contraction, beyond the likely action determining the PSPP, it is plausible to think of a triggering action on the sarcoplasmic reticulum which in turn leads to calcium release. (It has in fact been shown by Caputo and Di Polo[17], and Ashley et al.[18] that the calcium which activates the contractile system in barnacle fibers comes from intracellular stores, namely the sarcoplasmic reticulum.) Suarez-Kurtz and Sorenson[19] have come to a similar conclusion; they found a suppression of tension output when crab fibers bathed in either Ca^{2+} or Sr^{2+} media were treated with procaine, a suppressant of Ca^{2+} release from the sarcoplasmic reticulum.

From the evidence that EGTA-injected barnacle fibers can, in presence of Sr^{2+}, give rise to marked prolongation of the spike potential, it follows that: a) both Ca^{2+} removal and the presence of Sr^{2+} represent the conditions for the PSPP onset; b) the first condition seems to be attained either by 0Ca-ASW application or EGTA injection. With regard to the second point, Ca^{2+} removal is not to be interpreted as an intracellular calcium depletion, since 0Ca-ASW application has been shown to cause only a very little net loss of total intracellular calcium: less than 5% in the first hour[20]. However, no distinction was made among the various Ca^{2+} fractions inside the cell, and in which structures they are stored[21]. Therefore, it is difficult to recognize which calcium represents the net loss during 0Ca-ASW application, even though free myoplasmic Ca^{2+} is the most likely candidate.

As to the dependence of the development of the PSPP on the $[Ca^{2+}]_0$, a Ca-dependent membrane conductance effect may be tentatively considered. This view is supported by the observation that in leech neurons the increase in K-conductance after the spike requires the presence of external calcium[22]. Furthermore, the hypothesis that Ca^{2+} ions entering the fiber during the action potential are necessary for substained K-conductance finds also support from those experiments in which selective K-conductance increased upon intracellular injection of Ca^{2+}[23].

As far as the role played by Sr^{2+} in the PSPP onset is concerned, any interpretation would sound speculative because too little information is at present available. What has been shown is that Ba^{2+}, the other divalent ion which can enter the fiber during the activation and produce a spike in the absence of Ca^{2+}, can affect the membrane response in the way of producing PSPPs. While this contributes little to the understanding of the phenomenon, the evidence that the effect is not specific for a particular ion suggests that the adsorption of divalent ions on the outer side of the membrane could modify the field charge within it and so alter certain functions (namely, membrane conductance) mediated by the membrane.

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Excretion of acid hydrolases during molting in *Philosamia ricini*

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Summary. The level of the acid hydrolases β-glucuronidase (EC 3.2.3.11), acid phosphatase (EC 3.1.3.2) and acid protease (EC 3.4.4.) was studied during larval growth and molting in *P. ricini*. The level of activity of these enzymes remained low during larval growth; however, the level increased sharply at the time of molting and declined sharply thereafter in the newly ecdysed insect. Interestingly, the diminished activity of these enzymes was almost quantitatively recovered in the cast-off cuticle. The excretion of acid hydrolases through the cast-off cuticle has hitherto not been reported in insects during molting.

These observations suggest that during ecdysis the acid hydrolases are probably localized in the molting fluid present between the old and new cuticular layers. Since they cannot be transported to the hemolymph, they are excreted through the cast-off cuticle.

The level of acid hydrolases is known to increase during molting and metamorphosis in many insects[2-7]. The increase in acid hydrolase activity is an adaptive response to the autolytic or histolytic events occurring during metamorphosis. In insects, the fate of increased lysosomal enzymes once the histo-

lytic events come to an end is not very well known. In anurans it has been suggested that the increased lysosomal enzymes from degenerated tadpole tail are carried to the liver by the macrophages on the completion of metamorphosis. The degenerated mucosa epithelium of the intestine, together with the lysosomal enzymes it contains, is shed into the lumen of the intestine and then excreted from it[8,9].

Materials and methods. Philosamia ricini was reared as described by Pant and Lacy[10]. Larvae starved for 6 h and prepupae were picked up at random from insect colonies of known age and stage of development. They were weighed, chilled and homogenized with ice cold glass distilled water in a precooled Potter-Elvehjem homogenizer to give a 10% (W/V) tissue concentration. The homogenate was strained through nylon cloth to remove tissue debris and centrifuged at 3000 rpm for 10 min at 4–10 °C. The supernatant was employed for various enzymic assays. The same procedure was adopted for homogenizing the fresh larval excreta and cast-off cuticles.

β-Glucuronidase activity was assayed at pH 5.0 for 24 h at 37 °C by the method of Fishman[11] employing phenolphthalein mono-β-D glucuronic acid (0.001 M) as the substrate. Glycine buffer (0.2 M glycine in 0.2 M NaCl, pH 10.4) was used to stop the enzymic reaction as well as for color development. Acid phosphatase was determined as described earlier[5]. Acid protease was assayed at pH 5.6 according to Matsushita and Iwami[12] employing soluble casein as substrate. Tyrosine liberated during the enzymic reaction was determined using Folin-Ciocalteau's reagent. The reaction was carried out at 37 °C for 3 h. The results presented are means of duplicate determinations made on 3 individual samples of homogenate prepared from 3 lots of insects at various stages of larval development.

Results and discussion. Philosamia ricini is a lepidopteran insect which has 5 instars during its larval growth. During each instar ecdysis, the insect abandons feeding and becomes inert and motionless for about 24–36 h till the process is completed. The process of shedding the old cuticle and the formation of a new one in its place probably occurs concurrently during this time. The acid hydrolases were most active at the time of ecdysis (figs. 1–3). However, after ecdysis the activity declined sharply in the newly ecdysed larva. Fecal samples did not reveal any acid hydrolase activity whereas cast-off cuticles revealed significant activity (table). The decrease in the activity of acid hydrolases in the newly ecdysed larva can be almost quantitatively accounted for by the activity observed in the cast-off cuticles (table).

The excretion of acid hydrolases through the cast-off cuticle is unique for *P. ricini* and for insects in particular. The acid hydrolases perform an important function in insects during degeneration of larval tissues and formation of adult tissues in their place at the time of metamorphosis. In *Sarcophaga bullata,* prior to pupation, the larval midgut epithelium cells shrink in size and become filled with lysosomes as a prelude to degeneration and replacement by adult tissues. This shrinking in size and proliferation of lysosomes is the result of an increased titre of molting hormone[13]. Likewise, cuticular epithelial cells possess the property of retracting from the old cuticle (apolysis) and synthesizing a new one in vitro under the influence of β-ecdysone[14,15]. Thus, from the aforesaid observation, it is feasible that the enhanced activity of acid hydrolases of *P. ricini* during molting is due to the high titre of MH, and these enzymes probably participate in the histolysis (digestion) of the old cuticle. Since the acid hydrolases are excreted via cast-off cuticle, it is possible that they are localized in the molting fluid present between the old and new cuticular layers. Locke and Krishnan[16] described this fluid as 'molting gel' present in the molting space between the old and new cuticular layers. It has been reported that it contains several enzymes viz., chitinase[17], chitobiose[17] and proteinase[18] which are involved in the degradation of old cuticle. The cuticular hydro-

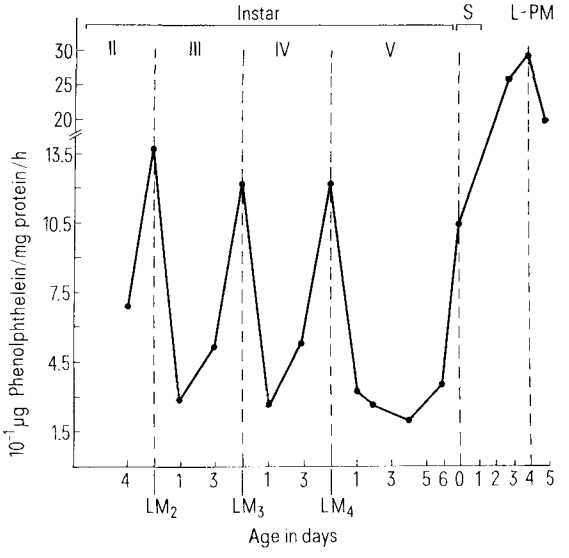

Figure 2. β-Glucuronidase activity during successive moltings of *P. ricini.*

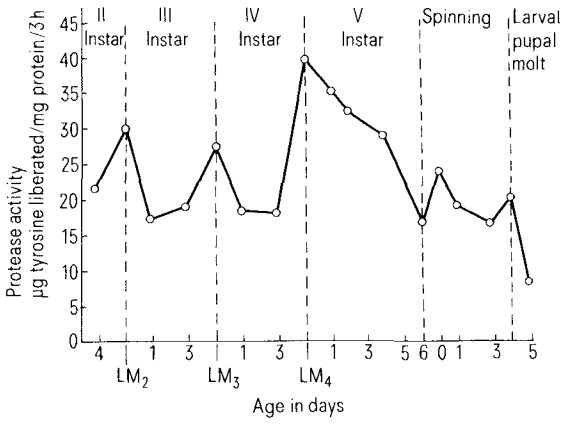

Figure 1. Variation in proteolytic activity during successive moltings of *P. ricini;* LM, larval molt.

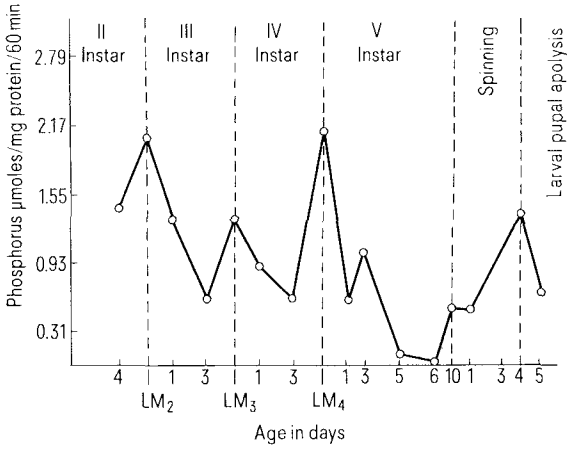

Figure 3. Variation in acid phosphatase activity during successive moltings of *P. ricini.*

Acid hydrolase activity during ecdysis and in cast-off cuticle of *P. ricini*

Molting stages	During ecdysis; total activity/insect			After larval-larval ecdysis; total activity/insect			Cast off cuticle; total activity/cuticle		
	a	b	c	a	b	c	a	b	c
LM_2	2.75	77.10	3.55	1.78	41.87	0.74	0.94	34.38	2.00
LM_3	8.85	338.56	14.94	6.14	228.17	3.30	3.00	101.66	8.33
LM_4	47.27	1686.17	52.96	13.81	1468.24	12.25	31.00	181.50	41.00

LM, Larval-larval molt; *a* acid phosphates activity (μmoles phosphorus released/60 min); *b* acid protease activity (μg tyrosine liberated/3 h); *c* β-glucuronidase activity (μg phenolthalein liberated/h).

lysate is then digested in multivesicular bodies and lysosomes and released into the cytosol[19]. At the time of resorption of molting fluid, appreciable amounts of pigment and products of the cuticular hydrolysate originating from the larval endocuticle and epidermis (but now present in molting fluid) are transferred across the integumentary epithelium and after passing through the hemolymph are taken up through the midgut epithelium into the closed lumen of the midgut[19]. In anurans, a definite mechanism for the excretion of lysosomal enzymes during metamorphosis is known[20]. An anuran tadpole, however, is an open system in the sense that it can take in the nutrients and other things it requires from its environment, and can excrete the waste products into it. An insect larva in molt, on the other hand, is a closed system. It has to meet its nutritional and other demands within the body and the undesirable excretory products formed are also retained within it because of its inability to dispose of them immediately.

The present study reveals that in *P. ricini* the disposal of acid hydrolases is achieved via the cuticle once their function is completed, the modus operandi being different from that in the anuran vertebrates. These observations also suggest that during larval ecdysis the acid hydrolases are probably localized in the molting fluid present between the old and new cuticular layers. However, the lysosomal localization of acid hydrolases has yet to be established. These enzymes probably participate in degradation and digestion of old cuticle. Since they cannot be transported to the hemolymph, they are excreted through the cast-off cuticle.

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DNA methylation in chicken brain and liver

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Summary. Adult chicken brain DNA is subject to a developmental alteration of methylation at *Hpa*II sites flanking 4, 1, 0.8 and 0.7 kilobase sequences. This change is not evident in adult chicken liver DNA and 1-day-old chick brain and liver DNAs, suggesting tissue specificity and age-dependency in DNA methylation.

5-Methylcytosine is the only minor base found in all eukaryotic DNAs so far investigated[1-4]. Although the biological significance of this modification is currently unknown, it has been suggested that methylation may be involved in gene expression, cell differentiation and development[5-7]. It is therefore of interest to compare the degree of DNA methylation in different tissues. This paper describes the analysis of DNA methylation in chicken brain and liver by using high-pressure liquid chromatography (HPLC) and restriction endonucleases.

Materials and methods. The brain and liver tissues were obtained from 6-month-old White Leghorn female chickens (*Gallus gallus* var. *domesticus*) and 1-day-old chicks killed under chloroform anesthesia. To isolate liver tissue depleted of blood, liver perfusion was performed with Hanks' balanced salt solution. The tissues were homogenized and lysed in a solution containing 8M urea, 1% sodium dodecyl sulfate (SDS), 1mM disodium ethylenedi-aminetetraacetate (Na_2EDTA), 1 M sodium perchlorate and 0.24 M phosphate buffer, pH 6.8. After extraction with a mixture of chloroform and isoamyl alcohol (24:1, v/v), the DNA from these tissues was isolated by the hydroxyapatite batch elution technique[8]. The absorbance at 260 and 280 nm was measured to assess DNA concentration and purity. The ratio of A_{260}/A_{280} was between 1.8 and 2.0.

Chick and chicken DNAs digested with enzymes which recog-