

The Journal of
ALLERGY
and
CLINICAL IMMUNOLOGY

VOLUME 49

NUMBER 1

Radioimmunosorbent assay of allergens

M. Ceska, R. Eriksson, and J. M. Varga *Uppsala, Sweden*

A method has been developed for the assay of allergens and may be summarized as follows: 1. Filter paper discs are activated with BrCN. 2. The allergens are coupled to the reactive groups on the paper disc carrier. 3. IgE immunoglobulin, present in the sera of allergic patients, then attaches itself to the allergen coupled to the paper discs. 4. ¹²⁵I-labeled anti-IgE antibodies thereafter interact with the IgE molecules, attached via the allergens to the paper carrier. Examples of the assay with the use of dog, cat, horse, cow, and timothy allergens are presented. The allergen assay method can conveniently be used to compare the "potency" of different allergen extracts and to check the extraction procedure, storage, and further treatment of allergens.

The quantitative assay of allergens has usually been carried out by means of skin tests¹ on allergic individuals. The method is used to locate allergen activity in fractions from a purification procedure. Over a limited range, the relation between wheal size and the logarithm of allergen concentration is approximately linear.² Using this method, a twofold increase in skin reactivity is produced by about a tenfold increase in allergen concentration.

The application of in vitro methods, for example, quantitative precipitin analysis^{3, 4} and histamine release from human leucocytes,⁵ for allergen assay has not been extensive.

Recently, an in vitro test was developed for the diagnosis of allergy⁶ in which the allergens are chemically coupled to an insoluble carrier (Sephadex). The coupled allergens were able to bind the respective IgE immunoglobulins present in the sera of allergic patients, which in turn could bind ¹²⁵I-labeled anti-IgE antibodies.

Since the radioactive uptake depends on the presence of allergens on the carrier, the principle could be used to quantitate allergens. From a systematic

From the Department of Biochemistry, Pharmacia AB.

Received for publication June 4, 1971.

Reprint requests to: M. Ceska, Pharmacia AB. Box 604, S-751 25, Uppsala 1, Sweden.

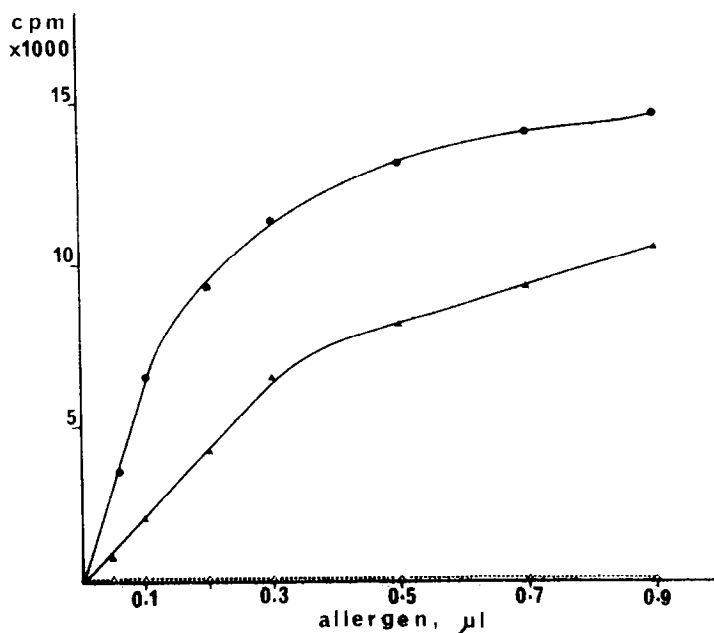


FIG. 1. Dose-response function with dog allergen. The method described in Methods section was used with sera No. 093 (●—●) and No. 120 (▲—▲) obtained from dog-sensitive persons, and a non-sensitive person (Δ — Δ). The points in the figure represent an average of three assays. The "Background" values, obtained without coupled allergens, were subtracted. Usually they are in the range of 300 to 500 c.p.m.; if higher, they are indicated in figures (e.g., Figs. 7 and 8). On the X axis the allergen content is indicated as microliters of allergen in 400 μ l of total volume.

study of the various parameters and suitable carriers, a method of allergen assay based on the principle outlined above has been developed. Preliminary results have been presented at a congress.⁷

MATERIALS AND METHODS

Materials

Commercially available chemicals of reagent grade were used. The human serum albumin is a product of KABI AB, Stockholm, Sweden. The allergen extracts were purchased from different allergen-producing firms (the sources of allergens will be specified on request) or prepared by us according to a published method.⁸ The ¹²⁵I-labeled anti-(Fc) IgE(ND) and sera of patients were kindly supplied by Dr. S. G. O. Johansson of the Blood Centre, University Hospital, Uppsala, Sweden. A Wallac Gamma Sample Counter (Wallac OY, Turku 5, Finland) was used to determine radioactivity. The pollen of *Betula lenta* was obtained from Sharp & Sharp, Inc., Everett, Washington.

Methods

Assay of allergens.

STEP 1: ACTIVATION OF PAPER DISCS. Ten grams of paper discs (diameter 5 mm.) were cut with a punch from Munktells No. OOH filter paper. Three other types of filter paper were also tested and gave essentially the same results. The discs were allowed to swell for 30 minutes in 200 ml. of water. BrCN solution, 200 ml. (5 per cent in water), was added and mixed with a mechanical stirrer for 3 minutes in a water bath at 19° C. NaOH (1M),

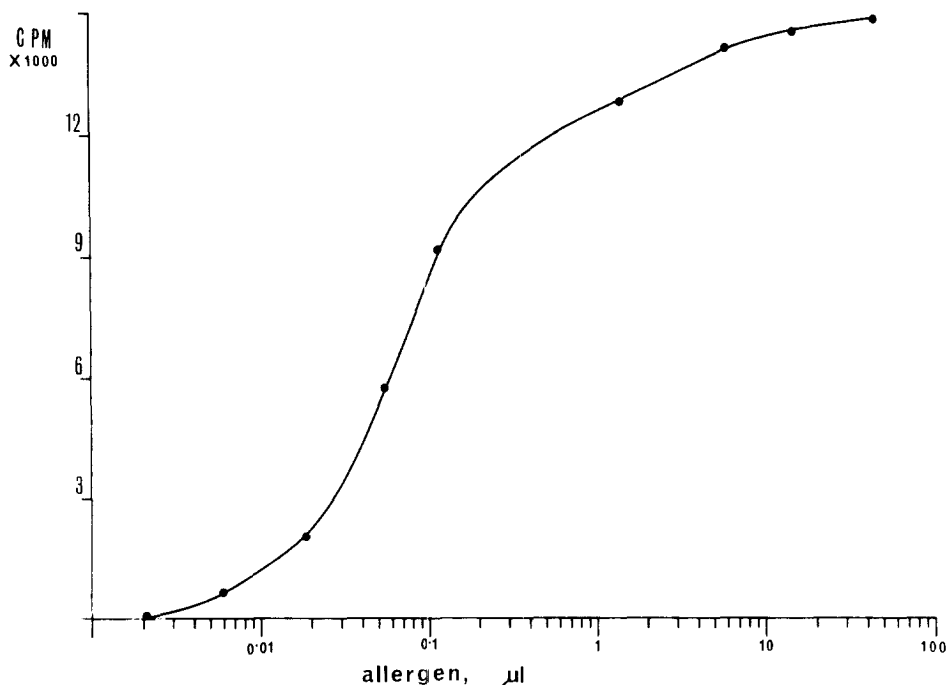


FIG. 2. Dose-response curve obtained with cat allergen.

40 ml., was added dropwise to maintain the pH in the range of 10.0 to 10.5. The suspension was immediately poured into 2 L. of cold NaHCO_3 solution (0.005M, 4° C.). After thorough mixing, the solution was decanted. The washing with 2 L. of NaHCO_3 solution was repeated five times. The paper discs then were washed four times with 500 ml. of acetone (reagent grade, 4° C.) and placed on a filter paper in the cold room (4° C.) for 3 hours. The drying was continued overnight in a vacuum-desiccator, over CaCl_2 , at 4° C. The paper discs were then stored at -20° C. No decrease in activity was detectable after 8 months of storage.

STEP 2: COUPLING OF ALLERGENS TO ACTIVATED PAPER. One or two activated paper discs were incubated with 0.2 to 0.4 ml. of allergen solution in plastic tubes covered with paraffilm at 4° C. on a horizontal shaker (about 100 r.p.m.). The concentration of allergens used is indicated in the figures. The coupling was terminated after about 20 hours. With high concentrations of allergens the response could be increased by about 10 per cent if the coupling time were extended to 2 to 3 days.

STEP 3: WASHING AFTER COUPLING THE ALLERGENS. After coupling, 2.5 ml. of NaHCO_3 solution (0.1M) was added, and the solution was drawn off by inserting a Pasteur pipette connected, to a water pump. β -Ethanamine solution (0.05M in 0.1M NaHCO_3) 1 ml., was added and agitated on a horizontal shaker (about 100 r.p.m.) for 3 hours. The solution was drawn off and the paper discs were then washed once with 2.5 ml. of NaHCO_3 (0.1M), three times with 2.5 ml. of acetate buffer (0.1M; pH 4.0), and twice with 2.5 ml. of "incubation buffer" (NaCl, 0.9 per cent; phosphate buffer, 0.05M; human serum albumin, 0.3 per cent; sodium azide, 0.05 per cent; final pH 7.4 at 20° C.).

STEP 4: INCUBATION WITH SERUM. Serum solution, 50 μ l, obtained from allergic patients, diluted 2:3 with "incubation buffer," was added to the paper discs and agitated on a horizontal shaker (100 R.P.M.) overnight at room temperature. After the incubation, the paper discs were washed three times with 2.5 ml. of "incubation buffer" containing one per cent Tween 20.

STEP 5: INCUBATION WITH ^{125}I -ANTI-(Fc) IgE(ND). ^{125}I -anti-(Fc) IgE(ND) (40,000 c.p.m. in "incubation buffer") 50 μ l solution was added and agitated overnight on a

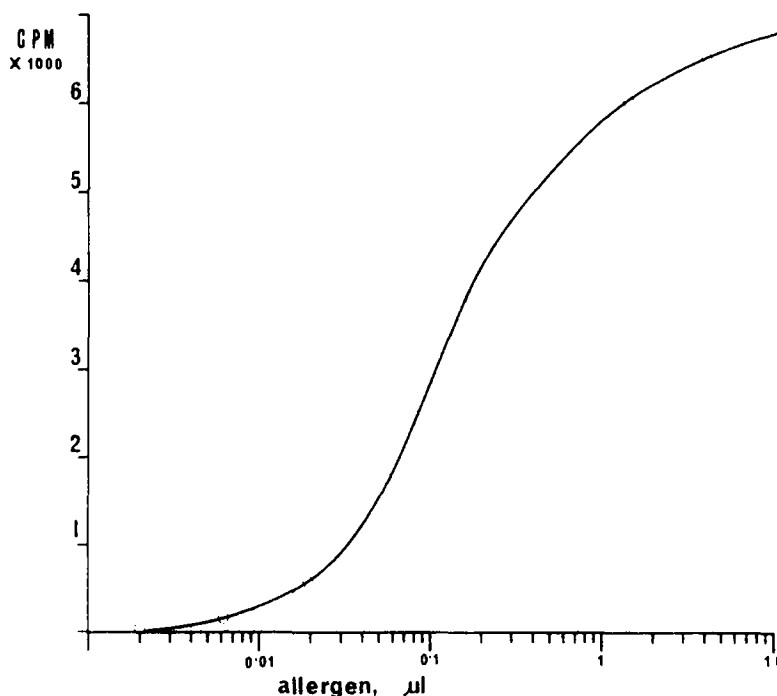


FIG. 3. Dose-response curve obtained with horse allergen.

horizontal shaker (100 R.P.M.) at room temperature. The paper discs were washed three times with a 2.5 ml. solution of 0.9 per cent NaCl and one per cent Tween 20 in water. The tubes were then closed with plastic caps and counted in a γ -counter.

RESULTS

Fig. 1 demonstrates the response obtained by the aforementioned method with an increasing dose of dog allergen. There was no positive response when sera from nonallergic individuals were used.

Serum from sensitive patients may or may not contain IgE antibodies to the different allergen determinants present in a crude allergen extract, and the IgE concentration in the serum may vary considerably from patient to patient. Therefore, the observed difference in the response using serum from different sensitive persons is not unexpected. The response to increasing allergen concentrations is nearly linear at lower concentrations, while at higher concentrations it approaches a saturation level.

With cat (Fig. 2) and horse (Fig. 3) allergens, the linear relationship between response and allergen concentration is limited to a narrow range of low allergen concentrations. At higher concentrations, the dose-response function is of a saturation type. With cow allergen, there is a linear relationship between the response and the logarithm of allergen concentration for increases in concentration up to a factor of 10^2 (Fig. 4). With crude timothy (*Phleum pratense*, Fig. 5) allergen extract, the response is of a nonsaturation type over the whole range of concentrations investigated. Using a major allergen frac-

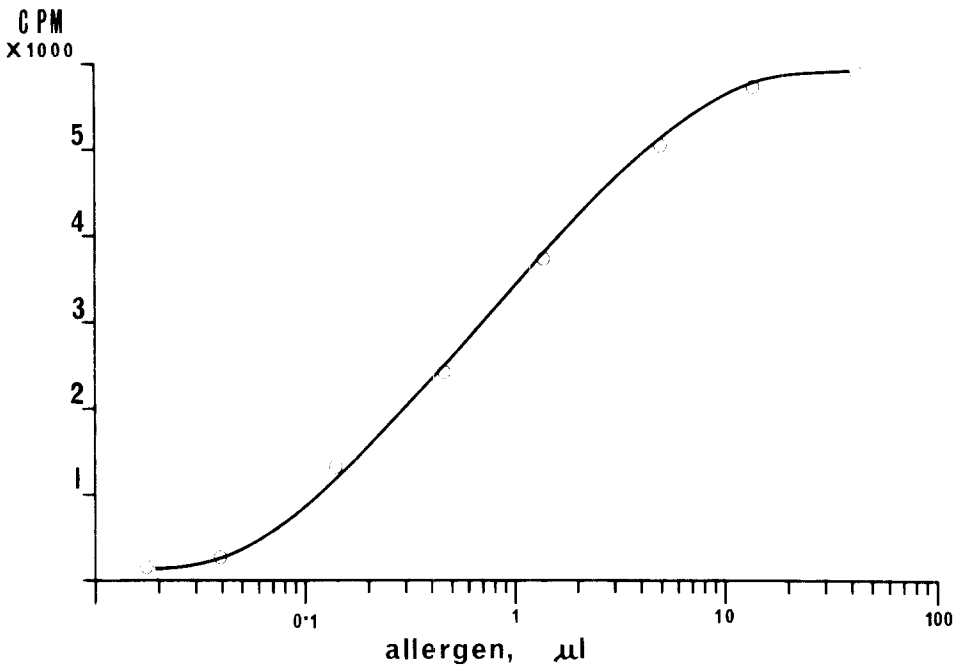


FIG. 4. Dose-response curve obtained with cow allergen.

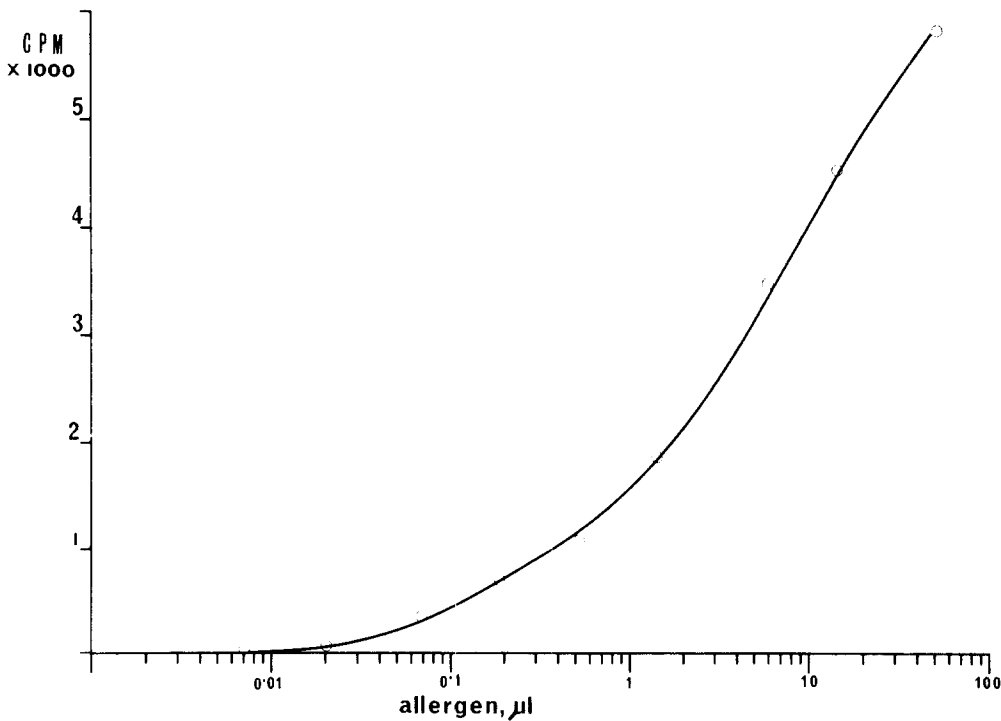


FIG. 5. Dose-response curve obtained with timothy allergen.

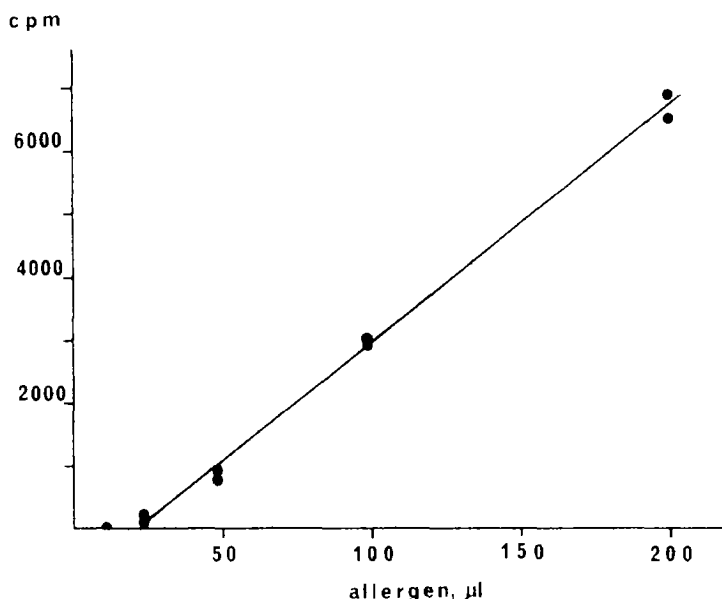


FIG. 6. Dose-response curve obtained with timothy allergen, purified by gel isoelectrofocusing.⁸ Fraction B (isoelectric point 4.8 to 4.9) was used.

tion, obtained from timothy allergen extract by isoelectrofocusing,⁸ the dose response function is linear (Fig. 6).

The "potency" of different timothy and birch allergens was compared by this assay method. Two commercial timothy pollen extracts displayed activities which differed by a factor of several hundred (*A* and *B* in Fig. 7). The activity of two commercial birch extracts (produced by an American and an European firm) were surprisingly similar (Fig. 8); our own birch extract was somewhat more active. The experimental freeze-dried timothy and birch extracts obtained from a firm for an investigational purpose had very low activities (Figs. 7 and 8).

DISCUSSION

The allergen assay presented here is based on the ability of IgE antibodies present in the sera of allergic patients to bind allergens. This principle has been used before to determine the concentration of IgE antibodies in serum.⁶ We have applied the same principle of antigen assay by using a simplified disc method.

The response of this method depends on the presence of an *allergen/IgE/¹²⁵I-anti-(Fc) IgE(ND)* complex coupled to the paper carrier. Accordingly, in an ideal case a linear relationship between the allergen concentration and the response can be expected over a range of allergen concentrations in which the availability of active groups on the sorbent, the IgE antibodies in the serum, and the labelled anti-(Fc) IgE(ND) are not limiting. The assays with crude dog allergen and purified timothy allergen exemplify this ideal situation (Figs. 1 and 6). Crude allergen extracts, however, may contain a large

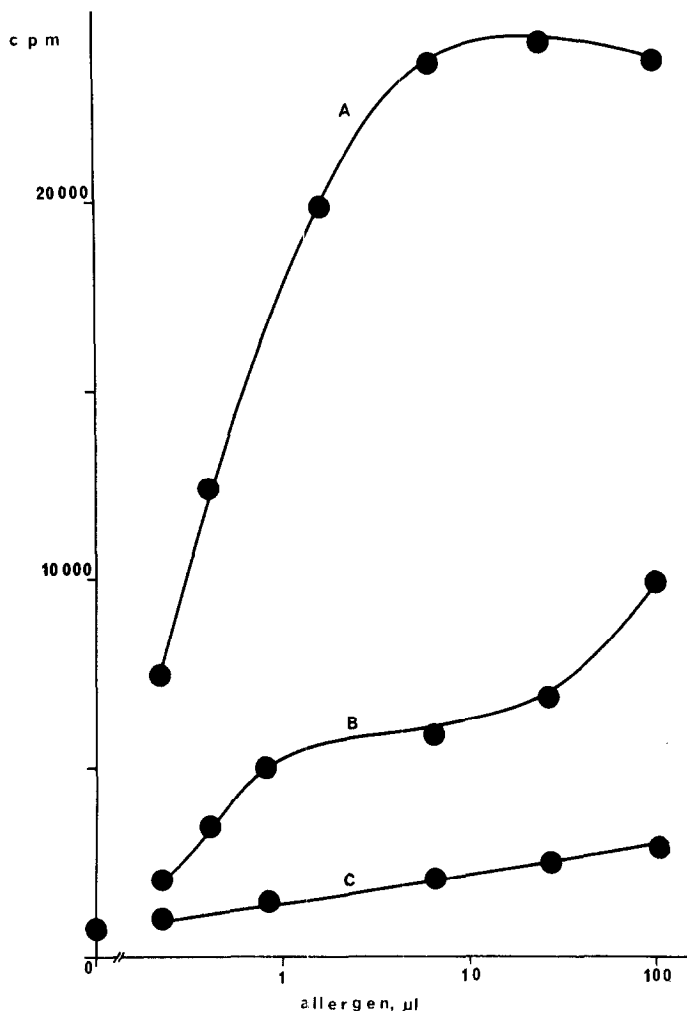


FIG. 7. A comparison of the "potency" of three timothy allergen extracts. The activities of two commercial timothy pollen extracts (**A** and **B**) and a freeze-dried experimental extract (**C**) obtained from a third firm were compared after dialysis.

number of major and minor allergenic determinants, in addition to the non-allergenic "ballast proteins" which make up the majority of total proteins. The occasionally observed augmented response at high antigen concentration (Fig. 7) may be a consequence of a competition between the allergenic and nonallergenic components for the active groups during the coupling procedure or of a threshold phenomenon which may appear in a nonequilibrium system. Concomitant presence of IgG antibodies may be another contributory factor in affecting the dose-response curves. Deviation of the dose response function from linearity may indicate increasing complexity of allergen extracts, the presence of nonallergenic components, a greater number of major and minor components, and/or the presence of blocking-type antibodies.

Despite the fact that with the majority of crude allergens the dose-response

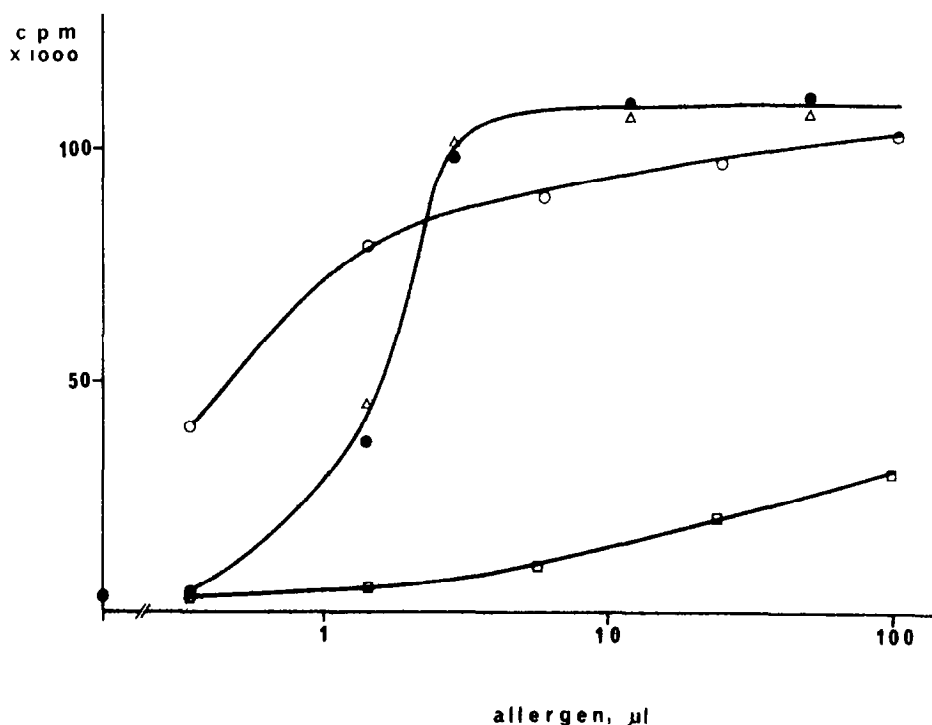


FIG. 8. A comparison of the "potency" of three birch pollen extracts. Own extract of the pollen of *Betula lenta* (O—O); commercial products of two firms (O—O; Δ — Δ); an experimental extract obtained from a third firm ($\square$ — $\square$).

functions are complex, the results are, however, reproducible with the use of a single allergen extract-serum combination. The reproducibility of the paper method depends on the allergen being tested, but in most cases the coefficient of variance is about 10 per cent. The method can therefore be conveniently used for the assay of allergens and for comparing the "potency" of different allergen extracts. The biological activity (which represents the total allergen content) of commercial extracts produced by different firms may differ from each other by a factor which may range from several hundred to more than a thousand (Fig. 7). Recently it was observed that other commercial allergen extracts (for example, house dust extracts) also differ greatly in their potency when tested with several sera of house-dust sensitive patients.

The present radioimmunosorbent assay method is much simpler to perform than the equivalent Sephadex radioimmunosorbent assay method. When paper discs are used, vertical rotation during incubation as well as centrifugation are avoided. The washing of the paper discs can easily be performed by a simple suction device.

A limitation of the present method is that it measures allergen activity associated only with IgE antibodies. It has to be remembered, therefore, that allergen activity which may be associated with other types of antibodies will escape detection. For such cases a similar paper radioimmunosorbent assay test could be set up, using the same paper disc-allergen complex, but, instead

of ^{125}I -anti-(Fc) IgE(ND), iodinated antibody to other classes of immunoglobulins may be used. In this way a test for blocking type of antibodies may, for example, be performed which may be especially useful when testing and following the treatment of subjects injected with pollen extracts.

If blocking-type antibodies are present in the tested serum, allergen dose-response curves may show lower values than those obtained in the absence of blocking-type antibodies. This would occur only when the concentration of the solidified allergen is limiting. Since in our studies we have been using sera of untreated allergic patients, their content of blocking-type antibodies may be low. It is obvious, however, that the blocking-type antibodies will have to be assayed especially in cases in which this method is being compared with other allergen-assaying methods in order to eliminate false negative values.

It has to be kept in mind that all radioimmunosorbent assay methods used for assaying allergens are measuring only the allergen-IgE antibody-binding capacity. In allergic diseases, however, other events also take place. Although beyond the scope of the present work, it is clearly important to establish the correlation between allergen activity as measured by the radioimmunosorbent assay method and other indices of allergen activity, such as leukocytes histamine release or skin testing.

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