

Mold allergy: Fractionation of allergens by Sephadex G-200 gel filtration

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*The fractionation of mold extracts by Sephadex G-200 revealed, with some molds, three peaks absorbing light at 280 m μ . Skin reactivity and precipitation were localized in the small and middle peaks, while the larger peak contained the mold pigments and ninhydrin staining components. In some molds the middle peak was absent, but in these cases the skin and precipitation activities were found in the first fractions of the larger peak. Individual variations were observed among the patients in their skin reactivity to the various molds. Some patients reacted to both the small and middle peaks, but others reacted to only one of them. It is evident, therefore, that mold allergens can be divided into two groups on the basis of molecular size. In the case of *Alternaria*, the thermostable antigens were all concentrated in the small peak; thus, they have a larger molecular size than the thermolabile antigens which came out in the middle peak.*

The aim of this work was to study the antigenic and allergenic components present in common airborne molds known to be responsible for symptoms in allergic individuals. Previous studies performed by us¹ with extracts of *Alternaria tenuis* by means of immunoelectrophoretic techniques revealed 6 antigenic components, 2 of which were thermostable. The peak of greatest skin reactivity in *Alternaria*-sensitive patients coincided with the location of the prominent thermostable area of precipitation and also with the zone of maximum carbohydrate and polypeptide staining. The reports of the presence of allergenic, low molecular weight, dialyzable components in *Alternaria* by Tatge² and of allergenic fractions which did not have the characteristics of typical polysaccharides or proteins by Sternberger and associates³ suggested that gel-filtration procedures, which separate components on the basis of their molecular weight, could be helpful in studying the allergenic materials in mold extracts. In this study the mold extracts have been fractionated by Sephadex G-200, and the

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fractions have been evaluated by skin testing in mold-sensitive patients. This is a preliminary report comparing a number of fungi with the use of a variety of immunological techniques. The large number of genera and species among the allergenic molds required an evaluation of the basic immunological similarities and differences among the most common genera in order to select the best representative from the group for a more detailed study. This study is now in progress.

MATERIALS AND METHODS

Isolation of molds

Molds were isolated by the exposure of plates of Mehrlich's medium at various localities in the San Juan metropolitan area. No attempt was made to make an extensive survey of airborne molds in the island. After exposing several hundred plates, it was found that the predominant genera were *Aspergillus*, *Penicillium*, *Hormodendrum*, and *Fusarium*. *Alternaria* was isolated on only a few occasions but was included in the study for the sake of comparison with our previous work. Our results did not differ markedly from those obtained in Puerto Rico by Pons and Belaval⁴ in 1958. For this work we selected 5 mold species: *Aspergillus*, *Penicillium*, *Hormodendrum*, *Fusarium*, and *Alternaria*. A large number of mold strains were tested in a 1:1,000 dilution in allergic subjects, and only those strains giving a wheal at least 20 mm. in diameter were selected.

Preparation of mold extracts

The molds were grown in a synthetic medium, devised by Steinberg and Thom⁵ and modified by Le Beau,⁶ having the following composition per liter and a pH of 7.2: Sucrose, 30 Gm.; K_2HPO_4 , 3 Gm.; KH_2PO_4 , 1.00 Gm.; KCl, 0.50 Gm.; $NaNO_3$, 3.00 Gm.; $MgSO_4 \cdot H_2O$, 0.50 Gm.; $FeSO_4$, 0.05 Gm.; $ZnSO_4 \cdot H_2O$, 0.0012 Gm.; $CuSO_4 \cdot 5H_2O$, 0.0005 Gm.; $MnSO_4 \cdot H_2O$, 0.0005 Gm.; $Na_2MoO_4 \cdot 2H_2O$, 0.0001 Gm. This medium was sterilized in shallow layers in one liter flasks, inoculated with mold spores, and incubated at room temperature for approximately 3 weeks. In order to decide on the method of extraction resulting in the greater number and concentration of antigenic components, three different methods of extraction were used, and the extracts were tested for antigenicity in rabbits.

The first method, which we will refer to as the standard method, consisted of separating the matts from the medium and killing and defatting them in petroleum ether for 48 hours. After decanting the ether, the dried matts were ground to a powder and the extract was prepared by shaking the powder for 48 hours in Coca's solution.

The second method, with minor modifications, was similar to that described by Prince and Morrow.⁷ The extract was prepared from the liquid medium after removal of the matts. The medium was concentrated by pervaporation to one tenth of its original volume and dialyzed. Acetone precipitation was carried out by adding 9 volumes of cold acetone to one volume of the dialyzed medium. The precipitate was redissolved in saline and dialyzed for removal of the acetone.

The third method was adapted after that of Schaffer, Molomut, and Center⁸ in which the entire mold culture, including the culture medium, was homogenized in a Waring Blendor and allowed to extract at 5° C. for 48 hours. The filtrate was dialyzed and buffered to a pH of 6.5 to 7.0. Kjeldahl nitrogen determinations were performed with the 3 extracts, and for some procedures they were adjusted to equivalent concentrations.

Immunization of rabbits

Groups of 5 rabbits each were hyperimmunized with the mold extracts. The first injections were given with the extract emulsified with an equal volume of complete Freund adjuvant. Subsequent injections were prepared with incomplete Freund adjuvant. The injections were given in the foot pads and intracutaneously at weekly intervals. Repeated trial bleedings were made until an optimum number of precipitin bands were obtained by testing the sera against the homologous extract by the gel-diffusion method of Ouchterlony.

Fractionation of mold extracts by Sephadex G-200 gel filtration

The mold extract was concentrated one hundred times with Carbowax. Two milliliters of the concentrated extract were passed through a column of Sephadex G-200 (Pharmacia Upsala Co., Sweden) of 2.5 cm. inside diameter and 36 cm. in length. Elution was done with phosphate buffer (pH 7.2), and 3 ml. fractions were collected in a Beckman, Model 132, fraction collector. The fractions were read in a Beckman DU Spectrophotometer at a wave length of 280 m μ .

The fractions were tested by gel diffusion against the homologous rabbit antisera. Ninhydrin staining was carried out on filter paper strips, placing 50 μ l from each fraction on the paper, drying in the oven at 100° C. for 5 minutes, and spraying with the ninhydrin reagent. Some fractions were tested for carbohydrate by means of the anthrone reaction. Portions of the fractions were sterilized by filtration through Swinney filters and used for intradermal tests in patients.

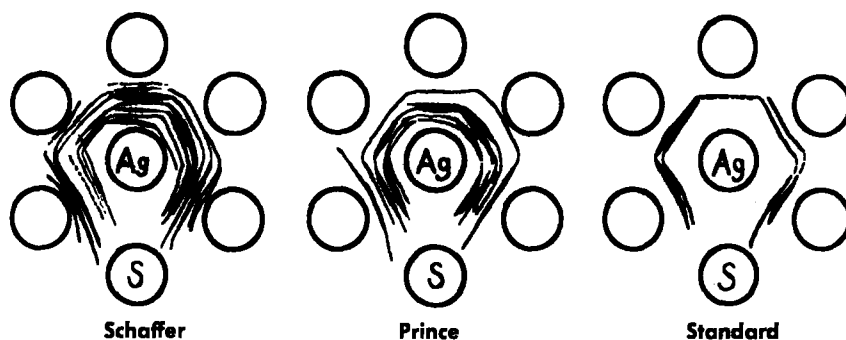


Fig. 1

Comparison of the antigenicity of mold extracts prepared by three different procedures. The peripheral wells contain antisera from different rabbits immunized with the homologous antigen placed at the central well. S = saline, Ag = *Aspergillus* extract.

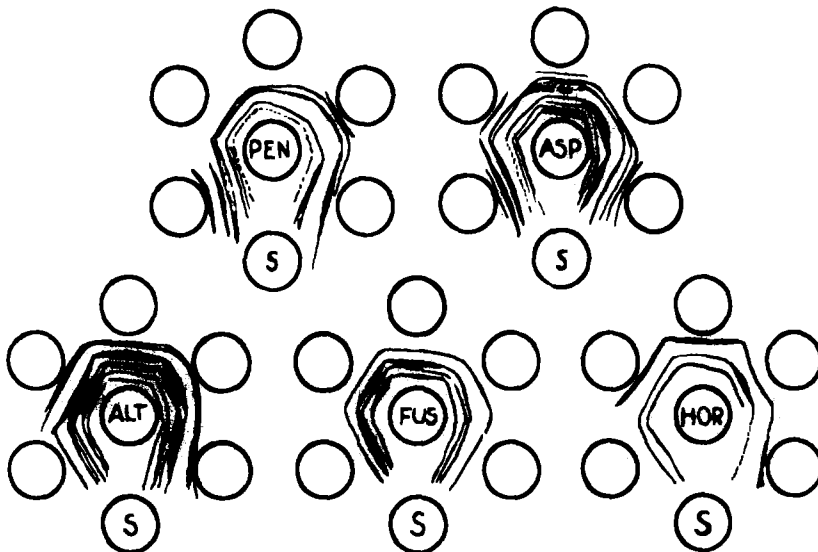
RESULTS**Antigenicity of extracts prepared by various procedures**

The antisera from rabbits immunized with extracts of *Aspergillus* prepared by the standard, modified Prince and Schaffer procedures were tested against the homologous extract by gel diffusion. The extracts were adjusted to a concentration of 0.2 mg. N per milliliter, but the bands obtained at that concentration were too faint. Sharp bands of precipitation were obtained when the extracts were concentrated 10 times by pervaporation. There were individual differences in immunologic response among the rabbits injected with a given extract, but it was evident that the pattern of response obtained with the Schaffer extract suggested a stronger immunogenic activity when compared with the Prince and Standard extracts. This can be readily seen in Fig. 1, where an average of 6 to 8 precipitin bands appear in the Schaffer extract versus 4 to 6 bands given by the Prince extract and 2 bands given by the Standard extract. In subsequent work the Schaffer procedure was used for the preparation of the extracts.

The patterns of precipitation reported in this, as well as in the following experiments, represent the optimum number of precipitin bands obtained after no less than 5 trial bleedings were carried out. The experiments with the final working antisera in all cases were repeated and found to be reproducible.

Antigenicity of the 5 molds selected for study

Our attempt to induce potent antisera by hyperimmunization of rabbits with 5 different fungi resulted in variations in the immunologic response to the

**Fig. 2**

Immunological response of rabbits to 5 different mold extracts. The peripheral wells contain the sera from the individual rabbits. The central wells contain the homologous mold extracts. Pen = *Penicillium*, Asp = *Aspergillus*, Fus = *Fusarium*, Hor = *Hormodendrum*, S = Saline.

different genera. All the extracts were prepared following the same procedure, and the immunization schedules were identical. The mold cultures were allowed to grow until sporulation was optimum. Figs. 2 and 3 give the results of testing the sera in Ouchterlony plates with the homologous and heterologous antigens, respectively. It may be seen that *Alternaria*, *Aspergillus*, and *Penicillium* gave fairly good antigenic stimulation, while the response to our strains of *Fusarium* and *Hormodendrum* was weaker. Sephadex fractionation of the *Hormodendrum* extract was postponed until a more potent antiserum could be obtained.

The testing of the antisera against the heterologous strains showed evidence of the presence of specific antigens in all the strains, but at the same time there was cross-reactivity in all cases with one or more of the other species.

Fractionation of extracts by Sephadex G-200 gel filtration

The results of the fractionation by Sephadex of *Aspergillus*, *Alternaria*, *Fusarium*, and *Penicillium* are shown in Figs. 4 to 7. In the case of *Alternaria* and *Fusarium*, two main peaks were obtained which absorbed light at $280\text{ m}\mu$, a small one which eluted first and a larger one which eluted last. *Aspergillus* and *Penicillium* also gave these two peaks, but in addition there was definite evidence of an intermediate peak which partly overlaps the larger peak. With all mold extracts, the fractions comprising the larger peak were characterized by brownish pigmentation, which suggests that the mold pigments are concentrated in this area. The *Aspergillus* fractions were stained with ninhydrin (see the slanted broken lines at the top of Fig. 4). Very marked ninhydrin staining was

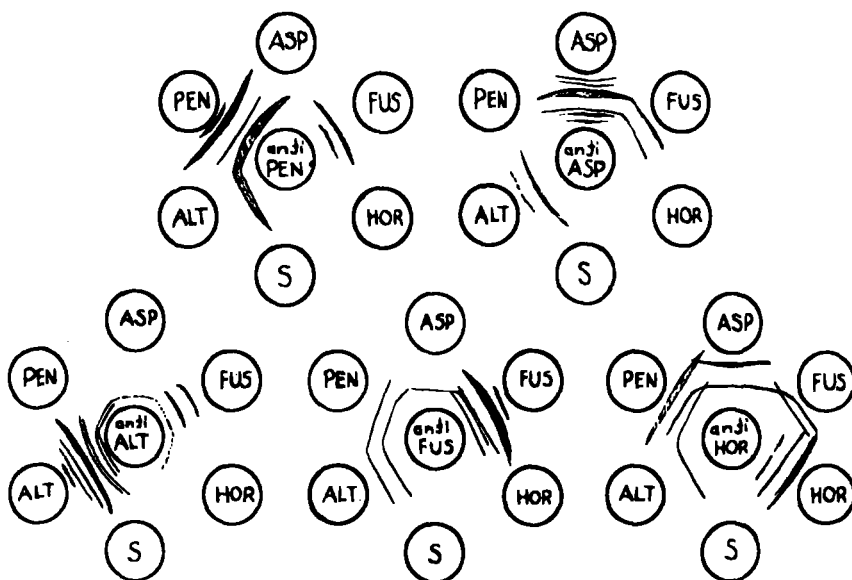
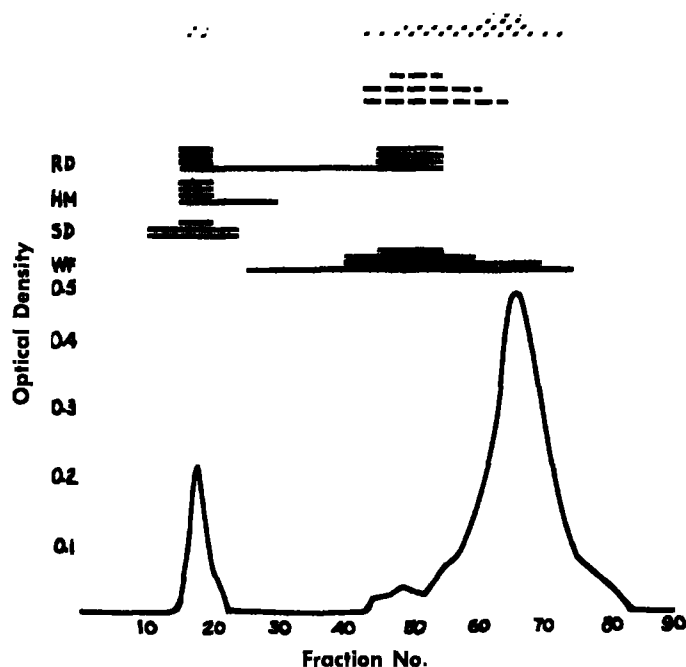


Fig. 3
Specific and cross-reactivities between different molds. The peripheral wells contain the mold extracts. The central wells contain the antisera. Alt = *Alternaria*, Fus = *Fusarium*, Hor = *Hormodendrum*, Pen = *Penicillium*, Asp = *Aspergillus*, S = Saline.

**Fig. 4**

Fractionation of *Aspergillus* extract by Sephadex G-200. The slanted broken lines at top of figure represent ninhydrin staining. The straight broken lines show the number of precipitin bands. The straight solid lines represent skin tests, with the number of lines meaning the approximate intensity from 1 + to 4 +.

obtained in fractions corresponding to the larger peak and a slight staining in the zone of the other peaks.

Gel-diffusion studies and skin testing in sensitive patients were carried out with the *Aspergillus* fractions. The results of the gel-diffusion studies are indicated in Fig. 4 by the straight broken lines. The location of 3 precipitin bands did not coincide with the fractions containing the larger peak but coincided with the intermediate peak. The straight black lines represent the results of the skin tests, the number of lines from 1 to 4 indicating the approximate intensity of the reactions. Two patients (R. D. and W. F.) exhibited marked skin reactivity with the fractions containing the intermediate peak, where precipitation reactions were also obtained. Three patients (R. D., H. M., and S. D.) also exhibited skin reactivity with the fractions containing the first peak, but these fractions failed to precipitate in the gel-diffusion plates. It is evident, therefore, that though allergenic activity is located in these 2 sets of fractions, individual patients may exhibit sensitivity with either, or sometimes both of them.

The results obtained with *Alternaria* extract are shown in Fig. 5. Two peaks resembling the small and large peaks of *Aspergillus* were obtained, but the intermediate peak was not discernible. Marked skin reactivity and precipitation was obtained, however, in the fractions corresponding to the region where the

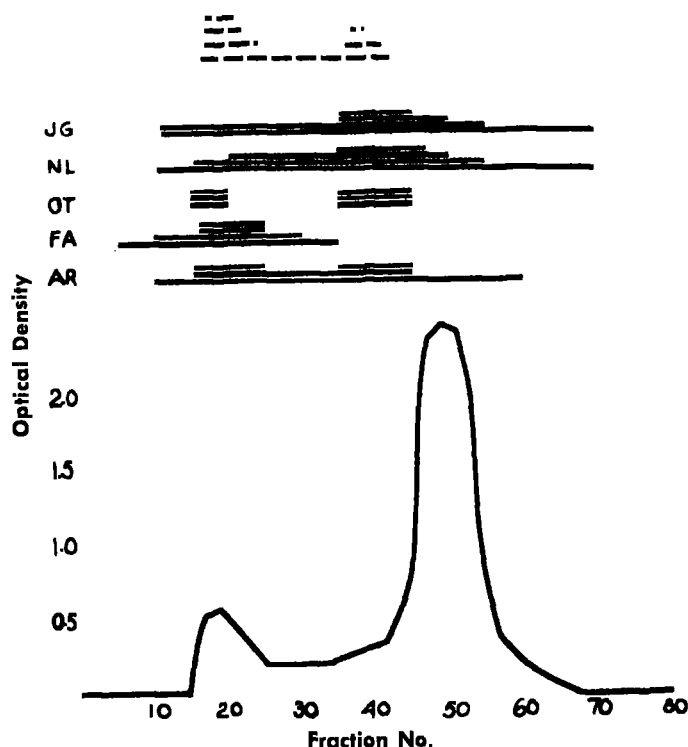
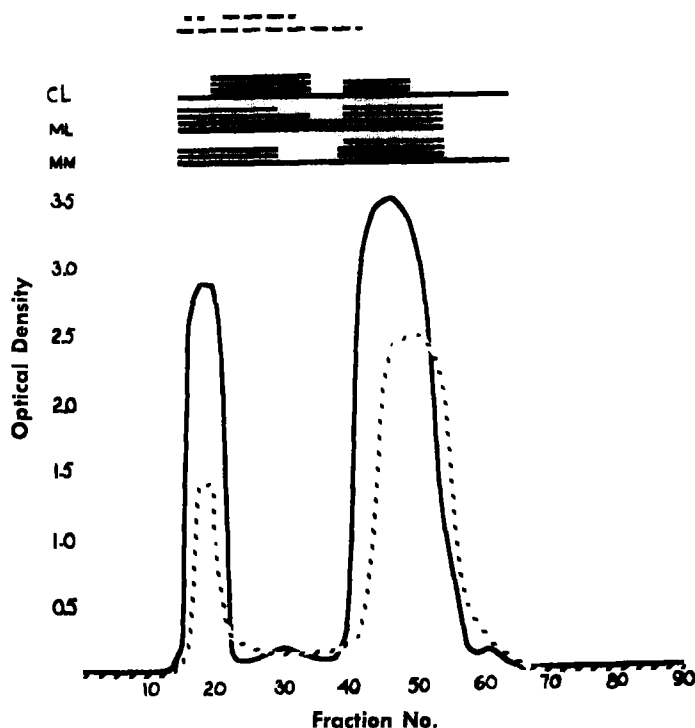


Fig. 5
Fractionation of *Alternaria* extract by Sephadex G-200. Broken and solid lines have same meaning as in Fig. 4.

intermediate peak and the large peak overlap in other species. *Alternaria* differed also from the other molds in the marked precipitation given by the fractions in the zone of the small peak. Four bands of precipitation as well as positive skin reactions in *Alternaria*-sensitive patients were obtained. In general, *Alternaria* followed the same pattern of skin reactivity as that given by *Aspergillus* extract.

The studies carried out with *Fusarium* extract resulted again in two peaks (Fig. 6). Gel-diffusion studies in this case gave precipitation in all the fractions from those containing the small peak to the first 4 of the approximately 20 fractions comprising the large peak. The skin tests showed reactivity in the fractions of the two peaks, but in some cases considerable reactivity was obtained also in the fractions eluting immediately after the first peak. These findings run parallel with the observation of precipitation in the gel studies made with these fractions.

The *Fusarium* fractions were examined also for carbohydrate by means of the anthrone reaction. The results, in terms of micrograms of carbohydrate per milliliter, are plotted in Fig. 6 by means of broken slanted lines. The peaks of greatest carbohydrate concentration coincided with the protein peaks, which

**Fig. 6**

Fractionation of *Fusarium* extract by Sephadex G-200. Broken and solid lines have same meaning as in Fig. 4. The broken slanted line represents the anthrone reaction.

suggests that the protein components in these peaks may be coupled to carbohydrate.

The results obtained with *Penicillium* extract are shown in Fig. 7. There is remarkable similarity with the other molds, but in this case the intermediate peak was quite definite and gave strong precipitation bands as well as skin reactivity. Four out of 5 *Penicillium*-sensitive patients reacted markedly to the components of the first peak.

Studies with the locally isolated *Alternaria* strain

An attempt was made to determine if the locally isolated strain of *Alternaria* behaved in an analogous way to the strain of *Alternaria* we had studied in Buffalo. The Buffalo strain possessed 2 thermostable components which remained stable and had skin reactivity even after autoclaving. It had 4 other thermolabile components.

The local strain gave rise to 7 bands of precipitation, 4 of which were still detectable after 10 minutes' autoclaving at 121° C. (Fig. 8, A). An additional experiment was performed which demonstrated that Sephadex fractionation may be useful in separating the thermolabile from the thermostable components of *Alternaria*. Fractions 17 to 20, comprising the small Sephadex peak, were

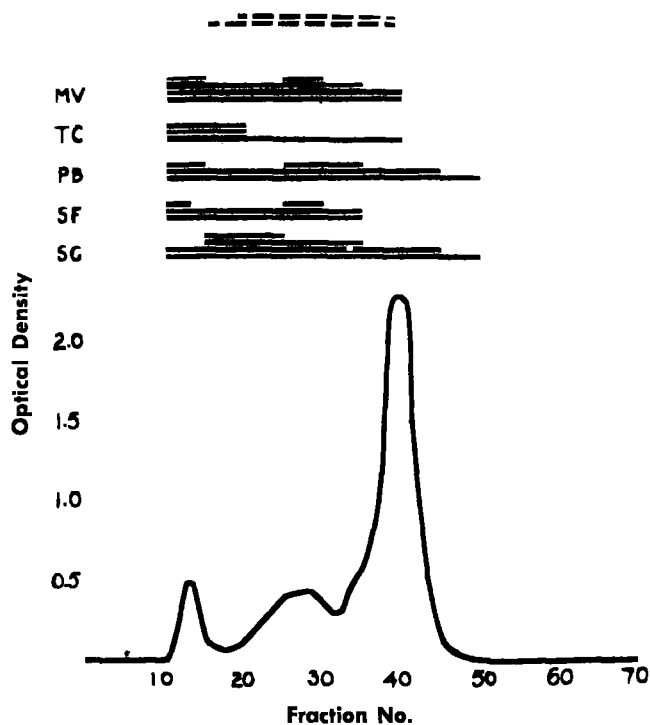


Fig. 7
Fractionation of *Penicillium* extract by Sephadex G-200. Broken and solid lines have same meaning as in Fig. 4.

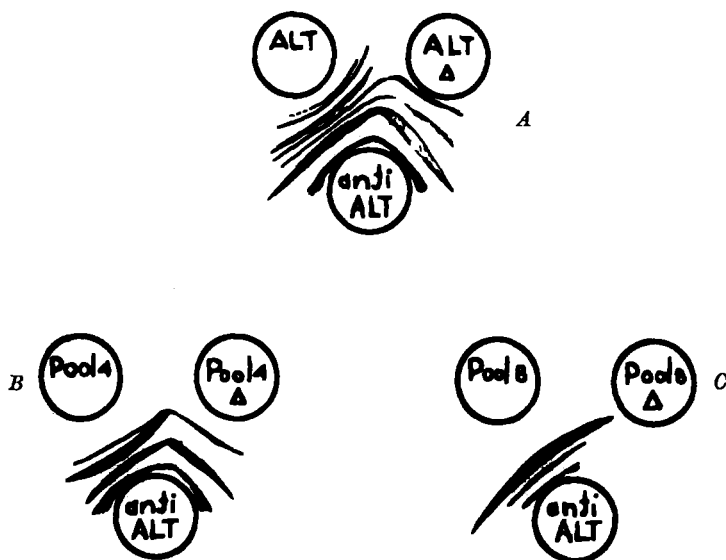


Fig. 8
Effect of heating on the antigens of *Alternaria*. *A*, Unheated and heated (Δ) whole extract. *B*, Unheated and heated (Δ) pooled Fractions 17 to 20 (Pool 4) obtained from a Sephadex G-200 column as described in the text. *C*, Unheated and heated (Δ) Fractions 36 to 42 (Pool 8) from the Sephadex column.

pooled (Pool 4) and divided into 2 equal portions. They were placed in sealed tubes, one of which was autoclaved for 10 minutes. The same procedure was followed with Fractions 36 to 42 representing the slope of the large peak, where 3 bands of precipitation had been obtained. These are labeled Pool 8. The results are shown in Fig. 8, *B* and *C*. They show that all the thermostable components were concentrated in the small peak (Pool 4). On the other hand, the 3 bands of precipitation given by the fractions in the slope of the large peak resulted from thermolabile antigens (Pool 8).

Our local strain of *Alternaria* also resembles the Buffalo strain in its behavior in serologic tests. The crude, unheated antigen is unable to fix itself directly to tannic acid-treated cells, but is able to do so after the antigen is heated. We are now evaluating the bis-diazotized benzidine test (BDB)⁹ in the detection of antifungal antibodies, and preliminary results suggest it may be useful for this purpose.

DISCUSSION

The allergenic molds isolated in Puerto Rico possess numerous extractable antigens, some peculiar to the individual genus but others shared among the various genera. In some cases as many as 8 different antigens were detectable by gel-diffusion studies, though, in general, the extracts have poor antigenicity in rabbits, and intensive immunization is required in order to induce good precipitin patterns. The presence of common antigens among different mold genera brings out the question as to whether or not these common antigens represent allergenic components. If this were the case, sensitization of a patient to one genus could cause symptoms of allergy when contact with many other different mold genera is made. This could also mean that hypersensitization with a single mold extract could be of benefit against a large number of molds. On the other hand, if the specific antigens are allergenic, a single genus could be responsible for the symptoms in a given patient. Obviously we must isolate and study these antigens individually in order to be able to answer this question.

The antigens are released into the liquid medium during the growth of the fungi and the best recoveries, in terms of number of precipitin bands, are obtained when the whole growth, matt and medium together, are homogenized and extracted.

The fractionation of the mold extracts by Sephadex G-200 resulted in similar protein, precipitin, and skin-reactivity patterns. Two peaks which absorbed light at 280 m μ were obtained, but in some cases (*Aspergillus*, *Penicillium*) a third, intermediate peak was present which partly overlapped the large peak which eluted last. Skin reactivity and precipitation were localized in the small and middle peaks. The larger peak gave negative skin reactivity and precipitation tests but had a very marked ninhydrin staining reaction suggestive of a high amino acid content, and the fractions appeared pigmented to the naked eye. There were individual variations among the patients in their reactions to the various molds, some reacting to both the small and intermediate peaks but others reacting to only one of them. When the middle peak was absent,

skin reactivity was observed in the first fraction of the large peak, where the overlapping intermediate peak was present in the other species.

The results seem to have demonstrated also that the allergenic components in common airborne molds can be divided into two groups on the basis of molecular size. These groups are not homogenous, and each contains anywhere from 1 to 4 antigens.

In the case of *Alternaria*, it was found that the 4 thermostable antigens came out from the column first, with the small peak, and are therefore of a larger molecular size than the 3 thermolabile antigens which came out later in the middle peak or, when this was absent, in the first fraction of the larger peak. It would require separation and skin testing of the individual components in order to identify the factor or factors responsible for skin reactivity.

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