

A STARCH BLOCK ELECTROPHORESIS STUDY OF AQUEOUS HOUSE DUST EXTRACTS

Wilton E. Vannier, M.D., Ph.D., and Dan H. Campbell, Ph.D., Pasadena, Calif.*

IN A PREVIOUS study,¹ a partially purified dust allergen fraction was found to consist predominately of a heterogeneous mixture of acidic polysaccharides with about 5 per cent polypeptide. This fraction contained about equal amounts of uronic acid (probably glucuronic acid), D-glucose, D-galactose, D-mannose, and lesser amounts of L-rhamnose and L-arabinose.

Rimington and co-workers² have studied the electrophoretic properties of a purified dust allergen fraction. They found their fraction to consist of two main peaks (phosphate buffer, pH 8, $\mu = 0.2$) of about equal skin reactivity. The material in the fast peak was colored while that in the slow was colorless. By removing the fast and slow materials from the ascending and descending limbs, respectively, of a free boundary cell and analyzing for total hexose and for nitrogen content, it was found that the two materials were chemically similar.

In general it has been found that aqueous dust extracts and their fractions are electrophoretically quite heterogeneous. The starch block electrophoresis method^{3, 4} provides a semipreparative procedure for fractionating the dust extracts predominately on the basis of the electrophoretic mobility of the components. With this in mind, we carried out the following experiments to study the relationship between the distribution of components produced by starch block electrophoresis and the distributions of the total carbohydrate, uronic acid, nitrogen, color, monosaccharide composition, and skin reactivity of four dust extracts. Furthermore, by varying the composition of the buffer used for the electrophoresis experiments, some information was obtained regarding the nature of the linkage between the polysaccharide and polypeptide portions of the dust extracts.

Two of the dust extracts studied were prepared in this laboratory and two were commercial preparations commonly used by allergists.

From the Division of Chemistry and Chemical Engineering, The California Institute of Technology, Pasadena, Calif. Contribution No. 2566.

This work was supported by the National Institute of Allergy and Infectious Diseases.

Presented, in part, before the Sixteenth Annual Meeting of the American Academy of Allergy, Hollywood, Fla., Jan. 11-13, 1960.

Received for publication Aug. 8, 1960.

*Staff Member, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases.

MATERIALS AND METHODS

Preparation of Dust Extract #110.—A pooled mixture of approximately equal parts of house dust* and mattress dust† was wet with 1.5 m. sodium hydroxide and, after twenty-four hours at 4° C., the liquid was removed with a mechanical press. The pH of the dust extraction mixture was 9.5 two hours after mixing the dust and the sodium hydroxide solution and 8.5 after twenty-four hours when the mixture was pressed. The residue was re-extracted with the sodium hydroxide solution and pressed as before, and the liquid was removed and pooled with the first liquid extract. The pooled solution was filtered through a cotton plug and then centrifuged with a Scharples Super-Centrifuge. The volume was reduced to one seventh of the initial volume by vacuum distillation, keeping the temperature of the dust solution below 40° C. The concentrated dust solution was dialyzed against distilled water and then pervaporated to dryness to recover the solid material.

Preparation of Dust Extract #125.—Mattress dust† was wet with water, frozen in the deep freeze, allowed to thaw, and the fluid expressed by a hydraulic press at 16,000 pounds per square inch. The extract was filtered through glass wool, concentrated to one fourth the initial volume by pervaporation, and centrifuged at about 2,000 g for about forty-five minutes. The supernatant was pervaporated to about one half the initial volume and an equal volume of saturated ammonium sulfate added. The mixture was allowed to stand at room temperature for approximately twelve hours, centrifuged as before, and the supernatant stored at 4° C. As the dust extract was needed, a portion was centrifuged, as above, and then exhaustively dialyzed against distilled water until the dialysate gave a negative test for ammonia with Nessler's reagent. The dust extract was dialyzed against 1 per cent sodium chloride for twenty-four to forty-eight hours and dialyzed against distilled water to remove the sodium chloride. The solution was concentrated for use by pervaporation.

Hollister-Stier Acetone Precipitated Dust Extract.‡—One gram of the dust extract was mixed with 100 ml. of distilled water, allowed to stand at room temperature twenty-four hours, and dialyzed against distilled water for four to five days with two or three changes of dialysis bath per day. The product was centrifuged to remove a small amount of insoluble material. About 30 per cent of the dust extract was found to be soluble and nondialyzable while about 5 per cent was water insoluble. The solution of nondialyzable material was concentrated for use by pervaporation.

Endo House Dust Concentrate.§—The material was supplied as a 2.5 per cent solution of dust extract in 50 per cent aqueous glycerol. The solution

*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. David McK. Pipes.

‡Obtained from Hollister-Stier Laboratories, Inc.

§Obtained from Endo Laboratories, Inc.

was dialyzed against distilled water, as before, and concentrated for use by pervaporation. All the material remained soluble; however, dry weight determinations indicated that about two thirds of the dust extract was dialyzable.

The approximate compositions of the materials are presented in Table I.

TABLE I. APPROXIMATE COMPOSITION OF DUST EXTRACTS

	DUST EXTRACT #110 (%)	DUST EXTRACT #125 (%)	HOLLISTER-STIER EXTRACT (%)	ENDO DUST EXTRACT (%)
Polypeptide	15	10	30	12
Nitrogen	2.5	1.5	4.4	2.0
Polysaccharide	85	90	70	88
Uronic acid	20	25	7.5	10
D-glucose	15	10	20	20
D-galactose	20	30	25	25
D-mannose	15	15	10	15
L-rhamnose	10	10	2	10
fucose	trace	none	none	none
L-arabinose	5	trace	5	5
xylose	trace	trace	none	none
Hexosamine	1.7	0.5	1	2

The polypeptide content is based on nitrogen analysis and the assumption that the polypeptide contained about 16 per cent nitrogen. The nitrogen analyses were carried out by digestion with sulfuric acid and hydrogen peroxide followed by nesslerization.⁵ Nitrogen standards and blanks were included and the optical density of the samples, after nesslerization and waiting at least thirty-five minutes, were determined at 440 $m\mu$ in the Beckman Model B spectrophotometer. The hexosamine determinations were performed using a modification of the Elson-Morgan procedure.^{6, 7} Known amounts (5 to 7 mg.), based on dry weight determinations of solutions of the dust fractions exhaustively dialyzed against distilled water, were hydrolyzed in sealed tubes with 4.0 ml. of 3 N hydrochloric acid for eight hours (at 105° C.).* Dische's carbazole method^{8, 9} was used for the uronic acid determinations. The results are reported as per cent equivalent glucuronolactone. In our previous study of a purified dust fraction,¹ we found that the uronic acid content determined by this method was in reasonable agreement with the total number of acidic groups determined by differential titration. The sugar composition indicated in Table I is based on hydrolysis and paper chromatography of the dust extracts. The samples (approximately 3 mg.) were hydrolyzed by heating in sealed tubes with 0.5N sulfuric acid for three hours at 105° C. The paper chromatography methods were the same as those previously described.¹ The relative sugar composition is based on a visual appraisal of the spot intensities in comparison with a standard sugar mixture of a similar sugar composition as the dust extract hydrolysates; it is therefore a very crude estimate. The sugar configurations indicated are based on the polarimeter measurements previously described.¹

*In our previous paper,¹ it was erroneously reported that the hydrolysis for the hexosamine determination was carried out in 0.5N sulfuric acid. In all cases we have used 3N hydrochloric acid.

Starch Block Electrophoresis.^{3, 4}—The apparatus consisted of two cubical Lucite buffer vessels (18 cm. on each edge) and a Lucite tray (1.5 cm. deep, 9 cm. wide, and 44 cm. long) that contained the starch block. Each of the buffer vessels, containing about 3 L. of solution, was divided into four compartments by perforated Lucite partitions. The outermost partitions had platinum wires intertwined through the perforations that were connected to the power supply. The other Lucite partitions were to diminish mixing of the solution in the buffer vessels and thus to keep electrode products away from the starch block. The tray was lined with a sheet of polyethylene and a double thickness of cloth towel placed at each end of the tray before pouring the starch. The ends of the cloth towels provided contact between the starch block and the solution in the buffer vessels. The potato starch was allowed to soak overnight in 1 per cent sodium chloride solution. Before pouring the block, the starch was washed, on a Büchner funnel, twice with 1 per cent sodium chloride and two to four times with the solution to be used as the supporting electrolyte for the electrophoresis run. During the course of washing the starch some of the lighter starch particles were removed by decantation. The washed starch was mixed with the electrophoresis solution to form a suspension of consistency such that it would just flow. The block was poured and the starch grains allowed to settle. The excess solution was removed with blotters. A hole, 1 cm. wide and 7 cm. long, was cut at right angles to the length of the starch block at a position of from 12 to 16 cm., depending on the particular experiment, from the end of the starch block to be attached to the negative terminal of the power supply. Starch was replaced to form a layer of 1 to 2 mm. thickness so the sample did not come in contact with the polyethylene sheet on the bottom of the tray. The sample to be fractionated, in a volume of 5 to 7 ml., was mixed with dry starch and placed in the hole cut in the starch block. After all the sample had been applied to the starch block, the hole was filled to the level of the top of the block with starch wet with the electrophoresis solution. The starch electrophoresis runs were carried out in a coldroom at 4° C. To ensure that the solution levels in each vessel were the same height, a rubber tube connected the two buffer vessels below the solution level. The starch block and the buffer vessels with the electrophoresis solution were allowed to equilibrate in the coldroom at least one hour before clamping the rubber tube connecting the buffer vessels and starting the current. After the current had run the desired length of time, the starch block was removed from the tray and cut into forty-four 1 cm. segments and each portion eluted with the same volume of distilled water. Each eluate was then analyzed and the distribution of various components or properties of the sample on the starch block studied.

Color Determination.—The optical density of each of the eluates was measured at 400 m μ in a Beckman Model B spectrophotometer. A 1 cm. Corex cell was used.

*Determination of Total Carbohydrate.*¹⁰—A 0.1 ml. volume of each eluate was mixed with 0.4 ml. of distilled water. Standards containing 10, 20, 30, 40,

50, 70, and 100 γ of galactose per 0.5 ml. and distilled water blanks were included in the determination. The method involves the addition of sulfuric acid and indole and heating in a boiling water bath to develop the color.¹⁰ The optical density of the samples was determined at 470 $m\mu$ in a Beckman Model B spectrophotometer. The data were plotted as milligrams of equivalent galactose per milliliter of eluate.

Uronic Acid Determinations.^{8, 9}—A 1.0 ml. volume of each eluate was analyzed for uronic acid following the carbazole sulfuric acid method of Dische.⁸ The optical densities of each of the eluates, standards (10, 20, 30, 40, 50, 70, and 100 γ of glucuronolactone) and blanks were determined at 535 $m\mu$ in the Beckman Model B spectrophotometer. Since a brown color is generated by heating the samples with the acid alone, the determinations were set up in duplicate and the carbazole added to only one series of tubes. The optical densities at 535 $m\mu$ determined for the samples with no added carbazole were subtracted from the values obtained for the corresponding samples in the complete test. The data were plotted as γ of equivalent glucuronolactone per milliliter of eluate.

*Nitrogen Determination.*¹¹—Two milliliter volumes of each eluate were evaporated to dryness in a vacuum desiccator over calcium chloride. A micro method, developed by Lanni and his co-workers,¹¹ was used, with slight modification, to determine the nitrogen content of each tube. A volume of 0.14 ml. of the diluted sulfuric acid, instead of 0.16 ml., was used for the digestion and the tubes were read at 440 $m\mu$ in the Beckman Model B spectrophotometer about 35 minutes after the addition of the Nessler's solution. Blanks and standards (10, 20, 30, 40, 50, and 60 γ of nitrogen as ammonium sulfate) were carried through the same analytical procedure. The data were plotted as γ of nitrogen per milliliter of eluate.

EXPERIMENTAL

Starch block experiments, in acetate buffer (pH 4.7, $\mu = 0.1$), were carried out with each of the four dust extracts. Varying amounts of the dust extract, in 5 to 6 ml. of distilled water, were applied to the starch block, 13 to 14 cm. from the end of the block connected to the cathode terminal of the power supply. Samples dialyzed against the acetate buffer gave the same kind of patterns as those in distilled water. There was no insoluble material obtained on dialysis of the dust extracts against the acetate buffer. The following amounts of dust extract, determined by analysis for dry weight, were applied to these starch blocks: extract #110, 154 mg.; extract #125, 180 mg.; Hollister-Stier extract, 68 mg.; and Endo extract, 82 mg. After the period of equilibration in the coldroom (4° C.), a potential of 300 volts (constant voltage output) was applied to the starch blocks for periods of 15.5 to 16.5 hours. Each one-centimeter segment of the block was eluted with 5 ml. and then with 2 ml. of distilled water. The pooled eluates were centrifuged and filtered to remove all the starch grains and the analyses for total carbohydrate, uronic acid, color, and nitrogen carried out on each eluate. The resulting data are

plotted in Figs. 1A, 2A, 3A, and 4A, along with the data from a blank or control starch block run. In the case of the blank starch block experiment, 5 ml. of distilled water was applied to the block and the current run as usual and all elutions and chemical analyses carried out as in the case of a dust extract sample.

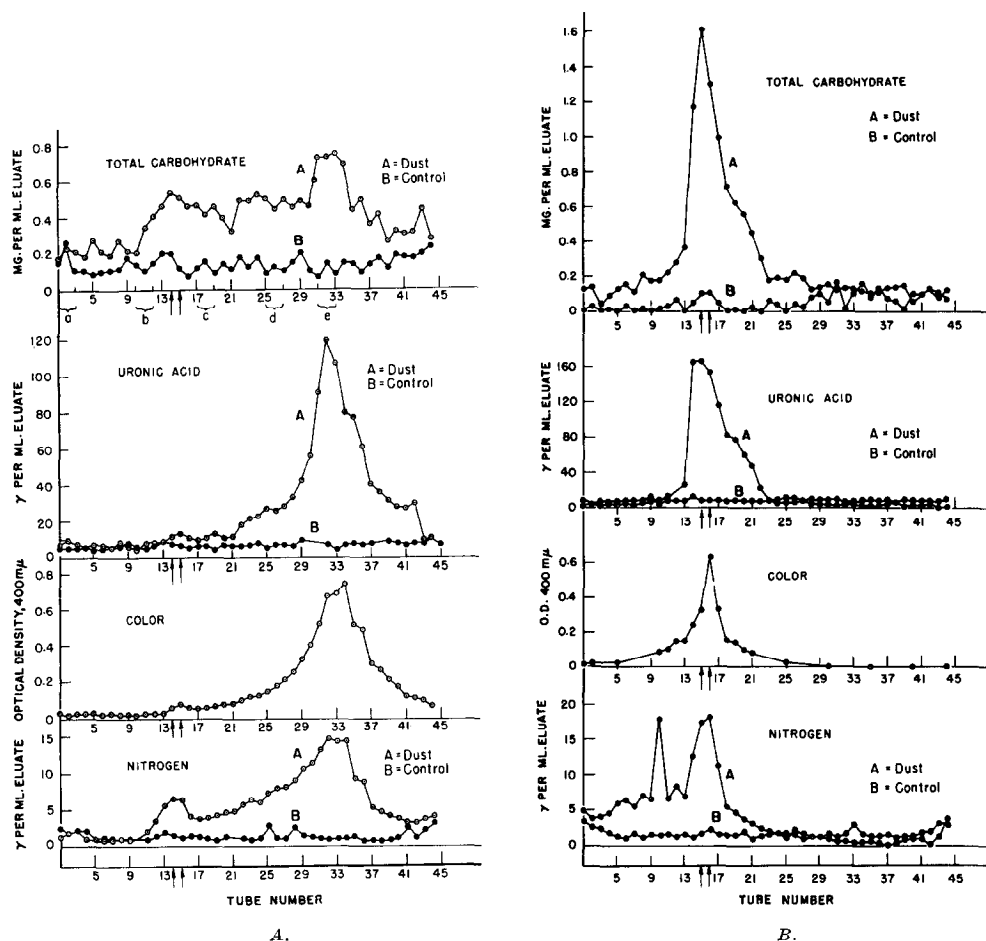


Fig. 1.—Dust extract #110, chemical analyses of starch block eluates. The positive electrode is to the right and the arrows indicate the position of application of the sample. A, Acetate buffer, pH 4.7, $\mu = 0.1$. The positions of the letters a, b, c, d, and e indicate the eluates pooled for chemical analyses and skin testing. B, KCl-HCl solution, pH 1.8, $\mu = 0.1$.

Starch block experiments in a KCl-HCl solution (KCl 44.7 Gm., concentrated hydrochloric acid [approximately 12.4N] 16.5 ml., and water to make 8 L. of solution; final pH 1.8, $\mu = 0.1$) were carried out with each of the four dust extracts. Each dust extract, in a volume of 5 to 6 ml., was dialyzed against the KCl-HCl solution and a small amount (approximately 5 to 8 per cent) of insoluble material that formed, at this low pH, was removed by centrifugation. The pH's of the dialyzed dust extracts were found to be within less than 0.1 pH unit of the pH of the KCl-HCl solutions. The sample was

placed 15 to 16 cm. from the cathode end of the starch block, except in the case of the Endo dust sample which was placed at a distance of 24 to 25 cm. The following amounts of dust extract, determined by dry weight analysis, were applied to the starch blocks: extract #110, 165 mg.; extract #125, 94 mg.; Hollister-Stier extract, 68 mg.; and Endo extract, 82 mg. After the equilibration period, a potential of 200 volts (constant voltage output) was applied to

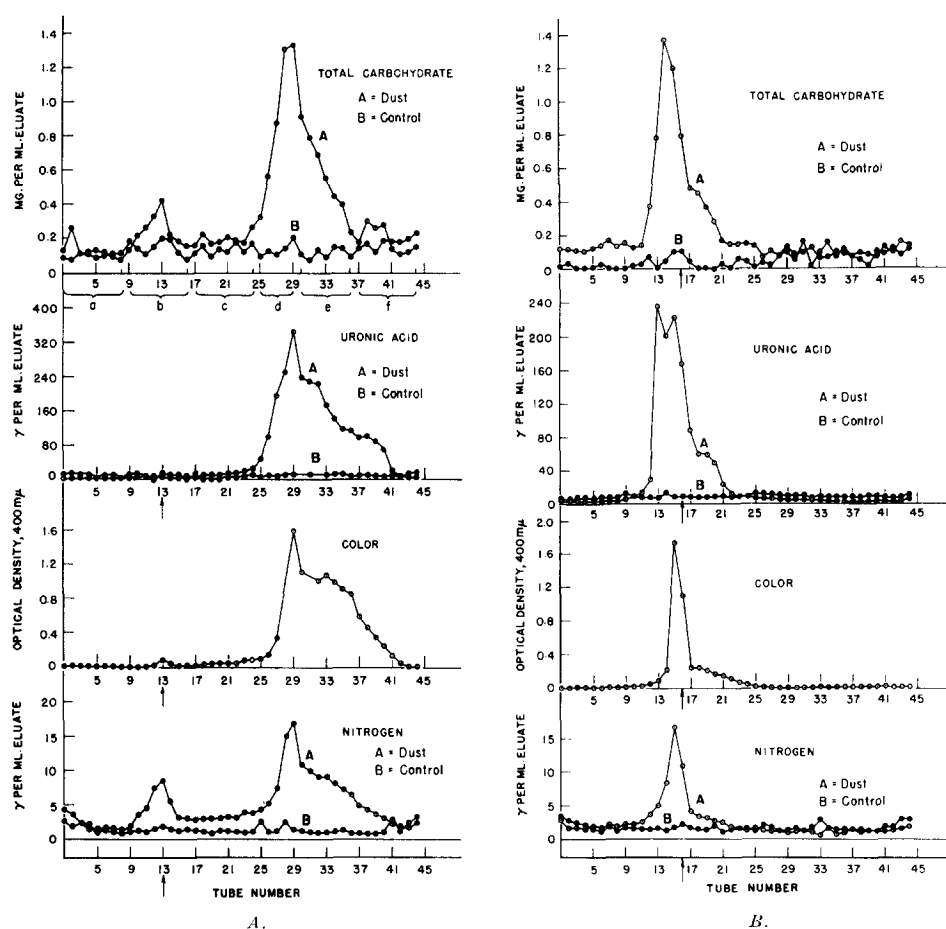


Fig. 2.—Dust extract #125, chemical analyses of starch block eluates. The positive electrode is to the right and the arrows indicate the position of application of the sample. A, Acetate buffer, pH 4.7, $\mu = 0.1$. The positions of the letters a, b, c, d, e, and f indicate the eluates pooled for chemical analyses and skin testing. B, KCl-HCl solution, pH 1.8, $\mu = 0.1$.

the starch block for 16 to 20 hours. As before, each one-centimeter segment was eluted with 5 ml. and then with 2 ml. of distilled water, the starch grains removed, and each eluate analyzed. The resulting data are plotted in Figs. 1B, 2B, 3B, and 4B, along with the data from a blank starch block run. In the case of the blank run, 5 ml. of the KCl-HCl solution, instead of a dust sample, was applied to the starch block and the conditions as well as all analyses were carried out as in the case of a dust sample.

In order to obtain material for hydrolysis and paper chromatography of the polysaccharides in various eluates and for skin testing, additional starch

block fractionations were carried out. In the case of dust extract #110, a starch run in acetate buffer with a 187 mg. sample was carried out for paper chromatography studies and another with a 60 mg. sample for skin testing. In each case the eluates were pooled, on the basis of individual total carbohydrate curves, yielding fractions **a**, **b**, **c**, **d**, and **e** as indicated on the total carbohydrate curve in Fig. 1, *A*. The fractions were dialyzed against distilled water and concentrated by pervaporation. The corresponding eluates from five starch block experiments in the acetate buffer, using varying amounts of the

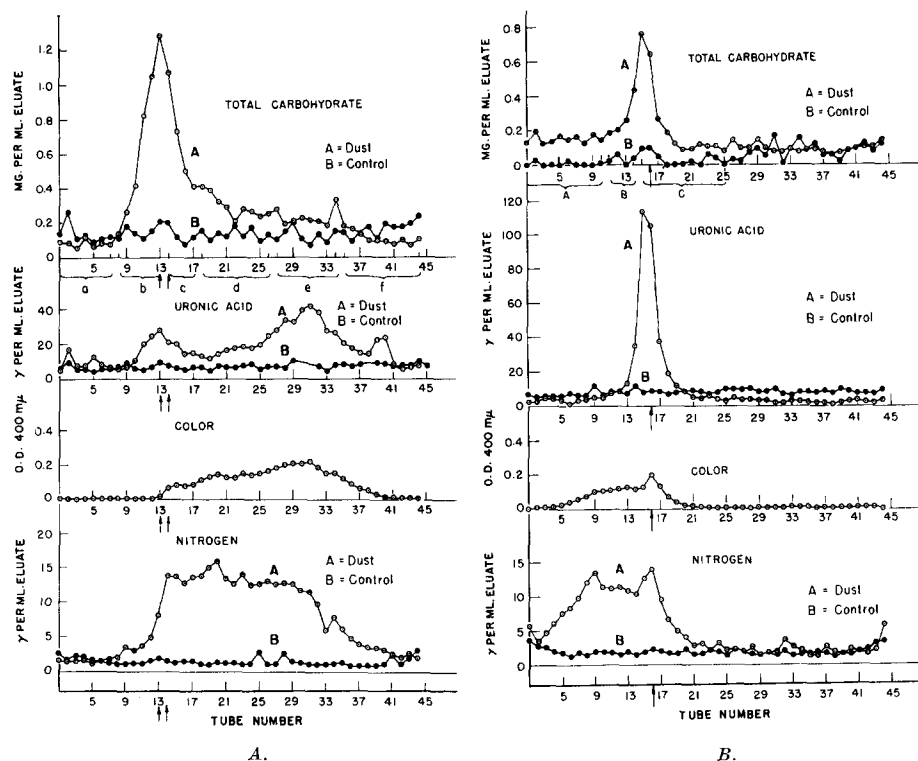


Fig. 3.—Hollister-Stier extract, chemical analyses of starch block eluates. The positive electrode is on the right and the arrows indicate the position of application of the sample. *A*, Acetate buffer, pH 4.7, $\mu = 0.1$. The positions of the letters **a**, **b**, **c**, **d**, **e**, and **f** indicate the eluates pooled for chemical analyses and skin testing. *B*, KCl-HCl solution, pH 1.8, $\mu = 0.1$. The positions of the letters *A*, *B*, and *C* indicate the eluates pooled for skin testing.

#125 dust extract (82, 82, 141, and 137 mg.) were pooled. Analyses for total carbohydrate were made on each set of eluates and the corresponding samples from each electrophoresis experiment pooled to yield fractions **a**, **b**, **c**, **d**, **e**, and **f** according to the position of the peaks as indicated in Fig. 2, *A*. These fractions were dialyzed and pervaporated, as before, and then skin tested. The relative monosaccharide composition was estimated by hydrolysis and paper chromatography. In the same way, the eluates from corresponding regions of five starch block runs, in acetate buffer, were pooled in the cases of the Hollister-Stier extract and the Endo dust extract. In each case, the corresponding regions of the starch blocks were pooled to form the fractions indicated in Fig. 3, *A* and Fig. 4, *A*. Samples of about 60 mg. were used in the case of the Hollister-Stier

material, while 70 mg. samples of the Endo extract were used. In both cases, the same acetate buffer (pH 4.7, $\mu = 0.1$) was used and a potential of 300 volts applied for about 15 to 16 hours. The one-centimeter segments of the starch blocks were eluted with 5 ml., 2 ml. then with an additional 2 ml. of distilled water in order to increase the yield of material. Samples of about 1 to 2 mg. were hydrolyzed for 3 hours (at 105° C.) in 0.5N sulfuric acid. The samples were neutralized with barium carbonate and analyzed by paper chromatography in two solvents (phenol-water and n-butanol-pyridine-water), in comparison with known sugars, as previously described.¹ The results are presented in Table II. Samples of the various fractions in 50 per cent aqueous glycerol and all

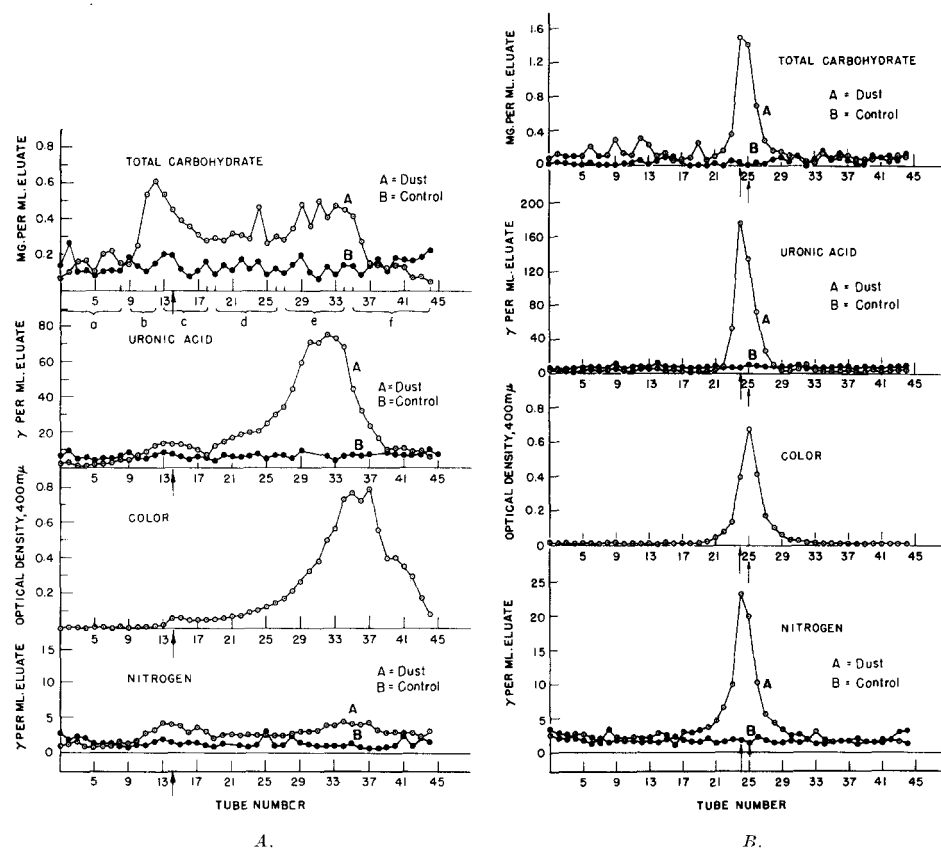


Fig. 4.—Endo extract, chemical analyses of starch block eluates. The positive electrode is on the right and the arrows indicate the position of application of the sample. A, Acetate buffer, pH 4.7, $\mu = 0.1$. The positions of the letters a, b, c, d, e, and f indicate the eluates pooled for chemical analyses and skin testing. B, KCl-HCl solution, pH 1.8, $\mu = 0.1$.

at the same concentration with respect to total carbohydrate were tested by direct scratch test in dust-sensitive individuals. In each case, a sample of unfractionated dust extract, at the same concentration as that of its fractions, was tested with the fractions. A dust extract and its fractions were always tested on the same individual at the same time; however, not all the four different dust extracts and their fractions were tested on the same individual at the same time. Therefore, only comparisons among the fractions of the same

TABLE II. ESTIMATION OF THE RELATIVE MONOSACCHARIDE COMPOSITION OF THE STARCH BLOCK FRACTIONS (pH 4.7)*

	GLUCOSE	GA-LACTOSE	MANNOSE	RHAM-NOSE	FUCOSE	ARABI-NOSE	XYLOSE
#110 fractions							
a	1	0	0	0	0	0	0
b	3	0	1	2	0	trace	0
c	3	0	2	1	0	1	0
d	3	3	2	2	trace	2	0
e	3	2	1	2	1	1	0
#125 fractions							
a	1	0	0	0	0	0	0
b	2	2	2	2	0	1	0
c	2	3	2	1	0	1	trace
d	2	3	1	1	0	1	1
e	2	2	2	2	0	trace	0
f	2	0	1	2	0	0	0
Hollister-Stier fractions							
a	1	0	0	0	0	0	0
b	2	3	2	trace	0	1	0
c	2	3	2	trace	trace	1	0
d	2	3	2	trace	trace	1	trace
e	2	2	trace	1	0	trace	trace
f	2	trace	trace	trace	0	0	0
Endo fractions							
a	1	0	0	0	0	0	0
b	2	2	1	1	0	trace	0
c	2	3	1	2	0	1	0
d	2	3	trace	2	trace	2	trace
e	2	2	0	1	1	1	0
f	2	1	0	1	0	0	0

*The relative amounts of the sugars were graded 0, trace, 1+, 2+, or 3+.

extract are valid. The same patient initials always indicate the same individual. The samples were keyed and randomized before testing to remove any possible bias in reading the skin tests. All the clinical testing was carried out by the direct scratch test method. The skin test data are presented in Tables III, IV, V, and VI. All the patients giving a 1+ reaction or better to any of the samples are included in the tables.

TABLE III. SKIN TEST DATA WITH #110 EXTRACT AND FRACTIONS OBTAINED BY STARCH BLOCK ELECTROPHORESIS (pH 4.7)*

PATIENT	#110	a	b	c	d	e
M. B.†	4		4	3	1	0
P. J.	4		3		3	1
M. B.	4		4		1	1
R. I.†	2		1	3	0	0
R. G.	3		1		1	1
A. G.	1		2		1	1
S. F.	2		1		0	0
P. B.	1		1		0	0
L. B.	2		?		0	?
T. B.	2		0		0	0
T. E.	2		0		0	0
J. C.	0		2		0	0
S. C.	1		0		?	0
R. L.	1		0		0	0
J. V. L.	1		0		0	0

*The concentration of the samples was 0.087 per cent, as equivalent galactose determined by the indole test for total carbohydrate, in 50 per cent aqueous glycerol. The skin reactions were graded 1+ through 4+. The symbol ? indicates a questionable reaction and 0 indicates a negative reaction. A blank space indicates no test was carried out. These were scratch tests.

†These test results were obtained with aqueous solutions of the dust extracts containing no glycerol.

TABLE IV. SKIN TEST DATA WITH #125 EXTRACT AND FRACTIONS OBTAINED BY STARCH BLOCK ELECTROPHORESIS (pH 4.7)*

PATIENT	#125	a	b	c	d	e	f
P. J.	0	0	?	2	3	0	0
C. B.	0	0	2	3	0	0	0
T. B.	2		0	2	0	0	0
G. P.	1		1	0	0	0	1
T. E.	1		0	0	0	0	2
A. G.	1		?	?	1	?	1
P. B.	1		0	1	0	0	1
S. F.	1		0	0	0	0	1
L. B.	?		2	?	0	0	0
J. C.	0	0	0	1	0	0	0
D. D.	?	0	1	0	0	0	0
J. V. L.	1		0	0	0	0	0
S. M.	1		0	0	0	0	0

*The concentration of the samples was 0.2 per cent, as equivalent galactose determined by the indole test for total carbohydrate, in 50 per cent aqueous glycerol. The skin reactions were graded 1+ through 4+. The symbol ? indicates a questionable reaction and 0 indicates a negative reaction. A blank space indicates no test was carried out. These were scratch tests.

TABLE V. SKIN TEST DATA WITH HOLLISTER-STIER EXTRACT AND FRACTIONS OBTAINED BY STARCH BLOCK ELECTROPHORESIS (pH 4.7)*

PATIENT	H. S.	a	b	c	d	e	f
L. B.	4	2	4	4	4	3	?
T. B.	4	2	4	4	3	0	0
R. G.	2	1	3	4	4	2	1
A. G.	3	1	3	4	3	1	2
P. J.	0	1	4	4	4	0	0
C. W.	3	0	4	3	1	0	0
K. N.	2	0	2	3	2	0	0
T. E.	2	0	2	4	0	0	0
S. F.	3	0	2	3	0	0	0
D. D.	1	0	2	4	0	0	0
A. B.	2	0	0	2	1	0	0
C. B.	0	0	2-3	2	0	0	0
J. C.	1	0	1	1	0	0	1
P. B.	0	1	0	1	0	0	1
S. H.	0	0	0	0	1	1	0
R. L.	0	0	0	1	0	0	0
R. A.	0	1	0	0	0	0	0

*The concentration of the samples was 0.2 per cent, as equivalent galactose determined by the indole test for total carbohydrate, in 50 per cent aqueous glycerol. The skin reactions were graded 1 through 4. The symbol ? indicates a questionable reaction and 0 indicates a negative reaction. These were scratch tests.

The curves, shown in Fig. 3, *B*, of the eluate analyses from the Hollister-Stier dust extract run on a starch block with the KCl-HCl solution (pH 1.8, $\mu = 0.1$) showed some relative separation of materials differing in nitrogen contents. In order to determine whether a correlation could be established between the relative nitrogen contents of the materials and the skin reactivity, four such starch block experiments were carried out using varying amounts of the dust extract (65, 65, 65, and 91 mg.). As before the samples were dialyzed against the KCl-HCl solution and a slight residue (5 to 8 per cent) was discarded. The current was applied at a potential of 200 volts for 16 to 17 hours, the starch block cut up in one-centimeter segments and eluted three times with 5 ml., 2 ml., and 2 ml. volumes of distilled water and then the three eluates from each segment combined and analyzed for total carbohydrate by the indole-sulfuric acid procedure. The eluates from the corresponding regions

TABLE VI. SKIN TEST DATA WITH ENDO EXTRACT AND FRACTIONS OBTAINED BY STARCH BLOCK ELECTROPHORESIS (pH 4.7)*

PATIENT	ENDO	a	b	c	d	e	f
A. G.	4	1	1	3	3	1	3
R. G.	2	1	2	3	2	3	2
L. B.	3	0	3	3	3	2	0
T. B.	2	0	3	4	4	0	0
P. J.	2	1	4	1	2	1	1
C. B.	2	0	2	2	2	0	0
C. A. S.	0	0	0	3	3	0	0
J. C.	2	0	1	2	0	0	0
K. N.	0	0	3	0	2	0	0
S. C.	1	0	0	0	3	1	0
P. B.	0	1	0	1	0	1	0
D. D.	?	0	?	1	1	?	0
S. M.	0	0	0	0	1	0	1
C. W.	0	?	0	2	?	?	0
S. F.	0	0	0	0	2	0	0
R. L.	0	0	1	0	0	0	0
G. P.	0	0	0	0	0	0	1
Sylvia M.	0	0	0	1	0	0	0
T. E.	1	0	0	0	0	0	0

*The concentration of the samples was 0.2 per cent, as equivalent galactose determined by the indole test for total carbohydrate, in 50 per cent aqueous glycerol. The skin reactions were graded 1+ through 4+. The symbol ? indicates a questionable reaction and 0 indicates a negative reaction. These were scratch tests.

of the starch blocks were pooled to form fractions A, B, and C as indicated in the total carbohydrate curve of Fig. 3, *B*. These fractions and a sample of the unfractionated Hollister-Stier extract were dialyzed against distilled water, concentrated by pervaporation, diluted to the same concentration, and then made up in 50 per cent glycerol and skin tested as previously described. The data are presented in Table VII.

TABLE VII. SKIN TEST DATA WITH HOLLISTER-STIER EXTRACT AND FRACTIONS OBTAINED BY STARCH BLOCK ELECTROPHORESIS (pH 1.9)*

PATIENT	H. S.	A	B	C
P. J.	2	3	4	3
T. B.	4	4	2	0
R. G.	3	3	2	1
L. B.	3	2	2	0
E. T.	2	2	0	0
A. G.	2	1	1	?
R. A.	1	0	1	0
P. B.	0	0	1	0
J. V. L.	0	1	0	0

*The concentration of the samples was 0.08 per cent, as equivalent galactose determined by the indole test for total carbohydrate (0.15 per cent by dry weight), in 50 per cent aqueous glycerol. The skin reactions were graded 1+ through 4+. The symbol ? indicates a questionable reaction and 0 indicates a negative reaction. These were scratch tests.

Two starch block runs were carried out with the #125 dust extract (180 mg. for the first run and 229 mg. for the second) in a KCl-HCl solution 7M in urea (pH 2.5). The solution was prepared by dissolving 3,360 Gm. of urea (reagent grade), 44.7 Gm. of potassium chloride, and 75 ml. of concentrated hydrochloric acid (12.5M) in water, and diluting to a final volume of 8 L. with distilled water. The samples were placed 15 to 16 cm. from the negative or cathode end of the starch blocks and a potential of 200 volts applied for

forty-eight hours. The color was distributed from the starting position to a distance of about 15 cm. toward the right or anode end of the block. In each experiment the starting position and a segment one centimeter wide on each side was cut out and eluted with 200 to 300 ml. of water on a Büchner funnel. This eluate was designated fraction A. The portion of the starch block from A extending 16 cm. to the right was also cut out and eluted in the same way. This was designated fraction B. The A and B fractions were dialyzed against distilled water for 6 days and the corresponding fractions, A and B, from the two starch block experiments were pooled, pervaporated to a volume of about 10 ml., and dialyzed against 10 L. of distilled water for an additional six days. In each case the dialysis bath was changed two to three times per day. Dry weight and nitrogen determinations showed a nitrogen content of 1.0 per cent for the A fraction and 1.5 per cent for the B fraction. The solutions were analyzed for residual urea by the diacetyl monoxime-arsenic acid method of Rosenthal.¹² The data obtained indicated that less than 5 per cent of the nitrogen in the dust fractions could be accounted for by the possible presence of urea. The solutions were also concentrated, adjusted to the same concentration in 50 per cent aqueous glycerol, and clinically tested. The data are presented in Table VIII.

TABLE VIII. SKIN TEST DATA WITH #125 EXTRACT AND FRACTIONS OBTAINED BY STARCH BLOCK ELECTROPHORESIS AT pH 2.5 IN 7M UREA*

PATIENT	#125	A	B
P. J.	2	2	2
A. G.	1	1	1
T. B.	2	0	0
E. T.	1	1	0
S. M.	1	0	0
J. V. L.	0	0	1
Sylvia M.	1	0	0
R. G.	1	0	?
L. B.	1	0	0

*The concentration of the samples was 0.28 per cent, as equivalent galactose determined by the indole test for total carbohydrate (0.36 per cent by dry weight), in 50 per cent aqueous glycerol. The skin reactions were graded 1+ through 4+. The symbol ? indicates a questionable reaction and 0 indicates a negative reaction. These were scratch tests.

DISCUSSION

The four dust extracts that we have studied by starch block electrophoresis are similar in general chemical composition (Table I) to the purified fraction previously reported.¹ The nitrogen contents of these materials are higher, however, and they are colored, whereas the purified fraction was colorless. Other differences demonstrated are a positive Elson-Morgan color reaction for hexosamine in the case of all four dust extracts and chromatographic evidence for the presence of xylose and fucose in some of the extracts, while the purified fraction appeared to be devoid of these components. Except for the fact that the Hollister-Stier extract has a higher nitrogen content than the other three extracts, the chemical composition of all the extracts is quite similar. Our #110 and #125 extracts have somewhat higher uronic acid contents than the other materials. Possibly this may be due to the fact that

our fractions were derived in part or totally from mattress dust as opposed to house dust. The #110 extract was obtained from half mattress and half house dust while the #125 extract was obtained entirely from mattress dust.

The eluate analyses from the starch block electrophoresis experiments carried out in acetate buffer (pH 4.7, $\mu = 0.1$) show considerable variation in the relative amounts of slow, nearly "isoelectric," materials and rapidly moving, negatively charged, materials. The #125 extract is largely composed of the rapidly moving fraction while the Hollister-Stier extract is largely composed of the slow moving or "isoelectric" fraction and the two other extracts, #110 and Endo, present an intermediate picture. This is shown by a comparison of the total carbohydrate curves in Figs. 1A, 2A, 3A, and 4A. The uronic acid components are almost exclusively associated with the fast peak in the case of the #110, #125, and Endo extracts, while in contrast the Hollister-Stier extract shows some association of uronic acid containing materials with the slow peak, as shown in the uronic acid curves of Figs. 1A, 2A, 3A, and 4A. The nitrogen curves in the same figures show that in the case of the #110 and #125 extracts the nitrogen is found to be primarily associated with the fast peak; however, there is some with the slow peak. In the case of the Hollister-Stier and Endo extracts, the nitrogen is found associated with both the fast and slow materials. The nitrogen content of the Hollister-Stier extract is much higher than that of the other extracts and this, in addition to the preponderance of slow material in this extract, possibly explains the much higher association of uronic acid containing components with the slow peak. The polypeptide moiety in the slow fraction of the Hollister-Stier extract possibly provides positive charges to slow down some of the materials in the complex, containing uronic acid, so that they remain in the slow peak. It should be noted that in the case of the Hollister-Stier extract the fast fraction has the higher nitrogen content, while in the case of the Endo extract the fast and slow fractions have more nearly equal nitrogen contents. The distribution of color is shown in the same figures (1A, 2A, 3A, and 4A). In general, the color is associated with the fast peak. Even in the case of the Hollister-Stier extract, where most of the material is in the slow fraction, the color is predominately associated with the fast moving materials. There is some evidence, especially from the #125 and Endo analyses (Figs. 2, A and 4, A), that the color is moving faster than the bulk of the material in the fast peak. This would suggest that the colored material is not linked through covalent bonds to most of the dust extract molecules. This is in agreement with the fact that we were able to easily separate the colored components from the bulk of the material by basic lead acetate precipitation in the preparation of our purified fraction.¹

The data from the paper chromatographic study of the relative monosaccharide composition of pooled eluates from the various regions of the starch blocks (as indicated on Figs. 1A, 2A, 3A, and 4A) are presented in Table II. The general pattern is about the same for all the extracts. Fraction **a**, in all cases, came from a base line region of the starch block and was found to contain only glucose. Fractions **c**, **d**, and **e** generally contain the greatest number of

different monosaccharides, with glucose, galactose, mannose, and rhamnose predominating. It is evident from both Tables I and II that the Hollister-Stier materials are lower in rhamnose than the other extracts. Arabinose was found in all extracts, particularly in fraction **d** which contained materials intermediate in mobility, on the starch block, between the slow and fast peaks and the slow edge of the fast peak. Lesser amounts of fucose and xylose were found especially in the **c**, **d**, and **e** fractions. The fraction **f** containing only the faster moving materials showed a much simpler sugar composition than the fractions **c**, **d**, **e**. Except for the #125 fraction, the mannose seems to be concentrated in the slower materials. It should be noted that these results were obtained by hydrolysis of an approximately constant amount of polysaccharide and that when the eluates were obtained from a region of the starch block low in dust polysaccharide the pooled eluates had to be concentrated more and the glucose contribution from the starch became greater. The method employed for hydrolysis and chromatography destroyed uronic acids, therefore the monosaccharide composition presented in Table II is incomplete and should include uronic acids following the distribution indicated in Figs. 1A, 2A, 3A, and 4A.

The skin test data for the #110 extract and its fractions are presented in Table III. Unfortunately there was not sufficient material of the **c** fraction for complete testing. Most of the activity seems to be confined to **b** and probably **c**, which are the trailing and leading portions of the slow peak. None of the fractions seem to be as active as the unfractionated extract, with the possible exception of **c** where data are lacking. Table IV lists the corresponding data for extract #125. In this case fraction **b**, the slow peak, and fraction **c**, material intermediate between the fast and slow peak, contain most of the activity. The #125 extract and its fractions are all less active than the other materials studied. This may be related to the observation that prolonged contact with ammonium sulfate solution destroys some activity.¹³ (The #125 extract was stored in ammonium sulfate at 50 per cent of saturation for many months.) The skin test data for the Hollister-Stier materials are presented in Table V. The most active materials are found, in this case, in both the trailing and leading edge of the slow peak (fractions **b** and **c**). These materials are both apparently a little more reactive than the unfractionated extracts. The intermediate material in fraction **d** seems to be of about the same activity as the unfractionated extract. In the case of the Endo extract and its fractions, most of the activity is confined to fractions **b**, **c**, and **d**, which contain the slow peak and some of the material of intermediate mobility. These three fractions seem to be of about the same activity as the unfractionated extract. It should be pointed out that statements about the relative reactivity of the various fractions are based on the relative intensity of scratch test reactions made at only one concentration. Much more precise ways to measure this would be to carry the reactions to end point, use the passive transfer technique or possibly an allergen neutralization or desensitization test.¹⁴ It is evident from these data that the materials contained in the fast peak, high in uronic acid and containing almost all the color, are much less active than the slow and

intermediate materials. The data also show considerable patient variation with regard to the fraction showing the greatest reactivity. This may be due to the possible presence of a multiplicity of materials of different allergen specificity moving at different rates on the starch block. This is in agreement with our previous observation¹ that some dust-sensitive individuals who gave strong reactions to the less purified extracts did not give any appreciable reaction to the purified fraction.

The problem of trying to correlate the skin reactivity of the fractions with the components as determined by chemical analysis is difficult. In general, it is fairly clear that a high uronic acid content or the presence of color is not necessary for activity. The question arises as to what are the differences evident in our monosaccharide composition data between fractions **d** and **e** of the Hollister-Stier and Endo extracts that could be correlated with the greater skin reactivity of **d** as compared with **e**. There is less mannose and arabinose in **e** than in **d**; however, these differences do not seem great. It is more probable that if the differences in activity are to be correlated with the monosaccharides present, the uronic acid content and the way the monosaccharides are linked to form polysaccharides are the determining factors. It is also possible that the determinant for allergen specificity resides in the polypeptide rather than the polysaccharide portion of the materials. Rimmington and colleagues² have suggested this on the basis of data correlating the loss of skin reactivity of their allergen fraction, on acid hydrolysis, with the appearance of free amino acids in the hydrolysate.

Dust allergen fractions have been found to be composed of a heterogeneous mixture of acidic polysaccharides with varying amounts of polypeptide components.^{1, 2, 15} There are a number of possibilities as to the type of bonding between the polysaccharide and polypeptide components. There may be no linkage between the materials, i.e., the polypeptides and polysaccharides may be present as separate independent molecules in solution. Ionic bonds, such as might exist between the carboxylate ion of a uronic acid residue and a protonated amino group of the polypeptide, may be involved. Covalent bonds such as amide, glycoside, or ester bonds may be present or the two types of materials may be held together by multiple hydrogen bonds, for example, between the carboxylate ion and hydroxyl group of the polysaccharide and the C—O and N—H groups of the amide linkages of the polypeptide.

Starch block electrophoresis experiments were carried out in a strongly acidic solution (KCl-HCl solution, pH 1.8, $\mu = 0.1$) to determine whether the materials were present as separate components in solution or whether they were linked through ionic bonds. In either case, it would be unlikely for the polysaccharide and polypeptide to remain together on the starch block at both pH 4.7 and pH 1.8. At the low pH, the polysaccharide, due to the protonation of its carboxylate ions, should become isoelectric and the polypeptide should become more positively charged, due to protonation of amino groups as well as less negatively charged, due to the protonation of its free carboxylate ions. The data from these experiments are presented in Figs. 1B, 2B, 3B, and 4B.

The extract #125 and the Endo extract show no separation of nitrogen from the carbohydrate. The #110 extract and the Hollister-Stier extract show evidence of movement of some of the nitrogen to the left (toward the cathode); however, in both these cases the carbohydrate peaks also extend to the left. The Hollister-Stier extract, more than the other extracts, shows the presence of materials of different nitrogen contents, under these conditions (pH 1.8). Eluates from various regions of the starch block, Fig. 3, *B*, were pooled and skin tested. The data are presented in Table VII. Fractions A and B and the unfractionated extract are of about equal activity while fraction C seems definitely less active. From these data it would appear that the materials of the higher nitrogen content were the most active; however, the starch block electrophoresis fractionation of this extract in acetate buffer (pH 4.7) yielded fractions (*d* and *e*) that were also relatively high in nitrogen content but were less active than fractions of lower nitrogen content (*b* and *c*) as shown in Fig. 3, *A* and Table V. It is possible that small amounts of nitrogenous materials have rapidly migrated to the left and off the end of the starch block; however, most of the polypeptide components have remained linked to the polysaccharides under these conditions. Therefore it is unlikely that the polypeptides are primarily present as separate components or are linked to the polysaccharides through ionic bonds.

In order to test the hypothesis that the polysaccharide and polypeptide moieties were linked by multiple hydrogen bonds, a starch block electrophoresis experiment was carried out in 7M urea at pH 2.5. The use of concentrated urea solution (6 to 7M) is the classical way to separate into subunits macromolecules that are held together by multiple hydrogen bonding. Concomitant electrophoresis at pH 2.5 should serve to separate any dissociated polypeptide from the polysaccharide. Fractions A and B obtained by the starch block electrophoresis of samples of #125 extract under these conditions had nitrogen contents of 1.0 and 1.5 per cent respectively. The unfractionated dust extract had a nitrogen content of 1.5 per cent. These data indicate that no separation of the two moieties was obtained and that the polysaccharide and polypeptide are probably not linked by multiple hydrogen bonds.

As a result of these experiments, it is evident that the polysaccharide-polypeptide bonding is primarily covalent.

The control experiments carried out with both the acetate buffer and the HCl-KCl solution show that some soluble carbohydrate as well as a little soluble nitrogenous material were extracted from the starch along with the dust extract fraction. In most cases this does not confuse the interpretation of the curves since the patterns obtained with the dust eluates were either fairly high or down close to base line. No peak should be considered significant, if it is based on one point alone. As an example, the high nitrogen content found in tube number 10 of Fig. 1, *B* probably has no significance.

The final pattern obtained by starch block electrophoresis is the end result of a number of processes. By far the most important of these is electrophoretic transport of the materials; however, electroosmosis, adsorption, and ion exchange also play some part.

SUMMARY

1. The approximate chemical compositions of four dust extracts, including the Hollister-Stier and Endo commercial preparations and two extracts prepared in our laboratory, were determined. These materials were found to consist of a heterogeneous mixture of acidic polysaccharides with varying amounts of polypeptide.

2. The materials were investigated by starch block electrophoresis in acetate buffer (pH 4.7, $\mu = 0.1$). In general, a fast (negatively charged) and a slow peak were found. The allergen activity was predominately associated with the materials of slow or intermediate mobility. In some cases the fractions seemed to be more active than the unfractionated extract.

3. No simple relation was evident between the monosaccharide composition or the nitrogen content of the fractions and the allergen activity.

4. The color moved slightly faster than the fast peak and was definitely separated from the more active fractions.

5. Experiments were presented that indicated that the polypeptide moiety of the dust extract was linked to the polysaccharides by covalent bonds.

The authors are indebted to Marie Stanley for superb technical assistance and to Drs. E. M. Heimlich and A. M. Targow for skin testing the dust allergen fractions. We wish to thank Mr. Morris Webb and the Hollister-Stier Laboratories for the gift of their acetone precipitated dust extract.

REFERENCES

1. Vannier, W. E., and Campbell, D. H.: The Isolation and Characterization of a Purified House Dust Allergen Fraction, *J. ALLERGY* **30**: 198, 1959.
2. Rimington, C., Stillwell, D. E., and Maunsell, K.: The Allergen(s) of House Dust; Purification and Chemical Nature of Active Constituents, *Brit. J. Exper. Path.* **28**: 309, 1947.
3. Kunkel, H. G., and Slater, R. J.: Zone Electrophoresis in a Starch Supporting Medium, *Proc. Soc. Exper. Biol. Med.* **80**: 42, 1952.
4. Bloemendal, H.: Starch Electrophoresis I. Starch Block Electrophoresis, *J. Chromat.* **2**: 121, 1959.
5. Lanni, F., and Campbell, D. H.: A Search for Heterologating Antibody, and the Significance of the Results to the Mechanism of Antibody Formation, *Stanford Med. Bull.* **6**: 97, 1948.
6. Winzler, R. J.: Determination of Serum Glycoproteins in *Methods of Biochemical Analysis*, edited by D. Glick, New York, 1955, Interscience Publishers, Inc., vol. II, p. 279.
7. Elson, L. A., and Morgan, W. T. J.: A Colorimetric Method for the Determination of Glucosamine and Chondrosamine, *Biochem. J.* **27**: 1824, 1933.
8. Dische, Z.: New Color Reactions for Determination of Sugars in Polysaccharides in *Methods of Biochemical Analysis*, edited by D. Glick, New York, 1955, Interscience Publishers, Inc., vol. II, p. 343.
9. Dische, Z.: A New Specific Color Reaction of Hexuronic Acids, *J. Biol. Chem.* **167**: 189, 1947.
10. Dische, Z.: New Color Reactions for Determination of Sugars in Polysaccharides in *Methods of Biochemical Analysis*, edited by D. Glick, New York, 1955, Interscience Publishers, Inc., vol. II, p. 320.
11. Lanni, F., Dillon, M. L., and Beard, J. W.: Determination of Small Quantities of Nitrogen in Serological Precipitates and Other Biological Materials, *Proc. Soc. Exper. Biol. Med.* **74**: 4, 1950.
12. Rosenthal, H. L.: Determination of Urea in Blood and Urine with Diacetyl Monoxime, *Anal. Chem.* **27**: 1980, 1955.

13. Lietze, A., and Reed, C.: Oregon State College, Corvallis, Oregon. Personal communication.
14. Richter, M., Harter, J. G., Schon, A. H., and Rose, B.: Studies on Ragweed Pollen. III. Estimation of the Minimum Number of Allergens in the Water Soluble Extract of Ragweed Pollen and a Critical Evaluation of the Neutralization Technique. The Demonstration of Two Different Reagins in Sera of Ragweed Sensitive Individuals, *J. Immunol.* **79**: 13, 1957.
15. Cayton, H. R., Furness, G., Jackson, D. S., and Maitland, H. B.: Studies on Cotton Dust in Relation to Byssinosis. III. Comparison of Cotton Dust and House Dust by Chemical and Skin Tests, *Brit. J. Indust. Med.* **9**: 303, 1952.