ELECTRON MICROSCOPIC AND BIOCHEMICAL CHARACTERIZATION OF COLLAGEN IN BLATTARIAN INSECTS

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ABSTRACT

The occurrence of collagen in the cockroach *Leucophaea maderae* has been demonstrated by electron optical and biochemical techniques. Electron micrographs of tissues of this and a related species (*Blaberus craniifer*) are presented and they show that collagenous-type fibers occur in the stroma of nonneural as well as neural organs of these insects. Hydroxyproline and hydroxylysine, amino acids considered to be "markers" for collagen, have been shown to be present in proteins extracted from material rich in neuroglandular tissue (corpus cardiacum plus corpus allatum). Trimmed carcasses of *Leucophaea maderae* have been shown to contain a protein or proteins soluble in hot trichloroacetic acid, with compositional characteristics similar to those of collagens in general, including diagnostic proportions of glycine, proline, hydroxyproline, and hydroxylysine. This collagen is not soluble in dilute acetic acid or in concentrated solutions of guanidinium chloride. It is measurably digested by bacterial collagenase.

INTRODUCTION

Insect organs are enveloped and, in some cases, partitioned by layers of connective tissue which separate the parenchyma from the circulating hemolymph. This stromal element has received particular attention in the case of the nervous system because its permeability properties bear on certain neurophysiological considerations. A combination of histochemical, electron microscopic, birefringence, and X-ray diffraction studies has led to the general conclusion that the noncellular component of the nerve sheath, called the neural lamella, consists of a matrix in parts of which are embedded collagen-like fibrils (see Smith and Treherne, 20). The suggestion by Smith and Treherne that this fibrous component is restricted to the sheaths around nervous structures is no longer tenable. A collagen-like material also forms part of the stroma of insect nonnervous tissues (17–19). The present study is intended to establish the degree to which this fibrous element is related to other known collagens.

In the course of comparative studies dealing with the fine structural properties of the neuroendocrine apparatus of insects, a suitable material was found which provided information on this question. Among various neural and nonneural organs of the cockroaches *Leucophaea maderae* and *Blaberus craniifer*, the corpora cardiaca and allata were selected for an ultrastructural as well as biochemical analysis. This neuroglandular organ complex has a particularly well developed stroma consisting of a substantial sheath from which numerous processes take their origin to permeate the parenchyma. Additional ultrastructural studies provided information on the stroma of another endocrine organ, the prothoracic gland, and of

several nonglandular tissues such as musculature and fat body.

Electron micrographs of the selected organs revealed that the connective tissue fibrils have a banding characteristic of collagen. Chemical studies were then undertaken to determine whether these fibers have a composition characteristic of the several classes of collagens, vertebrate and invertebrate.

MATERIAL AND METHODS

The insects used in this study (*Leucophaea maderae, Blaberus craniifer*) were taken from colonies that had been bred under controlled conditions in our laboratory for many years. Their diet consisted of dog chow and apples. For ultrastructural studies, an extensive number of electron micrographs was available covering 87 insect specimens that had been fixed at a variety of stages of their normal life cycles, as well as after various experimental procedures (starvation, castration, injection of certain chemicals, etc.). The following fixation procedures were used: (1) 1% osmium tetroxide, veronal acetate buffer, pH 7.8; (2) 2% osmium tetroxide, veronal acetate buffer, pH 7.4–7.8; (3) 2% osmium tetroxide, veronal acetate buffer, pH 7.6, followed by 10% formalin for 10 days; (4) 5% glutaraldehyde, cacodylate buffer, pH 7.0, followed by 2% osmium tetroxide, veronal acetate buffer, pH 7.4; (5) 1% sodium permanganate, veronal acetate buffer, pH 7.4. All fixations were carried out at 4°C. Among "staining procedures" used were uranyl acetate (0.25% in 50% ethanol) (30 min) and lead citrate (15 min), either alone or in combination. The Epon-embedded sections were studied in an RCA EMU 3G microscope at 100 kv.

Two separate experiments were performed to ascertain, by biochemical means, the presence of collagen in the tissues of *Leucophaea maderae*. In the first, excised neuroglandular tissue known to be rich in stroma was analyzed, and in the second, trimmed carcasses were examined in order to increase the yield.

Collagens are characterized by the presence of glycine as almost one-third of the total amino acid residues, although in several collagens, for example that of the cuticle of *Ascaris lumbricoides* (9), the glycine residues constitute about one-fourth of the total, and the proline residues somewhat less than one-third of the total. A second characteristic of collagens is the occurrence of pyrrolidine imino acids, proline and hydroxyproline, in amounts totaling approximately one-fifth to one-third of the total residues. In most collagens, proline occurs to a larger extent than hydroxyproline, but in the case of the collagen of the cuticle of *Lumbricus terrestris* (11), hydroxyproline is about 14 times more abundant than proline. Proline, however, is present in some quantity in other types of tissue proteins; hydroxyproline occurs only in collagen, and, among vertebrates, perhaps in elastin. Thus the occurrence of hydroxyproline becomes a "marker" or indicator for the presence of collagen.

Another amino acid that occurs in many collagens and in no other known tissue proteins is hydroxylysine. At most, however, only a few residues of hydroxylysine are present in collagen. Other features of most, but perhaps not all, collagens are the low content of tyrosine, the absence of trytophan and in some cases cysteine or cystine, and a characteristic distribution of other amino acids. Thus, an amino acid analysis of a purified protein enables one to relate it to collagens that are known; and an analysis of a given tissue, even though the components are not separated, may reveal the probable presence of collagen if hydroxyproline or hydroxylysine, or both, are present.

A physicochemical property of tropocollagen, the fundamental unit of collagen, is its solubility in weakly acidic media. This property is of great use in the extraction of certain collagens, which may be achieved by application of solutions of acetate or citrate at pH values of three to five. The bulk of tissue collagen does not dissolve under these conditions, however, and complete solution is accomplished only by denaturation ("gelatinization"). Denaturation and consequent solubilization of collagen occurs when a collagenous tissue is treated with concentrated solutions of guanidinium chloride or when it is heated in aqueous or aqueous-acidic media at relatively elevated temperature and pressure. Thus, autoclaving in the presence of water may cause insoluble tissue collagen to become converted to soluble gelatin.

In the present study, therefore, these and certain other considerations were applied in order to determine whether collagenous material was, indeed, present in the stroma of *Leucophaea maderae*. No attempt was made to obtain quantitative recovery of all collagenous fractions because of the extremely small quantities with which one is dealing in these species.

EXPERIMENT 1: Tissues (400 mg wet weight) from 200 adult specimens of *Leucophaea maderae* were pooled. Male and female animals were removed from stock colonies and the corpora cardiaca together with the attached corpora allata were separated from surrounding structures under a dissecting microscope and placed immediately into a freezer. At time of use, the tissue was thawed, homogenized briefly by hand with a ground-glass homogenizer, and divided into two equal portions. One portion was placed in a dialysis sack and dialyzed against three changes of 50 ml each of water at 4°C (for a total of 36 hr), and the combined dialysates were lyophilized. This material, containing the free amino acids, was labeled D. The

material remaining in the sack was centrifuged at 39,000 *g* for 10 min at 2°C, and a supernatant fraction (S) and a residue (R) were obtained. Fractions S and R were hydrolyzed separately by treatment with 6 N HCl at 110° for 22 hr. The second portion of homogenized tissue, equivalent to the tissue from 100 roaches, was also dialyzed as described, but, in this instance, the material remaining in the sack was transferred to a tube and autoclaved for 24 hr at 15 lb. of pressure. The autoclaved material was centrifuged as described, and the supernatant (autoclaved) fraction (AS) and a residue (autoclaved) (AR) were obtained. These fractions were also hydrolyzed in order to free amino acids by heating in 6 N HCl. The dialysate (D) and the four hydrolyzed fractions were analyzed for amino acid contents by the method of Piez and Morris (13).

EXPERIMENT 2: 400 specimens of *Leucophaea maderae* were removed from stock colonies on the day they emerged as adults. In order to reduce bulk and to minimize possible interference with the amino acid determination by chitinous material, wings, legs, and antennae were removed. Furthermore, in each case the abdominal cavity was opened so that we could examine whether the digestive tract was empty. If an animal already had ingested its recently shed cuticle, the foregut was removed. The trimmed carcasses were pooled, yielding a total weight of tissue of 472 g. The material was stored in a freezer until used.

At the time of use, the cockroach carcasses were thawed and then homogenized in 2 liters of cold 0.5 M acetic acid. The homogenate was stirred at 4°C for 48 hr, and then centrifuged at 13,200 *g* for 10 min at 2°C. The supernatant was decanted from the residue (R), and was made 1.7 M with respect to NaCl by addition of solid salt. This mixture was stirred for 2 hr at 4°C and left overnight. It was then centrifuged at 13,200 *g* for 20 min at 2°C. The resulting supernatant was filtered through glass wool and dialyzed against 0.02 M disodium hydrogen phosphate. A very small amount of precipitate formed in the dialysis sack, and was discarded. The supernatant was lyophilized, labeled A, and saved for analysis.

The residue of tissue (R) was extracted twice with 2 liters of 0.5 M acetic acid, and the supernatants were decanted and discarded. The washed residue was then mixed with 2 liters of 5 M guanidinium chloride, pH 7.6, which had been clarified previously by addition of Norit followed by filtration through Whatman No. 2 paper. The mixture of residue and guanidinium chloride solution was stirred for 3 days at 4°C. The material was then centrifuged at 13,200 *g* for 20 min at 2°C. The resulting supernatant was dialyzed against distilled water at 4°C until free of guanidinium chloride. Within 2 hr after initiation of dialysis, layers of fibrous material, cotton-like in appearance, appeared in the sack. At conclusion of dialysis, the material in the sack was centrifuged and the supernatant decanted and discarded. The fibrous material was washed, by suspension and centrifugation, three times with cold distilled water, labeled G, and saved for analysis.

The guanidinium chloride–extracted tissue residue (R) was suspended in 2 liters of cold distilled water, and the mixture stirred for 24 hr, then centrifuged. This washing of the residue was repeated twice. The washed residue was stirred with 200 ml of 5% trichloroacetic acid at 90° for 1 hr according to the procedure of Fitch, Harkness, and Harkness (6). The mixture was cooled, and then centrifuged at 13,200 *g* for 20 min at room temperature. The residue was reextracted once more with trichloroacetic acid, and a second extract obtained. Each of the extracts was dialyzed against water until it was free of trichloroacetic acid, which resulted in the appearance of fibrous material in each instance. The two portions of fibrous material were combined in a fraction (T). An aliquot of T was subjected to amino acid analysis.

RESULTS AND DISCUSSION

Insects, the most prominent group of invertebrates, should attract a good deal of attention in comparative studies of connective tissues. The structural and functional properties of insect stroma are of interest not only in comparison with those observed among vertebrates and nonarthropod invertebrates, but also because of the special requirements existing in the insect organism. Among these requirements are the need for support and compartmentalization of tissues in the absence of an internal skeleton, the requirement for separating the parenchymal tissues from the circulating hemolymph, and the need for flexibility in the changing architectural arrangements of organs in the course of growth and metamorphosis.

Ultrastructure

Figs. 1–4 demonstrate that acellular insect stroma of nonneural organs consists of a relatively structureless matrix plus a fibrous component, as does the neural sheath. From a structural point of view, the fibrils greatly resemble collagen as known in other groups of animals.

In the corpora cardiaca of *Leucophaea*, for example, the fibrillar elements of the stroma form an interlacing network in random orientation embedded in the matrix. In electron micrographs, individual fibrils appear, for the most part, as more or less oblique profiles. Their most conspicuous property is the existence of periodic cross-banding. Because of differences related to

fixation procedures and difficulties in obtaining precise calibrations, the axial periodicities measured in the present material should be considered as approximations. The values determined for most of the cases ranged between 570 and 610 A. In material postfixed in formalin for a prolonged period, the axial periodicity was approximately 520 A. In addition to some fluctuations in cross-banding patterns, the diameters of the fibrils vary over a wide range (in *Leucophaea* approximately between 150 and 1000 A). Similar observations were reported for the ganglionic sheath by Rehberg (15) who also pointed out the finely granular and filamentous structure of the matrix and its scarcity in the vicinity of the fibrils.

From data on invertebrates available in the literature, it can be concluded that their connective tissue fibrils show considerable variation in ultrastructure. This applies to fibril diameter as well as axial periodicity. For the latter, values as low as $\sim$170 A have been determined in certain forms of insects (14). In other species, including those used in the present study, periodicities of approximately 500–600 A are characteristic. The higher of these values holds for the onychophoran *Peripatopsis* (16), as compared with the somewhat lower measurement of 500 A for the xiphosuran *Limulus* (5). Thus, it appears, from various though still limited samples, that invertebrate collagens may show cross-bandings comparable to that in vertebrates, but that the periodicity measurements in the former tend to fall somewhat or considerably below 640 A.

Biochemistry

Biochemically, there is considerable similarity between the insect collagen analyzed in the present study and that of different species examined by other investigators. Of special significance are the observations, discussed below, that amino acids considered diagnostic of collagen occur in both classes of materials analyzed, i.e., the isolated neuroglandular tissues and the whole carcasses.

Table I shows the amino acid analysis of fractions of the tissue complex consisting of corpus cardiacum and corpus allatum. The data reveal that a water-insoluble residue of this neuroglandular tissue contained a significant amount of hydroxylysine and a smaller but detectable amount of hydroxyproline. Autoclaving of the tissue caused a portion of it to dissolve, and analysis of this portion showed that it contained approximately equal amounts of hydroxylysine but not of hydroxyproline. The residue after autoclaving still contained detectable amounts of hydroxylysine but not of hydroxyproline. Thus, one may conclude that the two amino acids considered markers for the presence of collagen occur in measurable amounts in the neuroglandular tissue of the cockroach, and that a portion of the protein containing these amino acids becomes soluble on autoclaving just as most other known collagens do. Because the amounts of material with which we had to deal were so small, we were unable to isolate, in these experiments, a purified protein exhibiting all of the compositional characteristics of other collagens. The data suggest that the protein or proteins in the tissue containing the marker amino acids had a somewhat larger content of hydroxylysine than of hydroxyproline. However, the significance of these data can be evaluated properly only after a purified collagen is obtained and analyzed.

Additional evidence that the neuroglandular

FIGURES 1–4 Collagen fibrils embedded in extracellular matrix of nonneural organs of cockroaches.

FIGURE 1 Connective tissue partition in corpus allatum of old castrated female of *Leucophaea*. 1% OsO_4, Epon, uranyl acetate. × 72,900.

FIGURE 2 Connective tissue sheath of corpus allatum of normal old adult female of *Leucophaea*. 1% OsO_4, Epon, uranyl acetate. × 47,700.

FIGURE 3 Connective tissue sheath of prothoracic gland of male last instar nymph of *Leucophaea*, fixed 12 days after molt. 2% OsO_4 followed by 10% formalin (10 days), Epon, uranyl acetate, and lead citrate. × 76,300.

FIGURE 4 Sheath of prothoracic gland of male last instar nymph of *Blaberus*, fixed 16 days after molt. 1% OsO_4, Epon, uranyl acetate, and lead citrate. × 40,500.

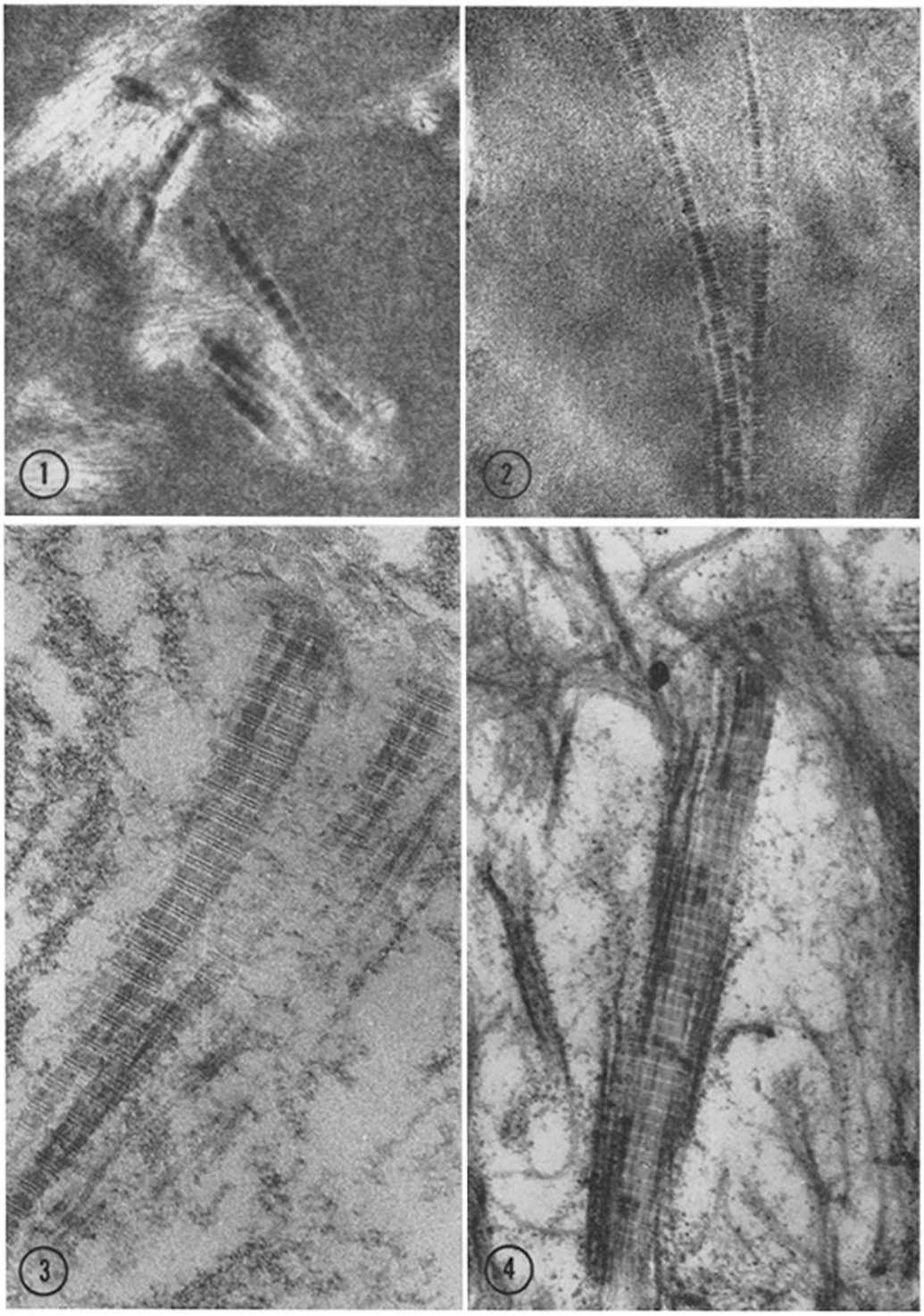

TABLE I

*Amino Acid Compositions of Various Fractions Obtained from Corpus Cardiacum plus Corpus Allatum**

Residues per 1,000 Total Residues

Amino acid	Free amino acids in dialysate (D)	Supernate after dialysis (S)	Residue after dialysis (R)	Supernate after autoclaving (AS)	Residue after autoclaving (AR)
Aspartic acid	9.0	108.7	92.1	107.4	75.1
Threonine	20.9	52.0	54.2	44.6	45.8
Serine	61.6	64.7	60.6	50.3	48.2
Glutamic acid	114.7	114.4	90.4	130.0	105.7
Proline	92.4	46.2	58.7	63.8	50.3
Glycine	119.2	80.9	118.5	120.7	82.5
Alanine	238.2	68.2	80.6	81.2	86.3
Valine	23.7	57.8	75.0	58.5	68.8
½ Cystine	—	—	—	5.0	4.7
Methionine	—	—	3.3	10.5	9.9
Isoleucine	10.9	39.3	52.3	42.6	53.8
Leucine	28.2	69.4	82.7	72.3	92.4
Tyrosine	12.6	20.8	28.7	24.8	34.6
Phenylalanine	—	33.5	38.1	34.0	43.5
Lysine	22.3	74.0	71.2	59.6	17.3
Histidine	27.7	19.7	20.6	20.3	6.1
Arginine	96.0	31.2	47.1	45.6	54.2
Cysteic acid	33.2	22.0	10.6	3.9	1.3
Methionine sulfoxide	60.0	9.7	22.9	8.1	10.7
Hydroxyproline	—	—	0.4	8.9	—
Hydroxylysine	—	—	6.2	7.8	3.3

* See text for description of fractions.

Threonine and serine values are uncorrected for destruction during hydrolysis.

tissue indeed contained a collagenous protein was obtained by treatment of fraction AS with a highly purified bacterial collagenase, prepared as described by Harper and Seifter (8). The enzyme promoted significant proteolysis of fraction AS. Thus, the enzyme caused an increase in ninhydrin reactivity indicating that 40 peptide bonds per 1,000 total residues had undergone scission. Since collagenase is known to act on collagen or denatured collagen exclusively, the conclusion that fraction AS contained a collagenous protein is reinforced.

Other interesting aspects of the analyses shown in Table I may be noted. Thus, under the conditions of the experiment, no hydroxyproline or hydroxylysine could be detected in D, the dialysate containing the free amino acids of the neuroglandular organs. Although not germane to the subject of the present communication, the finding of a considerable amount of free methionine sulfoxide in a single isomeric form also is of interest. This observation may be added to that of Lucas and Levenbook (10) who found the L(+) isomer of this compound in the blowfly, *Phormia regina*.

Trimmed carcasses of cockroaches were used in order to obtain larger amounts of collagenous protein. Table II shows amino acid compositions of fractions obtained in such an experiment. Evidently, whatever collagen was present in the carcass was not extracted either by dilute acetic acid or by concentrated solutions of guanidinium chloride. Apparently, therefore, the collagen is not of the "acid soluble" (tropocollagen) nor of the easily denaturable type. However, the table shows that a protein with the compositional characteristics of a collagen could be extracted by hot trichloroacetic acid, thus exhibiting a behavior in common with that of many other "insoluble" collagens. This material contained 235 residues of glycine per 1,000 total amino acid residues, a figure higher than that found for most other proteins, but considerably less than the average

TABLE II

Amino Acid Compositions of Fractions Obtained from Cockroach Carcasses
Residues per 1,000 Total Residues

Amino acid	Extracted by acetic acid (A)	Extracted by guanidinium chloride (G)	Extracted by trichloroacetic acid (T)
Aspartic acid	101.1	104.3	66.4
Threonine	50.5	51.7	40.1
Serine	49.6	55.0	62.5
Glutamic acid	93.5	119.8	86.5
Proline	50.2	48.1	88.1
Glycine	67.6	92.7	235.2
Alanine	102.3	86.8	104.7
Valine	75.3	60.8	44.0
½ Cystine	9.1	11.2	7.9
Methionine	7.4	9.6	3.8
Isoleucine	48.5	47.9	25.3
Leucine	82.5	69.9	35.3
Tyrosine	48.9	36.7	31.7
Phenylalanine	39.8	36.9	27.6
Lysine	63.4	75.9	20.7
Histidine	23.3	22.8	18.2
Arginine	48.0	60.2	34.5
Cysteic Acid	0.8	—	—
X*	2.1	—	—
Methionine sulfoxide	21.8	—	—
Methionine sulfone	5.9	9.5	—
Y‡	8.6	—	—
Hydroxyproline	—	—	41.2
Hydroxylysine	—	—	26.4
Ammonia	80.1	93.4	61.2

* X represents an unidentified peak emerging after cysteic acid.
‡ Y represents a component emerging in the chromatogram at a time identical with the time of emergence of a standard β-alanine.
Threonine and serine values are uncorrected for destruction during hydrolysis.

figure of 333 residues of glycine found for most other collagens. One can conclude that the protein is not pure, or that it is a collagen of the type with a smaller content of glycine. We have mentioned earlier that collagens with glycine contents comprising about one-fourth of the total amino acids have been found in other animals. It becomes instructive to look at the contents of the other amino acid residues in the trichloroacetic acid–soluble collagen of the cockroach. In the table it may be seen that, per 1,000 total residues of amino acids, this protein had about 88 residues of proline, 41 residues of hydroxyproline, and 26 residues of hydroxylysine. The evidence is strongly presumptive that the main protein in the extract is collagenous in nature. The distribution of other amino acid residues is also characteristic of most other collagens. The presence of about eight residues of half-cystine and 32 of tyrosine could again be evidence of contamination by a non-collagenous protein, or, indeed, could indicate that the collagen of this species does contain significant amounts of these amino acids.

One may also note that the protein extracted by trichloroacetic acid from the carcass contained more hydroxyproline than hydroxylysine. This contrasts with the possibility that the collagenous protein obtained directly from the neuroglandular tissue contained more hydroxylysine than hydroxyproline. Should further experiments substantiate the latter suggestion, one could infer that the carcass contains additional collagenous com-

ponents of amino acid composition somewhat different from that of the collagen in the neuroglandular tissues.

Additional evidence that the trichloroacetic acid–soluble protein is indeed collagenous in nature is the fact that the highly specific collagenase of *Clostridium histolyticum* was found to digest it measurably.

From the total amount of trichloroacetic acid–soluble protein obtained, one can estimate that probably less than 0.1% of the trimmed carcass is collagen. Obviously, this small amount of collagen in the cockroach must be restricted in location, presumably to the stroma of both nonneural and neural organs, some of which are illustrated in the electron micrographs presented here.

Biochemical studies of fibrous proteins of the collagen group carried out by other investigators are concerned primarily with animals other than insects (see Fitton Jackson, 7). In a qualitative manner, the presence of collagen in insect tissues was sought by examination for hydroxyproline, the chemical indicator of this protein. Ashhurst (1, 2) reported that paper chromatography of hydrolysates of nerve cords of *Locusta migratoria* and *Periplaneta americana* revealed the presence of hydroxyproline. This substance was also demonstrated in the nerve sheaths of the moth *Galleria mellonella* by colorimetric analysis (3).

Finally, a comment should be made concerning the protein (G) extracted from the carcass by guanidinium chloride. This material came out of solution rapidly as the guanidinium chloride was depleted by dialysis, appearing in copious layers having a fibrous appearance. Both from the amino acid analysis presented in Table II and from the fact that a separate analysis showed the presence of only 1 or 2% of *N*-acetylglucosamine, the material obviously is not chitinous. Because its amino acid composition suggests a resemblance to the composition of actin (4), further study of this protein will be undertaken to determine whether a relationship with actin indeed exists.

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