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Studies on Carotenoprotein in Aquatic Animals

I. Distribution of Carotenoprotein in Exoskeleton of Crayfish (*Cambarus clarkii*)

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(Figs. 1–6; Tables 1–2; Plates 1–3)

The pigments in crustacean exoskeleton are located in the pigmented layer of the endocuticle[1], and the various color appearances of crustacean are originally due to the carotenoids. The carotenoid and protein complex, crustacyanin, is well known as a kind of chromoprotein associated with the prosthetic group, astaxanthin. The various colors of carotenoprotein have been observed in many crustaceans[2,3]. Isolation from the exoskeleton and properties of carotenoproteins have been studied by many authors[4~10]. BUCHWALD and JENCKS[10] separated four kinds of pigmented carotenoproteins from the lobster shell. Presence of colorless carotenoprotein, which turns pink after boiling, was also suggested in the prawn[11]. Moreover, the color appearances of carotenoprotein range from red to purple as we find them in the seven colors in nature, and various colored carotenoproteins seem to coexist in a same animal. Furthermore, it may be pointed out that the simultaneous presence of protein-bound and unbound carotenoids in an exoskeleton can effectuate a far wider spectral range.

It is recognized that the adaptive color changes are regulated by hormones influenced themselves by the environmental conditions. Moreover, an actual formation of the carotenoprotein is under control[1,12,13].

The present paper deals in particular with the nature of three kinds of carotenoproteins in the exoskeleton of the freshwater crayfish, living in Hiroshima district and which is particularly rich in color variety.

EXPERIMENTALS AND RESULTS

Extraction of carotenoprotein: The crayfish (*Cambarus clarkii*) shown in Plate 1 were collected at the Umeda's Carp Breeding pond and its connected streams in Kannabe, Hiroshima Prefecture. The whole body was cleaned with a brush in order to remove all mossy substances, then stored at −20°C until the extracting of carotenoprotein. After removal of muscle and hypodermis from the exoskeleton as much as possible, a batch of exoskeleton was washed again and dried on a paper sheet overnight in a dark room. The exoskeletons were cut into small pieces and grinded. The granules were then suspended in 0.6 *M* ammonium sulfate solution by the method of CECCARDI and ALLEMAND[7]. The suspension was left overnight in a dark room, and was shaken from time to time. The resulting bluish extract was centrifuged, and the supernatant was obtained. The extraction was repeated and the clean bluish extracts were collected. All the preparations were done at room temperature and under weak

illumination in order to avoid slow fading and denaturation of carotenoprotein.

Absorption spectra of carapace: The microscopic photograph of a transparent fragment of uncalcified carapace which appeared newly after moult is shown in Plate 2. Plate 3 illustrates the same carapace after extraction with 0.6 *M* ammonium sulfate solution. In order to test the presence of carotenoprotein, absorption spectra both of the carapace and of its extract were determined. The uncalcified carapace was cut and

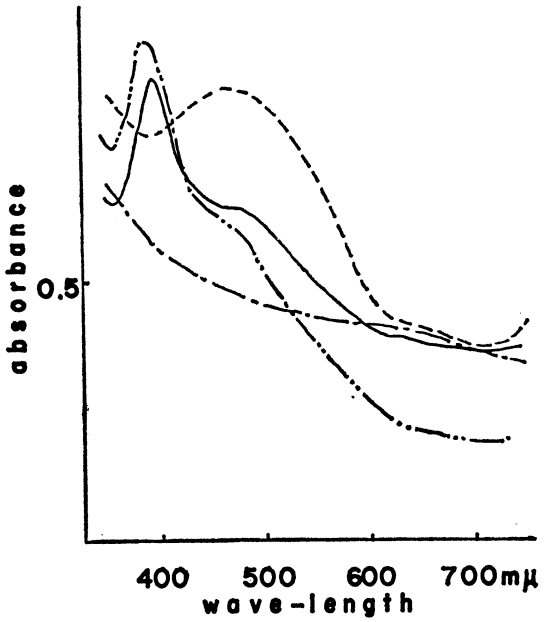


Fig. 1. Changes of absorption spectra of uncalcified carapace of crayfish after various treatments.
——: Original
- - -: Boiled
——: Extracted with 0.6 *M* $(NH_4)_2SO_4$
——: Extracted with aceton

pasted on an optical cell with water, then the spectrum was obtained by a Shimadzu IV–50A Spectrophotometer. The absorption spectrum of the carapace revealed a maximum absorption at 400 mμ (Fig. 1). After treatment with 0.6 *M* ammonium sulfate solution the spectrum showed a slight decline in absorbance at 660–700 mμ. The extract from the carapace was bluish and its absorption maximum was located at around 630 mμ. After extraction by means of acetone the colorless carapace showed no significant spectrum. The shift of absorption maximum from 400 mμ to 470 mμ was detected after boiling the uncalcified carapace at 100°C.

Cellulose acetate electrophoresis: The crude carotenoprotein was extracted with 0.6 *M* ammonium sulfate from three different colored parts, abdomen and tail (dark bluish color), cephalothorax (bluish or purplish color), and ambulatory legs (reddish color). The absorption spectra of these crude extracts are shown in Fig. 2. The extract from the part of abdomen and tail shows a broad peak at 620 mμ. The

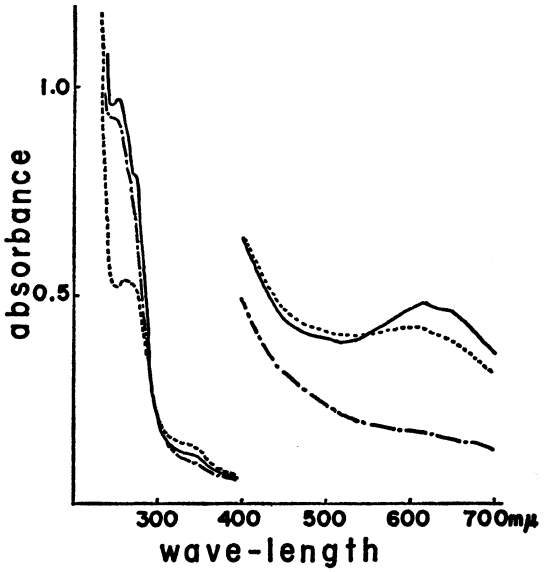
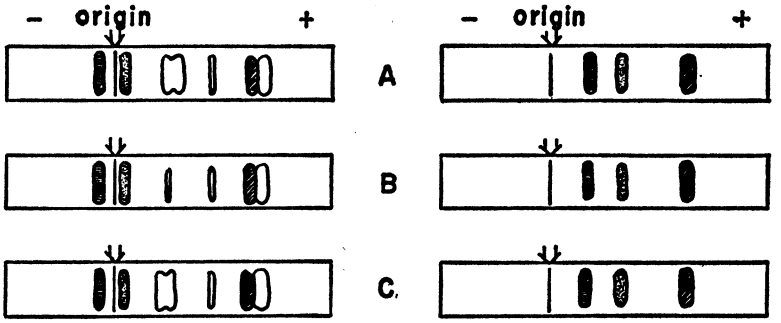


Fig. 2. Absorption spectra of extracts from the exoskeleton of crayfish.
——: Abdomen and tail
- - -: Cephalothorax
——: Ambulatory legs
Scale of absorbance in the ultraviolet region is one twelfth.

absorption ratios of 280 mμ to 630 mμ in the extracts were 20.0 in the abdomen and tail, 21.9 in the cephalothorax and 31.5 in the ambulatory legs. These ratios of the further purified carotenoprotein fractions with ammonium sulfate salting-out became 2.19, 2.19 and 7.62 respectively. The crude carotenoprotein fractions thus obtained



0.06 *M* veronal buffer
pH 8.6, 1 mA/cm, 30 mins.

0.05 *M* phosphate buffer
pH 7.3, 1 mA/cm, 60 mins.

Fig. 3. Cellulose acetate electrophoresis of carotenoproteins obtained from various colored exoskeletons.

: Colorless protein
: Blue carotenoprotein
A: Abdomen and tail
B: Cephalothorax
C: Ambulatory legs
: Purple carotenoprotein
: Red carotenoprotein

were dissolved in 0.06 *M* veronal buffer (pH 8.6) or 0.05 *M* phosphate buffer (pH 7.3), and were dialyzed against the same buffers. Such carotenoprotein fractions were plotted on the cellulose acetate films (6.0 × 1.5 cm) and were migrated on a Toyo electrophoretic apparatus Model SE–2. Three pigmented zones, red, purple, and blue, were found on the every electrophoretic diagrams as shown in Fig. 3. Three or four colorless proteins were also detected after staining with ponceau 3R. Three kinds of carotenoproteins were found in all parts of the exoskeleton although in different proportions. The percentage composition of these carotenoproteins in different parts was measured from the diagrams at pH 7.3 of phosphate buffer by employing an Ozumer Densitometer 82. The result is listed in Table 1. It appeared that the red carotenoprotein was relatively predominat in the ambulatory legs which are reddish in color, while rather secondary in the bluish carapace. At pH 9.8 of 0.1 *M* carbonate buffer or pH 5.0 of 0.1 *M* citrate buffer, the carotenoproteins turned red, and the red matter remained near the origin and colorless matters migrated during the electrophoresis.

Table 1. Percentage composition of three colored carotenoproteins in exoskeleton of crayfish.

	Red	Blue	Purple
Abdomen & tail	13.6%	57.0%	29.4%
Cephalothorax	10.2%	38.0%	51.8%
Ambulatory legs	24.1%	37.2%	38.7%

Salting-out with ammonium sulfate: The colorless protein in the exoskeleton extract was removed by the following procedure of precipitation and dissolution with ammonium sulfate. The solid ammonium sulfate was added into the extract to be a half saturation. The yellowish clean solution which was not precipitated through addition of ammonium sulfate (or even trichloroacetic acid) was discarded. The resulting bluish precipitates were collected by centrifugation and washed with 0.5 saturated ammonium sulfate solution, then redissolved in distilled water. The insoluble matter in distilled water was removed by centrifugation. The obtained crude carotenoprotein fraction was fractionated with different concentrations of ammonium sulfate into the following three fractions. Fraction A, soluble in 0.4 saturation but insoluble in 0.5 saturation, absorption maximum around 610 mμ; Fraction B, soluble in 0.35 saturation but insoluble in 0.4 saturation, absorption maximum around 655 mμ; Fraction C, soluble in 0.3 saturation but insoluble in 0.35 saturation, absorption maximum around 640 mμ. The colorless residue precipitated in less than 0.3 saturation was removed by centrifugation. These fractions were dissolved in a 0.5 *M* sodium acetate solution and applied to a gel filtration of Sephadex G–200 (45 cm in length, 1.0 cm in diameter). When the absorbances of the eluates were measured at 280, 475, 615, and 655 mμ, three different colored carotenoproteins, which were corresponding to those of the electrophoretic pattern, were fractionated. The percentage composition of three carotenoproteins in the above mentioned fraction A, B, and C are listed in Table 2. It was shown in the results of gel filtration that three kinds of carotenoproteins were contained in each fraction with different composition. The purple carotenoprotein was dominant in Fraction A, and the blue carotenoprotein in Fractions B and C. The proportion of the red carotenoprotein in Fraction C was comparatively higher than the

Table 2. Distribution of carotenoproteins in the fractions separated by salting-out with ammonium sulfate.

	Red	Blue	Purple
Fraction A	15.5%	33.9%	50.6%
Fraction B	11.0%	50.3%	38.7%
Fraction C	31.8%	42.5%	25.7%

These values were calculated from the elution patterns of gel filtration.

other fractions; however, the blue or the purple carotenoproteins were also present in rather considerable amounts. It appeared that the solubility in ammonium sulfate solution was in the following order, purple>blue>red carotenoproteins. During the process of the salting-out at 0.5 saturation and the dissolution at 0.3 saturation of ammonium sulfate, the carotenoprotein's absorption ratio of 280 mμ to maximum absorption in the visual region varied from 30.0 to 1.0. Therefore, these procedures seemed to be useful for preparative separation of carotenoproteins.

Purification of the carotenoproteins: The carotenoprotein mixture obtained by above procedure was transferred to a column of Sephadex G–200 of length 35 cm and diameter 1.1 cm, and eluted with 0.5 *M* sodium acetate solution. Red, blue and purple eluates appeared in this order (Fig. 4). Each eluate was precipitated by the addition of ammonium sulfate. The precipitated carotenoproteins were dissolved in a 0.05 *M* phosphate buffer solution (pH 7.0). After dialyzing against the same buffer the carotenoprotein was individually placed on the column of DEAE-cellulose (25 cm in length, 1.5 cm in diameter), and washed with 0.05*M* phosphate buffer solution until the colorless protein was eluted. Then elution was continued with a continuous gradient

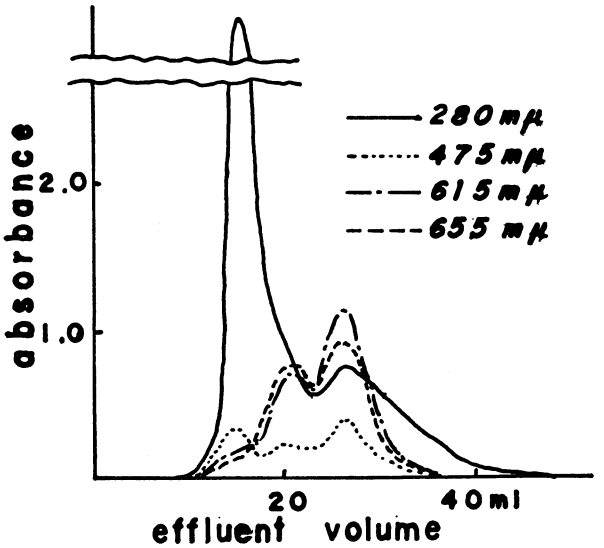


Fig. 4. Gel filtration of a mixture of carotenoproteins on a 35.0×1.1 cm bed of Sephadex G–200. One fraction volume is 2.0 m*l*.

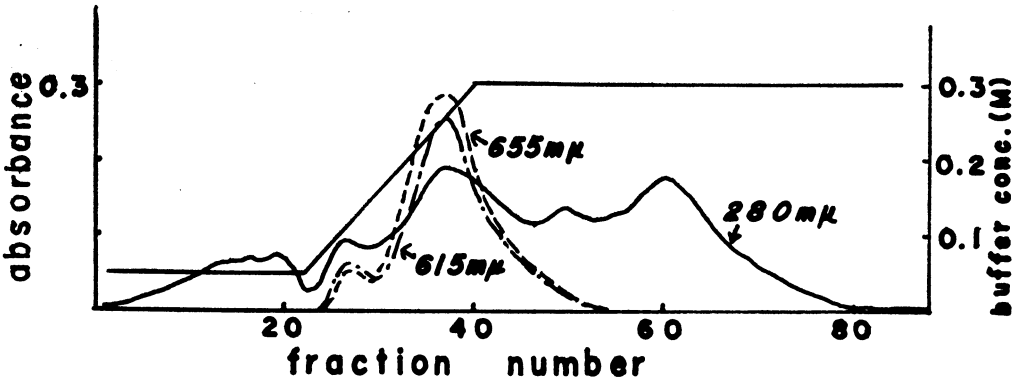


Fig. 5. Elution patten of gel filtered blue carotenoprotein on a DEAE-cellulose column (25×2 cm) with phosphate buffer gradient. One fraction volume is 2.0 m*l*.

phosphate buffer (0.05 to 0.3 *M*). The purple carotenoprotein was eluted with the lower concentration of the buffer. The blue carotenoprotein which occupied a zone under the top of the column was finally eluted with the 0.2 *M* buffer solution. One fraction 2.0 m*l* was collected continuously by a fraction collector and their absorptions at 280, 615, and 655 mμ were measured (Fig. 5). The red carotenoprotein was strongly absorbed at the top of the column, and not eluted with this buffer. The treated eluates showed a sole band in disc and cellulose acetate electrophoresis, and the absorption ratio of 280 mμ to maximum absorption in visual region for the blue and the purple carotenoproteins lay 0.35 and 0.43, respectively.

The absorption spectra of the blue and the purple carotenoproteins, and the crude

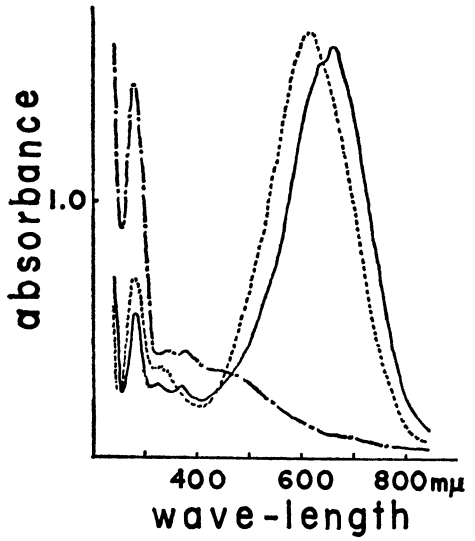


Fig. 6. Absorption spectra of purified carotenoproteins in 0.05 *M* phosphate buffer pH 7.3.
——: Blue carotenoprotein
- - -: Purple carotenoprotein
——: Red carotenoprotein

red carotenoprotein are shown in Fig. 6. Although the red carotenoprotein fraction was passed thrice through the column of Sephadex G–200, colorless proteins were still found in the fraction. The red carotenoprotein revealed no clear absorption maximum at the visual region as shown in the blue or the purple carotenoproteins, but a weak absorption was found around 475 mμ.

DISCUSSION

It has been confirmed by many investigators that the carotenoproteins are widely distributed among the invertebrates, and the protein-carotenoid complexes of various types could occur in wide variety of organisms[2]. In the crayfish various colored exoskeletons are observed. For example, the claws are brilliant red, the abdomen and tail are dark blue. The carapace, however, shows a great color variety, such as brown, blue, purple and yellow.

JENCKS and BUTEN[6] succeeded in separating the carapace pigments of the lobster into three fractions, α-, β-crustacyanins, and a yellow pigment with absorption maxima at 630, 590, and 410 mμ, respectively. CHEESMANN *et al.*[5] extracted the blue carotenoprotein with absorption maxima at 633 mμ from the lobster carapace. Moreover, α-, β-, γ-crustacyanins, and a yellow pigment were isolated by BUCHWALD and JENCKS[10]. Three carotenoproteins obtained from crayfish revealed different absorption maxima from the crustacyanins of the lobster.

In the presence of denaturing agents, the carotenoproteins obtained from the crayfish showed a behavior similar to that of the crustacyanins prepared by other workers[6,9]. By dissolution in acid or alkaline solutions, the blue and the purple carotenoproteins turned to reddish carotenoprotein. This denatured carotenoprotein differed from the naturally occuring red carotenoprotein which was obtained here. Many authors had already observed that the blue crustacyanins turn into reddish or purplish crustacyanins by denaturation[4,5,6,14,15,16]. A similar phenomenon was observed in this experiment. However, the absorption spectrum of the red carotenoprotein obtained here was different from the denatured crustacyanins reported by many authors[4,5,6,9,16] in the absorption spectra at visual region. When heated, the bluish carapace turned red, and the absorption maximum of uncalcified carapace shifted from 400 mμ to 475 mμ thus corresponding with that of heated carotenoprotein[14].

In the eluating pattern on the Sephadex column, the red carotenoprotein was the largest, the blue was medium and the purple the smallest in the molecular size.

In nature the various colored carotenoproteins are distributed over the different parts of the exoskeleton in different proportion. It was observed that the red carotenoprotein was found at a relatively higher rate in the claws than in the other bluish parts. The blue and the purple carotenoproteins could be almost completely extracted, the red carotenoprotein, however, could hardly be extracted by this procedure, in fact with 0.1 N NaOH, more pinkish protein, which seemed to be artifact, could be extracted from the reddish exoskeleton. Even after 0.1 N NaOH extraction a fairly reddish color remained in the residue. When the carotenoproteins were removed from the uncalcified exoskeleton of the crayfish, an orange-colored pigment remained, yet in the calcified exoskeleton, that remainder was fairly red tinted. Therefore, it has been suggested that the dissociated astaxanthin or its derived astacin might contribute to the reddish color of exoskeleton.

These results suggested that the various colored carotenoproteins and the unbound

carotenoids did coexist in the exoskeleton in different proportion. These color variations were probably controlled by the qualities and quantities of carotenoproteins and unbound carotenoids present. At least, immediately after ecdysis the carotenoproteins seemed to be the main pigment in the exoskeleton.

SUMMARY

1. The blue and the purple carotenoproteins with absorption maxima at 655 and 615 mμ in 0.05 *M* phosphate buffer at pH 7.3 were obtained from the exoskeleton of the freshwater crayfish (*Cambarus clarkii*). Besides the above carotenoproteins, the red carotenoprotein which revealed a weak absorption around 475 mμ in visual region was separated in the exoskeleton.

2. Three kinds of carotenoproteins were distributed in various colored exoskeleton in different proportions. In the claws and limbs, the red carotenoprotein was found in somewhat larger degree than in the bluish carapace.

3. A fair amount of carotenoid-pigment remained in the exoskeleton after extraction by aqueous solution. This suggested the coexistence of carotenoproteins and unbound carotenoids in the pigmented exoskeleton.

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水産動物のカロチノプロテインに関する研究

I. アメリカザリガニ甲殻における カロチノプロテインの分布

中川平介・鹿山　光・浅川末三

1. 0.6 *M* 硫安を用いてアメリカザリガニ甲殻より赤，紫，青の三種のカロチノプロテイン（以下 CP と略記）を得た．精製後の可視部の最大吸収波長は紫 CP 615 mμ，青 CP 655 mμ であった，赤 CP の最大吸収は前二者に比較して不明瞭であるが，475 mμ 付近にわずかな吸収を示した．

2. 甲殻における三種の CP の分布は部分によって異なり，尾部，頭胸部等の青い甲殻は青 CP 及び紫 CP を多く含み，赤い甲殻では赤 CP の占める割合が青い甲殻よりも大きいことを明らかにした．

3. CP を硫安水溶液で抽出後なお赤い色が甲殻に残ることから，甲殻の色は CP のみによるものではなく，非結合型のカロチノイドと CP との共存によるものと考察した．

Explanation of Plates

Plate 1. Photograph of crayfish (*Cambarus clarkii*)

Plate 2. Uncalcified carapace of the crayfish (×100)

Plate 3. Uncalcified carapace after extraction of carotenoprotein with 0.6 *M* ammonium sulfate solution (×100)

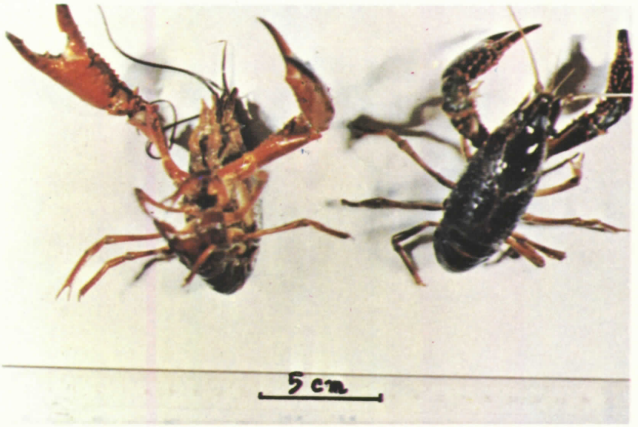

Plate 1

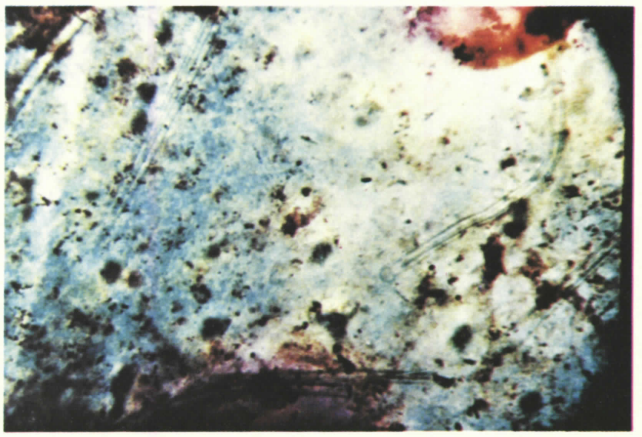

Plate 2

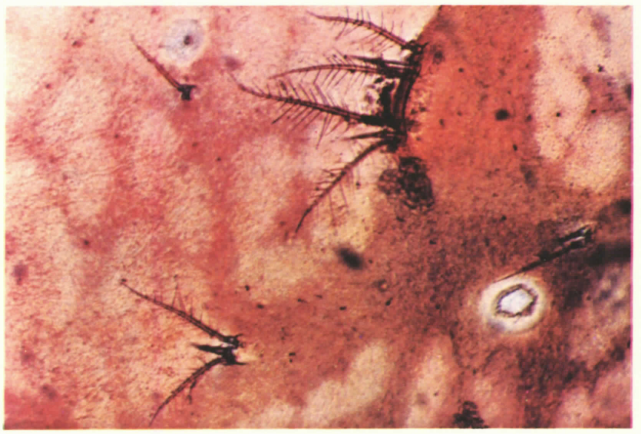

Plate 3