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RNA METABOLISM IN PUPAE OF THE OAK SILKWORM, *ANTHERAEA PERNYI*: THE EFFECTS OF DIAPAUSE, DEVELOPMENT, AND INJURY*

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Communicated by C. M. Williams, June 29, 1964

In this communication we report a partial characterization of the RNA of the oak silkworm, *Antheraea pernyi*—specifically, its separation by means of sucrose gradient centrifugation into the heavier ribosomal fractions, and the lighter "messenger" and "transfer" fractions. We have further determined the relative rates of RNA synthesis by studying the incorporation of labeled uridine into the several fractions during pupal diapause, during the early stages of adult development, and following injury to diapausing pupae. Finally, we record the effects of actinomycin D on RNA metabolism under each of these conditions. Two different tissues are compared, viz., the wing hypodermis and the fat-body.

Materials and Methods.—(1) *Management of experimental animals, injuries, and in vivo labeling:* Pupae of *Antheraea pernyi* employed in these experiments were in one of two different physiological states: (a) diapausing pupae maintained by a 12-hr daily photophase at 25°C; and (b) lengthily chilled pupae with development blocked by storage at 2–3°C. Upon exposure to 25°C the latter pupae showed visible initiation of development within 48 hr. The early stages of development

were distinguished as described by Williams and Adkisson.¹ Integumentary injuries were made in the facial region of diapausing pupae as described by Harvey and Williams.² Unanesthetized pupae were injected with labeled uridine through the mesothoracic dorsum, just lateral to the mid-line. Each animal received either 2.5 μ C of uridine-2-C¹⁴ or 50 μ C of uridine-H³ in 0.05 ml Pringle's insect Ringer's solution. For inhibition studies, 15 μ g of actinomycin D were injected into each animal.

(2) *Extraction of RNA*.³ In each experiment, fat-body or wing hypodermis from 2 to 25 animals was pooled. Tissues were washed in the Ringer's solution, centrifuged for 5 min at 1600 *g*, and resuspended in 1 ml 0.01 *M* sodium acetate buffer (pH 5.0) containing (for inhibition of RNase activity) 1.5×10^{-4} *M* copper chloride and 1% sodium dodecyl sulfate. After glass-to-glass homogenizing, the tissue homogenate was transferred to a 25-ml flask to which 2 ml buffer, 0.2 ml 10% sodium dodecyl sulfate, and 3 ml water-saturated phenol were added. The mixture was gently shaken at 2–3°C for 10 min, and then centrifuged for 5 min at 1600 *g* to separate the two phases. The aqueous phase was subjected to two or three additional extractions with phenol. After final extraction, the aqueous phase was dialyzed for 1–2 hr against the buffer at 0°C for removal of phenol and uric acid, an excretory product which accumulates in insect fat-body during diapause.⁴ The RNA was precipitated from the aqueous phase by the addition of 2 vol of cold ethanol and storage at –10°C for at least 3 hr. Finally the RNA was collected by centrifugation at 1600 *g* and redissolved in 1 ml acetate buffer.

(3) *Analysis of RNA*: Sedimentation analysis of RNA was accomplished by means of a sucrose gradient procedure.⁵ One ml acetate buffer containing the RNA was layered onto a 24 ml, 5–20% (w/v) sucrose gradient. This was centrifuged at 2°C in the SW 25 rotor of a Spinco model L ultracentrifuge for 12 hr at 25,000 rpm. Following centrifugation, bottom-to-top fractions were collected. Their optical densities at 260 *m* μ were recorded, and the radioactivities of the fractions were then determined by liquid scintillation counting.

Results.—(1) *Diapausing pupae*: Figure 1 shows two profiles of RNA from the fat-body of diapausing *Antheraea pernyi* pupae. The optical density profiles show two peaks of ribosomal RNA (28s and 16s rRNA) and a rather large amount of lighter, 4–8s material, probably to be identified as "transfer" RNA. There is scant rRNA in the tissues of diapausing animals, compared with the amounts found in the tissue of developing and injured animals (Table 1). Calculations of specific activities indicate that after 6–8 hr *in vivo* pulsing with labeled uridine, the amount of incorporation of radioactivity into rRNA is very low for both fat-body and wing hypodermis (Fig. 1; Table 2). When the pulse period was extended to 12 hr, a markedly enhanced incorporation of label into both ribosomal peaks occurred (Fig. 1). Likewise, after 13 hr, considerable incorporation of labeled uridine was demonstrated in 12s fractions, a region of relatively low total RNA (Table 2).

(2) *Injured pupae*: As previously pointed out,^{7–10} injury to diapausing pupae produces metabolic responses similar in many biochemical particulars to those observed at the initiation of adult development. The "injury" attendant to the injection is, in itself, sufficient to

FIG. 1.—RNA profiles from the fat-body of diapausing *Antheraea pernyi* pupae. Ten animals: 2.5 μ C uridine-2-C¹⁴/animal; 6- and 12-hr pulses.

TABLE 1

AMOUNTS OF RIBOSOMAL RNA PRESENT IN TWO TISSUES FROM PUPAE OF *Antheraea pernyi*

Fat-Body			Wing Hypodermis		
Pupal stage	Ribosomal RNA (OD units/animal)		Pupal stage	Ribosomal RNA (OD units/animal)	
	28s	16s		28s	16s
Diapause	0.03	0.03	Diapause	0.01	0.01
Days after injury			Days after injury		
1	0.12	0.11	1	0.04	0.03
2-14	0.11	0.12	2	0.06	0.04
Days of visible development			3-14	0.11	0.09
0	0.10	0.11	Days of visible development		
1	0.22	0.22	0	0.14	0.11
2	0.26	0.33	1	0.22	0.17
3	0.27	0.31	2	0.19	0.16
			3	0.29	0.21

TABLE 2

COMPARISON OF THE SPECIFIC ACTIVITIES (CPM/OD UNIT) OF THE RIBOSOMAL FRACTIONS (28s, 16s), AND A 12s FRACTION OF RNA*

	Pulse length (hr)	Specific Activities		
		28s	16s	12s
Wing hypodermis:	8	190	220	680
	13	1,500	2,600	7,000
Fat-body:	6	220	200	240
	8	680	365	800
	12	2,480	2,040	800
	13	1,000	2,750	3,500

* From diapausing pupae after the injection of labeled uridine for various lengths of time.

produce this metabolic response within 24 hr. The results from the 12-hr pulse, reported above, suggested that effects of injury on RNA synthesis are evident 12-13 hr after injection.

To document the stimulatory effect of injury on RNA synthesis, RNA profiles were obtained from pupae at various time intervals (1-14 days) after integumentary injury.² The optical density profile of RNA from the fat-body of pupae 1 day after injury shows a

fourfold increase in the amounts of both 28s and 16s rRNA, as compared with that present in diapausing animals (Table 1). The amount of rRNA in the fat-body continues to increase slightly for another 24 hr, and is then maintained at a steady, high level throughout the 14-day postinjury period (Table 1). In the wing hypodermis, the increase in amount of rRNA is even more dramatic. The data summarized in Table 1 indicate for this tissue that (i) a rapid synthesis continues through the third postinjury day, and that (ii) as a result of injury there is a rapid, nearly 10-fold increase in the amount of ribosomal RNA compared with that present during diapause.

Experiments with both uridine-2-C¹⁴ and uridine-H³ confirmed the notion that injury stimulates a rapid synthesis of rRNA in both fat-body and wing hypodermis. As noted above, there was considerable incorporation of radioactive uridine into rRNA 12 and 13 hr after injury. When this was examined in experiments on animals 1 day after injury, a 1-4-hr pulse period yielded specific activities which demonstrated considerable incorporation into rRNA in both fat-body and wing hypodermis (Table 3). Between 3 and 7 days after injury, the amount of incorporation of radioactive uridine into rRNA decreased to a low level in the case of a 4-hr pulse, and to virtually zero in the case of a 1-hr pulse. By 14 days after injury the amount of incorporation of label into the rRNA of both tissues had returned to the level characteristic of diapausing animals (cf. Tables 2 and 3). Labeling of the 12s fraction initially followed much the same pattern as that for rRNA. However, specific activities of the 12s fraction remained fairly high until

TABLE 3
COMPARISON OF THE SPECIFIC ACTIVITIES (CPM/OD UNIT) OF THE VARIOUS RNA FRACTIONS FROM THE TISSUES OF DIAPAUSING PUPAE AT DIFFERENT LENGTHS OF TIME AFTER INJURY

Wing Hypodermis					Fat-Body				
Days after injury	Pulse length (hr)	Specific Activities			Days after injury	Pulse length (hr)	Specific Activities		
		28s	16s	12s			28s	16s	12s
1	2	1,600	1,300	3,000	1	1	500	1,200	1,350
2	1	480	680	4,400	1	4	950	620	1,500
3	1	740	1,200	3,360	2	1	480	620	1,200
4	2	240	600	1,920	3	4	570	875	2,300
5	1	0	0	290	4	2	280	340	280
7	1	0	0	0	5	1	80	135	360
7	4	160	240	800	7	1	0	0	400
10	1	60	80	0	7	4	160	140	640
14	2	60	150	480	10	1	60	60	0
					14	2	35	30	770

at least 7 days after injury (Figs. 2a-d; Table 3), and had not yet returned to the diapause level by 14 days after injury (Fig. 2e; Table 3).¹¹

(3) *Developing pupae*: The changes in RNA metabolism associated with early adult development are not as dramatic as those associated with injury. It is

FIG. 2.—RNA profiles from injured *Antheraea pernyi* pupae. (a) Fat-body, 1 day after injury; 3 animals, 50 μ C uridine- H^3 /animal; 1-hr pulse. (b) Wing hypodermis; 2 days after injury; 5 animals, 2.5 μ C uridine-2- C^{14} /animal; 1-hr pulse. (c) Wing hypodermis; 3 days after injury; 5 animals, 2.5 μ C uridine-2- C^{14} /animal; 1-hr pulse. (d) Wing hypodermis; 7 days after injury; 3 animals, 2.5 μ C uridine-2- C^{14} /animal; 4-hr pulse. (e) Wing hypodermis; 14 days after injury; 4 animals, 50 μ C uridine- H^3 /animal; 2-hr pulse.

Fig. 3.—RNA profile from developing *Antheraea pernyi* pupae. Wing hypodermis; zero day of development; 4 animals, $2.5 \mu\text{C}$ uridine- 2-C^{14} /animal; 4-hr pulse.

useful to recall here that the pupae used in this series of experiments were just prior to the initiation of adult development, and that development was prevented by storing the animals at low temperature ($2\text{--}3^\circ\text{C}$). An examination of an RNA profile obtained from animals taken directly from the cold indicates that synthesis of rRNA is one of the biochemical preludes to visible development which can occur in the cold once diapause has been broken. Thus, we observe that the fat-body of these chilled pupae contains, just prior to visible development, as much 28s and 16s rRNA as is ever present in an injured animal, while the wing hypodermis contains even more ribosomal RNA than is usually found in an injured animal (Table 1; Fig. 3).

For both fat-body and wing hypodermis the rate of incorporation of labeled uridine into rRNA was relatively low compared with that occurring during the first 2 days after injury, and showed little variation between the zero day of visible development and 3 days of visible development (Table 4). That there was still some incorporation of label into fat-body rRNA on the third day of development, but no net increase of rRNA on this day, suggests that the rate of breakdown of rRNA must be roughly equal to its rate of synthesis.

The amount of incorporation of labeled uridine into the 12s (messenger?) fraction varied considerably in these experiments, probably for reasons already mentioned.¹¹ There tends to be a greater incorporation very early in adult development followed by a gradual decline. This trend was particularly clear in fat-body where the amount of incorporation reached a very low level by the third day (Table 4). In the case of wing hypodermis, this trend was somewhat less clear. The amount of incorporation was quite high on the first day of development. On the second day, both high and low specific activities have been obtained.¹² On the third day of development, the specific activities of the 12s fraction were quite low, but were noticeably higher than the values obtained from fat-body (Table 4).

(4) *The effect of actinomycin D on pupal RNA metabolism:* It is well known

TABLE 4

COMPARISON OF THE SPECIFIC ACTIVITIES (CPM/OD UNIT) OF THE VARIOUS RNA FRACTIONS FROM THE TISSUES OF DEVELOPING PUPAE AT DIFFERENT STAGES OF DEVELOPMENT

Developmental age (days)	Wing Hypodermis				Developmental age (days)	Fat-Body			
	Pulse length (hr)	Specific Activities				Pulse length (hr)	Specific Activities		
		28s	16s	12s			28s	16s	12s
0	4	120	285	3,200	0	4	160	169	3,200
1	2	120	425	1,400					
1	4	80	240	2,400	1	4	240	420	940
1	4	140	200	2,700					
					1	8	110	280	3,200
2	4	120	180	10,000	2	2	200	160	1,600
2	8	500	540	1,360					
3	4	100	140	960	3	4	80	100	280
3	4	350	350	800	3	4	310	780	730
					3	8	70	160	240

FIG. 4.—Effect of actinomycin D on RNA synthesis in *Antheraea pernyi* pupae. (a) Fat-body; 3 days after injury; 3 animals, $50\ \mu\text{C}$ uridine- H^3 /animal; 2-hr pulse preceded 30 min earlier by $15\ \mu\text{g}$ actinomycin D/animal. (b) Wing hypodermis; first day of development; 3 animals, $50\ \mu\text{C}$ uridine- H^3 /animal; 2-hr pulse preceded 30 min earlier by $15\ \mu\text{g}$ actinomycin D/animal. (c) Fat-body; third day of development; 2 animals, $50\ \mu\text{C}$ uridine- H^3 /animal; 6-hr pulse followed by $15\ \mu\text{g}$ actinomycin D/animal; animals sacrificed 2 hr after actinomycin D treatment. (d) Fat-body; third day of development; 2 animals, $50\ \mu\text{C}$ uridine- H^3 /animal; 6-hr pulse; control—no actinomycin D. Compare the amount of labeling in the 12s fraction (tubes 26–27) in (c) and (d).

that in a number of different organisms the antibiotic actinomycin D is an inhibitor of DNA-dependent RNA synthesis. Insect tissues seem to be no exception to this conclusion. Thus, the injection of actinomycin D 30 min prior to the injection of isotope blocks incorporation of uridine into both ribosomal fractions and the 12s fraction. This was true of both fat-body and wing hypodermis from injured as well as developing animals (Figs. 4a and b; Table 5).

An attempt was made to gain some information on the rate of turnover of the 12s (messenger ?) fraction of RNA. In a single experiment, developing pupae were allowed to incorporate labeled uridine for 6 hr. The controls were then sacrificed while the experimentals were injected with actinomycin D. The latter were sacrificed 2 hr after injection of actinomycin D. The resulting profiles of RNA from the fat-body show very little difference in the specific activities of the

TABLE 5
THE EFFECT OF ACTINOMYCIN D ON THE INCORPORATION OF
LABELED URIDINE INTO PUPAL RNA

	Pulse length (hr)	Actinomycin D: when administered in relation to injection of label	Specific 28s	Activities 16s	12s
Wing hypodermis:					
Injured					
Day 3	2	1 hr before	0	0	0
Day 3	2	1 hr before	0	0	0
Developing					
Day 1	2	1/2 hr before	0	0	0
Day 2	2	1/2 hr after	130	115	285
Fat-body:					
Injured					
Day 3	2	1 hr before	0	0	0
Day 3	2	1 hr before	0	0	0
Developing					
Day 1	2	1/2 hr before	0	0	0
Day 2	2	1/2 hr after	90	180	285
Day 3	6	No actinomycin D	70	113	650
Day 3	6	Additional 2 hr with actinomy- cin D	85	100	400

ribosomal fractions (Figs. 4c and d; Table 5). By contrast, the specific activity of the apparent messenger fraction was reduced by approximately one third in the animals treated with actinomycin D, suggesting a messenger turnover time of about 6 hr in the fat-body of developing pupae.

Discussion.—(1) *Ribosomal RNA in *Antheraea pernyi* pupae:* Our findings suggest that RNA profiles from insect tissues are basically similar to those from other organisms. Of particular interest is the finding of so little RNA present in the tissues of diapausing animals. This suggests that one of the earliest occurrences in the development of the adult is that of the synthesis of ribosomes. A buildup of the machinery for protein synthesis is also characteristic of the injury response; indeed, in the case of injury the synthesis of rRNA appears to be even more rapid than that occurring during adult development. Our findings on the injury response in *Antheraea pernyi* parallel those of Wyatt's studies of injury in the related saturniid, *Hyalophora cecropia*.⁴ He notes that the over-all rate of RNA synthesis reaches a maximum 24 hr after the injury and gradually declines to the diapause level after several days. In both species, protein synthesis does not reach a maximum until 7 days after the injury. In *Antheraea pernyi*, ribosomal RNA synthesis has ceased by 7 days after injury, but the rRNA population remains elevated for at least another week. The cause and effect relationship between the absence of much of the protein-synthesizing apparatus and the diapause state remains uncertain.

(2) *The evidence for messenger RNA in *Antheraea pernyi* pupae:* In microbial systems a short pulse of 1 min or less of uracil-C¹⁴ will specifically label messenger RNA (mRNA).¹³ It is difficult to apply the concept of rapid labeling to our experiments because the mRNA may have a much longer half life and because there has been a concomitant rapid manufacture of ribosomes, at least during the early stages of injury and development.

Scherrer, Latham, and Darnell¹⁴ report that the most rapidly labeling, metabolically unstable RNA in HeLa cells is heavy (35–45s). They found that much of

this heavy RNA was precursor to the 28s and 16s rRNA, although the results of their DNA-RNA hybrid experiments suggested that some of this heavy RNA was mRNA. Insect tissues seem to lack entirely this heavy fraction and there is evidence that our least stable fraction (12s) does not become incorporated into ribosomes. On the contrary, 5–7 days after injury this fraction is still in production at a time when rRNA synthesis has dropped once more to a minimum (Fig. 2*d*). In this light, Wyatt's finding that protein synthesis reaches a maximum 7 days after injury further suggests that our 12s fraction is a messenger class of RNA. The nature of mRNA in insects awaits elucidation, and we note that recent evidence from other systems indicates various size ranges for mRNA.^{15, 16}

(3) *Comparison of RNA metabolism in fat-body and wing hypodermis:* Our findings indicate, for the three physiological states studied, that the gross aspects of RNA metabolism are quite similar for the two tissues employed in spite of their very different biological roles in the life of the insect. In diapausing pupae, the fat-body possesses a greater amount of RNA and a higher rate of synthesis of RNA than the wing hypodermis. After injury, RNA synthesis in fat-body reaches a maximum within the first 24 hr after which there is no net synthesis. Meanwhile, in wing hypodermis, net RNA synthesis continues for 3 days following injury. The significance of this difference is unknown. In developing pupae, net RNA synthesis in fat-body ceases on the third day of visible development. In wing hypodermis on the other hand, net RNA synthesis is accelerated on the third day of visible development. This difference is not surprising since the fat-body is destined to break down while the wing hypodermis is at the outset of its major synthetic career. That there is so much RNA synthesis in fat-body during early adult development hints that this tissue is highly active synthetically at this time, presumably producing materials to be used elsewhere in the construction of the adult.

(4) *Theory and future studies:* It has been suggested by Clever and Karlson,¹⁷ that in stimulating metamorphosis, ecdysone acts directly on the control mechanism of protein synthesis. This suggestion finds support in studies on the mode of action of various other hormones (e.g., androgens,¹⁸ estrogens,^{19, 20} and thyroxine²¹), all of which suggest that the hormonal influences on growth and differentiation are mediated directly or indirectly via the informational and synthetic axis: DNA → RNA → protein. The action of ecdysone may be envisaged to result in the derepression of a certain portion of the genome allowing the production of messenger RNA for new "species" of proteins. The action of the hormonelike substance released after injury^{2, 4, 7} may be thought to derepress another portion of the genome resulting in the production of messengers and proteins perhaps not too different from those employed at the time of pupation. By the use of the DNA-RNA hybrid technique,²² the way now seems open for an evaluation of the qualitative distinctions between the genetic information made available at successive stages in metamorphosis and after injuries to diapausing pupae.

We are indebted to Professor Carroll M. Williams, and Drs. E. H. McConkey, John Hopkins, III, and Victor Brookes for advice and criticism, and to Miss Katherine Hodgkinson and Mr. Neil Miller for assistance.

* This study was supported, in part, by NSF grant G-19962 (R.H.B.) and, in part, by Professor Williams' NSF grant G-21760.

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THE MEASUREMENT OF CYCLIC ADENYLATE IN TISSUES*

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Communicated by Oliver H. Lowry, September 22, 1964

A diverse group of biochemical regulatory phenomena are currently believed to involve adenosine-3',5'-phosphate (cyclic adenyate or 3,5-AMP).¹⁻⁶ Most of the studies which have led to this belief concern effects produced by adding 3,5-AMP to isolated tissues or enzymes, or the production by tissue fractions of 3,5-AMP from added ATP. Although it is clear that determination of the endogenous concentrations of cyclic adenyate in intact mammalian tissue under various conditions would be highly desirable, only a few such measurements have been reported.^{7, 8} This is attributed to the extremely low levels in tissue and to technical limitations in-