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A MOLTING RHYTHM FOR SERUM PROTEINS OF THE COCKROACH, *BLATTA ORIENTALIS*

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Abstract—1. Polyacrylamide gel electrophoresis (PAGE) in 4% gels of *Blatta orientalis* larval hemolymph revealed 4 major proteins.

2. Maximum incorporation of [^{14}C]-leucine into total hemolymph protein occurs within 24 hr and is followed by negligible turnover in the next 24 hr.

3. Quantitative PAGE was used to monitor concentration changes during the molting cycle in synchronously molting cultures.

4. All 4 proteins show the same temporal rhythm of variation but different amplitudes.

5. The widely differing concentrations achieved are accounted for by different rates of synthesis.

INTRODUCTION

Using specific antisera, Kunkel & Lawler (1974) demonstrated that the concentration of a major hemolymph protein of larval cockroaches (LSP) fluctuates greatly during each molting cycle. The concentration of LSP rises steadily throughout the instar and begins a rapid decline shortly before ecdysis. The maximum concentration attained is a function of the length of the instar (Kunkel, 1975*a*).

LSP is a major serum protein in species throughout the order Dictyoptera and antigenically related proteins are also found in the closest relatives of cockroaches, the termites and preying mantis (Kunkel & Lawler, 1974).

The physiological role of LSP is not known but its characteristics, mentioned above, point to a unique role in the molting process. If so, the temporal pattern of change in LSP should stand in sharp contrast to that of the other hemolymph proteins which are not larval specific.

In this paper, molting cycle fluctuations of all 4 major hemolymph proteins of the cockroach, *Blatta orientalis*, are compared and contrasted. The rate of incorporation of radioactivity into protein is also described. It is concluded that the general pattern of change is the same for all 4 proteins and is not a property of LSP alone.

MATERIALS AND METHODS

Animals

Cultures of the Oriental cockroach, *B. orientalis*, were maintained at 30°C as described previously (Kunkel, 1966; Kunkel & Lawler, 1974).

Synchronized molting is achieved by controlling the availability of food. Newly molted animals are stored at 15°C until all animals in a culture have molted. The culture is then returned to 30°C and fed to initiate the next instar.

Animals were injected under light CO_2 anesthesia using a 10 μl Hamilton syringe or a 1 μl repeating injector (Unimetrics). Microcap capillary micropipets (Drummond) were used to collect hemolymph from the trochanter of an autotomized leg. The hemolymph sample was diluted into a convenient volume of PBS (0.15 M NaCl, 0.01 M phosphate buffer at pH 7.5).

Disc gel electrophoresis

Disc gel electrophoresis was carried out according to the standard method of Davis (1964) in which the gel buffer is at pH 8.9 and the reservoir buffer at pH 8.3 but the stacking gel was omitted. Preliminary experiments had determined that 4% acrylamide (acrylamide:BIS:20:1; 8 × 0.5 cm gels) provided optimum separation of the 4 hemolymph proteins.

The gels were scanned at 280 nm in a quartz cuvette immediately after electrophoresis and at 620 nm after fast green staining (Gilford 240 spectrophotometer with linear transport). The area under a peak was measured with a planimeter (Keuffel & Esser). The staining technique of Gorovsky *et al.* (1970) was modified: fast green (Fisher Scientific; 1% in distilled water) was freshly diluted 1:21 with 12.5% trichloroacetic acid (TCA). Each gel was immersed in 7 ml of this staining and fixing solution for 18 hr and destained by diffusion in three changes of 7.5% acetic acid.

Radioisotope techniques

The incorporation of radioactivity into hemolymph proteins was determined after injecting animals with 0.18 μCi of [^{14}C]-leucine (312 mCi/mmol, Schwarz/Mann). Hemolymph samples and solubilized gel slices were counted in 5 ml of a Triton X-100-based scintillation fluid (Benson, 1966). Gel slices (1–10 mm) were solubilized by overnight incubation at 60°C in glass scintillation vials containing 0.4 ml of 30% hydrogen peroxide (Young & Fulhorst, 1965). Vials were counted at 10°C in a Nuclear-Chicago Mark I spectrometer.

The incorporation of radioactivity into total protein was determined by TCA precipitation on to glass fiber discs (Bollum, 1966). The discs (23 mm diameter) were cut from a Whatman GF/A glass fiber sheet. Samples were applied and allowed to dry. The discs were treated with 4 ml/disc of cold 5% TCA for 15 min and sequentially washed with cold 5% TCA, warm ethanol:ether (3:1), cold ethanol:ether (1:1) and room temperature ether. After drying, the discs were counted in Liquifluor (Nuclear-Chicago).

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Production of $^{14}CO_2$ was monitored by absorption into 23 mm glass fiber filters soaked with 300 μl of 10% KOH and placed in the culture dish with the [^{14}C]-leucine-injected animals. After drying, the filters were counted in Liquifluor.

RESULTS

Kinetics of labeling

The rates of incorporation of [^{14}C]-leucine into total hemolymph protein were determined on selected days of the metamorphic 7th instar. On days 3 and 6, 25 animals of each sex were injected with 0.18 μCi of [^{14}C]-leucine. Hemolymph (5–10 μl) was collected from 5 animals of each sex at intervals after injection. The release of $^{14}CO_2$ in the culture dish as a result of the metabolism of [^{14}C]-leucine was also monitored. Even though the 7th instar is considerably longer in males than in females, there was no significant difference between the sexes on days 3 and 6; the data from both sexes were therefore combined (Fig. 1).

The labeling kinetics in Fig. 1(A) show that labeled proteins, as assayed by TCA-precipitable radioactivity, accumulate steadily in the hemolymph for the first 12 hr. After reaching a maximum concentration at 24 hr there is no significant decrease during the next 24 hr. By 24 hr, little [^{14}C]-leucine is available for protein synthesis, most of it having been metabolized in the first 24 hr (Fig. 1B).

The plateau that is reached at 24 hr on both days indicates that, after synthesis, little net removal of protein occurs. This suggests that at these times in the molt cycle the hemolymph is storing protein. The amount of radiolabeled protein present in the hemolymph at 24 hr after [^{14}C]-leucine injection can be used as a measure of the rate of synthesis. Figure 1(A) shows that the rate of synthesis of hemolymph protein on day 6 is almost double that on day 3.

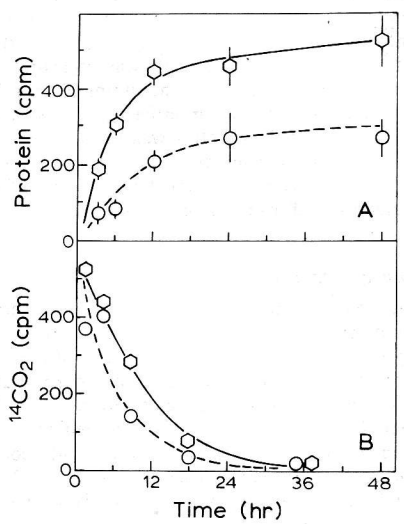

Fig. 1. (A) Incorporation of [^{14}C]-leucine into protein. Seventh instar animals were injected on day 3 (circles) and day 6 (hexagons) with 0.18 μCi of [^{14}C]-leucine (312 mCi/mmol). TCA-precipitable radioactivity per μl of hemolymph was determined. The vertical lines indicate the standard error. (B) Release of $^{14}CO_2$ after [^{14}C]-leucine injection. Filter discs impregnated with 10 N KOH were used to trap $^{14}CO_2$. The values are plotted at the midpoint of the collection intervals.

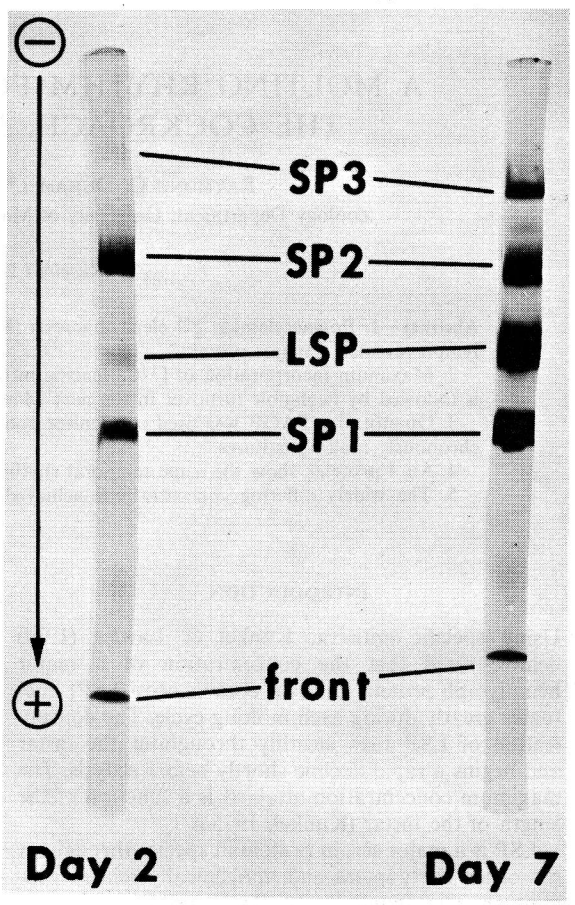

Fig. 2. Polyacrylamide gel electrophoresis of larval hemolymph. Samples (2 μl) from animals on day 2 and day 7 of the 5th instar were electrophoresed in 4% polyacrylamide gels. The gels were stained with fast green.

Quantitation of polyacrylamide gels

Disc electrophoresis in 4% acrylamide gels fully resolved the major hemolymph proteins. Gels illustrating the staining patterns on days 2 and 7 of the 5th instar are shown in Fig. 2. There are 4 bands, three of which correspond to proteins identified as sucrose gradient fractions by Kunkel & Lawler (1974). The Kunkel and Lawler terminology will be used: Serum Protein I (SP1) (R_f = 0.64), Serum Protein II (SP2) (R_f = 0.37) and Larval-specific Serum Protein (LSP) (R_f = 0.49). A fourth protein is here named Serum Protein III (SP3) (R_f = 0.25). The correspondence of the gel bands and the previously published sucrose gradient fractions was established using specific antisera in Ouchterlony double diffusion plates (not shown).

Protein concentration in gels was determined spectrophotometrically by scanning gels immediately at 280 nm and, after staining with fast green, at 620 nm. A wide range of concentration could thus be measured. The high correlation of u.v. absorption with fast green staining is demonstrated in Fig. 3. Using a purified sample of LSP, it was determined

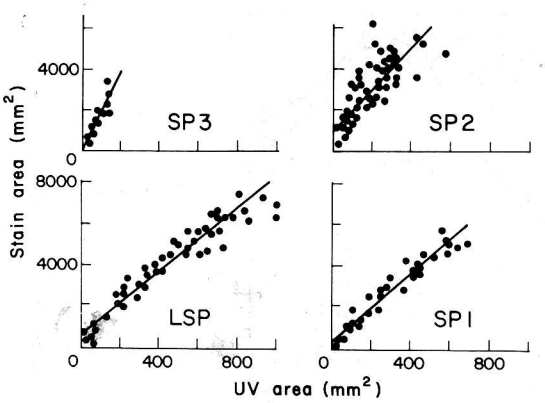

Fig. 3. Correlation of u.v. absorption with fast green staining in polyacrylamide gels. Disc gels of 5th instar hemolymph samples were scanned at 280 nm (u.v. area) and at 620 nm after staining with fast green (stain area). The straight lines represent the fitted least squares regression equations.

that 1 μg of LSP (dry weight) corresponds to 1200 mm^2 of stain area.

Pattern of change during the molting cycle

The concentrations of each of the 4 proteins were measured in 5th instar animals on the first 10 days after feeding (Fig. 4). The mean ecdysis time for this culture was 11.2 $\pm$ 0.3 (S.E.) days after feeding. On day 10, 5% of the animals had molted and as a result the sampling could no longer be considered random; the molted and pre-molt values on day 10 are plotted as separate points. Samples on day 0 (the day of feeding) were taken from animals stored an average of 3 days without food after the previous ecdysis.

Protein concentration in Fig. 4 is expressed in units of fast green stain (mm^2/μl) representing the area under a peak in a gel scan. Highly concentrated gel bands could only be read at 280 nm; u.v. absorption units were converted to units of fast green stain (620 nm) according to the linear relationships shown in Fig. 3.

The incorporation of [^{14}C]-leucine into the 4 proteins is also shown in Fig. 4. It is apparent that the rates of incorporation into each of the 4 proteins change substantially during the instar. These rate changes bring about corresponding changes in the concentrations of the 4 proteins.

The plotting of absolute concentrations in Fig. 4 does not draw attention to changes in the relative proportions of the 4 proteins; yet, these changes are substantial. For example, although SP2 increases in concentration from day 2 to day 6, the percentage of total hemolymph protein that it represents decreases from 50 to 20%. LSP shows the most pronounced change, increasing from 5 to 42% of the total protein. The two gels in Fig. 2 dramatize this shift in staining pattern from day 2 to day 7.

Superimposed upon the change in relative proportions is a temporal pattern of change in absolute concentration that is common to all 4 proteins. This becomes obvious when the concentration data are normalized as in Fig. 5. In each case the molting cycle consists on an accumulation phase (from day 1 to day 6), a plateau (from day 6 to day 8) and a decline that begins a few days before ecdysis and continues through ecdysis. The decline continues after ecdysis as judged from day 0 and day 1 animals which have, of course, recently molted from the 4th instar.

The concentration changes in Figs 4 and 5 are not due to mere changes in hemolymph volume. Hemolymph volume, measured by dilution of [^{14}C]-carboxyl inulin (Levenbook, 1958; Wharton *et al.*, 1965), increases steadily from 14 μl to 24 μl in the 5th instar without any rapid fluctuations (not shown).

DISCUSSION

The most significant finding of this study is that all 4 major proteins of the hemolymph in the cockroach, *B. orientalis*, show essentially the same basic pattern of variation during the 5th instar molting

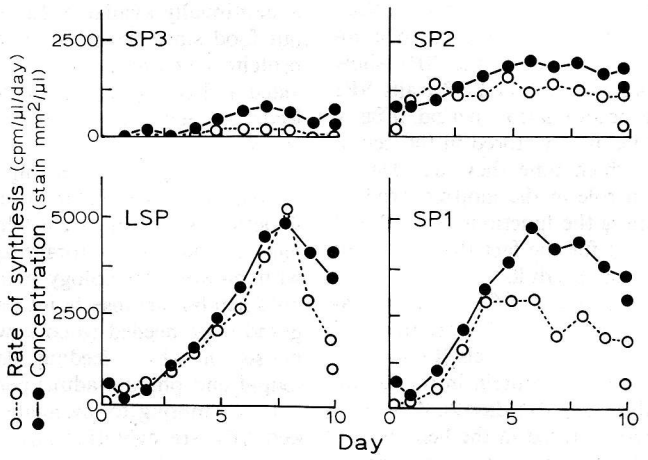

Fig. 4. The concentrations of SP1, SP2, SP3 and LSP (solid circles) were determined during the 5th instar (15 animals per data point). One-third of the animals were injected with 0.15 μCi of [^{14}C]-leucine (312 mCi/mmol) 24 hr before sampling; the protein bands were cut from the gels and counted (open circles). The free-standing data points on day 10 represent measurements on molted animals.

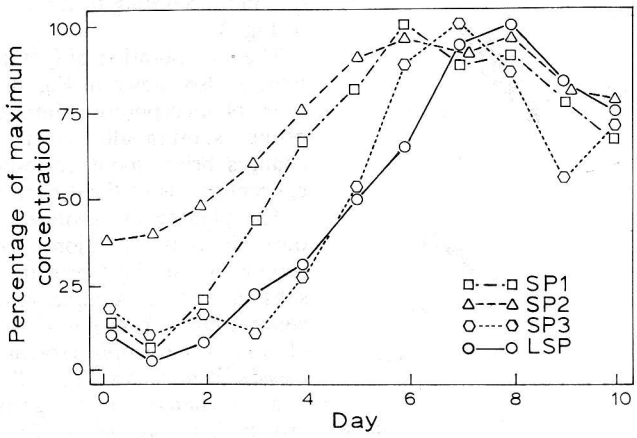

Fig. 5. Temporal patterns of change of the hemolymph proteins during the molting cycle of the 5th instar. The concentration data from Fig. 4 are normalized with respect to the concentration on the day in the molting cycle when each protein reached its maximum.

cycle (Fig. 5). The pattern consists of three phases: an accumulation period that begins one day after feeding, a plateau phase on days 6, 7 and 8 for SP1, SP2 and SP3 and on days 7 and 8 for LSP, and a decline phase that begins on day 8 and extends through the period of ecdysis.

It was shown previously (Kunkel & Lawler, 1974) using the quantitative Oudin test that LSP cycles at every instar. Using leg regeneration to modulate the length of the molting cycle, it was shown that the peak and decline of LSP concentration are events of the molting phase of the instar (Kunkel, 1975*a*: 1975*b*). The peak of LSP concentration follows about one day after the molting hormone, ecdysone, reaches its peak titer in the hemolymph (Kunkel, 1977). Using electrophoresis to quantitate all 4 proteins, we now show in this study that SP1, SP2 and SP3 also fluctuate during the molting cycle. We have also observed (not shown) that the 5th instar patterns of all 4 proteins are repeated in the 6th and 7th (metamorphic) instars of *B. orientalis*.

Although all 4 proteins fluctuate with the same rhythm, they do so with different amplitudes. Both LSP and SP1 increase almost 10-fold from their initially very low levels. In fact, LSP and SP1 show almost identical behavior except that LSP trails SP1 by one day during the accumulation period (Figs 4 and 5). They appear to be merely stored in the hemolymph until ecdysis at which time they are cleared. Although this suggests a role in the molting process, any speculation concerning the functions of LSP and SP1 also needs to account for the fact that LSP, but not SP1, falls to low levels in adults.

In contrast, the concentration of SP2 remains relatively constant. It shows less than 3-fold variation and is not permitted to fall to very low levels (Figs 4 and 5). As a result, SP2 is the major protein in the hemolymph shortly after ecdysis (e.g. on day 2). This suggests that SP2 is not merely stored in the hemolymph until ecdysis but that it has a continuing physiological role throughout the instar.

SP2 may be homologous to the major lipoproteins of holometabolous insects. Sedimentation of the *B. orientalis* SP2 in sucrose gradients is similar to that of the *Blattella germanica* SP2 (Kunkel & Lawler, 1974). The *B. germanica* SP2 has a particle weight of 5.1×10^5 dalton, contains 53% lipid (Kunkel & Pan, 1976) and resembles the lipoprotein of *Locusta* (Peled & Tietz, 1975) in amino acid composition.

SP3 is described here for the first time. Its absolute concentration is sufficiently high to merit inclusion with the other 3 major proteins, yet at no time does it account for more than 8% of the total protein. Its pattern of variation is virtually identical to that of LSP. It is not yet clear whether a corresponding protein is found in other cockroach species as is the case for LSP, SP1 and SP2.

The animal's feeding habits are pertinent to an understanding of its protein economy. Certainly the control mechanisms that regulate the hemolymph proteins involve the availability of food as that is the entire basis for synchronizing the molting cycles of cockroach cultures. Many insects do not feed for a period before and after ecdysis (Browne, 1975) and in cockroaches this period extends from several days before ecdysis to 24–36 hr after ecdysis even if food is continually available. In cockroaches stored without food since the previous ecdysis, the hemolymph proteins increase within 1 or 2 days after food is provided and continue to increase until the animals stop feeding which occurs several days before the next ecdysis.

Considering that feeding is incompatible with ecdysis and that substantial cuticle deposition occurs nonetheless, it is apparent why a protein storage system is required. A storage system designed for use with molting physiology could have evolved in the holometabolous insects into a storage system on the grand scale needed to cope with complete metamorphosis in which feeding (larval) and non-feeding (pupal and pharate adult) periods are obvious.

It is tempting to speculate, for example, that LSP and SP1 are evolutionarily related to the Dipteran storage proteins, calliphorin and lucilin (see Thomson, 1975, for review). LSP and SP1 share similar physical properties with calliphorin (to be published) but unfortunately, anti-LSP antisera do not cross-react outside the order Dictyoptera (Kunkel &

Lawler, 1974) nor do anti-calliphorin antisera cross-react outside the order Diptera (Munn & Greville, 1969).

The mechanisms controlling the rates of synthesis of the 4 proteins may be independently modulated as judged from the distinct [^{14}C]-leucine incorporation patterns (Fig. 4). Such temporal shifts in the synthesis of different protein species are classic features of developmental processes and merit further study, particularly in reference to the other developmental and hormonal events of the cockroach molt cycle (Kunkel, 1975*b*).

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