I

HOUSE DUST ALLERGENS

II

EXPERIMENTS ON THE DISTENSIBILITY
OF THE EXCISED RABBIT BLADDER

Thesis by

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Abstract

Part I:

The problem of the nature of house dust allergens has been investigated by studying the chemical and physical properties of active fractions of aqueous dust extracts. The fractions consisted largely of a heterogeneous mixture of acidic polysaccharides and in lesser amounts a polypeptide component (2-30%), probably linked to the polysaccharides. The polysaccharides contained uronic acid (probably mostly glucuronic acid), D-glucose, D-mannose, D-galactose and L-rhamnose and lesser amounts of a number of pentoses. Nitrogen free active polysaccharide fractions have not been obtained. The most purified active fraction contained 0.4% nitrogen. The relation between the chemical and physical properties of the fractions and current ideas of the origin of the dust allergens was discussed.

Part II:

Rabbit bladder stretching curves under constant pressure have been studied. A method, involving the use of eserine and acetylcholine, has been found to restretch the same bladder and obtain reproducible curves. The effect of temperature and epinephrine upon the bladder stretching curves was investigated.

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Part I

House Dust Allergens

Part I: House Dust Allergens

A. Introduction

House dust is the source of one of the most common allergens to be implicated as the cause of such allergic diseases as hay fever and asthma. The essential clinical features of the relationship are as follows:

(1) The symptoms of hay fever and asthma occur during or after inhalation of the house dust.

(2) Sensitive individuals give an immediate wheal and flare response upon skin test with house dust extracts. This skin response can be demonstrated in non-allergic individuals by passive transfer of serum from a house dust sensitive individual to selected skin sites of the non-allergic individual with subsequent direct skin testing at these sites.

(3) In most cases the symptoms of house dust allergy may be alleviated by immunization with gradually increasing doses of house dust extracts.

The first observations of the relationship between house dust and clinical allergy were made by Cooke in 1922 (1). Subsequent experiment and observation has suggested the concept that there is present in house dust a unique allergen or group of allergens specifically characteristic of house dust. House dust sensitivity cannot be completely explained on the basis of other known allergens that are

present in the dust e.g. pollens, animal danders and molds. This concept of the uniqueness of the house dust allergen is based on the fact that a correlation of house dust sensitivity with that of any other allergen has never been established. It is also based on studies (2) involving specific in vitro desensitization and the use of passively transferred skin reactions (Pruasnitz-Küstner reactions).

Allergen activity is found only in dust from houses, not in dust from barns, stores, empty buildings or the street. Studies have indicated that the same specificities are involved in sensitization to house dust in all parts of the world. The origin of the allergen characteristic of house dust is unknown. One of the theories is that the allergen is a material that is produced by molds or bacteria either directly or by acting on cotton, kapok or feathers. So far no mold or bacteria that has been cultured from house dust has been found to produce any dust allergen. There is some evidence that the "aging" of cotton is involved in the production of the allergen (3). Extracts from fresh cotton linters were found to be inactive while extracts from cotton that had been in contact with air for many months were active. Activity developed even if the cotton was autoclaved in a plugged vessel to kill any organisms that might be present. It was felt that the allergen activity developed fastest in those samples that had the freest access to the air. There is still a possibility that molds or bacteria from heat resistant spores may be involved in the

process. Other sources of the house dust allergen that have been suggested include desquamated epithelial scales, and rodent and insect feces.

Most of the work in the field of the chemistry of allergens has been directed toward the preparation of potent extracts for skin testing and hyposensitization rather than to determine the chemical nature of the active materials. The standardization of allergen extracts is an important practical problem that has forced allergists to consider the chemistry of these materials. In the course of clinical practice it is frequently necessary to know the relative potency of various preparations of the same allergen. A biological assay of the activity is time consuming and difficult. Attempts have therefore been made to correlate some easily determined property of the allergen extract with the specific activity. Determinations of the total nitrogen, amino nitrogen and the phosphotungstic acid precipitable material have been especially popular. Nitrogen determinations yielding nitrogen units of activity have been reasonably successful in the case of many pollens, however in the case of house dust they have been proven to be worthless. It is clear that what is required is the study of the chemistry of the active materials. If the chemical nature of the allergens were known it should not be too difficult to devise suitable analytical methods.

For many years aqueous extracts of house dust have been used for diagnostic tests and hyposensitization. One of the

first attempts to concentrate and study the chemical properties of the skin reacting materials in house dust was that of Boatner and Efron (4). They fractionated an aqueous extract of house dust with dioxane, then with ammonium sulfate and obtained a material that was a potent allergen and gave positive tests for both protein and carbohydrate.

Sutherland (5) 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 et al. (6) have carried out further purification by acetone fractionation of dust allergen prepared by Sutherland's method. Their work contains the most detailed study of the properties of potent dust allergen fractions so far published. Their most purified allergen fraction contained both carbohydrate and polypeptide as demonstrated by acid hydrolysis and paper chromatography. The analytical data they reported are as follows:

Total hexose (as galactose by the orcinol method)	40 - 60%
Nitrogen	5 - 7%
Reducing Power (as galactose)	1.5%
Ash	1 - 2%

In this paper, Rimington describes the results of studies by hydrolysis and paper chromatography that indicated

the presence of nine aliphatic amino acids (no aromatic amino acids) and galactose with a trace of glucosamine. Subsequent studies from Rimington's laboratory are presented as an unpublished communication in a paper by Cayton et al. (7) on a comparison of the properties of extracts of cotton dust and house dust. These later studies of Rimington with the same purified house dust fraction demonstrated the presence of arabinose, xylose and a small amount of rhamnose in addition to the galactose. The possibility of a trace of galacturonic acid was also mentioned. A more detailed amino acid analysis was also reported as follows:

Amino Acid	% of Total Amino Acids
aspartic acid	6
glutaric acid	15.5
glycine	22
serine	16
valine	5
leucine	7
arginine or lysine	5
proline	±

Rimington also reported in his paper (6) the presence of two main electrophoretic components (phosphate buffer, pH 8, $\mu = 0.2$) of about equal skin reactivity. The fast component was colored while the slow component was colorless. There was little difference in the hexose content and the nitrogen content between the fast and slow components. The data were

reported as follows:

	Slow Component	Fast Component
Hexose (orcinol) as galactose	59.0%	50.0%
Nitrogen	5.38%	5.69%
Color	colorless	colored

An experiment involving the partial hydrolysis (0.5 N HCl, $\frac{1}{2}$ hr., 100°C) of the purified allergen was carried out. This procedure liberated about 80% of the carbohydrate from the allergen as reducing sugar without liberating amino acids. The product (12% hexose and 10% N) maintained essentially undiminished allergen activity. Upon further heating free amino acids appeared in the hydrolysate and the activity immediately disappeared. On this basis Rimington concluded that the biologic activity was associated with the amino acid containing component rather than the carbohydrate component. The allergen was found to be not only stable to heat, as indicated by the above hydrolysis experiment, but also stable to all the enzymes tested (crystalline trypsin, pancreatin, taka-diastase, hyaluronidase and pectase). Rimington also reported low but definite inhibiting activity for the agglutination of A, B or O erythrocytes by specific antibody. There was no inhibition in the Rh system.

House dust extracts are poor antigens in experimental animals. A number of workers have produced precipitins (8) and shocked guinea pigs (9) with dust extracts, generally with the use of an adjuvant (alum or Freund). One of these

studies was especially interesting (10), in that it indicated that the components against which the animal was making precipitin antibodies were not the components responsible for skin reactivity. The supernatants from precipitin tubes in antibody excess still had unaltered skin reactivity.

In the present study our object has been to determine the chemical nature of the substance or substances present in house dust that are responsible for wheal production in the specifically sensitive individual. There have been two phases to the work. One involved the fractionation of aqueous extracts of house dust and mattress dust with subsequent direct skin testing of the fractions on dust sensitive individuals. The other phase involved chemical and physical studies of fractions known to be active.

B. Preliminary Investigations

Source of the Dust:

The dust used in this investigation was supplied by the members of the American Academy of Allergy. There were ten to fifteen batches of house dust of a few pounds each from various individual members of the Academy and a large pool of about 200 pounds of mattress dust from a mattress cleaning establishment in Atlanta, Georgia. The mattress dust was sent to Caltech by Dr. Mck. Pipes, a member of the Academy.

Clinical Testing of the Dust Fractions:

We were fortunate in having the cooperation of a number of allergists in the skin testing of our materials. Drs. Samuel Feinberg, Townsend Friedman, George Piness and Willard Small tested a number of the fractions. While Dr. Charles Sutherland was visiting Caltech he carried out preliminary skin tests on student volunteers at the Student Health Center. I have tested some of the materials at the Student Health Center and also, by scratch tests, on myself, as I am dust sensitive. For sterilization the samples were heated to 56°C. on three successive days prior to skin testing. This method of sterilization was used rather than Seitz filtration because it has been reported that the filter pads remove some of the activity (6).

1. Preparation of House Dust Extracts by the Methods of Rimington and Efron.

As a first step, batches of mattress dust were processed following the methods of Boatner and Efron (4) (Fig. 1) and Rimington et al. (6) (Fig. 2). We had originally hoped that the relative activity of materials from each step of the fractionation procedures could be tested; however we were unable to find facilities for having this rather large number of samples tested.

Drs. George Piness, Willard Small and Samuel Feinberg tested the final products from these fractionation procedures as well as a sample of purified dust allergen prepared by Dr. B. G. Efron and sent to us. Dr. Efron's dust extract was prepared by acetone and dioxane fractionation of an aqueous house dust extract. The material was precipitated between 40 and 75% acetone and then between 33 and 75% dioxane. The solvents were removed by dialysis.

The skin test results are presented in Figs. 3, 4 and 5. The test results indicated that these materials were potent allergens. There was no consistent pattern of reactivity to the three allergen preparations among the patients tested. In general the material prepared by Dr. Efron seemed to be a little more reactive than our materials however there ~~are~~ were a number of patients that were more reactive to our preparations. This general trend in reactivity may depend more on differences in the potency of the dust used as a starting material than on the method

Fig. 1

The Efron Procedure (4) for the Isolation of the House Dust Allergen

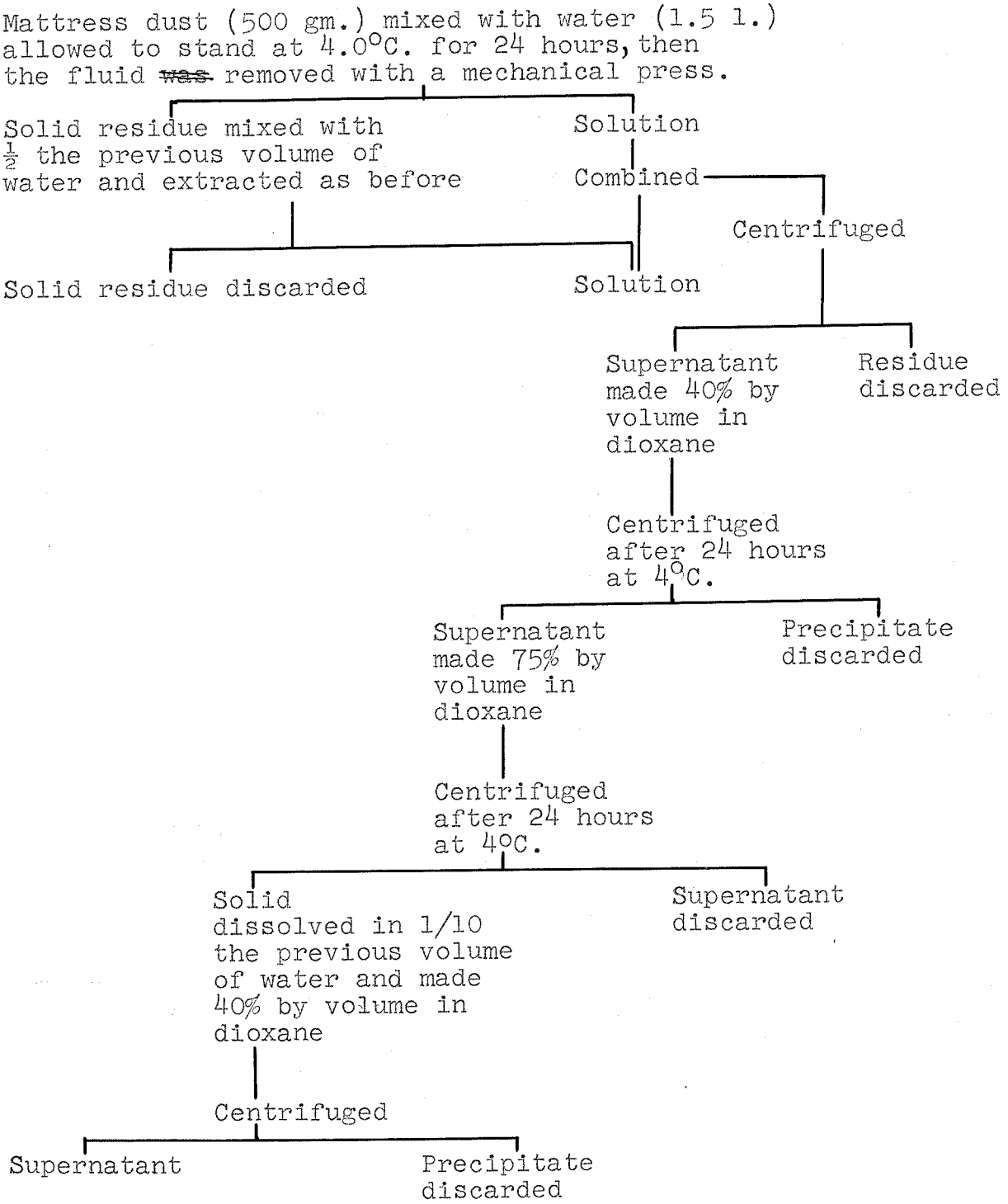


Fig. 1 (cont.)

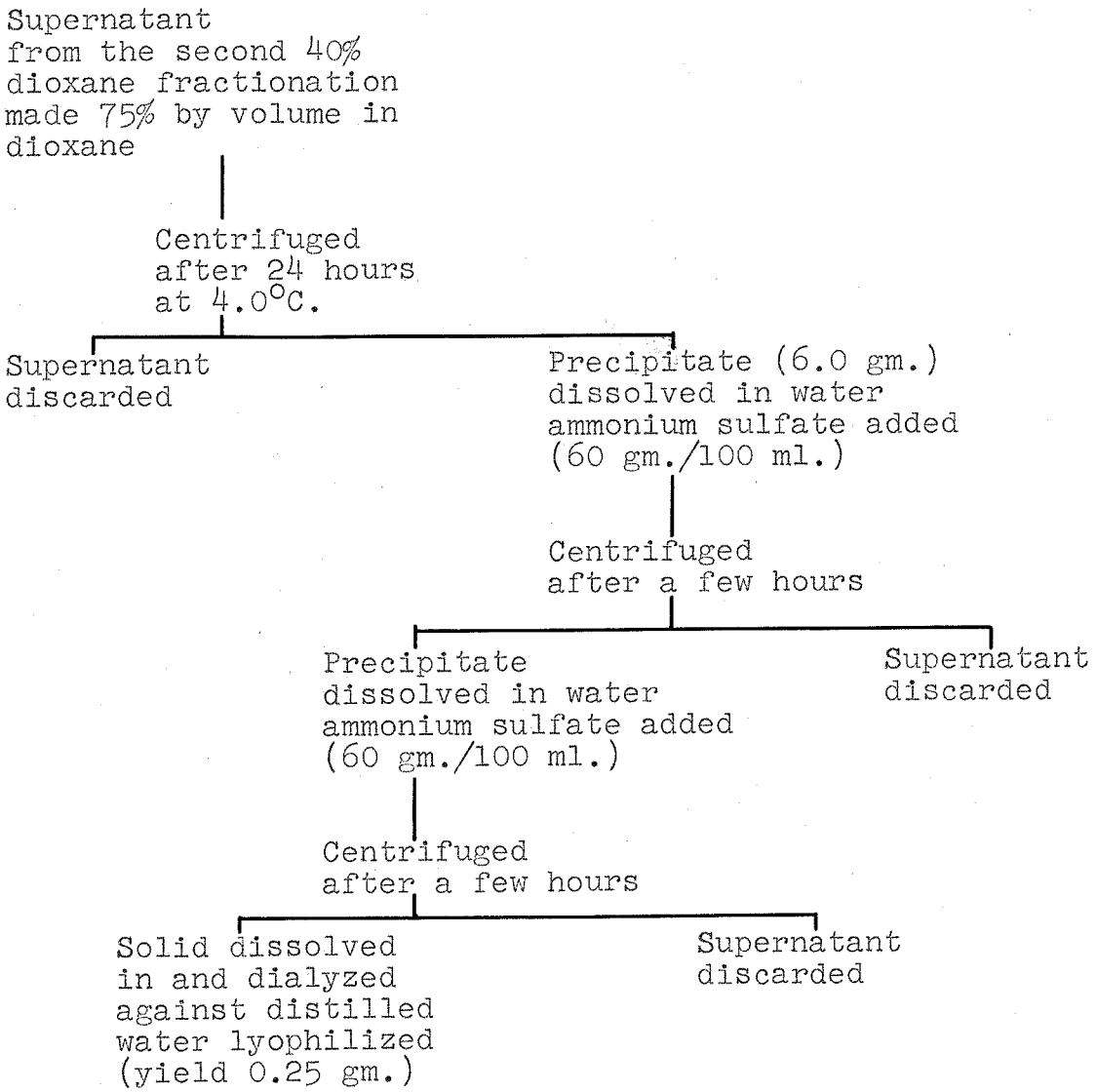

Supernatant from the second 40% dioxane fractionation made 75% by volume in dioxane
Centrifuged after 24 hours at 4.0°C.
Supernatant discarded
Precipitate (6.0 gm.) dissolved in water ammonium sulfate added (60 gm./100 ml.)
Centrifuged after a few hours
Precipitate dissolved in water ammonium sulfate added (60 gm./100 ml.)
Supernatant discarded
Centrifuged after a few hours
Solid dissolved in and dialyzed against distilled water lyophilized (yield 0.25 gm.)
Supernatant discarded

Fig. 2

The Rimington Procedure (6) for the Isolation of the House Dust Allergen

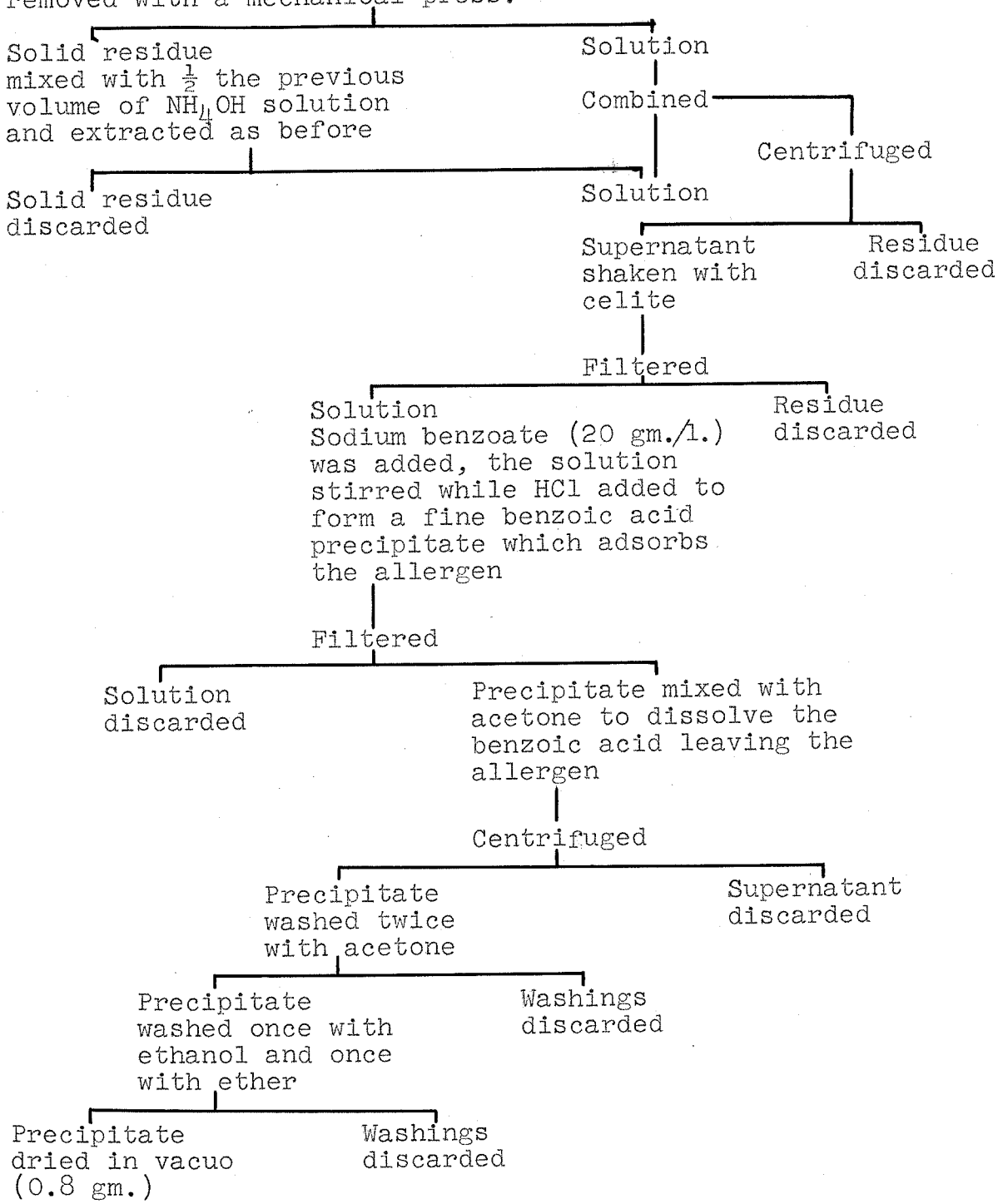


Fig. 2 (cont.)

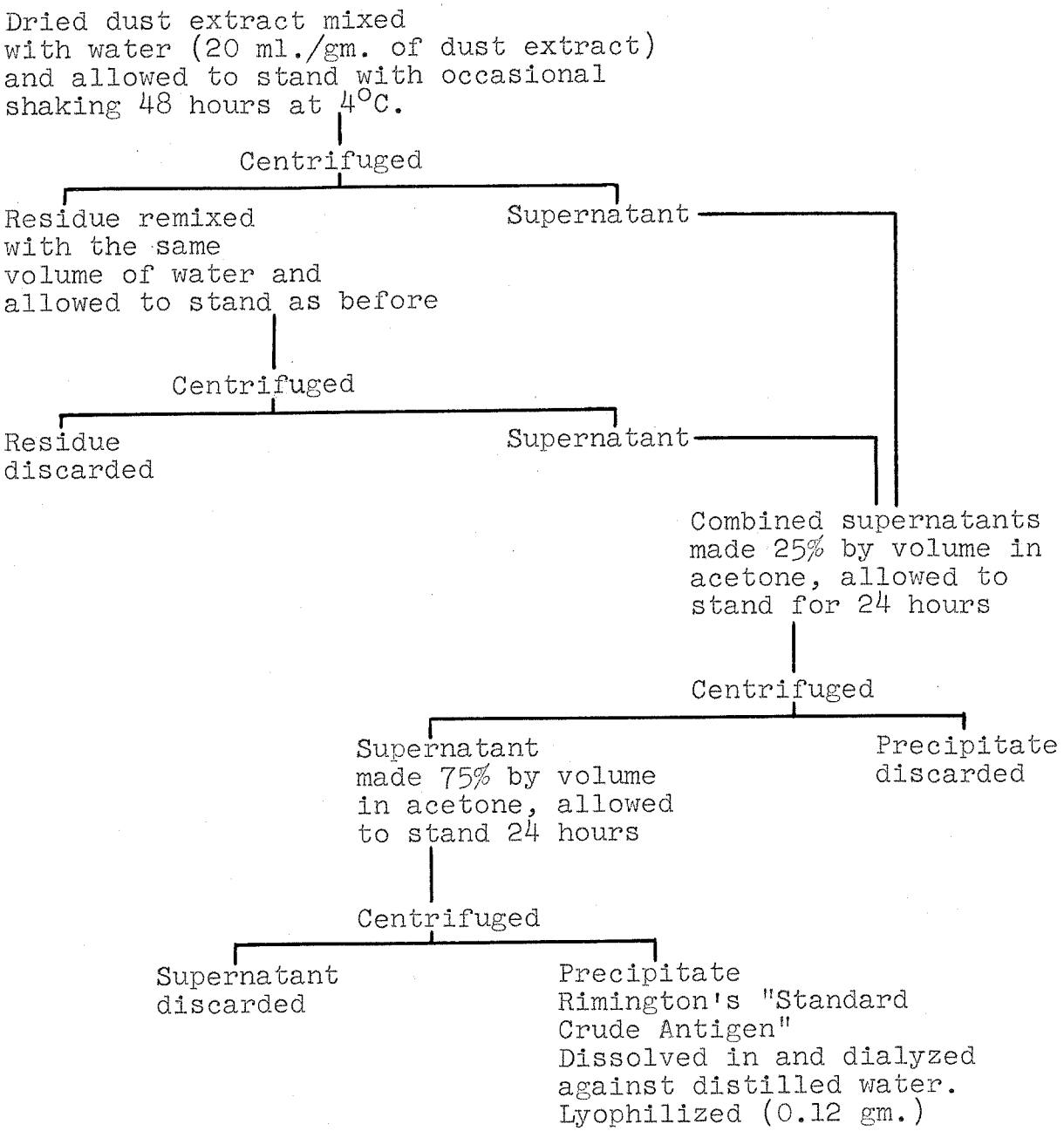

Dried dust extract mixed with water (20 ml./gm. of dust extract) and allowed to stand with occasional shaking 48 hours at 4°C.
Centrifuged
Residue remixed with the same volume of water and allowed to stand as before
Supernatant
Centrifuged
Residue discarded
Supernatant
Combined supernatants made 25% by volume in acetone, allowed to stand for 24 hours
Centrifuged
Supernatant made 75% by volume in acetone, allowed to stand 24 hours
Precipitate discarded
Centrifuged
Supernatant discarded
Precipitate Rimington's "Standard Crude Antigen" Dissolved in and dialyzed against distilled water. Lyophilized (0.12 gm.)

Fig. 3

Intradermal Skin Tests Reported by Dr. George Piness and Performed at the Los Angeles Children's Hospital

Patient	Preparation[1] and result of tests[2]				
	I	II	III	IV	V
1	3	3	4	3	2
2	3	4*	4*	2	3
3	3	2	4	2	2
4	4	4	4*	4	3
5	4	4	4*	4	2
6	4	4	4	4	2
7	4*	4	4*	4	3
8	4	3	4	3	2
9	4	4	4	4	2
10	3	4	4	3	3
11	4*	4	4*	3	2
12	3	4	4	3	3
13	4	4	4*	3	3
14	2	4	2	3	2
15	4*	4	4*	3	3
16	3	3	4	2	2
17	4*	4	4*	3	2
18	2	2	2	2	0
19	4	4	4*	4	3
20	3	2	+	0	0
21	4	3	3	+	0
22	4	4*	3	2	0
23	4	3	4	3	+
24	4	4	4*	3	3
25	3	2	2	0	0
26	2	2	2	0	0
27	3	3	4	2	3
28	3	3	3	2	2
29	4	4	4	2	2
30	3	2	2	0	0
31	2	2	2	0	0
32	2	2	2	0	0

[1]Samples tested:

I Dust extract prepared by the Efron procedure at Caltech.
II Dust extract prepared by the Rimington procedure at Caltech.
III Dust extract sent to us by Dr. Efron.
IV Dust extract prepared by Dr. Piness' laboratory.
V Commercial dust extract (Arlington).

[2]The reactions were graded 1 through 4. The asterisk indicates an unusually violent reaction. The antigens were used in a concentration of 0.1 percent, based on total solids.

Fig. 4

Intradermal Skin Tests Reported by Dr. Willard Small

Patient	Preparation[1] and result of test[2]			
	I	II	III	IV
M	1:100	0	1:1000	1:1000
Me	1:100	1:100	1:1000	1:10,000
S	0	0	1:100	Positive puncture test
So	?	?	1:1000	1:1000

[1] Tests were made with dilutions of 1:100 and 1:1000 of a stock solution. The stock solutions contained 0.5 mg. per ml. of dust extract in samples I, II and III and 50 mgm./ml. in the case of sample IV.

I Dust extract prepared by the Efron procedure at Caltech.
II Dust extract prepared by the Rimington procedure at Caltech.
III Dust extract sent to us by Dr. Efron.
IV Dr. Small's preparation.

[2] The results are expressed as the highest dilution which gave a positive reaction. Preparations I and II gave questionable reactions in patient So.

Fig. 5

Skin Tests (Scratch Tests) Reported by Dr. Samuel Feinberg

Patient	Preparation[1] and result of test[2] I	II	III
VH	1:150* (21---)	1:150 (11???)	1:150* (21---)
NB	_____	_____	1:150 (21?--)
FN	1:100* (1----)	1:100 (1?---)	1:4050 (22221)
MB	1:150* (21---)	1:100* (1----)	1:150* (21---)
CS	1:1350 (4321?)	1:1350 (4321?)	1:450 (221?-)
JM	0 (??---)	1:150* (11---)	1:100 (1?---)
LM	1:150 (21?--)	1:150 (31?--)	1:150 (21?--)

[1] Tests were made with varying dilutions of 1.0% solutions.

I Dust extract prepared by the Efron procedure at Caltech.
II Dust extract prepared by the Rimington procedure at Caltech.
III Dust extract sent to us by Dr. Efron.

[2] The results are expressed as the highest dilution which gave a positive reaction. An asterisk indicates the highest dilution reported (i.e. the end point was not reached) and _____ indicates that no test was made. The numbers in parenthesis indicate the grade of the reaction starting at a dilution of 1:100 and proceeding to the higher dilutions of 1:150, 1:450, 1:1350 and 1:4050. The symbol ? indicates a questionable reaction and 0 indicates a negative reaction.

of extraction and fractionation. In this case Dr. Efron's material was derived from house dust and our materials were derived from mattress dust.

Dr. Piness' skin test data deserve special comment because of the unusual violence of some of the reactions. His laboratory prepared 1:1000 solutions of the solid dust materials that we submitted. These were tested intradermally and compared with his own extract and a commercial preparation. Some of the children tested (indicated by an asterisk in Fig. 3) developed very large local reactions and a regional lymphadenopathy. Such reactions are not characteristic of allergy and the question arises as to the possibility of a nonspecific irritant being responsible. It may be that it is entirely a matter of concentration and that much more dilute solutions should have been used for intradermal testing, especially in children. Patients 30, 31 and 32 in Dr. Piness' series were considered to be nonallergic to house dust; however they gave a reaction to our materials. The result could be explained by a subclinical sensitivity to house dust or by the presence of a nonspecific irritant. It would be necessary to carry out passive transfer studies to distinguish the two possibilities. We tested our materials intradermally in normal guinea pigs and found that at a concentration of 0.1% (1:1000) there was no evidence of any irritant in the allergen preparations. The skin responses at this concentration were identical to those with normal saline.

There is some indication from these data that there are a number of different allergenic components present in the fractions. This aspect of the problem will be discussed in detail when the results of other fractionation studies have been presented.

2. Exploration of Other Fractionation Methods.

The Efron and Rimington procedures have been found to yield potent allergen materials. Since we were not able to check the relative potency of the products of the various steps in the fractionations, we do not know whether most of the activity of the original aqueous extracts has been concentrated in the final products or not. It seemed desirable to study various methods of extracting the dust and of fractionating the extracted materials. Possibly simpler methods could be found that would yield products as potent as those obtained by the Efron and Rimington procedures.

As a step in this direction the effect of the pH of the extracting solution was studied. A series of experiments was performed using a 145 ml. volume of extracting solution of varying pH and 50 grams of dry mattress dust. The pH was observed at various times after mixing. The liquid was removed with a mechanical press, centrifuged and then dialyzed. The pH and scratch test data are presented in Fig. 6. The results of the few scratch tests performed indicated that there was not much difference between the

Fig. 6

Extracting Solution	pH of Extracting Solution	pH after ½ hour	pH after 24 hours	pH after dialysis
0.1 N HCl	1.1		4.12	6.26
0.1 N HOAc	2.88	4.51	4.72	
1.0 N HOAc	2.32	3.62		
2.0 N HOAc	2.08	3.23	3.36	5.82
water	6.45	5.95	5.92	5.60
0.01 N NH_4OH	10.45		5.9	5.5
0.01 N NaOH	12.24		6.02	6.30
0.1 N NaOH	12.8	10.23	8.65	
0.15 N NaOH		11.12	9.4	
0.3 N NaOH		12.27	10.47	5.2

Scratch Test Results for Three of the Dialyzed Products

Extracting Solutions	Patients (1)	(2)	(3)	(4)	Concentration of the test soln.
water	1	0	1	1	7.6 mg./ml.
2 N HOAc	±	0	±	0	6.6 mg./ml.
0.3 N NaOH	1	±	±	1	11.0 mg./ml.

products with neutral and basic extracting solutions. The material extracted with 2 N acetic acid appeared to be less active. The pH of the extract after dialysis was independent of the pH of the extracting solution varying from 5.2 to 6.3.

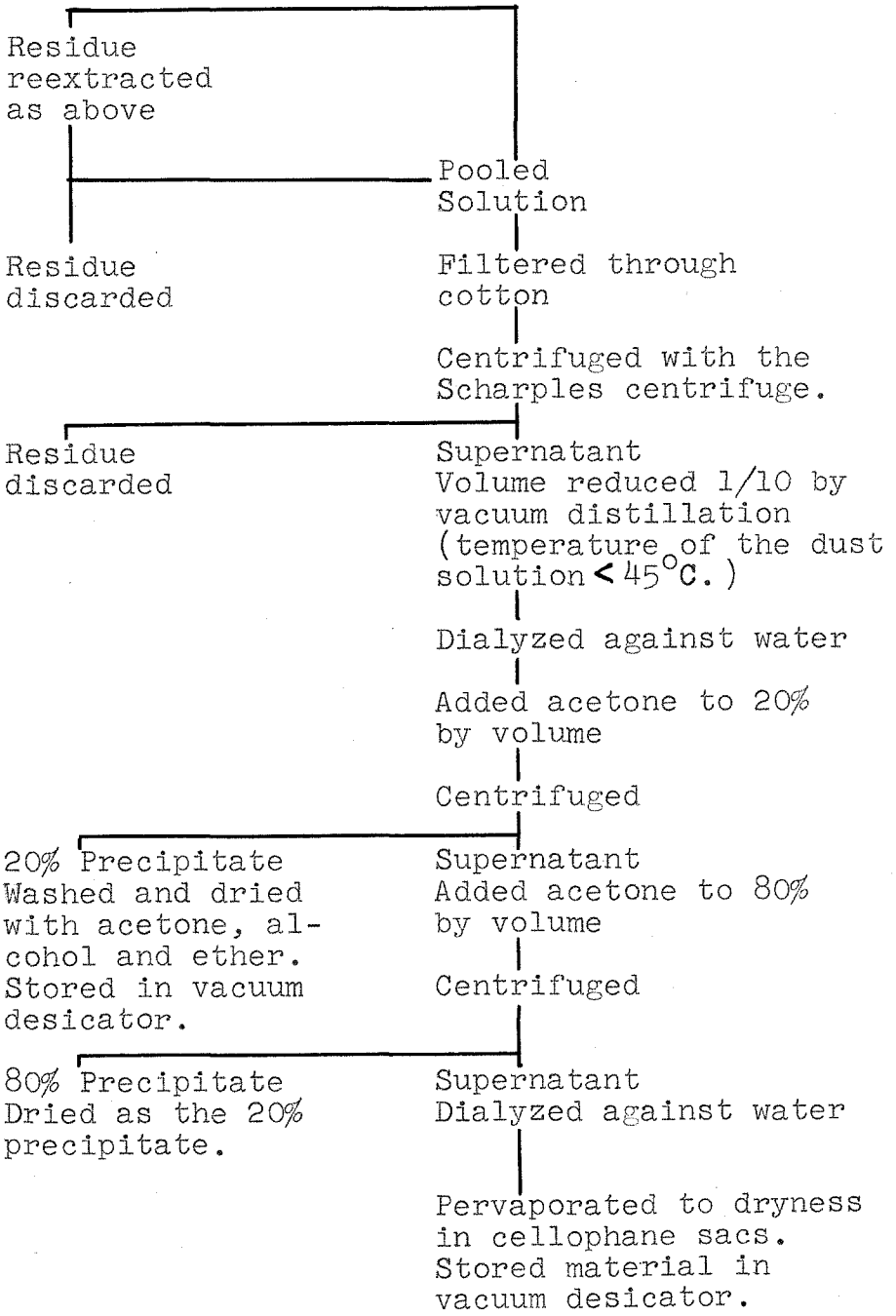
A large amount of house dust (two to three buckets full) was twice extracted with water and the centrifuged aqueous extract concentrated to 1/10 the initial volume by vacuum distillation (temperature of the dust solution kept less than 45°C.). The concentrated solution was then dialyzed, at 4°C., against distilled water for five days, with a total of nine changes of the dialysis bath. The dialyzed extract was then fractionated with acetone at 20 and 80% by volume in acetone. A schematic diagram of the procedure is given in Fig. 7. These materials were designated Crude Dust Fractions #105. In order to obtain more material the above procedure was twice repeated yielding Crude Dust Fractions #106 and #107. Preliminary scratch testing of these materials showed no significant difference in the skin reactivity of the material precipitated in 20% acetone and that precipitated in 80% acetone. Both fractions seemed to be as active as the products of the Rimington and Efron methods; however more extensive testing carried out by Dr. Feinberg in Chicago suggested that the Rimington and Efron type materials were more active (Fig. 10).

It seemed from the experiments in which dust samples were extracted at different pH's that more material was

Fig. 7

Preparation of Crude Dust Fractions #105

House dust wet with water containing a few drops of Tergitol. After 12 hours at room temperature the liquid was removed with a press.



obtained with a basic extracting solution. There was no indication of any loss of activity under basic conditions. An experiment was carried out to study the possible base inactivation of an active dust extract (Fig. 8). Three 50 mg. portions of an active dust fraction (#106 precipitate to 20% acetone) were subjected to basic conditions of varying degree by the addition of sodium hydroxide. One such sample was kept as a control being carried through the experiment with no added sodium hydroxide. The samples were kept approximately at pH 8.3, 10.4, 12.5 and the control at 6.5 for 41 hours. They were then neutralized by the addition of hydrochloric acid and all brought to the same final volume. Scratch tests on a single dust sensitive individual were performed. The results indicated that the allergen was not inactivated by any of the sodium hydroxide treatments. It would have been most desirable to test these materials on more dust sensitive individuals; however this was not done.

Since our experiments had indicated that the allergen activity was stable to strong base it was decided to prepare a crude dust extract using 1.5 N sodium hydroxide solution rather than water to extract the dust. A large amount (about two buckets full) of a 50/50 mixture of house dust and mattress dust was extracted with 5.5 liters of 1.5 N NaOH (Fig. 9). Two hours after mixing the pH was 9.5. After 24 hours, at 4°C., the liquid was removed with a mechanical press and the solid reextracted with 3 liters of the 1.5 N

Fig. 8

Sodium Hydroxide Inactivation Experiment

Sample	pH after 16 hours*	
1 no NaOH added, pH 6.95	6.51	
2 NaOH added until pH 8.15	6.85	8.58
3 NaOH added until pH 10.34	8.15	10.3
4 NaOH added until pH 12.10	9.85	12.7

Sample	pH after 20 hours		pH after 41 hours	
1	6.47		6.75	
2	7.43	8.55	7.35	8.35
3	9.05	10.55	9.51	10.72
4	12.5 no NaOH added		10.4	12.5

* In each column the pH before and after the addition of more sodium hydroxide solution is given. After 41 hours the samples were brought to neutrality by the addition of hydrochloric acid solution. The volumes were adjusted until they were equal.

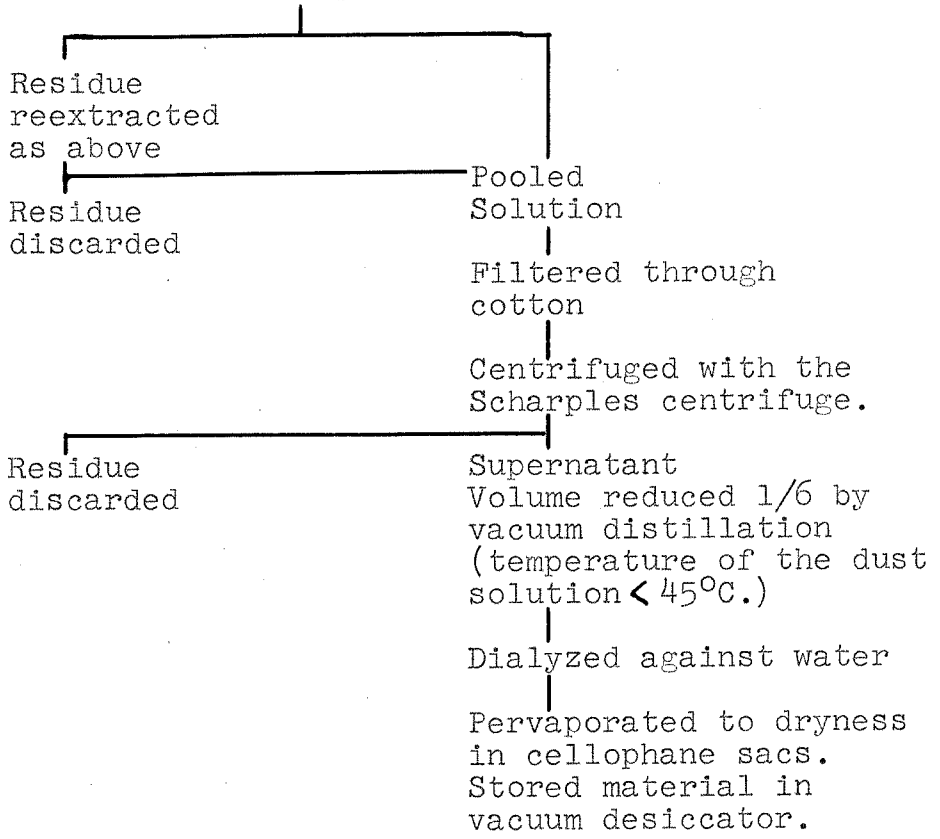
Scratch Test Results on One Dust Sensitive Individual

Sample	Response
1	1
2	2
3	1
4	1

Fig. 9

Preparation of Crude Dust Fraction #110

Dust (50/50 house dust and mattress dust) wet with 1.5 N NaOH (containing some toluene). After 24 hours at 4°C. the liquid was removed with a press.



sodium hydroxide solution. The mixture was allowed to stand at 4°C. and then the liquid removed with a mechanical press as before. The pH of the second extraction product was 9.4. The liquids were pooled yielding a final volume of 6.5 liters, filtered, centrifuged with the Scharples centrifuge and then concentrated by vacuum distillation to 950 ml. The concentrated aqueous extract was dialyzed against water. A small portion of the dialyzed extract was brought to 80% by volume in acetone and the precipitate washed with acetone to dry. Another small portion of the extract was pervaporated to dryness. Skin tests indicated that the two materials were of essentially the same activity and that they were about as active as our previous fractions prepared by acetone fractionation of a water extract (see Fig. 10 and Fig. 11). It should be remembered that there would have to be a five fold difference in activity before we could be reasonably sure of detecting it. The remainder of the aqueous extract was then pervaporated to dryness. This material has subsequently been used for a wide variety of experiments.

A series of experiments ~~were~~ was carried out involving the ammonium sulfate fractionation of this crude dust extract (#110). The object was to divide the material into fractions on the basis of ammonium sulfate precipitability and then to determine the relative skin reactivity of these fractions. First a water solution of the dust extract was fractionated

Fig. 10

Skin Tests (Scratch Tests) Reported by Dr. Samuel Feinberg

Preparation[1] and result of test[2]

Patient	I	II	III	IV	V
ES	1:4050* (44321)	1:150 (32?--)	1:450 (321??)	1:1350 (3211?)	———
AH	1:4050* (44221)	———	0 (?----)	0 (?----)	0 (?----)
FE	1:1350* (2222-)	1:1350* (1-11-)	1:1350* (2211-)	1:150 (11??-)	1:100 (1??--)
LC	0 (?-?--)	1:150 (21??-)	1:150* (11---)	1:1350* (11?1-)	0 (??---)
EB	1:4050* (33322)	1:4050* (33211)	1:4050* (22111)	1:450 (221??)	1:4050* (34322)
RC	1:150* (22---)	1:100* (1----)	1:150* (11---)	———	———
AB	1:4050* (33322)	1:4050* (32222)	1:150 (21?--)	1:1350* (2--2-)	1:1350* (211--)
VW	1:4050* (43444)	1:1350* (2211-)	1:4050* (22111)	1:1350* (2321-)	1:4050* (44444)
AF	1:4050* (22211)	1:4050* (11111)	1:450 (111??)	1:450* (111--)	1:4050* (22211)
JG	1:150 (21?--)	———	———	———	1:150 (42??-)

[1] Tests were made with varying dilutions of 0.75% solutions except in the case of sample V which was 1.0% and sample I whose concentration is unknown.

I Commercial house dust extract (Endo).

II Precipitate from an acetone fractionation (at 80% by volume acetone) of an aqueous house dust extract. Pooled samples #105, #106 and #107.

III #110 alkali extracted crude house dust material. Dried by pervaporation.

Fig. 10 (cont.)

IV Precipitate from acetone fractionation (at 80% by volume acetone) of the crude house dust extract #110.

V Dust extract sent to us by Dr. Efron.

[2] The results are expressed as the highest dilution which gave a positive reaction. An asterisk indicates the highest dilution reported (i.e. the end point was not reached) and _____ indicates that no test was made. The numbers in parenthesis indicate the grade of the reaction starting at a dilution of 1:100 and proceeding to the higher dilutions 1:150, 1:450, 1:1350, and 1:4050. The symbol ? indicates a questionable reaction and 0 a negative reaction.

Fig. 11

Scratch Tests on a Single Dust Sensitive Individual (WEV)

of Some of the Samples Tested

by Dr. Feinberg (Fig. 10)

Preparation[1]	Test Result[2]
II	2
III	1½
IV	1½

[1] The following preparations were tested:

II Precipitate from an acetone fractionation (at 80% by volume acetone) of an aqueous house dust extract. Pooled samples #105, #106 and #107.

III #110 alkali extracted crude house dust material. Dried by pervaporation.

IV Precipitate from acetone fractionation (at 80% by volume acetone) of the crude house dust extract #110.

[2] The solutions were 0.75%. The scratch tests were carried out in duplicate with identical results.

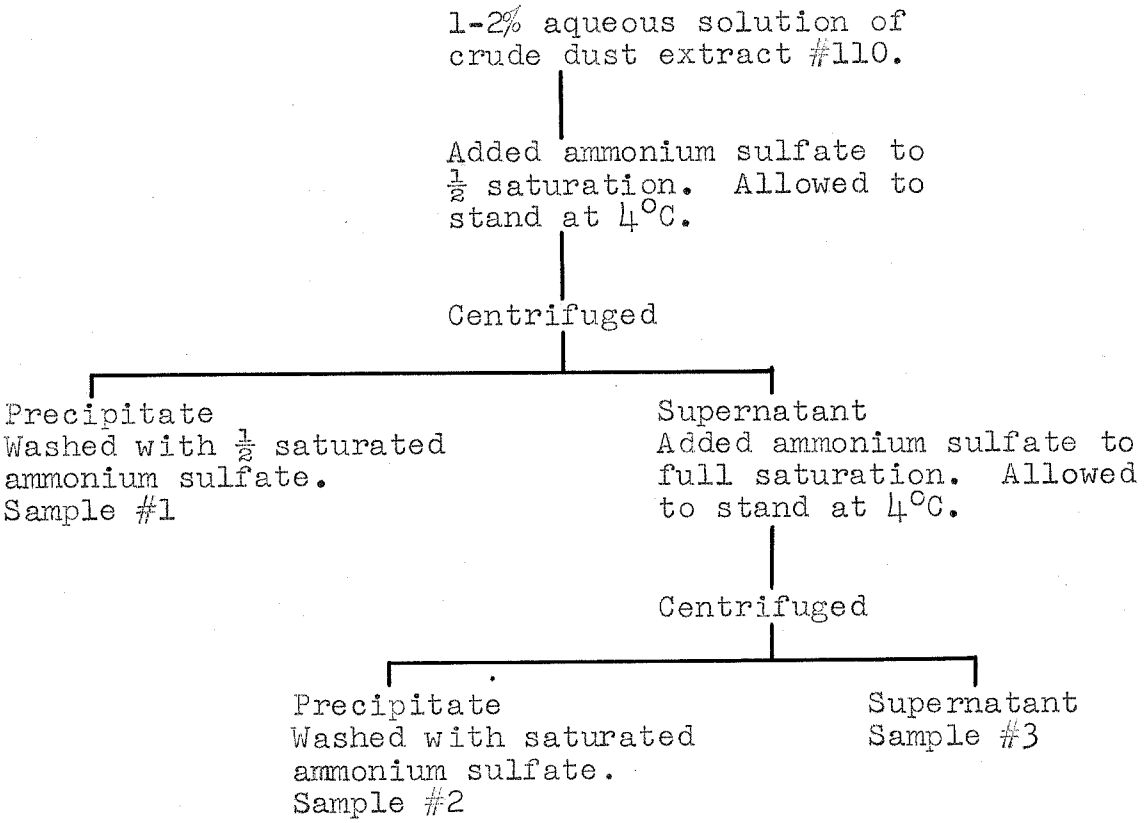
at one half and full saturation ammonium sulfate as indicated in Fig. 12. The precipitations were only carried out once and the solutions were unbuffered. The fractions were dialyzed against water and pervaporated to dryness. Subsequently ammonium sulfate fractionations were carried out with buffered solutions. The pH was also readjusted to the proper value after each addition of ammonium sulfate. The precipitations were twice repeated so as to minimize contamination of one fraction with another. This fractionation procedure is outlined in Fig. 15. Three buffers were used: barbital (pH 8.5), acetate (pH 4.0) and phthalate (pH 2.9). Data from the skin testing of these materials are presented in Figs. 13, 16, 17 and 18. In general the materials prepared by fractionation in barbital buffer at pH 8.5 are less active than those fractionated at pH 4.0 or 2.9. Considering all the data the fractions seem to be about equally active. It is interesting to note that some people seem to be more sensitive to one fraction while others show the greatest sensitivity to another fraction. For example, I seem to be more sensitive to the material soluble in the saturated ammonium sulfate. It is unfortunate that three different groups of patients were used by Dr. Feinberg rather than testing all the fractions on the same patients.

It is generally assumed that the allergenic components of house dust as well as other inhalent allergens are water soluble. All the fractionation procedures currently used

Fig. 12

Ammonium Sulfate Fractionation (Unbuffered)

Of Crude Dust Extract #110



The precipitates were dissolved in water and all the samples dialyzed against water to remove all the ammonium sulfate. The dust samples were pervaporated to dryness. The yield of dried material was approximately the same for each of the three samples.

Fig. 13

Skin Tests (Scratch Tests) Reported

by Dr. Samuel Feinberg

	Preparation[1] and result of test[2]		
Patient	Sample #1	Sample #3	III
VH	1:150* (12---)	1:150* (21---)	1:150* (21---)
NB	———	1:100 (1??--)	1:150 (21?--)
FN	0 (??---)	1:150* (21---)	1:4050* (22221)
MB	1:150* (21---)	———	1:150* (21---)
CS	1:4050* (44211)	1:1350 (2331?)	1:450 (221?-)
JM	1:150 (21??-)	1:100* (1----)	1:100 (1?---)
LM	1:450 (321?-)	1:100* (1----)	1:150 (21?--)

[1] Tests were made with varying dilutions of 0.75% solutions except in the case of sample III which was 1.0%.

Sample #1 Precipitate at ½ saturation of ammonium sulfate. Unbuffered fractionation of crude dust extract #110 (Fig. 12, Sample 1).

Sample #3 Material soluble in saturated ammonium sulfate. Unbuffered fractionation of crude dust extract #110 (Fig. 12, Sample 2).

III Dust extract sent to us by Dr. Efron.

[2] The data presented here were obtained at the same time and on the same patients as the data presented in Fig. 5.

Fig. 13 (contd.)

The data for preparation III is reproduced in both figures to facilitate comparison. The results are expressed as the highest dilution which gave a positive reaction. An asterisk indicates the highest dilution reported (i.e. the end point was not reached) and _____ indicates that no test was made. The numbers in parenthesis indicate the grade of the reaction starting at a dilution of 1:100 and proceeding to the higher dilutions of 1:150, 1:450, 1:1350 and 1:4050. The symbol ? indicates a questionable reaction and 0 indicates a negative reaction.

Fig. 14

Scratch Tests on a Single Dust Sensitive Individual (WEV) of Some of the Samples Tested by Dr. Feinberg (Figs. 5 and 13)

Preparation[1]	Test Result[2]	
I	0	0
II	0	0
III	?	1
Sample #1	1	1
Sample #2	2	2
Sample #3	2	2

[1] The following preparations were tested:

I Dust extract prepared by the Efron procedure at Caltech.

II Dust extract prepared by the Rimington procedure at Caltech.

III Dust extract sent to us by Dr. Efron.

Sample #1 Precipitate at ½ saturation of ammonium sulfate. Unbuffered fractionation of crude dust extract #110 (Fig. 12, Sample #1).

Sample #2 Precipitate at full saturation of ammonium sulfate. Unbuffered fractionation of crude dust extract #110 (Fig. 12, Sample #2).

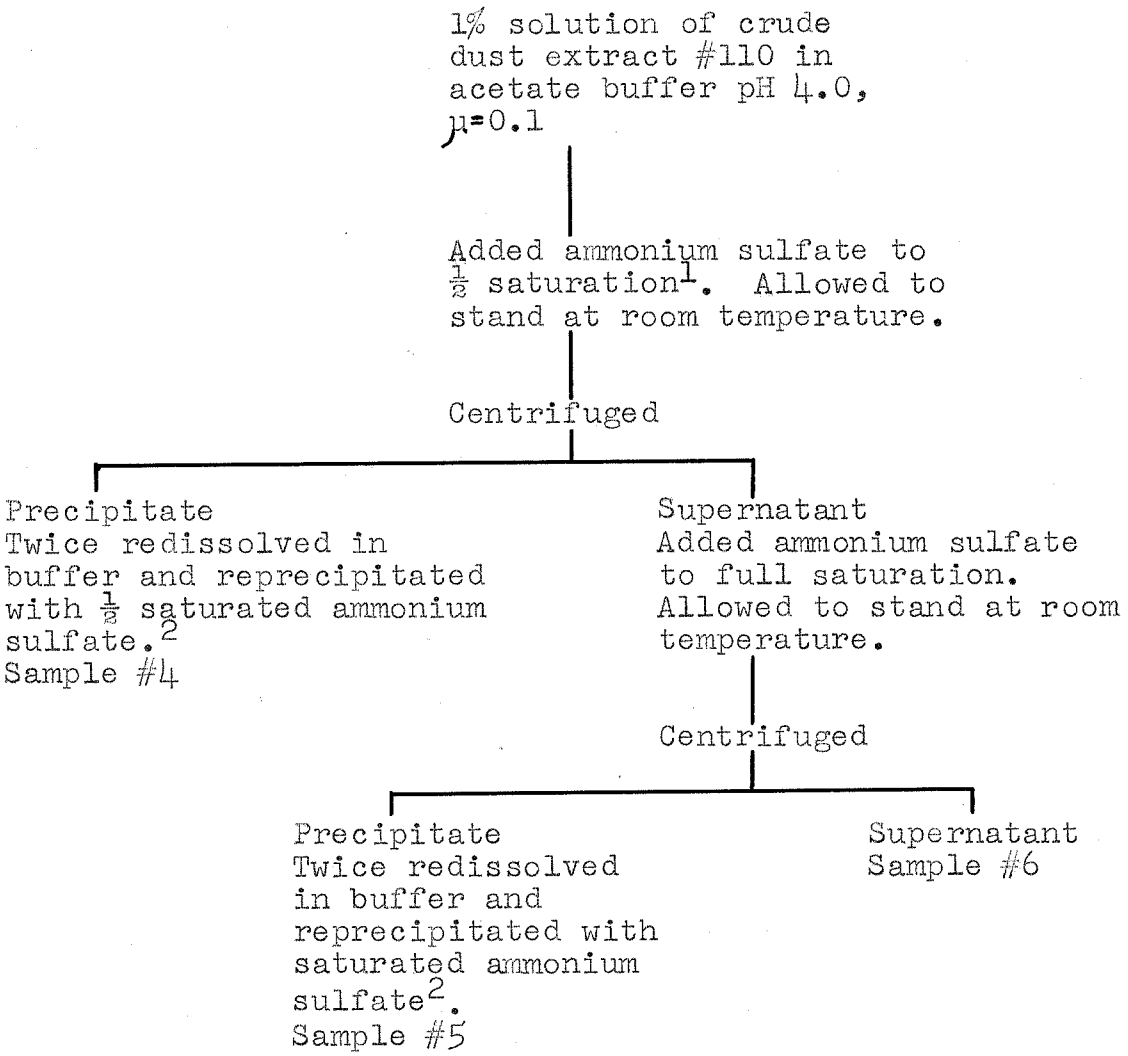
Sample #3 Material soluble in saturated ammonium sulfate. Unbuffered fractionation of crude dust extract #110 (Fig. 12, Sample #3).

[2] The solutions I, II and III were 1.0% while the others were 0.75%.

Fig. 15

Ammonium Sulfate Fractionation (Buffered) of

Crude Dust Extract #110



[1] After each addition of ammonium sulfate the pH was readjusted to the proper value by the addition of sodium hydroxide solution.

[2] The precipitates were dissolved in water and all the samples dialyzed against water to remove all the ammonium sulfate and buffer. The dust samples were pervaporated to dryness.

Fig. 16

Skin Tests (Scratch Tests) Reported

by Dr. Samuel Feinberg

	Preparation[1] and result of test[2]			
Patient	Sample #4	Sample #7	Sample #5	III
MM	0 (00000)	0 (00000)	1:100 (10000)	1:100 (10000)
GH	1:4050* (22211)	0 (?0000)	1:4050* (33333)	———
MB	1:150 (11?00)	0 (00000)	1:450 (111?0)	1:450 (111?0)
PS	1:100 (10000)	1:100 (1?000)	0 (00000)	1:450 (211?0)
LM	1:450 (21100)	0 (00000)	1:100 (10??0)	1:1350 (22110)
EK	1:1350 (1111?)	0 (00000)	1:1350 (1111?)	1:4050* (12111)

[1] Tests were made with varying dilutions of 0.75% solutions except in the case of sample III which was 1.0%.

Sample #4 Precipitate at ½ saturation of ammonium sulfate. Acetate buffered (pH 4.0) fractionation of crude dust extract #110 (Fig. 15).

Sample #7 Precipitate at ½ saturation of ammonium sulfate. Barbital buffered (pH 8.5) fractionation of crude dust extract #110.

Sample #5 Precipitate of full saturation ammonium sulfate. Acetate buffered (pH 4.0) fractionation of crude dust extract (Fig. 15).

III Dust extract sent to us by Dr. Efron.

[2] The results are expressed as the highest dilution which gave a positive reaction. An asterisk indicates the

Fig. 16 (contd.)

highest dilution reported (i.e. the end point was not reached) and _____ indicates that no test was made. The numbers in parenthesis indicate the grade of the reaction starting at a dilution of 1:100 and proceeding to higher dilutions of 1:150, 1:450, 1:1350 and 1:4050. The symbol ? indicates a questionable reaction and 0 indicates a negative reaction.

Fig. 17

Skin Tests (Scratch Tests) Reported

by Dr. Samuel Feinberg

	Preparation[1] and result of test[2]			
Patient	Sample #8	Sample #6	Sample #9	III
PC	0 (?0000)	1:150 (22000)	1:100 (10000)	1:150 (22??0)
MD	0 (??000)	1:4050* (22221)	1:4050* (22111)	1:4050* (43322)
JS	1:100 (10000)	1:150 (21?00)	1:450 (22100)	1:4050* (44443)
RC	0 (?0000)	0 (00000)	1:100 (1?000)	1:450 (22100)

[1] Tests were made with varying dilutions of 0.75% solutions except in the case of sample III which was 1.0%.

Sample #8 Precipitate at full saturation ammonium sulfate. Barbital buffered (pH 8.5) fractionation of crude dust extract #110.

Sample #6 Material soluble in saturated ammonium sulfate. Acetate buffered (pH 4.0) fractionation of crude dust extract #110.

Sample #9 Material soluble in saturated ammonium sulfate. Barbital buffered (pH 8.5) fractionation of crude dust extract #110.

III Dust extract sent to us by Dr. Efron.

[2] The results are expressed as the highest dilution which gave a positive reaction. An asterisk indicates the highest dilution reported (i.e. the end point was not reached) and _____ indicates that no test was made. The numbers in parenthesis indicate the grade of the reaction starting at a dilution of 1:100 and proceeding to higher dilutions of 1:150, 1:450, 1:1350 and 1:4050. The symbol ? indicates a questionable reaction and 0 indicates a negative reaction.

Fig. 18

Scratch Tests on a Single Dust Sensitive Individual (WEV) of Materials Obtained by Ammonium Sulfate Fractionation in Buffered Solutions of Crude Dust Extract #110

Preparation[1]	First Fractionation[2]		Second Fractionation	
Phthalate Buffer (pH 2.9)				
½ SAS ppt.	____	____	0	0
SAS ppt.	____	____	1	1
SAS soluble	____	____	2	2
Acetate Buffer (pH 4.0)				
½ SAS ppt.	0 (Sample #4)	0	0	0
SAS ppt.	? (Sample #5)	0	0	?
SAS soluble	2½ (Sample #6)	2	2	2
Barbital Buffer (pH 8.5)				
½ SAS ppt.	0 (Sample #7)	0	0	0
SAS ppt.	0 (Sample #8)	0	0	0
SAS soluble	0 (Sample #9)	0	1	1½

[1] All solutions were 0.75%. The scratch tests were carried out in duplicate. The same sample was tested in skin

Fig. 18 (contd.)

areas remote from each other so as to minimize the effect of possible differences in skin reactivity at different sites.

[2] The first fractionation samples were tested by Dr. Feinberg (See Figs. 16 and 17). The procedure for the second fractionation was identical to that used for the first except that a sample was fractionated at pH 2.9 in phthalate buffer as well as the acetate and barbital buffers previously used.

are based on the preparation of an aqueous extract which is then fractionated by various methods. In some cases, as for example that of pollens, the crude material is first extracted with a nonpolar solvent to remove lipids and then a higher yield of allergen is obtained by aqueous extraction. This does not seem to be true in the case of house dust. The aqueous extracts obtained before such a solvent extraction seem to be about the same in amount of material and potency as those obtained afterwards (11). The water solubility of the inhalent allergens is consistent with current ideas of the mechanism by which the allergen gains entrance to the body i.e. by solution in the nasal and bronchial secretions and absorption of the dissolved material. It is possible that material of the same immunological specificity as the active water soluble allergen remains in the insoluble dust residue. If this were so it might be possible to solubilize some of the water insoluble residue and thus obtain more active material.

Two methods were used to solubilize some of the water extracted dust residue. One was the alkaline thioglycolate method used by Pillemer et al. (12) to dissolve keratin. The thioglycolate solution reduced the disulfide links in the keratin to free sulfhydryl groups. The reduced protein was soluble and thus could be used for studies of the immunological specificity of various keratins. If the dust allergen were a protein and if it were present in the dust residue in an insoluble form it seemed possible that this

thioglycolate procedure might solubilize additional allergen. A fifty gram portion of house dust was twice extracted with water and then mixed with 500 ml. of 0.5M thioglycolate solution. The thioglycolate solution was prepared by adjusting an aqueous solution of thioglycolic acid to pH 10.7 by the addition of sodium hydroxide. The final volume was such as to give a 0.5M solution. The mixture was liquid but filled with suspended solid material. It was allowed to stand at room temperature for 48 hours with occasional agitation, the fluid removed by centrifugation and the supernatant dialyzed against 1% sodium chloride solution. The final solution contained 5 mgm. of organic material per ml.

The other method that was used in an attempt to extract water soluble active materials from the dust residue was that of Fuller (13), devised to extract specific polysaccharides from hemolytic streptococci. As before a fifty gram sample of house dust was twice extracted with water. The residue was mixed with 180 ml. of formamide and heated at 125 to 145°C. for 45 minutes. After being allowed to cool the fluid was removed with a mechanical press and then centrifuged. The combined residues from the press and the centrifugation were extracted with water. This aqueous extract was dialyzed against 1% sodium chloride solution. The final solution contained 5 mgm. of organic material per ml. The formamide supernatant was made 80% by volume in acetone. The precipitate was washed and dried with acetone

then mixed with water. Not all the acetone precipitate was soluble in water. The mixture was centrifuged and the supernatant dialyzed against 1% sodium chloride solution. As before the final solution was diluted to 5 mgm. per ml. of organic material.

The materials from the thioglycolate and formamide extraction were scratch tested on four dust sensitive individuals and found to be completely inactive. Active dust fractions have not been subjected to the severe conditions used in these extraction procedures. Therefore it is possible that any insoluble active material that remained was destroyed during the extraction procedure. The hot formamide seems especially severe, however the group specific polysaccharides of hemolytic streptococci do not lose their specificity under these conditions. On the basis of the base stability of the allergen (p. 19) it seems unlikely that the alkaline conditions involved in the thioglycolate extraction inactivated any allergen. Active allergen was either not present in the water insoluble residue, not solubilized by these procedures or inactivated. Possibly a better way to look for insoluble allergen specificity would be to attempt to deplete a serum from a dust sensitive individual of reagin antibody by absorption with exhaustively washed house dust.

3. Preparation of Crude Dust Extracts #121 and #125.

In order to have a large supply of crude dust extract for various chemical and physical studies two batches of

dust were processed. In the first procedure (Crude Dust Extract #121, Fig. 19) two buckets full of house dust were extracted three times with water. The aqueous extract was filtered, centrifuged, concentrated by vacuum distillation and dialyzed against water. The crude dust material was pervaporated to dryness and stored in a vacuum desiccator over calcium chloride.

Subsequent to the preparation of this material experiments carried out by Dr. Silver and Dr. Campbell indicated that freezing and thawing the wet dust facilitated the extraction of the allergen. Therefore this modification was incorporated into the procedure used in the next large scale dust extraction (Crude Dust Extract #125, Fig. 20). Twelve to fifteen buckets full of mattress dust were wet with a minimum amount of water and then frozen and allowed to thaw. The liquid was removed with a hydraulic press at a pressure of 20,000 p.s.i. and then filtered and concentrated to about 1/6 the original volume by pervaporation. Previously the extracts have been concentrated by vacuum distillation. This method is quicker than pervaporation but requires constant attention. Pervaporation is the method of choice as it requires almost no attention and therefore may be carried out on a twenty-four hour basis. The concentrated dust extract was dialyzed against water and then made up to one half saturation in ammonium sulfate by the addition of an equal volume of saturated ammonium sulfate (at room temperature). After twelve hours, the

Fig. 19

Preparation of Crude Dust Fraction #121

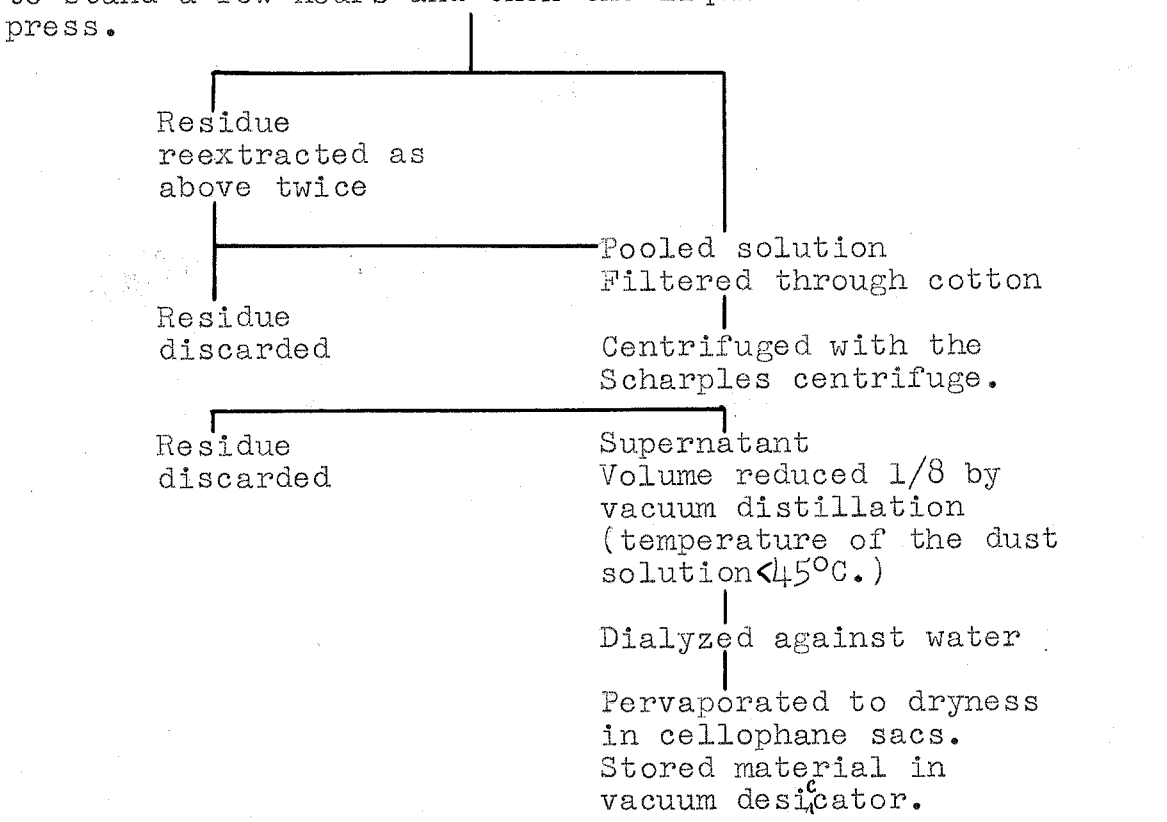


Fig. 20

Preparation of Crude Dust Extract #125

Mattress dust wet with a minimum amount of water and frozen; thawed and the liquid removed with a press.

- Solid discarded
- Solution
  Filtered through glass wool.
  Pervaporated to approximately 1/6 the original volume.
  Centrifuged
  - Residue discarded
  - Supernatant
    Dialyzed against water.
    Pervaporated to ½ the volume before dialysis.
    Added an equal volume of saturated ammonium sulfate.
    Allowed to stand 12 hours at room temperature.
    Centrifuged
    - Residue discarded
    - Supernatant
      Stored at 4°C. in the ½ saturated ammonium sulfate solution.

mixture was centrifuged. The supernatant was stored at 4°C. in the half saturated ammonium sulfate solution.

These materials were later used for various chemical determinations, electrophoretic and ultracentrifuge studies and for fractionation by zone electrophoresis.

4. Electrophoretic Studies of Crude Dust Fractions.

A number of free boundary electrophoresis experiments were carried out to follow the changes in electrophoretic pattern during the course of fractionation. As an example of the type of patterns obtained the results for the #121 crude dust material (Fig. 19) and the #105 dust fraction soluble in 80% acetone (Fig. 7) will be presented. These particular examples were chosen to be discussed because the data were the most complete in the case of these materials.

The large free boundary electrophoresis apparatus (with a 12 ml. cell) built in this laboratory (14) was used to obtain patterns of the crude dust extract #121 (Fig. 19) and of the fraction of this material soluble in saturated ammonium sulfate. A 0.8% solution of the crude dust extract was prepared in acetate (pH 4.0) and phthalate (pH 2.8[3]) buffers and the samples dialyzed against the corresponding buffers for 24 hours in the cold room. The buffers were prepared as follows:

Acetate buffer (pH 4.0, μ=0.1)

sodium acetate trihydrate	81.7 gm.
glacial acetic acid	150.0 ml.
water to make a final volume of	6.0 liters

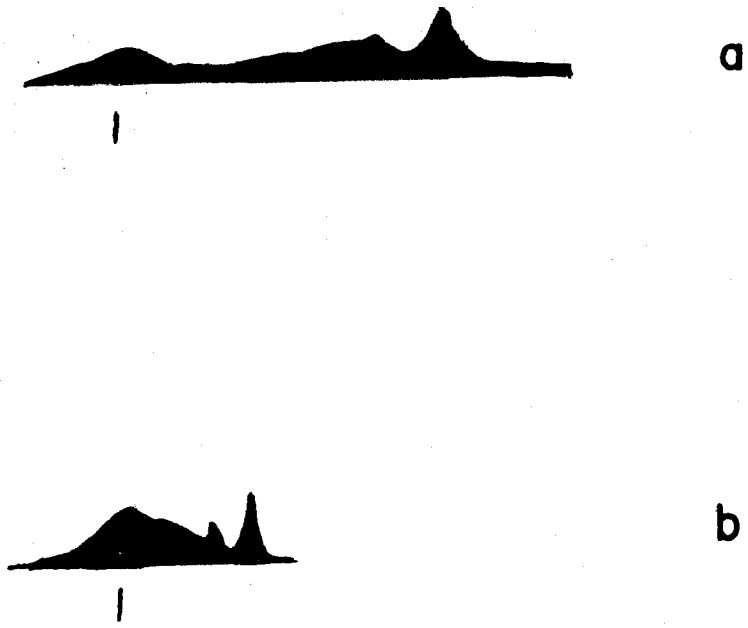
Phthalate buffer (pH 2.8[3], μ=0.1)

potassium hydrogen phthalate	61.2 gm.
concentrated hydrochloric acid	13.1 ml.
potassium chloride	22.36 gm.
water to make a final volume of	6.0 liters

The phthalate buffer was stored in the cold room and filtered before use. A current of 15 mamps. was allowed to pass through the cells for two hours. The patterns are reproduced in Fig. 21. The material is quite heterogeneous electrophoretically. There are four peaks; however the curve does not come back to the base line between the peaks indicating the presence of material of intermediate mobility. The mobilities of the faster components are much less at the lower pH. There is no evidence of any material becoming positively charged.

The fraction of the #121 dust extract soluble in saturated ammonium sulfate was prepared following the procedure outlined in Fig. 15. This material was examined by electrophoresis following the same procedure as described above for the whole dust extract (except that the concentration of the solution was 0.75% instead of 0.8%). The patterns from the electrophoresis experiments using acetate (pH 4.0) and phthalate (pH 2.8[3]) are reproduced in Fig. 22. This fraction is electrophoretically less heterogeneous than the whole extract. In the acetate buffer, almost all the material is distributed among three distinct symmetrical peaks rather than being more or less continuously distributed

Fig. 21

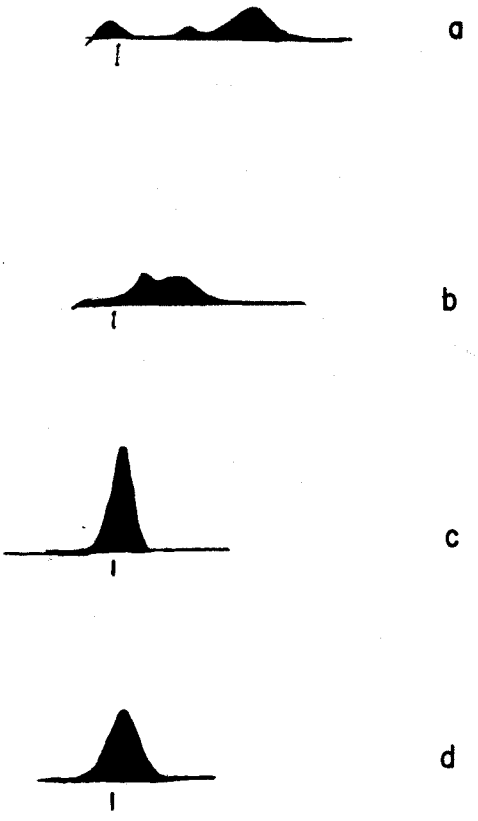


Electrophoretic patterns of crude dust fraction #121.

a. Acetate buffer, pH 4.0, μ=0.1, after two hours at 15 mamps.

b. Phthalate buffer, pH 2.8, μ=0.1, after two hours at 15 mamps.

Fig. 22



Electrophoretic patterns of the fraction of the #121 extract soluble in sat. A. S.

a. Acetate buffer, pH 4.0, μ=0.1, after two hours.

b. Phthalate buffer, pH 2.8, μ=0.1, after two hours.

c. KCl HCl solution, pH 1.8, μ=0.1, after two hours.

d. KCl HCl solution, pH 1.8, μ=0.1, after four hours.

throughout the cell. This difference in heterogeneity is more evident at higher pH's where the material separates well, than in the case of phthalate buffer (pH 2.8[3]) where more of the material is isoelectric or very slowly moving. The set of electrophoresis patterns reproduced in Fig. 22 provides a good example, though perhaps a rather obvious example, of the importance of using a number of different buffers to investigate the electrophoretic heterogeneity of a material.

In order to determine whether any of the material could be given a net positive charge at still lower pH's, an electrophoresis experiment was carried out using a mixture of potassium chloride and hydrochloric acid (pH 1.7[5]) instead of the acetate or phthalate buffer. This solution was prepared as follows:

potassium chloride	16.78 gm.
concentrated hydrochloric acid	6.19 ml.
water to make a final volume of	3.0 liters

The starting boundaries were compensated to the middle of the cell and the current (15 mamps.) started. Pictures were taken after two hours and four hours and are reproduced in Fig. 22. All the sample appears as one symmetrical peak. There was a very slight movement to the anode during the course of the run. Therefore there is still a slight net negative charge. The sample was observed during the first two hour period so we can be sure there were no positively

charged components that had split away from this main peak and left the cell before the first picture. This result makes it seem unlikely that the nitrogen present in the fractions is present as a separate protein or polypeptide mixed in with the carbohydrate. It seems more reasonable that the nitrogen containing components are chemically bonded to the carbohydrate. More evidence on this point will be presented in connection with the starch zone electrophoresis experiments (p. 130).

The #121 crude dust extract (Fig. 19) and the fraction of the #105 crude dust extract that was soluble in 80% (by volume) acetone (Fig. 7) were further fractionated with ammonium sulfate and acid acetone. The fractionation procedure is outlined in Fig. 23. All the precipitations and centrifugations were carried out at room temperature. The materials separated by this procedure were examined by free boundary electrophoresis in the Perkin-Elmer Model 38 Electrophoresis Apparatus (15). A 2.0 ml. cell with an open system was used in these experiments. Distilled water solutions (0.8%) of the various fractions were dialyzed against acetate buffer (pH 4.7, μ=0.1 preparation on p. 122). The fractions #121 - IV and #105 - IV were run at much lower concentrations because only a very small amount of material was obtained. In the case of these fractions, all the material available was pervaporated to 3.0 ml. and dialyzed against the acetate buffer. A current of 6.0 mamps. was allowed to pass through the cell for 4500 seconds and then

Fig. 23

Fractionation Procedure Used for the Following:

(1) #121 crude dust material.

(2) #105 dust fraction soluble in 80% acetone.

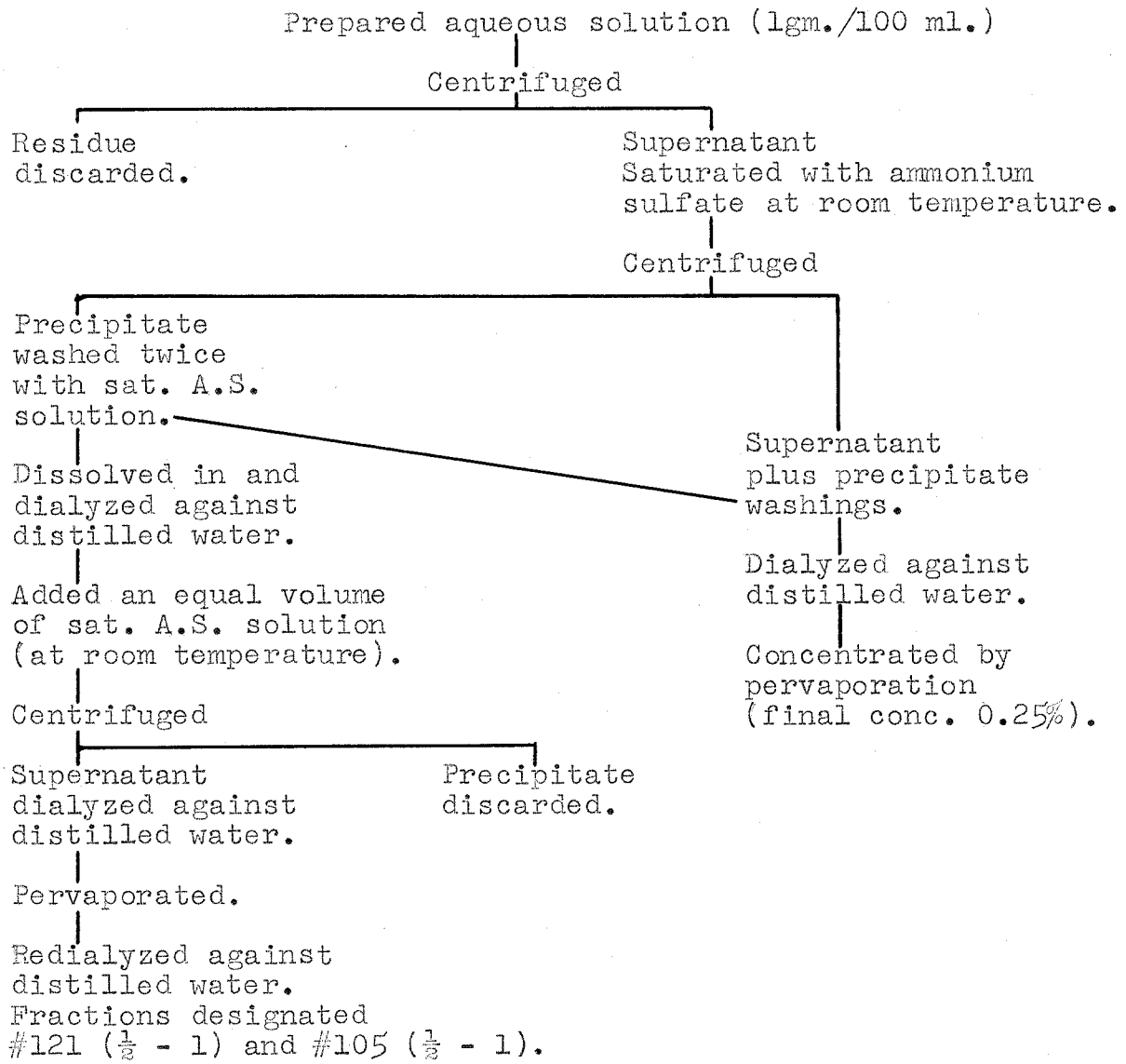
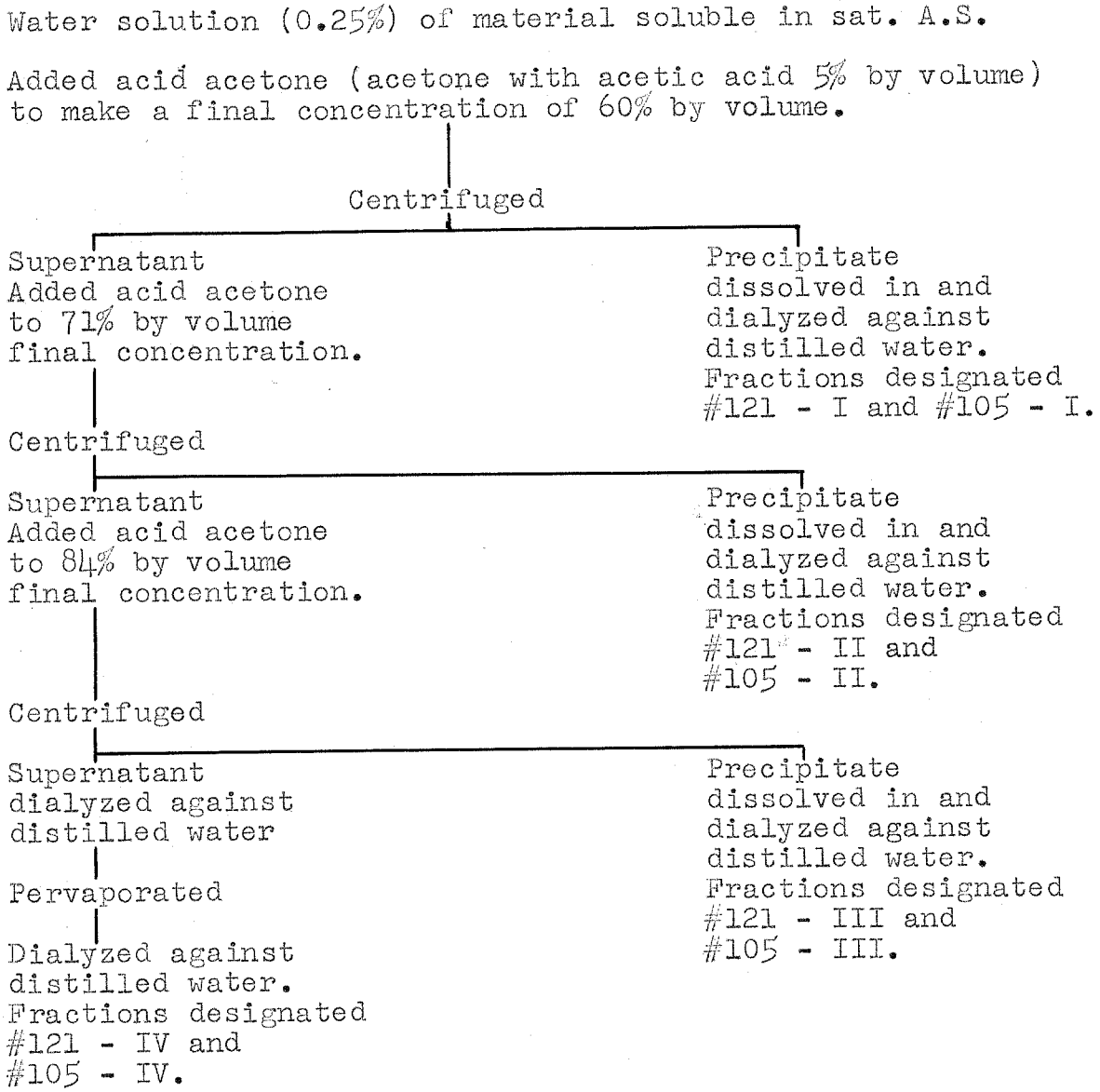


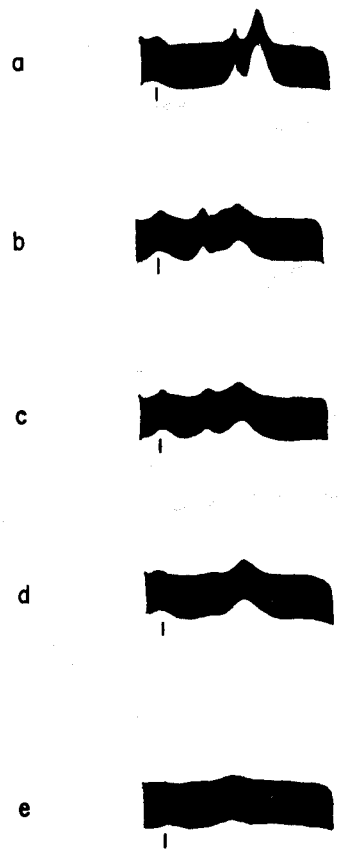
Fig. 23 (contd.)


Water solution (0.25%) of material soluble in sat. A.S.
Added acid acetone (acetone with acetic acid 5% by volume) to make a final concentration of 60% by volume.
Centrifuged
Supernatant
Added acid acetone to 71% by volume final concentration.
Precipitate dissolved in and dialyzed against distilled water. Fractions designated #121 - I and #105 - I.
Centrifuged
Supernatant
Added acid acetone to 84% by volume final concentration.
Precipitate dissolved in and dialyzed against distilled water. Fractions designated #121 - II and #105 - II.
Centrifuged
Supernatant dialyzed against distilled water
Precipitate dissolved in and dialyzed against distilled water. Fractions designated #121 - III and #105 - III.
Pervaporated
Dialyzed against distilled water. Fractions designated #121 - IV and #105 - IV.

the pictures were taken. Shorter time intervals were required by sample #121 - IV and #105 - IV because at the lower concentration diffusion would obliterate the peaks in 4500 seconds. The patterns are presented in Figs. 24 and 25.

In each figure, the reproductions of the electrophoretic patterns are arranged so that the starting boundaries fall in line and thus the mobilities of the components may be easily compared. The mobilities of the main peaks are tabulated in Fig. 26. The materials soluble in half saturated ammonium sulfate but precipitated at full saturation, fractions #121 ($\frac{1}{2}$ - 1) and #105 ($\frac{1}{2}$ - 1), contain two fast components with mobilities of about -9 and -11 x 10^{-5} ($cm.^2$ $sec.^{-1}$ $volt^{-1}$). The #121 materials soluble in saturated ammonium sulfate contain the -9 x 10^{-5} component and another slower component (-4.8 to -5.4 x 10^{-5}). The #105 fractions soluble in saturated ammonium sulfate contain the -9 x 10^{-5} component as well as -12 x 10^{-5} and -7 x 10^{-5} components. In general the fractions soluble in saturated ammonium sulfate appear to be less heterogeneous than the crude dust extracts or the #121 ($\frac{1}{2}$ - 1) and #105 ($\frac{1}{2}$ - 1) materials. The peaks appear symmetrical and there is little evidence of base line elevation between the peaks. Fractional precipitation with acid acetone provides a relative separation of these components. The more soluble fractions (III and IV) consist almost entirely of the -9 x 10^{-5} component.

Fig. 24



Electrophoretic patterns of the #121 dust extract fractions.

Acetate buffer, pH 4.7, μ=0.1

a. #121 ($\frac{1}{2}$ - 1) 4500 sec.

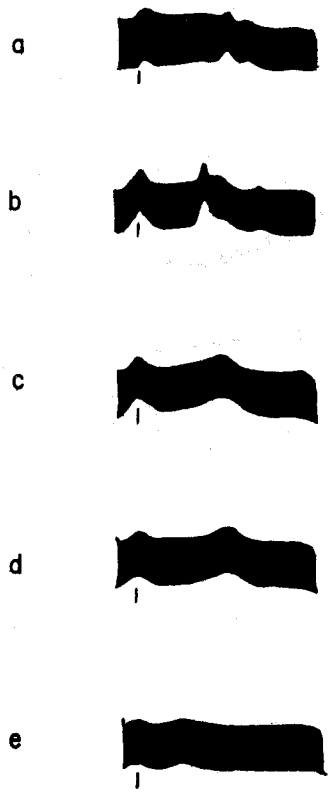
b. #121 - I 4500 sec.

c. #121 - II 4500 sec.

d. #121 - III 4500 sec.

e. #121 - IV 3500 sec.

Fig. 25



Electrophoretic patterns of the #105 dust extract fractions.

Acetate buffer, pH 4.7, μ=0.1

a. #105 ($\frac{1}{2}$ - 1) 4500 sec.

b. #105 - I 4500 sec.

c. #105 - II 4500 sec.

d. #105 - III 4500 sec.

e. #105 - IV 2500 sec.

Fig. 26

Mobilities of Main Electrophoretic Components

of Crude Dust Fractions

Acetate Buffer pH 4.7, μ=0.1

Material	Peak	Mobility ($cm.^2 sec.^{-1} volts^{-1}$)
#110 crude dust extract	fast	-10.2×10^{-5}
	slow (shoulder on the fast peak)	-8.2
#121 fraction soluble in sat. A. S.	fast	-9.1
	slow	-5.4
#121 ($\frac{1}{2}$ - 1)	fast	-11.1
	slow	-8.7
#121 - I	fast	-8.5
	slow	-4.8
#121 - II	fast	-8.5
	slow	-4.8
#121 - III		-8.8
#121 - IV		-10.2
#105 ($\frac{1}{2}$ - 1)	fast	-11.5
	slow	-9
#105 - I	first small peak	-12.4
	leading shoulder	-8.7
	large peak	-6.9
#105 - II		-8.6
#105 - III		-9.2
#105 - IV		-8.8

In addition to these peaks some of the fractions contain considerable material of slower mobility indicated by the elevation of the base line back to the peak near the starting position. It is not clear how much of the area of the stationary peak is due to the presence of uncharged or very slightly changed material and how much is due to the ϵ salt boundary. On the basis of the results obtained with crude dust extract #110 (Fig. 9) in starch zone electrophoresis experiments (p.122) it would seem likely that a considerable portion of the stationary boundary is due to the presence of a fraction of the dust extract with a very low net charge. It should be remembered, however, that an uncharged material should be found on the starch block behind the point of sample application due to the electroosmotic effect. The charge on the starch grains is certainly dependent upon the pH of the solution and probably also on the nature of the buffer. Thus the contribution of electroosmosis to the position of an uncharged dust fraction on the starch block will depend on the buffer used. Possibly the material that is found at the point of sample application is the material that migrates with a mobility of about -5×10^{-5} in free boundary electrophoresis. In starch electrophoresis, there is also the complication due to the possibility of absorption of the dust extract on the starch. It has been shown that the material that remains near the starting position in starch electrophoresis (pH 4.7, acetate) is qualitatively different from the material that

rapidly migrates with a net negative charge. This material does contain some uronic acid and should have a net charge at pH 4.7 unless the acid groups are blocked in some way. The problem could be solved by experimentally determining the importance of electroosmosis (with an uncharged marker) and of absorption in starch electrophoresis or by fishing out material from the region of the ϵ boundary of the descending limb in the course of a free boundary electrophoresis experiment and determining whether there is any dust extract present. Until such experiments are carried out we cannot decide the question as to whether the stationary peaks in free boundary electrophoresis contain dust material or whether they are just due to the buffer ion gradients.

None of the electrophoretic experiments that have been carried out on any of dust materials have provided evidence for any fundamental difference between the ascending and descending patterns. In all cases, as more dilute solutions are used the patterns tend to become enantiographic. In all cases, the descending patterns are reproduced in the figures.

Electrophoretic patterns of the crude dust fraction #110 and the #121 supernatant to saturated ammonium sulfate (prior to acid acetone fractionation) are reproduced in Fig. 27. The #121 whole supernatant pattern is entirely consistent with the patterns obtained by the acid acetone fractionation of this material. The #110 crude extract

Fig. 27

a

b

Electrophoretic patterns (Acetate buffer, pH 4.7, μ=0.1).

a. Crude dust extract #110, after 3600 sec.

b. #121 material soluble in sat. A. S., after 4500 sec.

pattern is reproduced for comparison with the #121 crude extract pattern (Fig. 21) and because this material was used for an extensive starch zone electrophoresis study. The patterns of #110 and #121 crude dust extracts are similar. Acetate buffers were used in both cases; however the #110 material is at pH 4.7 and #121 at pH 4.0. The #121 sample was run twice as long (two hours instead of one hour) and shows greater resolution of the two fast components. A comparison of the #110 free boundary electrophoresis pattern and the starch zone electrophoresis pattern (p.123) as determined by analyses of the eluates for total carbohydrate shows a considerable difference in the relative size of the mobile and stationary peaks. The two experiments were carried out in the same buffer. The discrepancy may be due to an improper identification of the peaks due to electroosmosis as discussed above, to differences in the refractive index increments of the materials or to additional factors present in the case of the starch electrophoresis such as absorption by the starch.

5. Sedimentation Studies of Crude Dust Fractions.

The object of this study was to examine the sedimentation behavior of a number of the crude dust fractions. Having found that the patterns generally showed single slowly moving peaks, it was decided to examine a series of materials prepared by fractional precipitation with acid acetone to determine whether this fractionation procedure,

based on solubility differences, yielded products of varying sedimentation coefficient. Two of the fractions (#121 - II and #105 -.II) were selected for studies of the concentration dependence of the sedimentation coefficients.

The products of the ammonium sulfate and acid acetone fractionation of crude dust extracts #105 and #121 were examined in acetate buffer (pH 4.7, μ = 0.1). These were the same fractions whose preparation and electrophoretic analysis were discussed in the preceding section. The buffer used was the same as that for the electrophoresis experiments. Aqueous solutions of the materials (0.5 - 1.0%) were dialyzed against the acetate buffer for two days. All the samples were run in a synthetic boundary cell because the sedimentation coefficients were so low that boundaries did not readily form in the regular cell. The buffer which the sample had been dialyzed against was used to form the boundary in the cell. The runs were performed in a Spinco Model E Ultracentrifuge. In all cases, the experiments were carried out at full speed (59,780 r.p.m. or approx. 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 in the cell were determined and the uncorrected sedimentation coefficients calculated. By the use of Svedberg's tables (16) and the assumed value 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 for $\bar{v}$ was based on Säverborn's (17) determination of the partial specific volumes of various polyuronides. For all the experiments, except those involving a study of the concentration dependence of Sw,20, the sample concentrations were determined by nitrogen analysis and calculations based on the previously determined nitrogen contents of the fractions. This was an unfortunate choice as the nitrogen contents were not accurately known. In the case of the samples for the concentration dependence studies, the indole method for the determination of total carbohydrate (described on p.120) was used to determine the concentration of the most concentrated solutions. Dilutions of these solutions of known concentration were then made with acetate buffer. A stock solution (in distilled water) of fraction #121 ($\frac{1}{2}$ - 1), whose concentration was known from dry weight determinations, was used as a standard in the total carbohydrate determination.

Some of the Schlieren patterns are reproduced in Fig. 28. Symmetrical peaks with no evidence of base line elevation were obtained for all the acid acetone fractions except #105 - I which had a shoulder on the leading edge. The #105 ($\frac{1}{2}$ - 1) fraction also had a shoulder on the leading edge that did not resolve into a separate peak. The #121 ($\frac{1}{2}$ - 1) fraction was the only one that resolved into two distinct peaks. In this case, the peaks were of about equal size and completely resolved. For comparison a pattern of the #125 crude dust extract run under the same conditions is

Fig. 28

a b c

d e

Sedimentation patterns, Acetate buffer, pH 4.7, μ = 0.1.

a. #121 - II, 2.0% solution, after 48 min.

b. #105 - I, 0.6% solution, after 89 min.

c. #105 ($\frac{1}{2}$ - 1), 0.7% solution, after 24 min.

d. #121 ($\frac{1}{2}$ - 1), 1% solution, after 96 min.

e. #125 crude extract, 0.8% solution, after 48 min.

presented in Fig. 28.

The sedimentation data are presented in Fig. 29. The Sw,20 values obtained for the acid acetone fractions indicate that the fractionation procedure has yielded a series of materials of differing sedimentation coefficient and that the more soluble materials have lower sedimentation coefficients.

The concentration dependence data for the #121 - II and the #105 - II fractions are plotted in Fig. 30 and Fig. 31. The values of the sedimentation coefficients at infinite dilution ($\overset{\circ}{S}w,20$) were estimated by extrapolating 1/Sw,20 vs. C plots. These values are given in the table and are plotted on the Sw,20 vs. C graphs for the two fractions. The concentration dependence is roughly similar to that observed for serum albumin. If one takes the concentration dependence exhibited by the #121 - II material as characteristic of the fractions and estimates from this the $\overset{\circ}{S}w,20$ values of the acid acetone fractions, a similar series of sedimentation coefficients is obtained varying from 2.31 to 1.28 for the #121 fractions and from 1.98 to 1.24 for the #105 fractions. The differences in sedimentation values obtained among the various fractions ~~is~~ are much greater than could be accounted for on the basis of variation in concentration.

There are errors involved in these experiments. The points in the concentration dependence studies do not all fall on a smooth line. The large discrepancies are probably due to small leaks in the cell during the run. Smaller

Fig. 29

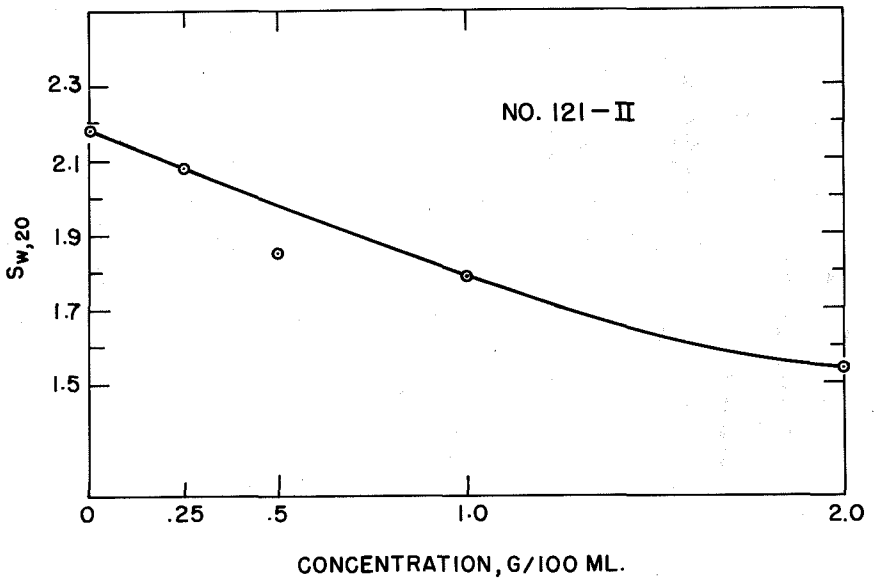
Sedimentation Coefficients of Crude Dust Fractions

Acetate Buffer pH 4.7, μ = 0.1

Fraction	Concentration, %		Sw,20
#121 - I	0.7		2.00
#121 - II	0.5		1.57
#121 - III	0.8		1.36
#121 - IV	1.0		1.14
#105 - I	0.6		1.73
#105 - II	0.6		1.36
#105 - III	0.6		1.14
#105 ($\frac{1}{2}$ - 1)	0.7		2.21
#121 ($\frac{1}{2}$ - 1) slow peak	~1.0		1.77
#121 ($\frac{1}{2}$ - 1) fast peak	~1.0		2.85
#121 - II	2.0		1.54
"	1.0		1.79
"	0.5		1.85
"	0.25		2.08
		$S^{\circ}_{w,20}$	= 2.18
#105 - II	1.5		1.41
"	0.75		1.99*
"	0.37		1.76
"	0.19		1.78
		$S^{\circ}_{w,20}$	= 1.89

* High value probably caused by a leak in the cell.

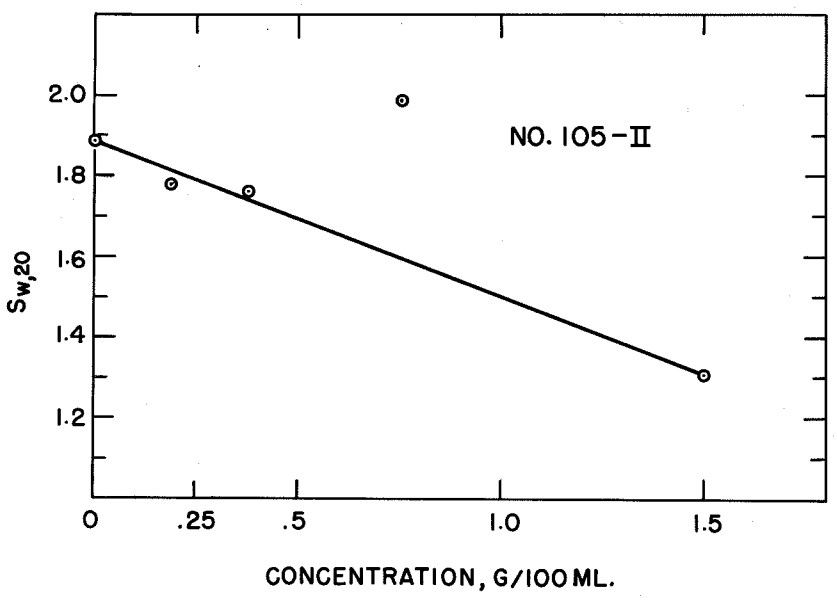
Fig. 30.



Crude Dust Fraction #121 - II

Concentration Dependence of Sw,20 with Extrapolation to $S\mathring{w},20$.

Fig. 31.



Crude Dust Fraction #105 - II

Concentration Dependence of Sw,20 with Extrapolation to $S\mathring{w},20$.

discrepancies may be due to inaccuracies involved in estimating the boundary positions in the cell from the projections of the patterns. The Sw,20 values for the fractions #121 - II and #105 - II determined with the other fractions do not fall close enough to the curves obtained in the concentration dependence experiments. These discrepancies are probably due to errors involved in the estimation of the concentrations as well as possible leaks and projection errors. The data are not sensitive to errors in $\tilde{v}$.

6. Chemical Study of the Crude Dust Fractions.

Survey of Precipitants:

In the course of fractionating aqueous solutions of house dust extracts many precipitating agents have been used. Organic solvents such as acetone, dioxane, methanol and ethanol and salts, commonly ammonium sulfate, are the most frequently used materials. These act by diminishing the amount of water available for solution of the dust material and thus precipitating the least water soluble of the components of the dust extract. It seemed desirable to examine a wide variety of materials to see if other reagents might not also be useful for precipitating the dust allergen material. Previous experiments (p. 33) had indicated that the materials soluble in 80% acetone and saturated ammonium sulfate were active. Therefore it seemed particularly important to find reagents for fractionating these more soluble materials. In order to accomplish this a series of

small scale tests was carried out. Because of this special interest in precipitating agents for the more soluble materials the tests were carried out with a dilute solution (0.4%) of the material in crude dust fraction #110 (Fig. 9) that was soluble in saturated ammonium sulfate.

The solution for these tests was prepared from 75 ml. of a 1.2% water solution of the crude dust extract #110. Solid ammonium sulfate was added to full saturation at 4°C. The mixture was allowed to stand overnight at 4°C. and then centrifuged. The precipitate was redissolved in water and then reprecipitated by the addition of more ammonium sulfate. After centrifugation, the two supernatants were pooled and exhaustively dialyzed against distilled water. The solution was pervaporated to reduce the volume. The final concentration was 0.4%. The tests were carried out using 0.1 ml. volumes of the dust solution and reagent.

Acetone, absolute methanol and absolute ethanol did not yield any precipitate on the stepwise addition of up to eight volumes of solvent. The addition of acid acetone (95% acetone and 5% acetic acid, by volume) yielded some precipitate at two volumes and successively greater amounts with the addition of the acid acetone solution up to six volumes. It was on the basis of this result that this solvent was used to fractionate the materials examined in the electrophoresis and ultracentrifuge study (p. 53). It is perfectly reasonable that the acid form of the polysaccharides would be less soluble in water than the salt form.

Probably other water miscible organic solvents would also precipitate the dust material if acidified.

As it is believed that much of the dust material consists of acidic polysaccharides, it seemed desirable to look for insoluble heavy metal salts of these materials. A variety of saturated or almost saturated solutions of metal salts were tested in one volume amounts. The salts tested were as follows:

Barium chloride
Calcium chloride
Magnesium chloride
Basic lead acetate
Silver nitrate
Cupric chloride
Mercuric chloride
Lead acetate
Zinc sulfate.

The basic lead acetate solution immediately yielded a large amount of precipitate, while the lead acetate solution gave only a slight amount of precipitate. The basic lead acetate solution was prepared according to the procedure of Hawk, Oser and Summerson (18). None of the other salts yielded any precipitate.

Various protein precipitants were tested, however none of them precipitated any of the dust extract. One volume amounts of the following reagents were tested:

Picric acid

Tannic acid

Phosphotungstic acid

Trichloacetic acid

Sulfosalicylic acid.

The trichloracetic acid solution was 50% (W/V) and the phosphotungstic acid solution was prepared according to the procedure of Hawk, Oser and Summerson (19). The other solutions were saturated or almost saturated. One possibility as to the nature of the nitrogenous components is that we simply have a mixture of proteins with the polysaccharides. If this is true none of the proteins are precipitable by such classical protein precipitants as trichloracetic acid and phosphotungstic acid. In all of these experiments it is probable that if a much more concentrated dust solution had been used some of the material would have been precipitated. It would be much more useful to find something to precipitate the dust extract from dilute solution than from concentrated solutions. If the solution is concentrated enough the addition of almost any water soluble material would precipitate the dust extract.

Determination of Total Reducing Substances:

An experiment was carried out with a crude dust fraction exploring various conditions for acid hydrolysis of the material. The dust fraction that was used, #121 ($\frac{1}{2}$ - 1), was that portion of crude dust extract #121 (Fig. 23) that was

soluble in half saturated ammonium sulfate and precipitated at full saturation. The preparation of this material is described on page 52. Nine different sets of conditions were used in this experiment. Three of the samples were hydrolyzed with 0.1 N hydrochloric acid, three with 1.0 N hydrochloric acid and three with 0.5 N sulfuric acid. In the case of each acid, one sample was heated for one hour, one for two hours and the third for three hours. All the samples were heated in sealed tubes in an oven at 100 to 105°C. Each tube contained 5.1 mg. of the dust fraction in a total volume of 3 ml. After heating, the samples were allowed to cool to room temperature and then neutralized with sodium hydroxide solution. Some of the tubes contained dark brown insoluble material and therefore the samples were filtered into 10 ml. volumetric flasks. The tubes and filters were carefully washed with water, the washings being collected in the volumetric flasks. Each flask was brought to a final volume of 10.0 ml. by the addition of water.

A 5.0 ml. aliquot from each sample was analyzed for total reducing sugar by the method of Hagedorn-Jensen (20) as described by Kabat and Mayer (21). In this procedure the sugar is heated with an excess of ferricyanide solution. Reduction of the ferricyanide to ferrocyanide takes place and the excess ferricyanide is determined by the addition of an excess of potassium iodide to liberate an amount of iodine equivalent to the ferricyanide excess. The iodine is determined by titration with a thiosulfate solution.

The amount of sugar present is proportional to the difference between the amount of ferricyanide in the blank and that left in the sample with the sugar. The reaction of the sugar with the ferricyanide is not stoichiometric and therefore it is necessary to include standards. Different sugars give different results with regards to the completeness of the reaction. In this experiment glucose has been used as the standard sugar and the results are given in terms of mg. of equivalent glucose. The solutions used for the procedure were prepared as follows:

Potassium Ferricyanide Solution.

Potassium ferricyanide	8.25 gm.
Sodium carbonate, anhydrous	10.6 gm.

Water added to a final volume of 1.00 liters.

Solution kept in the dark.

Potassium Iodide Solution.

Potassium iodide	5.0 gm.
Zinc sulfate ($7H_2O$)	10.0 gm.
Sodium chloride	50.0 gm.

Water added to a final volume of 200 ml.

Filtered.

Acetic Acid Solution.

Glacial acetic acid	5.0 ml.

Water added to a final volume of 100 ml.

Starch Solution.

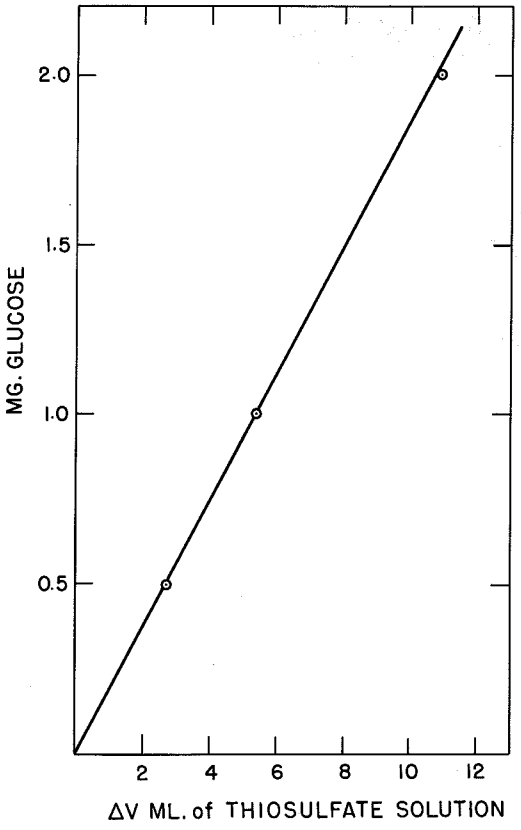
Soluble starch	1.0 gm.
Saturated water solution of sodium chloride.	100 ml.

Sodium Thiosulfate Solution.

Sodium thiosulfate	0.7 gm.
Water added to a final volume of	500 ml.

There was only one sample for each set of hydrolysis conditions; however the standards (glucose 0.5, 1.0 and 2.0 mg. made up to 5.0 ml. with water) were determined in duplicate and the blank (5.0 ml. of water) in triplicate. The 5.0 ml. sample aliquots, standards and blanks were placed in identical one inch diameter test tubes with 5.0 ml. of the potassium ferricyanide solution and heated in a boiling water bath for 15 minutes and then cooled in running water. Five ml. of the potassium iodide solution and three ml. of the acetic acid solution were added. The iodine liberated was titrated with the sodium thiosulfate solution in a calibrated 25 ml. buret using a few drops of the starch solution as an indicator. The data for the glucose standards are plotted in Fig. 32. The difference between the volume of thiosulfate used for the blank (average) and the amount used for each glucose standard (average) is Δ V. The amount of equivalent glucose in each sample was determined by reference to the standard curve and the percent reducing sugar calculated. The data are presented in Fig. 33. Of the conditions used in this experiment the 0.5 N sulfuric acid gave

Fig. 32



Glucose Standard Curve

Mg. of Glucose plotted against thiosulfate volume, ΔV.

Fig. 33

Sample	Thiosulfate Volume, ml.	ΔV*, ml.	mg. Glucose	% Equivalent Glucose
Blank	21.55			
Blank	21.57			
Blank	21.57			
0.1 N HCl				
1 hour	13.53	8.03	1.50	59
2 hour	14.28	7.28	1.36	52
3 hour	13.08	8.48	1.58	62
1 N HCl				
1 hour	13.55	8.01	1.50	59
2 hour	13.84	7.72	1.44	56
3 hour	14.00	7.56	1.41	55
0.5 N H_2SO_4				
1 hour	12.37	9.19	1.71	67
2 hour	12.85	8.71	1.62	63
3 hour	13.76	7.80	1.45	57
Glucose				
0.5 mg.	18.94	2.68	0.5	
0.5 mg.	18.83			
1.0 mg.	16.23	5.32	1.0	
1.0 mg.	16.26			
2.0 mg.	10.61	10.89	2.0	
2.0 mg.	10.74			

* ΔV is the difference between the average blank and the volume of thiosulfate used for each sample.

the higher yield of equivalent reducing sugar. The sulfuric acid solutions also were less brown in color and contained less insoluble material than either of the hydrochloric acid solutions. In general the most reducing sugar is obtained after one hour and further heating diminishes the yield. An exception to this is seen in the case of the 0.1 N hydrochloric acid samples. There is probably an error involved in the three hour sample; however it would have to be repeated to be certain of this.

In the course of another experiment, a 5.1 mg. sample of the dust fraction #121 ($\frac{1}{2}$ - 1) was analyzed directly (i.e. without prior hydrolysis) for reducing sugar content by the method described above. The result was 18% equivalent glucose. This rather high result could be due to two factors: hydrolysis during the course of the reduction or very highly branched polysaccharide structures. The relative importance of these two effects is unknown.

The remainder of each of the dust hydrolysate solutions was heated just to dryness under an infrared lamp and a fan. The residues were twice extracted with pyridine to dissolve the sugars and separate them from the salt by the method of Malpress and Morrison (22). Each time the hydrolysate residue was mixed with a small volume of pyridine (1-2 ml.) and gently heated (about 90°C.) for 10 minutes. The two pyridine solutions were pooled for each sample, centrifuged and then the supernatants taken to dryness in a desiccator. About 0.1 ml. of water was added to each

sample and mixed with the residue. The resulting solutions were chromatographed on sheets of filter paper following the general procedure described on page 80 . Two sheets of filter paper were used. Each had eleven spots, the nine samples and two spots with known sugars (L-rhamnose, D-ribose, D-galactose and D-maltose). One sheet was developed in the pyridine-butanol-water (pyridine 2 parts, n-butanol 3 parts and water 1.5 parts by volume) solvent for 17 hours and the other sheet was developed in the phenol (saturated with water at room temperature) solvent for 23 hours. The sheets were allowed to dry in the hood at room temperature and then sprayed with 3,5 dinitrosalicylate solution (p. 83) and heated in the oven at about 105°C. for 5 to 7 minutes. The pattern of sugar spots obtained from the nine hydrolysates was essentially identical in both solvent systems. In the pyridine-butanol-water solvent a spot remained at the point of application with an upward tail and in the phenol-water solvent there was a spot about half way between the point of application and the maltose level. These slow spots are di- or oligosaccharides, uronic acids or a combination (possibly an aldobiuronic acid). The spray used to locate the spots indicates reducing substances and does not distinguish between sugars and uronic acids. The difference in position of the slow spot in the acid phenol solvent and the basic pyridine solvent suggests that an acidic material was present. On the basis of a later more detailed chroma-

tographic study (p. 87) the other spots were rhamnose, fucose, mannose, glucose and galactose.

Paper Chromatography of Hydrolysates of the Crude Dust Fractions:

A sample (51 mg.) of the crude dust fraction #121 ($\frac{1}{2}$ - 1) was hydrolyzed by heating two hours under reflux with 20 ml. of 0.5 N sulfuric acid. The preparation of this dust fraction is described on page 52 . After the solution had been allowed to cool it was neutralized with barium carbonate and filtered to remove the barium sulfate and the excess barium carbonate. The precipitate was washed with water on the filter. The filtrate and wash water were concentrated to a final volume of 1.3 ml. by vacuum distillation. A large volume (75 ml.) of absolute methanol was added and after two hours the insoluble material was removed by centrifugation. About 30 ml. of water was added to the methanol supernatant and the volume reduced to about 5 ml. by vacuum distillation. The solution was placed in a 10 ml. beaker and the volume further reduced to about 0.5 ml. by placing the sample under an infrared lamp and a fan. This solution was then used to spot paper chromatograms.

Paper chromatography was carried out using the ascending method as described by Williams and Kirby (23). Sheets of Whatman No. 1 filter paper (36 X 40 cm.) were rolled into cylinders 40 cm. in length. The paper was held by four staples. The cylinders did not buckle even if the solvent

front reached the top of the sheet. The experiments were carried out in pipet jars (6 X 18 inches) covered with glass plates. The glass plates were sealed to the tops of the pipet jars with rings of Lubriseal (stopcock grease). The filter paper cylinders were supported above the solvent for about six hours prior to starting the run so that the paper could equilibrate with the solvent vapor. A bent iron wire and a magnet were used to support the paper cylinder over the solvent. The magnet was placed on top of the glass plate and it supported the iron wire with the attached paper cylinder against the lower surface of the glass plate. When the magnet was removed the paper cylinder dropped into the solvent in the bottom of the pipet jar and the run began. About 200 ml. of solvent were placed in the jar and reached to a depth of about 3/4 of an inch. The sample spots were applied two inches from the bottom of the paper cylinder. It is important not to apply too much material to the chromatogram. The spots were between 0.5 and 0.7 cm. in diameter. If the paper is overloaded with sample the spots smear. In some cases I have noted that the Rf of a sugar in a mixture of other sugars will be lower than the Rf of the sugar alone. This is probably due to the high total sugar concentration changing the local properties of the developing solvent and thus influencing the Rf. This poses a problem in identifying minor components in a sugar mixture. If the total sugar concentration is too high an incorrect Rf will be obtained and if it is too low minor

components will be missed. In general we have attempted to solve the problem by using high enough concentrations to locate as many sugars as possible and guessing at their identity on the basis of the Rf values while bearing in mind the effect of concentration on the Rf. Then a mixture of known sugars was prepared to correspond to the best guess as to the nature of the unknown mixture and the two mixtures, known and unknown, compared by chromatography on the same sheet of filter paper in a number of different solvents. Identity under such conditions was taken as good evidence for the correctness of the original guess as to the nature of the sugars in the unknown mixture. There may be some variation ($\pm$ 0.02) in the Rf of a given sugar from run to run. For this reason it is necessary to compare known and unknown sugars on the same chromatogram. It is desirable to carry out these comparisons in duplicate or triplicate for additional confidence in the result.

Two solvents were used most extensively in this work. One was a solution of pyridine, n-butanol and water and the other was a solution of phenol saturated with water. These solvents were prepared as follows:

Pyridine-Butanol-Water Solvent (24).

Pyridine	66 ml.
n-Butanol	100 ml.
Water	50 ml.

Phenol-Water Solvent (25).

Phenol	200 gm.

Water added until two phases separated (about 70 ml.) Mixed, centrifuged, pipeted 200 ml. of the water saturated lower phenol phase into the pipet jar.

The Rf values of a number of known sugars in these two solvent systems were determined and are given in Fig. 34 and Fig. 35.

The chromatograms were allowed to run until the solvent front was within two to three inches of the top of the paper. The sheets were then removed from the jars and allowed to dry at room temperature in the hood. Two spray reagents were used to locate the spots on the chromatograms. One of these was an alkaline solution of sodium 3,5 dinitrosalicylate (26) that was prepared as follows:

3,5 Dinitrosalicylic acid	0.25 gm.
Sodium hydroxide	2.0 gm.
Water added to make a final volume of	50.0 ml.

The chromatogram was sprayed with this solution and then heated in an oven at about 105°C. for five to seven minutes. Reducing sugars appear as brown spots against a yellow background. There seems to be no change in the appearance of the chromatogram over a period of a few months. The other spray reagent that was used was an alcohol solution of p-anisidine hydrochloride (27). Reagent grade p-anisidine was obtained from the stockroom, dissolved in a minimum amount of diethylether and concentrated hydrochloric acid slowly added until no more p-anisidine hydrochloride was precipi-

Fig. 34

Rf Values of Known Sugars in the Pyridine-Butanol-Water Solvent

Sugar	Rf
L-rhamnose	0.52
D-ribose	0.44
L-fucose	0.44
D-lyxose	0.44
D-xylose	0.41
D-mannose	0.37
D-arabinose	0.37
D-fructose	0.35
D-glucose	0.33
D-galactose	0.30
D-glucosamine·HCl	0.27
D-maltose	0.24

Fig. 35

Rf Values of Known Sugars in the Phenol-Water Solvent

Sugar	Rf
L-fucose	0.61
L-rhamnose	0.54
D-ribose	0.54
D-arabinose	0.49
D-lyxose	0.47
D-mannose	0.40
D-xylose	0.39
D-galactose	0.39
D-glucose	0.34
D-maltose	0.30

tated. The dark purple precipitate was dissolved in absolute ethanol and heated with Norite for ten mintues and then filtered. Upon gradual cooling white crystals were formed. The mixture was allowed to stand for a few hours in the deep freeze (-10°C.) and then the crystals collected by filtration. The mass of crystals was washed with cold absolute ethanol and then quickly dried between filter paper and finally dried in a vacuum oven at 50°C. The white crystalline product was stored in a vacuum desiccator over calcium chloride. The alcoholic solution of the p-anisidine hydrochloride was prepared as follows:

p-Anisidine hydrochloride	0.50 gm.
Ethanol (95%)	15.0 ml.
n-Butanol	20.0 ml.

Mixed the ethanol with the p-anisidine hydrochloride until dissolved, then added the n-butanol.

The chromatogram was sprayed with this solution and then the excess solvent removed with an electric hair dryer. After all the solvent had been removed, the chromatogram was heated in an oven at 105°C. for five to seven minutes. The sugar spots showed up in different colors depending upon the class of sugar. Aldohexoses were brownish olive green, methyl pentoses light yellow and aldopentoses red purple. The background was a faint tan. The colors lasted for only a few hours and therefore these chromatograms could not be used for a permanent record.

The crude dust hydrolysate was chromatographed using

the Pyridine-Butanol-Water solvent and the Phenol-Water solvent. One set of chromatograms was sprayed with the 3,5 dinitrosalicylate reagent while another set was sprayed with the p-anisidine hydrochloride solution. The Rf data for the two solvent systems are presented in Fig. 36 and Fig. 37. On the basis of the Rf data from the dust hydrolysate and comparison with the data for known sugars (Fig. 34 and Fig. 35) a tentative identification of the sugars was made. The color obtained by the use of the p-anisidine hydrochloride spray was also used in making this identification. The following sugars were believed to be present in the hydrolysate: rhamnose, fucose, arabinose, mannose, glucose and galactose. Some material was left at the point of spot application in both of the solvent systems.

A mixture of known sugars was prepared, for comparison with the crude dust hydrolysate, as follows:

D-mannose	10 mg.
D-glucose	10 mg.
D-galactose	10 mg.
L-rhamnose	5 mg.
L-fucose	5 mg.
D-arabinose	2.5 mg.
Water	1 ml.

The amounts of the sugars used in the mixture were based on visual appraisal of the relative intensities of the spots on the paper chromatograms. Four chromatograms were pre-

Fig. 36

Chromatography of Crude Dust Hydrolysate[1]

Pyridine-Butanol-Water Solvent

Sugar[2]	Position of spot, cm.	Rf[3]	Rf of known sugar	Class of sugar[4]
rhamnose	15.18	0.51	0.52	methylpentose
fucose	12.50	0.42	0.44	methylpentose
mannose arabinose	11.15	0.38	0.37 0.37	hexose plus pentose
glucose	10.00	0.34	0.33	hexose
galactose	8.55	0.29	0.30	hexose

Solvent front 29.60 cm.

[1] Hydrolysate of crude dust fraction #121 ($\frac{1}{2}$ - 1).

[2] Tentative identification of sugar spots based on comparison of Rf's with those of known sugars and color reaction with p-anisidine hydrochloride.

[3] The Rf is the ratio of the distance the spot moved to the distance the solvent front moved.

[4] Class of sugar indicated by color reaction with p-anisidine hydrochloride spray.

Fig. 37

Chromatography of Crude Dust Hydrolysate[1]

Phenol-Water Solvent

Sugar[2]	Position of spot, cm.	Rf[3]	Rf of known sugar	Class of sugar[4]
fucose	18.10	0.59	0.61	methylpentose
rhamnose	16.48	0.54	0.54	methylpentose
arabinose	14.68	0.48	0.49	pentose
mannose	11.35	0.37	0.40	hexose
galactose			0.39	
glucose	9.15	0.30	0.34	hexose

Solvent front 30.78 cm.

[1] Hydrolysate of crude dust fraction #121 ($\frac{1}{2}$ - 1).

[2] Tentative identification of sugar spots based on comparison of Rf's with those of known sugars and color reactions with p-anisidine hydrochloride.

[3] The Rf is the ratio of the distance the spot moved to the distance the solvent front moved.

[4] Class of sugar indicated by color reaction with p-anisidine hydrochloride spray.

pared with two spots of the hydrolysate and two spots of the mixture of known sugars on each sheet. One sheet was developed in the pyridine-butanol-water solvent and another in the phenol-water solvent, both already described. Two new solvents were used for the other two chromatograms. They were prepared as follows:

Butanol-Water Solvent.

n-Butanol	200 ml.
Water	200 ml.

Shaken vigorously, removed 200 ml. of top butanol rich phase with pipet.

Pentanol-Propanol-Water Solvent (28).

t-Pentyl alcohol	133 ml.
n-Propyl alcohol	33 ml.
Water	50 ml.

The sugars do not move as far in these solvents as they do in the pyridine-butanol-water and phenol-water solvents. Therefore after the chromatograms had been allowed to dry in the hood they were rerun in the same solvent. This process was repeated again so that each chromatogram was run a total of three times in these two solvents. Each time the solvent was allowed to rise to within two to three inches of the top. The butanol-water solvent required about 24 hours for each run while the t-pentyl alcohol solvent required about 40 hours. No attempt was made to have the solvent front reach the same height each time. After the sheets had dried they were cut in two and one set of spots

(including both the known and unknown mixtures) was sprayed with 3,5 dinitrosalicylate and the other with p-anisidine hydrochloride. Good agreement was obtained for each set of spots in all four solvents. The results support our original identification.

Color Reactions for Uronic Acids:

A number of color tests for uronic acids have been carried out with the crude dust fraction #121 ($\frac{1}{2}$ - 1) following the methods of Dische (29). These experiments provide presumptive qualitative evidence for the presence of uronic acid residues, probably glucuronic acid, in the dust material.

Carbazole Reaction for Total Uronic Acids (29,30):

The procedure involves heating the polysaccharide solution with concentrated sulfuric acid and then adding carbazole. A red purple color is formed by the reaction of the carbazole with the products of the reaction of the uronic acid and the sulfuric acid. Pentoses do not give a positive reaction. On this basis Dische believes that the product of the uronic acid that reacts with the carbazole is not furfuraldehyde but some intermediate. According to Dische (30) this reaction is more specific for uronic acids than is the Tollens naphthoresorcinol reaction.

One ml. volumes of the following solutions were used in the test:

1) 0.051% distilled water solution of crude dust fraction #121 ($\frac{1}{2}$ - 1). One ml. contained 510 γ of dust extract.

2) 0.01% distilled water solution of glucuronolactone (Corn Products Refining Co.). One ml. contained 100 γ of the glucuronolactone.

3) 0.01% distilled water solution of galacturonic acid. One ml. contained 100 γ of the galacturonic acid. (Nutritional Biochemical Corporation).

4) Distilled water was used for the reagent blank.

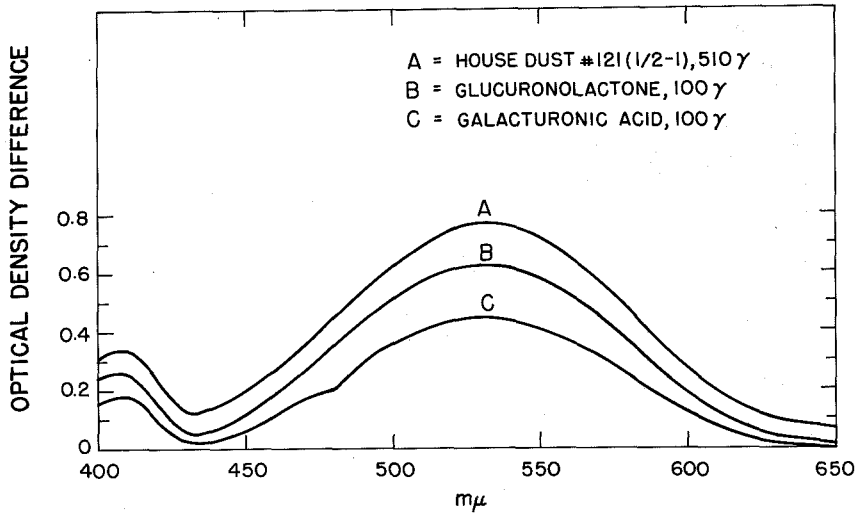
Procedure:

The test samples (1 ml.) were placed in 15 X 125 mm. Pyrex test tubes. It is important to include tubes (internal standards) containing the sugar or polysaccharide solution and sulfuric acid but no carbazole so that the absorption contributed by the reaction with the sulfuric acid alone may be subtracted from the spectra. The test was set up in duplicate so as to have enough solution to wash the spectrophotometer cell between determinations. To each one ml. sample 6 ml. of concentrated sulfuric acid was added and mixed with a small stirring rod, while cooling the tube in an ice bath. The tubes were warmed to about room temperature and then heated in a boiling water bath for 20 minutes. After heating, the tubes were cooled in tap water. To each of the test solutions, except the internal standards, 0.2 ml. of a 0.1% ethanol(95%) solution of carbazole was added. The same volume of pure ethanol(95%) was added to each of

the internal standard tubes. The solutions were mixed and after one hour the absorption spectra were determined in a Cary Spectrophotometer. The addition of these solutions was made at 15 minute intervals so that the spectra of each type of sample could be determined close to one hour after the addition of the reagent.

The absorption spectra for the reagent blank and the corresponding internal standard were subtracted from the spectra of the test solutions. The resulting curves are presented in Fig. 38. On the basis of these curves it appears that about one fifth of the dust material is uronic acid. This is not a quantitative result because it is not certain that the uronic acid incorporated in the polysaccharide has the same reactivity and absorption properties as the free uronic acid or lactone used as a standard. It is obvious by a comparison of the curves for glucuronolactone and galacturonic acid that different uronic acids yield quantitatively different absorption curves. It is not known which uronic acids are present in the dust; however the thioglycolic acid and cysteine experiments discussed in the following sections indicate that of the three commonly occurring uronic acids (glucuronic, galacturonic and mannuronic acids) glucuronic acid most probably is the main constituent. Another error in the uronic acid determination is that not quite all the effect of the presence of hexose is removed by subtracting the internal standard. Some of the products of the reaction of hexose and sulfuric acid

Fig. 38



Carbazole Test for Uronic Acid

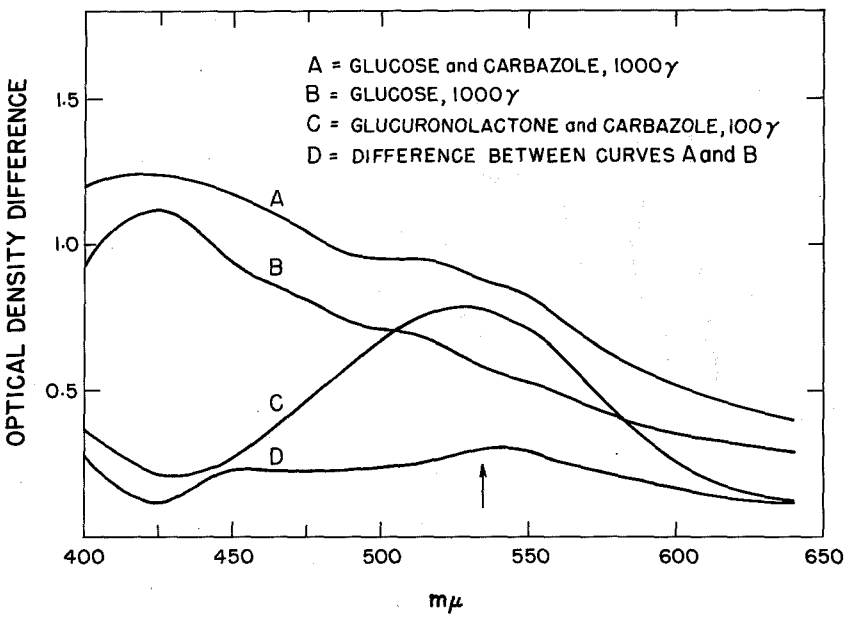
Crude Dust Fraction #121 ($\frac{1}{2}$ - 1)

react with the carbazole and produce absorption in the same region as the products from uronic acids. The following experiment demonstrates this latter effect. The test was carried out as described above using one ml. of water (reagent blank), one ml. of water containing 1000 γ of glucose, and one ml. of water containing 100 γ of glucuronolactone. The usual internal standards were included for the saccharide solutions, as described above. The spectra of the resulting solutions were determined in the Beckman Model B Spectrophotometer. The curves are presented in Fig. 39. The internal standard for the glucuronolactone had an optical density of less than 0.01 throughout the range measured and was therefore not plotted. The vertical arrow indicates the wavelength at which measurements are normally made for the determination of uronic acids. It is clear that the use of the internal standard corrects for most but not all of the color contribution due to the presence of hexoses in the uronic acid determination.

Color Reaction for Glucuronic Acid with Thioglycolic Acid and Sulfuric Acid in the Presence of Mannose (29,31).

The material to be tested is mixed with mannose, heated with sulfuric acid and the thioglycolic acid is added. The color (maximum about 535 mμ) develops over the course of a day or so. This is a modification of a general reaction of saccharides with sulfuric acid and thioglycolic acid. According to Dische, only glucuronic acid shows the charac-

Fig. 39



Glucose Interference in the Carbazole Test for Uronic Acids

teristic color in the presence of mannose.

0.8 ml. volume of the following solutions was used in the test:

1) 0.051% distilled water solution of crude dust fraction #121 ($\frac{1}{2}$ - 1). The test volume contained 408 γ of dust extract.
2) 0.01% distilled water solution of glucuronolactone. The test volume contained 80 γ of the glucuronolactone.
3) 0.01% distilled water solution of galacturonic acid. The test volume contained 80 γ of the galacturonic acid.
4) Distilled water was used for the reagent blank.

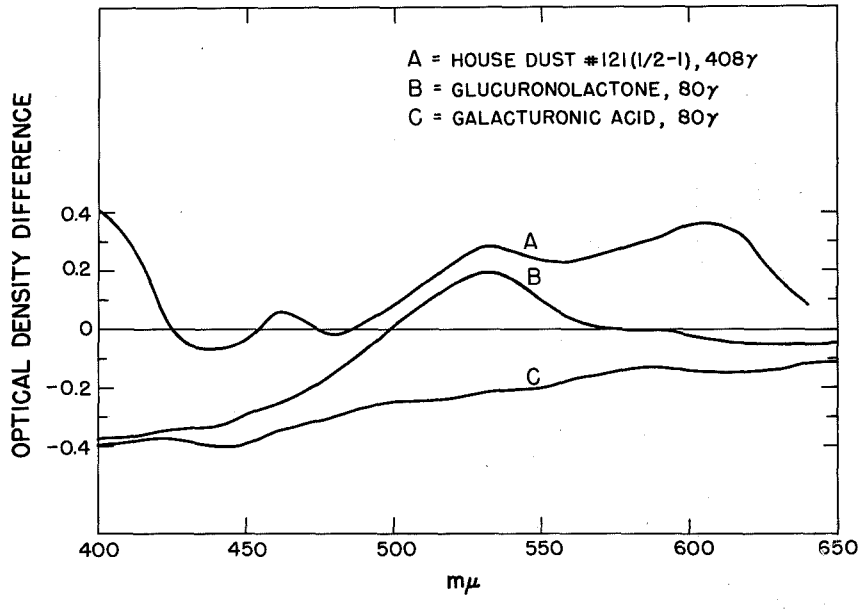
Procedure:

Internal standards were included for each of the saccharide solutions and the test was carried out in duplicate so as to have sufficient solution to wash the spectrophotometer cell between each determination. The test samples (0.8 ml.) were placed in 15 X 125 mm. Pyrex test tubes and a 0.2 ml. volume of a 0.2% solution of mannose in water was added to each tube. Then 4.5 ml. of a diluted sulfuric acid solution (6 volumes of concentrated sulfuric acid and one volume of water) were added to each tube while being cooled in an ice bath. The samples were mixed with a glass stirring rod, warmed to room temperature and then heated in a boiling water bath for three minutes. After cooling to room temperature in tap water, a 0.1 ml. volume of a 2.5% aqueous solution

of thioglycolic acid (freshly prepared) was added to each test solution, except the internal standard. The same volume of water (0.1 ml.) was added to each of the internal standards. The solutions were mixed and allowed to stand at room temperature for 24 to 28 hours before determining the absorption spectra in the Cary Spectrophotometer. The curves for the reagent blank and the corresponding internal standard were subtracted from the absorption spectra curves of the test solutions. The resulting curves are presented in Fig. 40.

The dust curve shows an absorption maximum (535 mμ) corresponding to that of the glucuronolactone curve. There is no such maximum in the galacturonic acid curve. The reagent blank with the mannose shows considerable absorption as does the internal standard. When the sum of these absorption curves is subtracted from the test solution curve a negative result is obtained in some regions of the spectrum. This is probably due to a lack of additivity of the effects as well as an improper choice of internal standard. By the procedure used I have subtracted the absorption due to the mannose acid reaction twice. The internal standard should have had dust material and acid only i.e. no mannose. The reagent blank absorption curve is fairly flat in the region of the glucuronic acid absorption maximum (535 mμ) and the general shape of the curves and position of the maximum would be only very slightly influenced by this effect.

Fig. 40



Thioglycolic Acid Test for Glucuronic Acid

Dische (29) reports that polyuronides that contain galacturonic or mannuronic acid do not give the reaction and that all those that contain glucuronic acid give a positive reaction with the single exception of heparin which exhibits anomalous behavior.

The conclusion from this experiment is that a large portion of the uronic acid present in the dust material is glucuronic acid. The presence of other uronic acids is not excluded by this experiment.

Cysteine Reaction for Galacturonic Acid (29, 32)

In the procedure, the saccharide solution is mixed with sulfuric acid and then a solution of cysteine is added. A green blue color develops in the presence of galacturonic acid. None of the other saccharides, including mannuronic acid and glucuronic acid, produce the characteristic color.

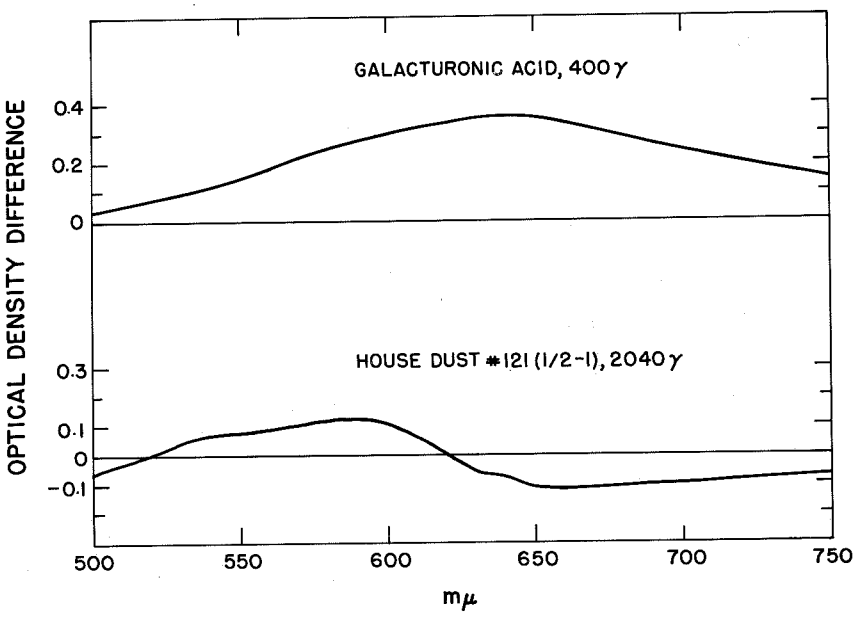
One ml. volumes of the following solutions were used in the test:

1) 0.204% distilled water solution of crude dust fraction #121 ($\frac{1}{5}$ - 1). The test volume contained 2040γ of dust extract.
2) 0.04% distilled water solution of galacturonic acid. The test volume contained 400γ of galacturonic acid.
3) 0.01% distilled water solution of glucuronolactone. The test volume contained 100γ of glucuronolactone.
4) Distilled water was used for the reagent blank.

Procedure:

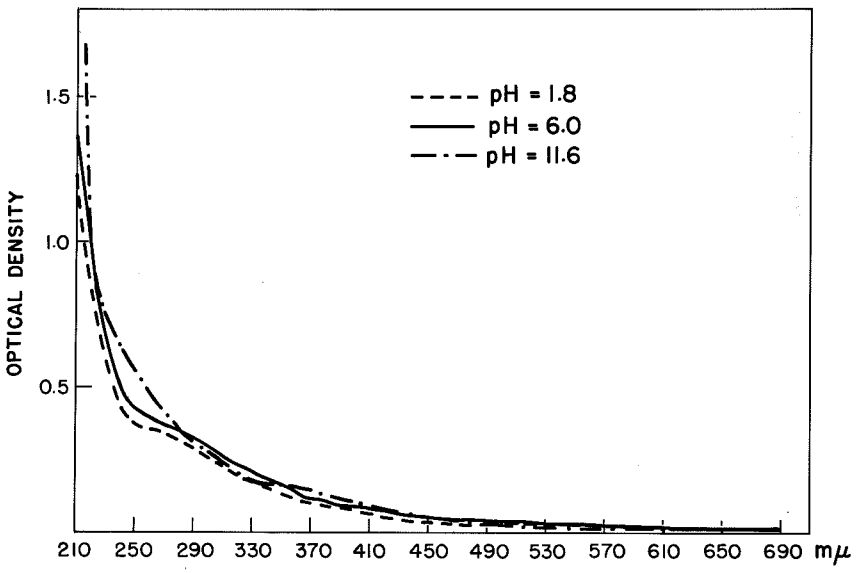
Internal standards were included for each of the saccharide solutions and the test was carried out in duplicate so as to have sufficient solution to wash the spectrophotometer cell between each determination. The test samples (1.0 ml.) were placed in 15 X 125 mm. Pyrex test tubes and 4.0 ml. volumes of concentrated sulfuric acid were added with mixing to each tube while being cooled in tap water. The samples were not cooled in an ice bath as the heat liberated by mixing the water and sulfuric acid, under these conditions, aids the reaction. This is the only heating operation to which the samples are subjected in this procedure. After the samples had been mixed and cooled to room temperature, 0.1 ml. of a 2.5% aqueous solution of cysteine hydrochloride was added to each test solution except the internal standards. The same volume of water (0.1 ml.) was added to each of the internal standards. The solutions were mixed and allowed to stand at room temperature for 48 to 52 hours. The visual absorption spectrum of each sample was determined in the Cary Spectrophotometer. The curves for the reagent blank and the corresponding internal standard were subtracted from the test solutions. The resulting corrected curves for the dust solution and the galacturonic acid solution are presented in Fig. 40a. The curves for the glucuronolactone (100%) and the reagent blank were almost completely superimposable i.e. essentially no absorption was contributed by the glucurono-

Fig. 40a



Cysteine Reaction for Galacturonic Acid

Fig. 41



Absorption Spectra of the Crude Dust Fraction #105 ($\frac{1}{2}$ - 1)

Concentration 0.1 mg./ml.

lactone. A comparison of the curves for the dust extract and the galacturonic acid indicated that there probably is little if any galacturonic acid in the dust extract. The peak in the galacturonic acid curve comes at about the minimum in the dust curve. Dische (29) states that the polyuronides containing galacturonic acid that he tested gave this reaction in about the same intensity as equivalent amounts of free galacturonic acid.

The carbazole reaction described above provides evidence for the presence of uronic acid residues in the dust fractions and more specifically the thioglycolic acid reaction indicated that a large part of this uronic acid is glucuronic acid. The cysteine reaction suggests that there is little if any galacturonic acid present. On the basis of the height of the carbazole peak (Fig. 38) it would appear that about one quarter to one fifth of the dust extract is composed of uronic acid residues.

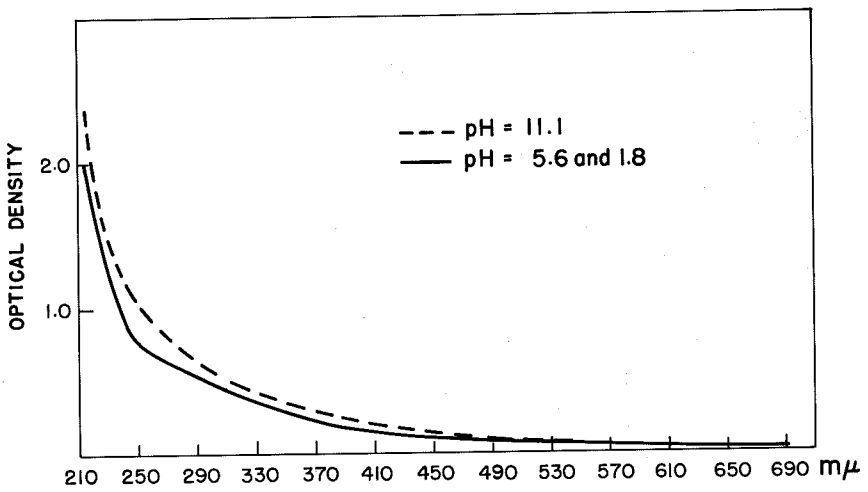
Absorption Spectra of the Crude Dust Fractions - pH Dependence:

Solutions of dust extracts in water have a brown color. House dust extracts have more of a black brown color while mattress dust extracts exhibit a lighter yellow brown color. The solid materials isolated from house dust or mattress dust by pervaporation to dryness or lyophilization of aqueous solutions show the same difference in color. The color of an aqueous solution of house dust varies with the pH of the solution. In alkaline solution the color is the darkest

brown. As the solution becomes more acid the color changes to a lighter brown color. The color changes are reversible. The absorption spectra of the crude dust fractions #105 ($\frac{1}{2}$ - 1) and #121 ($\frac{1}{2}$ - 1) (preparation described on p. 52) were determined in the Cary Spectrophotometer at three pH's. Distilled water solutions of these materials were examined at their natural pH (5.6 and 6.0) and then a few drops of dilute hydrochloric acid added to lower the pH to 1.8. After determining the spectra in acid solution, a few drops of dilute sodium hydroxide solution were added to make the dust solution alkaline (pH 11.1 and 11.6) and the spectra redetermined. The absorption curves are shown in Fig. 41 and Fig. 42. There are no very definite absorption peaks. The absorption gradually increases starting at about 450 mμ and proceeding into the ultraviolet region. The curves show an increase in absorption at the higher pH, however the visual effect is much more striking than the slight elevation of the curves would indicate.

The nature of the material responsible for the absorption is unknown. Aromatic amino acids would contribute to the 260 to 290 mμ absorption; however they are not in very high concentration. Carbohydrates present in food and other complex mixtures characteristically undergo a browning reaction. The spectra in these cases (33) also show the gradual rise in absorption in the ultraviolet; however there is generally a peak at about 280 mμ presumably due to furfuraldehyde or a related compound. Wolfrom et al.(34)

Fig. 42



Absorption Spectra of the Crude Dust Fraction #121 ($\frac{1}{2}$ - 1)

Concentration 0.2 mg./ml.

have studied the browning obtained by heating xylose in water. They attributed the high absorption in the 220 mμ region to the formation of the conjugated diene or enol structures that are postulated as intermediates in the formation of furfuraldehyde. During the course of heating the xylose solution the first spectral change is an increase in absorption at 220 mμ; then later the peak at 277 mμ characteristic of furfuraldehyde develops. Browning reactions in carbohydrates have also been attributed to reactions between the carbohydrate and amino acids, peptides or proteins (Maillard reaction). The nature of these reactions is still not clear and their relation to the browning observed in carbohydrates is even less well understood. It is possible that reactions such as these may be responsible for the color of house dust extracts.

The problem of the distribution of the colored material among the various electrophoretic components will be discussed in connection with the zone electrophoresis experiments (p.122).

Nitrogenous Components of Crude Dust Fractions:

One of the most important problems in establishing the antigencity or allergenicity of a polysaccharide is to be certain that the immunological response is due to the polysaccharide itself and not to the presence of a protein or polypeptide. The protein or polypeptide may be present as an impurity mixed with the polysaccharide allergen, the protein or polypeptide may be the true allergen and the

polysaccharide the impurity or the allergen may be a complex molecule containing protein or polypeptide and polysaccharide moieties. In order to gather data on this problem, the nitrogen content of some of the crude dust fractions was determined, a hexosamine determination was carried out on three of the dust fractions and attempts were made to separate protein or polypeptide components from the crude dust materials by zone electrophoresis.

Determination of the Nitrogen Content of Crude Dust Fractions:

Aqueous solutions of the crude dust fractions were dialyzed extensively against distilled water, centrifuged and then accurately measured volumes of the solutions pipeted into previously dried (100°C.) and weighed beakers or test tubes. The water was slowly removed in a vacuum desiccator over calcium chloride and the samples finally dried in an oven at 100°C. After the beakers or test tubes had cooled they were weighed and thus the concentrations of the dust fraction in the solutions established. Accurately measured volumes of the solutions or the dried residues in the test tubes, were then digested with sulfuric acid and hydrogen peroxide. The digests were diluted with water and Nessler's solution added. The nitrogen contents of the samples were determined by reading the optical densities (440 mμ) of the solutions and comparison with suitable blanks and standards. The exact procedure used was that in common practice in this laboratory and is des-

cribed on page 121. The nitrogen contents of the various fractions were calculated and tabulated in Fig. 43. The values vary from 0.3 to 5%. I believe that in general materials derived from house dust, as opposed to mattress dust, have higher nitrogen contents. As an illustration of this the #105 ($\frac{1}{2}$ - 1) material is derived entirely from house dust and had a nitrogen content of 5% while the #125 material was derived entirely from mattress dust and had a nitrogen content of 1.7%. The color also seems to be correlated with the nitrogen content and the source of the dust. The house dust extracts are darker than those from mattress dust. This relation between color and nitrogen content will be discussed further in connection with the zone electrophoresis experiments. The more water soluble materials (requiring higher acetone or ammonium sulfate concentrations for precipitation) seem to have lower nitrogen contents than the more insoluble materials.

Determination of the Hexosamine Content of Three of the Crude Dust Fractions:

The Elson-Morgan procedure as modified by Rimington (35, 36) was employed. The first step was the hydrolysis of the materials to liberate the hexosamine. It is necessary to try various conditions for hydrolysis and determine what procedure gives the highest yield of hexosamine. Three sets of hydrolysis conditions were used: 3 N HCl for 4 hours, 3 N HCl for 8 hours and 4 N HCl for 12 hours all at 100 to

Fig. 43

Nitrogen Content Data for Crude Dust Fractions:

Fractions*	% Nitrogen
#105 ($\frac{1}{2}$ - 1). Material soluble in half sat. A.S. and precipitated at full sat.	5.0
#105-I. Material soluble in sat. A.S. and precipitated by 60% acid acetone.	1.3
#105-II. Material soluble in sat. A.S. and precipitated by 60-71% acid acetone	2.1
#105-III. Material soluble in sat. A.S. and precipitated by 71-84% acid acetone	2.3
#121 ($\frac{1}{2}$ - 1). Material soluble in half sat. A.S. and precipitated at full sat.	3.0
#121-I. Material soluble in sat. A.S. and precipitated by 60% acid acetone.	0.5
#121-II. Material soluble in sat. A.S. and precipitated by 60-71% acid acetone.	0.6
#121-III. Material soluble in sat. A.S. and precipitated by 71-84% acid acetone.	0.6
#121-IV. Material soluble in sat. A.S. and soluble in 84% acid acetone.	0.3
#110. Crude Dust (Fig. 9).	2.5
#110. Material soluble in sat. A.S. Preparation described on page 70.	2.2
#125. Crude Dust (Fig. 20).	1.7

* The starting material for the #105 fractions listed here was the material soluble in 80% acetone (Fig. 7). The preparation of these fractions is described on page 52 and in Fig. 23. The starting material for the #121 fractions was the crude dust product #121 (Fig. 19). The preparation of these fractions is described on page 52 and in Fig. 23.

105°C. in sealed tubes. The samples for hydrolysis were set up as follows:

Tube No.	
1	3 N hydrochloric acid, heated 4 hours 1.5 ml. 6 N HCl 1.5 ml. of water containing 10.0 mgm. of #110 crude dust extract.
2	3 N hydrochloric acid, heated 8 hours Same as Tube No. 1.
3	4 N hydrochloric acid, heated 12 hours 2.0 ml. of 6 N HCl 1.0 ml. of water containing 10.0 mgm. of #110 crude dust extract.
4	3 N hydrochloric acid, heated 4 hours 1.5 ml. of 6 N HCl 1.5 ml. of water containing 2.16 mgm. of #105 ($\frac{1}{2}$ - 1) crude dust extract.
5	3 N hydrochloric acid, heated 8 hours Same as tube No. 4
6	4 N hydrochloric acid, heated 12 hours 2.0 ml. of 6 N HCl 1.0 ml. of water containing 2.16 mgm. of #105 ($\frac{1}{2}$ - 1) crude dust extract.

7 3 N hydrochloric acid, heated 4 hours
1.5 ml. 6 N HCl
1.5 ml. of water containing 5.1 mgm. of #121 ($\frac{1}{2}$ - 1) crude dust extract.

8 3 N hydrochloric acid, heated 8 hours
Same as tube No. 7

9 4 N hydrochloric acid, heated 12 hours
2.0 ml. of 6 N HCl
1.0 ml. of water containing 5.1 mgm. of #121 ($\frac{1}{2}$ - 1) crude dust extract.

All the tubes were sealed and heated in an oven at 100 - 105°C. Tubes 1, 4 and 7 were heated four hours, tubes 2, 5 and 8 were heated eight hours and tubes 3, 6 and 9 were heated twelve hours. After the samples had cooled, it was observed that there was considerable black precipitate in all of the tubes. The most was present in tubes 1, 2 and 3, while the least was found in tubes 4, 5 and 6. The tubes were opened and the samples filtered into 10 ml. volumetric flasks. The tubes and filters were washed with a little water into the corresponding volumetric flasks. The samples were neutralized with 5 N sodium hydroxide solution and each made up to a final volume of 10.0 ml. with water.

In the Elson-Morgan procedure for the determination of hexosamine, acetylacetone in alkaline solution acetylates the hexosamine and then with heating a compound is formed by ring closure (an oxazole or a pyrrole) that couples with

p-dimethylaminobenzaldehyde to yield a red colored product that may be determined spectrophotometrically.

The solutions used for the procedure were prepared as follows:

Acetylacetone Solution

Acetylacetone	1.0 ml.
0.5 N sodium carbonate solution	50.0 ml.

This solution should be freshly prepared before use.

Ehrlich's Reagent

p-dimethylaminobenzaldehyde (recrystallized once from 50% aqueous methanol)	0.24 gm.
concentrated hydrochloric acid	9.0 ml.
absolute methanol	9.0 ml.

Glucosamine Standard Solution

glucosamine hydrochloride	11.6 mgm.
water	100 ml.

The test was carried out in 15 X 125 mm. Pyrex test tubes (marked at the 10.0 ml. level) using a 1.0 ml. aliquot from each of the samples in the 10.0 ml. volumetric flasks. Three one ml. volumes of water were carried through the test for reagent blanks as well as four different volumes of the standard glucosamine hydrochloride solution (1.0, 0.5, 0.2 and 0.1 ml.) with sufficient added water to make a final volume of 1.0 ml. To each test tube was added 1.0 ml. of the acetylacetone solution, the solutions mixed and the tubes heated in a boiling water bath for fifteen minutes. After cooling the tubes in tap water, a 5.0 ml. volume of

ethanol(95%) was added to each tube and the solutions mixed. One ml. of Ehrlich's reagent was then added to each tube and ethanol(95%) added to bring each solution to the 10.0 ml. mark. The solutions were mixed and allowed to stand at room temperature for one half hour. The optical density of each solution, in the same cuvette, was then determined at 530 mμ in the Beckman Model B Spectrophotometer. The data are presented in Fig. 44. A standard curve was prepared using the glucosamine hydrochloride data and the hexosamine contents of the dust fractions are reported as percent equivalent glucosamine. The maximum value obtained for percent equivalent glucosamine content under the three hydrolysis conditions was taken as the hexosamine content of the sample. The nitrogen content, hexosamine content and the percent of the total nitrogen contributed by the hexosamine are tabulated for the three crude dust fractions in Fig. 45. It has been reported that amino acids in the presence of carbohydrate may give a false positive hexosamine reaction (37). The dust fractions undoubtedly contain amino acids (p. 5 and p. 158) and carbohydrate. This experiment does not give information as to how much of the total hexosamine determined is really due to the amino acid carbohydrate reaction. Such information may be obtained by carrying out the reaction in excess acetylacetone and carefully controlling the pH (37). In any case, the conclusion may be drawn that only a small amount of the nitrogen present in the dust fractions is due to the presence of hexosamine.

Fig. 44

Hexosamine Content of Crude Dust Fractions:

Sample	Tube No.	Optical Density (530 mμ)	γ Equivalent Glucosamine	% Equivalent Glucosamine
Reagent blank		0.025		
" "		0.025		
" "		0.025		
Glucosamine hydrochloride				
0.116 mg.		0.492		
0.058 mg.		0.281		
0.023^2 mg.		0.122		
0.011^6 mg.		0.067		
#110 crude dust (1.0 mg.)				
3 N HCl heated 4 hours	1.	0.112	17.3	1.7
3 N HCl heated 8 hours	2.	0.102	15.2	1.5
4 N HCl heated 12 hours	3.	0.079	10.8	1.1
#105 (½ - 1) crude dust (0.216 mg.)				
3 N HCl heated 4 hours	4.	0.057	6.7	3.1
3 N HCl heated 8 hours	5.	0.060	7.3	3.4
4 N HCl heated 12 hours	6.	0.055	6.2	2.9
#121 (½ - 1) crude dust (0.51 mg.)				
3 N HCl heated 4 hours	7.	0.061	7.5	1.5
3 N HCl heated 8 hours	8.	0.067	8.5	1.6^5
4 N HCl heated 12 hours	9.	0.050	5.4	1.0^5

Fig. 45

Comparison of Nitrogen and Hexosamine Contents of Crude Dust Fractions

Material	% Hexosamine	% Nitrogen	% of total N from Hexosamine
#110	1.7	2.5	5.2
#105 ($\frac{1}{2}$ - 1)	3.4	5.0	5.4
#121 ($\frac{1}{3}$ - 1)	1.6	3.0	3.7

Zone Electrophoresis Study of a Crude Dust Extract:

The crude dust extract #110 (Fig. 9) was studied by the method of starch zone electrophoresis (38) which involves placing a concentrated solution of the sample in a block of potato starch wet with buffer. An electric current is applied to the starch block and the resulting movement of the dust extract depends upon the electrophoretic effect due to the charge on the dust extract molecules and the electroosmotic effect due to the charge on the starch grains with a resulting flow of solvent. After the electric current has been allowed to pass through the starch block for a suitable length of time, the block is cut and the dust extract eluted from the different portions of the block. Chemical analyses are then carried out on the eluates to study the electrophoretic distribution of the various components of the sample. In the case of the house dust extract, the color was measured by determining the optical density at 400 mμ, the total carbohydrate content was estimated by means of the indole reaction, the uronic acid content was estimated by the carbazole reaction and the nitrogen distribution was determined by digestion and Nesslerization.

Methods:

Starch Electrophoresis: Potato starch (Mallinckrodt) was washed with 1% sodium chloride solution the day prior to the starch electrophoresis run and allowed to stand overnight in the salt solution. The apparatus consisted of two buffer vessels and a lucite tray 1.5 cm. deep, 9 cm. wide and 44 cm.

long that contained the starch block. The tray was supported at each end by a buffer vessel. These were cubical lucite boxes (about 18 cm. on each edge) containing three liters of buffer and divided into four compartments by perforated lucite partitions. The two partitions most remote from the starch tray had platinum wire intertwined through the perforation. The wires served as electrodes to connect the buffer vessels to the power supply. The other two lucite partitions were to diminish mixing of the buffer and thus to keep electrode products away from the starch block. A double thickness of cloth towel was used at each end of the tray to connect the starch block to the buffer solution. The starch electrophoresis run was carried out in the cold room at about 4°C. to minimize heating of the block during the run. It was necessary to be sure that the apparatus was level so that all the movement of the sample was due to the passage of the current and not to the siphoning of buffer from one vessel to the other. To insure that buffer levels in each vessel were the same height, a rubber tube connected the two buffer vessels below the buffer level. Prior to starting the current this was closed with a pinch clamp.

The potato starch that had been soaking overnight in 1% sodium chloride solution was washed three or four times with the salt solution on a Buechner funnel and then washed three or four more times with the buffer which was to be used in the experiment. When acid solutions were used (phthalate buffer and the KCl HCl mixture) a brownish material collected

on top of the filter cake. This was scraped off each time and greatly diminished in amount with successive washings until after about four washings little more appeared.

After the starch had been washed thoroughly with the buffer a thick suspension of the starch in buffer was prepared. The suspension was free of lumps and thick enough so that it was just possible to pour it. The tray was lined with a sheet of polyethylene and the cloth wicks to connect with the buffer vessels carefully placed at each end of the tray. Then enough of the starch suspension was quickly poured into the tray to fill it to the top. The starch grains settled to the bottom and clear buffer appeared on top. This excess buffer was removed with blotters and at the same time any differences in thickness of the starch corrected. The starch block was allowed to stand for about thirty minutes and then the excess buffer again removed with blotters. Buffer was removed until the surface of the block had lost its shiny appearance and became dull.

A hole (1 cm. X 7.5 cm.) was cut through the starch block 13 to 15 cm. from one end with the aid of a metal stamp. Enough buffer wet starch was replaced to make a 2 mm. thick covering for the bottom of the hole. A thick putty like paste was prepared with dry starch and five ml. of a concentrated distilled water solution of crude house dust extract #110. The hole in the starch block was filled with the starch dust sample mixture and a thin layer of the buffer wet starch placed on top. The area over the sample was

firmly pressed to insure good contact with the starch block. The block and tray were wrapped with the ends of the polyethylene sheet to prevent loss of water from the block during the run. The tray was placed in the cold room on the buffer vessels with the sample end toward the cathode. The cloth wicks were placed in the buffer and the clamp applied to close off the rubber tubing connection between the two buffer vessels. The current (about 70 mamps. at 300 volts, constant voltage) was allowed to pass through the starch block for approximately 16½ hours.

After the current had been turned off, the starch block was removed from the tray and cut into forty-four one cm. segments. The cuts were made perpendicular to the direction of motion of the dust extract in the electric field. Each segment of the starch block was placed in a 50 ml. test tube, mixed with 5 ml. of water and then allowed to stand for about one hour. The starch settled to the bottom of the tubes and the supernatants were decanted. Each starch sample was re-extracted with 2 ml. of water and the two eluates from the same segment of the starch block pooled. The solutions were then centrifuged three times to free them from starch grains. Chemical analyses were carried out on each of these eluates.

Color Determination:

The optical density of each of the eluates was measured at 440 mμ in a Beckman Model B Spectrophotometer. A corex 1 cm. cell was used.

Indole Determination of Total Carbohydrate (29):

In this test the sugar was heated with sulfuric acid and indole and the absorption from the resulting brown color determined in a spectrophotometer at 470 mμ. According to Dische, all saccharides produce a brown color in this reaction with the single exception of amino sugars. A galactose solution was used for a standard and the data reported in terms of equivalent galactose.

To a volume of 0.1 ml. of each eluate was added 0.4 ml. of water and 5.0 ml. of diluted sulfuric acid (3 volumes of acid mixed with one volume of water). The tubes were cooled in an ice bath while the sulfuric acid was added and the solutions mixed. Then a 0.2 ml. volume of a 1.0% solution of indole in ethanol(95%) was added to each tube and the solutions mixed. The tubes were heated in a boiling water bath for 10 minutes, cooled and the optical density of each solution determined in a Beckman Model B Spectrophotometer using the same cuvette for each sample. One ml. volumes of water were carried through the procedure for reagent blanks. Standards containing varying amounts of galactose (10 to 100γ) were also included. The data for the standards and blanks were plotted and the sugar content of the eluate samples estimated from the standard curve. The data are plotted in terms of equivalent galactose.

Carbazole Determination of Uronic Acid (29):

The uronic acid contents of one ml. volumes of the

eluates were estimated by the carbazole method as described on page 91 . The optical densities of the solutions were determined at 535 mμ in the Beckman Model B Spectrophotometer. The data are plotted in terms of equivalent glucuronolactone.

Nitrogen Determination:

Two ml. volumes of the eluate solutions were placed in 12 mm. X 100 mm. Pyrex test tubes and the water removed by storage in a vacuum desiccator over calcium chloride for three or four days. To each tube 0.14 ml. of sulfuric acid solution (prepared by diluting 1.2 volumes of water with 1 volume of concentrated sulfuric acid) was added and the tubes heated over a Fisher burner for 30 to 45 minutes. During the last twenty minutes of the digestion, the tubes were heated strongly so that condensation of acid occurred over two thirds the height of the tube. After the tubes had been allowed to cool, one drop of 30% hydrogen peroxide was added to each tube and the tubes heated for another fifteen minutes. If the samples were not colorless after one peroxide treatment another drop was added and they were heated again for fifteen minutes. When the tubes were cool, a 50 ml. volume of water was added to each tube and a 2.0 ml. volume of Nessler's solution. The solutions were mixed and after forty-five minutes the optical density, in a single cuvette, was determined at 440 mμ. Four reagent blanks and duplicate standards (of 10, 20, 40 and 60γ of nitrogen as ammonium sulfate) were carried through each determination. The

nitrogen content of each tube was determined by reference to a standard curve obtained by plotting the data for the standards and reagent blanks.

Starch Electrophoresis Experiment I:

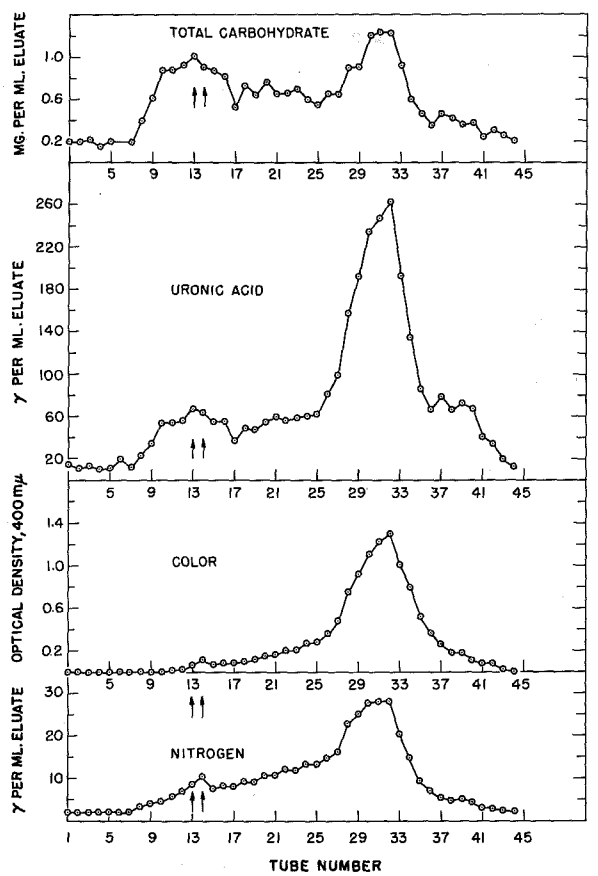
The first starch electrophoresis experiment was carried out using acetate buffer pH 4.7, μ = 0.1.
The buffer was prepared as follows:

glacial acetic acid	38 ml.
sodium acetate trihydrate	108.8 gm.
water to make	8.0 liters

The sample was 5.0 ml. of a 4.6% distilled water solution of crude dust extract #110. It was placed in the starch block 13 to 14 cm. from the left end. The current (300 volts, 70 mamps.) was allowed to pass through the starch block for 16 2/3 hours. The eluates were prepared and analyzed as described above. Curves showing the distribution of total carbohydrate, uronic acid, color and nitrogen along the starch block are presented in Fig. 46. The tube numbers plotted correspond to the distance along the starch block in cm. The arrows indicate the initial position of the sample.

A comparison of the shapes of the four curves indicates that the faster moving material has a higher uronic acid content and demonstrates a good correlation between the color and the nitrogen content. There is no evidence for the separation of a nitrogen rich moiety from the bulk of the polysaccharide. The nitrogen distribution follows that of

Fig. 46



Starch Zone Electrophoresis Experiment I

Crude Dust Fraction, #110

Acetate Buffer pH 4.7, $\mu = 0.1$

the uronic acid more closely than that of total carbohydrate. The uronic acid determination was carried out without internal standards (see p.92 and Fig. 39) to correct for absorption due to the reaction of the other saccharides with the sulfuric acid. This is an error that was corrected in subsequent starch electrophoresis runs. The uronic acid data from the second starch electrophoresis experiment were plotted both with and without the correction from the internal standard to demonstrate that the general shape of the curve was unaffected by the correction (Fig. 47)

Starch Electrophoresis Experiment II:

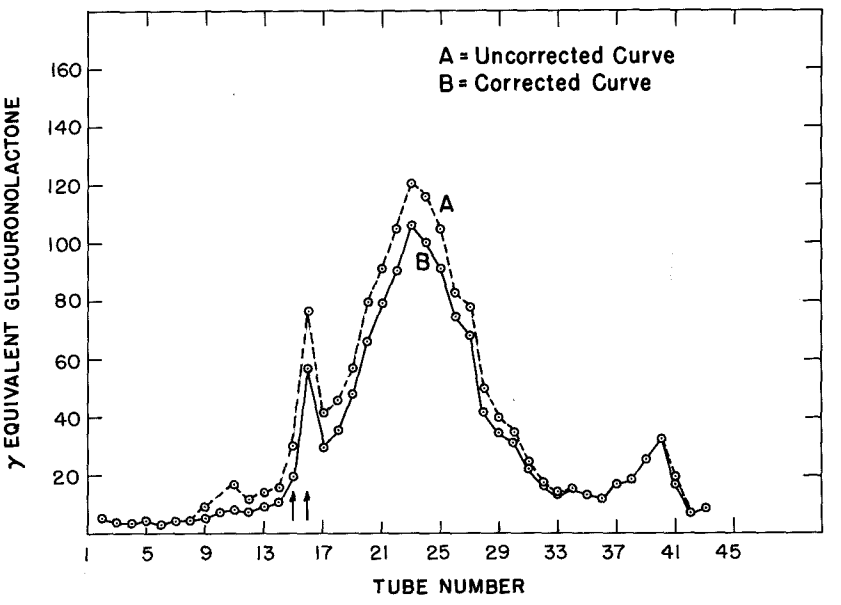
This experiment was carried out following the same procedure as in the previous experiment except that a more acid buffer was used. If the link between the protein moiety and the polysaccharide were a simple ionic bond, possibly the lower pH might break the attachment and permit the separation of the polypeptide or protein from the polysaccharide by electrophoresis.

The buffer was prepared as follows:

potassium hydrogen phthalate	102.1 gm.
potassium chloride	22.33 gm.
concentrated hydrochloric acid	30.0 ml.
water to make a final volume	8.0 liters

After the buffer solution had been mixed it was stored in the cold room (4°C.) and filtered before use. This buffer solution had a final pH of 2.7 (24°C.) and ionic strength of 0.1. The

Fig. 47



Comparison of Uronic Acid Estimate With and Without the Internal Standard Correction for Starch Electrophoresis Experiment II.

sample of crude dust extract in distilled water (200 mg./5.0 ml.) was placed 15 to 16 cm. from the cathode end of the block and the current (300 volts and 100 to 150 mamps.) allowed to pass through the starch block for 16 2/3 hours. The eluates were prepared and analyzed as described above. The resulting data are plotted in Fig. 48. The results are about the same as those from the first experiment in acetate buffer except that at the lower pH the dust extract did not move as far. Again the uronic acid is concentrated in the right end (anode end) of the total carbohydrate distribution; however the nitrogen peak is now at the starting position rather than with the uronic acid peak. This represents some separation of materials high in uronic acid from those high in nitrogen. The color and nitrogen are fairly well correlated as before; however the nitrogen distribution curve extends further to the left relative to the color curve than in the case of the acetate experiment.

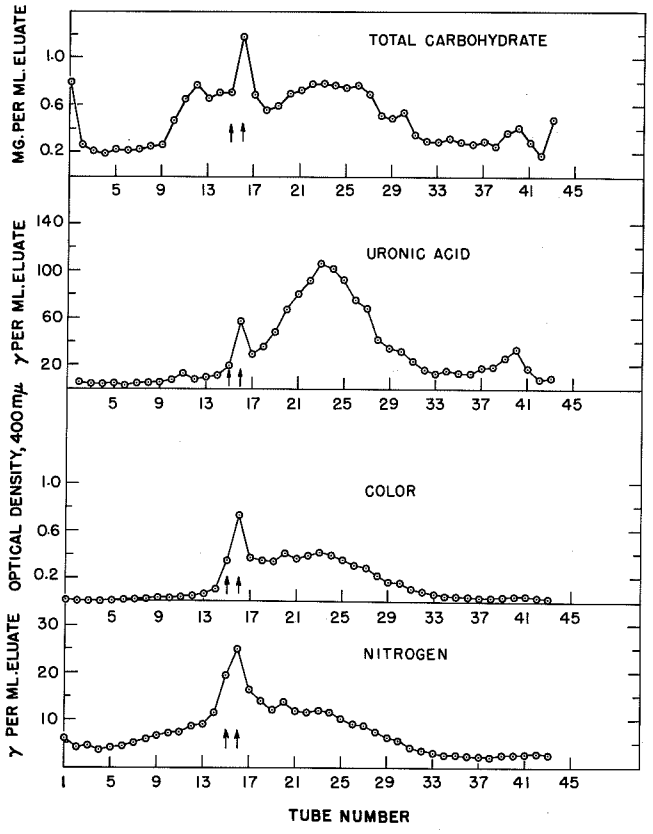
Starch Electrophoresis Experiment III:

In this experiment the crude dust extract was run at even lower pH than the previous experiment. A solution of hydrochloric acid and potassium chloride was prepared as follows:

concentrated hydrochloric acid	16.5 ml.
potassium chloride	44.7 gm.
water to make a final volume of	8.0 liters

The final pH was 1.8 and ionic strength 0.1. A solution of crude dust extract #110 (150 mgm./5 ml.) was dialyzed against

Fig. 48



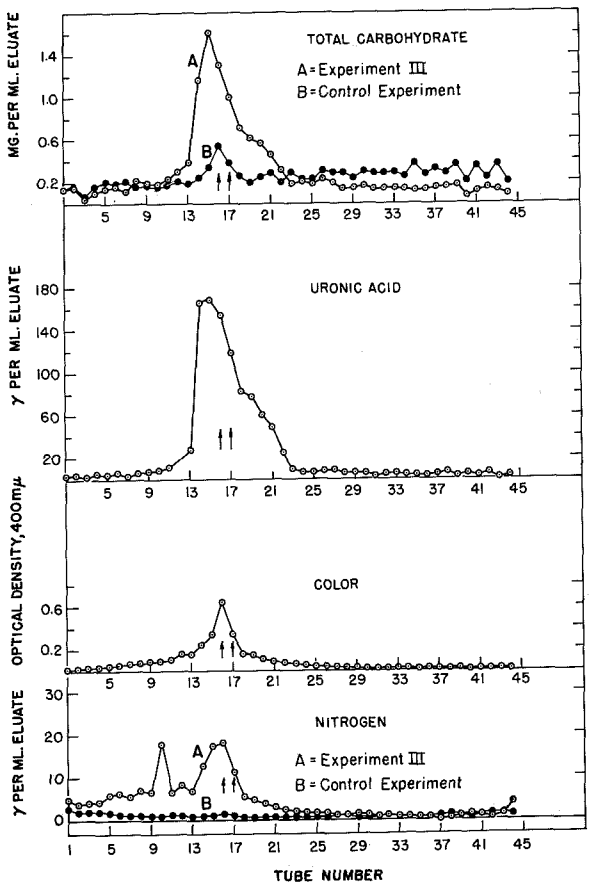
Starch Zone Electrophoresis Experiment II

Crude Dust Fraction, #110

Phthalate Buffer pH 2.7, μ = 0.1

the potassium chloride hydrochloric acid mixture. A small amount of the dust material was insoluble under these conditions; therefore the sample was centrifuged and the supernatant used for the starch experiment. The pH of the dialyzed dust solution was 1.9. The sample was placed 15 to 16 cm. from the left end (cathode end) of the starch block. A current of about 200 mamps. and 300 volts (constant voltage) was passed through the starch block for 3 1/3 hours and the current was then reduced to about 170 mamps. and 200 volts for 13½ hours. The eluates were prepared and analyzed as previously described. The data for the experiment are presented in Fig. 49. A control starch run was carried out identical to this one except that a 5.0 ml. volume of distilled water was used instead of the dust sample. The eluates from the control starch electrophoresis experiment were analyzed for nitrogen and total carbohydrate to provide a suitable base line to aid in the interpretation of the data from the dust starch experiment. The data for experiment III as well as the control experiment are plotted in Fig. 49. Under the conditions of this experiment there is very little movement of the dust extract. The uronic acid and total carbohydrate distributions are essentially identical. All the peaks fall at the same position. The nitrogen peak at tube 10 is probably an error. Aside from this tube, there is a shift to the left in the nitrogen distribution further than that found in the previous experiment with phthalate buffer at pH 2.7. This is a relative separation of

Fig. 49



Starch Zone Electrophoresis Experiment III

Crude Dust Fraction, #110

KCl, HCl Solution pH 1.8-1.9, μ = 0.1

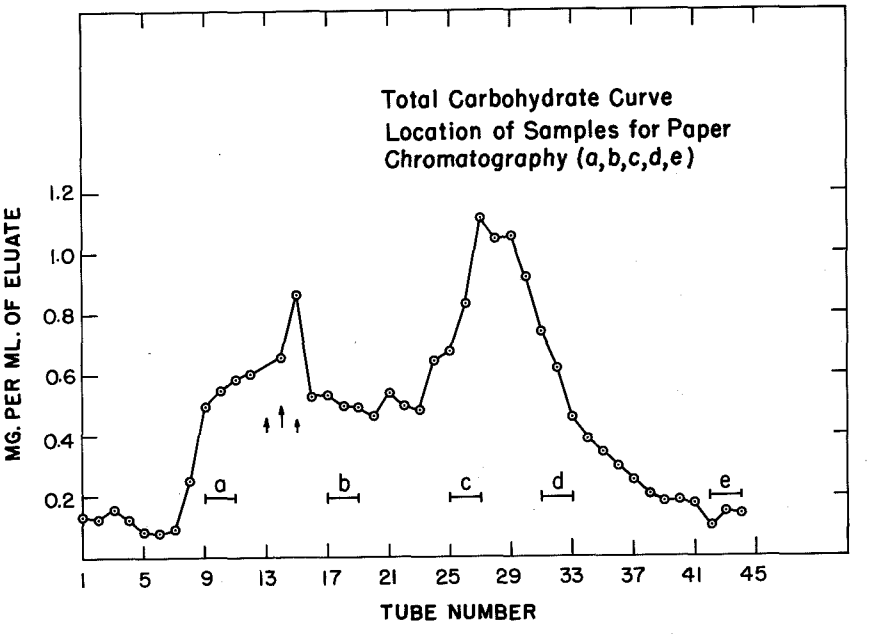
materials high in nitrogen content from those high in uronic acid. A good deal of the nitrogen remains in line with the other peaks and indicates the presence of materials whose uronic acid nitrogen ratio are roughly the same at the three different pH's.

The experiments at pH 2.7 and 1.9 suggest that some of the nitrogen may be separated from the main carbohydrate component under the conditions of these experiments; however a great deal of the nitrogen remains bound with the polysaccharide, especially the polysaccharide with the higher uronic acid content. The distributions obtained for total carbohydrate and uronic acid at pH 4.7 and 2.7 demonstrate a considerable variation in the uronic acid contents of the polysaccharide present.

Starch Electrophoresis Experiment IV:

Having found a marked heterogeniety as to uronic acid content among the polysaccharides separated by starch electrophoresis, it was decided to look for differences in other sugar residues. A starch electrophoresis experiment was carried out with crude dust extract #110 (200 mg./5 ml.) and acetate buffer exactly as described in experiment I. The eluates were obtained and analyzed for total carbohydrate by the indole reaction as usual. The curve obtained (Fig. 50) was very similar to that of experiment I and indicated that the method of fractionation is fairly reproducible. This is in agreement with the general experience

Fig. 50



Starch Zone Electrophoresis Experiment IV

Crude Dust Fraction, #110

Acetate Buffer pH 4.7, μ = 0.1

Location of Samples Taken for Paper Chromatography Indicated.

of this laboratory in serum fractionation by starch electrophoresis.

Groups of three eluates from five different regions of the starch block were pooled as follows:

Sample	Tubes Pooled
a	9, 10, 11
b	17, 18, 19
c	25, 26, 27
d	31, 32, 33
e	42, 43, 44

The samples pooled are indicated on the total carbohydrate plot (Fig. 50). Sample e was included in order to have a control to estimate the contribution of glucose from starch on the final chromatogram. The samples (each about 17 ml.) were made 0.5 N in sulfuric acid by the addition of 6 N sulfuric acid (approximately 1.6 ml.) and boiled under reflux for three hours. The samples were neutralized with barium carbonate, after centrifugation to remove the barium sulfate and excess barium carbonate, the samples were concentrated to dryness and the residues extracted with a small volume (approximately 0.3 ml.) of pyridine for 10 minutes at 95 to 98°C. The samples were centrifuged and a chromatogram was spotted with the pyridine supernatants. Each spot received four applications of approximately 5λ each of the pyridine supernatant. A mixture of known sugars (glucose, galactose, mannose, arabinose and rhamnose) was applied to

each of two spots. The pyridine extraction procedure was based on the method of Malpress and Morrison (22) for separating sugars from electrolytes for paper chromatography. In other cases it has not been necessary to use any procedure to remove electrolytes; however due to the large amount of sodium acetate present in the samples from the buffer it was necessary in this case.

A more detailed discussion of the methods of hydrolysis and paper chromatography used has been previously presented on page 80. The chromatogram was developed in the pyridine, butanol, water solvent and sprayed with the p-anisidine hydrochloride reagent to locate the spots. The result is as follows:

Sample	Sugars present
a	mainly glucose
	some rhamnose
b	mainly glucose
	some rhamnose
	trace arabinose and mannose
c	glucose
	galactose
	rhamnose
	arabinose
	trace mannose
d	glucose
	galactose

mannose

rhamnose

arabinose (less than in sample c)

e faint glucose spot

The chromatogram indicates considerable qualitative difference in the sugar residues obtained from different parts of the starch block. The last material, with the most uronic acid, has at least five different kinds of sugars present in addition to the uronic acid, while the slow material has only glucose and rhamnose with the uronic acid.

No experiments have been performed to determine the distribution of skin reactivity among the eluates from the starch blocks. Dr. Don Silver carried out an analogous experiment (at Caltech) using paper electrophoresis and a partially purified dust extract. The dust fraction used in this experiment was prepared as follows:

(1) An aqueous extract of mattress dust was centrifuged and the supernatant concentrated by vacuum distillation.

(2) The concentrated solution was saturated with ammonium sulfate and the precipitate removed by filtration (at room temperature). The filtrate was dialyzed free of ammonium sulfate and concentrated.

(3) The dust fraction was precipitated with 3 volumes of acetone (with added sodium chloride to aid precipitation), redissolved in water and repre-

cipitated. A water solution of this material was dialyzed and concentrated by pervaporation and a small sample then used for the paper electrophoresis experiment.

Duplicate paper strips were run. One was cut up in one cm. segments and eluted with water and an orcinol test for carbohydrate carried out on each eluate. The other strip was cut up in corresponding one cm. segments and used directly in scratch tests on a sensitive patient's skin. The results are presented in Fig. 51. On the basis of this experiment, it would appear that the skin activity is associated with both the fast and slow components. It should be pointed out that this result is based on one experiment involving one dust sensitive patient; however it is in agreement with the experiments of Rimington (p. 5) in which he found the activity to be about equally distributed between the slow and fast components (isolated from a free boundary electrophoresis experiment).

Fig. 51

Paper Electrophoresis: 25 mg. of partially purified dust extract in acetate buffer pH 4.1, μ = 0.1, 5 hours at 500 volts and 15 mamps. Sample applied at 1 cm. on the strip

Cm. along strip	H_2SO_4 orcinol reaction	Scratch test	Control (glycerol and saline)	Color along strip
1	±	0	0	+
2	±	0	0	0
3	0	0	0	0
4	0	0	0	0
5	0	0	0	0
6	+	2+	0	0
7	0	0	0	0
8	0	0	0	0
9	0	0	0	0
10	+	3+	0	0
11	+	2+	0	+
12	+	0	0	+
13	±	0	0	+
14	±	0	0	+
15	0	0	0	+
16	0	0	0	+
17	0	0	0	0
18	0	0	0	0
19	0	0	0	0
20	0	0	0	0

Part C: Isolation and Characterization of a Purified Polysaccharide Fraction

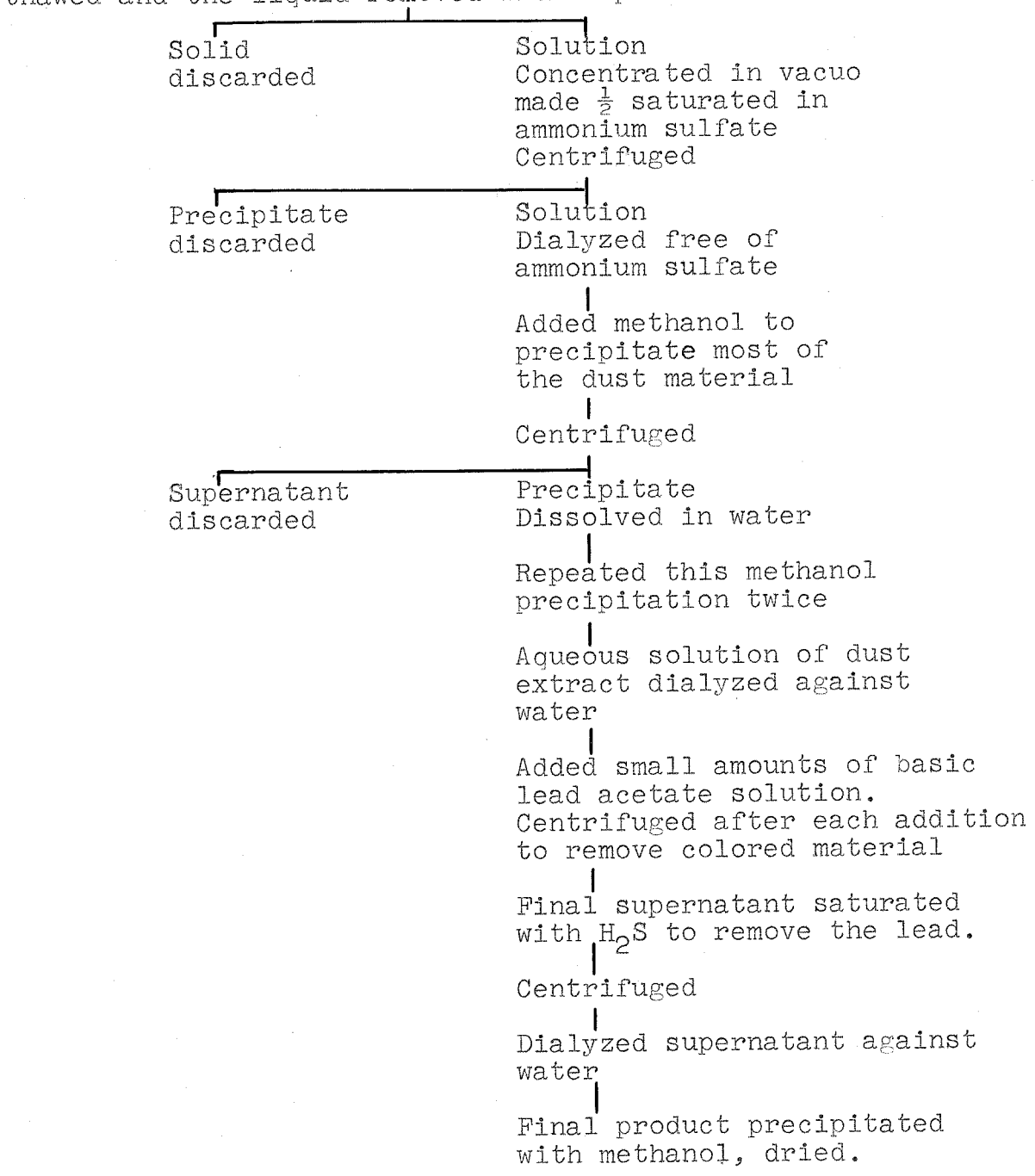
One of the key questions in the study of the dust allergen problem is whether the biological activity is associated with a protein or polypeptide component or with a polysaccharide. If the allergen is a complex molecule containing both polypeptide and polysaccharide components, then the problem is to determine whether both are required for the allergen activity and whether the specificity is associated with the polysaccharide or polypeptide portion. In order to study this problem and to obtain a less heterogeneous dust allergen fraction an attempt was made to obtain materials with as low nitrogen contents as possible.

1. Isolation and Clinical Evaluation of a Purified Dust Fraction.

A fractionation scheme was devised by Dr. Dan Campbell that yielded materials low in both nitrogen and color. The procedure is outlined in Fig. 52. An aqueous extract of dust was fractionated at half saturation with ammonium sulfate. The material soluble at this salt concentration was dialyzed free of ammonium sulfate and precipitated three times with methanol. An aqueous solution of this material was dialyzed against water and colored material removed by fractional precipitation with basic lead acetate solution (18). The colored components were precipitated first on the addition of the basic lead acetate solution. The lead

Fig. 52

Basic Lead Acetate Fractionation



was removed from the decolorized fraction with hydrogen sulfide and centrifugation. The supernatant was dialyzed against distilled water and the final product obtained by methanol precipitation. The precipitate was dried and stored in a vacuum desiccator over calcium chloride. Two dust fractions (I and II) were obtained by Dr. Campbell using this method. The prime difference in the preparation of the two products was that more colored material was removed from fraction I by the addition of basic lead acetate than in the case of fraction II. Fraction I was almost white, while fraction II was light tan in color.

The Hollister-Stier Laboratories carried out a series of skin tests on house dust sensitive patients to compare the reactivity of the lead acetate purified fraction I with two commercial preparations. The data are presented in Fig. 53. 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. Many of the patients gave impressive reactions to all three preparations. Therefore purified fraction I does exhibit good allergen skin reactivity in many of the patients. There were some individuals that reacted to the commercial preparations that did not react to the purified material. This could be due to the particular relationship between the concentrations of materials of the same allergen specificity, in the three preparations, and the patient's threshold of skin reactivity.

Fig. 53

Skin Tests Reported by Hollister-Stier Laboratories

	Preparation[1] and test result[2]				
	Endo	Caltech Dust		H-S Dust	
Patient	1/1000	1/500	1/1000	1/500	1/1000
L	3	1	1	1	1
CD	2	1	1	2	1
FB	3	3	2	3	3
S	2	2	1	2	1
Y	2	1	2	1	1
MW	3	2	1	2	?
F	4	4	?	3	?
Y	2	3	0	1	0
DM	3	2	2	2	0
K	1	0	0	1	0
H	4	3	2	3	1
HRS	3	2	1	1	0
DH	3	1	0	0	0
DL	4	?	0	4	3
G	1	1	0	1	?
A	2	2	1	2	2

[1] The dust preparations were the following:

Endo, House Dust Concentrate, Endo Laboratories Inc.
Caltech Dust, Lead Acetate Purified Material I
H-S Dust, Hollister-Stier Dust Extract.

[2] The skin reactions were graded 1 through 4. The symbol ? indicates a questionable reaction and 0 indicates a negative reaction.

Another possible explanation is that more than one allergen specificity is involved and that there are materials with allergen specificities in the crude commercial preparations that are not present in our more purified material. The patients were probably selected because they gave good reactions to the commercial preparations, so it is not known whether the patients that react to the crude preparations but not to the more purified materials are indeed sensitive to the specific dust allergen(s).

2. Physical Properties of the Purified Fractions I and II.

The allergen fractions I and II were amorphous white and tan powders, both readily soluble in water to produce clear solutions. The solution was essentially colorless in the case of fraction I and of a slightly brownish color in the case of fraction II. A determination of the ash content of fraction II was carried out by Dr. Campbell and found to be 4%. The ash content of other fractions that had been electrodialyzed was essentially zero.

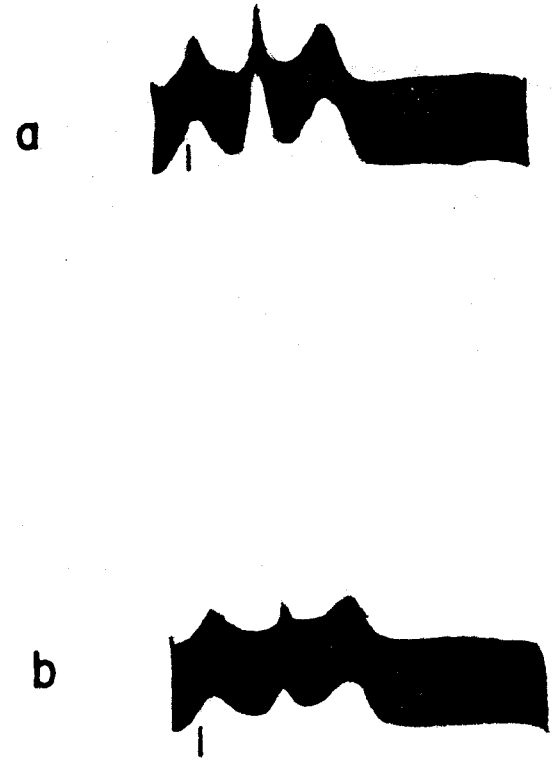
The optical rotation of a 1.0% solution of fraction I in water was determined ($[\alpha]_D^{26^\circ} = +63^\circ$). This result was subject to considerable error due to the small amount of material available for the determination.

An electrophoretic analysis of the two materials was carried out in the Perkin-Elmer Electrophoresis Apparatus as described on page 51 . The preparation of the acetate buffer (pH 4.7, $\mu = 0.1$) used is given on page 122. Repro-

ductions of the patterns obtained are presented in Fig. 54. The patterns are quite similar for the two fractions. The fast peak in both samples has a mobility of -9×10^{-5} ($cm.^2\ sec.^{-1}\ volts^{-1}$). In the case of the fraction I sample the mobility of the slower peak was -4.7×10^{-5} and in the case of the fraction II material the mobility was -5.1×10^{-5}. On the basis of these single determinations and the errors involved in the method it is not certain that these slower peaks have different mobilities. Area analysis indicates that about 60% of the material is present in the fast peak (neglecting the ϵ boundary). The possibility that the stationary boundary contains uncharged or slightly charged dust material has been discussed on page 58.

Sedimentation analyses were carried out following the procedure described for the crude dust fractions (p. 61). Solutions (approximately 2%) of the purified materials in acetate buffer (pH 4.7, $\mu = 0.1$) were prepared and dialyzed for two days against the buffer. The concentrations were then determined by the indole method for total carbohydrate. The other solutions were obtained by dilution with buffer. One of the sedimentation patterns obtained for each of the materials is presented in Fig. 55. Each pattern shows a single fairly symmetrical peak. The sedimentation coefficients for the four concentrations are tabulated in Fig. 56 and plotted in Fig. 57 and Fig. 58. The $S^{\circ}_{w,20}$ values were calculated as before. The fraction I material had the

Fig. 54



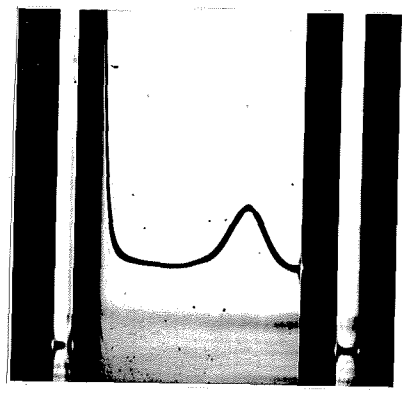
Electrophoretic Patterns of the Purified Fractions

Acetate Buffer, pH 4.7, μ = 0.1

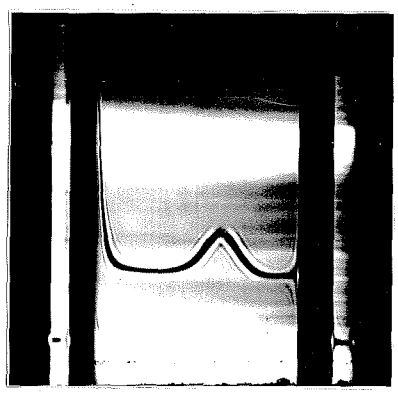
a. Purified Fraction I, conc. 1%, 3000 sec.

b. Purified Fraction II, conc. 0.8%, 4100 sec.

Fig. 55



a



b

Sedimentation Patterns, Acetate Buffer, pH 4.7, μ = 0.1

a. Purified Fraction I, conc. 1%, 48 min.

b. Purified Fraction II, conc. 1%, 48 min.

Fig. 56

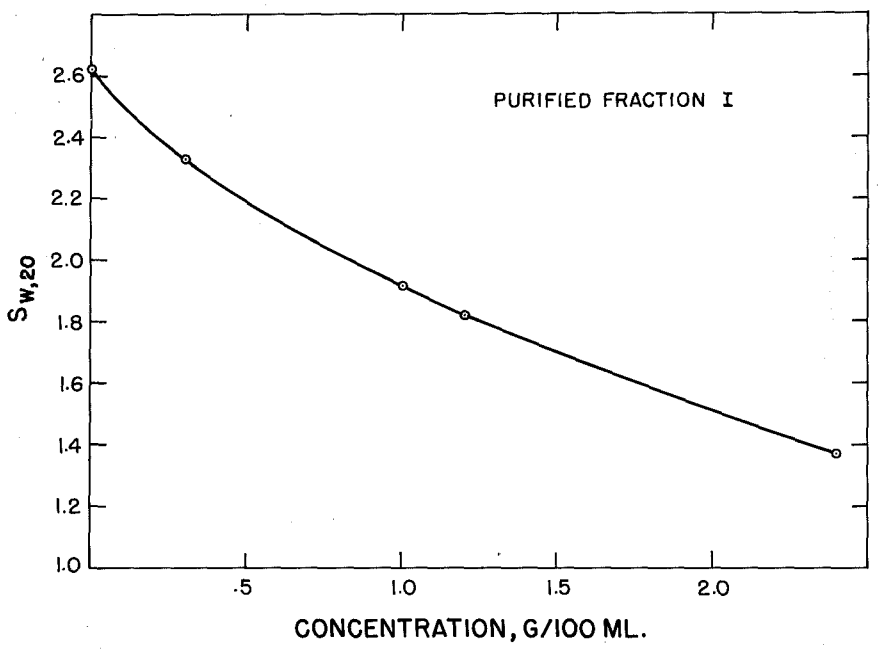
Sedimentation Coefficients of the Purified Fractions

Acetate Buffer pH 4.7, μ = 0.1

Fraction	Concentration, %		$S^{\circ}_{w,20}$
I	2.4		1.37
"	1.2		1.82
"	0.6		3.39*
"	0.3		2.33
		$S^{\circ}_{w,20}$ =	2.62
I	1.0		1.92
II	2.8		1.76
"	1.4		1.79
"	0.7		1.95
"	0.35		2.02
		$S^{\circ}_{w,20}$ =	2.12

* High value probably caused by a leak in the cell.

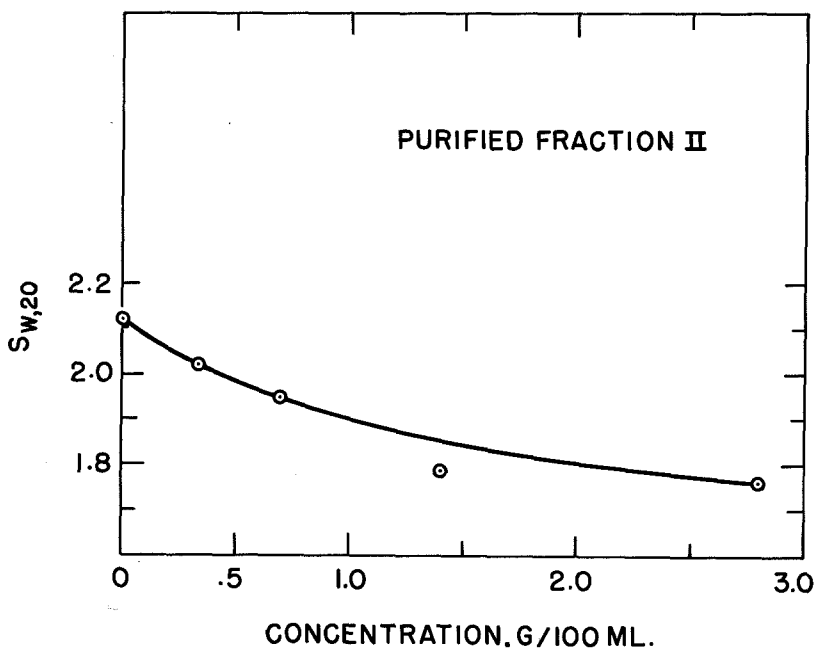
Fig. 57



Purified Fraction I

Concentration Dependence of Sw,20 with Extrapolation to $S^{o}_{w,20}$.

Fig. 58



Purified Fraction II

Concentration Dependence of Sw,20 with Extrapolation to $\mathring{S}_{w,20}$.

higher value ($S^{\circ}_{w,20}$ = 2.62) and showed more concentration dependence than fraction II ($S^{\circ}_{w,20}$ = 2.12).

3. Chemical Composition of the Purified Fractions I and II.

Nitrogen Analysis:

The nitrogen contents of the purified fractions were determined by digestion and Nesslerization of small carefully weighed samples (~15 mg.). The fractions had been stored under vacuum in a desiccator with calcium chloride for many months and were presumed dry. The procedure for the digestion, Nesslerization and colorimetric estimation has been described on page 121. Both of the purified fractions had a nitrogen content of 0.43%.

Hexosamine Estimation:

Weighed samples of the purified fractions (~12 mg.) were hydrolyzed in sealed tubes in an oven (~105°C.) with 3.0 ml. of 0.5 N sulfuric acid for eight hours. The hexosamine contents of the hydrolysates were determined following the Elson-Morgan procedure described on page 108.

The hexosamine determinations were carried out on duplicate aliquots of each hydrolysate. One of the set of duplicates was analyzed as previously described (using 2% acetylacetone) while 5% acetylacetone was used in the analysis of the other set of duplicates. The false positive hexosamine reaction, due to the presence of amino acids and sugars, makes much less of a contribution at the higher con-

centration of acetylacetone (37).

The fraction I sample yielded a value not significantly different from zero at both acetylacetone concentrations. A value of about 1% was obtained for the equivalent glucosamine content of the fraction II sample. This result was obtained at both the higher and lower acetylacetone concentrations indicating that this was not a false positive reaction.

Titration of a Sample of Purified Fraction II:

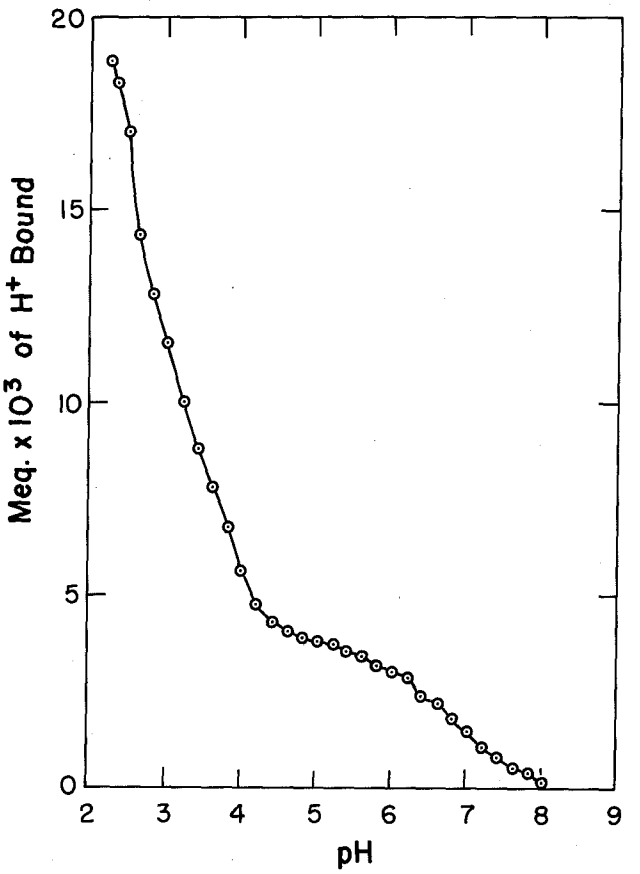
A carefully weighed sample of the purified dust fraction II (11.1 mg.) was dissolved in 5.0 ml. of CO_2 free distilled water. The pH of the solution was measured with a Beckman Model G pH Meter and found to be 5.3$\frac{7}{}$. A small volume (0.15 ml.) of 0.0212 N sodium hydroxide solution was added to bring the pH to 8.1$\frac{5}{}$. Small volumes of a standard hydrochloric acid solution (0.021 N) were added from a calibrated 2 ml. buret and pH readings made after each addition. A total of thirty-five additions and readings were made. From pH 2.3 to lower values a 0.21 N standard hydrochloric acid solution was used so that the volume would not become too large. A blank titration was carried out with 5.0 ml. of CO_2 free distilled water in the same manner as in the case of the dust fraction. The final volume in the dust titration was 8.0 ml. while the volume for the blank was 7.9 ml. Curves showing the relation between the pH and the millequivalents of H^+ 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 given in Fig. 59. The shape of the curve is consistent with the idea that carboxyl groups are being titrated between pH 2.0 and 5.5. If we extrapolate the curve to pH 2 and 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 690. If the material were a polyhexose, one out of four of the residues would have a carboxyl group. The uronic acid color reactions carried out with crude dust material (p. 103) indicated that about one fifth of the material was uronic acid. This is good agreement considering the errors involved in the uronic acid experiment and that different dust fractions were used in the two experiments. It should be pointed out that free boundary electrophoretic analysis of these materials indicates gross heterogeneity with respect to charge density and that the titration and uronic acid results are average values over this heterogeneous population of materials.

Paper Chromatography of Hydrolysates of the Purified Dust Fractions:

In the initial stages of this research project, a variety of experiments were carried out to determine the best

Fig. 59



Titration Curve of a Sample (11.1 mg.)

of the Purified Fraction II

conditions for the hydrolysis of the dust extracts. Various concentrations of aqueous sulfuric and hydrochloric acids were used at different temperatures and for varying times. In all cases, the material was either incompletely hydrolyzed or it was decomposed, yielding a dark brown solution, sometimes with a brownish black precipitate. Experiments were carried out using a sulfonated polystyrene resin (Amberlite I.R. 120) with varying amounts of added aqueous sulfuric acid. The addition of resins such as this has been reported to increase the rate of acid hydrolysis of starch and glycogen (39). Samples of purified fraction II were heated in solutions of sulfuric acid in methanol to degrade the material, followed by a short period of hydrolysis in dilute aqueous sulfuric acid to convert the methyl glycoside to free sugars. The dried dust material was initially insoluble in the methanol sulfuric acid solution; however after an hour or so of heating, almost all of it had gone into solution. Both of these procedures were an improvement over hydrolysis with aqueous acid solutions alone; however even with these methods some decomposition of the material occurred.

Soon after these studies were undertaken, it became evident that the main problem was due to the decarboxylation and decomposition of the uronic acid components. Uronic acids are very sensitive to decarboxylation in acid solution and the quantitative degradation of these materials to monosaccharide residues would be extremely difficult,

if possible at all, unless an enzyme were found that would accomplish this. Therefore it was decided to neglect the decomposition problem and to proceed with the hydrolysis of the materials with aqueous sulfuric acid solutions (0.5 N) and determine what sugars could be isolated and characterized from these hydrolysates. It was hoped that other degradation experiments could be carried out under conditions more favorable for the isolation of uronic acids and that the uronic acid components could then be chemically characterized. The most promising procedure to achieve this result would seem to involve heating the dried dust fraction with 90% formic acid. Studies have been carried out with alginic acid under these conditions and it was stated that decarboxylation did not occur (40). A 45% yield of mannuronolactone was isolated from the hydrolysis mixture. Such an experiment has not yet been carried out with any of the dust extract fractions.

Five mg. samples of both of the purified dust fractions were hydrolyzed by refluxing in 2.0 ml. volumes of 0.5 N sulfuric acid for two and ten hours. The samples were prepared and chromatographed as described in the section on the paper chromatographic studies of the crude dust fractions (p. 80). The butanol-pyridine-water and the phenol-water solvents were used for development and the sodium 3,5-dinitrosalicylate and p-ansidinehydrochloride sprays were used to locate the spots. The time of hydrolysis did not make any difference in the appearance of the chromatograms. The patterns of sugar spots with the two purified dust fractions

were identical except that the fraction II material showed a spot that behaved like lyxose in the two solvents. Both of the fractions had spots corresponding to glucose, galactose, mannose, arabinose and rhamnose. There was no evidence for the presence of fucose in either of the purified fractions. Samples of the fraction I material were hydrolyzed in 0.5 N sulfuric acid for 51 and 114 hours. These hydrolysates were chromatographed following the same procedure and yielded patterns almost identical to those obtained after the shorter periods of hydrolysis.

As in the case of the paper chromatographic study of the crude dust fractions, a series of comparison chromatograms in a number of different solvents was prepared. A sample of purified fraction I (12 mg.) in 18 ml. of 0.5 N sulfuric acid was hydrolyzed by refluxing for ten hours. The hydrolysate was neutralized and prepared for chromatography as before. A solution of known sugars was prepared to correspond to the tentative identification and relative amounts of materials found on the previous chromatograms. The solution was as follows:

glucose	10 mg.
galactose	10 mg.
mannose	10 mg.
rhamnose	5 mg.
arabinose	2.5 mg.
lyxose	2.5 mg.
water	1.0 ml.

The lyxose had only been found in the fraction II hydrolysate; however it was included. Chromatograms were prepared with duplicate hydrolysate and known sugar mixture spots and

developed in the four solvent systems previously used: (1) butanol-pyridine-water, (2) phenol-water, (3) butanol-water and (4) pentanol-propanol-water. Single runs were made with the first two of these solvents, while triple runs were made with the last two solvent systems in the same manner as previously described. The hydrolysate was also compared with the known sugar mixture in another solvent system prepared as follows:

ethyl acetate	100 ml.
water	60 ml.
acetic acid	50 ml.

The sugar spots are fairly mobile in this solvent; therefore, only one run was required in this case. The chromatograms were sprayed with the p-anisidine hydrochloride solution. The positions of the spots were measured in the case of the solvent systems that required only one run. The other two solvents that required triple developing runs could not be used for Rf measurements. The Rf data are tabulated in Fig. 60. Tracings from the chromatograms are reproduced in Fig. 61. The data support our initial tentative identification of the sugars found in the purified fraction I hydrolysate (i.e. glucose, galactose, mannose, arabinose and rhamnose).

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. Glucose, galactose, mannose, glucuronolactone and the galacturonic acid were individually carried through the hydrolysis and chromatography procedure. The hexoses were examined

Fig. 60

Chromatography of Purified Fraction I

Pyridine-Butanol-Water Solvent

Sugar	Rf (known sugar)	Rf (hydrolysate)
galactose	0.30	0.29
glucose	0.35	0.33
mannose	0.40	0.39
arabinose	0.38	0.36
rhamnose	0.54	0.52

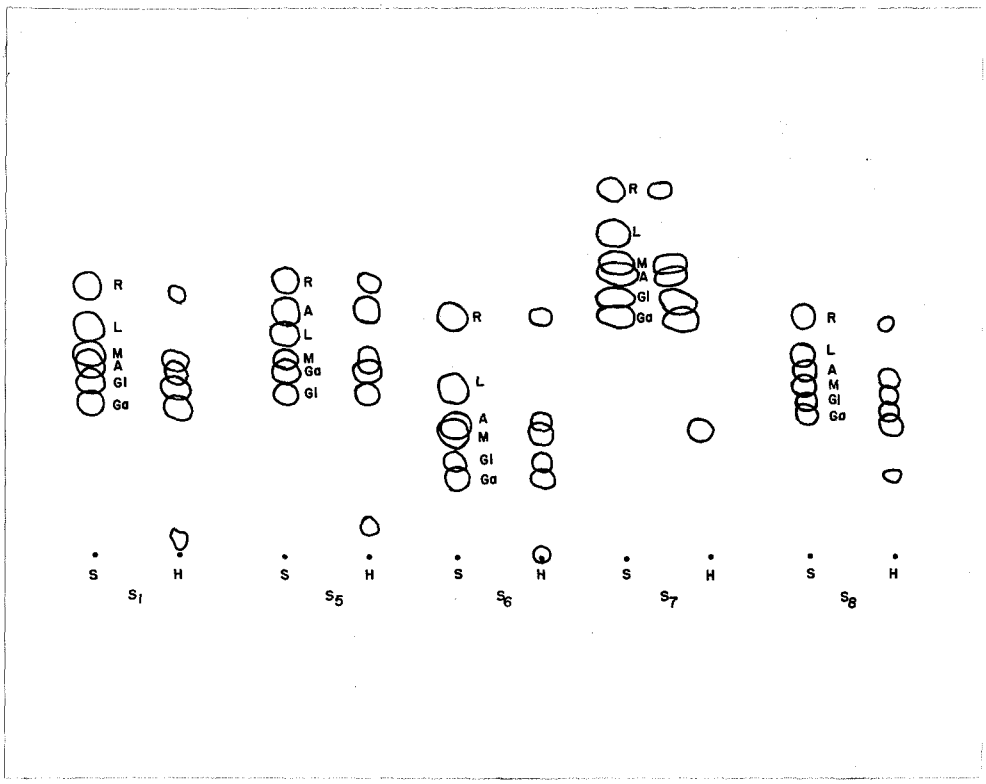
Phenol-Water Solvent

Sugar	Rf (known sugar)	Rf (hydrolysate)
glucose	0.31	0.32
galactose	0.35	0.36
mannose	0.38	0.39
arabinose	0.47	0.48
rhamnose	0.53	0.54

Ethyl Acetate-Acetic Acid-Water Solvent

Sugar	Rf (known sugar)	Rf (hydrolysate)
galactose	0.28	0.26
glucose	0.30	0.29
mannose	0.33	0.32
arabinose	0.36	0.35
rhamnose	0.47	0.46

Fig. 61



Comparison Chromatography: Purified Fraction I Hydrolysate and Known Sugars

S Standard
H Hydrolysate

● Origin

Solvents	Sugar Spots	
S_1 pyridine-butanol-water	Rhamnose	R
S_5 phenol-water	Arabinose	A
S_6 butanol-water	Mannose	M
S_7 pentanol-propanol-water	Glucose	Gl
S_8 ethyl acetate-acetic acid-water	Galactose	Ga

chromatographically and found to be identical to samples not carried through the procedure. Both of the uronic acid samples were destroyed during this method of hydrolysis and sample preparation.

Chromatograms of purified fraction II hydrolysate were prepared and developed in the butanol-pyridine-water solvent. Some were then sprayed with a 0.1% ninhydrin solution (in 95% ethanol) and then heated in an oven for a few minutes at 110°C. Five purple spots were found. Three of these were close to the origin while the other two were at the hexose level. These spots were probably due to the presence of amino acids in the dust hydrolysate. Rimington has studied the amino acid composition of the dust fraction he isolated and his data were presented in the introduction (p. 5). The other chromatograms were sprayed with reagents to detect the presence of hexosamines on paper chromatograms (25). The solutions were prepared as follows:

Acetylacetone Reagent.

Solution 1.

acetylacetone	0.5 ml.
n-butanol	5.0 ml.

Solution 2.

potassium hydroxide aqueous solution 50% (w/v)	5.0 ml.
ethanol (95%)	20 ml.

0.5 ml. of Solution 2 mixed with 10 ml. of Solution 1 just before use.

p-Dimethylaminobenzaldehyde Reagent.

p-dimethylaminobenzaldehyde (recrystallized once from 50% aqueous methanol)	0.1 gm.
ethanol (95%)	3.0 ml.
concentrated hydrochloric acid	3.0 ml.
n-butanol	18.0 ml.

The chromatograms were sprayed with the freshly mixed acetylacetone reagent, heated in the oven at 105°C. for five minutes, sprayed with p-dimethylaminobenzaldehyde reagent and reheated in the oven at 105°C. for five minutes. There appeared a very faint rose colored spot at the point of sample application that extended up the chromatogram to the level of the glucosamine standard spot. This suggests the presence of some partially hydrolyzed materials containing a small amount of hexosamine. The result is consistent with the direct analysis of the fraction II material for hexosamine previously described (p. 148). Hexosamines frequently occur in the more difficultly hydrolyzable portions of a polysaccharide because the amino group becomes protonated and the resulting positive charge slows the rate of hydrolysis. Chromatograms of the fraction I hydrolysates were not sprayed with either the ninhydrin or the hexosamine reagents.

Preparation of Derivatives of Sugars Isolated from House Dust Fractions:

In order to provide more definite evidence for the

identity of the sugars found in the dust allergen fractions, an attempt was made to prepare derivatives of the sugars from the dust fractions and to compare them by melting point and mixed melting point with the same derivatives prepared from authentic samples. As sufficient material was not available to carry out these studies using the purified fractions I and II, it was necessary to use crude dust fractions as the source of the sugars for derivatization. The experiments are presented in connection with the studies involving the purified fractions because they are intended to provide additional evidence to support the chromatographic identification of the sugars present in the purified fractions. The purified fractions consist of a part of the population of materials found in the crude fractions and it seems unlikely that corresponding spots on chromatograms from the two types of material would be due to the presence of different components.

The preparation of a number of different types of sugar derivatives was explored, namely benzimidazoles, osotriazoles, substituted hydrazones and phenylhydrazides. No difficulty was experienced in the preparation of these derivatives on a 50 - 100 mg. scale. On proceeding to a 2 - 3 mg. scale it became most difficult to isolate crystalline products. Preliminary experiments indicated that the substituted hydrazones would be the easiest derivatives to prepare on this scale. The methods used to prepare these derivatives were based on the procedures given by Cheronis

and Entrikin (41) and Van der Haar (42).

Some of the substituted hydrazones 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 hydrolysates involved the use of ascending paper chromatography at elevated temperatures. It had previously been found that milligram amounts of sugars may be separated on a single sheet of filter paper at temperatures of 50 to 60°C. (43).

Fractionation of a Hydrolysate by Paper Chromatography at 50-55°C.:

Approximately 400 mg. of pooled crude dust extract 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 twenty ml. volume of water was added to the methanol solution and the volume 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 x 37 cm. sheet of Whatman No. 1 filter paper. A 3-4 cm. wide pad of six thicknesses of filter paper was stapled at the top of each chromatogram and the sheet folded into a cylinder and stapled. Eight such chromatograms were prepared. They were developed at 50-55°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 chromatogram and the cylinders were then removed from the jars and dried at room temperature before the next run.

Strips, 3 cm. wide, were cut from both edges of each chromatogram and the position of the sugar bands located by use of the sodium 3,5 dinitrosalicylate spray reagent. With the strip from each edge of chromatogram as a guide, the remainder of the chromatogram 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 for four hours with 100 ml. of methanol in a Soxhlet extraction apparatus. Each of the methanol extracts was mixed with 25-30 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 stream of nitrogen to a final volume of 1-2 ml. A small sample of each fraction was then chromatographed in the butanol-pyridine-water solvent following the usual procedure. The sugars present in each fraction, as determined

by the paper chromatograms, are listed in Fig. 62.

The separations achieved are not perfect but certainly a great improvement. It is probable that with better control of the experimental conditions (especially constant and uniform temperature), greatly improved separations could be achieved. Fraction III was the first dust material from which xylose has been obtained. This result was checked by repeating the chromatography in the butanol-pyridine-water solvent as well as using the phenol-water solvent. Known samples of arabinose, ribose, xylose and lyxose were run for comparison. The spot definitely corresponds to xylose and not to ribose or lyxose in these solvents.

Optical rotation measurements were made using the aqueous solutions of the fractions I through VI. The angles were small and difficult to measure accurately and the concentrations of the various sugars were unknown. All the solutions had a + rotation and the data were consistent with the following forms of the sugars identified by paper chromatography i.e. D-glucose, D-galactose, D-mannose, D-xylose, L-arabinose, D-fucose and L-rhamnose. These are all the common forms of those sugars except in the case of the D-fucose which is not frequently found. The sign of rotation of this fraction should be redetermined.

Preparation of Derivatives of Mannose: Mannose phenylhydrazone.

Because of the insolubility of the mannose phenylhydrazone, phenylhydrazine has been used to quantitatively precip-

Fig. 62

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 arabinose
V	glucose galactose
VI	galactose trace of glucose

itate mannose from solutions of sugar mixtures. It was not therefore necessary to use the hydrolysate fractions in order to prepare this derivative. A 0.5 gm. sample of crude dust extract #110 (Fig. 9) was hydrolyzed by refluxing 2 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, concentrated to 7 ml. by vacuum distillation and the concentrate extracted twice with 5 ml. volumes of chloroform to remove any lipid components. Previous experiments had shown that about 2% 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 heat lamp, decolorized by heating with Norite and centrifuged to remove the Norite. A 0.06 ml. volume of 50% 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 and 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 184-186°C. All the melting point determinations have been carried out on a micro hot stage melting point apparatus. A sample of known D-mannose (100 mg.) in 0.4 ml. of water was mixed with 0.06 ml. of 50% aqueous acetic acid and 0.06 ml. of freshly distilled phenylhydrazine. An immediate white precipitate

was formed. After one half hour at room temperature and one hour in an ice bath the precipitate was collected by centrifugation, washed three times with cold water and recrystallized from aqueous ethanol. The product was washed three times with cold water and dried in a vacuum oven at 50°C. (m.p. 184-185°C.). Small amounts of the dust product and the mannose phenylhydrazone were intimately mixed on a microscope cover glass and the mixed melting point found to be 184-185°C., (Lit. m.p. D-mannose phenylhydrazone 199°C. recryst. from 60% alcohol, 190-191°C. recryst. from 90% alcohol).

Mannose p-bromophenylhydrazone.

The mannose phenylhydrazone (approximately 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 0.1-0.2 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 mixed. After two minutes a precipitate formed. The mixture was cooled in an ice bath, the precipitate collected by centrifugation, washed three times with cold water and dried in a vacuum oven at 50°C. (m.p. 198-199°C.). A known sample

of D-mannose (10 mg.) was mixed with 15 mg. of sodium aceta 12.4 mg. of p-bromophenylhydrazine hydrochloride and 0.3 ml. of 50% aqueous alcohol. The product was recovered as in the case of the dust derivative (m.p. 199-200°C.). The mixed melting point was 199-201°C. (Lit. m.p. D-mannose p-bromophenylhydrazone recryst. from 50% alcohol, 208°C.).

Preparation of Derivatives of Galactose:

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 which was removed by centrifugation. The supernatant was heated on a boiling water bath with 3-4 drops of 50% 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 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 a completely normal dust hydrolysate pattern except that mannose was completely absent.

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% aqueous acetic acid

and 0.06 ml. of freshly distilled α-methyl-α-phenylhydrazine were added and after 10 minutes the precipitate that formed was removed by centrifugation. The precipitate was mixed with a few 0.1 ml. volumes of 30% alcohol 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 (m.p. 181-183°C.).

A known sample of D-galactose (10 mg.) was mixed with 0.4 ml. of water, 0.1 ml. 50% aqueous acetic acid and 0.1 ml. of freshly distilled α-methyl-α-phenylhydrazine yielding a white precipitate in one minute. After 10 minutes at room temperature, the precipitate was collected by centrifugation, washed three times with water and recrystallized from ethanol(95%) 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 (m.p. 179-181°C.). The mixed melting point with the dust product was 181-183°C. (Lit. m.p. D-galactose α-methyl-α-phenylhydrazone recryst. from 30% alcohol, 190-191°C.)

The galactose α-methyl-α-phenylhydrazone derived from the dust extract was decomposed by heating with 0.1 ml. Formalin, 0.3 ml. 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 prepared. Only one sugar spot was found and this was at the galactose level.

Galactose o-tolylhydrazone:

An attempt was made to prepare this derivative from the galactose obtained by the decomposition of the dimethyl-d-phenylhydrazone; however this was unsuccessful. Therefore half of the galactose fraction VI (Fig. 62), obtained by paper chromatography at 50-55°C., was used. This material had been shown to be almost all galactose by paper chromatography. The solution was concentrated to approximately 0.2 ml. under a stream of nitrogen and heat lamp and 10 mg. of sodium acetate, 12 mg. of o-tolylhydrazine hydrochloride and 0.3 ml. of 50% aqueous alcohol 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 reducing the volume by about 20% 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%), washed with cold ethanol(95%), and dried (vacuum oven 50°C.). The product melted at 167-169°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 171-172°C. The mixed melting point with the dust product was 170-171°C.

Preparation of a Derivative of Arabinose: Arabinose diphenylhydrazone:

Fraction III (Fig. 62) 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. About one third of this material available was used for this experiment. 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 mgm. of diphenylhydrazine hydrochloride and 0.2 ml. 50% aqueous alcohol were added and the solution heated for 15 minutes in a boiling water bath. The mixture was allowed to cool, the volume reduced by about 20% under a stream of nitrogen and heat lamp, and the sample then cooled in an ice bath. The resulting crystalline product was collected by centrifugation, recrystallized from absolute ethanol and then from ethanol(95%) and dried (m.p. 197.5-199°C.). L-arabinose diphenylhydrazone was prepared from a known sample of L-arabinose by the procedure described above. The known derivative melted at 198.5-199.5°C. The mixed melting point with the dust product was 198.5-200°C. (Lit. m.p. L-arabinose diphenylhydrazone recryst. from 95% ethanol, 204°C.).

Other Derivatives:

Two unsuccessful attempts have been made to obtain derivatives of the rhamnose. These involved the attempted

preparation of the β-napthylhydrazone and the phenylhydrazide. In neither case was any crystalline product obtained. Some difficulty was experienced with these derivatives in the case of experiments with samples of known rhamnose; however after a few trials the known derivatives were prepared. No attempts have been made to prepare glucose derivatives as yet.

Part D: Discussion.

A wide variety of fractionation procedures have been employed in an attempt to separate the water soluble non-dialyzable components of house dust and mattress dust into fractions of differing skin reactivity. It has been found by previous workers (4) that the least soluble fraction that is first precipitated by the addition of organic solvents or ammonium sulfate is less active than the more soluble components. Attempts to separate these more soluble materials into fractions definitely active or inactive for all dust sensitive patients have not been successful. It has been found however that some of the fractions obtained would not react with all the individuals that were sensitive to crude dust extracts and that different fractions would react to different groups of crude dust sensitive patients. It may be concluded therefore that there are materials of differing specificity with regard to skin reactivity in the dust extracts. The major difficulty associated with these experiments is the unreliability of skin tests as an estimate of allergen concentration. Even under the best conditions it is necessary that there be a five fold difference in concentration to be sure of having a distinguishable difference by skin test (44). A more detailed study of the allergen specificities to be found in house dust extracts is a matter of great importance to the study of house dust allergy; it would however, require an elaborate program for the clinical testing of materials. Skin test titrations should be carried to end

points and passively sensitized skin sites should be used.

An allergen may be defined as the substance that induces a specific sensitivity in an individual and subsequently produces an abnormal specific reaction on contact. All our experiments have been concerned with the material that reacts in the skin of a previously sensitized individual rather than the material that induces the sensitization. It may be that the requirements to fulfill the two functions are different. In the case of the precipitin type antigen antibody reaction it has been found that a higher molecular weight, combination with a protein or some other special requirement is frequently necessary for the induction of antibody formation but unnecessary for reaction with the preformed antibody.

One of the central problems is that of whether the specific activity is due to the presence of a polypeptide component, to a polysaccharide alone or is due to a complex molecule containing both moieties. In these studies it has not been possible to obtain an allergen fraction that was free of nitrogen. Purified fraction I contained 0.4% nitrogen while Rimington's active partial hydrolysis product (6) contained 10 % nitrogen and 12% carbohydrate by orcinol determination. In neither case has allergen activity been found to be associated with a carbohydrate or polypeptide component alone. The starch zone electrophoresis studies provide evidence that the carbohydrate and polypeptide components are linked together rather than being present as

separate components. There was some evidence from the starch electrophoresis experiment at pH 1.8 (Experiment III) that a portion of the nitrogen had separated from the carbohydrate peak; however even in this case most of the nitrogen remained associated with the carbohydrate. The various fractionation procedures that have been used have yielded active products with a wide range of nitrogen content. Part of the variation in nitrogen content is a reflection of heterogeneity of the dust components and experimental evidence shows that the variation is partly dependent on the kind of dust used to prepare the extract. Mattress dust in general yields extracts of lower nitrogen content and less intense color than house dust. The fractionation studies based on solubility have indicated that the more soluble components, in general, have the lower nitrogen contents, while the starch electrophoresis studies have indicated that the more rapidly moving components have the higher nitrogen content. The uronic acid content is also highest in the rapidly moving components and is undoubtedly responsible for the high mobility. All the data available at the present time indicates that both the slow and fast components are active by skin test. Starch electrophoretic fractionation of the lead acetate purified materials that are low in nitrogen would seem to offer the best chance to obtain an active material considerably lower in nitrogen content.

Rimington (6) 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 (1-2%) in some of the fractions. Rimington also found a hexosamine by paper chromatography of a hydrolysate of his purified allergen.

Most of the material present in the dust fractions is polysaccharide. By hydrolysis and paper chromatography D-glucose, D-galactose, D-mannose, L-rhamnose and L-arabinose have been identified as the sugars most commonly present and in greatest amount. The configurations are based on rough optical rotation measurements of the eluates from the chromatograms prepared at 50-60°C. Certain fractions, upon hydrolysis, have yielded xylose, lyxose and fucose. The identification of these sugars is based on paper chromatography in a number of different solvents and careful comparison with known samples, except in the case of galactose, mannose and arabinose where derivatives and mixed melting points with derivatives of known samples confirmed the chromatographic identification. The fact that derivatives have not been obtained for the other sugars does not imply an error in chromatographic identification, only that the work has not proceeded that far as yet. The derivatives that were obtained are quite insoluble and crystallize fairly readily on a 1-5 mg. scale. The presence of lyxose should be rechecked as this sugar is somewhat rare in nature and the chromatographic evidence for its presence is less extensive than for most of the other sugars.

The acidic nature of the polysaccharides has been shown by titration 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. Hydrolysis in formic acid followed by chromatography and possibly derivatization may provide additional evidence on this point.

On the basis of these results, the composition of the purified fraction I was roughly estimated to be as follows:

polysaccharide components	(96-98%)
D-glucose	20%
D-galactose	20%
D-mannose	20%
L-rhamnose	10%
L-arabinose	5%
uronic acid (probably glucuronic acid)	20-25%
polypeptide components	2-4%

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

The fractions have been shown to be heterogeneous mixtures of acidic polysaccharides. The heterogeneity, with respect to sedimentation properties and the rough correlation between solubility and sedimentation, has been demonstrated by fractional precipitation and by demonstrating differences in the sedimentation coefficients of the fractions. The heterogeneity with respect to electrophoretic properties and correlation between the electrophoretic mobility and

differences in chemical composition has been demonstrated. The starch zone electrophoresis experiments showed that the fast component is much more complex, either a substance of greater complexity (more different kinds of components) or more different substances present or both. The slow component appears to be simpler. The fraction contains only glucose, rhamnose and the uronic acid and is lower in nitrogen content than the fast component. If both of these fractions are active, as all evidence would suggest that they are, the slow component appears to be the material most suitable for further study to determine what chemical structures are responsible for the allergen skin reactivity. Possibly the slow fraction may be further fractionated by solubility methods. The use of two methods of fractionation based on different properties would be very much more effective than one alone. If these materials arise by the slow chemical alteration and degradation of large molecules (e.g. naturally occurring polysaccharides or polysaccharide protein complexes), it is to be expected that a heterogeneous population of materials would be produced.

Dr. Michael Heidelberger tested the purified fraction I material for cross reactivity with various pneumcoccus polysaccharides. There was a strong cross reaction with the following type specific substances VII, XII, XV, XVIII. There were more moderate but very 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 component sugars of the type specific substances that gave the strongest reaction are as follows:

Type	Components
VII	galactose glucose rhamnose glucosamine
XII	galactose glucose N-acetylhexosamine
XV	?
XVIII	glucose rhamnose

The dust fraction gave a moderate cross reaction with type specific substance II and VIII both containing 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 fraction reacts with many of those antipneumococcus sera that show cross reactions with glycogens and polyglucoses. It would be particularly interesting to examine the supernatants from such a test to see if there were any loss of allergen skin reactivity associated with particular type specific antisera.

Rimington (6) has reported that his purified allergen specifically inhibited both A and B hemagglutination. Dr. Elvin Kabat tested purified fraction I and found it to be devoid of either of these activities.

The dust fractions have been found to consist largely of a heterogeneous mixture of polysaccharides. A fairly large number of sugar residues ~~has~~ have been identified as being present in the mixture. How many of the different residues are present in any particular species of molecules is unknown as is the number and distribution of different species of molecules present. The specific allergen activity or group of activities has not been proven to be associated with any particular polysaccharide or with the polysaccharides as a group. It seems unlikely that another component would be carried through all the fractionations (based both on solubility and electrophoretic mobility) unless it were linked to a polysaccharide, though this is possible. The problem of whether the specific allergen activity requires the presence of a nitrogen containing component has not been solved.

If the dust allergen activity can be produced under sterile conditions from autoclaved samples of cotton linters (3) it is difficult to see how a complex polysaccharide with many different residues 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. Cotton fibers consist largely of cellulose; however there are also other constituents present (45). Waxes, proteins, pigments and pectins have been demonstrated. The pectins (partially methyl-esterified polygalacturonic acid with arabinose and xylose)

are present to the extent of about 1% in the mature fibers. Possibly some of the arabinose and xylose found 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. There is always the possibility that some unknown substance is altered during the "aging" process and becomes the active allergen.

The experiments involving the production of dust allergen activity by the "aging" of cotton linters under sterile conditions should be repeated as there is the possibility that mold or bacteria contaminated the samples and were involved in the production of the allergen. Polysaccharides containing the types of residues found in the dust extract fractions have been isolated from a wide variety of bacteria (e.g. pneumococci). Experiments have been presented demonstrating the importance of molds in the production of the kapok allergen (46). It is therefore natural to suspect that bacteria and molds might be involved in the production of the dust 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, a good deal more has been learned about the nature of the materials that have been used clinically for the past thirty years.

Summary:

(1) It has not been possible to fractionate the soluble dust extracts into active and inactive components. All of the fractions show activity with some of the patients sensitive to crude dust extracts. Evidence for the presence of materials of more than one allergen specificity was obtained. It is probable that the same allergen specificity is to be found associated with molecules of differing physical properties.

(2) The gross heterogeneity of the crude dust fractions with respect to electrophoretic properties, sedimentation properties and chemical composition has been demonstrated.

(3) The dust extracts were found to consist largely of a heterogeneous mixture of acidic polysaccharides containing uronic acid (probably with glucuronic acid), D-glucose, D-mannose, D-galactose, L-rhamnose and lesser amounts of a number of pentoses. Fractions of dust extracts with nitrogen contents indicating a polypeptide composition of from 2 to 30% have been obtained. Some of the fractions contained small amounts (~2%) of hexosamine. Sedimentation coefficients (Sw,20) of from 1 to 3 have been obtained for various fractions.

(4) The composition and some of the physical properties of a purified dust allergen fraction have been studied. The composition was found to be roughly 2 to 4% polypeptide and 96 to 98% polysaccharide, containing about equal amounts of uronic acid (probably glucuronic acid), D-glucose, D-mannose,

D-galactose with lesser amounts of L-rhamnose and L-arabinose. This material showed at least two main components by electrophoresis and had a $S^{o}_{w},20$ value (sedimentation coefficient corrected to water at 20°C. and extrapolated to infinite dilution) of 2.6 Svedbergs. No molecular weight determinations were carried out.

(5) The relation of the composition and the properties of the house dust allergen fractions to current ideas as to the origin of the allergen was discussed.

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Part II

Experiments on the Distensibility of the Excised Rabbit Bladder

Part II: Experiments on the Distensibility of the Excised Rabbit Bladder

Most of the studies that have been carried out in bladder physiology have been concerned with the effects of stimulation or cutting nerves to the bladder, measurements of electrical changes in the bladder and pressure volume measurements made on bladders in living animals with varying degrees of nerve connection. Some of this work has been carried out on patients with different types of nerve and spinal lesions and some on anesthetized animals. One of the central problems in these investigations has been the role of the sympathetic nervous system in the normal functioning of the bladder. The problem of management of patients with spinal cord lesions has provided a strong stimulus to the study of bladder function.

The only studies carried out on excised bladders, that I have found are those of Osborne (1) and Kesson (2). Osborne measured pressure radius relations using a dog bladder, as well as cat and monkey bladders. The experiments were carried out in a medium of moist air. The results were compared with those obtained using toy rubber balloons. Both the rubber balloons and the bladder showed deviations from the type of curve expected for perfect elasticity, however the bladder showed the greater differences. In order to obtain consistent bladder curves it was necessary to use the bladders at least twenty-four hours after removal from the

animal. Osborne recognized the problem of the difference in properties of the various types of fibers in the bladder, some being elastic and some not. He concludes that only when the muscle is fully inhibited can the pure elasticity of the walls play a dominant part. The bladders showed some hysteresis on deflation. This could be made small by not approaching the elastic limit too closely and by carrying out inflation and deflation by very small increments. Osborne does not tell exactly how he handled his bladders to inhibit the muscle. Kesson studied excised cat bladders in a plethysmograph measuring the rate of change of volume with change of pressure as a function of pressure. Both of these experiments were carried out by making the measurements at a constant time after the application of the pressure.

The purpose of the present experiment was to investigate the change in bladder volume as a function of time under a constant pressure. The rabbit bladder was chosen because of the ready availability of the material.

The Method: The apparatus used is diagramatically represented in Fig. 1. The vessel containing the bladder was a two liter separatory funnel. The pressure applied to the bladder was controlled by the level of the two liter aspirator bottle. Both the separatory funnel and the aspirator bottle were filled with Ringer-Locke solution of the following composition:

Fig. 1

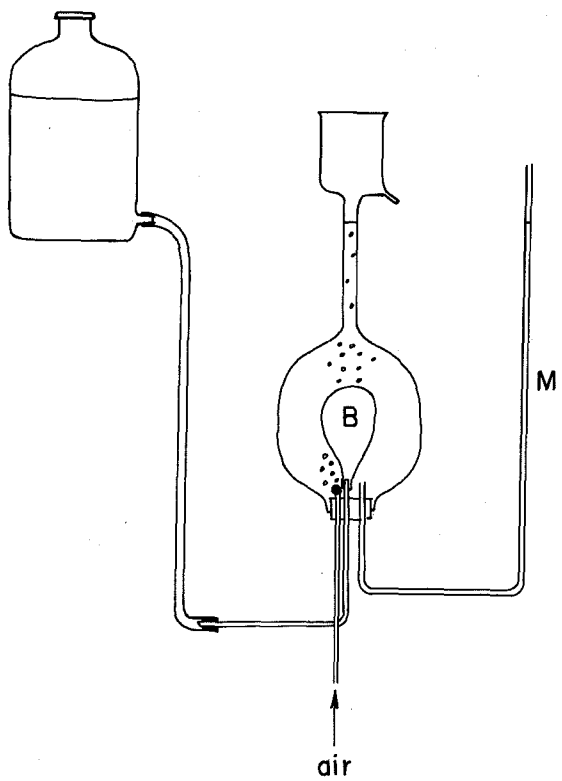


Diagram of the Apparatus

B Bladder
M Manometer tube

sodium chloride	0.9%
calcium chloride	0.024%
potassium chloride	0.042%
sodium bicarbonate	0.02%

The bladder was aerated during the course of the experiment by passing moist air through the solution from a gas dispersion tube. Due to the passage of the air bubbles through the solution the fluid level in the separatory funnel could not be easily measured. Therefore a small glass manometer tube was used to more accurately measure the fluid level. The difference between the height of the solution in the aspirator bottle and in the manometer tube measured by the pressure applied to the bladder. The temperature was measured by means of a small thermometer placed inside the separatory funnel near the bladder. Temperatures above that of the laboratory were maintained by the use of a heat lamp, while lower temperatures were maintained by the application of cold water to the outside of the separatory funnel. During the course of a run at the elevated temperatures, the temperature variation was about $1^{\circ}C.(\pm 0.5^{\circ})$ while at the lower temperatures the variation was at least twice as great.

The volume change of the bladder during the experiment was measured by the volume of solution that had to be removed (or added in some cases) to maintain a constant pressure on the bladder. It was important that the temperature be kept constant during the experiment as volume changes of the solution would be interpreted as changes in volume of the

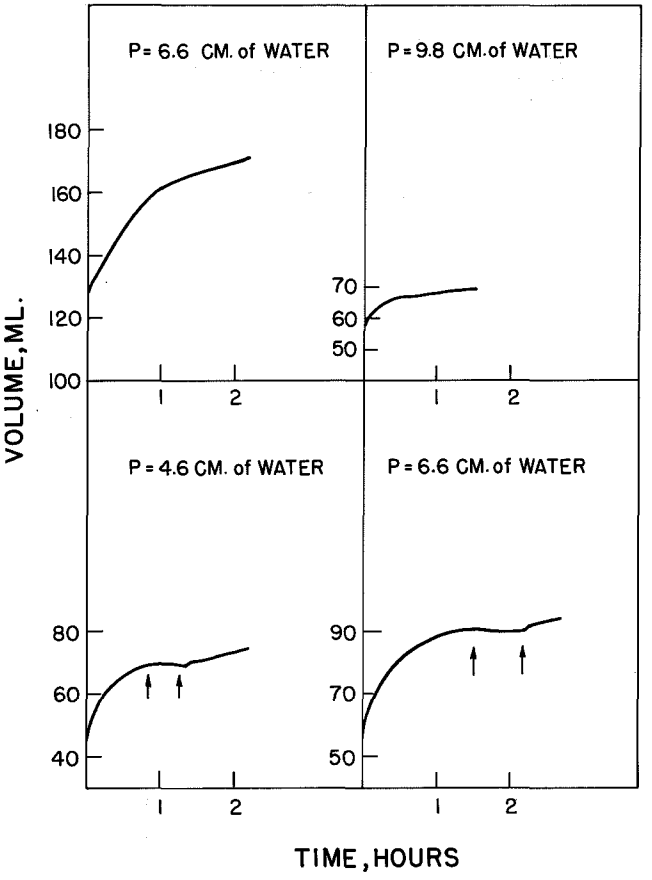
bladder. Foaming of the solution, due to the passage of air through the solution containing traces of protein, was controlled by the occasional addition of a small drop of capryl alcohol.

The bladder was removed from the rabbit as soon as possible after death (generally less than one hour). The ureters were ligated and the bladder securely tied to the glass catheter. It was important that the bladder be treated gently and tied securely. The bladder was gently filled with Ringer-Locke solution and connected to the bottle, care being taken to avoid the trapping of air in the bladder. It was essential that the bladder not be over distended during the course of setting up the experiment. The bladder was set up under no pressure and the tube to the bottle clamped. The apparatus was then arranged as in Fig. 1, and the solution levels adjusted to proper heights to give the correct pressure on the bladder when the clamp was released. The bladder was allowed to accommodate itself to the new environment for 30-40 minutes. The clamp was then removed and the bladder expanded under the pressure. Solution was rapidly removed to attempt to keep the pressure constant. After about two minutes the solution levels became constant enough so that it was easy to maintain them at the proper height. This was taken as zero time for the graphs. The levels were then kept at the correct height by removing or adding solution throughout the course of the experiment. The volume of solution removed was noted for each five minute

interval, except when the change was very slight, then longer time intervals were recorded. After the last reading the rubber tube was clamped close to the glass catheter to the bladder and the apparatus taken apart and the volume of solution in the bladder at the end of the experiment directly determined. The volume change data collected during the experiment were then used to calculate the volume of the bladder throughout the experiment. It was found during the course of the first experiment that one of the greatest problems was the occasional presence of slow leaks; therefore the bladder was filled with Ringer-Locke solution containing methylene blue so that any leak would become immediately evident.

Preliminary Experiments: A number of experiments were carried out in order to determine what shapes of curves were obtained at different pressures and to assess the degree of variability from bladder to bladder under a constant set of experimental conditions. Some of the curves obtained are reproduced in Fig. 2. It seemed possible that the initial period of slow stretching observed at 6.6 cm. of water might be prolonged by performing the experiment at a lower pressure e.g. 2.0 cm. of water. This was found not to be the case. A similar shape of curve was obtained at all the pressures investigated (2.0 to 9.8 cm. of water). The pressure determines the final volume that is approached but not the shape of the stretching curve. It is customary

Fig. 2.



Stretching Curves from Four Bladders

The arrows indicate the time during which the tube to the bladder was clamped.

P = pressure in cm. of water

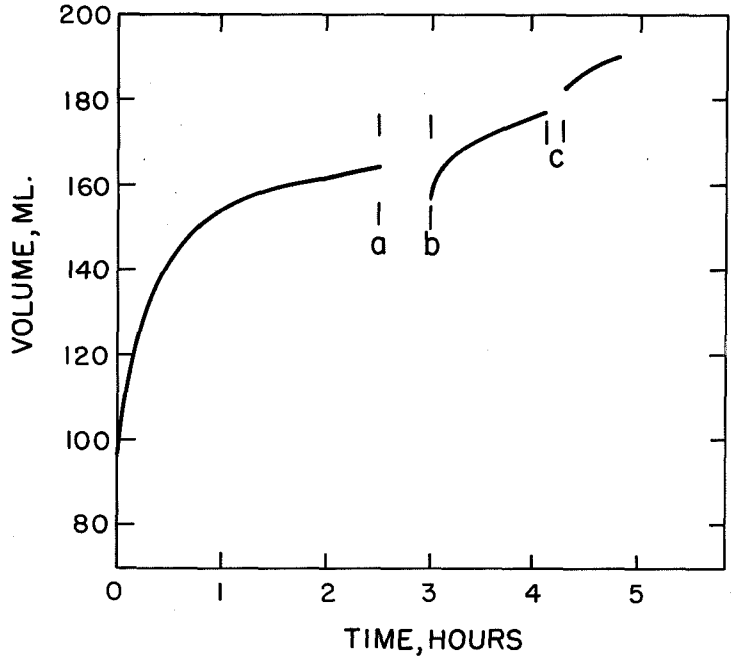
Temperature 35°C.

in bladder pressure volume studies to make the volume measurement at a standard time after the application of the pressure.

In one experiment (Fig. 3) the pressure was increased after the curve had leveled off under the influence of the constant initial pressure. In this case the rate of stretching immediately increased and then began to level off to approach a new volume. It was not determined whether the final volume that was approached at the higher pressure is the same volume that would have been obtained if the bladder had been initially stretched at the higher pressure. During the course of this experiment the pressure was released and the bladder allowed to rest under a slight negative pressure (i.e. the fluid level in the bottle was lower than the level in the manometer tube) for one half hour. Only a very slight recovery in bladder volume was observed. This ease of adjustment to a new length or larger volume is characteristic of smooth muscle and is ascribed to the plastic properties of the muscle.

In the case of two of the bladder experiments presented in Fig. 2, the tube to the bladder was clamped close to the glass catheter after the volume had shown a tendency to level off. This is indicated in Fig. 2 by the arrows. This allowed the bladder to be relieved of the stimulus of the constant pressure while maintaining it at essentially the same volume. During the period that the tube was clamped the bladder volume fell by about 1-2 ml. When the former

Fig. 3



Bladder Stretching Curve at 25°C.

The pressure was 6.6 cm. of water to a and from b to c.

The bladder was allowed to rest from a to b.

At point c the pressure was raised to 9.8 cm. of water.

pressure was restored by opening the clamp the bladder rapidly expanded to about the volume that it would have attained during the same time interval if the constant pressure had been applied throughout. This result was similar to that obtained by Maas (3) in stretching the foot muscle of Helix pomatia by weights and then supporting the weight during the rest period. Possibly during this rest period there is the opportunity for some recovery of the elastic components at the expense of the plastic components, and when the pressure is resumed the partially recovered elastic materials rapidly stretch again.

Great variation in the shape of the curves obtained from different bladders under the same conditions of temperature and pressure was observed. The variation seemed to be greater than that which could be ascribed to differences in size and structure of the individual bladders and appeared to be related rather to the initial tonic state of the bladder. Osborne (1) had stated that he was not able to obtain consistent results in his experiments unless the bladders were kept 24 hours prior to carrying out the experiments. Various pretreatment procedures were explored in an attempt to find a method of obtaining consistent results under the same set of experimental conditions but with different bladders. These procedures involved various combinations of storage at 4°C. for a few days in Ringer-Locke solution and treatments with acetylcholine and all were uniformly unsuccessful.

Having been unable to obtain consistent results with different bladders, a procedure was devised to permit the repeated use of the same bladder. This involved the use of lower pressure (2.0 cm. of water), shorter periods of stretching (one hour or less) and pretreatment before each stretching experiment with eserine and acetylcholine. The procedure was as follows:

1) The bladder was removed as soon as possible after death, ureters ligated and washed carefully with Ringer-Locke solution.
2) The bladder was placed in Ringer-Locke solution containing eserine (1:10,000) for 10 minutes then washed in fresh Ringer-Locke solution and placed in Ringer-Locke containing acetylcholine (1:10,000) for 10 more minutes.
3) The bladder was washed as before and the first stretching experiment carried out at 2.0 cm. of water pressure and 35°C.
4) Following this stretching experiment the eserine acetylcholine treatment was repeated and the bladder stretched again with repetition of the eserine and acetycholine treatment after this and each succeeding experiment.

It was found that good agreement was obtained between the second and third and sometimes the fourth experiment with the procedure described. The series of experiments was carried out as rapidly as possible so that the entire series

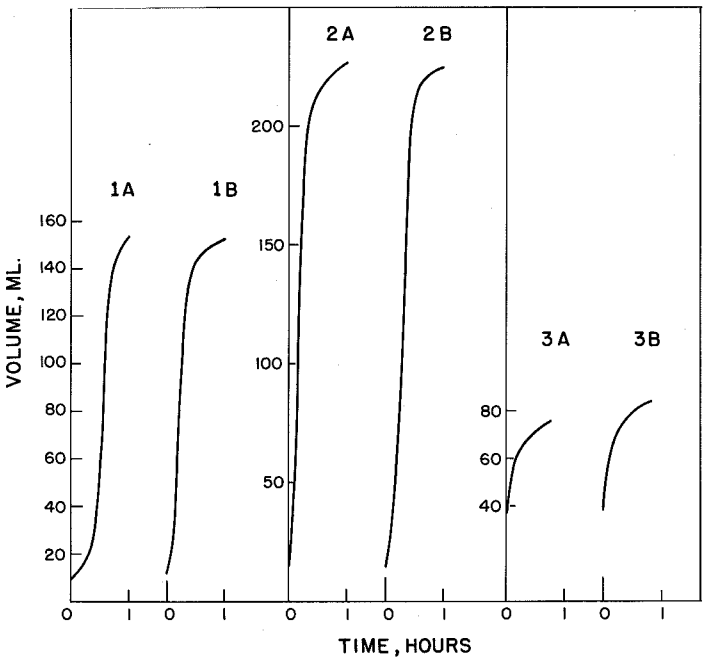
would be completed in six or seven hours after the death of the rabbit.

In such a series of experiments when the results became nonreproducible, it was because the bladder lost its tone and stretched more rapidly than in the previous experiments. Therefore in those experiments in which the effect of different sets of conditions was being tested, the experiment was arranged so that the bladder should exhibit more tone in the second experiment than in the first. In this way confusion between loss of tone due to the experimental conditions and aging of the preparation was avoided. The second and third stretching curves from three series of experiments (with three different bladders) are presented in Fig. 4. The reproducibility is fairly good. Having found a method of obtaining consistent results under the same set of conditions, it was now possible to study the effect of different variables on the stretching curves.

Temperature Effect:

A series of four experiments was carried out to demonstrate the effect of temperature on the stretching curves. The data are presented in Fig. 5. The first three sets of experiments involved a temperature difference of 14°C. (21-35°C.) and definitely showed increased tone at the lower temperature. In the last experiment (No. 7) there was an eight degree difference in temperature and no difference in the shape of the curve. It is not known whether this result is due to aging and loss of tone by the bladder or whether

Fig. 4



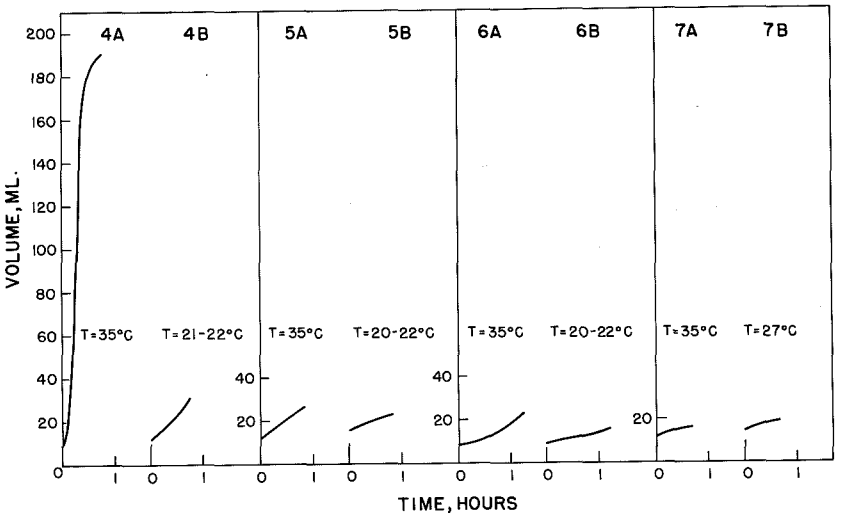
Bladder Stretching Curves Demonstrating the Reproducibility Obtained after the Eserine Acetylcholine Procedure

The curves from three bladders (1, 2 and 3) are presented.

Pressure 2.0 cm. of water

Temperature 35°C.

Fig. 5



Bladder Stretching Curves Demonstrating the Effect of Temperature

The curves from four bladders (4, 5, 6 and 7) are presented.

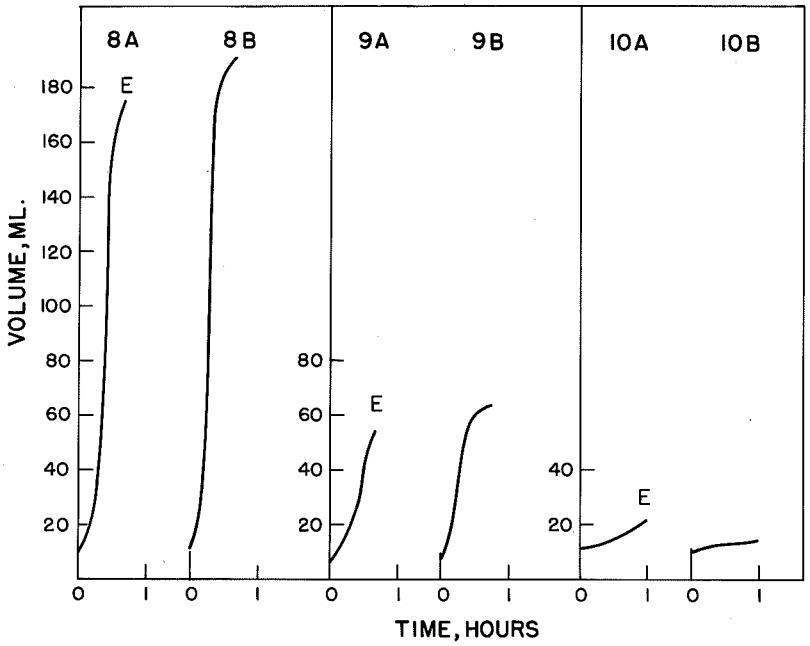
Pressure 2.0 cm. of water

there is really little difference between the resistance to stretching at 35° and 27°C.

Epinephrine Effect:

An experiment to study the effect of epinephrine on this system was carried out. After the first stretching, the bladder was placed in the eserine and acetylcholine solutions, each for 10 minutes as usual, and then placed in a Ringer-Locke solution that contained epinephrine (1:10,000) for 10 minutes. The second stretching experiment was carried out at 2.0 cm. of water pressure and 35°C. After this experiment the bladder was treated with the eserine and acetylcholine as usual and then placed in plain Ringer-Locke solution for 10 minutes in order to provide the proper control for the period the bladder remained in the epinephrine solution before the second stretching experiment. The bladder was then used for the third stretching experiment. The data are presented in Fig. 6. Consistent results were not obtained. We should expect the epinephrine to relax the bladder so the second curve should show the greatest tone. This is the case only in the third experiment (No. 10). In experiment 8 there was essentially no effect and in experiment 9 the effect was just the reverse. It is probable that the properties of the bladder are determined by the eserine and acetylcholine treatment and that the epinephrine has little effect.

Fig. 6



Bladder Stretching Curves Demonstrating the Effect of Epinephrine

The curves from three bladders (8, 9 and 10) are presented.

Pressure 2.0 cm. of water

Temperature 35°C.

Discussion:

The factors involved in the stretching of a bladder are many. They may be divided into two groups, the active effects of the smooth muscle and possible nerve influences on the tone of the bladder and the passive effect of the connective tissue structures. In the experiments presented differences in initial tone of the bladder seemed to be particularly important in determining the shape of the stretching curve. The eserine and acetylcholine treatment restored the bladder to near its initial tone so that more reproducible stretching could be obtained. Part of the resistance to stretching was probably due to the contractile mechanism of the muscle and part due to the passive resistance to deformation, both elastic and plastic, offered by the tissues. The fact that the first stretching curve was different from the second and third indicates that factors were involved in the resistance to stretching in this first experiment that were not reversible by stimulating the contractile mechanism with acetylcholine. Whatever mechanisms we postulate to be causing the resistance to stretching in these experiments have to be reversible by the eserine acetylcholine treatment. In the experiment presented in Fig. 3 in which the pressure was released for one-half hour, the bladder showed very little recovery. The action of the eserine and acetylcholine was necessary for appreciable recovery to take place. Therefore the resistance to stretching was probably mainly associated with the contractile

mechanism rather than unrelated connective tissue structures. The relative amounts of the resistance to stretching, by the contractile mechanism, to be ascribed to active processes and to purely passive resistance is unknown. The experiments in which the tube to the bladder was clamped demonstrated some gain in bladder resistance to stretching at constant volume. This has been ascribed by Maas (3) and others, in the case of the foot muscle of Helix pomatia, to the recovery of elastic components by the distortion of plastic components. This elastic recovery may be recovery of either the contractile structures or connective tissue structures or both.

The temperature effect observed, i.e. an increase in resistance to deformation associated with lowering the temperature, is a property of many materials and is generally associated with increased intermolecular interactions at lower temperature. The concept of muscle viscosity has evolved from such observations with muscle preparations.

The results with epinephrine were not consistent but suggest that epinephrine has little effect on the muscle due to the more overpowering effect of the eserine and acetylcholine. Normally epinephrine has a marked effect on mammalin smooth muscle and in the case of the bladder musculature would have been expected to cause relaxation or loss of tone.

There were a number of possible sources of error in the experiments. The greatest problem was the development of

small leaks. The methylene blue inside the bladder greatly aided the detection of the leaks. As an added check the volume of solution initially placed in the bladder, the volume change of the bladder during the experiment and the final volume of the solution in the bladder were measured and discrepancies here provided an indication of leaks. Other errors were due to variation in temperature and pressure as well as not keeping up with the volume changes. The solution to these latter problems was vigilance.

Summary:

1) The changes in volume of excised rabbit bladders as a function of time under constant pressure were measured.

2) Great individual variation was found in the bladder stretching curves.

3) It was found that reproducible bladder stretching curves could be obtained by restretching the same bladder with intervening eserine and acetylcholine treatment.

4) Increased resistance to stretching was demonstrated at 21°C. as compared with 35°C.

5) Epinephrine was found not to produce a consistent effect under these conditions.

6) Some of the problems and factors involved in deciding the relative importance of the various structures of the bladder in producing the resistance to stretching measured are discussed.

References

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Propositions:

1) House dust fractions have been found to be poor antigens for precipitin production in rabbits. It is proposed that the method of Partridge and Morgan (1) for enhancing the antigenicity of polysaccharides should be explored in an attempt to produce high precipitin titers. This method involves the "coupling" of the polysaccharides to a protein derived from the Shiga somatic antigen.

2) Basic ion exchange resin particles should strongly bind house dust allergen fractions and be specifically agglutinated by very low concentrations of anti house dust precipitating antibody. This might provide a method for following the development of blocking antibody in response to hyposensitization procedures.

3) An attempt should be made to synthesize materials with dust allergen specificity by oxidation (e.g. with NO_2) of various polysaccharides.

4) It is proposed that the following procedure be explored in an attempt to demonstrate the specific coprecipitation of uronic acid containing components of the house dust allergen materials by specific reagin containing serum fractions.

a) Mix allergen and reagin fractions, at pH 6-7.

b) Precipitate a globulin fraction with cold alcohol solution, pH 6-7, and centrifuge.

c) Wash the precipitate with cold alcohol solution, pH 6-7, and mix with cold alcohol solution buffered at pH 2.8 and centrifuge.

d) Determine the uronic acid content of the supernatant. Compare allergic serum fractions with normal serum controls.

5) A relative separation of the Wassermann reagin antibody and the Treponema pallidum immobolizing antibody, in syphilitic rabbit serum, may be obtained by the method of electrophoresis-convection (2).

6) It is proposed that the method of starch zone electrophoresis may be used to prepare specifically purified precipitating antibody in those cases in which the electrophoretic mobility of the antigen and antibody are sufficiently different at pH 2.7-3.0 to permit resolution. The method would involve solution of the specific precipitate in a buffer of sufficiently low pH to dissociate the antigen antibody precipitate (e.g. glycine buffer, pH 2.8) and the separation of the two components by starch electrophoresis in the same buffer. The purified antibody could then be eluted from the starch block. In some cases, the method could be modified to obtain specifically purified nonprecipitating antibody.

7) The use of membrane electrodes (3) should provide

a suitable method for studying hapten (e.g. sulfanilate or arsanilate ion) binding by specific antibody pseudoglobulin fractions.

8) The use of glass strips or squares (for two dimensional procedures) coated with various adsorbents, called chromatostrips (4), should provide a convenient method for the separation and tentative identification of small quantities of phospholipids and phosphatidic acids. Such methods would be useful in the study of the biochemistry of cardiolipin.

9) The possibility of derivitizing milligram amounts of sugars by oxidation (iodine and potassium hydroxide) and formation of a salt with S-1-naphthylmethylthiuronium iodide (5) should be explored.

10) It is proposed that the possibility of a direct action of serotonin and antigen (after passive sensitization) on the contractile mechanism of smooth muscle be explored by the use of nerve free smooth muscle preparations from placental tissue, smooth muscle cells in tissue culture or from amniotic smooth muscle.

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