

THE ISOLATION AND CHARACTERIZATION OF A PURIFIED HOUSE DUST ALLERGEN FRACTION*

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THE importance of house dust sensitivity in clinical allergy has been appreciated since the early observations of Cooke.¹ Even though house dust is a very heterogenous mixture clinically, house dust sensitivity appears to be due to a unique allergen or group of allergens.

For many years aqueous extracts of house dust have been used for diagnostic tests and hyposensitization. Boatner and Efron² were among the first to attempt to concentrate and study the chemical properties of the skin-reacting materials in house dust. They fractionated an aqueous extract of house dust with dioxane and then with ammonium sulfate, and they obtained a material that was a potent allergen and gave positive tests for both protein and carbohydrate. Sutherland,³ in 1942, prepared potent extracts of house dust by extraction of the dust with 0.01 N ammonia solution and adsorption of the active material on benzoic acid. The material was found to consist largely of carbohydrate. Rimington and colleagues⁴ have carried out further purification by acetone fractionation of dust allergen prepared by Sutherland's method. Their purified allergen fraction contained both carbohydrate and polypeptide. Additional information regarding the composition of this material has been presented as an unpublished communication in a paper by Cayton and associates⁵ on a comparison of the properties of extracts of cotton dust and house dust. The purified dust allergen fractions were found to contain 40 to 60 per cent hexose (equivalent galactose by the orcinol method) and 5 to 7 per cent nitrogen. Hydrolysis and paper chromatography indicated the presence of nine aliphatic amino acids, galactose, arabinose, xylose, and a very small amount of rhamnose and glucosamine. The possibility of a trace of galacturonic acid was also mentioned. Rimington⁴ also reported the presence of two main electrophoretic components (phosphate buffer, pH 8, $\mu = 0.2$) of about equal skin reactivity and equal nitrogen and hexose contents. On the basis of partial

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hydrolysis studies in which about 80 per cent of the carbohydrate was liberated without loss of amino acids and without loss of skin reactivity, Rimington concluded that the biologic activity was associated with the component containing amino acid rather than the carbohydrate component. On further hydrolysis, skin reactivity was lost when amino acids appeared in the hydrolysate.

We have fractioned aqueous extracts of house dust* and mattress dust† with ammonium sulfate and a variety of organic solvents. In general, most of the fractions were of a similar order of activity by direct skin test. The least soluble materials had the higher nitrogen content (2 to 5 per cent), while the more soluble fractions had lower nitrogen contents (0.3 to 2 per cent). In this article, we describe the isolation and characterization of an active dust allergen fraction,‡ low in both nitrogen and color and consisting predominantly of a heterogenous mixture of acidic polysaccharides.

ISOLATION AND CLINICAL TESTING OF PURIFIED DUST FRACTION

A mixture of mattress dust and house dust was wetted with water, frozen, and thawed, and the fluid was removed with a large mechanical press. The aqueous extract was filtered, concentrated *in vacuo*, and then fractionated at one-half saturation in ammonium sulfate. The material soluble at this salt concentration was dialyzed free of ammonium sulfate and then precipitated three times with methanol. An aqueous solution of the methanol-precipitated material was dialyzed against water, and colored material was removed by

TABLE I. SKIN TESTS REPORTED BY HOLLISTER-STIER LABORATORIES

PATIENT	ENDO 1/1,000	PREPARATION* AND TEST RESULT†			
		CALTECH DUST		H-S DUST	
		1/500	1/1,000	1/500	1/1,000
L.	3	1	1	1	1
C. D.	2	1	1	2	1
F. B.	3	3	2	3	3
S.	2	2	1	2	1
Y.	2	1	2	1	1
M. W.	3	2	1	2	?
F.	4	4	?	3	?
Y.	2	3	0	1	0
D. M.	3	2	2	2	0
K.	1	0	0	1	0
H.	4	3	2	3	1
H. R. S.	3	2	1	1	0
D. H.	3	1	0	0	0
D. L.	4	?	0	4	3
G.	1	1	0	1	?
A.	2	2	1	2	2

*The dust preparations were as follows: Endo, House Dust Concentrate, Endo Laboratories, Inc.; Caltech Dust, the authors' purified dust extract; H-S Dust, Hollister-Stier Dust Extract. The dilutions were made on a dry weight basis.

†The skin reactions were graded 1+ through 4+. The symbol ? indicates a questionable reaction, and 0 indicates a negative reaction. These were intradermal skin tests.

*Collected and donated by various members of the American Academy of Allergy.

†A pool of about 200 pounds of mattress dust from a mattress-cleaning establishment in Atlanta, Georgia, was sent to us by Dr. McK. Piples.

‡This is the same dust fraction as the extract C discussed by Follensby, Lowell, and Schiller (J. ALLERGY 27: 103, 1956).

fractional precipitation with basic lead acetate solution.⁶ The solution was centrifuged after each addition of basic lead acetate to remove colored material. The lead was removed from the decolorized fraction by means of hydrogen sulfide and centrifugation. The supernatant was dialyzed against water, and the final product was obtained by methanol precipitation. The precipitate was dried and stored in a vacuum desiccator over calcium chloride. A schematic diagram of this fractionation procedure is presented in Fig. 1.

Dust was wetted with a minimum amount of water and frozen; then it was thawed and the liquid was removed with a press.

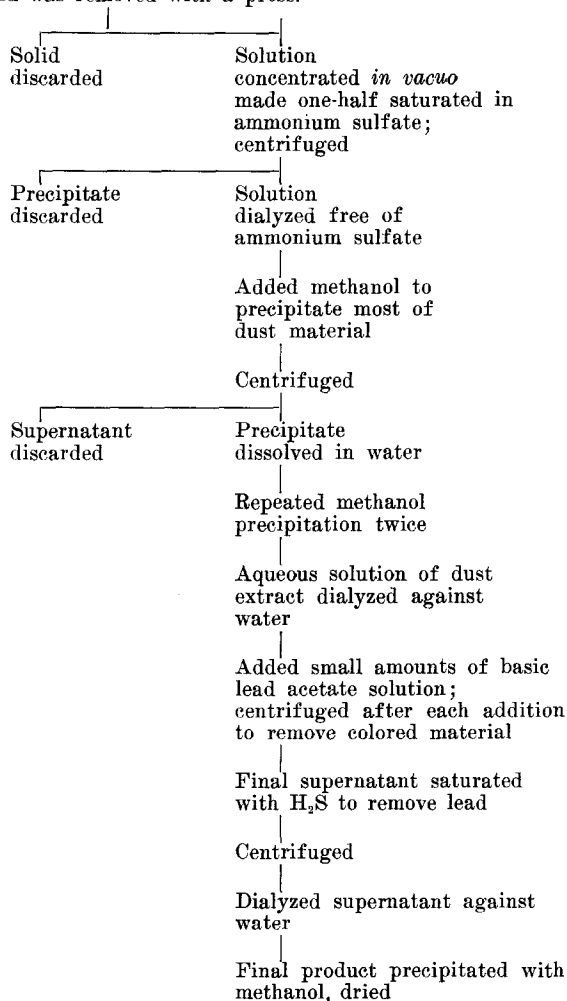


Fig. 1.—Method of preparation of the purified house dust allergen fraction.

The Hollister-Stier Laboratories carried out a series of skin tests on patients sensitive to house dust to compare the reactivity of the lead-acetate-purified fraction with two commercial preparations. The data are presented in Table I.

PHYSICAL PROPERTIES OF PURIFIED FRACTION

The allergen fraction was an amorphous white powder which was readily soluble in water to produce a clear, colorless solution.

The optical rotation of a 1 per cent solution of the material in water was determined ($[\alpha]_D^{26} = +63^\circ$). This result was subject to considerable error due to the small amount of material available for the determination.

An electrophoretic analysis of the material was carried out in the Perkin-Elmer electrophoresis apparatus, Model 38. A 2 ml. cell with an open system was used in these experiments. A 1 per cent solution of the dust fraction in acetate buffer (pH 4.7, $\mu = 0.1$) was prepared, and the solution was dialyzed against a 1 liter volume of the buffer. A current of 6.0 Ma. was allowed to pass through the cell for 3,600 seconds, and then pictures were taken by the Longworth scanning procedure. A reproduction of the pattern obtained from the descending limb is presented in Fig. 2. The fast peak has a mobility of -8.9×10^{-5} (cm.² sec.⁻¹ volts⁻¹), while the mobility of the slower peak is -4.6×10^{-5} . Area analysis indicates that about 60 per cent of the material is present in the fast peak (neglecting the ϵ boundary). The stationary or salt boundary (ϵ boundary) probably contains uncharged material as well as the usual salt gradient; however, the magnitude of this effect is not known.

Sedimentation studies were carried out in the Spinco Model E ultracentrifuge; buffer of the same composition as that employed for the electrophoresis experiment was used. A number of runs were made at different allergen concentrations, so that an $S_{w,20}^\circ$ value (sedimentation coefficient extrapolated to infinite dilution) could be obtained. All the samples were run in a synthetic boundary cell, since the sedimentation coefficients were so low that boundaries did not readily form in a regular cell. The buffer against which the sample had been dialyzed was used to form the boundary in the cell. In all cases, the experiments were carried out at full speed (59,780 r.p.m. or approximately 250,000 g) with the temperature of the rotor controlled close to its initial temperature after gaining full speed. Schlieren pictures were taken every eight minutes. From projections of these pictures, the positions of the concentration gradients at various times were determined and the uncorrected sedimentation coefficients were calculated. By the use of Svedberg's tables⁷ and the assumed value of 0.6 for the partial specific volume ($\bar{v}$) of the dust material, the sedimentation coefficients were corrected to standard conditions. The value of 0.6 was based on Säverborn's⁸ determination of the partial specific volumes of various polyuronides. One of the sedimentation patterns obtained is reproduced in Fig. 2. Each pattern shows a single symmetrical peak. The corrected sedimentation coefficients ($S_{w,20}$) are plotted in Fig. 3, and the extrapolation to infinite dilution to obtain $S_{w,20}^\circ$ (2.6S) is shown. In order to obtain a more nearly linear extrapolation of the $S_{w,20}$ values to $S_{w,20}^\circ$, a plot of the reciprocal ($1/S_{w,20}$) of the $S_{w,20}$ values versus concentration was used to evaluate $S_{w,20}^\circ$ rather than the direct plot shown in Fig. 3.

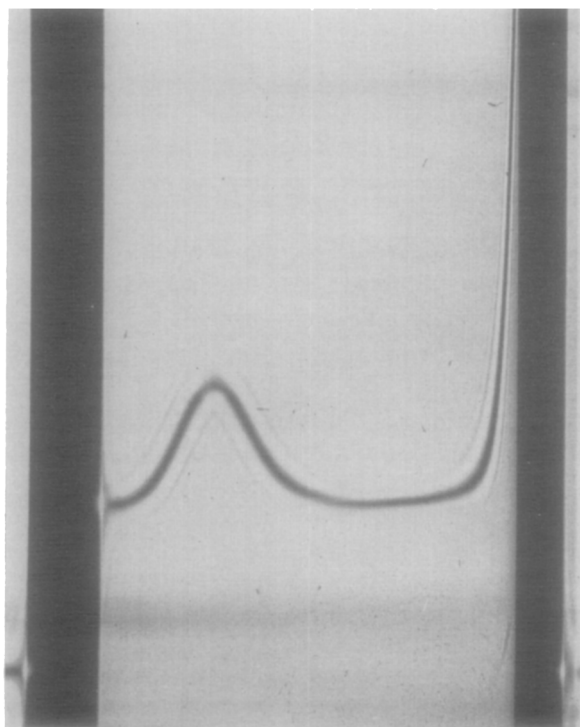
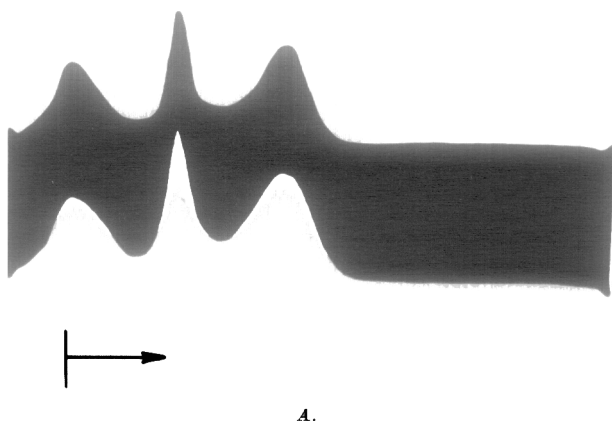


Fig. 2.—Electrophoretic and sedimentation patterns of a 1 per cent solution of the purified dust fraction in acetate buffer, pH 4.7, μ 0.1. *A*, Descending electrophoretic pattern after 3,600 seconds. *B*, Sedimentation pattern after 2,880 seconds. The vertical line on the electrophoretic pattern indicates the position of the starting boundary, and the arrow shows the direction of motion of the boundaries.

CHEMICAL COMPOSITION OF PURIFIED FRACTION

Elementary Analysis.—A sample of the purified dust fraction was submitted to a commercial laboratory* for analysis. The sample contained 38.37 per cent carbon, 5.98 per cent hydrogen, 1 per cent nitrogen, 7.9 per cent ash, and 54.65 per cent oxygen by difference after ash correction. With the ash correction, and neglecting the nitrogen content, this leads to an empirical formula of the type $[C_{1.09}(H_{2.04}O_{1.00})]_n$. A phosphorus analysis was carried out by the method of Allen.⁹ The material was found to contain 0.1 per cent phosphorus. It has been found in the case of other dust fractions that the ash content may be reduced to zero by electrodialysis.

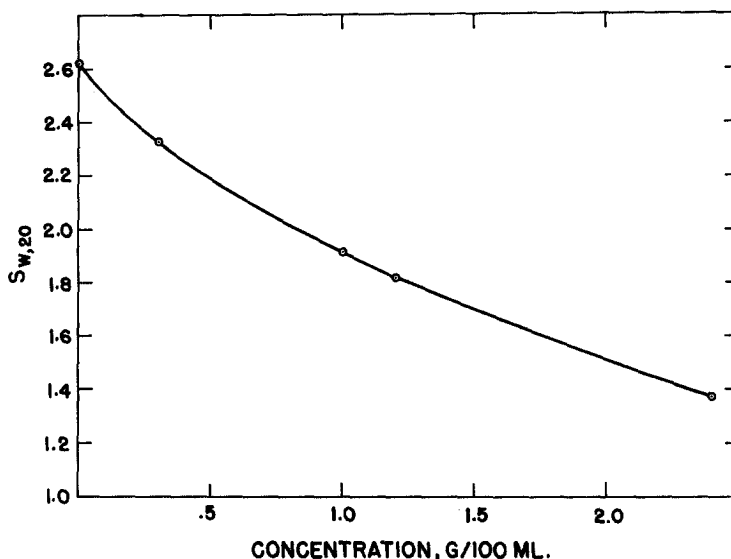


Fig. 3.—Concentration dependence of the sedimentation coefficient ($S_{w,20}$) of the purified dust fraction (acetate buffer, pH 4.7, μ 0.1) with extrapolation to infinite dilution to obtain $S^{\circ}_{w,20}$.

Hexosamine Estimation.—The Elson-Morgan procedure as modified by Rimington^{10, 11} was employed. Weighed samples of the purified fraction (about 12 mg.) were hydrolyzed in sealed test tubes in an oven (105° C.) with 3 ml. of 0.5 N sulfuric acid for eight hours. It had previously been determined by the use of crude dust extracts that these hydrolysis conditions were suitable for the hexosamine determination. The crude dust extracts had been found, by this method, to contain about 2 per cent equivalent hexosamine, while the purified material was found to have 0 per cent equivalent hexosamine.

Determination of Total Reducing Substances.—Weighed samples (about 3 mg.), dissolved in 0.5 N sulfuric acid, were hydrolyzed by heating in sealed tubes in an oven at about 105° C. It was found that a maximum yield of reducing substance was obtained after heating for about 105 minutes. The

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samples were analyzed by the method of Hagedorn-Jensen¹² as described by Kabat and Mayer.¹³ D-glucose was used as the standard substance for the determination. The maximum yield of reducing substances obtained was 80 per cent (equivalent glucose).

Titration of a Sample of the Purified Dust Fraction.—A carefully weighed sample of the dust fraction (11.6 mg.) was dissolved in 5 ml. of 1 per cent sodium chloride solution. The pH of the solution was measured with a Beckman Model G pH meter and found to be 4.8. A small volume (0.1 ml.) of dilute sodium hydroxide solution was added to make the solution alkaline, and then small volumes of standard hydrochloric acid solution (0.0206 N) were added from a calibrated 2 ml. buret and pH readings were made after each addition. From pH 2.5 to pH 1.7, a more concentrated standard hydrochloric acid solution (0.206 N) was used, so that the volume would not become too large. A blank titration was carried out with 5.0 ml. of 1 per cent sodium chloride solution in the same manner as in the case of the dust fraction. Curves showing the relationship between the pH and the milliequivalents of hydrogen ion added were plotted for both the dust sample and the blank. The difference between the two curves for any particular pH represents the number of milliequivalents of hydrogen ion bound by the dust sample. A plot of the hydrogen ion bound as a function of pH is shown in Fig. 4. The shape of the curve is consistent with the idea that carboxyl groups are being titrated between pH 2.0 and pH 5.5. If we take the number of milliequivalents of hydrogen ion bound between pH 2.0 and pH 5.5 as the acid-binding capacity of the dust sample, then the calculated equivalent weight is 750. If the material were a polyhexose, one out of four of the residues would have a carboxyl group.

Color Reactions for Uronic Acids.—A number of color tests for uronic acids have been explored according to the methods of Dische.¹⁴

The carbazole reaction^{14, 15} for uronic acids was carried out with a 500 γ sample of the purified dust fraction. The corrected spectra obtained with the dust sample and samples of D-glucuronolactone and D-galacturonic acid are presented in Fig. 5. It is not possible to make a quantitative estimate of the uronic acid content of the dust polysaccharides on the basis of such data, as free uronic acid and polysaccharide uronic acid do not necessarily give equivalent color production. The curves obtained with the dust polysaccharide indicate the presence of uronic acid.

A color reaction test for glucuronic acid with thioglycollic acid and sulfuric acid in the presence of mannose^{14, 16} was carried out. A 500 γ sample of the purified dust fraction was used for this test with suitable blanks and controls. The resulting spectra showed an absorption maximum at 535 $m\mu$; however, this absorption peak was obscured by a much larger absorption maximum at 610 $m\mu$ (Fig. 6, curve A). This latter peak is probably due to the presence of other carbohydrate components of the dust fraction. A fraction was obtained from a crude house dust extract that contained the uronic acids with much less of the other sugar components. The dust extract was hydrolyzed by heating in dilute sulfuric acid; then it was neutralized, and the acidic carbohydrate components were removed from the solution by adsorption on a basic

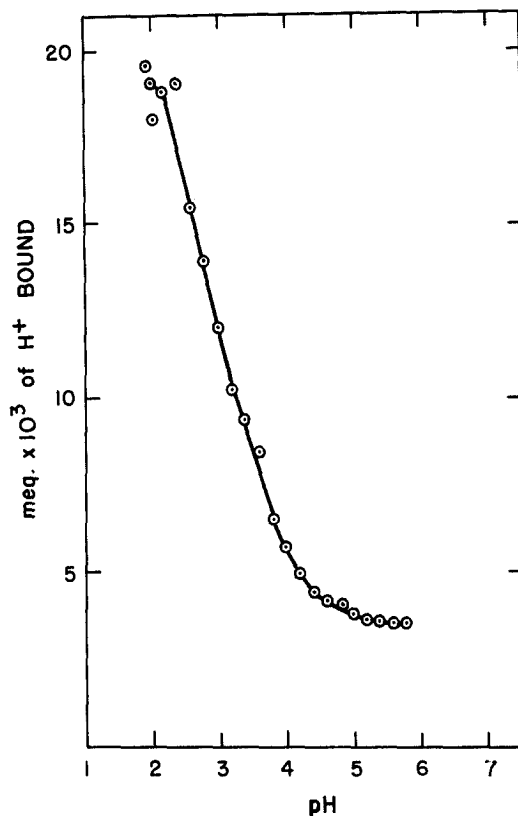


Fig. 4.—Titration curve of a sample (11.6 mg.) of the purified dust fraction.

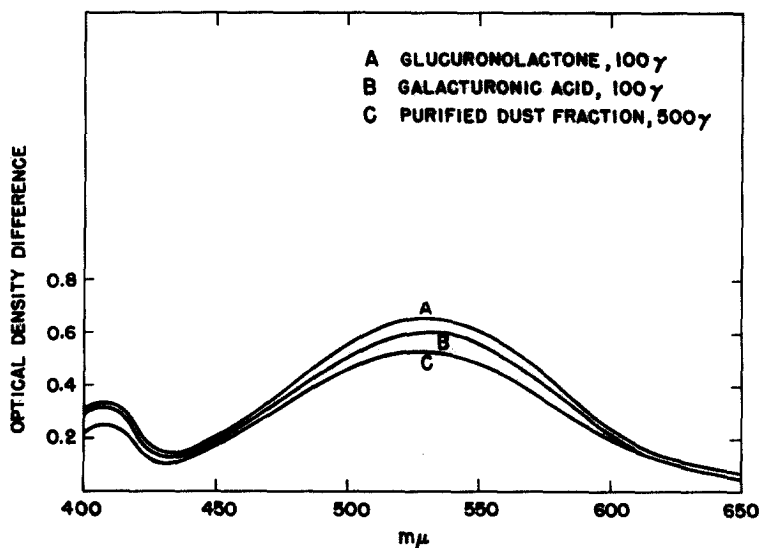


Fig. 5.—Carbazole test for uronic acid.

ion exchange resin column. The column was washed to remove the neutral sugars and the acidic sugar components eluted with dilute acid. This fraction showed an absorption peak at $535\text{ m}\mu$ free from the interference of the $610\text{ m}\mu$ absorption (Fig. 6, curve B).

The cysteine color reaction for galacturonic acid^{14, 17} was used to test for the presence of galacturonic acid in the dust fraction. A 2.0 mg. sample of the purified dust fraction was tested by this method and found to be completely negative.

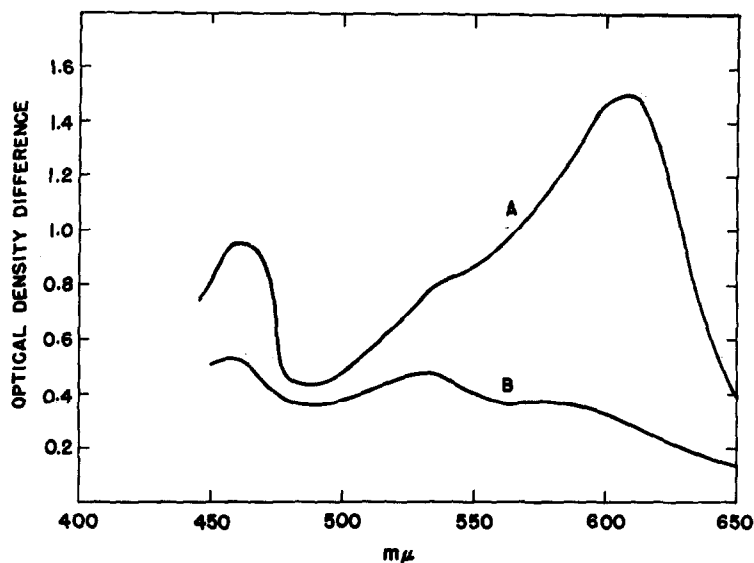


Fig. 6.—Thioglycollic acid test for glucuronic acid. Curve A, 500γ of purified dust fraction. Curve B, Acidic hydrolysis components from an ion exchange resin column.

Hydrolysis and Paper Chromatography.—Five milligram samples of the purified dust fraction were hydrolyzed by refluxing in 2 ml. volumes of 0.5 N sulfuric acid for two and ten hours. The samples were neutralized with barium carbonate and filtered to remove the barium sulfate and the excess barium carbonate. To concentrate the filtrates to about 0.2 ml., the samples were placed under an infrared heat lamp and a fan. These solutions were used to spot paper chromatograms.

Paper chromatography was carried out by the ascending method described by Williams and Kirby.¹⁸ Sheets of Whatman No. 1 filter paper (36×40 cm.) were rolled into cylinders 40 cm. in length and held by four staples. The experiments were carried out in pipette jars (6×18 inches) covered with glass plates. The hydrolysates were compared with each other and with known sugars in two solvent systems: pyridine-*n*-butanol-water (2, 3, 1.5 by volume)¹⁹ and water-saturated phenol.²⁰ A 1.6 per cent solution of *p*-anisidine hydrochloride in ethanol 1.5 parts and *n*-butanol 2 parts, by volume,²¹ was used as the spot-locating reagent. The dried sheets were sprayed with the *p*-anisidine solution and then heated in an oven at about 105°C . for five to ten minutes.

It was found that the length of time of hydrolysis (two hours versus ten hours) had no effect on the appearance of the chromatograms. The spots from the dust hydrolysate were compared with the spots from known sugars on the same chromatogram. On this basis, a trial mixture of known sugars was prepared as follows: D-glucose, 10 mg.; D-galactose, 10 mg.; D-mannose, 10 mg.; L-rhamnose, 5 mg.; L-arabinose, 2.5 mg.; and water, 1.0 ml. The relative amounts of the sugars used in the standard solution were based on a visual appraisal of the relative intensities of the spots on the paper chromatogram. A comparison of the sugar spots from the dust hydrolysate with those of the standard solution in the pyridine-*n*-butanol-water and the phenol-water solvents showed agreement in those two solvent systems. As a further check on the identity of the sugar components, these solutions were compared by chromatography in three additional solvent systems: *n*-butanol saturated with water, tert.-pentanol-*n*-propanol-water (4, 1, 1.5 by volume),²² and ethyl acetate-acetic acid-water (2, 1, 1.2 by volume).²³ The Rf's of the sugars were low in the butanol-water and pentanol-propanol-water solvents. Therefore, the chromatograms were run three times in these solvents. The sheets were dried after each run. As with the first two solvents, the sugar spots from the two solutions showed good agreement as to both position and size of the spot on the chromatogram and color when sprayed with the p-anisidine hydrochloride reagent.

A control experiment was carried out to determine the effect of the hydrolysis conditions and the method of preparation of the samples for chromatography on known sugars. D-glucose, D-galactose, D-mannose, D-glucuronolactone, and D-galacturonic acid were individually carried through the hydrolysis and preparatory procedure. The hexoses were examined chromatographically and were found to be identical to samples not carried through the procedure. There was no evidence of any epimerization. Both the D-glucuronolactone and D-galacturonic acid samples were destroyed during this method of hydrolysis and sample preparation.

PREPARATION OF DERIVATIVES OF SUGARS ISOLATED FROM CRUDE HOUSE DUST FRACTIONS

In order to provide more definite evidence for the identity of the sugars found in the purified dust allergen fraction, derivatives of sugars isolated from house dust extracts were prepared and compared by melting point and mixed melting point with the same derivatives prepared from authentic sugar samples. As sufficient material was not available to carry out these studies with the purified fraction, it was necessary to use crude dust extracts as the source of the sugars for preparation of derivatives. In general, it has been found that substituted hydrazones are easier derivatives to prepare on a 2 to 3 mg. scale than benzimidazoles or osotriazoles. The methods used to prepare the substituted hydrazones were based on the procedure given by Cheronis and Entrikin²⁴ and Van der Haar.²⁵ Some of the hydrazines are more or less specific precipitants for a particular sugar under a given set of conditions. In these cases, the

derivative could be prepared from a hydrolysis mixture without preliminary separation of the sugars. In other cases it was necessary to make a separation of the sugars before the preparation of the derivative. The method used to fractionate the sugar hydrolysate involved the use of ascending paper chromatography at elevated temperatures (50 to 60° C.).^{21, 26}

Fractionation of a Hydrolysate by Paper Chromatography at 50 to 60° C.—A sample of pooled crude dust extract (ca. 400 mg.) was hydrolyzed by refluxing two hours with 180 ml. of 0.5 N aqueous sulfuric acid. The hydrolysate was neutralized with barium carbonate and concentrated just to dryness. The residue was extracted by mixing with four successive volumes of methanol (100 ml., 50 ml., 50 ml., and 20 ml.). A 20 ml. volume of water was added to the methanol solution, and the volume was reduced to approximately 10 ml. under vacuum. The solution was further concentrated with a stream of nitrogen under a heat lamp to a final volume of 1.2 ml. A 0.1 ml. volume of this concentrated sugar solution was used to make a thin streak 5 cm. from the narrow edge of a 40 × 37 cm. sheet of Whatman No. 1 filter paper. A pad, 3 to 4 cm. wide, of six thicknesses of filter paper was stapled at the top of each chromatogram, and the sheet was folded into a cylinder and stapled. Eight such chromatograms were prepared. They were developed at 50 to 55° C. three times in water-saturated *n*-butanol (saturated at room temperature). The solvent fronts were allowed to ascend to wet the filter paper pads at the top of the chromatograms, and the cylinders were then removed from the jars and dried at room temperature before the next run.

TABLE II. COMPONENTS FOUND IN HYDROLYSATE FRACTIONS OBTAINED BY HIGH TEMPERATURE PAPER CHROMATOGRAPHY

FRACTION	COMPOSITION
I	Rhamnose
II	Fucose trace of rhamnose
III	Arabinose, xylose
IV	Mostly mannose, some araminose
V	Glucose, galactose
VI	Galactose, trace of glucose

Strips 3 cm. wide were cut from both edges of each chromatogram, and the position of the sugar bands was located by use of a spray reagent. With the strip from each edge of the chromatogram serving as a guide, the remainder of the chromatograms was cut in strips so as to provide the maximum separation of the sugars. The corresponding strips from each of the eight chromatograms were extracted with 100 ml. of methanol in a Soxhlet extraction apparatus. Each of the methanol extracts was mixed with 25 to 30 ml. of water and concentrated to a final volume of 5 to 10 ml. by vacuum distillation. The resulting solutions were further concentrated with a heat lamp and a stream of nitrogen to a final volume of 1 to 2 ml. A small sample of each fraction was then chromatographed in the pyridine-*n*-butanol-water solvent following the previously described procedure. The sugars present in each fraction, as determined by the paper chromatograms, are listed in Table II. The same sugars

that were found in the purified dust fraction were found in the crude extract, except that two additional sugars (xylose and fucose) are present in the crude dust hydrolysate.

Optical rotation measurements were made with the aqueous solutions of the fractions I through VI. All the solutions had a positive rotation, and the data were consistent with the presence of the following forms of the sugars in the purified dust fraction: D-glucose, D-galactose, D-mannose, L-arabinose, and L-rhamnose.

Preparation of Derivatives of Mannose: Mannose Phenylhydrazone.—Because of the insolubility of mannose phenylhydrazone, phenylhydrazine has been used for quantitative precipitation of mannose from solutions of sugar mixtures. It was not necessary, therefore, to use the hydrolysate fractions in order to prepare this derivative. A 0.5 Gm. sample of crude dust extract was hydrolyzed by refluxing two hours with 200 ml. of 0.5 N sulfuric acid. The hydrolysate was neutralized with barium carbonate, filtered to remove the barium sulfate and excess barium carbonate, and concentrated to 7 ml. by vacuum distillation; the concentrate was extracted twice with 5 ml. volumes of chloroform to remove any lipid components. Previous experiments had shown that about 2 per cent of the weight of the dust fraction could be extracted from the hydrolysate with chloroform. The sample was concentrated to 1 ml. under a stream of nitrogen and a heat lamp, decolorized by heating with Norite (decolorizing carbon), and centrifuged to remove the Norite. A 0.06 ml. volume of 50 per cent aqueous acetic acid (v/v) and a 0.06 ml. volume of freshly distilled phenylhydrazine were added to the dust hydrolysate solution. The solution immediately became turbid and after one-half hour at room temperature, followed by one-half hour in an ice bath, a brown precipitate had formed. The precipitate was removed by centrifugation, washed three times with cold water, and dried in a vacuum oven at 50° C. The product was found to melt at 184 to 186° C.* A sample of known D-mannose (100 mg.) in 0.4 ml. of water was carried through the same procedure. The product melted at 184 to 185° C. A mixed melting point of the two materials was found to be 184 to 185° C. (literature melting point† D-mannose phenylhydrazone 199° C. recrystallized from 60 per cent ethanol).

Mannose p-Bromophenylhydrazone.—The mannose phenylhydrazone (about 6 mg.) was heated in a boiling water bath with 0.06 ml. of benzaldehyde and 0.12 ml. of water for one hour to decompose the phenylhydrazone and release the mannose. The product was cooled in an ice bath, and the benzaldehyde phenylhydrazone removed by centrifugation. The supernatant was extracted three times with an equal volume of diethyl ether and concentrated under a stream of nitrogen and a heat lamp to a final volume of approximately 0.1 to

*All the melting point determinations have been carried out on a micro hot-stage melting point apparatus.

†All the literature melting points are from Brown, C. A., and Zerban, F. W.: *Physical and Chemical Methods of Sugar Analysis* (ed. 3, New York 1941, John Wiley & Sons, Inc., pp. 685-688), except those for the 2,5 dichlorophenylhydrazones which are from Mandl and Neuberg.²⁷

0.2 ml. An equal volume of absolute ethanol, 6.5 mg. of sodium acetate, and 5.4 mg. of *p*-bromophenylhydrazine hydrochloride were added and the solution was mixed. After two minutes a precipitate formed. The mixture was cooled in an ice bath; the precipitate was collected by centrifugation, washed three times with cold water, and dried in a vacuum oven at 50° C. (melting point 198 to 199° C.). A known sample of D-mannose (10 mg.) was mixed with 15 mg. of sodium acetate, 12.4 mg. of *p*-bromophenylhydrazine hydrochloride, and 0.3 ml. of 50 per cent aqueous ethanol. The product was recovered as in the case of the dust derivative (melting point 199 to 200° C.). The mixed melting point was 199 to 201° C. (literature melting point D-mannose *p*-bromophenylhydrazone recrystallized from 50 per cent ethanol, 208° C.).

The supernatant from which the mannose phenylhydrazone precipitated was centrifuged to remove additional precipitate that had formed and extracted with diethyl ether to remove excess phenylhydrazine. A small sample of this solution was chromatographed. The paper chromatogram showed almost complete depletion of the mannose spot. Over the course of a few days, more precipitate formed and was removed by centrifugation. The supernatant was heated on a boiling water bath with 3 or 4 drops of 50 per cent aqueous acetic acid and 0.03 ml. of benzaldehyde for an hour. An immediate turbidity appeared when the benzaldehyde was added. The mixture was cooled in an ice bath, and the precipitate was removed by centrifugation. The supernatant was extracted four times with two volumes of diethyl ether to remove the excess benzaldehyde. The extracted solution was concentrated and rechromatographed. The paper chromatogram showed the usual dust hydrolysate pattern, except that mannose was completely absent.

Preparation of Galactose Derivatives: Galactose α -Methyl α -Phenylhydrazone.—The sugar solution, depleted in mannose, was concentrated to a volume of 0.3 ml.; 0.1 ml. of 50 per cent aqueous acetic acid and 0.06 ml. of freshly distilled α -methyl α -phenylhydrazine were added, and after ten minutes the precipitate that formed was removed by centrifugation. The precipitate was mixed with a few 0.1 ml. volumes of 30 per cent ethanol and gently heated. Only part of the solid dissolved. The less soluble portion was washed with cold water, dried, and washed with diethyl ether. This material was recrystallized from aqueous ethanol, dissolved in a minimum of absolute ethanol and precipitated by the addition of diethyl ether. The product was washed with diethyl ether and dried (melting point 181 to 183° C.).

A known sample of D-galactose (10 mg.) was mixed with 0.4 ml. of water, 0.1 ml. 50 per cent aqueous acetic acid, and 0.1 ml. of freshly distilled α -methyl α -phenylhydrazine, yielding a white precipitate in one minute. After ten minutes at room temperature, the precipitate was collected by centrifugation, washed three times with water, recrystallized from ethanol (95 per cent), and dried. The product was dissolved in a minimum amount of absolute ethanol and precipitated by the addition of diethyl ether, washed with diethyl ether, and dried (melting point 179 to 181° C.). The mixed melting point with the

dust product was 181 to 183° C. (literature melting point D-galactose α -methyl α -phenylhydrazone recrystallized from 30 per cent ethanol 190 to 191° C.). The galactose α -methyl α -phenylhydrazone derived from the dust extract was decomposed by heating with 0.1 ml. of formalin and 0.3 ml. of water in a boiling water bath for one hour. The solution was taken just to dryness under a stream of nitrogen and a heat lamp. The residue was extracted with two 0.2 ml. volumes of water. The water extract was concentrated to 0.1 ml., and a paper chromatogram was prepared. Only one sugar was found, and this corresponded to galactose in Rf.

Galactose o-Tolylhydrazone.—An attempt was made to prepare this derivative from the galactose obtained by the decomposition of the α -methyl α -phenylhydrazone, but this was unsuccessful. Therefore, one-half of the galactose fraction VI (Table II), obtained by paper chromatography at 50 to 55° C., was used. This material had been shown by paper chromatography to be almost all galactose. The solution was concentrated to approximately 0.2 ml. under a stream of nitrogen and a heat lamp, and 10 mg. of sodium acetate, 12 mg. of *o*-tolylhydrazine hydrochloride, and 0.3 ml. of 50 per cent aqueous ethanol was added. The solution was heated in a boiling water bath for fifteen minutes and then allowed to cool. A small volume (0.2 ml.) of water was added, and the solution became cloudy. After the volume was reduced by about 20 per cent under a stream of nitrogen and a heat lamp and cooling in an ice bath, yellow crystals were obtained. The product was washed twice with cold water, recrystallized from ethanol (95 per cent), washed with cold ethanol (95 per cent), and dried (vacuum oven 50° C.). The product melted at 167 to 169° C.

D-galactose *o*-tolylhydrazone was prepared from a known sample of D-galactose (10 mg.) by the procedure described above. The known derivative melted at 171 to 172° C. The mixed melting point with the dust product was 170 to 171° C. (literature melting point D-galactose *o*-tolylhydrazone recrystallized from 95 per cent ethanol, 176° C.).

Preparation of a Derivative of Arabinose: Arabinose Diphenylhydrazone.—Fraction III (Table II) from the high-temperature paper chromatography fractionation was used as the starting material for the preparation of this derivative. Paper chromatography had indicated that the fraction contained about equal amounts of arabinose and xylose. The amount used contained roughly 5 mg. of arabinose in a volume of 0.1 ml. An equal volume of absolute ethanol, 5 mg. sodium acetate, 9 mg. diphenylhydrazine hydrochloride, and 0.2 ml. 50 per cent aqueous ethanol were added, and the solution was heated for fifteen minutes in a boiling water bath. The mixture was allowed to cool, the volume was reduced by about 20 per cent under a stream of nitrogen and a heat lamp, and the sample was then cooled in an ice bath. The resulting crystalline product was collected by centrifugation, recrystallized from absolute ethanol and then from 95 per cent ethanol, and dried (melting point 197 to 199° C.). L-arabinose diphenylhydrazone was prepared from a known sample of L-arabinose by the procedure described above. The known derivative melted at 198.5 to 199.5° C.

The mixed melting point with the dust product was 198.5 to 200° C. (literature melting point L-arabinose diphenylhydrazone recrystallized from 95 per cent ethanol, 204° C.).

Preparation of a Derivative of Rhamnose: Rhamnose 2,5 Dichlorophenylhydrazone.—A sample of crude dust hydrolysate (about 600 mg.) was fractionated by filter-paper chromatography as previously described, except that the procedure was carried out at room temperature instead of at 50 to 55° C. and a different solvent system was used (*n*-butanol-ethyl acetate-methanol-water: 6, 1, 1,3 by volume). A portion (about 5 mg.) of the material recovered from the chromatograms at the rhamnose level was used to prepare the 2,5 dichlorophenylhydrazone.²⁷ The material was contained in a 0.1 ml. volume of water to which was added 3 mg. of sodium acetate and 0.1 ml. of 95 per cent ethanol. A 0.2 ml. volume of a 50 per cent aqueous ethanol solution containing 2.5 per cent acetic acid and 4.5 mg. of 2,5 dichlorophenylhydrazine was added to the sugar solution and heated, with mixing, in a boiling water bath for ten minutes. The solution was cooled, a 0.2 ml. volume of water was added, and the solution was concentrated under a stream of nitrogen and a heat lamp. There was formed a gummy precipitate, which was discarded. Further concentration yielded a crystalline product which was washed three times with cold water and dried and then washed three times with cold diethyl ether and dried. The product was recrystallized from 50 per cent aqueous ethanol, yielding a product that melted at 163.5 to 165.5° C. A known sample of L-rhamnose (about 2 mg.) was carried through the same procedure and yielded a product melting at 168 to 169° C. A mixed melting point was 165 to 168° C. (literature melting point L-rhamnose 2,5 dichlorophenylhydrazone, 171° C.).

Preparation of a Derivative of Glucose: Glucose 2,5 Dichlorophenylhydrazone.—A sample of material (about 4 mg.), chromatographically identical with pure glucose, was isolated from the same dust hydrolysate as the rhamnose sample by repeated preparative paper chromatography in the *n*-butanol-ethyl acetate-methanol-water solvent system. The sample was mixed with 4.5 mg. of 2,5 dichlorophenylhydrazine in 0.1 ml. of methanol and heated for ten to fifteen minutes in a boiling water bath. After being dried and washed with diethyl ether, the material was dissolved in 0.05 ml. of methanol and precipitated by the addition of 1 ml. of diethyl ether and cooling at -15° C. The product was collected by centrifugation, washed with diethyl ether, and then recrystallized twice from a 50 per cent ethanol-carbon tetrachloride solution. The resulting material melted at 148 to 150° C. A known sample of glucose was carried through essentially the same procedure and yielded a product melting at 154 to 155° C. A mixed melting point was 150 to 154° C. (literature melting point D-glucose 2,5 dichlorophenylhydrazone, 160° C.). An aqueous solution of the glucose isolated from the dust hydrolysate was compared in optical rotation and found to be identical with an authentic sample of D-glucose.

DISCUSSION

A purified dust allergen fraction has been isolated from crude dust extracts. This material has been found to give impressive skin reactions with

many, but not all, of those persons who react to the crude dust extracts. A detailed comparative analysis of the data is impossible because the concentrations of the preparations tested are unknown and because complete titrations to end points were not carried out. The fact that many of the patients gave strong reactions to all three of the dust fractions tested indicates that our purified fraction does exhibit good allergen skin reactivity in many dust-sensitive persons. It has been found that some of those persons who reacted to the commercial preparations did not react to our purified material. This could be due to the particular relationship, in the various preparations, between the concentrations of materials of the same allergen specificity and the patient's threshold of skin reactivity. Another more likely explanation is that more than one allergen specificity is involved and that there are materials with allergen specificities in the less purified commercial preparations that are not present in our material. It is reasonable that clinical house dust allergy may be associated with a number of different allergen specificities. It is also possible, however, that some of the reactions obtained to the less purified fractions are due to the presence of other known allergens (for example, animal danders or pollens in the original house dust). The type of dust used to prepare the extracts may also be a factor. Our starting material contained large amounts of mattress dust which may be deficient in some of the allergen specificities normally found in house dust.

The purified dust allergen fraction consists predominantly of a heterogeneous mixture of acidic polysaccharides. This heterogeneity is indicated by the presence of two mobile peaks on free boundary electrophoresis. It is probable that there is also present uncharged material that contributes to the ϵ boundary. The patterns obtained by sedimentation analysis consist of single symmetrical peaks. Upon more detailed analysis of the time dependence of the boundary spreading by the method of Baldwin and Williams,^{28, 29} however, it is clear that the allergen fraction consists of a population of materials of varying sedimentation coefficient. The concentration dependence of the sedimentation coefficient, as shown in Fig. 3, is fairly small. This indicates that the molecules are probably fairly symmetrical but does not suggest a choice as to the correct model (random coil, short rod, or a compact globular structure).

The polysaccharide nature of the dust allergen fraction is indicated by the high yield of equivalent glucose (80 per cent) on hydrolysis and by the paper chromatography studies. By hydrolysis and paper chromatography, D-glucose, D-galactose, D-mannose, L-rhamnose, and L-arabinose were tentatively identified as the sugars present in greatest amount. The configurations indicated are based on rough optical rotation measurements of the eluates from the high-temperature chromatograms (see Table II). The identification of these sugars has been confirmed by the preparation of substituted hydrazone derivatives and the comparison, by melting point and mixed melting point, of these dust derivatives with the same derivatives prepared from authentic sugar samples. Substituted hydrazones are not ideal derivatives in that they tend to decompose on melting, and the melting point depends on both the solvent

used in recrystallization and the age of the derivative. Due to the small amounts of material involved, the number of times that the products could be recrystallized was limited. In a number of cases further recrystallization failed to yield any product. These difficulties account for the fact that the melting points of the derivatives are considerably lower than that reported in the literature, even in the case of derivatives prepared from known pure sugars. No melting point depressions were observed on mixing the dust derivative with the known derivative. This proved that the tentative identification based on paper chromatography was correct. The possibility of preparing other derivatives, such as benzimidazoles and osotriazoles, was explored. No difficulty was experienced in the preparation of these other derivatives on a 50 to 100 mg. scale; on proceeding to a 2 to 3 mg. scale, however, it was found that crystalline products could not be consistently isolated.

The acidic nature of the polysaccharides has been shown by titration (Fig. 4) and electrophoresis. The evidence that the acid components are uronic acids and, more specifically, largely glucuronic acid rests on reactions in concentrated sulfuric acid with various reagents to yield characteristic absorption spectra. The absorption curves obtained with the carbazole reaction (Fig. 5) indicate the presence of uronic acid and suggest that the uronic acid content is of the order of 20 per cent. This is in agreement with the result obtained by titration (that is, an equivalent weight of 750). The color reaction with thioglycollic acid (Fig. 6) suggests the presence of glucuronic acid, while the cysteine reaction indicates that all the uronic acid present could not be galacturonic acid. These experiments provide presumptive qualitative evidence for the presence of uronic acid residues, probably glucuronic acid, in the dust fraction.

Rimington^{4, 5} has studied the amino acid composition of his dust allergen fraction and, on the basis of his studies, it seems likely that most of the nitrogen present is due to a polypeptide component. We have obtained evidence for the presence of a small amount of hexosamine (1 to 2 per cent) in some crude dust fractions; however, the purified dust fraction appeared to be free of hexosamine.

On the basis of these results, the composition of the purified dust allergen fraction (neglecting the ash content) was roughly estimated to be as follows:

Polysaccharide components	95%
D-glucose	20%
D-galactose	20%
D-mannose	20%
L-rhamnose	10%
L-arabinose	5%
Uronic acid (probably glucuronic acid)	20%
Polypeptide components	5%

The relative amounts of the sugars were based on a visual appraisal of the intensities of the spots and were therefore very crude estimates. The general

chemical composition of this purified fraction is similar to that of most crude dust fractions, except that the latter generally have higher nitrogen contents, some hexosamine by the Elson-Morgan color reaction, and fucose and xylose as demonstrated by paper chromatography.

One of the central problems is that of whether the specific dust allergen activity is due to the presence of a polypeptide component, to a polysaccharide alone, or to a complex molecule containing both moieties. The purified fraction described in this article had a nitrogen content of about 1 per cent, while Rimington's active partial hydrolysis product⁴ contained 10 per cent nitrogen and 12 per cent carbohydrate by orcinol determination. In neither case has allergen activity been found to be associated with a carbohydrate or polypeptide component alone. Rimington carried out an experiment in which he followed, by paper chromatography and skin test, the partial acid hydrolysis of his purified allergen fraction. With 80 per cent of the total carbohydrate liberated as free reducing sugar, there was no loss of skin reactivity; however, sudden loss of activity occurred when, upon further hydrolysis, amino acids were split off. The data were interpreted as indicating that the polypeptide portion of the allergen fraction was responsible for the skin activity. It is possible that the data could be explained as well on the basis of continued polysaccharide hydrolysis and uronic acid decarboxylation.

Dr. Michael Heidelberger tested the purified fraction for cross-reactivity with various pneumococcus polysaccharides. There was a strong cross-reaction with type-specific substances VII, XII, XV, and XVIII. There were more moderate but definite cross-reactions with twelve of the other type-specific substances. Some of the moderate reactions were negative at low concentration but became positive at higher concentrations. This was interpreted as indicating that the material was heterogeneous. The dust fraction gave a moderate cross-reaction with type-specific substances II and VIII, both of which contained glucuronic acid, glucose, and rhamnose. There was only a very slight cross-reaction with type III (polycellobiuronic acid). The general conclusion drawn was that the dust allergen fraction reacts with many of those antipneumococcus sera that show cross-reaction with glycogen and polyglucoses.

Rimington⁴ has reported that his purified allergen specifically inhibited both A and B hemagglutination. Dr. Elvin Kabat³⁰ tested our purified fraction and found it to be devoid of either of these activities.

The problem of the origin of the specific house dust allergen activity is most intriguing. If the dust allergen activity can be produced under sterile conditions from autoclaved samples of cotton linters,^{31, 32} it is difficult to see how a complex polysaccharide or polypeptide could be built up under these conditions. It is much more likely that only a minor change, possibly involving air oxidation, is necessary to convert an inactive material to an active one. A change in a preformed polymer involving some kind of degradative process (possibly oxidative) would tend to produce a polydisperse mixture such as has been found. It is entirely possible that many of the components of such a

polydisperse mixture would possess activity. Cotton fibers consist largely of cellulose, but other constituents are also present.³³ Waxes, protein, pigments, and pectins have been demonstrated. The pectins (partially methyl-esterified polygalacturonic acid with arabinose and xylose components) are present to the extent of about 1 per cent in mature fibers. Possibly some of the arabinose found in the purified fraction and the arabinose and xylose found in less purified materials comes from this source. Some of the uronic acid present could be galacturonic acid; however, it is not likely to be all galacturonic acid, in view of the negative test.

Polysaccharides containing residues of the types found in the house dust fraction have been isolated from a wide variety of bacteria (for example, pneumococci and soil bacteria).³⁴ Experiments have demonstrated the importance of molds in the production of kapok allergen.³⁵ It is therefore natural to suspect that bacteria and molds might be involved in the production of the dust allergen. They might either produce the allergen directly or promote the degradation of cellulose or other materials to form the active allergen. More detailed studies with systems in which inactive known materials develop allergen activity, both under sterile conditions and in the presence of bacteria and molds, might aid in solving the problem of the origin and nature of the house dust allergens.

Even though the problem of the chemical nature of the specific house dust allergen has not been solved, these studies have revealed new information about the nature of the materials that have been used clinically for the past thirty years.

SUMMARY

1. An active house dust allergen fraction, low in nitrogen and colorless, has been isolated.
2. The material has been found to show activity with most, but not all, persons sensitive to less purified commercial preparations. This suggests the presence of more than one allergen specificity.
3. The dust fraction was found to be heterogeneous by electrophoretic and sedimentation analysis. Two main peaks were demonstrated by electrophoresis. The average sedimentation coefficient ($S_{w, 20}$) was found to be 2.6S.
4. The chemical composition was found to be roughly 5 per cent polypeptide and 95 per cent polysaccharide, with about equal amounts of uronic acid (probably glucuronic acid), D-glucose, D-galactose, and D-mannose as well as lesser amounts of L-rhamnose and L-arabinose.
5. The relationship of the composition and physical properties of the dust allergen fraction to current ideas concerning the origin of the house dust allergen was discussed.

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