THE MOULTING FLUID OF THE CECROPIA SILKWORM

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INTRODUCTION

For a period prior to each moult an insect is surrounded by two cuticles—the old cuticle about to be shed enveloping the new cuticle in process of formation. As first noted by Malpighi (1669), the space between the old and new cuticles is at this time occupied by a transparent fluid, the so-called 'moulting fluid'. The latter has subsequently been found to play an important role in the process of moulting. Thus the old cuticle, when finally shed, consists of only its outermost layers, the innermost portion having undergone dissolution while in contact with the moulting fluid (Plotnikow, 1904; Tower, 1906). Indeed, in the case of hard-bodied insects, the actual shedding of the exuviae becomes mechanically possible only after the old cuticle has been weakened in this manner (Plotnikow, 1904; Tower, 1906; Eidmann, 1924).

In addition to this primary action on the old cuticle, certain investigators have suggested that the moulting fluid serves also as a lubricant in the casting of the skin (Plotnikow, 1904; Verson, 1911; Eidmann, 1924; Hoop, 1933; Schürfeld, 1935). This function appears unlikely for, as Causard (1898) and Wigglesworth (1933, 1948a, b) point out, the fluid is ordinarily resorbed before the shedding of the exuviae takes place.

The secretion of the moulting fluid begins in synchrony with other early events in the process of moulting. Among the first of these is the detachment of the hypodermis from the overlying cuticle (Tiegs, 1922; Hoop, 1933; Wigglesworth, 1933; Kühn & Peipho, 1938; Paillot, 1939; Dennell, 1946). In species where the hypodermal cells are laced to the cuticle by cytoplasmic processes, the latter are retracted from the cuticular pore canals (Tower, 1906). The hypodermal cells elongate and generate, as it were, a surface tension in the hypodermis as a whole. In consequence, the hypodermis retracts from the overlying cuticle to form the exuvial space. The driving force almost certainly lies in the hypodermis itself, rather than in the traction of attached musculature. In fact, as Tower (1906), Kühn & Piepho (1938), and Way (1950) have observed, the sites where muscles insert on the hypodermis are the last, rather than the first regions to lose their attachments to the old cuticle. As the hypodermis retracts, the exuvial space is flooded with the moulting fluid. Meanwhile, the hypodermis begins to elaborate

its new cuticle whose outer boundary is, therefore, in continuous contact with the moulting fluid.

The origin of the moulting fluid has been debated in the older literature. Verson (1890, 1902, 1911) described its production by 'exuvial glands' located beneath the integument of the thorax and abdomen—a view subsequently shared by numerous investigators, including Tower (1902), Eidmann (1924), Poisson (1924), Ertogroul (1929), Wachter (1930), and Wigglesworth (1933). Yet Blunck (1923), Hoop (1933), von Buddenbrock (1930), Schürfeld (1935), Wigglesworth (1947, 1948b) and Way (1950) found that the dermal glands discharge their contents long after the moulting fluid has already appeared. Moreover, the glands are known to be atrophic or absent in the pupal stage of most insects, yet the moulting fluid appears promptly at the outset of adult development (Plotnikow, 1904; Schulze, 1912; Willers & Dürken, 1916; Deegener, 1928; Hoop, 1933). Thus the production of the moulting fluid by special exuvial glands seems to be a discredited theory: there is now general agreement that the principal constituents of the fluid are secretory products of the hypodermis itself.

The literature contains only scanty reference to the properties of the moulting fluid. In histological fixatives its behaviour resembles that of proteins (Tower, 1902; Plotnikow, 1904; Deegener, 1928). Schulze (1912) observed an acid reaction to litmus, Verson (1902) and Plotnikow (1904) an alkaline reaction, and Blunck (1923) and Wigglesworth (1933) a neutral reaction. Wigglesworth's (1933, 1948b) description of the fluid as 'salt-free' was apparently based on negative spot-tests for chloride.

The presence of enzymes in the moulting fluid has been inferred from its capacity to digest the old endocuticle (Tower, 1906; Wigglesworth, 1933, 1948b). For this function the minimal requirements would appear to be a proteinase and a chitinase. Why these enzymes attack the old cuticle and spare the new cuticle has been a recurrent problem (Chauvin, 1949; Richards, 1951). Hamamura, Iida & Otsuka (1940) report the presence in 'moulting fluid' of proteinase, chitinase, invertase and amylase. However, the determinations were performed on extracts of dried, pulverized, larval exuviae—a material which, in our experience, is singularly devoid of moulting fluid.

From this résumé it is evident that only limited insight into the properties of moulting fluid can be gained from a study of the literature. In view of its intimate relation to the process of moulting, the fluid merits more detailed consideration than it has received heretofore. As a first step in this direction we have studied the properties and kinetics of the moulting fluid during the pupal-adult transformation of the Cecropia silkworm.

MATERIALS AND METHODS

Pupae of the giant silkworm, *Platysamia cecropia*, were used as experimental animals. The insects were reared in large numbers and stored at $5 \pm 1°$ C. for at least 3 months prior to use. As described by Williams (1946), such chilled pupae initiate adult development about 2 weeks after being placed at $25°$ C. The first

visible indication of adult development is the initiation of the pupal-adult moult; i.e. the above-mentioned retraction of the hypodermis from the overlying pupal cuticle and the simultaneous appearance of the fluid-filled exuvial space. These changes occur rapidly and throughout the entire animal.

From individuals at various stages in adult development moulting fluid was withdrawn from the exuvial space for study or analysis. Each insect was anaesthetized with carbon dioxide; a disk of pupal cuticle was then excised from the tip of the abdomen, and the moulting fluid withdrawn into fine pipettes. Up to o·1 ml. of moulting fluid could be obtained from a single individual. These procedures were performed under the dissecting microscope. Considerable care was exercised to avoid puncturing the hypodermis and thereby contaminating the colourless moulting fluid with the yellow blood. Additional methods will be described in the specific procedures reported below.

I. THE PUPAL CUTICLE

(1) *Structure*

When a fresh fragment of mature pupal cuticle is viewed in hand-cut sections, two conspicuous layers are evident: a dark brown outer layer, the exocuticle, and a white, lamellated inner layer, the endocuticle. In histological sections a further extremely thin and inconspicuous layer is visible; namely, the epicuticle on the outermost surface of the exocuticle. In the fresh pupal cuticle the exocuticle varies in depth from 6μ in the thinnest regions to 16μ in the thickest. The depth of the endocuticle varies from 36 to 46μ. About four-fifths of the cuticle is therefore endocuticle.

In addition to these well-recognized cuticular components, we have consistently observed that a membrane suddenly appears between the old cuticle and the retracting hypodermis at the outset of the moulting process. We have been unable to discover the precise mode of origin of this membrane, though it apparently arises at the line of juncture of the cuticle and the retracting hypodermis. It is a thin, homogeneous, transparent structure, which is appressed to, but separable from, the overlying cuticle throughout the premoult period. The exuvial space and moulting fluid develop internal to the membrane. If the pupal cuticle is carefully cut away at any stage in adult development, the membrane is exposed and may be teased from the insect as a continuous sheet which extends over the entire insect including the wings. In histological sections its appearance, thickness, and staining properties resemble those of the basophilic basement membrane underlying the hypodermis. The membrane persists as a discrete structure throughout adult development, and is finally shed as an inner lining to the pupal exuviae.

A search of the literature fails to reveal any previous reference to this membrane, for which we suggest the name, 'ecdysial membrane'.* For our present purposes

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* We find the ecdysial membrane to be a conspicuous structure in moulting Lepidoptera. The membrane is present, not only at the pupal-adult moult, but also at the larval-larval and larval-pupal moults. It is possible that an ecdysial membrane underlies the moulted cuticle in other Orders, and is shed as an inner lining of the exuviae.

the ecdysial membrane is of interest because it is interposed between the moulting fluid and the old cuticle.

(2) Chemistry of the pupal cuticle

(a) The exocuticle

The exocuticle is a complex of chitin and protein that undergoes pigmentation and 'tanning' with quinones during the first few days following the pupal moult (Pryor, 1940; Pryor, Russell & Todd, 1947). Once this sclerotization has taken place, the chitin and protein can no longer be hydrolysed by chitinase or proteinase.

(b) The endocuticle

By means of fine forceps the endocuticle was stripped from pupal cuticles and tested separately from the exocuticle for its chemical constituents. The results summarized in Table 1 confirm the presence of both chitin and protein.

Table 1. *Chemical tests on pupal endocuticle*

Test	Specificity	Reaction
Xanthoproteic	Protein; specific for aromatic groups	+
Millon's	Protein; specific for phenolic groups as in tyrosine	+
Sakaguchi	Protein; specific for arginine	+
Ninhydrin reaction on the water extract of endocuticle	Specific for α-amino-acids	+
Nitroprusside	Specific for sulphhydryl groups	o
Chitosan	Specific for chitin	+

(3) Changes in the pupal cuticle

Ten chilled Cecropia pupae were provided with transparent facial windows in order to observe the onset and progress of adult development (Williams, 1946); a sliver of pupal cuticle was then excised from the side of the last abdominal segment of each individual, the wound being sealed with melted paraffin. By means of a camera lucida, the thickness of the exo- and endocuticle was measured under the compound microscope in fresh hand-cut sections. In this manner the initial thickness of the cuticle was calibrated for each individual. The ten pupae were then stored at 25° C. At successive stages during the 3-week period of adult development, slices of pupal cuticle were again removed from the last abdominal segment and measured according to the above-mentioned procedure. Changes in the thickness of the cuticle were judged by comparison with the initial control measurements for each individual.

The results summarized in Table 2 show that the moulting fluid is present during 19 of the 21 days of adult development at 25° C. During the first two-thirds of this period, the overlying cuticle does not change in thickness. However, during the succeeding 6 days, the pupal endocuticle undergoes rapid erosion and dissolution, and by the end of this period has completely disappeared. Meanwhile the pupal exocuticle shows no obvious alterations. Thus, the exuviae shed by the adult moth at the time of emergence consist of the epicuticle, the exocuticle, and the ecdysial membrane.

Table 2. *Time-table for adult development of* Platysamia cecropia *at* 25° C.

(After the termination of pupal diapause)

Stage of development	Days after initiation of development	Thickness of pupal endocuticle (%)
Earliest hypodermal retraction and secretion of moulting fluid	0	100
Contours of face fully formed	2	100
Initiation of pink eye pigment; contours of genitalia fully formed but without pigment or hairs	6	100
Generalized pink eye pigment; no hairs	7	100
Generalized reddish brown eye pigment; no hairs	8	100
Complete brown eye pigment; silky hairs	9	100
Coarse white hairs; no cuticular pigmentation	11	100
Coarse white hairs and tan cuticular pigment	14	—
Face with red hairs; generalized but incomplete wing pigment	17	80
Complete wing pigment; generalized softening of pupal cuticle	18	40
Moulting-fluid resorbed	20	0
Adult emergence	21	—

That the dissolution of the endocuticle is due to the moulting fluid was demonstrated in experiments of the following type:

The cuticle of the last two abdominal segments was removed from developing animals without injury to the underlying adult tissues. A sliver of normal pupal cuticle was then implanted into the moulting fluid and the defect in the pupal cuticle capped over with a plastic cover-slip and sealed with melted paraffin. After the emergence of the hosts, the cuticular implants were removed from the exuviae, sectioned by hand, and examined microscopically.

The endocuticle had disappeared from the implants as well as from the pupal exuviae of the hosts, thus demonstrating a direct action of the moulting fluid on the pupal cuticle. This result argues in favour of the presence in the moulting fluid of a proteinase and chitinase.

II. Physical properties of the moulting fluid

(1) *Consistency*

When first elaborated the moulting fluid is a colourless gel resembling egg albumen in consistency. Consequently, the term, moulting *fluid*, is, at this stage, a misnomer, and one could properly speak of the moulting *gel*. This physical state persists for approximately two-thirds of adult development during which period the moulting gel has no obvious effects on the overlying pupal cuticle (Table 2). By approximately the fourteenth day, adult development has progressed to a stage signalled by the initiation of the tan pigmentation of the underlying adult cuticle. The moulting gel now changes to a moulting *fluid*, and simultaneously begins to act on the pupal endocuticle. By the twentieth day the endocuticle has disappeared leaving the unaltered exocuticle as a thin and crisp residue.

The initial gel-like character of the moulting fluid seems to be a new observation. The transition from gel to sol is of special interest since it occurs at approximately

the same time that the moulting fluid begins to act on the overlying cuticle. We shall hereafter refer to the fluid during the first two-thirds of adult development as 'early-moulting fluid'; during the final one-third, as 'late-moulting fluid'.

(2) Cellular content

Moulting fluid at various stages in development was suspended in a hanging drop of Glaser-Locke solution. No cells were observed in the fluid at any stage. Similar treatment of blood showed the presence of numerous leucocytes.

(3) Solubility

The moulting fluid at all stages is completely miscible with water. Addition of ethyl alcohol caused a conspicuous precipitation in the early moulting fluid and a scanty precipitate in the late moulting fluid.

(4) Refractive index

Measurements of refractive index were performed in order to ascertain whether the moulting fluid might approximate a binary mixture of protein and water, since under this circumstance the index would provide a simple means of determining the protein content of the fluid. The underlying assumption proved to be false, however, so that the measurements, recorded in Table 3, fail to permit calculations of this type.

Table 3. *Properties of moulting fluid and of blood*

	Moulting fluid		Blood	
	Early	Late	Early	Late
Refractive index	1·3383	1·3377	1·3492	1·3431
H_2O content (%)	95·8±0·6	94·5±0·4	85	92
Total nitrogen (mg./ml.)	5·1	6·0	12·4*	9·4*
Protein nitrogen (mg./ml.)	4·7	1·1	8·3*	5·5*
Non-protein nitrogen (mg./ml.)	0·4	4·9	4·1*	3·9*
Protein concentration (g./100 ml.)†	2·96	0·71	5·2*	3·4*
pH	7·35	7·4 to 7·55	6·40	6·65
Redox potential (mV.)‡	+380 to +400	+360 to +390	+330 to +350	+306 to +308
Glucosamine (mg./ml.)	0·145	0·800	0·700	0·800
N-acetylglucosamine (mg./ml.)	0·322	2·600	0	1·200

* Unpublished data provided by William Chefurka.
† Protein nitrogen × 6·25; the assumption is made that this conversion factor is valid for insect proteins.
‡ With reference to potential of calomel electrode.

III. CHEMICAL PROPERTIES OF THE MOULTING FLUID

(1) Water content

Moulting fluid or blood was withdrawn from pupae at various stages in development, placed on previously weighed plastic slips, and reweighed as quickly as possible. A Roller-Smith microtorsion balance, permitting direct readings to ±0·005 mg., was used. The slips were then dried to constant weight in a desiccator.

The water content of the moulting fluid and blood was found to vary, as indicated in Table 3. It will be observed that the moulting fluid, at all stages, is more dilute than the blood, and that the transition from gel to sol is accompanied by only a minor decrease in water content.

(2) Chemical composition

The results of a number of qualitative tests are summarized in Table 4. The chemistry of the blood seems to remain qualitatively constant during the course of adult development. In contrast, the moulting fluid undergoes definite alterations,

Table 4. *Chemical composition of moulting fluid and blood*

Test	Moulting fluid		Blood	
	Early	Late	Early	Late
Xanthoproteic for protein	+	o	+	+
Millon's for protein	+	o	+	+
Biuret for protein	+	o	+	+
Sakaguchi for protein	+	+	+	+
100° C. for protein	+	o	+	+
Ninhydrin for α-amino-acids	+	+	+	+
Tyrosine for tyrosinase	+	o	+	+
Catechol for tyrosinase	+	o	+	+
Fehling's for reducing sugars	+	+	+	+
Molisch's for furfural precursors	+	+	+	+
$FeCl_3 + Na_2CO_3$ for o-dihydroxyphenols	o	o	o	o
Nitroprusside for sulphhydryl	o	o	o	o
For chloride*	+	+	+	+
For calcium†	+	+	+	+
For potassium‡	+	+	+	+

* See Feigel (1943), p. 112.   † Wenger & Duckert (1948), p. 215.
‡ Wenger & Duckert (1948), pp. 231–2.

of which the most conspicuous are (1) a decrease in protein content to levels undetectable in the spot tests utilized; and (2) a disappearance of the enzyme tyrosinase. The positive tests for chloride, potassium and calcium are of interest in that Wigglesworth's (1933, 1948b) description of the moulting fluid of *Rhodnius* as 'salt free' was based on the apparent absence of chloride.

(3) Total nitrogen content

Moulting fluid was analysed for total nitrogen by the micromethod of Umbreit, Burris & Stauffer (1949). Samples consisting of o·2 ml. moulting fluid were diluted to 10 ml. with water, and tests performed on o·5 ml. aliquots.

Results recorded in Table 3 show that the total nitrogen of moulting fluid increases from o·51 % in early development to approximately o·60 % in late development. This is equivalent to an absolute increase of o·9 mg. nitrogen per ml. of moulting fluid. The corresponding values for blood, according to the detailed but unpublished measurements of William Chefurka, are 1·2 and o·9 % nitrogen.

(4) Protein content

The proteins of moulting fluid were precipitated with trichloracetic acid, centrifuged, digested, and analysed for nitrogen by the Conway microdiffusion technique (1947); the titrations were performed with a microburette described by Black (1949).

The results of a total of eight analyses are summarized in Table 3. It is evident that the protein content of moulting fluid decreases markedly during the course of adult development; in late moulting fluid the concentration was one-fourth that of early fluid. Evidently, in the early fluid, over 90 % of the total nitrogen is in protein; in the late fluid less than 20% is in protein. Blood at all stages contains a higher concentration of protein than the moulting fluid (Table 3).

(5) pH

The pH of moulting fluid and blood was measured with a Beckman pH meter in conjunction with a one-drop glass electrode and a small calomel electrode. Results summarized in Table 3 show that at all stages the moulting fluid is slightly alkaline, and that the hydrogen-ion concentration in the blood is nearly ten times that in the moulting fluid.

(6) Oxidation reduction potential

The redox-potential of moulting fluid was examined because pronounced changes in potential during the course of development might activate enzymes that had been held inactive or render some component of the endocuticle available to enzymes already present.

The cuticle of the last two abdominal segments was removed from unanaesthetized developing pupae, and standardized microplatinum and calomel electrodes inserted directly into the moulting fluid. In determinations performed on blood, a few drops were removed with fine pipettes, dispensed into small vessels, and promptly measured.

Results obtained with the Beckman instrument and recorded in Table 3 show that the moulting fluid possesses a slightly higher potential than the blood, and that only minor alterations occur during the course of adult development.

IV. ENZYMATIC ACTIVITY OF THE MOULTING FLUID

(1) Chitinase

As far as is known, the chitinases are the only biological reagent capable of hydrolysing the polymer, chitin, into its monomer, N-acetylglucosamine. Since the chitin-containing endocuticle undergoes dissolution in the presence of the moulting fluid, a detailed study was performed of the chitinase activity of moulting fluid.

(a) N-acetylglucosamine and glucosamine

Moulting fluid and blood were first assayed for the products of the chitinase reaction; namely, N-acetylglucosamine and the deacetylated derivative, glucosamine.

0.2 ml. of moulting fluid or of blood was diluted with 1.0 ml. of phosphate buffer, pH 7.1; the glucosamine content of 0.5 ml. aliquots was then assayed by the colorimetric procedure of Elson & Morgan (1933). Since this test is insensitive to N-acetylglucosamine, and to partial breakdown products of chitin, the latter were hydrolysed to glucosamine using 4 N-HCL, according to the method of Palmer, Smyth & Meyer (1937). The solutions were brought to pH 6.0–6.4 with 6 N-NaOH, diluted to 5, 10, or 25 ml. and then re-analysed for glucosamine. The analytical procedure was accurate to within ± 5 %.

The results, recorded in Table 3, show that both glucosamine and N-acetyl-glucosamine are present in the moulting fluid at all stages. During the period of destruction of the old endocuticle, their concentrations in the moulting fluid increase five- and eightfold, respectively, and detectable concentrations of N-acetylglucosamine appear for the first time in the blood. The recruitment of N-acetylglucosamine, over and above its concentration in the blood, is strong evidence that the late-moulting fluid contains a chitinase.

(b) *Chitinase activity*

Tests for chitinase were performed on purified crayfish chitin most kindly provided by Prof. A. Glenn Richards and on crayfish chitin reprecipitated by the method of Karrer & Hofmann (1929). Both materials showed negative reactions when tested for proteins and reducing sugars; when hydrolysed with 12 N-hydrochloric acid, the yield of glucosamine conformed to theory.

0·1 or 0·2 ml. of moulting fluid was diluted with 1 ml. of phosphate buffer at pH 7·1 and added to tubes containing weighed amounts of chitin. In control preparations the moulting fluid was inactivated by boiling. Parallel tests were performed on the insect blood. The tubes were incubated for 5 days at 37·5° C., their contents filtered, and assayed for glucosamine and N-acetylglucosamine by the technique described above.

Each sample was corrected for the amount of glucosamine and N-acetylglucosamine initially present in the moulting fluid, as determined in control experiments utilizing heated moulting fluid. The difference, representing *in vitro* formation of the breakdown products, was taken as a measure of chitinase activity.

The results recorded in Table 5 are typical of a series of experiments of this type. Chitinase activity was in all cases much higher in late than in early moulting fluid. In the experiments described in Table 5, the difference was 13½-fold.

Table 5. *Chitinase activity of early and late moulting fluid when incubated 5 days with chitin*

Stage of donor	Chitin substrate (mg.)	Vol. moulting fluid added (ml.)	Glucosamine + N-acetylglucosamine (mg.)		
			Unheated moulting fluid	Heated moulting fluid	Difference
Early (5th to 11th day)	6·7	0·2	0·126	—	0·040
	8·3	0·2	0·140	—	0·054
	5·4	0·2	—	0·086	—
Late (12th to 19th day)	8·9	0·2	1·360	—	0·560
	11·0	0·2	1·520	—	0·720
	10·4	0·2	—	0·800	—

When a single sample of moulting fluid was tested in graded dilutions, the chitinase activity was found to be proportional to the concentration of moulting fluid. In all these procedures the enzyme appeared to be remarkably stable. Thus, when the incubation period was extended from 5 to 11 days, the final concentration of breakdown products was greatly enhanced. Parallel tests on the insect's blood failed to reveal any chitinase activity at any stage in adult development.

(2) *Proteinase*

The proteinase activity of moulting fluid was measured in terms of its hydrolytic action on gelatin.

(a) *Viscosity measurements*

A series of Ostwald 5 ml. viscosimeters was first calibrated at 37° C. with distilled water and with a standard 2% solution of Difco gelatin. 5 ml. of gelatin were then placed in each of a series of tubes, along with a crystal of thymol to prevent bacterial growth. The temperature was held constant at 37° C. by the use of a water-bath.

A precise volume of moulting fluid was then added to each tube, mixed with the gelatin, and measurements of the latter's viscosity begun immediately. The efflux time was recorded at frequent intervals until constancy was observed. Parallel experiments were performed on the insect's blood and on moulting fluid which had previously been heated and centrifuged. Comparisons were also made with the action of trypsin on the test system.
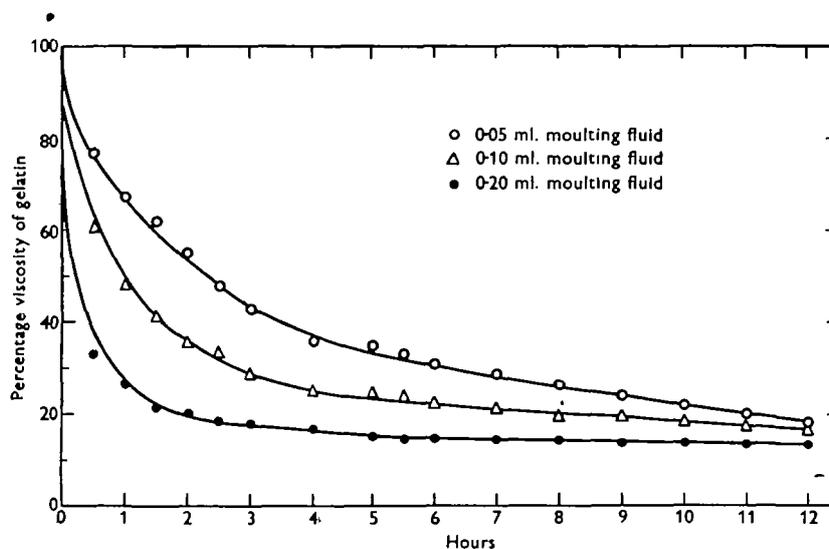

Fig. 1. Percentage change in the viscosity of a standard gelatin solution when incubated with late moulting fluid. On the vertical axis 0% corresponds to the viscosity of distilled water; 100%, to the initial viscosity of the gelatin solution.

Early moulting fluid, obtained from animals during the first 2 weeks of adult development, had no significant effects on the viscosity of the gelatin. In contrast, the late moulting fluid, obtained from animals during the third week of adult development, showed considerable proteolytic activity.

A typical experiment is recorded in Fig. 1, where it will be observed that the late moulting fluid decreased the viscosity of gelatin, over a period of 12 hr., to a value within 20% of that of distilled water. The rate of change in viscosity was proportional to the concentration of moulting fluid. 0·2 ml. of the moulting fluid showed approximately the same proteinase activity as 0·3 ml. of 0·45% trypsin.

Late moulting fluid, previously heated to 100° C., was without significant effects after incubation with gelatin for 20 hr. Negative results were also obtained with the insect's blood at all stages in adult development.

(b) Formol titrations

At the end of each series of viscosity measurements the degree of degradation of the gelatin was determined by formol titration (Koch & Hanke, 1948). These measurements, when suitably corrected for the free amino groups initially present in the gelatin and the moulting fluid, confirmed the proteinase activity of the late moulting fluid.

V. RESORPTION OF THE MOULTING FLUID

As noted in Table 2, the moulting fluid is resorbed during the final 2 days of adult development. Consequently, the surface of the adult moth at the time of emergence is only superficially moist. In experiments in which the disappearance of the fluid was witnessed through plastic windows in the pupal cuticle, the antennal 'cases' of the pupa were found to be the first sites to empty of fluid.

Resorption proceeds at all levels of the insect. The exit of the moulting fluid is accompanied by the entrance of air; the latter first appears in the antennal 'cases' and gradually fills the entire exuvial space. The fluid obviously disappears into the underlying insect, apparently through any region of the integument with which it is in contact. Drops of moulting fluid, trapped between a plastic window and the cuticle of the underlying insect, appeared to be resorbed *in situ*, regardless of their position. Moreover, the fluid underwent normal resorption when isolated anterior or posterior ends of pupae were caused to metamorphose. The ability to resorb the moulting fluid is, evidently, a generalized property of the newly formed integument —presumably of the hypodermis itself.

This conclusion is in agreement with that of Wigglesworth (1933) and suggests, in turn, that the moulting fluid may have a more dynamic relation to the insect than has been demonstrated heretofore. This possibility was tested in the following manner:

The pupal cuticle was removed from the last two abdominal segments of a series of previously chilled pupae, and the opening sealed to a plastic slip by means of melted paraffin. After the insects had initiated adult development, each pupa was anaesthetized with carbon dioxide and the plastic slip carefully removed. To the moulting fluid 0·1 ml. of glycine solution was then added containing 10 μg. of ^{14}C carboxyl-labelled-DL-glycine (activity of 4052·7 CPM) and 40 μg. of unlabelled DL-glycine. The opening in each animal was finally resealed with a plastic slip.

At specific intervals thereafter, animals were sacrificed and assayed for radioactive protein. In this procedure the pupal cuticle was removed and the moulting fluid washed away in a stream of distilled water. The insect was then homogenized in distilled water to a total volume of 5·0 ml. An equal volume of 10% trichloracetic acid was added to precipitate the protein, the homogenization repeated, and the protein collected by centrifugation. The protein was then washed twice on the centrifuge in 5% trichloracetic acid, twice in methyl alcohol, and twice in ether. The final protein fraction was thoroughly

dried, layered in 'infinite thickness' on weighed disks, and assayed for radioactivity by means of a gas-flow Geiger counter.*

The results recorded in Fig. 2 show that the tagged amino-acid promptly entered the organism and was incorporated into the proteins of the moth. The adult protein showed maximal radioactivity on the second day following injection, and subsequently underwent a decrease. The latter we attribute, in part, to the metabolism of the protein and to the resulting loss of ^{14}C as carbon dioxide.
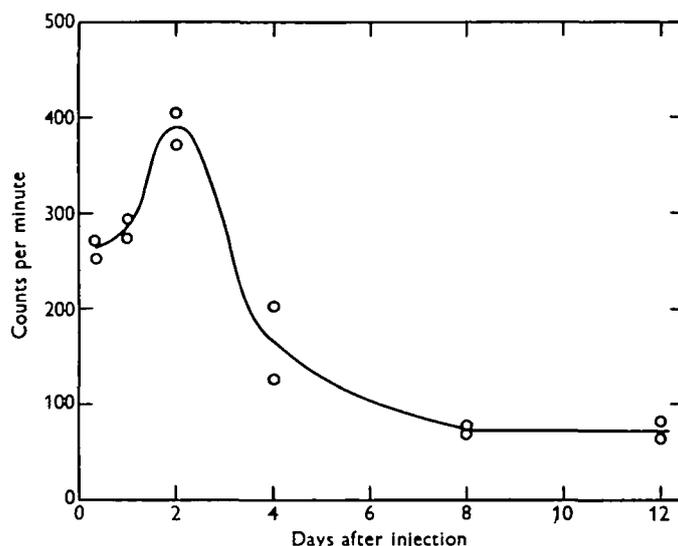

Fig. 2. Radioactivity of proteins extracted from developing Cecropia after the injection of ^{14}C-labelled DL-glycine into the moulting fluid of animals on the second day of adult development.

DISCUSSION

As the experimental results indicate, the moulting fluid at all stages shows physical, chemical and enzymatic properties which differ quantitatively and, in several respects, qualitatively from those of the blood. The moulting fluid is therefore a distinctive fluid compartment of the moulting insect—as distinctive as the blood itself. The insect is 'immersed' in this special fluid during the premoult period: all integumentary surfaces are bathed by the moulting fluid. Consequently, the status of the organism at this time is not unlike that of an aquatic insect breathing through spiracles at the water surface.

The moulting fluid, as we have seen, is secreted at the outset of the moulting process. As the hypodermis detaches itself from the pupal cuticle and retracts, the intervening space is simultaneously flooded with moulting fluid. As Hinton (1946, 1948) has emphasized, this detachment and retraction of the hypodermis is the real

moult; it is merely a technicality that the old discarded cuticle continues to be present for a period thereafter.*

As considered in the Introduction, all the evidence points to the hypodermis as the source of at least the principal constituents of the moulting fluid. Thus the hypodermis, while engaged in secreting and organizing the new cuticle, is called upon to perform yet another task, the synthesis and secretion of the moulting fluid.

More or less abrupt changes in the moulting fluid take place during the course of adult development. The early moulting fluid, present during the first two-thirds of adult development, is a gel with no demonstrable proteolytic activity and only a trace of chitinase activity. The late moulting fluid, present during the final third of adult development, is a sol with considerable enzymatic activity. The obvious function of the early fluid is to protect the delicate underlying insect—a role analogous to that of the amniotic fluid of the mammal. The late moulting fluid is an agent for dissolving the bulk of the old cuticle and permitting the escape of the mature insect.

Most of the observed differences between early and late fluids appear to be secondary to the striking increase in the fluid's enzymatic activity on about the fourteenth day of adult development. The action of the proteinase on the old endocuticle, and on the proteins of the moulting fluid itself, can account for the following changes in the fluid: the gel–sol transformation, the decrease in protein nitrogen, the increase in non-protein nitrogen, and the slight increase in pH. Indeed, all of these changes are encouraged when a pure proteinase, such as trypsin, is incubated with a gelatin solution. The action of chitinase on the old endocuticle is obviously responsible for the considerable increase in glucosamine and N-acetyl-glucosamine; these compounds, in turn, contribute to the increase in non-protein nitrogen.

Acting jointly on the overlying cuticle, the chitinase and proteinase of the late fluid lead to the hydrolytic breakdown of the old endocuticle. But the old, sclerotized exocuticle is spared by virtue of the innate resistance to enzyme action of its tanned protein-chitin complex. The enzymatic reactions reduce the thickness of the old cuticle by approximately 80%, corresponding to the depth of the endocuticle. Indeed, the reduction is almost 100% along the predetermined 'rupture' or ecdysial lines where the exocuticle is extremely thin or absent. Meanwhile, as demonstrated in the tracer studies, the breakdown products of the old endocuticle are continuously reclaimed from the moulting fluid and utilized by the adult moth.

The mechanism for the total resorption of the moulting fluid during the final 2 days of development is unknown. The resorption, as we have seen, appears to take place through the integument as a whole. Provided that the integument is

* A tardy appreciation of this fact has led to considerable confusion in the literature, especially with respect to the pupal-adult transformation. Thus it is commonly assumed that if one looks inside a 'pupa', one encounters an adult moth more or less fully formed. But, in point of fact, such a finding gives assurance that the pupal stage ended days, weeks, or months earlier, when the hypodermis retracted from the pupal cuticle and began to secrete the adult cuticle. Thereafter, one is dealing not with a pupa but with a developing adult. In short, the stage of an insect is not always obvious on external examination and can only be identified with certainty in terms of the type of cuticle to which the hypodermis is attached.

permeable to water, a passive process such as osmosis would favour the resorption of the fluid, since the osmotic pressure of the blood is apparently much higher than that of the moulting fluid. Under this point of view, the final resorption would signal the end of a pre-existing steady state in which active secretion had continuously counterbalanced osmotic resorption. Yet we find that precocious resorption of the moulting fluid fails to occur in animals placed at 5° C.; that is, under conditions greatly inhibiting the active metabolic process while only slightly impairing the passive osmotic process. It is clear that the resorption of the moulting fluid is poorly understood and merits further study.

Finally, there remains to be considered the basis for the moulting fluid's selective action on the overlying old cuticle while the underlying new cuticle is spared. In connexion with this classical problem, it has not previously been appreciated that the early moulting fluid is extremely low in enzymatic activity. Thus, in the case of the Cecropia silkworm, the new cuticle completes two-thirds of its development in contact with moulting fluid incapable of digesting protein. Moreover, we have observed that the enzymatic activity of the moulting fluid rapidly increases in synchrony with the sclerotization of the underlying new exocuticle—a tanning of the chitin protein complex sufficient, in itself, to account for its resistance to enzymatic attack.

Further insight into these events was obtained in endocrinological experiments in which Cecropia pupae were caused to undergo a second pupal moult by the implantation of active corpora allata (Williams, 1952). In such preparations the new pupal cuticle underwent precocious pigmentation and sclerotization in certain localized regions, while other regions remained unsclerotized. It is of particular interest and importance that the moulting fluid became enzymatically active, and attacked the old cuticle in precisely those zones overlying the precociously sclerotized new cuticle.

Experiments of this type suggest a causal relation between the sclerotization of the new cuticle and the activation of enzymes in the overlying moulting fluid. Perhaps the secretion of enzymes into the moulting fluid is coupled to the secretion of the dihydroxyphenol or the quinone which tans the exocuticle. Alternatively, the early moulting fluid may already contain precursors of proteinase and chitinase which are activated by some by-product of the sclerotization reaction. Though the present study failed to reveal any such pro-enzymes in early moulting fluid, it is worth recalling that all extracellular proteinases, thus far characterized, are secreted as pro-enzymes.

SUMMARY

1. The initiation of adult development in the pupa of the Cecropia silkworm is accompanied by a retraction of the hypodermis from the pupal cuticle; the exuvial space is simultaneously flooded with the moulting fluid.

2. During the first two-thirds of adult development the moulting fluid is a dilute, aqueous, proteinaceous gel resembling egg albumen; at this time it is without effects on the pupal cuticle.

3. On approximately the fourteenth day of adult development the gel is converted into a sol and shows a considerable increase in chitinase activity and the first demonstrable proteolytic activity.

4. The late, active moulting fluid then begins to hydrolyse the protein and chitin in the overlying pupal endocuticle.

5. By the twentieth day the old endocuticle has disappeared. The tanned protein-chitin complex of the sclerotized exocuticle is not attacked.

6. Finally, the moulting fluid is completely resorbed into the underlying insect. The residual pupal exocuticle forms the bulk of the exuviae.

7. Moulting fluid and blood are compared in respect to the following properties: consistency, cellular content, solubility, refractive index, water content, chemical composition, total nitrogen, protein nitrogen, pH, redox potential, and enzymatic content.

8. The moulting fluid shows numerous quantitative and qualitative differences from the blood. The fluid is, evidently, a distinctive secretory product of the underlying integument—presumably of the hypodermis itself.

9. A dynamic state exists between the moulting fluid and the underlying insect. Radioactive glycine, injected into the moulting fluid, was promptly resorbed and incorporated into the protein of the adult moth.

10. Several lines of evidence suggest that the secretion or activation of the moulting fluid enzymes are synchronized with the sclerotization of the underlying new cuticle. This synchronization, it is suggested, may serve to protect the new cuticle from the hydrolytic action of the moulting fluid.

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