

**THE STRUCTURE AND HISTOCHEMISTRY OF THE CUTICLE OF
LARVAE AND ADULTS OF *ANAX IMMACULIFRONS* RAMBUR
(ANISOPTERA: AESHNIDAE)**

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Received February 27, 1985 / Revised and accepted March 10, 1988

Histological and histochemical studies on the cuticle of *A. immaculifrons* from the third larval instar to the imago revealed 3 types of cuticular organisation. The first type was present in larval instar 3 to 9; instars 10 to 13 had type-II, while the imago had type-III. The type-I cuticle had 3 layers, i.e. epi-, exo- and endocuticle. The protein of the epicuticle as well as that of the exocuticle is stabilized by disulphide linkages. The type-II epicuticle resembled its counterpart in type-I in the mode of hardening but the exocuticle had undergone hardening involving both aromatic substances and organic sulphur (R-S-R). Lying between the exocuticle and the endocuticle was a new layer, the mesocuticle. This new layer had undergone primary tanning. The type-III cuticle differed from the first two, both in its histology and histochemistry. The epicuticle was bilaminar, having an outer lipid layer and an inner lipoprotein lamina. The inner epicuticle as well as exocuticle had undergone hardening by aromatic tanning. However, the mesocuticle resembled its homologue in the preceding stage in chemical composition. The protein from the 3 types of cuticle was extracted and analysed by electrophoretic and chromatographic methods. These results substantiated those obtained by histochemical procedures. Changes in the proportion of the exoskeleton to body weight in larvae and adults were also recorded. The results are discussed in relation to the evolution of aerial life.

INTRODUCTION

Odonata are the earliest known pterygote insects, representing the link between the wingless and winged hexapods (MARTYNOV, 1925). Hence they may give information on the changes that may have taken place in the insects in relation to the evolution of wings and aerial life. KRISHNAN & SUNDARA RAJULU (1964) observed that a major problem for flying insects would be the

reduction of skeletal weight by evolving a mechanically more rigid cuticle and a suitable mode of hardening making the exoskeleton light in weight. Two kinds of hardening of the cuticle in insects are -S-S- bonds found in apterygote insects like *Machilis variabilis* and *Smynthurus punctatus* (KRISHNAN & SUNDARA RAJULU, 1964; KRISHNAN, 1969) and aromatic tanning which occurs in the pterygotes (see DENNELL, 1958).

KRISHNAN & SUNDARA RAJULU (1964) found that hardening by disulphide linkages made the cuticle heavier while aromatic tanning rendered the exoskeleton lighter in weight. For apterygote insects, a heavy exoskeleton may not be a problem as they are cursorial or fossorial. However, with the evolution of wings, the hexapods have to bear the entire body weight on their wings. Hence phenolic tanning probably evolved simultaneously with the evolution of wings (see LOWER, 1957). In that case we have to look for a stage of transition from disulphide linkages to phenolic tanning from among the paleopterygote odonates. Hence the present investigation was undertaken.

MATERIAL AND METHODS

The materials used in the present study were the larvae and adults of *Anax immaculifrons* Rambur, available in the scrub jungles around Coimbatore city, Tamilnadu, India. These were obtained by collecting eggs and rearing the larvae in the laboratory, as outlined by VARADARAJ (1978).

The material was fixed in Susa. To perform histochemical tests, frozen sections of 15-20 μm thick were prepared according to CARLTON (1938). For staining, 5-8 μm thick sections were cut by the conventional paraffin wax embedding method.

Information concerning the types of forces involved in stabilising the structural protein of different layers of the cuticle was obtained by the methods of TRIM (1941) and BROWN (1950), which consist of treating the protein under test with various reagents. The effect of the reagents on the different layers of the cuticle was examined by incubating 15-20 μm thick frozen sections with the reagents in well slides with cover slips ringed with petroleum jelly to prevent evaporation. After 12 h, parts of the cuticle were removed, washed rapidly in distilled water and examined.

Stains, histochemical tests and solubility tests employed are given in the relevant tables.

For electrophoretic analyses, the protein from the cuticle was extracted following the method of HACKMAN (1953). Details of the electrophoretic analyses have been given elsewhere (VARADARAJ, 1978). The electropherograms were scanned with a Biochem-Densitometer for measuring the percentage concentration of different fractions. The amino acid concentration of different fractions was determined by the two-dimensional paper-partition method of BLOCK et al. (1958).

RESULTS

Histological and histochemical studies were conducted on the cuticle of all the instars of *A. immaculifrons* except the first two, where it was very hard to make out any histological details of the cuticle, and its chemical nature could not be made out unambiguously. The results of such studies on instars from the third to

the imago revealed three types of organisation. These were called type-I, II and III.

TYPE-I CUTICLE

Type-I cuticle was present in larval instars 3 to 9. Externally it was pale yellowish brown in colour and hard on the dorsal side. It had two layers in unstained sections; an outer thin homogenous hyaline membrane of about 2-4 μm and an inner, much thicker, uniform translucent layer without any striations. The epidermis was a syncytial layer with epidermal glands having ducts.

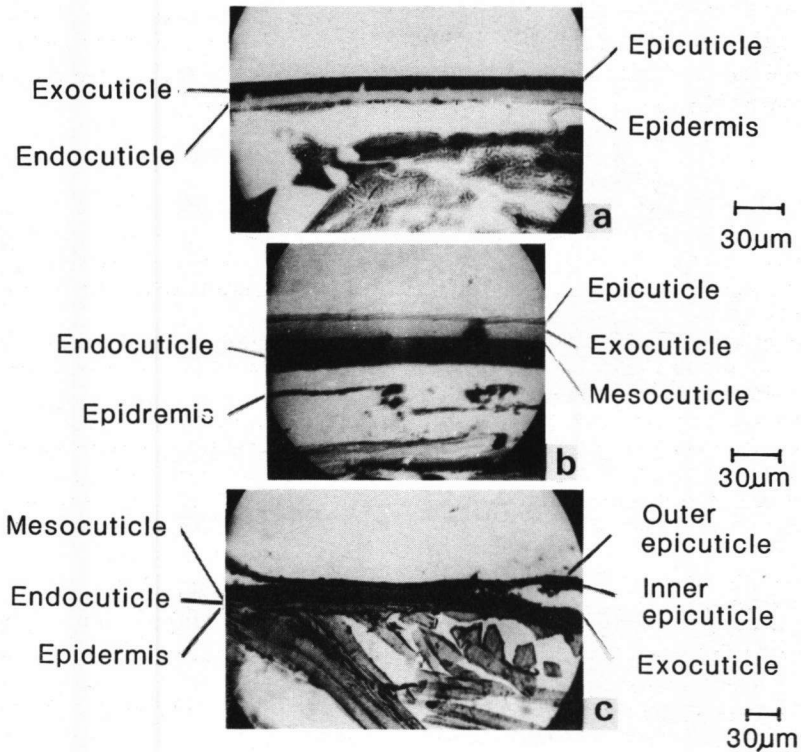


Fig. 1. Transverse section: (a) Type-I; — (b) Type-II; — (c) Type-III.

In Mallory's triple stain the outermost homogenous thin membrane and the outer half of the inner part of the cuticle were refractory. However, the lower half of the cuticle took a blue colour (Figs 1, 2; Tab. I). From the staining reactions, it was inferred that the thin outer layer was homologous with the epicuticle and the

broader part to the procuticle of other insects like *Sarcophaga faculata* and *Periplaneta americana* (DENNELL, 1947; DENNELL & MALEK, 1954). The refractory outer half of the procuticle would be homologous with the exocuticle while the inner half would correspond to the endocuticle. The presence of protein with disulphide linkages in the epi- and exocuticle was indicated by histochemical tests (Tab. I). The solubility reactions shown by the epi- and exocuticle to mineral acids before and after treatment with lytic reagents confirmed the occurrence of -S-S- bonds in these layers.

There was no lipid layer in the epicuticle as it showed a negative reaction to lipolytic agents (Tab. I).

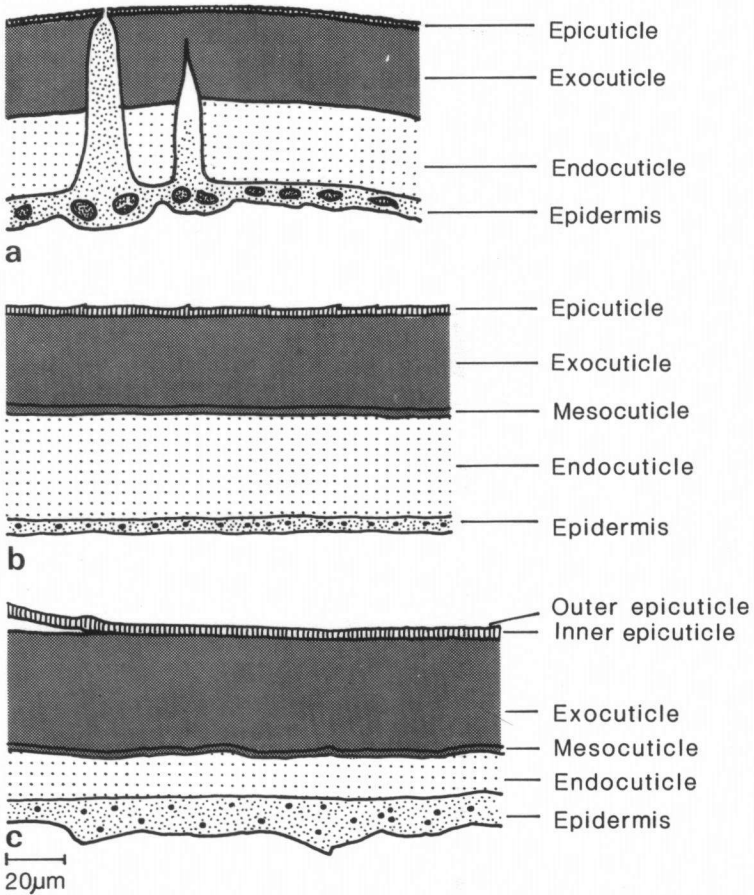


Fig. 2. Diagrammatic transverse section of cuticle, based on Mallory's stain: (a) Type-I; — (b) Type-II; — (c) Type-III.

Table I
Staining, solubility and histochemical reactions shown by different types of cuticle of *Anax immaculifrons*

Stains/ Tests	Reference	Type-I				Type-II				Type-III			
		Epicu- ticle	Exocu- ticle	Endocu- ticle	Epicu- ticle	Exocu- ticle	Mesocu- ticle	Endocu- ticle	Outer epi- cuticle	Inner epi- cuticle	Exocu- ticle	Mesocu- ticle	Endocu- ticle
Mallory's triple stain	MALLORY, 1938	ref.	ref.	blue	ref.	ref.	red	blue	blue	ref.	ref.	red	blue
after diaphanol	KENNAUGH, 1957	ref.	ref.	blue	ref.	ref.	blue	blue	blue	ref.	ref.	blue	blue
after alkaline sodium sulphide	BROWN, 1950	red	red	blue	red	red	red	blue	blue	ref.	ref.	red	blue
Conc. mineral acids	FEARON, 1946	res.	res.	dis.	res.	res.	dis.	dis.	blue	res.	res.	sw.	dis.
after diaphanol	KENNAUGH, 1957	dis.	dis.	dis.	res.	dis.	dis.	dis.	—	dis.	dis.	dis.	dis.
after alkaline sodium sulphide	BROWN, 1950	res.	res.	dis.	dis.	dis.	dis.	dis.	—	res.	res.	sw.	dis.
50% mineral acid	FEARON, 1946	—	—	—	res.	res.	res.	sw.	—	res.	res.	dis.	dis.
after diaphanol	KENNAUGH, 1957	—	—	—	res.	sw.	dis.	sw.	—	sw.	sw.	dis.	dis.
after alkaline sodium sulphide	BROWN, 1950	—	—	—	sw.	sw.	res.	sw.	—	res.	res.	sw.	dis.
Acrolian Schiff test	DUIJIN, 1961	+	+	+	+	+	+	++	—	+	+	+	+
Bromophenol blue test	PEARSE, 1968	+	+	++	+	+	+	++	—	ref.	ref.	+	++
Biuret test	FEARON, 1946	—	—	+	—	—	+	++	—	ref.	ref.	+	++
Millon's test	PEARSE, 1968	—	—	—	—	+	++	—	—	++	++	+	—
Chromaffine test	LISON, 1936	—	—	—	—	+	—	—	—	+	+	—	—
Nitroprusside test	LILLIE, 1965	—	—	—	—	—	—	—	—	—	—	—	—
Lead acetate test	PEARSE, 1968	+	+	—	++	+	—	—	—	—	—	—	—
Fast-green-Van Gieson stain	LILLIE, 1965	—	—	+	—	—	—	+	—	—	—	—	+
Boiling water	FEARON, 1946	—	—	sw.	—	—	—	sw.	—	—	—	—	sw.
Sudan Black-B in ethanol	BAKER, 1956	—	—	—	—	+	—	—	++	—	—	+	—
in acetone at 60° C	BERENBAUM, 1958	—	—	—	—	—	—	—	++	+	+	—	—
Nile blue	CAIN, 1947	—	—	—	—	—	—	—	++	—	—	+	—
Liebermann-Burchardt test	LISON, 1936	—	—	—	—	—	—	—	+	—	—	+	—
Chitosan test	CAMPBELL, 1929	—	+	+	—	+	+	+	—	—	—	+	+

+ Positive reaction; ++ intense positive reaction; no reaction; dis.: dissolves; ref.: refractory; res.: resists; sw.: swells

The complete dissolution of the endocuticle in concentrated mineral acids suggested the presence of a collagen-like protein in the layer. This inference was confirmed by swelling reactions and histochemical tests (Tab. I).

TYPE-II CUTICLE

The type-II cuticle was present in larval instars 10-13. It resembled the type-I cuticle in the absence of lamellations in the entire cuticle (Figs 1b, 2b).

The staining reactions were the same as in the type-I cuticle, except that there was a new layer between the exo- and endocuticle in the type-II which recalled the mesocuticle of insects like *P. americana* (DENNELL & MALEK, 1955). In the type-II cuticle, the epicuticle resembled its counterpart in the type-I in all respects and reacted positively to histochemical tests for -S-S- bonds (Tab. I). However, the exocuticle was hardened by aromatic tanning as well as by disulphide linkages, as indicated by the histochemical tests and solubility reactions. From the solubility and histochemical tests it was inferred that the mesocuticle had undergone what was termed primary tanning, as in the case of the mesocuticle of *P. americana* (DENNELL & MALEK, 1955). The endocuticle in all the tests recalled strongly its homologue in the earlier larval stages where the indications were for the presence of collagen-like protein (Tab. I).

TYPE-III CUTICLE

Type-III cuticle was present in the imago. It showed two major homogenous layers, an outermost thin layer measuring 5-7 μm and an inner thick part (Figs 1c, 2c). The outer layer was faintly yellowish brown, while the outer part of the inner zone was deeply amber coloured. The two principal layers might correspond to the epi- and procuticle of the type-I and II cuticles, with the amber coloured outer half of the procuticle possibly homologous with the exocuticle, and the colourless inner half corresponding to the endocuticle of the cockroach (DENNELL & MALEK, 1954).

Stained sections revealed the presence of two layers which might be homologous to the outer and inner layers of the epicuticle of *Sarcophaga falcuata* (DENNELL, 1947). The outer thin layer was lipoidal and the lipid was of the nature of sterol, as revealed by a positive Libermann-Burchardt test (Tab. I). The inner layer was lipoprotein as it showed positive reactions to quinone tests and tests for bound lipids. Tests for organic sulphur in the epicuticle of the imago were all negative (Tab. I). Hence the nature of the protein in the epicuticle of the imago was totally different from that in the larvae.

Solubility reactions revealed that the exocuticle had undergone aromatic tanning. The results of the histochemical tests substantiated this inference (Tab. I).

In *A. immaculifrons* adults there existed a layer corresponding to the mesocuticle of *P. americana* (DENNELL & MALEK, 1955). From the results re-

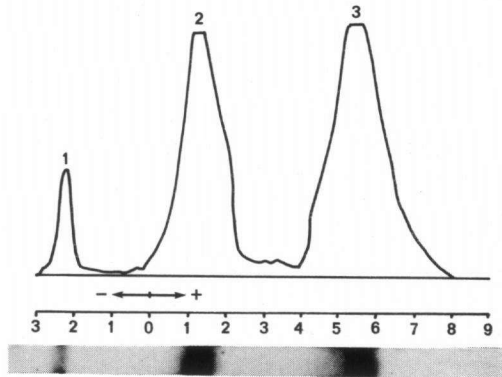


Fig. 3. Electrophoretic pattern of the proteins extracted from the type-I cuticle of *Anax immaculifrons*. The curve above the pherogram was obtained with a Biochem-Densitometer. (Scale line in cm; + and - stand for anodic and cathodic fields respectively).

Table II
Mobility properties of the proteins extracted from the type-I cuticle of *Anax immaculifrons*

Fraction	Mobility (cm)	% of the fraction out of the total concentration
1 Cathodic	2.1-2.5	12
2 Anodic	0.8-1.6	41
3 Anodic	5.1-5.7	47

corded in Table I, it may be noted that the mesocuticle was found to have undergone preliminary tanning, involving aromatic substances like that of the type-II cuticle. Similarly the protein of the endocuticle of the type-III cuticle resembled collagen like that of the type-II cuticle in its swelling, solubility and staining properties (Tab. I).

ELECTROPHORETIC AND CHROMATOGRAPHIC STUDIES ON THE PROTEIN COMPONENT

The results of histochemical studies indicated that the cuticle contained more than one kind of protein and the protein component of each type of cuticle might differ. It is desirable to find out the precise number and nature of these proteins by the application of electrophoretic and chromatographic methods.

Figure 3 shows an electropherogram of the proteins of the type-I cuticle. The mobility properties of these three proteins are given in Table II. It can be seen that one of these proteins is of much lower concentration than the other two. The proteins from these bands were extracted from about 50 electropherograms and the extracts pooled separately. Each pooled protein was hydrolyzed in 6 N hydrochloric acid and analyzed using a two-dimensional chromatographic method (BLOCK et al., 1958). The results are given in Table III. The first fraction had 12 amino acids, including two sulphur-containing ones, fraction 2 had high concentrations of proline and hydroxyproline, in addition to glycine which is a characteristic feature of col-

Table III
Amino acid composition of the electrophoretically separated protein fractions from the type-I cuticle in *Anax immaculifrons*

Amino acids	Protein fraction		
	1 <i>Cathodic</i>	2 <i>Anodic</i>	3 <i>Anodic</i>
ALIPHATIC			
Alanine	++	+	+
Glycine	+	++	+
Isoleucine	++	+	++
Leucine	+	++	++
Serine	+	+	+
Threonine	+	+	—
Valine	+	+	+
AROMATIC			
Phenylalanine	—	—	—
Tyrosine	—	—	—
SULPHUR-CONTAINING			
Cysteine	+	—	—
Cystine	—	—	++
Methionine	++	+	+
HETEROCYCLIC			
Hydroxyproline	—	++	—
Proline	—	++	+
ACIDIC			
Aspartic acid	++	+	+
Glutamic acid	++	+	—
BASIC			
Arginine	—	+	+
Histidine	—	+	+
Lysine	+	+	+

— absent; + intense band; ++ very intense band

lagen. The third fraction had a high concentration of cystine.

The type-II cuticle had five proteins (Fig. 4). A comparison of the mobility properties of these proteins with those of type-I (Tabs IV, V) indicated that the cathodic (1) and first anodic (2) fractions in both cases occupied the same position. Fraction 5 from the type-II cuticle resembled fraction 3 of the type-I cuticle. The third and fourth fractions did not have any homologue in type-I cuticle. These similarities and dissimilarities were reflected in the amino acid constitution of the respective proteins (Tab. V). Fractions 3 and 4 both contained large quantities of the aromatic amino acids but, whereas fraction 4 was rich in cystine as well, this was absent from fraction 3.

Only three protein components were noticed in the type-III cuticle; all in the anodic field (Fig. 5). The first and the second fractions resembled in their mobility properties and amino acid composition those of the first and second anodic fractions from the type-II cuticle (Tabs IV-VII). Fraction 3, which was unique to the type-III cuticle, had only the aromatic amino acids and none of the sulphur-containing ones (Tab. VII).

Table IV
Mobility properties of the proteins extracted from the type-II cuticle of *Anax immaculifrons*

Fraction	Mobility (cm)	% of the fraction out of the total concentration
1 Cathodic	2.0-2.4	14
2 Anodic	0.8-1.1	16
3 Anodic	2.4-3.0	29
4 Anodic	3.9-4.2	19
5 Anodic	5.1-5.4	22

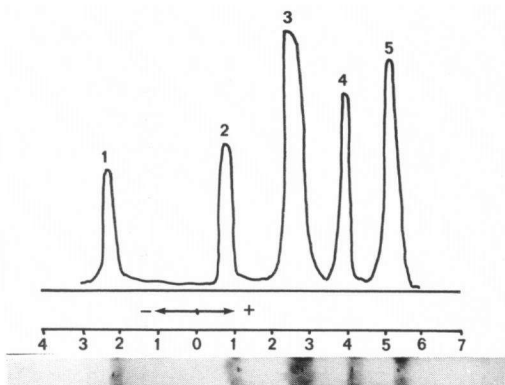


Fig. 4. Electrophoretic pattern of the proteins extracted from the type-II cuticle of *Anax immaculifrons*. The curve above the pherogram was obtained with a Biochem-Densitometer. (Scale line in cm; + and — stand for anodic and cathodic fields respectively).

WEIGHT OF EXOSKELETON

An incidental observation made in the course of the study was the change in the weight of the exoskeleton with advancing instars. The exoskeletal weight was measured as outlined by VARADARAJ (1978). The body weight of ten third

Table V
Amino acid composition of the electrophoretically separated protein fractions from the type-II cuticle in *Anax immaculifrons*

Amino acids	Protein fraction				
	1 <i>Cathodic</i>	2 <i>Anodic</i>	3 <i>Anodic</i>	4 <i>Anodic</i>	5 <i>Anodic</i>
ALIPHATIC					
Alanine	++	+	+	+	+
Glycine	+	++	+	+	+
Isoleucine	+	+	+	+	++
Leucine	+	++	+	++	++
Serine	+	+	+	+	+
Threonine	+	+	+	+	—
Valine	+	+	+	—	+
AROMATIC					
Phenylalanine	—	—	++	++	—
Tyrosine	—	—	++	++	—
SULPHUR-CONTAINING					
Cysteine	+	—	—	—	—
Cystine	—	—	—	++	++
Methionine	++	+	—	—	+
HETEROCYCLIC					
Hydroxyproline	—	++	—	—	—
Proline	—	++	—	+	+
ACIDIC					
Aspartic acid	++	+	+	+	+
Glutamic acid	++	+	—	—	—
BASIC					
Arginine	—	+	+	+	+
Histidine	—	+	+	+	+
Lysine	+	+	+	+	+

— absent; + intense band; ++ very intense band

instar individuals was 1.431 g, and this gradually increased to 9.953 g by the adult stage (Tab. VIII). However, the exoskeletal weight of third instar larvae was 62.2% of the weight of the insect.

Up to the 9th instar the percentage weight of the exoskeleton ranged between 61.4% and 69.3%. From the 10th instar up to the 13th instar there was a lower proportion of exoskeletal weight (54.0% to 59.5%), in the adults it was only 44.9%. Thus, while the total body weight was increasing with advancing age,

Table VI
Mobility properties of the proteins extracted from the type-III cuticle of *Anax immaculifrons*

Fraction	Mobility (cm)	% of the fraction out of the total concentration
1 <i>Anodic</i>	1.0-1.6	32
2 <i>Anodic</i>	2.7-3.1	26
3 <i>Anodic</i>	6.9-7.2	42

the proportion of the weight of the exoskeleton to the body weight was decreasing in the later part of development.

DISCUSSION

In insects aerial life needs a high energy supply, low skeletal weight and a mechanism to conserve water (RAMSEY, 1935; KROGH & ZEUTHEN, 1941; PRINGLE, 1957; WEIS-FOGH, 1961; MACHIN et al., 1962; WIGGLESWORTH, 1964). Studies on the cuticle weight of *A. immaculifrons* showed that indeed the exoskeleton does form a lower proportion of body weight in the adult, as compared to the larval stages. The entire cuticle in the early larval stages (upto 9th instar) was hardened by disulphide linkages. From the 10th to the 13th instar the cuticle showed the advent of phenolic tanning, which was present either independently as in the mesocuticle, or in combination with disulphide linkages (R-S-R) as in the exocuticle. In the imago the organic sulphur was totally eliminated and the mode of hardening of the cuticle involved only aromatic substances. Thus the transformation from the type of hardening of the cuticle with -S-S- bonds, which was found in the apterygote insects, to quinone tanning of the pterygote cuticle takes place in *A. immaculifrons*.

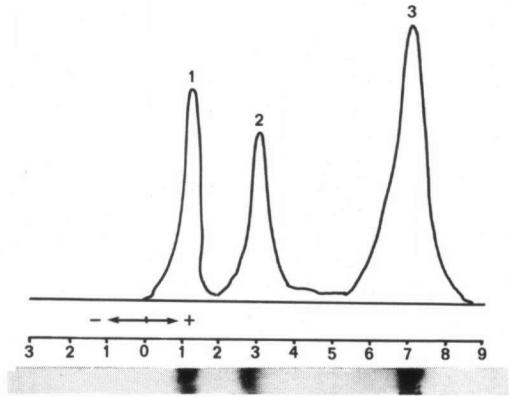


Fig. 5. Electrophoretic pattern of the proteins extracted from the type-III cuticle of *Anax immaculifrons*. The curve above the pherogram was obtained with a Biochem-Densitometer. (Scale line in cm; + and — stand for anodic and cathodic fields respectively).

It was known from the works of LOWER (1958), KRISHNAN & SUNDARA RAJULU (1969) and KRISHNAN (1969) on the cuticle of apterygote insects such as *Ctenolepisma longicaudata*, *Machilis variabilis* and *Smynthurus punctatus* that disulphide linkages having the amino acid cystine made the cuticle heavy. In contrast to this, tyrosine, which takes part in the phenolic tanning, is a very light amino acid. Correspondingly, the quinone tanning would render the cuticle hard without adding weight. The presence of a lighter mechanism of hardening in the adult cuticle in *A. immaculifrons* was obviously an adaptation to aerial life.

Table VII
Amino acid composition of the electrophoretically separated protein fractions from the type-III cuticle in *Anax immaculifrons*

Amino acids	Protein fraction		
	1 <i>Anodic</i>	2 <i>Anodic</i>	3 <i>Anodic</i>
ALIPHATIC			
Alanine	+	+	+
Glycine	++	+	+
Isoleucine	+	+	+
Leucine	++	+	++
Serine	+	+	—
Threonine	+	+	+
Valine	+	+	+
AROMATIC			
Phenylalanine	—	++	++
Tyrosine	—	++	++
SULPHUR-CONTAINING			
Cysteine	—	—	—
Cystine	—	—	—
Methionine	+	—	—
HETEROCYCLIC			
Hydroxyproline	++	—	—
Proline	++	—	+
ACIDIC			
Aspartic acid	+	+	—
Glutamic acid	+	—	+
BASIC			
Arginine	+	+	+
Histidine	+	+	+
Lysine	+	+	+

— absent; + intense band; ++ very intense band

Conservation of water is another major problem for the pterygote insects. The works of RAMSEY (1935), BEAMENT (1945, 1960, 1961a, 1961b), WAY (1950), HOLDGATE & SEAL (1956), BEAMENT et al. (1956) and JACKSON & BAKER (1970) indicated that the chief mechanism of water-proofing in insects is the presence of a free lipid layer on the cuticle. In *A. immaculifrons*, the epicuticle was proteinaceous and monolayered in all the aquatic instars. In the imago, in contrast, the epicuticle was bilaminar; the outer layer was lipoidal while the inner lamina had a lipoprotein complex together with aromatic substances. Thus the cuticle showed adaptation in quality and quantity to the difference in the mode of life of the larvae and adults.

Table VIII
Weight of the exoskeleton in *Anax immaculifrons*

Instars	Body weight (g/10 individuals)	Weight of skeleton (g/10 individuals)	% body weight
3	1.431	0.890	62.2
4	2.245	1.378	61.4
5	3.075	1.946	63.3
6	3.678	2.413	65.6
7	4.433	2.997	67.6
8	5.076	3.304	65.1
9	6.147	4.260	69.3
10	6.768	4.026	59.5
11	7.210	3.893	54.0
12	7.554	4.279	58.8
13	8.009	4.701	58.7
Imago	9.953	4.469	44.9

ACKNOWLEDGEMENTS

The author is thankful to Thiru. G. RAJENDRAN, President and Thiru. G. RAJASEKAR, Secretary, Chikkaiah Naicker College, Erode for their interest and encouragement.

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