The Subimaginal Cuticle of the Mayfly *Callibaetis* sp. (Ephemeroptera)[1, 2]

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ABSTRACT

Mayflies are the only group of insects in which there is usually another molt after a functionally winged stage is attained. Doubt has been expressed in the literature whether the cuticle of the subimago is a true cuticle, and hence whether the subimago represents a true instar. Examination of stages from late naiad through subimago to imago of a species of *Callibaetis* shows that the subimago has a normal cuticle and that its exuvia is also normal. During the subimaginal stage there is a normal molt cycle producing the adult cuticle. Therefore the subimaginal stage represents a true instar. That the ecdysial membrane seems to arise by modification of the subcuticle is suggested from the series of histochemical tests employed. Other details of the subimaginal cuticle are given, including that the staining reactions of intersegmental membranes are distinctive, that both the subcuticle and the ecdysial membrane are visibly fibrous, and that the subcuticle is composed of at least two different materials.

Mayflies are the only insects which normally molt after acquiring functional wings. This is one of the reasons why they have long been the subject of much interest and speculation among biologists. It should be noted, however, that this peculiar molt does not occur in all the Ephemeroptera. In one genus it does not occur at all, it is partial in some, and it occurs only in the male in others (Needham et al. 1935). The aquatic (immature) stages of the Ephemeroptera are called naiads. The term "subimago" is applied to the winged stage between the last aquatic instar and the final form of the insect, the adult or imago. There is some doubt expressed in the literature as to the true nature of the subimaginal "cuticle." After it has been cast, various authors describe it as a "delicate pellicle," "shroud," or "skin," but seldom as a cuticle. Lameere (1917) stated that the cuticle of the subimago divides to form an outer layer which is shed and an inner layer which is retained as the imaginal cuticle. Needham et al. also do not refer to the shedding of the subimaginal "cuticle" as a true molt but as "a

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partial shedding of skin after the adult form has been assumed;" "it is only a casting of the delaminated hairy outer cuticle." Neither Lameere nor Needham et al. presented any direct evidence to substantiate their statements.

The subimaginal period is extremely short, lasting from a few minutes in some species to 24 hours or more in others. Judging from the number of rings in the Palmen body of an adult mayfly, which Needham et al. state as representing successive molts, the mayfly *Callibaetis* appears to undergo approximately 15 molts. Since the interval between hatching from the egg and attainment of the adult stage in *Callibaetis* is 5 to 6 weeks (Needham et al.), each naiadal instar, therefore, averages 2 to 3 days. The subimaginal period of *Callibaetis* was observed to last approximately 12 hours under laboratory conditions. Either the subimaginal cuticle is not a complete cuticle, or the adult cuticle is secreted in a few hours, or the adult cuticle is already present when the subimago emerges from the last naiadal instar. In order to ascertain which of these possibilities was true and in order to learn more about the nature of the subimaginal cuticle, this research was undertaken.

MATERIALS AND METHODS

Callibaetis, probably *ferrugineus* (Walsh),[3] was chosen for this study because it is a pond-water mayfly, therefore comparatively easy to rear in the laboratory, and it has a comparatively short life span with sometimes two generations per summer.

Naiads of various ages were collected during the summer of 1960 from the shallow water of a small lake a few miles north of St. Paul, Minnesota. They were brought to the laboratory and kept at room temperature in pans of aerated water.

For histological study, specimens were severed at the thorax-abdomen intersegmental membrane and fixed in Bouin's fluid (picro-formol) for 5 to 18 hours; those tissues which were to be treated with enzymes were fixed in Carnoy's fluid. It was not possible to determine precisely the developmental age within a naiadal instar; they could only be designated as "young" and "old." Specimens of the subimaginal stage were fixed at approximately hourly intervals from emergence until the ecdysis to the imago. Imagos, like the subimagos, were fixed at known intervals after the last ecdysis. Tissues were dehydrated by means of the butanol series, and imbedded in 20 parts paraffin (m.p. 54°–56° C.) to one part bayberry wax. Head and thoraces were handled separately from the abdomens and were set aside for possible later studies. Each abdomen was sectioned longitudinally at 5μ and the ribbon was divided and placed on five slides. One of the five slides of each specimen was stained with Mallory's triple stain (omitting the orange G). This histological stain was used for observing the various layers of the cuticle. A second slide was stained by the PAS (periodic acid-Schiff) test. A third was stained with Gomori's aldehyde-fuchsin stain following, in general, the procedure outlined by Halmi (1952), with the precautions listed by Elftman (1959), but omitting the use of hematoxylin. A fourth slide was stained with alcian blue following the method described by Baldwin and Salthouse (1959).[4] The fifth slide was reserved for confirmative tests.

The methods used for the trypsin, pepsin, and hyaluronidase extractions are those given by Pearse (1960).[5] Hyaluronidase was used at a concentration of 1 mg./ml. in 0.85 percent saline solution, trypsin at a concentration of 0.2 mg./ml. in 0.05 M borate buffer at pH 8.9, and pepsin at 2 mg./ml. in 0.2 N HCl at pH 1.6.

In general, the terms used in this paper are those used by Richards (1958a).

RESULTS

Changes in Old Cuticle

Thickness.—The fully formed cuticle of *Callibaetis* in all three stages examined is very thin, averaging around 2.5μ. It is perhaps slightly thicker than this in the last naiadal instar. Endo- and mesocuticle are present, the former being twice the thickness of the latter. An exocuticle is only slightly developed in *Callibaetis*. It is seen only in the latter stages of an instar and then it is usually confined to the spines on the surface of the cuticle. The epicuticle is extremely thin; it was ignored in this study since its development could not be readily followed. Just prior to ecdysis, as a result of the action of the molting fluid, the endocuticle is reduced to about half its previous thickness. Here and in the exuvia the cuticle is approximately 1.5μ thick (fig. 5).

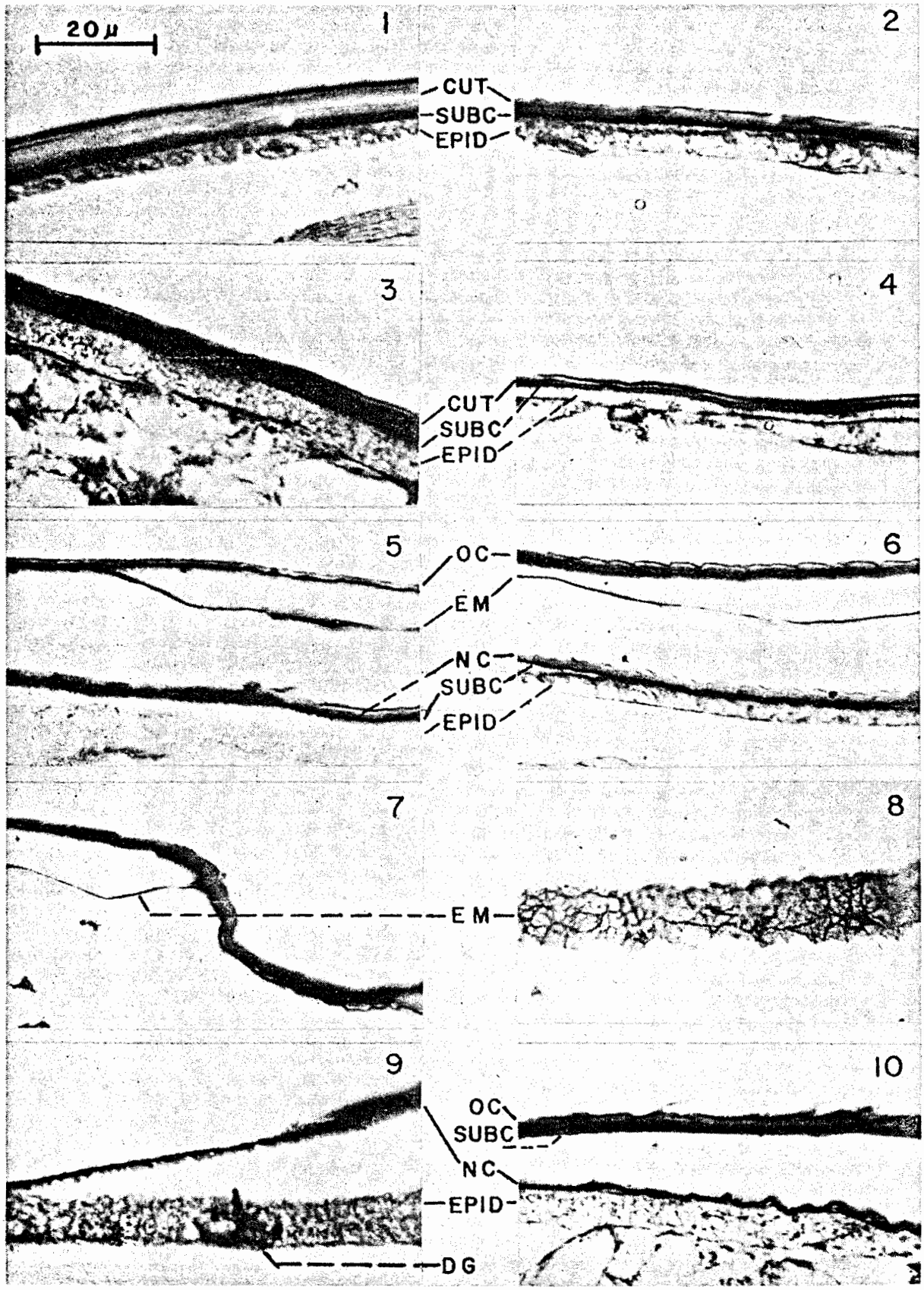
The cuticle of the imago presents an interesting case. It continues to increase slightly in thickness until shortly after the molt (fig. 3), but then it decreases in thickness until it is one-half to one-third as thick by 24 hours after the ecdysis (fig. 4) (the endocuticle is still thicker than the mesocuticle). A possible explanation for this phenomenon will be presented below in the discussion of the epidermis.

Separation of Cuticle from Epidermis.—In sectioning the specimens, the cuticle was often unavoidably separated from the underlying epidermal cells. As a result it was difficult to determine at what time the subimaginal cuticle separated from the epidermis. Since the new imaginal cuticle appears at 3 to 4 hours after emergence of the subimago, one would asume that the separation had probably occurred shortly prior to this. However, at about 7 hours after emergence the subimaginal cuticle is still joined to the new cuticle in the regions immediately posterior to the intersegmental membranes,

[3] Identification by Dr. Lewis Berner, University of Florida.

[4] Alcian blue from Allied Chemical, New York. Nuclear fast red (counterstain) from G. T. Gurr, Ltd., London.

[5] Trypsin and pepsin from Difco Laboratories, Detroit, Michigan. Hyaluronidase from Nutritional Biochemical Corp., Cleveland, Ohio.


20 μ
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and is essentially the same thickness as in the newly emerged subimago. At 8 hours, the endocuticle of the subimaginal cuticle begins to thin, and at 10 hours it is completely free from all underlying structures. It should be noted that the cuticles are two to three times thicker in the regions that separate last, and that in these regions instead of the endocuticle being twice the thickness of the mesocuticle as elsewhere, the mesocuticle is approximately three times thicker than the endocuticle; this is the region marking the separation of primary segments.

Subcuticle.—The subcuticle is seen as a distinct layer between the cuticle and the epidermis after certain staining methods such as aldehyde-fuchsin and PAS (Schmidt 1956); it was found to be readily stained by alcian blue in all stages except the imago (figs. 1, 2). The subcuticle is present at all stages in *Callibaetis*. When the cuticle separates from the epidermis in molting, the subcuticle remains attached to its inner surface, and seems to give rise to the ecdysial membrane. In regions where the cuticle has separated from the epidermis as a result of sectioning, the subcuticle can sometimes be seen to adhere to the epidermis, sometimes to the cuticle, and sometimes split and adhering to both. When the subcuticle is thus split, fibrous material can often be seen filling the space between the old cuticle and epidermis. This may represent the subcuticle in a torn condition.

Ecdysial Membrane.—An ecdysial membrane is present between the new and old cuticles of a last naiadal instar. It varies in thickness at all stages and at all times from less than 0.5μ to 1μ. Its normal position appears to be appressed to the old cuticle, though it is often seen free as in figures 5 and 6. It does not appear to separate from the intersegmental membrane as is shown in figure 7. The ecdysial membrane is part of the last naiadal exuvia. It is sometimes separated from the endocuticle of the exuvia, and it is sometimes contiguous with it.

In the subimago, the ecdysial membrane is first seen 8 hours after emergence, i.e., three-fourths of the way through the instar. It seems to arise from what had been the subcuticle—both having identical histochemical reactions; these are described in a later section. No significant differences in thickness or staining reactions were noted at any time between the subcuticle and ecdysial membrane. It should be noted here, however, that even after the ecdysial membrane is formed, a histochemically identical layer still remains attached to the inner surface of the old cuticle.

The ecdysial membrane is more difficult to find in the subimaginal exuvia than it was earlier. It is possible that during drying it becomes adherent to the inner surface of the exuvia.

Pore Canals.—Pore canals are seen clearly only in the aldehyde-fuchsin preparations. They are extremely numerous with much too small a diameter to be measured with the light microscope. They are seen passing from the epidermal cells part way through the cuticle.

Surface Spines.—The cuticle of the last naiadal instar appears serrated in longitudinal section (fig. 6). From the tip of each caudally directed "tooth" projects a short spine approximately 1μ long. These spines appear about 5μ to 8μ apart. Similarly directed and distributed spines are present on the subimaginal cuticle. Here, however, the spines are considerably longer (9μ to 12μ). In surface view, the subimaginal cuticle appears to be surface-sculptured into somewhat hexagonal-shaped areas; one or two of the 9μ- to 12μ-long spines arise from each of the "hexagonal" regions. There are no spines present on the surface of the imaginal cuticle.

In the last naiadal instar only, there is a ring of long amber-colored spines (30μ to 40μ long) encircling the body and projecting caudad at the posterior margin of each abdominal segment.

Intersegmental Membranes.—The endocuticle of the intersegmental membranes is always thicker than the mesocuticle, even after action by the molting fluid. In fact, they do not appear to be acted on by the molting fluid at all, being the same thickness in the old last naiadal instar and in the exuvia as in the young last naiadal instar. Because of this, the intersegmental membranes of the last naiadal exuvia are thicker than the rest of the exuvia (fig. 7). The intersegmental membranes of the subimaginal exuvia, however, are as thin as or thinner

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All figures are longitudinal sections from the abdomens of *Callibaetis*. They are all at the same magnification. Fig. 1 is stained with alcian blue; the others are stained with aldehyde-fuchsin.

FIG. 1.—Dorsal surface of a young last naiad. Note the darkly stained subcuticular layer between the cuticle and epidermis. FIG. 2.—Dorsal surface of newly emerged subimago. FIG. 3.—Ventral surface of imago approximately 4 hours after emergence. FIG. 4.—Ventral surface of imago approximately 24 hours after emergence. Observe how the epidermis and cuticle have thinned. FIG. 5.—Dorsal surface of subimago at ecdysis. FIG. 6.—Ventral surface of an old last naiad. The new (subimaginal) cuticle has formed and the last naiadal cuticle with its ecdysial membrane is about to be shed. FIG 7.—Ventral surface of the last naiadal exuvia. The intersegmental membrane is seen in the right half of the photograph. It is thicker than the rest of the cuticle and does not possess a free ecdysial membrane. FIG. 8.—Surface view of the ecdysial membrane of a last naiadal instar showing the apparent fibrous nature of the membrane. Green filter. FIG. 9.—Ventral surface of subimago just prior to molting illustrating a dermal gland. Note the duct of the dermal gland. New (imaginal) cuticle has been separated from the epidermis in sectioning. Green filter. FIG. 10.—Dorsal surface of subimago approximately 3 hours after emergence. New (imaginal) cuticle is beginning to form on the epidermis.

CUT = cuticle; SUBC = subcuticle; EPID = epidermis; OC = old cuticle; EM = ecdysial membrane; NC = new cuticle; DG = dermal gland.

than the rest of the exuvia. The explanation for this point possibly has to do with the fact that the last naiadal exuvia is shed in water whereas the subimaginal exuvia is shed in air. The intersegmental membranes of the latter could therefore come to be of lesser thickness than those of the last naiadal exuvia by dehydration.

Formation of New Cuticle

As mentioned earlier, the last naiadal instars could not be aged with any precision. As a result, the development of the subimaginal cuticle in that instar could not be readily followed. In the subimago, however, the imaginal cuticle first becomes visible at 3 to 4 hours after emergence, i.e., one-third to one-fourth of the way through the instar. It is then a very thin membrane less than 0.5μ thick (fig. 10). By 8 hours after emergence a definite endo- and mesocuticle can be seen. The new cuticle continues to increase in thickness through ecdysis and into the imaginal stage.

Epidermis

The nuclei of the epidermal cells are more or less round and granular. They appear the same in all stages (except the old imago) demonstrating no obvious process of breakdown and build-up. No mitotic figures were seen at any stage. Granules are seen in the cytoplasm of the epidermal cells in many of the preparations. These granules appear to follow no developmental process and possibly represent a fixation artifact.

The epidermis of a last naiad appears to remain approximately 4μ thick throughout the instar. In the subimago, the epidermis increases gradually from this thickness until 7 to 10 hours after emergence when it is at its thickest—approximately 5μ; it then decreases slightly until ecdysis. By 24 hours after the molt to the imago, the epidermis has sharply decreased in thickness to 2.5μ to 1μ (fig. 4). The nuclei of the epidermis are now darker-staining, smaller, more elongate, and more granular than previously. They are against the basement membrane while the remainder of the epidermis appears transparent. The thinning of the cuticle (as was mentioned earlier) and the epidermis is possibly due to (1) the loss of cuticular water to the external environment of the mayfly, or (2) the loss of cuticular water or of other cuticular or epidermal constituents to the internal environment of the mayfly (Lower 1959) demonstrated that certain cuticle constituents can be utilized by a starving insect). Male imagos, under laboratory conditions, were observed to live approximately 24 hours; the last specimen described above, a male imago 24 hours old, was very near death and possibly undergoing starvation since adult mayflies are thought not to feed. Females live a week or more under laboratory conditions.

Dermal Glands.—Dermal glands, when present, are round or oval shaped with ducts extending out through the cuticle as seen in figure 9. No dermal glands were seen in the epidermis of the young last naiadal instar; some were seen in the old last naiadal instar, but they were very few in number and appeared to be empty. In the subimago, they become gradually more and more prevalent until 8 hours after emergence when they appear to stop increasing in numbers. They continue to increase in size, however, as the epidermis increases in thickness. They also have some material in them at this time. They appear to be more numerous in the anterior abdominal segments than elsewhere. Twelve hours after emergence of the subimago they are at their largest and contain more material than previously. There were no dermal glands seen in the one specimen that was fixed halfway through the shedding of the subimaginal cuticle. Also, there were no dermal glands seen in the imago.

Ecdysis

The wing pads of the last naiadal instar appear darker in color than those of earlier instars. One-half to one hour prior to molting the naiad takes on a silvery, sheenlike appearance and becomes very active, darting rapidly here and there just below the surface of the water. If the naiad darts toward the bottom of the rearing pan and ceases all muscular movement it immediately floats to the surface, apparently bouyed up by a gaseous layer between the old and new integuments. At all other stages of naiadal life the mayfly remains on the bottom of the rearing pan amongst aquatic plants. Eaton (1883), Causard (1898), and Daggy (1938) note the presence of this gas between the new and old cuticles of emerging mayflies. Causard speculates that the gas is derived from tracheae. Daggy theorizes that it may be a layer of carbon dioxide being given off from the body wall and trapped between the two integument layers as they are separating, and that it apparently functions in holding the naiad at the surface while transformation is taking place. An analysis of this gas would be a profitable area of investigation.

At emergence, the naiad suddenly darts across the surface of the water, withdraws from the exuvia and flies to the screen on top of the rearing pan. Some naiads do this in less than 15 seconds, others take somewhat longer.

The subimaginal ecdysis is no less dramatic than that of the last naiad. Again for 30 minutes to an hour prior to emergence, the subimago becomes quite active. The abdomen begins twitching from side to side, a couple of the legs begin flexing, then one or two of the others—there being no particular sequence to any of these movements. While these events occur, the wings separate slightly and are held more or less vertical, whereas at all other stages the wings are held rooflike over the body. Suddenly the mayfly lowers its wings to slightly less than a horizontal position, the cuticle bursts anteriorly along the ecdysial line, and the mayfly quickly withdraws from the cuticle and walks away

from it. From the time the subimago begins to lower its wings until it walks away from the exuvia takes approximately 15 to 30 seconds.

Histochemistry

Subcuticle and Ecdysial Membrane.—The subcuticle and ecdysial membrane will be discussed jointly since they seem to represent different stages in the development of the same structure, and also since their histochemical reactions are identical. The subcuticle and ecdysial membrane at all stages of *Callibaetis* are positive to the PAS test and are also stained by aldehyde-fuchsin; the latter is always a much stronger reaction. The PAS test is negative if the oxidation with periodic acid is omitted. Identical reactions have been previously noted by Schmidt (1956) for the subcuticle of all six insects of the five orders he examined. The ability of materials to stain with aldehyde-fuchsin closely parallels their giving a positive reaction to the PAS test (Scott and Clayton 1953; Halmi and Davies 1953). As the PAS test is widely considered as specific for certain carbohydrate constituents of tissues, stainability with aldehyde-fuchsin must suggest the probable presence of carbohydrate. In paraffin sections, the only PAS-positive materials are glycogen, or various mucoid substances such as the neutral mucopolysaccharides, muco- or glycoproteins or perhaps a complex of these; acid mucopolysaccharides are PAS negative (Pearse 1960). It has been shown by Schmidt (1956) that muco- and glycoproteins are probably present in the subcuticle.

The subcuticle and ecdysial membrane are also alcian blue-positive. Alcian blue staining, however, is extremely weak in the imaginal subcuticle and in the area under the thick part of the cuticle immediately posterior to each intersegmental membrane (recall that this is the area which separates last in molting). When alcian blue is used under carefully controlled conditions, positively stained areas suggest the presence of acidic carbohydrates, specifically, the acid mucopolysaccharides (Lison 1953; Mowry 1956; Wagner and Shapiro 1957). Further evidence is provided by the fact that the metachromasia produced to toluidin blue 0 and the positive reaction with alcian blue have been shown to parallel one another closely (Wagner and Shapiro 1957). The subcuticle and ecdysial membrane, however, demonstrated only very weak metachromasia. No explanation can be offered for this. On digestion by hyaluronidase, the alcian blue-positive material in the subcuticle and ecdysial membrane remained intact. The only acid mucopolysaccharides known to be fast to hyaluronidase are epithelial mucin and chondroitin sulfuric acid Type B (Pearse 1960). Of these two substances, it would seem more probable that the former would be present in the subcuticle.

As acid mucopolysaccharides are PAS-negative, apparently there are at least two different materials in the subcuticle and ecdysial membrane. One of the materials, which is PAS-positive, may be either glycogen, a neutral polysaccharide, muco- or glycoprotein, or a complex of these; and the other, which is PAS-negative but alcian blue-positive, is an acid mucopolysaccharide. This latter material is present in the imaginal subcuticle in extremely small amounts.[6]

Table 1.—Summary of the histochemical reactions of the cuticular materials.

	Subcuticle and ecdysial membrane	Imago subcuticle	Cuticle	Intersegmental membrane	Epidermis	Basement membrane	Dermal glands
PAS and aldehyde-fuchsin	+++	+++	+	+++	+	+	+
PAS, no previous oxidation	—	O	—	—	—	—	—
alcian blue	+++	+	—	+++	—	—	—
alcian blue after hyaluronidase	+++	O	—	+++	—	—	—
metachromasia	+	O	—	+	—	O	—
metachromasia after hyaluronidase	+	O	—	+	—	O	—
pepsin and trypsin	*	O	**	**	#	O	#

* ecdysial membrane—intact subcuticle—inconclusive
** intact
\# dispersed
+++ very strongly positive
++ moderately positive
\+ weakly positive
— no reaction
O no observation

The fibrous material that is sometimes observed between the old cuticle and epidermis when they have separated in sectioning is positive to aldehyde-fuchsin and alcian blue. It probably also stains with PAS but so weakly that it cannot be readily observed. It should be noted that not all structures are stained identically with PAS, aldehyde-fuchsin, and alcian blue. The tracheal lining, gut lining, lining of the oviducts, chorion, connective tissue of fat body, basement membrane, and muscle sarcolemma all stain positively by the PAS test and aldehyde-fuchsin; of these structures only the first three are stained by alcian blue. It may be significant that of the structures listed, the first three are those in which one would most expect to find an epithelial mucin.

The ecdysial membrane remained fast to pepsin and trypsin digestion. The subcuticle appeared to remain fast but the results here were inconclusive.

Cuticle.—The cuticle proper is negative to alcian blue and only very weakly positive to the PAS test

[6] Recently a new rubber-like protein, "resilin," has been described from elastic ligaments and cuticles of several insects (Weis-Fogh 1960). Resilin is recorded as negative to the PAS reaction and as showing no metachromasia with toluidin blue. Accordingly, it does not seem likely that it is involved in the membranes being studied here.

and to aldehyde-fuchsin. This weak PAS positivity could possibly be due to the presence of some tyrosine, which would give a reaction.

Intersegmental Membranes.—The staining reactions of the intersegmental membranes contrast sharply with the rest of the cuticle. They are strongly positive to the PAS test and to aldehyde-fuchsin. The thicker inner portions of the membranes are alcian blue-positive; the thin outer layer is alcian blue-negative. This condition exists in all stages examined except for the imago where the intersegmental membranes are almost completely alcian blue-negative; such a condition is comparable to the almost complete lack of any alcian blue staining in the imaginal subcuticle.

Epidermis.—The cytoplasm is stained weakly positive to the PAS test and to aldehyde-fuchsin. This result may be due to the presence of some glycogen, but, since no diastase digestions were performed, no definite conclusions can be reached. The basement membrane is positive to PAS and aldehyde-fuchsin. It is negative to alcian blue, apparently lacking any acid mucopolysaccharides.

Dermal Glands.—The dermal glands, like the basement membrane, are positive to PAS and aldehyde-fuchsin. A small percent of them are very weakly positive to alcian blue. The possible function of these glands will be briefly discussed later.

A summary of the histochemical tests together with the results are presented in table 1.

DISCUSSION

The subimago and imago of *Callibaetis* obviously each represent a true instar (defining instar as a stage in the life cycle of an insect separated from another stage by a molt). Shortly after emergence to the subimago the cuticle begins to separate from the epidermis, and the imaginal cuticle begins to form. After spending approximately 12 hours as a subimago, which is one-fourth to one-sixth the length of each aquatic instar, the mayfly sheds the subimaginal cuticle and emerges as an imago.

One of the more interesting things observed in this study was the subcuticular layer and its fate. As was described above, the ecdysial membrane seems to arise from the subcuticle. This situation contrasts with evidence presented by Schmidt (1956). He suggested that the subcuticle he found in *Cecropia* and the ecdysial membrane found in the same insect by Passoneau and Williams (1953) were two different structures. His evidence was threefold. First he showed that the two structures were of different thicknesses. This could be rationalized, however, on the basis of their being different stages in a developmental process. Secondly, he pointed out that there was evidence that the ecdysial membrane of *Cecropia* was homogeneous with conventional or phase contrast optics (Richards 1955), whereas he found the subcuticle to be fibrous. It is interesting in this respect that both layers appear to be fibrous in *Callibaetis* under the light microscope (fig. 8). Thirdly, the ecdysial membrane of *Cecropia* had been shown by Richards (1955) to contain chitin, whereas Schmidt demonstrated that the subcuticle was disrupted by pepsin and trypsin digestion thereby indicating that it does not contain chitin. In the mayfly, as mentioned earlier, the ecdysial membrane is fast to these enzymes. The subcuticle appears to be fast, although the tests were inconclusive. It would be necessary to follow *Cecropia* through molting to be certain of the relationship between these two layers in that insect; Schmidt did not do this.

Malek (1958), with *Schistocerca*, reported that the ecdysial membrane is formed when the innermost laminae of the old endocuticle are "sclerotized" (due possibly to premature secretion or leakage of sclerotizing compounds intended for the new cuticle) and subsequently separated off from the endocuticle by action of the molting fluid. This is consistent with the report that in the pupa of the moth *Anagasta* (=*Ephestia*) an ecdysial membrane forms in only those genetic stocks in which dissolution of endocuticle by molting fluid occurs (Richards 1958b). For a similar phenomenon to be true in *Callibaetis*, it would have to be assumed that the subcuticle represents the inner laminae of the endocuticle that are distinct in their histochemical reactions from the other laminae. While the cuticle increases in thickness in an instar, the inner portions of the subcuticle would have to be continuously formed anew as the outer portions continuously lose their unique staining reactions and become an unidentifiable part of the endocuticle. There is, however, another possible explanation as to the origin of the ecdysial membrane that is also consistent with the observations made in this research. It is possible that the subcuticle at all stages in an instar represents the anlage of the ecdysial membrane, i.e., the subcuticle that is present under the cuticle when it is first secreted is the same subcuticle, structurally and functionally, as that under the cuticle after it has reached its maximum thickness. The endocuticle could increase in thickness as a result of material passing through the subcuticle rather than some sort of conversion of the subcuticle to endocuticle. This latter explanation does not eliminate the possibility that the physical or structural makeup of the ecdysial membrane results from a process such as that described by Malek. All that would be required is that the subcuticle, instead of dispersing as Schmidt suggested, persists and separates off with the old cuticle in the initial stages of molting. The question is not readily settled.

Malek stated that "the ecdysial membrane may best be defined as that layer which is first apparent between the old cuticle and the epidermis at the outset of molting, and which is not subsequently dissolved by the molting fluid." Locke (1958), however, showed that what he called the ecdysial membrane in the tracheae and tracheoles in *Rhodnius* is lost in the molting fluid. Ecdysial membranes previously reported in the literature are said to be struc-

tureless or nonfibrous when viewed under the light microscope (Richards 1955; Lower 1957); they may, however, show a fibrous structure in electron micrographs (Richards 1955). The ecdysial membrane of *Callibaetis,* as already noted, is fibrous under the light microcope (fig. 8). Therefore, in view of this and other recorded differences (Richards 1955; Malek 1958), it would appear that the ecdysial membrane could best be defined as any layer which is apparent between the old and new cuticles at some time in the molting cycle. It remains to be determined whether or not such membranes have the same origin, morphology, and composition in different species of insects (to date ecdysial membranes have been recorded only for several moths, the honey bee, a locust, and now this mayfly; no such membrane is to be seen in cockroaches, *Rhodnius,* or *Tenebrio*).

Schmidt (1956) suggested that (1) the subcuticle functions in fastening the cuticle to the epidermis, and that (2) it possibly functions in the loosening of the cuticle at the time of molting. Possibly, the PAS-positive material of the subcuticle is responsible for the first of Schmidt's suggestions, and the alcian blue-positive material is responsible for the second. This latter statement is derived from the knowledge that the cuticular region that separates last from the epidermis during molting is a region of very little alcian blue-positive material compared to the rest of the cuticle. After the cuticle has completely separated from the epidermis then more alcian blue-positive material is seen in this region. A third possible function of the subcuticle (at least in *Callibaetis*) is that after it changes into an ecdysial membrane, it might conceivably function as a lubricating layer aiding the process of molting. The almost complete lack of an acid mucopolysaccharide in the imaginal subcuticle could possibly be correlated with the fact that the imago has no need of a lubricating layer, only an adhesive layer provided by the aldehyde-fuchsin- and PAS-positive material.

Baldwin and Salthouse (1959) showed with alcian blue staining that the majority of dermal glands in *Rhodnius prolixus* secrete a mucin at the time of molting which they suggest serves as a lubricant in the molting process. The morphological development of the dermal glands in *Callibaetis,* as reported earlier, would tend to support this suggestion; the histochemical data, however, do not. The majority of the dermal glands are negative to alcian blue; the others are only very weakly positive. They are, however, strongly positive to aldehyde-fuchsin and PAS. If they did secrete a lubricating material at molting, it would appear not to be an acid mucopolysaccharide. Since the dermal glands increase their size and stainability at the time when the new cuticle begins to increase in size and when the old cuticle begins to be digested, it is possible that they function somehow in one of these processes.

From the evidence presented in this paper for *Callibaetis,* it cannot be assumed that the same developmental process occurs in all the Ephemeroptera. As was mentioned in the introduction, not all mayflies undergo a complete subimaginal molt. Also, some mayflies, such as *Caenis* and *Ephoron,* spend only a few minutes as subimagos. Though these few minutes probably represent a true instar, it would seem that the imaginal cuticle must be formed (or at least partially formed) during the last naiadal instar. It would be exciting to examine one or both of these genera to see if this were true.

ADDENDUM

Since submittting this manuscript we have learned of experimental work by Dr. C. M. Williams which has certain intriguing aspects relative to our findings. Williams (personal communication) has found that when adult Cecropia moths are induced to molt again by being joined in parabiosis with pupae supplying ecdysone, the abdomen of the adult molts again in a fairly normal manner but the cuticle of the second adult is nearly or completely devoid of scales. These data parallel closely what we found in the subimaginal-imaginal stages of *Callibaetis.* That is, the cuticle of the subimago is setose whereas that of the imago is smooth and shiny; this change is recorded as general for mayflies (Needham et al. 1935).

It therefore appears that when an adult molts again—regardless of whether the molt is normal or induced—it can form a normal cuticle but cannot form setae or scales. This gives another point suggesting that the subimago represents a true instar, and, further, that it is a true first adult instar (as opposed to the possibility of being a last nymphal instar or "hyper-nymphal instar" which it might otherwise be considered since subimagos have not been shown to mate and lay eggs). On the basis of cuticle development, then, the subimago of mayflies compares with the normal adult of *Cecropia* and other insects whereas the true imago of the mayfly compares with the induced second adult stage of Williams' Cecropia moths.

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