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MOULTING-CYCLE REGULATION OF HAEMOLYMPH PROTEIN CLEARANCE IN COCKROACHES: POSSIBLE SIZE-DEPENDENT MECHANISM

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Abstract—The clearance of exogenously administered proteins from the haemolymph of larval *Blatta orientalis* was measured during the intermoult and at ecdysis. Two large proteins, radiolabelled *B. orientalis* larval-specific protein ($M_r = 508{,}000$; $R_S = 68$Å), and *E. coli* β-galactosidase ($M_r = 464{,}000$; $R_S = 69$A) and a smaller protein, radiolabelled bovine serum albumin ($M_r = 66{,}300$; $R_S = 53$ Å), were injected into the haemolymph and their clearances measured at various times during the moulting cycle. During the intermoult, [^{3}H]bovine serum albumin was rapidly cleared ($T_{1/2} = 4.5$ h) while [^{14}C]larval-specific protein and β-galactosidase showed no detectable depletion from the haemolymph in 48 h. In moulting animals, however, both high-M_r proteins were rapidly cleared (larval-specific protein, $T_{1/2} = 41$ h; β-galactosidase, $T_{1/2} = 9.2$ h). The $T_{1/2}$ for depletion of endogenous larval-specific protein at moulting is approx. 24 h. These data suggest that cockroach haemolymph proteins remain confined and accumulate within the haemolymph during the intermoult because of their large size, but during moulting the size barrier no longer functions and the haemolymph is depleted of proteins. Neither the size barrier nor the clearance mechanism exhibit pronounced protein specificity since both mechanisms are responsive to foreign proteins as well as homotypic proteins.

INTRODUCTION

The concentrations of the major haemolymph proteins in larval cockroaches fluctuate in synchrony with the moulting cycle (Kunkel and Lawler, 1974; Duhamel and Kunkel, 1978). The haemolymph proteins accumulate during intermoult periods, but are depleted from the haemolymph at moulting. This cycling pattern was first observed for larval-specific protein, which exhibits the greatest degree of fluctuation (Kunkel and Lawler, 1974), but a similar pattern of change has also been demonstrated for the other major haemolymph proteins, serum protein I, serum protein II and serum protein III (Duhamel and Kunkel, 1978). Larval-specific protein, so named because it is absent from old adults, exhibits the greatest amplitude of change; it is virtually undetectable in the haemolymph 48 h after moulting, but steadily accumulates to become the most abundant protein just before the next moult. Serum protein I, which closely resembles larval-specific protein in physical properties, but is present in adults, exhibits almost the same quantitative degree of change as larval-specific protein in each larval moulting cycle. Serum protein II, a lipoprotein, also fluctuates during the larval moulting cycles, but does not attain such a high concentration as larval-specific protein or serum protein I, nor is it as severely depleted during moulting. Serum protein III is a relatively minor protein of undetermined physical properties, perhaps unique to *Blatta orientalis*, but it also fluctuates with the same pattern as the major proteins.

These fluctuations indicate the presence of a developmentally regulated mechanism for first accumulating and later depleting proteins from the haemolymph, but the basis for this mechanism is not known. Despite the quantitative differences in the amplitude of the concentration changes for individual proteins, the common pattern suggests the possibility of a general mechanism applicable to all haemolymph proteins. One physical property that larval-specific protein, serum protein I and serum protein II have in common with each other and with insect haemolymph proteins in general is their large size: larval-specific protein, $M_r = 508{,}000$ (Duhamel and Kunkel, 1983); serum protein I, $M_r = 620{,}000$ and serum protein II, $M_r = 450{,}000$ (Kunkel and Pan, 1976). The M_r of serum protein III has not been determined. It is possible, therefore, that these proteins are restricted to the haemolymph during the intermoult period because of their large size and cleared from the haemolymph during moulting when the size barrier no longer functions. To test this hypothesis the clearance of injected radiolabelled larval-specific protein was compared to that of two foreign proteins of different size, *E. coli* β-galactosidase, $M_r = 464{,}000$ (Craven *et al.*, 1965) and bovine serum albumin, $M_r = 66{,}300$ (Brown, 1977).

MATERIALS AND METHODS

Animals

Synchronously moulting cultures of *B. orientalis* were raised as previously described (Kunkel, 1966; Duhamel and Kunkel, 1978).

Quantitation of larval-specific protein, serum protein I and serum protein II in larval serum

Larval serum was collected and the concentrations of major haemolymph proteins were determined by

disc gel electrophoresis and quantitative staining with Fast Green as previously described (Duhamel and Kunkel, 1978).

Preparation of [^{14}C]larval-specific protein

Larval-specific protein was radiolabelled *in vivo* by injecting late 7th-instar animals with [^{14}C]amino acids (algal hydrolysate, 100 μCi/ml; Schwartz-Mann Biochemicals Co.). Fifty animals were injected with 0.2 μCi each. Haemolymph was collected after 30 h and larval-specific protein was purified by QAE-cellulose chromatography as previously described (Duhamel and Kunkel, 1983) except that removal of lipoprotein prior to QAE chromatography was accomplished by precipitation upon dialysis against distilled water rather than by sedimentation in preparative sucrose gradients. The resulting [^{14}C]larval-specific protein had a specific activity of 40 dpm/μg.

Preparation of [^{3}H]bovine serum albumin

Bovine serum albumin (fraction V; Sigma Chemical Co.) was externally labelled with [^{3}H]*N*-ethylmaleimide (NEM) in 50 mM Tris buffer (pH 8.5) for 30 min at 30°C. To stop the reaction a large excess of unlabelled NEM was added and the solution incubated for 30 min more, after which the solution was dialyzed extensively against distilled water at 4°C. The resulting [^{3}H]NEM-bovine serum albumin had a specific activity of 1130 dpm/μg, corresponding to an average of 0.61 mol NEM per mol bovine serum albumin.

Clearance studies

The clearance of endogenous larval-specific protein was estimated by interpolation between the concentration present in freshly moulted animals and that present in pre-moult animals 24 h before the mean moulting time for the population. It represents a minimal estimate, since secretion into the haemolymph may also occur during this period.

To measure the clearance of exogenous proteins, animals were injected immediately after moulting while under carbon dioxide anaesthesia with 2–4 μl of protein dissolved in buffered saline. At fixed time intervals after injection, haemolymph was collected and the haemocytes removed by centrifugation. Injection times were staggered so that all animals were bled at the same time in order to minimize variation due to changes in haemolymph volume or circadian rhythms. The amounts injected per animal were: 21 μg [^{14}C]larval-specific protein (25 cpm/μg); 20 μg β-galactosidase (57 U/mg); 15 μg [^{3}H]bovine serum albumin (170 cpm/μg). In one experiment, [^{14}C]larval-specific protein and β-galactosidase were injected together at 0.5, 24 and 48 h before sampling, using 5 animals per time point. In a separate experiment, [^{3}H]bovine serum albumin was injected alone at 0.5, 2.5, 5.5, 24 and 48 h before sampling, using 5 animals per time point. The radiolabelled proteins in serum were quantitated by liquid scintillation counting in a Triton X-100-based mixture (Benson, 1966).

The amount of β-galactosidase present in serum collected at various times after injection was estimated from measurements of enzyme activity performed according to the method of Craven *et al.* (1965). To evaluate the validity of enzyme activity as a measure of residual enzyme protein in the haemolymph, control experiments were performed to test for the presence of inhibitors of β-galactosidase activity in cockroach haemolymph at various times during the moulting cycle. No inhibitory activity was detected (not shown).

RESULTS

Cyclic accumulation and depletion of native haemolymph proteins

The concentrations of larval-specific protein and serum protein I were followed during the last three larval instars of *B. orientalis* as were the concentrations of larval-specific protein and serum protein I in the adult instar. These data are depicted in Fig. 1. Figure 1 demonstrates that the pattern consisting of intermoult accumulation followed by depletion at, or shortly before, ecdysis is repeated in the 6th and 7th larval instars. After the metamorphic moult to the 8th instar, the haemolymph remains depleted of larval-specific protein while serum protein I attains pre-moult levels by the 4th day following moulting. In this experiment, no samples were taken prior to day 4 of the 8th instar and, therefore, the depletion phase of serum protein I was not observed (compare with days 0–4 of instars 5, 6, and 7), but depletion of serum protein I at the metamorphic moult was confirmed in independent experiments (not shown).

Clearance of proteins injected into the haemolymph

The clearance rates of exogenously administered proteins were measured at selected times in the moulting cycle (Table 1). Three proteins were injected: [^{14}C]larval-specific protein, a homotypic protein of high M_r (508,000); unlabelled β-galactosidase, a foreign protein also of high M_r (464,000); and [^{3}H]bovine serum albumin, also a foreign protein, but of relatively low M_r (663,000). During the intermoult period, on days 4 and 7, the low-M_r protein is rapidly cleared with a half-life of 6 h, while the two large proteins show no clearance (Table 1), measured on days 4 and 7 for larval-specific protein and day 7 for β-galactosidase. During moulting, however, [^{14}C]larval-specific protein is cleared with a half-life of 41 h while β-galactosidase is cleared at a rate approaching that of [^{3}H]bovine serum albumin in the pre-moult period. The clearance of endogenous larval-specific protein at moulting was even more rapid than injected [^{14}C]larval-specific protein (Table 1).

DISCUSSION

In a previous report, a repeated pattern of intermoult accumulation of larval-specific protein followed by depletion at moulting was demonstrated for all instars of *Blattela germanica* (Kunkel and Lawler, 1974). Proteins immunologically cross reactive to *B. germanica* larval-specific protein were found to be widely distributed across the entire order with indications of similar moulting-cycle related changes in other cockroach species. A detailed examination of the kinetics of change in concentration of the four haemolymph proteins of 5th-instar *B. orientalis* de-

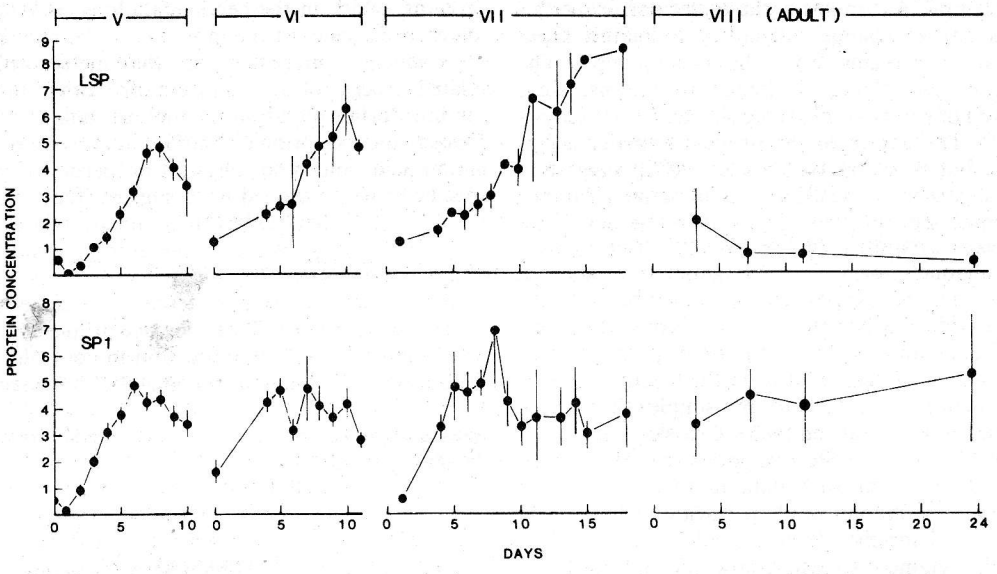

Fig. 1. Haemolymph protein concentrations in the 5th-, 6th- and 7th-larval instars, and in the adult instar of *Blatta orientalis*. The data for the 5th instar are taken from Duhamel and Kunkel (1978). The protein concentrations of larval-specific protein and serum protein I were determined as the integrated density of the respective gel bands stained with Fast Green. The concentration units are in $mm^2 \times 10^{-3}$. For larval-specific protein, 1200 mm^2 of stain area corresponds to 1 $\mu g/\mu l$ (Duhamel and Kunkel, 1978).

tectable by disc gel electrophoresis revealed that not only larval-specific protein, but also serum protein I, serum protein II and serum protein III exhibit the same pattern of moulting-cycle related changes in this species (Dunhamel and Kunkel, 1978). The data in Fig. 1 confirm that, in *B. germanica*, this cycle is repeated in 6th- and 7th-instar *B. orientalis* larvae. The data for the two most abundant larval proteins, larval-specific protein and serum protein I, are shown in Fig. 1 to illustrate the rapid and dramatic clearance of both proteins from the haemolymph at each moult. The larval-specific nature of larval-specific protein is also illustrated by the failure of larval-specific protein to re-accumulate in adults, while serum protein I returns to pre-adult levels.

The clearance of endogenous larval-specific protein proceeds with a $T_{1/2}$ of approx. 24 h (Table 1). It should be noted that this is a minimal estimate since it reflects net disappearance from the haemolymph, which may be mitigated by a competing process of synthesis and release into the haemolymph. There is evidence, however, that synthesis of larval-specific protein during the moult is minimal (Duhamel and Kunkel, 1978). Clearance of exogenously administered radiolabelled larval-specific protein exhibits the same pattern of change as the concentration of endogenous larval-specific protein, with little or no clearance during the intermoult (days 4 and 7), but moderately rapid clearance when injected into newly moulted animals ($T_{1/2} = 40.7$ h on day 11). It is not clear why radiolabelled larval-specific protein exhibits less rapid clearance than endogenous larval-specific protein. Clearance of injected *E. coli* β-galactosidase, a foreign protein of approximately the same size as larval-specific protein also follows the same pattern. β-galactosidase exhibits little detectable clearance during the intermoult, but rapid clearance ($T_{1/2} = 9.2$ h) at moulting. In contrast, bovine serum albumin, a much smaller foreign protein, is rapidly cleared even during the intermoult ($T_{1/2} = 4.5$ and 6.5 h on days 4 and 7, respectively).

These data suggest that a size-dependent molecular barrier is maintained during the intermoult which allows large macromolecules to accumulate in the

Table 1. Half-life clearance rates of injected and endogenous proteins from the haemolymph of 5th-instar *Blatta orientalis*

Protein	Half-lives (hours)		
	Day 4 (pre-moult)	Day 7 (pre-moult)	Day 11 (post moult)
Endogenous larval-specific protein*	—	—	<24†
Injected [^{14}C]larval-specific protein*	>500	>250	40.7
Injected *E. coli* β-galactosidase‡	—	>500	9.2
Injected [^{3}H]bovine serum albumin§	4.5	6.5	—

*M_r = 508,000 (Duhamel and Kunkel, 1983).
†Estimate of clearance based on depletion of endogenous larval specific protein after ecdysis. This is a minimal estimate since secretion into the haemolymph may also occur during this period. Clearance of endogenous larval specific protein cannot be estimated for days 4 and 7 because the concentration is increasing during this time.
‡M_r = 464,000 (Craven *et al.*, 1965).
§M_r = 66,300 (Brown, 1977).

haemolymph. At moulting, the molecular properties of this barrier change sufficiently to permit rapid removal of proteins from the haemolymph. The timing of this change is linked to ecdysis, since depletion of protein is observed shortly before ecdysis (Fig. 1). The nature of the proposed barrier is unknown, but the work of Crossley (1972) suggests a possible role for pericardial cells in active clearance of haemolymph proteins. If so, then the junctional complexes separating the haemolymph from the tortuous channels formed by invagination of the cell membrane of pericardial cells, described by Crossley, may provide the barrier function during the intermoult. Lane and Skaer (1980) also hypothesize that insect tissues are sealed off from the haemolymph by tight junctions analogous to, but simpler than, those described in vertebrate epithelia. Crossley proposed a limit of 120 Å for the effective pore-size of this barrier which is consistent with data in this report since bovine serum albumin would permeate such a barrier while larval-specific protein and β-galactosidase would be retained. Crossley, however, did not make observations in the context of the moulting cycle and did not report on moulting cycle-related changes. Moulting cycle-related changes in these structures, therefore, may account for the changes in clearance reported here.

Molecular size is clearly not the sole determinant of haemolymph clearance as indicated by the differences in clearing rates between injected [^{14}C]larval-specific protein and β-galactosidase (Table 1) and by the differences in the amplitude of concentration changes among the haemolymph proteins. Larval-specific protein exhibits the greatest degree of change, followed closely by serum protein I; both fall to very low levels shortly after moulting (Fig. 1 and Duhamel and Kunkel, 1978). Serum protein II, in contrast, exhibits a more modest rise and a less drastic decline than larval-specific protein and serum protein I, although it exhibits the same pattern of change (Duhamel and Kunkel, 1978). The basis for this difference is unknown, but is is noteworthy that serum protein II is a lipoprotein (Kunkel and Pan, 1979) and may, therefore, be processed differently by the proposed clearing mechanism than larval-specific protein and serum protein I.

The physiological fate of proteins cleared from the haemolymph at moulting is also unclear, but the timing of events suggests the possibility that haemolymph proteins are accumulated and confined in the haemolymph compartment during the intermoult for later utilization in the building of new cuticle or other tissue reconstruction at moulting. This cycle of accumulation and depletion of putative "storage" proteins, which in the hemimetabolous cockroach is linked exclusively to the moulting cycle, may be the phylogenetic antecedent of the metamorphosis-related process of accumulation of storage proteins for use during pupation in holometabolous insects. Indeed, the exceptionally high content of aromatic amino acids and other physical properties of larval-specific protein have led us to suggest (Duhamel and Kunkel, 1983) that the larval-specific protein of cockroaches strongly resembles the storage proteins of holometabolous insects (Munn *et al.*, 1971). By analogy, then, a developmentally regulated size-dependent barrier similar to the one proposed for the moulting cockroach may function in controlling the utilization of proteins in holometabolous insects.

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