold for R_2 and 300 fold for R_3 . For comparison of the relative potencies of the different house-dust extracts, only the results obtained in the strong reactors have been used here, because preparation R_4 was not investigated in the weak reactors. The potencies were evaluated as the mean ($n = 16$) concentration in the group from end point titration (i.e. a 7 mm diameter wheal size). The value of R_1 was arbitrarily converted to unity, in order to establish the potency ratio of the four allergen preparations. The results are shown in Table 4. A more detailed account of the *in vivo* results will be given in a separate communication.

DISCUSSION

No attempts have been published so far to standardize the widely used, but chemically poorly defined house dust allergens by means of the RAST-inhibition technique. Although the poor correlation reported between *in vivo* skin test results and *in vitro* direct RAST assay in house dust allergy was not encouraging, it was decided to examine RAST inhibition as part of an extensive multicenter program on the standardization of house dust extracts.

The results obtained by RAST-inhibition with four different reference preparations showed that the method was reproducible, but not very accurate. Duplicate determinations often showed large variations, which could not be attributed to unequally charged paper discs, because direct RAST assays with these discs showed only small intra-assay variance (about 5%). The cause for this has not been elucidated. However, it has recently been suggested that IgE binding to house dust may be indirect (Berrens, Guikers & Bruynzeel, 1979), so that interference by unknown serum factors may strongly influence the results of RAST inhibition. This might well explain the different maxima of RAST inhibition observed with the individual sera and the serum pools.

Comparison of the results with the individual sera and the pools brings out one of the most striking effects of RAST-inhibition. Although with one serum and with the pools an almost identical potency sequence was found ($R_1 > R_4 > R_2 > R_3$), the sequence in serum 77951 deviated ($R_1 > R_2 > R_4$). Furthermore, in one other individual serum sample, not presented here, the entirely different sequence $R_2 > R_4 > R_1$ was observed. This illustrates the serious drawback of using a pool of sera in RAST inhibition, because in a mixture the individual serum characteristics are masked by dilution. On the other hand, a serum pool has the advantage of featuring a large diversity of antibody specificities. Results obtained with such a serum pool for the standardization of an allergenic preparation may, however, not be representative for its subsequent application in individual patients.

Skin tests with the different house dust preparations revealed that house dust allergic patients could be classed as either strong or weak reactors. In each group the dose-response curves for skin-titration differed from one patient to another. This indicates that particular characteristics of individual patients were being established rather than characteristics of the allergenic preparations. An entirely similar conclusion was reached by Sherago, Berkowitz & Reitman (1950), who evaluated the results of a much more extended program of house dust allergen standardization, sponsored at the time by the American College of Allergists. From the averaged results among the group of strong reactors, however, a sequence of relative potencies was roughly established, but this did not coincide satisfactorily with the sequence determined by RAST inhibition (Table 4). Furthermore, the sequence obtained by skin-testing also depended on the method of evaluation, as will be discussed elsewhere (Kersten *et al.*, in prep.).

In an attempt to standardize ragweed pollen extracts by skin testing, Arbesman *et al.* (1977) found this method unsuitable because of large variation in the reaction pattern among individual patients. By means of RAST inhibition, using a pool of sera of highly ragweed allergic patients, they were then able to establish the relative potencies of different ragweed extracts based on the Antigen E content. Amazingly, RAST inhibition was then proposed as an *in vitro* standardization of ragweed extracts, despite the fact that their own results showed it to have poor clinical relevancy. The results of these authors—like ours with house dust extracts—strongly suggest that mechanisms other than those mediated by IgE must be involved *in vivo*.

An unexpected—and ununderstood—outcome of the present studies was that the potency sequence in RAST inhibition apparently did not depend on the origin or extent of purification of the allergen batch insolubilized on the discs. Furthermore, the observation that the most highly purified preparation, R_4 , although more active than its parent product R_2 , was not the most potent of the series, underlines the previous recommendation not to push purification too far for extracts to be used in allergy practice (Berrens, 1971).

We do not consider it a disadvantage that the serum pools did not come from the patients actually skin-tested with preparations not collected within their own area. If standardization is going to be imposed on allergen producers, this is probably the practical situation they will have to face.

It has repeatedly been pointed out that, in house dust allergy, mechanisms other than antibody-mediated reactions must be involved (Berrens, 1974; Berrens, Schoonenwolf & Bruynzeel, 1978). One of these mechanisms is reflected in the observed *in vitro* complement consumption by house dust allergens; on this basis, an independent method of allergen standardization has been developed (Berrens & Guikers, 1975). The technique has also been applied in this study on R_1 – R_4 preparations. Surprisingly, the potency sequence observed with this method roughly ran parallel to the RAST-inhibition data presented here. These results will be discussed in more detail in a subsequent communication.

Acknowledgements

The authors are indebted to Mr C. L. H. Guikers and Mr W. van den Boogaard for skilled technical assistance. Mr G. T. Hoek (Haarlems Allergenen Laboratorium b.v., Haarlem, The Netherlands) kindly provided the R_1 -preparation. The R_3 -preparation was donated by Dr E. H. Relyveld (Institut Pasteur, Garches, France). We are greatly indebted to Dr W. D. Brighton, National Institute for Biological Standards and Control, London (England), for submitting his preliminary RAST-inhibition results. The initiation of this work was encouraged by the Paul Ehrlich Institute, Frankfurt am Main (Federal Republic of Germany) and by the Foundation Study Centre for Allergy Projects, Amsterdam (The Netherlands).

REFERENCES

- Aas, K. & Johansson, S. G. O. (1971). The radioallergosorbent test in the *in vitro* diagnosis of multiple reagenic allergy. *Journal of Allergy and Clinical Immunology* 48, 134–142.
- Arbesman, C. E. & Eagle, H. (1939). The assay of ragweed pollen extracts. *Journal of Allergy* 10, 521–536.
- Arbesman, C. E., Wypych, J. I. & Reisman, R. E. (1977). Evaluation of RAST inhibition as a method for the standardization of ragweed pollen extracts. *International Archives of Allergy and Applied Immunology* 53, 310–318.
- Berrens, L. (1970). The allergens in house dust. *Progress in Allergy* 14, 259–339.
- Berrens, L. (1971). Introduction to round table conference on allergen standardization. *International Archives of Allergy and Applied Immunology* 41, 222–230.
- Berrens, L. (1974). Inhalant allergens in human atopic diseases: their chemistry and modes of action. *Annals of the New York Academy of Sciences* 221, 183–198.
- Berrens, L. & Guikers, C. L. H. (1975). Value of fluid phase complement consumption as a means of *in vitro* allergen standardization. *Proceedings of the IABS-WHO Symposium on the Standardization and Control of Allergens Administered to Man, Geneva*, pp. 235–248. Basel: Karger.
- Berrens, L., Schoonenwolf, D. A. & Bruynzeel, P. L. B. (1978). Complement consumption and

- IgE binding by house dust allergen in the serum of atopic patients. *Allergologia et Immunopathologia* 6, 45-54.
- Berrens, L., Guikers, C. L. H. & Bruynzeel, P. L. B. (1979). Possible indirect binding of IgE in house dust RAST. *Annals of Allergy* 43, 38-43.
- Brighton, W. D. & Topping, M. D. (1977). Standards from house dust, in: *Allergy and Clinical Immunology. Proceedings IXth International Congress of Allergology, Buenos Aires 1976, Excerpta Medica International Congress Series* 414, 161-166.
- Ceska, M., Ericksson, R. & Varga, J. M. (1972). Radioimmunosorbent assay of allergens. *Journal of Allergy and Clinical Immunology* 49, 1-9.
- Gleich, G. J., Larson, J. B., Jones, R. J. & 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, 158-169.
- Hogarth-Scott, R. S., McNicol, R. N., Williams, H. E. & Johansson, S. G. O. (1973). Diagnosis of allergy *in vitro*: a comparison between skin-sensitivity testing and serum levels of specific IgE antibody in children. *Medical Journal of Australia* 1, 1293-1297.
- Kersten, W. (1978). Die Korrelation von Hauttest-Provokations-test-RAST (Radio-Allergo-Sorbent-Test) beim Hausstaub-allergen. *Allergologie* 1, 81-86.
- Laroche, Cl., Ky, N.-T., Relyveld, E. H. & Roche, L. (1967). Traitement de l'allergie respiratoire par une préparation retard d'extrait de poussière de maison adsorbée sur hydroxyde d'alumine. Etude préliminaire sur 30 cas. *La Presse Médicale* 75, 2667-2669.
- Pascual, H. C., Mohan Reddy, P., Nagaya, H., Lee, S. K., Lauridsen, J., Gupta, S. & Jerome, D. (1977). Agreement between radio-allergosorbent test and skin test. *Annals of Allergy* 39, 325-327.
- Scherago, M., Berkowitz, B. & Reitman, M. (1950). Standardization of dust extracts. I. Standardization on the basis of equal molecular size. *Annals of Allergy* 8, 437-452.
- Wide, L., Bennich, H. & Johansson, S. G. O. (1967). Diagnosis of allergy by an *in vitro* test for allergen antibodies. *Lancet* ii, 1105-1107.
- Wüthrich, B. & Kopper, E. (1975). Nachweis von spezifischen IgE-Serumantikörpern mit dem Radio-Allergo-Sorbent Test (RAST) und seine Bedeutung für die Diagnostik der atopischen Allergie. *Schweizerische Medizinische Wochenschrift* 105, 1337-1345.
- Yman, L., Ponterius, G. & Brandt, R. (1975). RAST-based allergen assay methods. *Proceedings of the IABS-WHO Symposium on the Standardization and Control of Allergens Administered to Man, Geneva*, pp. 151-165. Basel: Karger.