

Allergy to Flea Bites. V. Preliminary Results of Fractionation, Characterization, and Assay for Allergenic Activity of Material Derived from the Oral Secretion of the Cat Flea, *Ctenocephalides felis felis*¹

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Preliminary investigations of material obtained from *in vitro*-collected oral secretion of fleas have revealed several substances showing allergenic activity related to flea-bite hypersensitivity. The oral secretion was found to contain peptides, amino acids, aromatic compounds, phosphorus, and fluorescent materials. Gel filtration fractionation of the material showed that its allergenic activity was produced by a high-molecular-weight fraction (molecular weight of about 4000-10,000) and by a highly fluorescing aromatic fraction of molecular weight probably below 1,000. The low-molecular-weight aromatic fraction was further fractionated and its allergenic activity is suspected to be associated with a bright blue fluorescing material having an R_f of 0.6 in butanol-acetic acid, and migrating toward the anode on paper electrophoresis at pH 2.3.

In the preceding paper (this issue) we described the method used in obtaining the oral secretion of the cat flea, *Ctenocephalides felis felis* (Bouché) and the ability of the material to induce flea-bite hypersensitivity in guinea pigs. Evidence that this allergenic material is a hapten has been presented in several reports (Benjamini *et al.*, 1960b; Benjamini *et al.*, this issue).

The present report presents the results of our preliminary studies to isolate and characterize the allergenic material present in flea oral secretion. Generally, the studies were carried out by subjecting the material to paper chromatography, paper electrophoresis, gel filtration column chromatography, and cellulose column chromatography.

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METHODS AND MATERIALS

Flea Oral Secretion

The oral secretion was obtained as described in the preceding paper (this issue). Various pooled fractions were used and designated as Prep. I, Prep. II, etc.

Assay of Flea-Bite Hypersensitivity

An assay for activity of oral secretion as related to flea-bite hypersensitivity is described in the preceding paper (this issue). Since several techniques were reported in that paper, the specific method used here is described. The sample was dissolved in 0.87% saline in a concentration equivalent to at least 10 mg/ml of oral secretion. This solution was filtered through an ultra-fine sintered glass funnel (porosity never over 2.5 microns) into a sterile vial. An equal volume of Freund's complete adjuvant² was added.

² Bacto adjuvant, complete Freund (Difco Labs., Inc., Detroit, Michigan).

(Concentrations are expressed as the concentration of the aqueous solution *prior* to addition of adjuvant.) This mixture was vigorously shaken to give an emulsion and 0.1 ml was injected intradermally into a rear foot pad of each of five guinea pigs (white or albino, weighing 250–350 g). The injections were repeated 7 days later and on the 14th day following the first injections, the guinea pigs were challenged with flea bites. The challenge was performed according to the method described by Benjamini *et al.*, 1960a.

Location of Materials on Paper

The paper chromatograms and electrophoretograms were first examined for the presence of fluorescing materials using a long-wave ultraviolet lamp.³ It was found that the paper had to be exposed for several minutes to ultraviolet light before all fluorescing materials became visible. Stains used included: (a) 0.2% ninhydrin in acetone; (b) diazotized sulfanilic acid; (c) the phosphate reagent (Smith, 1960 a, b, and c); and (d), the peptide hypochlorite-starch, KI test of Pan and Dutcher (1956).⁴

Paper Chromatography

The two-dimensional ascending paper chromatographic method of McGreer *et al.* (1961) was used. Approximately 1 mg of oral secretion was spotted at one corner of an 18" × 24" sheet of Whatman #1 paper and developed overnight using Solvent I (isopropanol:ammonium hydroxide:water—8:1:1). After air drying, the chromatogram was turned at right angles and developed overnight in Solvent II (*n*-butanol:acetic acid:water—4:1:1). In place of Whatman #1 paper, Whatman #4 was used in some of the chromatograms, but no change was noted in the relative positions of the materials resolved.

³ Model BLF-6, Ultraviolet Products, Inc., San Gabriel, California.

⁴ Abbreviations used for these reactions: fluorescence (F); ninhydrin (N or NIN); diazotized sulfanilic acid (DSA); phosphate (PO₄); the peptide hypochlorite-starch KI test (Pep); faint (ft); blue (B); red (R); pink (Pi); quenching fluorescence (Q); yellow (Y); white (W); orange (Or).

Preparative paper chromatographic separation of oral secretion was performed by linear spotting of the material along 6 inches on a 9" × 24" sheet of Whatman #4 in approximately 2 μ l amounts per application at 0.5 cm intervals. The material was dried with a hair dryer and overspotted along the origin until the entire sample was applied. On each side of the sheet, side markers of the material were spotted equidistant from the edge of the paper to the six-inch line. Approximately 15 mg of oral secretion were applied to a single sheet. The chromatogram was run descending in Solvent II overnight and, after air drying, the fluorescent bands were marked. The side markers were cut from the sheet and treated with various stains. Areas of resolved material were cut from the unstained chromatogram and eluted by using either water or Solvent III (isopropanol:5% acetic acid—3:1). Completeness of elution from the strips was ascertained by examining them under ultraviolet light followed by staining with ninhydrin. The eluates were concentrated *in vacuo* over sulfuric acid and sodium hydroxide.

Paper Electrophoresis

Paper electrophoresis was performed using the "Standard, medium-scale horizontal Karler-MISCO apparatus."⁵ Whatman #3 sheets 6" × 12" were used; buffer levels in the reservoirs were adjusted to 14 cm (approximately 800 ml of buffer per vessel); 5 × 6 inch Whatman #3 wicks were used with a 1 inch overlap between the wicks and the paper electrophoretogram. The paper was moistened with the buffer and the cover and current applied for at least 15 minutes prior to application of the samples. The samples were applied about 2 cm apart to the center of the sheet in volumes of no more than 10 μ l. About 1 mg of oral secretion was used. The runs lasted approximately 3 hours; the power supply was set at 500 volts and read about 11 milliamps and 25 milliamps for the pH 2.3 buffer and the pH 5.6 buffer respectively. All runs were performed at room temperature. The pH 2.3 buffer was prepared by diluting

⁵ Microchemical Specialties Co., Berkeley, California.

25 ml formic acid (90.2%) and 14 ml acetic acid to 4 liters. The pH 5.6 buffer was prepared by diluting 25 ml pyridine and 5 ml acetic acid to 1 liter.

At the completion of the run, the wicks were removed and the electrophoretogram was dried in the horizontal position under a hair dryer.

Gel Filtration Column Chromatography

The method of Gellotte (1960) for Sephadex gel filtration column chromatography was used. Sephadex G-25, medium grade (100–200 mesh)⁶ was used with water as the eluting solvent. The same column was used for all experiments. The internal diameter of the column was 3 cm and the bed of gel was 75 cm high. The column was water-jacketed and maintained at 4° C. A refrigerated Buchler fraction collector⁷ was used for collecting the fractions. Five ml fractions were collected at a flow rate of approximately 2 ml per minute, the required pressure being maintained by nitrogen. The fractionation was followed by: (1) location of the chloride ion (one drop of each fraction was combined with one drop of 1% silver nitrate on a glass plate and observed for the white precipitate of silver chloride against a dark background); (2) determinations of the optical density of the fractions at 280 m μ ; and (3) ascending paper chromatography in Solvent II of concentrates of aliquots removed from various fractions. At the completion of each column fractionation, the column was washed with over 500 ml water prior to the application of another sample.

Cellulose Column Chromatography

Cellulose columns were prepared as follows: A small pad of glass wool was placed in the bottom of a 25 mm $\times$ 150 mm test tube having an 8 mm hole in the bottom. The tube was packed very tightly with about 20 g of

dry, sifted cellulose powder⁸ in 1 cm sections to a height of 75–90 mm. The column was pre-washed with 50 ml of Solvent III. Only columns giving flow rates of 10–30 seconds per drop were used. For fractionation, the sample (15 mg oral secretion/ml) was applied in 2 ml of Solvent III and eluted with Solvent III. Soon after the oral secretion was applied, 4 major fluorescent bands could be seen when the column was observed under a long-wave ultraviolet lamp. The bands were: 1) blue; 2) yellow; 3) white; and 4) white, in the order of their elution from the column. Four fractions were collected manually, each fraction containing only one of these fluorescent bands. The eluent was changed to water after fluorescent band 3 was eluted from the column. Each of the 4 fractions from a number of columns was pooled and concentrated by rotary evaporation followed by lyophilization.

Solvent III was used as the eluent for the cellulose column chromatography of the Sephadex S-3 fraction. Three ml fractions of the eluate were collected by a fraction collector and then were pooled on the basis of their optical density at 280 m μ , their Folin-Lowry color (Lowry *et al.*, 1951), and the paper chromatographic behavior in Solvent II of aliquots removed from various tubes. (The Folin-Lowry color was developed using 0.1 ml aliquots from alternate tubes; the final volume of the tubes after Folin-Lowry color development being 3.5 ml.) The pooled fractions were concentrated by rotary evaporation and stored in a small volume of water in the frozen state.

RESULTS

Paper Chromatographic Characterization of Oral Secretion

A two-dimensional paper chromatographic map of the oral secretion, Prep. I and Prep. IV, is shown in Fig. 1. The major spots which were identified in subsequent fractionations are numbered (1–11). Their fluorescence and reactivity to various stains are given in Table I. Spots designated IV in the figure were found in Prep. IV but were absent in Prep. I.

⁶ Pharmacia Fine Chemicals, Inc., Sales Office, Box 1010, Rochester, Minn.

⁷ Model 3 R-4004C, Buchler Instruments, Inc., 514 W. 147th St., New York 31, N.Y.

⁸ Whatman Standard Grade Ashless Cellulose Powder.

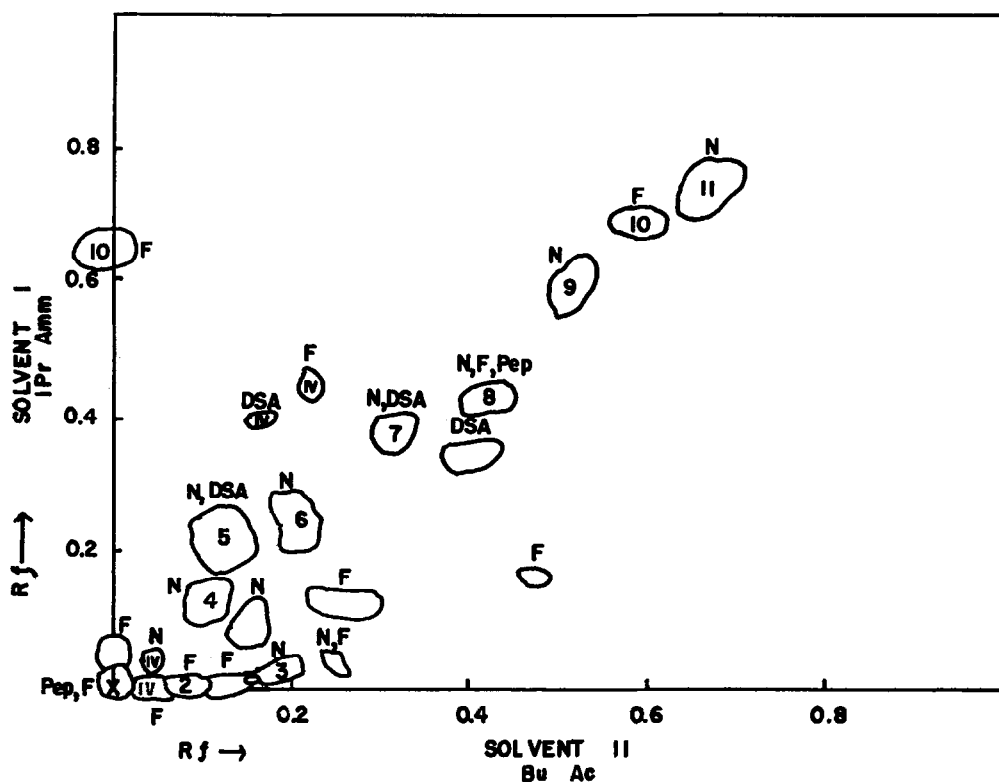


FIG. 1. Two-dimensional paper chromatographic map of flea oral secretion. Prep. I and Prep. IV. (Spots designated IV were present only in Prep. IV.)

TABLE I
Fluorescence and Stain Reactions of Flea Oral Secretion
Following Paper Chromatography

Spot number ^a	Reaction			
	F	NIN	DSA	Pep
1	Yellow	Faint	Orange	++++ ^b
2	Bright blue	—	—	—
3	—	+++	—	—
4	—	++	—	—
5	—	+++++	Bright red	—
6	—	+++	—	—
7	—	+++	Faint pink	—
8	Blue	++	—	—
9	—	+++	—	+++ ^c
10	Blue	—	—	—
11	—	+++	—	—

^a Spot number refers to that indicated in Fig. 1.

^b Plus signs indicate relative intensity of the stained spots.

^c The peptide reaction may be due to a material running very near to Spot 9.

Paper Electrophoretic Characterization of Oral Secretion

The paper electrophoretic patterns of oral secretion, Prep. I, using the pH 2.3 buffer and the pH 5.6 buffer are shown in Fig. 2, along with the reactions of the areas to various stains.

Gel Filtration Fractionation of Oral Secretion

Oral secretion, Prep. I and Prep. II were fractionated on a Sephadex G-25 column. Prep. I was fractionated on a single column at a concentration of 23 mg/ml. Prep. II was fractionated on three separate columns at concentrations of 13.6 mg/ml, 40.3 mg/ml and 40.8 mg/ml. Equivalent cuts from the three separate columns were pooled. The weight of oral secretion given in Table II is that weight which was soluble in water. (Prep. II contained 5–10% water-insoluble material even on re-extraction of the residue with water.) A typical elution pattern characterized by the optical density at 280 m μ is shown in Fig. 3. Fractions were pooled as shown in Fig. 3 on the basis of paper chromatographic behavior in Solvent II and the optical density at 280 m μ . The chromatographic pattern in Solvent II of each pooled fraction is super-imposed on the graph (Fig. 3). Fraction S-1 represented material of molecular weight greater than 4,000; Fraction S-2 was material of less than 4,000 molecular weight which eluted before or with chloride ion and contained most of the ninhydrin staining material. Fraction S-3 contained practically all of the fluorescing material and substances absorbing at 280 m μ . Fraction S-4, absorbing weakly at 280 m μ , was located by its bright blue fluorescence under the ultra-violet light.

Table II contains the weights obtained from the pooled fractions, the percent of each fraction from the total weight of the oral secretion placed on the columns, and the results of assays for induction of flea-bite hypersensitivity by fractions obtained from Prep. II. The concentration of each fraction in 0.87% sodium chloride used for assay was as follows:

Percent of total oral secretion in fraction $\times 20$
 100 mg/ml

Thus each sample assayed contained the amount of that fraction in 1 ml originally present in 20 mg/ml of unfractionated oral secretion. The fractions and the starting material were coded and identified only after the assay was completed. Results of two-dimensional paper chromatography of 0.02

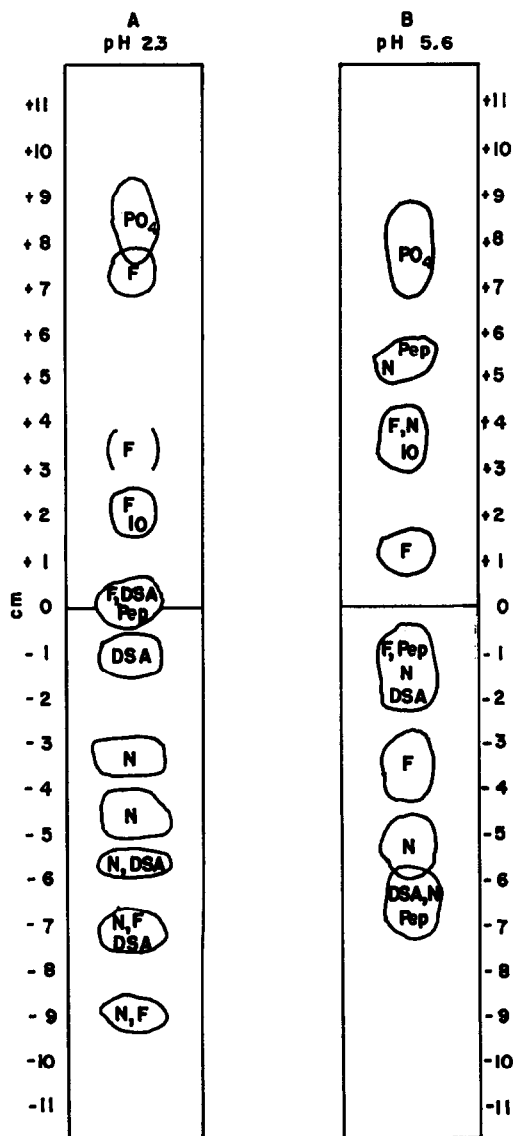


FIG. 2. Paper electrophoretograms of flea oral secretion, Prep. I. Spot 10 refers to Fig. 1.

of each of the fractions obtained from Prep. II are given in Table IV. (Cellulose column chromatography was performed on fraction S-3 obtained from oral secretion, Prep. I, and is presented.)

Cellulose Column Chromatography of Oral Secretion

Two experiments with cellulose column chromatography were performed using 360 mg of oral secretion, Prep. III and 360 mg of

TABLE II
Results from Sephadex G-25 Fractionation of Flea Oral Secretion

Oral secretion Prep. I mg used: 150			Oral secretion Prep. II mg used: 602		
Fraction ^a	Weight recovered (mg)	Percent of total weight	Weight recovered (mg)	Percent of total weight	Allergenic activity in guinea pigs
S-1	38.9	25.9	55.4	9.2	Active
S-2	57.9	38.6	344.0	57.2	Inactive
S-3	24.0	16.0	135.0	22.4	Active
S-4	26.3	17.5	65.8	10.9	Inactive
Total	147.1	98	600.2	99.7	

^a Fractions pooled as indicated in Fig. 3.

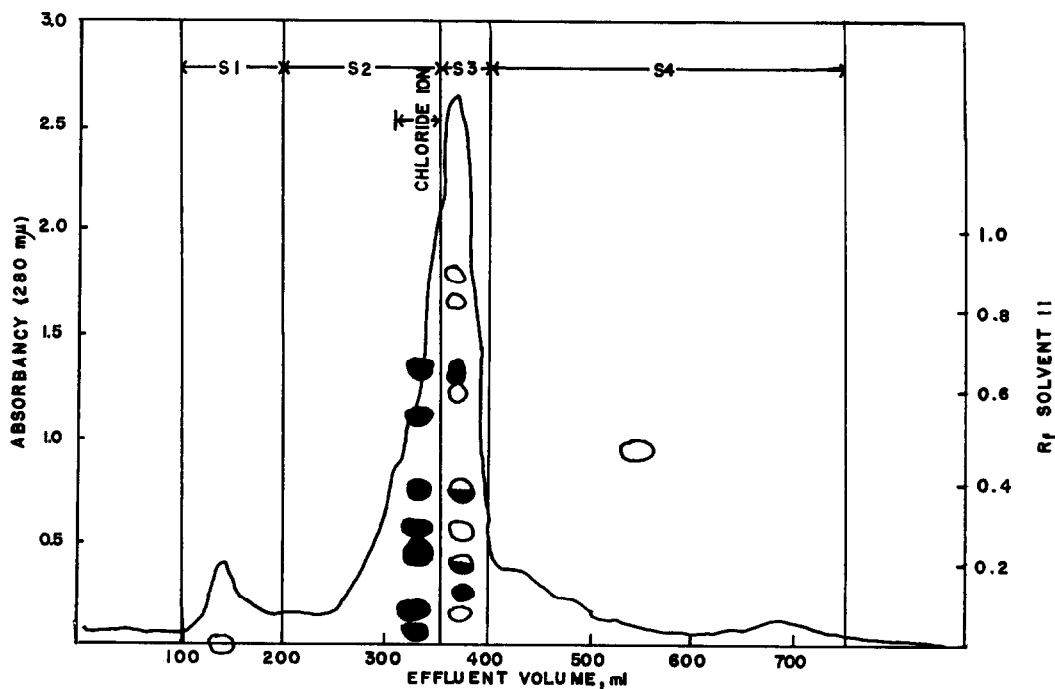


FIG. 3. Elution pattern of flea oral secretion fractionated on Sephadex G-25 column. Eluent: water. Pooled fractions designated S-1, S-2, S-3, S-4. Paper chromatographic pattern in Solvent II of pooled fractions: closed circles (●) ninhydrin positive spots; open circles (○) fluorescing spots; half-closed circles (◐) ninhydrin positive and fluorescent spots.

oral secretion, Prep. IV, respectively. Yields of the fractions C-1, C-2, C-3, C-4 are given in Table III together with the results of assays for induction of flea-bite hypersensitivity by those fractions obtained from the experiment

in which Prep. IV was used. Table IV gives the results of two-dimensional paper chromatography of 0.01 of each of the fractions from the experiment in which Prep. IV was used.

TABLE III
Results of Cellulose Column Chromatography of Flea Oral Secretion

Oral secretion Prep. III mg used: 360 percent recovered: 80			Oral secretion Prep. IV mg used: 360 percent recovered: 68		
Fraction	Color of major fluorescent band	mg Obtained	Assay of allergenicity in guinea pigs		
			mg Obtained	Concn. mg/ml	Activity
C-1	Blue	86	77	7.7	Active
C-2	Yellow	102	71	7.1	Slight
C-3	White	46	44	4.4	Slight
C-4	White	56	54	5.4	Active

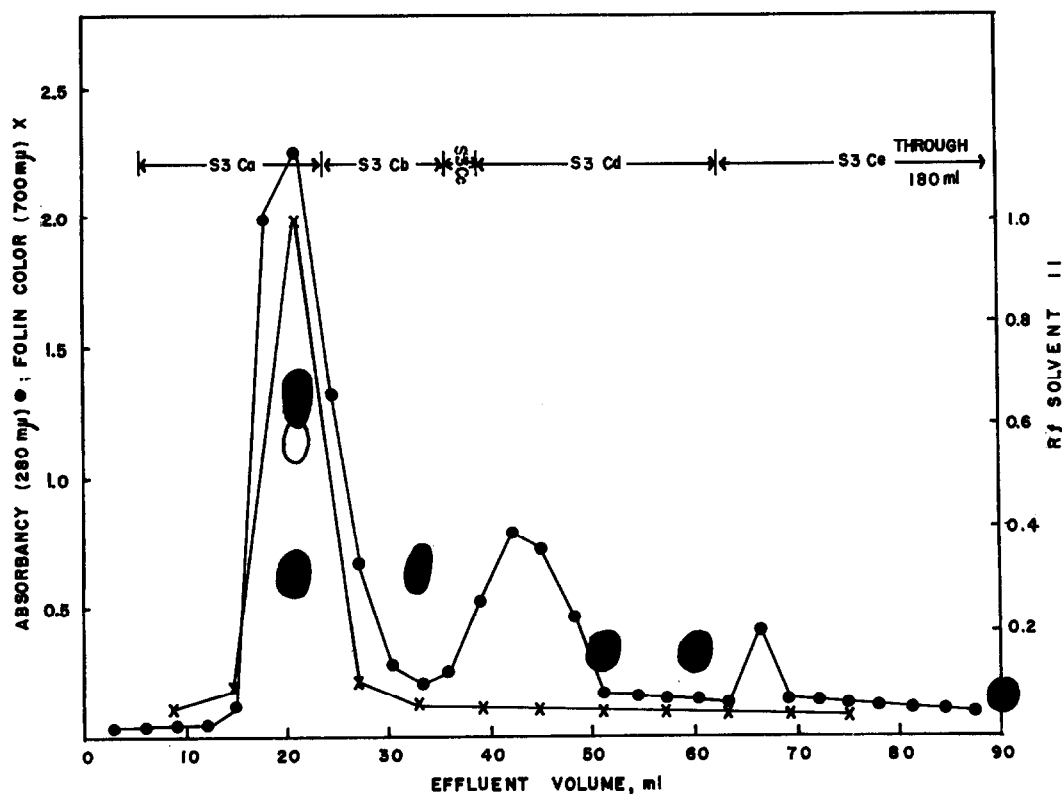


FIG. 4. Cellulose chromatographic elution pattern of fraction S-3 of flea oral secretion from Sephadex G-25. Pooled fractions designated S-3 Ca, etc. Eluent: isopropanol:5% acetic acid—3:1. Paper chromatographic pattern in Solvent II of aliquots from various tubes: open circle (○) fluorescent; closed circles (●) ninhydrin positive spots.

*Cellulose Column Chromatography of
Sephadex Fraction S-3*

Twenty-four mg of Sephadex fraction S-3 (Prep. I) in 2 ml of Solvent III were chromatographed on a cellulose column. Figure 4 shows the elution pattern characterized by the optical density at 280 m μ and by the Folin-Lowry color. The paper chromatographic pattern in Solvent II of aliquots removed from various tubes is superimposed on the graph (Fig. 4). The eluates were combined as indicated in Fig. 4 and were designated S-3 Ca, S-3 Cb, etc. (The two non-fluorescing ninhydrin positive spots obtained in fractions S-3 Cd and S-3 Ce (Fig. 4) were fluorescent on chromatography of the starting material.) Fraction S-3 Ca was found to be active when assayed for induction of flea-bite hypersensitivity. Other fractions were not assayed.

*Preparative Paper Chromatography of
Oral Secretion*

Oral secretion, Prep. IV (30 mg) was subjected to paper chromatography. Figure 5 shows the chromatographic pattern as revealed by fluorescence, staining with ninhydrin, and with diazotized sulfanilic acid. The unstained areas of the chromatograms were cut as indicated in the figure (P-1, P-2, P-3) and eluted with water. The fluorescent area in P-3 (having an R_f of 0.62) was not completely eluted and was re-eluted with Solvent III. This fraction is designated P-3'. Each fraction was assayed for induction of flea-bite hypersensitivity at a concentration equivalent to 15 mg/ml of original oral secretion. Fractions P-2 and P-3' were active; P-3 was weakly active.

*Preparative Paper Chromatography of
Cellulose Column Chromatographic
Fraction C-1*

A portion of fraction C-1 (35.65 mg) from cellulose column chromatography of oral secretion, Prep. IV, was subjected to paper chromatography using 2 sheets of Whatman #4 paper. The chromatographic pattern obtained is shown in Fig. 6. The eluted areas were designated C-1 Pa, C-1 Pb, etc. The

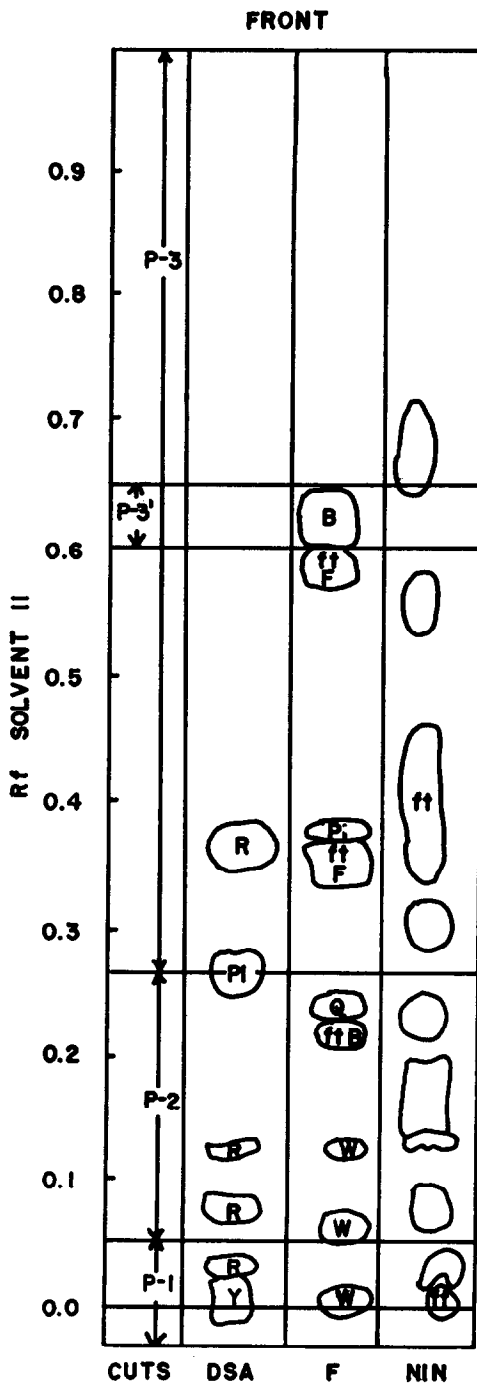
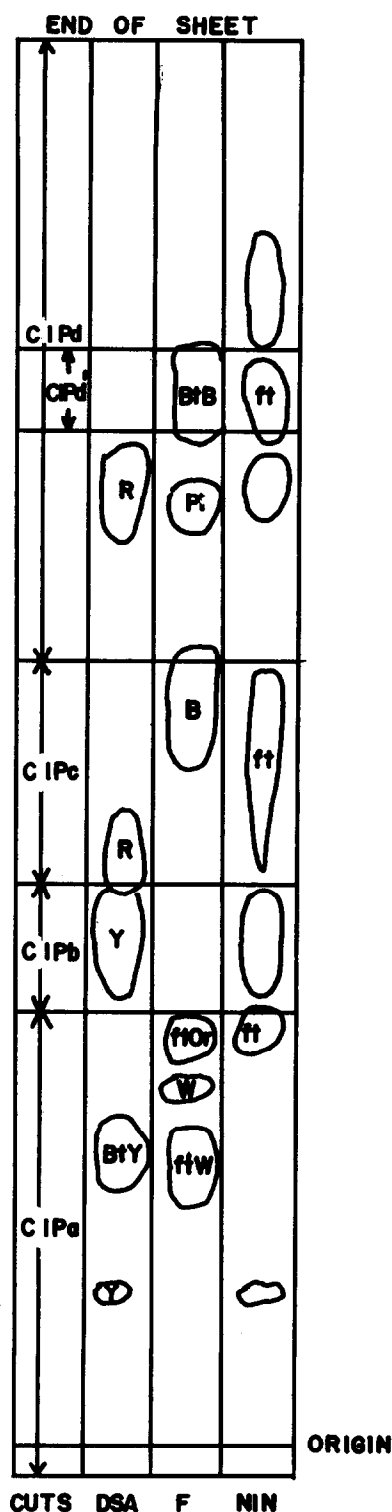


FIG. 5. Preparative paper chromatographic fractionation of flea oral secretion. For explanation, see text.



fluorescent area, *C-1 Pd'*, in fraction *C-1 Pd* was incompletely eluted with water and was therefore re-eluted with Solvent III. This eluate (*C-1 Pd'*) was contaminated with rust from staples used to attach the paper to the elution wick and was not assayed. Assays for induction of flea-bite sensitivity of *C-1 Pa*, *C-1 Pb*, and *C-1 Pc* were negative; *C-1 Pd* was active.

DISCUSSION

Characterization by Paper Chromatography and Paper Electrophoresis

Paper chromatograms and paper electrophoretograms of oral secretion which were stained with various reagents revealed the presence of peptides, ninhydrin positive materials, aromatic compounds yielding color with diazotized sulfanilic acid, phosphates, and fluorescent materials. Approximately 22 spots could be seen on a two-dimensional chromatogram of oral secretion (Fig. 1). These materials have not been identified. Some of the ninhydrin positive spots are amino acids but their identities on the chromatographic map have not as yet been unequivocally established. Although most of the spots occurred in all of the oral secretion preparations, some spots, designated IV in Fig. 1, occurred in oral secretion, Prep. IV but not in Prep. I. No cross-correlation has been attempted as yet between the electrophoretic mobilities and the paper chromatographic patterns of material in the oral secretion except that Spot 10 corresponds to the fluorescent spot at +2 cm using the pH 2.3 buffer and +4 cm using the pH 5.6 buffer (designated 10 in Fig. 2). Electrophoresis studies revealed that most of the fluorescing materials carry a charge and therefore this technique offers a good separation method. Spot 10 can be separated from materials which stain positive to ninhydrin by electrophoresis at pH 2.3. It therefore possesses a negative charge at a pH below that of any carboxyl group of the amino acids.

FIG. 6. Preparative paper chromatographic fractionation of fraction *C-1* obtained from cellulose column chromatography of flea oral secretion. For explanation, see text.

Because of the large number of spots resulting from chromatography of the oral secretion and fractions thereof, the variation in composition of different preparations, and, in some cases, the difficulty in judging the amount of material to chromatogram, it was not always possible to identify even the major spots with complete certainty. However, an attempt has been made to ascertain the presence of 11 major spots in most of the fractions in order to show the effectiveness of fractionation and to determine whether any of the spots could be implicated in allergenic activity. The results are shown in Table IV. It should be noted in Table IV, with reference to Fig. 1, that the presence of spots in each fraction and the activity of the fraction is designated by either a plus sign (+) indicating the fraction was found allergenically active, a minus sign (—) indicating the fraction

had no activity, and a plus-minus sign ($\pm$) indicating that some of the animals in the assay group for the particular fraction showed slight reactivity when challenged with bites. Most of the materials obtained have been assayed only one time at a single concentration. Repeated assays will be necessary to lend more confidence to the results obtained. As Table IV reveals, Spot 10 is the only spot which was present *only* in active fractions. (However, assays for induction of hypersensitivity by extracts of whole fleas which contain Spot 10 were not always positive.) Work now is in progress to ascertain the allergenic activity of Spot 10.

Gel Filtration Fractionation Studies

A fraction of high molecular weight and showing allergenic activity was isolated from oral secretion. This fraction (*S-1*) represented

TABLE IV
Correlation between Characterization of Fractions of Flea Oral Secretion by Paper Chromatography and Induction of Flea-Bite Hypersensitivity in Guinea Pigs by these Fractions

Fraction ^a	Number of spot on paper chromatographic map, Fig. 1 ^b										
	1	2	3	4	5	6	7	8	9	10	11
<i>S-1</i>	+										
<i>S-2</i>	—		—	—	—	—	—		—		—
<i>S-3</i>	+	+			+			+		+	+
<i>S-4</i>								—			
<i>C-1</i>						+		+	+	+	+
<i>C-2</i>				$\pm$		$\pm$		$\pm$	$\pm$	$\pm$	$\pm$
<i>C-3</i>			$\pm$	$\pm$							
<i>C-4</i>	+	+		+							
<i>P-1</i>	—	—	—								
<i>P-2</i>				+	+	+	+	+			
<i>P-3</i>									$\pm$	$\pm$	$\pm$
<i>P-3'</i>										+	
<i>C-1 Pa^c</i>											
<i>C-1 Pb</i>						—					
<i>C-1 Pc</i>								—			
<i>C-1 Pd</i>									+	+	+
No. active assays											
Total assays	3/5	2/3	1/3	4/5	2/3	3/5	1/2	4/6	4/5	6/6	5/6

^a *S* fractions, Sephadex G-25 fractions (Fig. 3); *C* fractions, cellulose column fractions; *P* fractions, fractions eluted from preparative paper chromatograms (Fig. 5); *C-1 P* fractions, cellulose column fraction refractionated by paper chromatography (Fig. 6).

^b The presence of the Spot in the Fraction is indicated with the result of the assay of the fraction for induction of flea-bite hypersensitivity: Active (+); Inactive (—); or weakly Active ($\pm$).

^c Inactive, no spots.

about 10% of the total weight of the material fractionated and, on paper chromatography, it stayed chiefly at the origin, fluoresced, and reacted strongly to the peptide stain. The molecular weight of the fraction can be assumed to be over 4,000 since the Sephadex G-25 used in the fractionation excludes from its inner pores molecules of that size, and the *S-1* fraction comes through the column immediately after the external volume of the column. However, the molecular weight of the material is suspected to be less than 10,000 since dialysis experiments with oral secretion revealed that all of the allergenic activity was dialyzable (preceding paper, this issue). The homogeneity of this material, its degradability to a smaller, but still allergenically active fraction and its allergenic activity compared to that of the low-molecular-weight allergenic fraction (*S-3*) are yet to be studied.

The gel filtration column provided a very good method of separating the fluorescing material from most of the ninhydrin positive material and from most of the inorganic salts. The fluorescing materials are probably aromatic and heterocyclic in nature since such materials were reported by Gellotte (1960) to be adsorbed onto the gel and to be eluted from the column after the chloride ion when water is the eluent. The low-molecular-weight fluorescing materials (fraction *S-3*) represented about 20% of the weight of the material placed on the column. Since allergenic activity was not found in the *S-2* or *S-4* fractions, this fractionation step effected a five-fold purification of the low-molecular-weight active material. In this respect oral secretion differs from fractions of low molecular weight obtained from extracts of whole fleas. These latter preparations show allergenic activity in both the aromatic and non-aromatic low-molecular-weight fractions.

Cellulose Column Chromatography

This fractionation was performed both with and without prior fractionation on Sephadex. In the fractionation without previous gel filtration chromatography, the separation of materials was good although, as in gel filtration, contamination occurred between adja-

cent fractions (See Table IV). The experiment in which Sephadex fraction *S-3* was resolved on the cellulose column included determination of allergenic activity only of the first fraction, *S-3 Ca*. Thus the absence of activity in other fractions cannot be excluded. The allergenic activity found in fraction *S-3 Ca* implicated Spots 10 and 11 as active haptens.

Paper Chromatographic Separation of Oral Secretion

The absence of activity in the origin area of the paper chromatogram of whole oral secretion was contradictory to our findings that the active Sephadex fraction *S-1* chromatographed chiefly in this area. However, when the peptide spray was applied to the eluted paper, a strong reaction resulted. Thus the activity may have been in the origin area of the paper but not eluted from it.

We observed an interesting phenomenon in regard to the adsorptive properties of Spot 10 on paper. When the purified material was placed on a dry electrophoretogram and electrophoresed, much of the material did not move, whereas when the material was spotted on a wet electrophoretogram all of the fluorescence migrated to the expected location.

Elution of areas from paper chromatographic separations of whole oral secretion and from paper chromatographic separation of the *C-1* fraction of the cellulose column fractionation of whole oral secretion showed that allergenic activity was present in the fast-moving material.

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