THE JOURNAL OF IMMUNOLOGY
Vol. 95, No. 1

Printed in U.S.A.

THE ROLE OF COLLAGEN IN THE INDUCTION OF FLEA BITE HYPERSENSITIVITY[1]

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Received for publication September 21, 1964

It has been recently demonstrated that at least one allergen involved in hypersensitivity to bites of fleas is haptenic in nature. This has been shown by experiments in which guinea pigs were sensitized to flea bites by injections of *in vitro*-collected oral secretion of fleas and subsequently challenged with bites of fleas. This preparation could not induce flea bite hypersensitivity, unless injected in combination with adjuvants; studies on the size of the allergens utilizing dialysis showed that activity[2] was freely dialyzable (1). Experiments utilizing G-50 Sephadex showed that activity was associated with a molecular weight of less than 10,000. Furthermore, when G_{50} Sephadex was used, activity was associated with a molecular weight of >4000 as well as <4000 (2). Chemical analyses of the *in vitro*-collected oral secretion failed to reveal the presence of proteins (3). Injections of the preparation in saline into guinea pigs failed to induce skin reactivity as shown by subsequent challenges with this preparation. Stability experiments showed that allergenic activity related to flea bite hypersensitivity was stable to heat (95°C, 4 hr) (1), to treatment with 6 N HCl at 110°C for 24 hr and that the hydrolyzed material did not lose its capacity to induce flea bite hypersensitivity following gas chromatography (4). From the above, it can be realized that at least one allergen related to flea bite hypersensitivity is of low molecular weight and exhibits the properties of a hapten.

Assuming that the haptenic material collected *in vitro* is similar to that secreted by a flea during the biting process, studies on the mechanism of rendering the hapten a complete antigen were initiated.

Preliminary investigations toward this end (5) showed that from a biopsy taken from guinea pigs following bites, allergenicity related to flea bite hypersensitivity was found both in saline-extractable and saline-nonextractable portions of the skin. This was shown by experiments in which guinea pigs, injected with preparations of such biopsies, reacted to challenges given by bites of fleas. Furthermore, allergenicity was nondialysable and was capable of inducing flea bite hypersensitivity when injected without use of adjuvants. A hypothesis was formulated suggesting that the skin of the host contributes a carrier for the flea hapten, thus playing a role in the mechanism of flea bite hypersensitivity. The purpose of the present study was to test this hypothesis, to isolate components of the skin which may play a role in the induction of flea bite hypersensitivity and to ascertain their physical and chemical characteristics.

MATERIALS AND METHODS

Insects. Cat fleas, *Ctenocephalides felis felis* (bouche) were used. They were reared according to the method of Hudson and Prince (6).

Guinea pigs. White or albino guinea pigs weighing 250 to 300 g were used.

Exposure to bites. Starved 3-day-old fleas, 1000 in number and confined in round plastic capsules (5 cm in diameter, 2.5 cm high) were allowed to feed for 20 min on the shaved abdomen of each guinea pig.

Preparation of neutral salt-soluble and acid-soluble collagens. All operations of extraction and purification were carried out at 4°C.

Following exposure to flea bites, the exposed skin area was immediately biopsied, weighed and

[1] This work was supported by Grant No. AI-3966-04 from the National Institutes of Health, United States Public Health Service.

[2] Throughout this article, the term "activity" denotes the ability of a given preparation to induce flea bite hypersensitivity as evaluated by skin reactions in response to challenges given with bites of fleas.

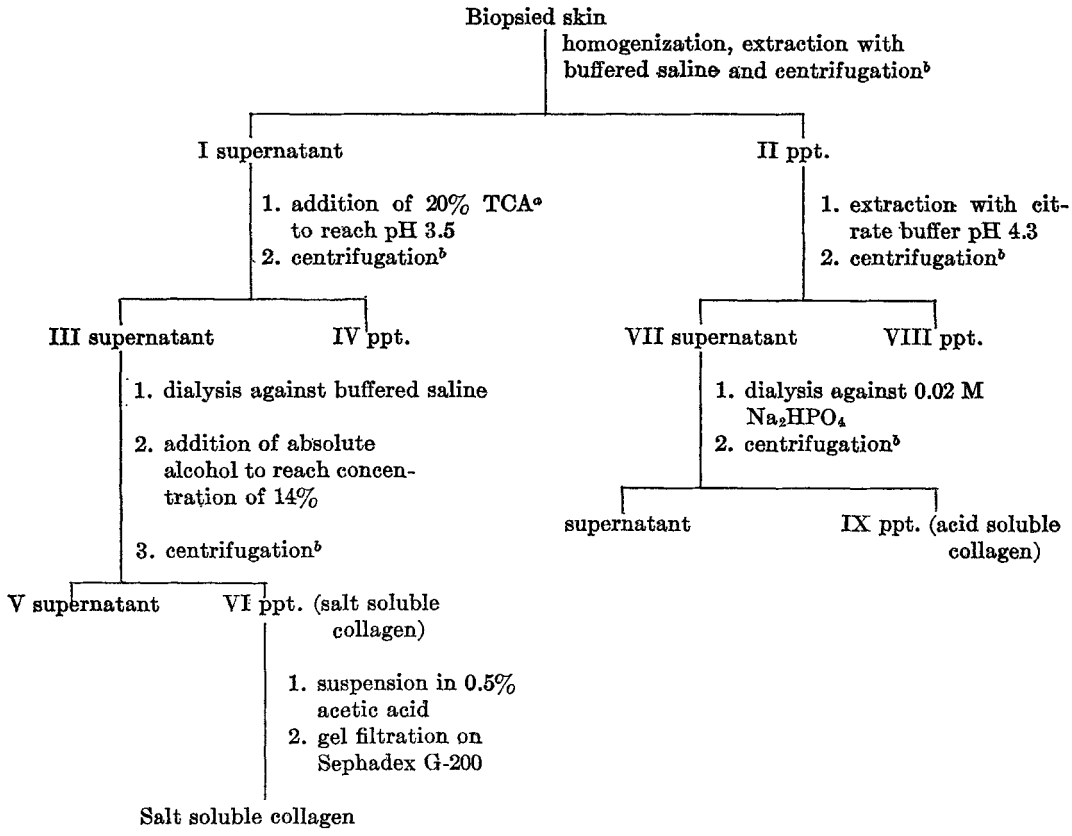

Figure 1. A schematic diagram describing the purification of salt-soluble and acid-soluble collagens. Fractions assayed for their capacity to induce hypersensitivity to flea bites are marked by Roman numerals.

homogenized with an all-glass homogenizer in 0.45 M saline buffered with 0.01 M phosphate buffer, pH 6.8, henceforth referred to as buffered saline. The ground skin was suspended in a volume of extraction medium yielding a concentration of 100 mg/ml. The preparation was shaken overnight and was then centrifuged at 8000 rpm for 1 hr to remove the coarse particles, followed by an additional 3 hr centrifugation at 17,000 rpm to remove the fine particles. The precipitates were combined and used as the source for the extraction of acid-soluble collagen. The supernatent resulting from the 17,000-rpm centrifugation was filtered through a medium Millipore filter to remove the fat layer. The method of Gross (7) was used for the purification of salt-soluble collagen from this supernatant. In a few cases, further purification was achieved by passing the salt-soluble collagen obtained by the above method through a column of Sephadex G-200, using buffered saline as the eluant.

Acid-soluble collagen was extracted with citrate buffer, pH 4.3, from the insoluble portion of the skin left after the extraction with buffered saline, and was purified according to the method of Gallop (8).

Identical procedures of extraction and purification were followed with normal skin (unexposed to flea bites), its fractions serving as controls.

A schematic diagram of the purification of salt soluble collagen and acid soluble collagen is shown in Figure 1.

Chemical analyses of skin fractions. The purification of salt- and acid-soluble collagens was followed by chemical analyses. Aliquots were taken from each of the fractions in the purifica-

TABLE I

Determination of total amino acids, proline and hydroxyproline of fractions obtained in the course of isolation of salt-soluble and acid-soluble collagens.

Fraction	Total Amino Acids Content	Proline		Hydroxyproline	
		Amount	Per cent total amino acids	Amount	Per cent total amino acids
	μmoles of leucine equivalents	*μmoles*		*μmoles*	
Salt-soluble					
I[a]	32.5	2.7	8.3	0.54	1.7
III	18.0	2.5	13.9	0.54	3.0
VI	1.7	0.28	16.4	0.18	10.6
I Control	60.0			0.76	1.3
III Control	15.2	1.47	9.7	0.42	2.7
VI Control	1.0	0.14	14.0	0.11	11.0
Acid-soluble					
VII	11.1	0.85	7.6	0.70	6.3
IX	8.0	1.42	17.8	1.41	14.2
VII Control	30.0			2.24	7.4
IX Control	7.0	1.25	17.8	1.03	14.7

[a] Roman numerals refer to the fractions as shown in Figure 1.

tion procedure, lyophilized and hydrolyzed in sealed tubes with 6 N HCl at 110°C for 24 hr. The hydrolysates were subjected to quantitative protein determinations using the ninhydrin technique described by Troll and Cannan (9). In these analyses, protein estimation was based on a standard consisting of leucine, and amounts of protein were expressed in terms of leucine equivalents. Proline and hydroxyproline analyses were carried out according to the methods described by Chinard (10) and by Neuman and Logan (11), respectively. In addition to these analyses, the final fractions, i.e., salt-soluble collagen and acid-soluble collagen, were subjected to amino acid analysis using the dinitrophenyl (DNP) method as described by Fraenkel-Conrat *et al.* (12) and to electron microscopic examination.

In vitro reconstitution of salt-soluble collagen. Preparations of purified salt-soluble collagen were suspended in water and incubated at 37°C overnight. A heavy precipitate formed; the supernatant was removed after centrifugation at 5000 rpm for 1 hr and the precipitate consisting of reconstituted salt-soluble collagen (13) was taken up with water to a concentration equivalent to 100 mg of skin/ml.

Assay of bitten skin fractions for their capacity to induce flea bite hypersensitivity

1. Sensitization. Each fraction to be assayed was adjusted to a concentration representing 500, 100 or 10 mg of skin/ml. Actual amounts of injected protein are expressed in terms of μmoles of leucine equivalents which were determined by the quantitative amino acids analyses as stated earlier. An aliquot of 0.25 ml was used for injection, mixed either with an equal volume of Freund's complete adjuvant (FCA) (Difco laboratories, Detroit, Michigan) or buffered saline. Guinea pigs in groups of five were given two intradermal injections each at weekly intervals.

2. Challenge with flea bites. This was performed 2 weeks following the initial injection according to the method described by Benjamini *et al.* (14). Skin reactions were evaluated on the basis of the diameter and the intensity of the erythematous areas at the bite site 24 hr after the bite. The following scale was used for rating the skin reactions produced by bites of fleas: − = no reaction; + = slight erythema approximately 1 mm in diameter; ++ = erythema of approximately 2 mm in diameter; +++ = moderately intense erythema, 3 mm in diameter; and ++++ = intense erythema more than 3 mm in diameter.

It is of importance to note at this point that the sizes of reactions following challenges with bites which were observed throughout the present experiments are typical of reactions involved in flea bite hypersensitivity induced by bites of fleas, by whole flea extracts, or by preparations of *in vitro*-collected saliva of fleas. The validity of the skin reaction induced by bites of fleas was ascertained histopathologically and reported in a recent paper by Larrivee *et al.* (15), where 24-hr reactions witness intense monocytic infiltration in the dermis at the bite site whereas no such infiltration occurred 24 hr after bites of nonsensitive guinea pigs.

RESULTS

Amino acid determination of the fractions isolated

Progress in the purification of salt-soluble collagen and acid-soluble collagen was followed by the determination of the percentage of proline and hydroxyproline of the total amino acid con-

TABLE II

Amino acid composition of salt-soluble and acid-soluble collagens[a]

Amino Acids	Salt-Soluble Collagen	Acid-Soluble Collagen
Glycine	36.2	35.1
Proline	14.6	16.4
Hydroxyproline	8.2	9.2
Glutamic + aspartic[b]	11.2	10.7
Alanine	7.9	10.1
Arginine	5.5	5.0
Leucine + valine[b]	5.1	4.3
Serine	3.0	3.7
Lysine + tyrosine[b]	2.8	3.4
Phenylalanine	1.5	2.1
Threonine	1.5	1.0

[a] The values represent an average of three determinations, and are given as moles/100 moles of amino acids. No corrections were made for amino acid decomposition which might have occurred during hydrolysis.

[b] No attempt was made to separate these amino acids.

TABLE III

Assay of saline-extractable and nonextractable fractions derived from flea-bitten and normal guinea pig skin for their capacity to induce flea bite hypersensitivity[a]

Fraction	Dosage Injected	No. of Reacting Animals/ No. Treated	Degree of Response to Challenge with Flea Bites
	μmoles of leucine equivalents		
I[b] with FCA	5.4	4/5	+++
I Control with FCA	10.0	1/5	+
II with saline		5/5	+++
II Control with saline		1/5	+

[a] All fractions were made up to a concentration equivalent to 100 mg of skin/ml; 0.25 ml of the preparation mixed with either Freund's complete adjuvant (FCA) or buffered saline was injected. Protein content is expressed in terms of μmoles of leucine equivalents of its hydrolysate.

[b] Roman numerals refer to the fractions as shown in Figure 1.

tent (the latter expressed in terms of μmoles of leucine equivalents). Results of two typical preparations, one prepared from guinea pig skin exposed to flea bites, the other from normal guinea pig skin, are summarized in Table I where the increases in the relative contents of proline and hydroxyproline are shown for each purification step.

Amino acid composition of salt-soluble collagen and acid-soluble collagen

The results of amino acid analyses of salt-soluble and acid-soluble collagens as determined by the DNP method are summarized in Table II. These results agree with those reported by several workers (16–18), establishing the collagenous nature of the proteins.

Activity of various fractions obtained during the purification of salt-soluble collagen

Activity of skin extracted with 0.45 M saline (Fractions I and II, Figure 1). Biopsies from normal and flea-bitten skin were extracted with 0.45 M buffered saline at a ratio of 1 g of skin/10 V of saline. The ability of each fraction to induce flea bite hypersensitivity was assayed by injections of 0.25 ml of each fraction mixed with an equal volume of Freund's complete adjuvant (Fraction I) or of buffered saline (Fraction II). Results shown in Table III demonstrate the capacity of both the saline-extractable as well as the saline nonextractable fractions derived from flea-bitten skin to induce flea bite hypersensitivity. The equivalent fractions derived from normal skin failed to induce sensitivity.

Activity of saline extracts of normal and flea-bitten skin following treatment with trichloroacetic acid and alcohol (Fractions III to VI, Figure 1). The buffered saline extract (Fraction I, Fig. 1) was placed in a dialysis bag which in turn was immersed in approximately ten times its volume of cold buffered saline. A 20% trichloroacetic acid (TCA) solution was added dropwise to the outside medium until the pH reached 3.5. This resulted in the formation of a heavy granular precipitate inside the bag within 30 to 60 min. The contents of the bag were then centrifuged. The resulting precipitate and supernatant were dialyzed against large volumes of buffered saline in order to remove the TCA and readjust the pH of the fractions to neutrality for injection purposes. Controls consisted of samples obtained from saline extract of normal skin which were processed in an identical way. Each fraction was made up to a concentration corresponding to

TABLE IV

Assay of fractions obtained from TCA and alcohol fractionations of saline extracts of control and flea-bitten guinea pig skin for their capacity to induce flea bite hypersensitivity[a]

Fraction	Dosage Injected	No. of Reacting Animals/No. Treated	Degree of Response to Challenge with Flea Bites
	μmoles of leucine equivalents		
III[b]	3.0	5/5	++++
	0.3	2/5	++
III Control	2.5	1/5	+
	0.25	1/5	+
IV		2/5	+
IV Control		1/5	+
V		0/5	−
		0/5	−
V Control		0/5	−
		0/5	−
VI	1.5	4/5	++
	0.3	4/5	+++
	0.03	5/5	+
VI Control	0.7	2/5	+
	0.14	1/5	+
	0.01	0/5	−

[a] All fractions were made up to a concentration equivalent to 100 mg of skin/ml. For injection, 0.25 ml of each preparation was mixed with Freund's complete adjuvant. Protein content is expressed in terms of μmoles of leucine equivalents of its hydrolysate.

[b] Roman numerals refer to the fractions as shown in Figure 1.

100 mg of skin/ml; 0.25 ml of this concentration and 0.25 ml of one tenth of this concentration were assayed for activity related to flea bite hypersensitivity by injection with an equal volume of FCA. Results are shown in Table IV.

Absolute alcohol was added dropwise to Fraction III (Fig. 1) until a final concentration of 14% alcohol was reached. The precipitate which was allowed to form overnight was recovered by centrifugation, and was taken up in 0.5% acetic acid to concentrations representing 500, 100 and 10 mg of skin/ml. The concentration of the supernatant was readjusted with buffered saline to represent 100 and 10 mg of skin/ml. Each fraction was assayed for activity by injections of 0.25 ml in combination with an equal volume of FCA. Results are shown in Table IV.

TABLE V

Assay of fractions obtained by citrate extraction of the saline-insoluble portion of guinea pig skin, and by phosphate precipitation of the extract for their capacity to induce flea bite hypersensitivity[a]

Fraction	Dosage Injected	No. of Reacting Animals/ No. Treated	Degree of Response to Challenge with Flea Bites
	μmoles of leucine equivalents		
VII[b]	1.85	5/5	+++
	0.185	4/5	+++
VII Control	5.0	0/5	−
	0.5	0/5	−
VIII		5/5	++
		0/5	−
VIII Control		0/5	−
		0/5	−
IX	1.3	3/5	++
	0.13	1/5	+
IX Control	1.2	0/5	−
	0.12	0/5	−

[a] All fractions were made up to a concentration equivalent to 100 or 10 mg of skin/ml; 0.25 ml of each fraction mixed with an equal volume of Freund's complete adjuvant was injected. Protein content is expressed in terms of μmoles of leucine equivalents of its hydrolysate.

[b] Roman numerals refer to the fractions as shown in Figure 1.

Data presented in Table IV show that flea bite hypersensitivity could be induced by the fraction which was nonprecipitable with TCA (Fraction III). Furthermore, the data show that, following alcohol precipitation of the above fraction, activity was recovered in the precipitate.

Activity of various fractions obtained during the purification of acid-soluble collagen

Activity of citrate extract of the saline-insoluble portion of the skin (Fraction II, Figure 1) and of its phosphate precipitation product (Fractions VII to IX, Figure 1). The residue left after the saline extraction of bitten skin (Fraction II, Fig. 1) was extracted with 0.1 M citrate buffer, pH 4.3. The extract (Fraction VII, Fig. 1) was separated from the citrate-insoluble residue (Fraction VIII, Fig. 1) by centrifugation at 17,000 rpm for 1 hr and was then dialyzed for 48 hr in the cold against a large volume of 0.02 M dibasic sodium

TABLE VI

Effect of degree of fibrillation of collagens carrying activity related to flea bite hypersensitivity on their capacity to induce the hypersensitivity[a]

Preparation	Adjuvant	Dosage Injected	No. of reacting Animals/No. Treated	Response
		μmoles of leucine equivalents		
Salt-soluble collagen obtained from bitten skin (Fraction VI, Fig. 1)	FCA[b]	0.3	5/5	+++
		0.03	3/5	++
	Saline	0.3	3/5	+
		0.03	3/5	++
Reconstituted salt-soluble collagen obtained from bitten skin	FCA	0.3	4/5	+
		0.03	3/5	++
	Saline	0.3	4/5	++++
		0.03	4/5	+++
Acid-soluble collagen obtained from bitten skin (Fraction X, Fig. 1)	FCA	1.3	3/5	++
		0.13	2/5	+
	Saline	1.3	5/5	++++
		0.13	2/5	+

[a] Protein content is expressed in terms of μmoles of leucine equivalents of its hydrolysate.
[b] FCA = Freund's complete adjuvant.

phosphate, resulting in the formation of a precipitate in the dialysis bag. Following centrifugation at 17,000 rpm for 1 hr, the precipitate was collected and resuspended in buffered saline (Fraction IX, Fig. 1). Controls were prepared from Fraction II of normal skin by the same procedure. Each of the above fractions was made up to a concentration equivalent to 100 mg and 10 mg of skin/ml and was assayed by injection of 0.25 ml of the fraction mixed with an equal volume of Freund's complete adjuvant. Results summarized in Table V show that the major portion of the activity resided in the citrate extract (Fraction VII) of bitten skin. Sodium phosphate precipitation of this extract resulted in the formation of an active precipitate.

Effect of degree of fibrillation of collagens carrying activity related to flea bite hypersensitivity on their ability to induce hypersensitivity to flea bites.

The finding that the salt-soluble collagen as well as the acid-soluble collagen obtained from skin biopsies following exposure to bites of fleas carried activity related to flea bite hypersensitivity (Tables IV and V, respectively) raised two questions: first, can the salt-soluble collagen carrying the activity form a more integrated fibril, and second, how would this change in size affect its capacity to induce the hypersensitivity? In order to obtain information regarding these questions, activity-carrying salt-soluble collagen was allowed to reconstitute to form a fibril. The preparation was made up to concentrations equivalent to 100 or 10 mg of skin/ml of water; 0.25 ml of salt-soluble collagen, of reconstituted salt-soluble collagen and of acid-soluble collagen were thoroughly mixed with equal volumes of Freund's complete adjuvant or buffered saline, and assayed for their capacity to induce flea bite hypersensitivity. Results summarized in Table VI show that of all the preparations tested, the reconstituted salt-soluble collagen derived from flea-bitten skin exhibited the highest capacity to induce flea bite hypersensitivity when injected in saline.

DISCUSSION

In a recent communication from this laboratory, the role of skin components was implicated in the mechanism of induction of hypersensitivity to flea bites (5). Since collagenous proteins are major constituents of animal skin, attention was focused on their participation in this mechanism. Thus, salt-soluble and acid-soluble collagens derived from flea-bitten guinea pig skin were

purified and assayed for their capacity to induce flea bite hypersensitivity upon injection into guinea pigs.

Tables III and IV depict the assay results of various fractions obtained during the purification of normal and flea-bitten salt-soluble collagen and acid-soluble collagen. Salt-soluble collagen from bitten skin is clearly demonstrated to be the fraction in the neutral salt extract of the skin, which is responsible for the induction of flea bite hypersensitivity. The identity of salt-soluble collagen was established by the DNP-amino acid analysis as shown in Table II, where the amino acid composition of the preparation is in agreement with that found by other workers (16–18). Furthermore, the collagenous nature of salt-soluble collagen was confirmed by electron microscope examination.

The finding that salt-soluble collagen derived from flea-bitten skin was involved in sensitization suggested that acid-soluble collagen might similarly be involved. Table V shows that this is indeed the case: acid-soluble collagen derived from flea bitten skin had the capacity to induce flea bite hypersensitivity. This fact may account in part for the activity residing in the fraction which is nonextractable with a neutral salt solution (Fraction II, Fig. 1).

From data presented in Tables IV and V it can be concluded that both salt-soluble and acid-soluble collagens, isolated from biopsies following bites of fleas, carry activity related to flea bite hypersensitivity, thus implicating these proteins in the sensitization mechanism to bites of fleas.

Although the identity of the allergen secreted by the flea into guinea pig skin during the bite is unknown, studies on *in vitro*-collected oral secretion of fleas showed that the latter contains an allergen or allergens haptenic in nature (2, 4). Assuming that the saliva collected *in vitro* is similar to that injected by the flea into the skin, then the flea introduces into the skin a hapten. For the induction of hypersensitivity this hapten has to be associated with a high molecular weight carrier, as is usually the case in the mechanism of sensitization by haptens. Since activity related to flea bite hypersensitivity was found to reside in acid-soluble and salt-soluble collagens isolated from flea-bitten skin, it is reasonable to assume that the flea hapten is now associated in some form with these proteins, and that through this association hypersensitivity to flea bites is induced. In this connection it is important to mention that flea bite hypersensitivity could be induced by injection of salt-soluble collagen which was allowed to react *in vitro* with *in vitro* collected oral secretion of fleas (19). The nature of the association between the hapten and collagen is not clear. On the one hand it may be due to adsorption of the hapten to the fibrous protein. This possibility is not too likely, in view of the fact that these fibrous proteins from the bitten biopsy retained their activity related to flea bite hypersensitivity even after treatment at the various pH's which were used for their isolation; nevertheless the possibility of adsorption can not be excluded. On the other hand the possibility exists that the hapten has been chemically bound with moieties of salt-soluble or acid-soluble collagens, or both.

Table VI shows data obtained from an experiment designed to compare the capacity of different preparations of collagens derived from flea-bitten skin to induce flea bite hypersensitivity. As may be realized from the data, salt-soluble collagen, acid-soluble collagen and reconstituted salt-soluble collagen could induce hypersensitivity. Salt-soluble collagen induced strong sensitivity when injected in combination with FCA. Sensitivity was also induced by its injection in saline. Acid-soluble collagen induced strong sensitivity when administered in saline; oddly, when it was injected with FCA only weak but significant sensitivity was induced. Similarly, reconstituted salt-soluble collagen induced a high degree of sensitivity when injected in saline and, as in the case of acid-soluble collagen, weaker sensitivity was induced when reconstituted salt-soluble collagen was injected in combination with FCA. From data presented in Table VI it can be seen that sensitivity is best induced by acid-soluble collagen and by reconstituted salt-soluble collagen derived from flea-bitten skin, when these preparations were injected in saline. At present we can not offer an explanation for the phenomenon of retardation of sensitization by FCA.

By examining the quantitative aspects connected with the capacity of salt-soluble, acid-soluble and reconstituted salt-soluble collagens to induce flea bite hypersensitivity (Table VI), it can be seen that the lowest quantity of acid-soluble collagen which was capable of inducing flea bite hypersensitivity was 1.3 μmoles of

leucine equivalents when injected in saline. The lowest quantity of salt-soluble collagen capable of induction was 0.03 μmoles of leucine equivalent when injected either in saline, or in combination with FCA. However, excellent sensitization was achieved when 0.03 μmoles of leucine equivalent of reconstituted salt-soluble collagen were injected in saline, whereas even a larger amount of acid-soluble collagen (0.13 μmoles of leucine equivalent) injected in saline failed to sensitize. This phenomenon may be explained by considering the degree of organization of the collagen fiber *in situ*: Newly formed collagen forms a looser, less stable fibrillar array than older collagen (20). Since cold neutral solutions are weaker dispersing agents for collagen than are acidic media, they can extract only the more recently formed, poorly integrated fibrils (salt-soluble collagen), whereas more integrated fibrils (acid-soluble collagen) are extractable by acidic media only. It is, therefore, reasonable to assume that a given quantity of poorly integrated fibrils (salt-soluble collagen) would possess a larger surface area for interaction with the flea hapten than would the same quantity of the more integrated fibril (acid-soluble collagen). Stated in a different manner, less salt-soluble collagen would be required to associate with a given quantity of hapten than would acid-soluble collagen.

On the basis of the foregoing, the possible role of collagen fibrils in the induction of hypersensitivity to the insect hapten can be related to the state of their *in situ* organization: the poorly integrated salt-soluble collagen which possesses a larger surface area than the more integrated acid-soluble collagen may serve as a better "trap" for the hapten secreted by the flea, following penetration into the dermal layer of the skin, than does acid-soluble collagen. On the other hand, although not as efficient in "trapping" the hapten as salt-soluble collagen, acid-soluble collagen serves as an excellent carrier for the hapten. Combining these two phenomena suggests that salt-soluble collagen carrying the flea hapten which subsequently forms a fibril should be a strong sensitizer. This is indicated by the activity of reconstituted salt-soluble collagen obtained from flea-bitten skin (Table VI).

Acknowledgments. We wish to thank the staff of the San Francisco Field Station Communicable Diseases Center Activity, United States Public Health Service, for their encouragement of this work and the generous use of their laboratory facilities.

We are grateful to Mr. H. Koehler and Mr. E. A. Benjamini, Research and Development, Fairchild Semiconductor Division, Palo Alto, California, for the electron microscope examination of our collagen fractions. We are also indebted to Dr. J. D. Young of our laboratory for her keen interest and valuable advice.

SUMMARY

Salt-soluble and acid-soluble collagens were isolated from guinea pig skin following bites of fleas, and were shown to possess the capacity to induce flea bite hypersensitivity upon injection into recipient guinea pigs. Less salt-soluble collagen was required for induction of sensitivity than was acid-soluble collagen. Flea hapten associated with salt-soluble collagen did not hinder the latter's *in vitro* reconstitution. The *in vitro* reconstituted product served as a better carrier for the hapten than the nonreconstituted form.

REFERENCES

1. Benjamini, E., Feingold, B. F., Young, J. D., Kartman, L. and Shimizu, M., Exp. Parasit., *13:* 143, 1963.
2. Young, J. D., Benjamini, E., Feingold, B. F. and Noller, H., Exp. Parasit., *13:* 155, 1963.
3. Feingold, B. F. and Benjamini, E., Ann. Allerg., *19:* 1275, 1961.
4. Benjamini, E., Young, J. D., Leung, C. and Feingold, B. F., Unpublished data.
5. Benjamini, E., Feingold, B. F. and Kartman, L., Exp. Parasit., *14:* 75, 1963.
6. Hudson, B. W. and Prince, F. M., Bull. W. H. O., *19:* 1126, 1958.
7. Gross, J., J. Exp. Med., *107:* 247, 1958.
8. Gallop, P. M., Arch. Biochem. Biophys., *56:* 486, 1955.
9. Troll, W. and Cannan, R. K., J. Biol. Chem., *200:* 803, 1953.
10. Chinard, F. P., J. Biol. Chem., *199:* 91, 1952.
11. Neuman, R. E. and Logan, M. A., J. Biol. Chem., *184:* 299, 1950.
12. Fraenkel-Conrat, H., Harris, J. I. and Levy, A. L., *Methods of Biochemical Analysis*, Edited by D. Glick, Vol. 2, p. 359, Interscience Publishers Inc., New York, 1955.
13. Wood, G. C. and Keech, M. K., Biochem. J., *75:* 588, 1960.

14. Benjamini, E., Feingold, B. F. and Kartman, L., Exp. Parasit., *10:* 214, 1960.
15. Larrivee, D. H., Benjamini, E., Feingold, B. F. and Shimizu, M., Exp. Parasit., *15:* 491, 1964.
16. Courts, A., Biochem. J., *81:* 356, 1961.
17. Rubin, R. L., Pfahl, D., Speakman, P. T., Davison, P. F. and Schmitt, F. D., Science, *139:* 37, 1963.
18. Hafter, R. and Hormann, H., Z. Physiol. Chem., *330:* 169, 1963.
19. Michaeli, D., Benjamini, E., Feingold, B. F. and Miner, D., Unpublished data.
20. Gross, J., J. Exp. Med., *108:* 215, 1958.