

**Allergy to Flea Bites. IV. *In Vitro* Collection and
Antigenic Properties of the Oral Secretion of
the Cat Flea, *Ctenocephalides felis felis*
(Bouché)¹**

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The *in vitro* collection of oral secretion of cat fleas *Ctenocephalides felis felis* and the allergenic properties of the collected material are described. The reported experiments demonstrate that the oral secretion contains a substance or substances responsible for the induction of flea-bite hypersensitivity, and that this substance or substances are haptenic in nature.

Most workers have assumed quite logically that it is the salivary secretion of hematophagous insects which contains the material responsible for sensitization of man and animals to their bites. Early work with mosquitoes resulted in the hypothesis that the bite reaction was due to regurgitation of substances in the esophageal diverticula (Schau-dinn, 1904; Hindle, 1914; and Roxburgh, 1927). However, the work of Hecht (1928), Pawlowsky and Shtein (1928), and Manalang (1931) suggested that the salivary secretion was the sole agent responsible. More recently, Gordon and Crewe (1947) stated that the substance causing both immediate and delayed reactions to the bites of *Aedes aegypti*

"... is contained in the salivary fluid and is injected into the tissues by the mosquito throughout the act of feedings." McKiel (1959) noted that the sensitizing substance in mosquito bites is limited to the head and thorax regions and he concluded that it must be a part of the secretion of the salivary glands.

An indirect experimental approach to the problem was made by Lester and Lloyd (1928), who showed that no reaction was produced by the bite of a tsetse fly from which the salivary glands had been removed. This was confirmed by Gordon and Crewe (1947) who failed to obtain a typical wheal when a tsetse fly, from which the glands had been removed, was allowed to probe for 5 minutes and to engorge on an individual who was extremely sensitive to bites of *Glossina*. Recently, A. Hudson and coworkers (1960) demonstrated that when the main salivary duct of *Aedes stimulans* was cut, subsequent feeding on man failed in every case to produce wheals or irritation, in contrast to typical reactions elicited by bites of control mosquitoes feeding on an adjacent area of the subject's arm. This was taken as evidence that the source of antigenic material is in the salivary glands.

A more direct approach to the problem was made by Nuttall and Shipley (1903),

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who injected emulsions of salivary glands of *Culex pipiens* under the skin of guinea pigs. This failed to produce a visible reaction. Cornwall and Patton (1914) produced hyperemic patches and a wheal reaction in the skin of a human subject by injecting an emulsion of the salivary glands of *Anopheles jamesi* and *A. rossii*. Metcalf (1945) observed that emulsions from salivary glands of *A. quadrimaculatus* in saline solution, when injected intradermally into the forearms of two human subjects, produced typical hyperemic wheals that persisted for 24 to 48 hours. Still dealing with dissected salivary glands, Rockwell and Johnson (1952) modified the technique somewhat when they prepared saline extracts of glands from *A. quadrimaculatus*. These were used in skin tests in sensitive persons and were part of a series of tests in which similar extracts of other portions of the mosquitoes were used. The salivary gland extracts produced skin reactions that were more severe than those obtained with extracts of legs and wings, but less than those elicited by extracts of abdomens.

It was recently reported by Hudson *et al.* (1960) that flea-bite sensitivity could be induced in guinea pigs by prior exposure to bites of these insects. Subsequently, Benjamini and coworkers (1960a) demonstrated that the same sensitization phenomenon could be elicited in guinea pigs by intradermal injections of extracts prepared from whole fleas, and by intraperitoneal injections of these extracts in combination with the complete Freund's adjuvant.⁵ It was suggested that these extracts contained the substance responsible for the bite reaction and induction of hypersensitivity to flea bites. However, certain drawbacks in working with extracts of whole flea bodies make it difficult to isolate the particular allergen in relative purity. Extracts of whole fleas contain, in addition to the allergen in question, all compounds naturally present in these insects. Thus, even though several purified fractions, capable of inducing hypersensitivity, were obtained by

electrophoretic and salt precipitation fractionation of whole flea extract, it was felt that these at best represented a complex that would render a precise biochemical and immunochemical characterization very difficult. We decided to attempt the *in vitro* collection of oral (salivary) secretion. (Theoretically, it can be assumed that such a preparation should resemble the secretion released during the natural blood-feeding of the insect). If direct experimental test could be made to demonstrate that an allergen responsible for the bite reaction is actually present in the oral secretion, this preparation should be a better starting material than extracts of whole fleas for the isolation of the particular allergen or allergens involved.

A preliminary report of some of this work already has been published (Benjamini *et al.*, 1960b; Feingold and Benjamini, 1961). The present paper is a detailed account of the *in vitro* collection of oral secretion of cat fleas and of certain findings regarding its immunochemical properties related to flea bite hypersensitivity. A further study of the physical, chemical, and immunochemical properties and the fractionation of the secretion is the subject of another communication (Young *et al.*, in press).

MATERIALS AND METHODS

Method of In Vitro Collection of Oral Secretion of Fleas

The fleas we used were cat fleas *Ctenocephalides felis felis* (Bouché) reared in our laboratory according to the method described by Hudson and Prince (1958). The flea feeding apparatus described by Kartman (1954) was adapted for the studies. Preliminary experiments were conducted on the ability of the fleas to feed through a skin of the vole *Microtus californicus* and through a highly purified commercial animal membrane (Silverlight)⁶ used by Tarshis (1958). Approximately 1000 unfed fleas, 24 to 72 hours old, were placed in a feeding chamber 1½" in diameter and 1¼" high, one end of which was covered with the particular membrane,

⁵ Bacto adjuvant, complete Freund (Difco Labs. Inc., Detroit, Michigan.) Use of trade names is for identification purposes only and does not constitute endorsement by the U.S.P.H.S.

⁶ Silverlight membrane, Julius Schmidt Inc., New York, N. Y.

following rinsing in distilled water, and the other end with nylon chiffon screen. The fleas were allowed to feed for 20 minutes on a 1% solution of Evan's blue dye in saline maintained at 37° C. Later they were anesthetized with CO₂ and feeding percentages were determined by crushing each individual flea on white filter paper to check for the presence of dye in the gut. These tests showed that over 90% of the fleas had fed either through the vole skin or Silverlight.

The above experiments were followed by tests in which 10,000 fleas were fed in a larger feeding chamber. For this purpose, a slightly modified feeding device was constructed out of plexiglass. The fleas were confined in a chamber 5" in diameter and 2" high, one end of which was covered with Silverlight membrane and the other end with nylon chiffon. The feeding chamber was placed in a plexiglass dish 6" in diameter and 2" deep, containing approximately 10 ml of Evan's blue solution equilibrated to 37° C. over a water bath. The cage containing the fleas was supported in the dish so that the membrane touched the surface of the solution very lightly. With this arrangement, again over 90% of the fleas fed during a 20 minute period. Similar tests, using a 0.5% solution of Evan's blue in distilled water yielded comparable results. On the basis of these tests, the larger feeding chamber and the greater number of fleas were used for the *in vitro* collection of oral secretion in distilled water.

It was assumed that while the fleas fed they introduced their oral secretion into the feeding medium (distilled water in the reported experiments). However, the possibility exists that the materials secreted during *in vitro* feeding may differ in quality and quantity from those secreted during *in vivo* feeding. Nevertheless, as will be described later, antigenic activity related to flea-bite hypersensitivity was discovered in the oral secretion collected in the manner described above.

Extensive observations were made while the fleas were feeding through the Silverlight membrane on distilled water, and during hours following the intake of distilled water. Several of us observed a few thousand fleas

under a stereoscopic microscope, but in no case was defecation observed, even as long as 6 hours following the intake of water. On the other hand, when hungry fleas were fed through the membrane on citrated or heparinated human blood, they defecated shortly after feeding. The inclination of the fleas to probe and release oral secretion even on such surfaces as glass, enamel, and filter paper easily could be observed under the stereoscope. In instances where the fleas had probed and secreted a greenish solution from the proboscis, if illumination of the microscopic field were changed to long-wave ultraviolet light, the secretory product had a bluish fluorescence similar to that observed in oral secretion collected through Silverlight membrane. In several instances, the actual act of secretion was observed both under regular and ultraviolet illuminations.

To ascertain further the purity of *in vitro*-collected oral secretion, we allowed very concentrated oral secretion solutions to diffuse through Silverlight membrane into distilled water, then examined the water for fluorescence under ultraviolet illumination. When a new membrane was used, the degree of fluorescence in the distilled water was undetectable. However, when a washed membrane was used, through which 10,000 fleas previously had fed for 2 hours, a low degree of fluorescence was detected presumably resulting from passage of materials through the membrane into the water. Nevertheless, the amount of fluorescence found in the water at the end of a 5-hour period was practically negligible when compared with that obtained from feeding 1,000 fleas on distilled water for only 45 minutes. Although it is fully recognized that materials could pass through the membrane from the inside of the feeding chamber into the distilled water if given sufficient time, the results of the above tests suggest that this diffusion would be minimal under the conditions described in which the feeding time of about 10,000 fleas was limited to 2 hours or less.

In addition to the above experiments, the *in vitro*-collected oral secretion was analyzed for the presence of digestive enzymes, proteins, and polysaccharides of high molecular

weight. Results of preliminary analyses were negative, a further indication that the *in vitro*-collected material was not contaminated with feces or products of regurgitation.

The oral secretion of large numbers of fleas was collected *in vitro* in the manner described above. The preparation was lyophilized and was found to be greenish in color and to have a somewhat gummy consistency when exposed to air. We soon realized that the dried material was highly hygroscopic and took appropriate steps for handling it. Controls consisted of empty feeding cages placed on distilled water in the feeding dishes so that the membrane was in contact with water for a period of time identical to the feeding time. Lyophilization of controls resulted in no detectable yield.

The accumulation of *in vitro*-collected material was standardized on the basis of the number of fleas feeding per hour. Thus, for example, the feeding of 50,000 fleas for 2 hours equals 100,000 flea-hours of feeding which is equivalent to the number of flea-hours obtained from 200,000 fleas feeding for 30 minutes. The number of flea-hours was correlated with the total weight of lyophilized material. On the average, it was found that 1,000,000 flea-hours yielded approximately 500 mg of lyophilized material. This average was derived on the basis of 10 batches of *in vitro*-collected material that showed a deviation of approximately $\pm 30\%$ as a result of feeding from 1 to 2 hours. (Early experiments in which smaller feeding cages and fewer fleas were used yielded approximately 1 mg per million flea-hours.)

Methods of Induction of Flea-Bite Hypersensitivity with Oral Secretion

Animals. Animals used were albino or white guinea pigs weighing 250–350 g.

Preparation of Inocula. The lyophilized, *in vitro*-collected oral secretion was dissolved in saline, passed through a fritted glass filter (porosity never higher than 2.5 microns), mixed with saline or with saline combined with adjuvants, and then injected into guinea pigs. (Reports on the individual experiments specify which preparation was used.)

Sensitization. The guinea pigs were sen-

sitized to flea bites, by exposure to fleas or by inoculation, according to the procedure described by Benjamini *et al.* (1960a).

Challenges. Challenges with flea bites were given by exposing the shaved, depilated abdomen of the animal for 15 to 20 minutes to bites of 20 unfed fleas, 2 to 3 days old. Challenges with whole flea extracts or with *in vitro*-collected oral secretion were carried out by intradermal injections of 0.05 ml of the test material in saline. The allergic response of guinea pigs to flea bites is manifested first by delayed skin reactions which, after 2 to 5 additional days of exposure, are accompanied by immediate skin reactions (Benjamini *et al.*, 1960a; Benjamini *et al.*, 1961). Thus the criterion for the induction of hypersensitivity, used in the present experiments, was the appearance of delayed skin reactions after the first challenge by flea bites. Furthermore, the animals were additionally exposed for 5 to 7 days until animals which had not responded to the original challenge manifested delayed skin reactions. This was done to ascertain the immunological responsiveness of the test animals to flea bites.

Dosage. Dosages used for injections are specified for each experiment. It should be noted, however, that in some experiments the yield of the lyophilized oral secretion was low. Consequently, the dosage of oral secretion was in terms of flea-hours (1,000,000 flea hours corresponding approximately to 500 mg of lyophilized material). In other experiments the dosages were expressed in units of weight.

RESULTS

Skin Reactivity Elicited by Fleas Biting Through Silverlight Membrane

Since the oral secretion of fleas was collected by letting them feed through Silverlight membrane, the question was raised as to whether the membrane could act as a barrier to deposition of oral secretion and/or the active allergenic substance. Accordingly, an experiment was performed in which 20 fleas were allowed to bite for 5 minutes through the membrane applied to the skin of sensitive animals. The animals responded to the challenging bites with both immediate

and delayed skin reactions identical to those elicited by fleas biting through nylon chiffon screens. These results indicated that the membrane did not prevent the fleas from probing, feeding, and eliciting skin reactions. It is important to note at this point that the only portion of the flea that penetrated through the membrane and into the host was the stylet, indicating that whatever material caused the skin reaction passed into the host through that organ. This procedure presumably eliminated the possibility that fecal and other excretory products were responsible for the bite reaction, since such materials could not pass through the membrane and penetrate the host's skin during the short period of exposure (5 minutes). (It was interesting to note that the percentage of fleas feeding on the guinea pigs through the membrane amounted to approximately 5%, as compared with over 90% feeding through nylon chiffon. The percentage feeding through the membrane increased to 20% when saline solution at 37° C was smeared on the shaved abdomens of the animals.)

Sensitization of Animals with In Vitro-Collected Oral Secretion in Saline

Oral secretion, corresponding to 4000 flea-hours, in 0.5 ml saline was injected intradermally, three times at weekly intervals, into each of five guinea pigs. On the 4th week, the animals were challenged with flea bites. No skin reactivity resulted, indicating that hypersensitivity to flea bites was not induced (Table I, assay number 1). Concurrently with flea bite challenges, the animals were challenged also with intradermal injections of 0.05 ml of 1 mg/ml whole flea extract in saline (prepared according to Benjamini *et al.*, 1960a). Results of these challenges were negative, indicating that the oral secretion was incapable of inducing hypersensitivity to the whole flea extract. This suggested that the oral secretion was lacking in complete antigens capable of inducing antibody synthesis.

On the other hand, it was suspected that the failure of the oral secretion to sensitize might have been due to extremely low concentrations of antigens. Accordingly, a new

experiment was performed in which each of five guinea pigs was injected intradermally three times at weekly intervals with a dosage of oral secretion corresponding to 20,000 flea-hours per injection. On the 4th week the animals were challenged in a manner identical to the one described above. All results were negative, suggesting that the failure of the *in vitro*-collected oral secretion to induce hypersensitivity was qualitative rather than quantitative (Table I, assay number 2).

Sensitization by In Vitro-Collected Oral Secretion in Combination with Silverlight Membrane

Throughout these experiments it was noted that Silverlight membrane, which normally did not fluoresce under long-wave ultraviolet light, fluoresced intensely after fleas had fed through it. Since the pale green-blue fluorescence was similar to that obtained when *in vitro*-collected oral secretion was observed under similar conditions, it was suspected that all or a portion of the oral secretion somehow was retained by the membrane. Accordingly the membrane, through which oral secretion equivalent to 20,000 flea-hours had been obtained, was extracted with saline and the filtered extract injected intradermally three times at weekly intervals into each of 8 guinea pigs (each injection corresponded to 2500 flea-hours per animal). Each of five control animals was challenged with flea bites. All results were negative (Table I, assays number 5, 6).

The experiment was repeated except that the membrane through which the fleas had fed, and the control membrane were finely ground, extracted with saline, and the residues (instead of the saline extracts) were inoculated intradermally, each injection equivalent to 4000 flea-hours per animal. When the animals were challenged with flea bites on the 4th week, 11 of 13 animals, injected with membrane through which fleas had fed, responded with typical delayed skin reactions (Table I, assay number 7). Of eight animals injected with residues of the control membrane none responded to challenging bites (Table I, assay number 8). The experiment then was repeated with intraperi-

toneal injections. Results shown in Table I (assays number 9, 10) indicate that flea-bite hypersensitivity was induced in three of five animals injected with residues of the membrane through which fleas had fed. The data also show that the control membrane did not induce hypersensitivity to flea bites.

The preceding experiments suggested that the *in vitro*-collected oral secretion could achieve full antigenicity only if it combined

with some unknown non-extractable portion of the Silverlight membrane. To confirm this, *in vitro*-collected oral secretion equivalent to 20,000 flea-hours was lyophilized and resuspended in 2.5 ml of saline containing the residue of a saline extract of a new 3-inch square of shredded Silverlight membrane. The preparation was allowed to stand in the refrigerator overnight. Then, 0.5 ml of the suspension (corresponding to 4000 flea-hours)

TABLE I
*Summary of Experiments on the Induction of Hypersensitivity to Flea Bites in Guinea
Pigs with Oral Secretion of Ctenocephalidis felis felis (Bouché)*

Assay Number	Inocula	Route of injection	Dosage per injection	Number weekly injections	Number sensitized/ no. tested
1	Oral secretion	ID ^a	4,000 FH ^b	3	0/5
2	Oral secretion	ID	20,000 FH	3	0/5
3	Oral secretion	ID	0.5 mg	3	0/4
4	Oral secretion	IP ^c	0.5 mg	3	1/5
5	Membrane ^d extract	ID	2,500 FH	3	1/8
6	Control membrane extract	ID	—	3	0/5
7	Membrane residue ^e	ID	4,000 FH	3	11/13
8	Control membrane residue	ID	—	3	0/8
9	Membrane residue	IP	4,000 FH	3	3/5
10	Control membrane residue	IP	—	3	0/5
11	New membrane plus oral secretion	ID	4,000 FH	3	4/5
12	Oral secretion plus adjuvant ^f	IP	2,000 FH	3	12/15
13	Control plus adjuvant	IP	—	3	1/14
14	Oral secretion plus adjuvant	IP	0.5 mg	3	13/15
15	Oral secretion plus adjuvant	ID (foot pad)	0.4 mg	2	20/20
16	Control plus adjuvant	ID (foot pad)	—	2	1/5
17	Oral secretion + adjuvant (incomplete)	IP	4,000 FH	3	1/5
18	Oral secretion + adjuvant (incomplete)	ID (foot pad)	0.4 mg	2	0/5
19	Heated oral secretion + adjuvant	IP	4,000 FH	2	10/10
20	Heated oral secretion + adjuvant (open tube)	IP	0.25 mg	2	2/4
21	Heated oral secretion + adjuvant (closed tube, N ₂)	IP	0.25 mg	2	5/5
22	Dialyzed oral secretion + adjuvant	IP	4,000 FH	3	0/10
23	Dialyzed oral secretion + adjuvant	ID	0.4 mg	2	0/5
24	Dialyzed oral secretion + adjuvant	IP	0.25 mg	2	1/5
25	Dialyzed oral secretion + adjuvant	ID (foot pad)	0.4 mg	2	2/5
26	Dialysate oral secretion + adjuvant	ID (foot pad)	0.4 mg	2	4/5

^a ID Intradermal injection in abdomen of animal unless indicated otherwise.

^b FH flea-hour—for definition see text.

^c IP Intraperitoneal injection.

^d Membrane—Silverlight membrane through which oral secretion of fleas was collected.

^e Membrane residue—residue following saline extraction of Silverlight membrane through which oral secretion was collected.

^f Freund's adjuvant, complete unless indicated otherwise.

TABLE II
*Induction of Flea-Bite Hypersensitivity in Guinea Pigs by Varying Dosages of
 in vitro-Collected Oral Secretion of Cat Fleas in Combination with Freund's
 Complete Adjuvant Following 1 Injection per Week for 2 Weeks*

Route of injection	Mg/ml	MI/injection	Mg/animal/ injection	No. reacting/ no. used
Intraperitoneal	2.50	0.5	1.250	5/5
	2.00	0.5	1.000	4/5
	0.50	0.5	0.250	5/5
	0.25	0.5	0.125	5/5
	0.20	0.5	0.100	2/5
	0.05	0.5	0.025	5/5
	0.02	0.5	0.010	4/5
	0.002	0.5	0.001	0/5
Intradermal (foot pad)	5.0000	0.1	1.00000	5/5
	4.0000	0.1	0.80000	4/5
	0.4000	0.1	0.08000	1/5
	0.0400	0.1	0.00800	4/5
	0.0040	0.1	0.00080	2/5
	0.0004	0.1	0.00008	1/5

was injected intradermally into each of five animals. This was repeated two more times at weekly intervals. On the 4th week each animal was challenged with bites of 20 fleas. Four of five animals thus treated responded with typical delayed reactions (Table I, assay number 11).

*Sensitization by In Vitro-Collected Oral
 Secretion in Combination with
 Freund's Adjuvant*

The preceding experiments suggested that although the substance or substances responsible for the bite reaction are present in the *in vitro*-collected oral secretion of fleas, these may be incomplete antigens or haptens. To confirm this hypothesis, experiments were conducted on the induction of flea-bite hypersensitivity by injection of *in vitro*-collected material in combination with Freund's complete⁷ or incomplete⁸ adjuvants.

a. *In vitro*-collected oral secretion in saline was shaken well with an equal volume of Freund's complete adjuvant. Three intraperitoneal injections were given at weekly intervals, each injection corresponding to 2000 flea-hours. On the 4th week the animals were challenged with flea bites. The usual

delayed skin reactions were elicited in 12 of 15 animals (Table I, assay number 12). In comparison, only one of 14 control animals responded (Table I, assay number 13). These controls consisted of runs identical to those in which oral secretion was collected, except that no fleas were placed in the feeding chambers.

b. An experiment similar to the above was performed, but using Freund's incomplete adjuvant. Of five animals injected, only one reacted to challenging bites (Table I, assay number 17).

c. A solution of 1 mg/ml of the dried lyophilized *in vitro*-collected oral secretion in saline was shaken well with an equal volume of Freund's complete adjuvant. Three intraperitoneal injections of 0.5 mg each were given to each animal at weekly intervals. On the 4th week, 13 of 15 animals responded with typical delayed skin reactions to flea bite challenges (Table I, assay number 14).

Similar experiments were conducted with intradermal injections into the foot pads of the animals. The inocula consisted of 0.1 ml of *in vitro*-collected oral secretion in saline mixed with equal volumes of Freund's complete adjuvant (0.4 mg oral secretion per injection), and two injections were given at weekly intervals. On the 3rd week, all of 20 test animals reacted to flea bite challenges

⁷ Containing *Mycobacterium butyrecum*

⁸ Without *Mycobacterium butyrecum*.

whereas only 1 of 5 control animals reacted, and that only weakly (Table I, assays number 15, 16.)⁹

When another group of animals was injected identically to the above, but using Freund's incomplete adjuvant, none of the 5 test animals responded to flea bite challenges (Table I, assay number 18).

d. Two experiments were performed to determine the lowest dosage of oral secretion which, when injected in combination with Freund's complete adjuvant, would induce hypersensitivity to flea bites. One test consisted of 2 intraperitoneal injections and the other of 2 intradermal injections into the foot pads of guinea pigs at weekly intervals. Results shown in Table II indicate that hypersensitivity could be induced by as little as 0.01 mg per intraperitoneal injection, or by as little as 0.008 mg per intradermal injection. Although sensitivity was induced in some animals inoculated intradermally with dosages below 0.008 mg, the magnitude of the reactions was low. Similar results were obtained when dosage was correlated with induction of hypersensitivity by intradermal injections of whole flea extracts or by bites of fleas. Induction of hypersensitivity proved to be independent of dosage, provided the dosage did not fall below a certain threshold (Benjamini *et al.*, 1960a).

Correlation Between Hypersensitivity Induced by Flea Bites, Whole Flea Extract, and In Vitro-Collected Oral Secretion of Fleas

Experiments were carried out to determine whether cross reactivity exists between hypersensitivity induced by flea bites, whole flea extract, and *in vitro*-collected oral secretion. For these studies, five animals were sensitized by bites of 20 fleas each until delayed reactions appeared (5 to 7 days following the initial exposure). In another group, five animals were injected intradermally, twice at weekly intervals with 0.5 ml of 4

mg/ml whole flea extract in saline. Five additional animals were injected intradermally, twice at weekly intervals with 0.5 ml of 5 mg/ml of *in vitro*-collected oral secretion in combination with Freund's complete adjuvant. Another group of five animals was similarly injected each with 0.5 ml of 5 mg/ml *in vitro*-collected oral secretion in saline (without adjuvant). Controls consisted of five animals that were not injected. On the 3rd week following the initial injection, and on the 7th day following the initial bite exposure, each animal was challenged with flea bites, intradermal injections of 0.05 ml of 1 mg/ml extract of whole flea in saline, and 0.05 ml of a 5 mg/ml preparation of *in vitro*-collected oral secretion in saline.

Results shown in Table III indicate immunological cross reactivity between flea bites, whole flea extract, and the *in vitro*-collected material. The animals sensitized with flea bites responded with delayed skin reactions to challenges given with whole flea extract or with the *in vitro*-collected material. Similarly, animals sensitized with whole flea extract reacted to challenges given with flea bites and with the *in vitro*-collected material. Also, the animals sensitized with the oral secretion in combination with Freund's complete adjuvant reacted to challenges given with flea bites and with whole flea extracts. However, those animals injected solely with the *in vitro*-collected oral secretion in saline (without adjuvant) did not respond to any of the challenges.

Allergenicity of Oral Secretion Following Dialysis

The oral secretion from large numbers of fleas was dialyzed in the cold for four days against distilled water. The nondialysable portion was concentrated by lyophilization, resuspended in saline and Freund's complete adjuvant (1:1 v/v saline:adjuvant) and injected intraperitoneally three times at weekly intervals, into each of 10 guinea pigs at a dosage corresponding to 4000 flea-hours per injection. Results in Table I (assay number 22) show that none of the treated animals responded to challenging bites, although even at half this dosage, oral secretion controls

⁹ Since this report was written, several hundred animals were successfully sensitized to flea bites by such injections, with over 95% success on challenge. Less than 5% of control animals reacted to flea bite challenges.

TABLE III

The Immunological Relationship between Sensitization with Flea Bites, Whole Flea Extract, in Vitro-Collected Oral Secretion in Combination with Freund's Complete Adjuvant and in Vitro-Collected Oral Secretion in Saline

Sensitizing material	Challenging material			
	Flea Bites	Whole flea extract	<i>In vitro</i> -collected oral secretion	Saline
Flea Bites	++++ ^a	++	++	—
Whole flea extract	++++	++++	+++	—
<i>In vitro</i> -collected oral secretion in saline	—	—	—	—
<i>In vitro</i> -collected oral secretion in combination with Freund's adjuvant	++++	++	++	—
Control	—	—	—	—

^a Number of plus signs indicates severity of reactions.

(not dialyzed) induced hypersensitivity to flea bites when similarly injected (Table I, assay number 12).

A similar experiment was performed with intradermal injections of the nondialysable portion of oral secretion. Each of five animals was injected with a dosage equivalent to 0.4 mg per animal of whole, nontreated oral secretion (the material was injected in combination with Freund's complete adjuvant). None of these animals responded to the bite challenges (Table I, assay number 23).

The nondialysable portion of the oral secretion was also injected intraperitoneally in combination with Freund's complete adjuvant twice at weekly intervals at a dosage corresponding to 0.25 mg per injection. Only one of the five injected animals responded to the challenging bites (Table I, assay number 24).

To confirm further the dialysability of the substance responsible for the bite reaction, 5 ml of a solution of 8 mg/ml *in vitro*-collected oral secretion in saline was allowed to dialyze in the cold for 4 days against distilled water. Both the nondialysable portion and the dialysate were recovered and lyophilized. The dialysate was redissolved in 5 ml distilled water, and the nondialysable portion was redissolved in 5 ml saline. Each portion was mixed with an equal volume of Freund's complete adjuvant and 0.1 ml of the mixture was injected intradermally, twice at weekly intervals into the foot pad of each

guinea pig (0.4 mg starting material per injection). Data in Table I (assays number 25, 26) indicate that the nondialysable portion lost most of the activity (even those animals which reacted did so only mildly) whereas activity was recovered in the dialysate. This experiment is further evidence that the substance or substances responsible for the bite reaction are of relatively low molecular weight and are haptenic in character. This was further confirmed by molecular sieving, using gel filtration work that is reported in detail in our next communication (Young *et al.*, in press).

Thermal Stability of the Allergenicity of In Vitro-Collected Oral Secretion

Oral secretion in saline was heated in a closed tube for 4 hours at 95° C, then combined with Freund's complete adjuvant. This preparation was injected twice, at weekly intervals, into each of 10 animals at a dosage equivalent to 4000 flea-hours per injection. When challenged by flea bites, all of the treated animals responded with delayed skin reactions (Table I, assay number 19).

Another experiment was performed in which a solution of 1 mg/ml of oral secretion in saline was heated for 4 hours at 95° C in an open test tube. The material then was mixed with an equal volume of Freund's complete adjuvant and 0.5 ml was injected intraperitoneally, twice at weekly intervals, into each of four animals. Results shown in Table I

(assay number 20) indicate that of four animals injected, only 2 reacted to the challenging bites. In a similar experiment, the preparation was sealed in tubes under N_2 atmosphere and heated. All of five animals treated with this preparation reacted to challenges (Table I, assay number 21).

These experiments demonstrate the relative heat stability of the allergenic component in the oral secretion, but also suggest that the substance may undergo changes upon heating in the presence of air. (It is of interest that the oral secretion not only retained its allergenicity when heated in closed tubes but that this allergenicity was greatly enhanced when judged by the degree of skin reactivity in the challenged animals. At present, this phenomenon remains unexplained.)

DISCUSSION

Data presented here support the hypothesis that the *in vitro*-collected oral secretion of cat fleas contains the substance or substances responsible for hypersensitivity to flea bites. The possibility that the oral secretion was contaminated with extraneous flea materials appears minimal.

The results strongly suggest that the principle responsible for the bite reaction is not a complete antigen but rather, is haptenic in character. This conclusion is suggested by considerations listed below:

No sensitivity to flea bites could be induced by injections of oral secretion in saline, whereas intradermal challenges of animals hypersensitive to flea bites resulted in positive delayed skin reactions.

Sensitivity to flea bites was induced by injections of the oral secretion in combination with adjuvants or "carriers" such as Freund's complete adjuvant, or finely ground Silverlight membrane.

With the exception of three low grade responses, sensitivity to flea bites could not be induced by the nondialysable portion of the oral secretion. On the other hand, sensitivity was induced by the dialysate, suggesting that the substance or substances responsible for the bite reaction are of relatively low molecular weight and are therefore not proteins or polysaccharides

of high molecular weight. (This is supported by data from chemical and physical analyses of the *in vitro*-collected oral secretion as presented in the communication by Young *et al.*, in press.)

Flea-bite hypersensitivity was induced by oral secretion which had been heated for 4 hours at 95° C prior to injection. Thus the substance or substances responsible for the bite reaction are relatively heat stable.

Injection of oral secretion in saline could not induce sensitivity to flea bites, to whole flea extract, or to oral secretion. Sensitivity was induced only when oral secretion was inoculated in combination with Freund's complete adjuvant. These results again suggest that the allergenic materials present in oral secretion are not proteins or polysaccharides of high molecular weight, but are haptenic in nature.

The haptenic substance or substances responsible for the bite reaction appear to possess a high degree of affinity for fibrous proteins such as those present in Silverlight membrane. That the substance has a strong tendency to combine with or adsorb on other proteins is suggested by the hypothesis that in the whole flea extract the hapten is bound to proteins. This postulation is supported by the finding that whole flea extract could induce flea-bite hypersensitivity when injected intradermally in saline (without adjuvants), and by the observation that this property was not lost following dialysis (Benjamini *et al.*, 1960a).

It was reported by Hudson *et al.* (1960) and Benjamini *et al.* (1960a), that a flare-up of previously nonreactive bite sites occurred when guinea pigs reacted to flea bites following sensitization by bites of fleas. This phenomenon may possibly be explained by the hypothesis that, during *in vivo* feeding by the flea, a portion of the elements responsible for the bite reaction combine, or are adsorbed by the skin of the host (analogous to the retention of the hapten by Silverlight membrane when fleas feed *in vitro*). When the animal becomes reactive, these bite sites function in a manner analogous to a bite, thus eliciting skin reactions.

The facts presented here do not constitute definitive proof that the material or materials secreted during *in vitro* feeding are identical to those secreted during *in vivo* feeding. However, they do show that *in vitro*-collected oral secretion can induce an immunological response specifically related to flea-bite hypersensitivity, and that the substance or substances responsible for this phenomenon are haptens rather than complete antigens. In this connection, it was recently demonstrated in our laboratory that flea-bite hypersensitivity could be induced by the low-molecular-weight portion of saline extracts of whole fleas, and that mosquito-bite hypersensitivity could be induced by the low-molecular-weight portions of saline extracts of whole mosquitoes, when these portions were injected into guinea pigs in combination with Freund's complete adjuvant (unpublished).

In view of our findings to date, we propose the tentative hypothesis that, during the act of feeding on its mammalian host, a hapten released in the flea's oral secretion combines with substances in the host's skin and thus achieves full antigenic properties. It is this allergenically active conjugate which is responsible for the induction of flea-bite hypersensitivity.

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