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Role of ecdysone, pupariation factors, and cyclic AMP in formation and tanning of the puparium of the fleshfly *Sarcophaga bullata*

(tyrosine hydroxylation/transcriptional and translational inhibitors/anterior retraction factor/pupariation tanning factor)

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ABSTRACT **Two pupariation factors, anterior retraction factor (ARF) and puparium tanning factor (PTF), are absent from the hemolymph of larvae at the time of tanning accelerated by ARF/PTF, cyclic AMP, or dopamine. ARF and PTF are not involved in derepression of dopa decarboxylase (aromatic L-amino-acid decarboxylase, aromatic L-amino-acid carboxy-lyase, EC 4.1.1.28) synthesis initiated by ecdysone. Tanning is entirely inhibited by injection of two transcriptional inhibitors, actinomycin and BrdUrd, and two translational inhibitors, puromycin and cycloheximide. Retraction activity is more severely inhibited by the transcriptional than by the translational inhibitors. A tanning response is initiated by cyclic AMP in the presence of the transcriptional but not the translational inhibitors. Dihydric tanning substances (dopa, dopamine) initiate tanning in the presence of both types of inhibitors. Release of ARF and PTF from the central nervous system is inhibited by the four inhibitors. ARF totally reverses the inhibitory effects on retraction, whereas PTF does not reverse inhibition of tanning. These data are interpreted to mean that PTF is concerned with the regulation of two components of the tanning response: (*i*) acceleration of synthesis of a particular protein (associated with the tyrosine hydroxylation complex), and (*ii*) activation via cyclic AMP of a component of the tyrosine hydroxylating system.**

In recent years, factors other than ecdysone have been postulated to be involved in morphogenesis of the puparium in higher Diptera (1–4). Unequivocal evidence for the existence and actual functional significance of such factors was developed by several workers in this laboratory. Contraction of the elongated larva into a barrel-shaped puparium and its subsequent tanning are two important aspects of pupariation clearly regulated by protein neurosecretion. The hormones initiating these processes have been called "anterior retraction factor" (ARF) and "puparium tanning factor" (PTF), respectively (5).

In a preceding paper, we showed that cyclic AMP (cAMP) or one of several catecholamines will accelerate tanning of the puparium of the fleshfly *Sarcophaga bullata* (6). cAMP was postulated to be the second messenger of the tanning hormone, PTF. Cyclic nucleotides do not appear to be involved in the mode of action of ARF.

In this paper we will present experimental data concerned with two related questions: (*i*) How is the sequential order of release of the various hormones from their endocrine sources related to the sequence of responses in the target tissue? (*ii*) What are the modes of action of the pupariation factors and how does cAMP mediate in the transmission of the hormonal stimulus initiated by PTF?

It must be stressed that ARF and PTF are recent discoveries, whereas the literature on the regulation of puparium formation by the prothoracic gland hormone, ecdysone, is extensive and sophisticated. Karlson and Sekeris (7) and Fragoulis and Sekeris (8) have applied many of the conceptual and technological innovations of modern biochemistry to their studies on the molecular biology of ecdysone-dependent dopa decarboxylase (aromatic L-amino-acid decarboxylase, aromatic L-amino-acid carboxy-lyase, EC 4.1.1.28) synthesis in hypodermal cells of third-instar larvae of *Calliphora erythrocephala*. However, their elegant hypothesis deals with only one aspect of pupariation: synthesis of a particular gene product, dopa decarboxylase. Regulation of processes, such as the mobilization of tyrosine at a precise moment in the ontogeny of the larva, as well as regulation of the stereotyped behavior pattern (anterior retraction) prior to puparium formation and the morphogenetic events, contraction and shrinkage of the cuticle, are not included in the Karlson–Sekeris model. Admittedly, these investigators recognized a relationship between ecdysone and the tyrosinase activator (9), but the dopa decarboxylase system was the only one thoroughly investigated.

The results described in this paper are consistent with the hypothesis that protein synthesis in the central nervous system (CNS) is required for release of the pupariation factors ARF and PTF into the hemolymph. One aspect of the mode of action of PTF is acceleration of protein synthesis in the target tissue. The other aspect of PTF action is a cAMP-mediated stimulation of a component of the tyrosine hydroxylation system.

MATERIALS AND METHODS

All experiments were performed with larvae of the fleshfly *S. bullata* (Parker).

The general procedures for rearing *S. bullata*, for injecting larvae, and for selecting the stage for injection have been described (10, 11). Most injections were made into larvae 3–4 hr before the formation of the puparium (pupariation). In *Sarcophaga* larvae, the region around the hind spiracles starts to tan at that time. This we call "red-spiracle larva." Most injections were made at the beginning of this period; this is about 10 hr after the "critical period" for ecdysone release.

The earlier data on the fluxes of ecdysone and dopa decarboxylase activity prior to pupariation were based on the chronological age of the larvae (12–14). Clearly, this is an inadequate tactic as one usually encounters great variation in the rates of development of individual larvae in any one culture, and variation between cultures is not unusual. For synchronizing larval development before pupariation and for gauging the period to pupariation before the red-spiracle stage, Ohtaki's water treatment was used (15). Pupariation is inhibited in many fly larvae when they are in contact with water. On transfer to dry condition—e.g., sawdust—pupariation then occurs after a definite period.

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Abbreviations: ARF, anterior retraction factor; PTF, puparium tanning factor; cAMP, cyclic AMP; CNS, central nervous system.

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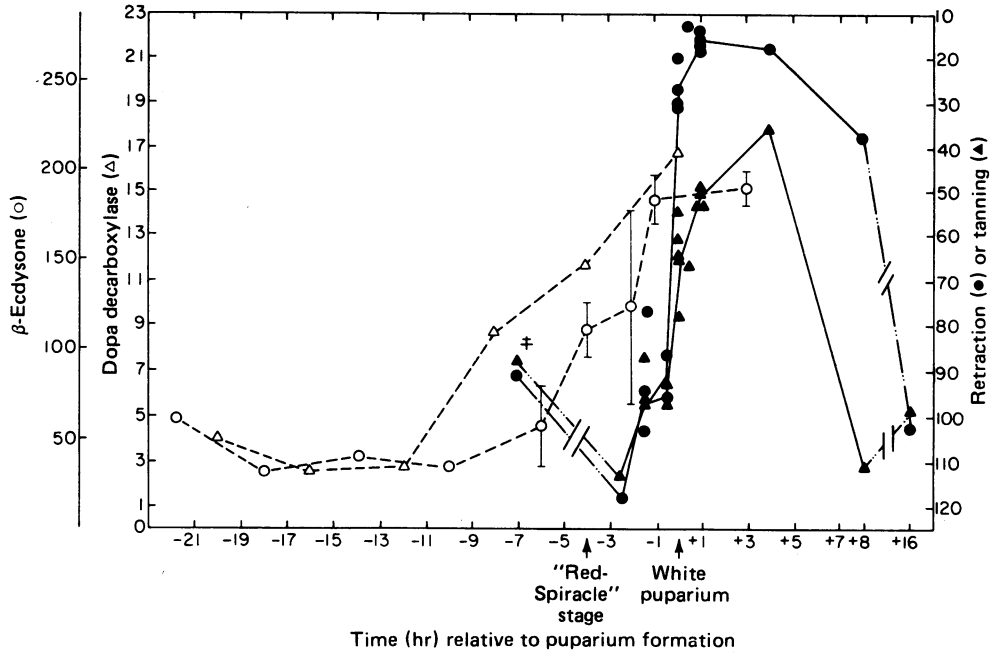

FIG. 1. Comparison of the titers of ecdysone (pg ecdysone equivalent per larva) and dopa decarboxylase [nmol dopa decarboxylated per larva in 30 min with the Shaaya and Sekeris assay (13)] and the relative activities of ARF (retraction time) and PTF (tanning time) in 5 μl of hemolymph per larva, expressed as a percentage of the time that retraction and tanning commences in the controls. ‡, Points refer to larvae taken at an uncertain time before the red-spiracle stage. Vertical lines, ±SEM.

Dopa decarboxylase was determined by a technique based on the method used by Shaaya and Sekeris (12). Radioimmunoassays for ecdysone (16) were kindly performed for us by John D. O'Connor of the University of California, Los Angeles.

RESULTS

Absence of ARF and PTF Activity in Hemolymph of Larvae at Time of Tanning Accelerated by cAMP, Dopamine, or ARF/PTF. We know that ARF and PTF activities appear in the hemolymph of normal puparia at the time of tanning (11). The question arises whether release of these hormones is dependent on either cAMP or dopamine. If this relationship does indeed exist, then the appearance of the two pupariation factors should be premature in larvae treated with cAMP or dopamine. This also raised the question of whether ARF and PTF accelerate the release of ARF and PTF.

Donor larvae were injected with 5 μl of "active blood" (containing ARF and PTF) from puparia about 1 hr after beginning of tanning, 50 μg of cAMP, or 100 μg of dopamine and bled 30 min after accelerated tanning had started. The hemolymph collected from these animals was injected into other red-spiracle larvae. The hemolymph from donors in the process of tanning was entirely devoid of either ARF or PTF activity. These results make it abundantly clear that release of ARF and PTF from the CNS was not dependent on prior synthesis of either cAMP or dopamine.

Changes in Levels of Ecdysone, Dopa Decarboxylase, and ARF/PTF Activity in Relation to Pupariation. Although generalities about the release of hormones can be inferred by extrapolation from experiments done on related species, interpretation of these data will always be fraught with uncertainty. Thus, it was essential that exact data be obtained for the timing of ecdysone release and the appearance of dopa decarboxylase and ARF/PTF activity in the hemolymph relative to the red-spiracle stage in *S. bullata*.

The ecdysone titer began to rise sharply 12–16 hr after termination of the water treatment, about 6 hr before the red-spiracle stage (Fig. 1). Dopa decarboxylase activity (determined on the same material) was unexpectedly high prior to the release of ecdysone. However, high levels of enzymatic activity preceding ecdysone release have also been reported in *Musca domestica* (17) and *Sarcophaga peregrina* (2).

The data obtained for this paper on the activities of ARF/PTF in the hemolyph differ from earlier data (11) in some details, but it is quite evident that the activity of both factors was low at all stages prior to initiation of pupariation, rose to a maximum during the first 2 hr of tanning, was maintained for several hours, and then waned.

Thus, no matter what the exact relationship between the titers of ecdysone released and dopa decarboxylase activity is, an increase in the levels of both occurred at about the critical period for ecdysone release, long *before* the red-spiracle stage. On the other hand, neither ARF nor PTF could be detected in the hemolymph until well *after* the beginning of the red-spiracle stage.

The Effects of RNA and Protein Synthesis Inhibitors on PTF-Dependent and cAMP-Dependent Tanning and on ARF-Dependent Retraction. In the following experiments, two inhibitors of RNA synthesis [BrdUrd (5-bromo-2′-deoxyuridine) and actinomycin D] and two inhibitors of protein synthesis (puromycin and cycloheximide) were injected into red-spiracle larvae. Tanning was inhibited by all four inhibitors (Table 1). In most experiments, cycloheximide and puromycin had no effect on retraction or caused a delayed retraction of the order of 150%. Inhibition of retraction with BrdUrd and actinomycin D was more severe, inhibition being complete in the vast majority of cases.

Use of inhibitors of protein and RNA synthesis as diagnostic probes for *in vivo* studies has limitations in that one is never sure whether the effect of the inhibitor is restricted to one biochemical reaction. Furthermore, the mode of action of BrdUrd on differentiating systems is unclear. Walther *et al.* (18) proposed that binding of the regulatory proteins to BrdUrd-containing DNA is altered, thus resulting in inhibition of transcription of *certain* genes.

Table 1. Inhibitory effects of BrdUrd, actinomycin D (Act D), cycloheximide (Cyclo), or puromycin (Puro) on tanning, and reversal by cAMP, dopa, or dopamine

cAMP effects			Dopa effects			Dopamine effects		
Injection	Tanning time, min ± SEM (*n*)	% of control	Injection	Tanning time, min ± SEM (*n*)	% of control	Injection	Tanning time, min ± SEM (*n*)	% of control
Water	196 ± 10 (13)	—	0.025 M HCl	218 ± 10 (14)	—	Water	196 ± 10 (13)	—
cAMP	74 ± 6 (13)	38	Dopa	70 ± 4 (15)	32	Dopamine	72 ± 7 (14)	37
BrdUrd	Never tan	—	BrdUrd	Never tan	—	BrdUrd	Never tan	—
BrdUrd + cAMP	63 ± 3 (16)	32	BrdUrd + dopa	63 ± 3 (17)	29	BrdUrd + dopamine	76 ± 5 (11)	39
Water	215 ± 8 (13)	—	0.025 M HCl	205 ± 9 (13)	—	Water	215 ± 8 (13)	—
cAMP	73 ± 4 (14)	34	Dopa	71 ± 5 (16)	34	Dopamine	98 ± 7 (13)	46
Act D	Never tan	—	Act D	Never tan	—	Act D	Never tan	—
Act D + cAMP	97 ± 9 (13)	45	Act D + dopa	100 ± 9 (15)	49	Act D + dopamine	98 ± 8 (15)	46
Water	210 ± 9 (12)	—	0.025 M HCl	223 ± 3 (11)	—	Water	210 ± 9 (12)	—
cAMP	77 ± 5 (11)	37	Dopa	72 ± 6 (11)	32	Dopamine	89 ± 8 (12)	42
Cyclo	Never tan	—	Cyclo	Never tan	—	Cyclo	Never tan	—
Cyclo + cAMP	Never tan	—	Cyclo + dopa	100 ± 11 (11)	45	Cyclo + dopamine	107 ± 13 (12)	50
Water	217 ± 9 (11)	—	0.025 M HCl	197 ± 8 (12)	—	Water	211 ± 11 (12)	—
cAMP	79 ± 4 (11)	36	Dopa	85 ± 9 (11)	43	Dopamine	53 ± 5 (11)	27
Puro	Never tan	—	Puro	Never tan	—	Puro	Never tan	—
Puro + cAMP	Never tan	—	Puro + dopa	78 ± 8 (13)	40	Puro + dopamine	92 ± 8 (15)	44

Injection volume was 5 μl per larva. Doses were: for cAMP effects, 50 μg for all agents except 0.5 μg for actinomycin D (Act D); for dopa effects, 40 μg for dopa, 50 μg for BrdUrd, 0.5 μg for Act D, 5 μg for cycloheximide (Cyclo), and 50 μg for puromycin (Puro); for dopamine effects, 100 μg for dopamine, 50 μg for BrdUrd, 0.5 μg for Act D, 5 μg for Cyclo, and 50 μg for Puro.

Ohtaki (19) claimed that actinomycin D and puromycin did not inhibit pupariation if given 4 hr after administration of ecdysone to water-treated *S. peregrina* larvae. However, he injected the inhibitors at least 4 hr before we did. Furthermore, the doses he used were 20% of those used in this study (10 μg of puromycin compared to 50 μg; 0.1 μg of actinomycin D compared to 0.5 μg). The doses used in our study were comparable to those used in studies of the effects of several inhibitors on protein synthesis in *Lucilia cuprina* (20).

The hemolymph of larvae injected with any of the above inhibitors was entirely devoid of PTF and ARF activities at the time when retraction and tanning would normally have occurred (4 hr after injection). PTF activity never appeared in specimens whose tanning was inhibited, whereas ARF activity appeared only in specimens that eventually retracted. Dilution of the inhibitors in the hemolymph of the donors was sufficiently high so that inhibitor carried over from the donor animals could be disregarded. In contrast, specimens injected with DL-α-methyl-α-hydrazion-β-(3,4-dihydrophenyl)propionic acid monohydrate, which formed normal-shaped puparia that never tanned, developed PTF and ARF activities at the time of pupariation (1, 11).

The deleterious effects of both transcriptional and translational inhibitors were reversed by simultaneous injection with dihydric metabolites of tyrosine such as dopa and dopamine (Table 1). Significantly, tyrosine did not accelerate tanning. This suggests that the entire enzymatic machinery for the transformation of dopa to the tanning substrate *N*-acetyldopamine must be intact when PTF is released into the hemolymph and that some hormonally sensitive aspect of the transformation of tyrosine to dopa must be dependent on RNA and protein synthesis.

In the *in vivo* test situation used in these experiments, the inhibitors might have their effects on more than one locus. Thus, if synthesis and release of hormone is the only hormone-sensitive site, then coinjection of hormone with inhibitor should elicit a normal response from the hormone-responsive tissue. Hemolymph from normal puparia 30–60 min after beginning of tanning (which contains maximal titers of ARF and PTF) was injected simultaneously with the inhibitors. ARF totally reversed the inhibitory effects on retraction, whereas PTF had absolutely no effect on the inhibition of tanning (Table 2).

Reversal of inhibition with cAMP was more selective. Tanning was initiated with cAMP in the presence of the transcriptional inhibitors but not in the presence of the translational inhibitor (Table 1). These data suggest that cAMP is not concerned with transcription but exerts its effect outside the nucleus of the cell.

Reversal of tanning inhibition with dopa, dopamine, and cAMP did not lead to complete tanning. The response at first resembled that of the accelerating agent alone—tanning was premature and appeared in the still-crawling larva, but hardening and darkening of the cuticle stopped at a stage resembling 2-hr normal tanning, when sclerotization is still confined to the

Table 2. Effect of injection of active hemolymph (containing ARF and PTF) on inhibitors of tanning [DL-α-methyl-α-hydrazion-β-(3,4-dihydrophenyl)propionic acid monohydrate (αMDH)] or retraction and tanning (BrdUrd, actinomycin D, cycloheximide, and puromycin

Inhibitor	*n*	Anterior retraction time, min ± SEM
αMDH	10	217.5 ± 16.3
+ Hemolymph*	7	45.0 ± 0
BrdUrd	15	None
+ Hemolymph*	12	37.5 ± 2.3
Actinomycin D	16	None
+ Hemolymph*	17	36.0 ± 2.1
Cycloheximide†	15	Times not taken
+ Hemolymph*	14	34.4 ± 1.9
Puromycin†	15	197.0 ± 6.4
+ Hemolymph*	11	38.2 ± 2.3

Tanning was inhibited under all experimental conditions.
* Retraction was normal and was normally accelerated.
† Retraction (when it occurred) was abnormal and late. See *text*.

Table 3. Inhibiting effects of bromodeoxyuridine (BrdUrd) on tanning, and counteracting effect of thymidine*

Injections per larva	Tanning time, min ± SEM (*n*)	% of control
Water	208.0 ± 7.1 (12)	—
BrdUrd, 40 μg	Never tan	—
Thymidine, 500 μg	215.0 ± 8.7 (13)	103
BrdUrd, 40 μg + thymidine, 500 μg	224.5 ± 9.3 (10)	108
Water	174.0 ± 7.3 (14)	—
BrdUrd, 40 μg	Never tan	—
Thymidine, 500 μg	210.0 ± 8.4 (11)	121
BrdUrd, 40 μg + thymidine, 500 μg	188.0 ± 4.3 (15)	108

* BrdUrd did not inhibit retraction in this experiment.

outer layers of the cuticle. (Normally, tanning spreads slowly inwards throughout almost the entire thickness of the cuticle.) We suggest that the incompleteness of tanning is due to toxicity of the inhibitors. Inhibitor-treated larvae developed irregular dark patches in the cuticle after about 4 hr, and occasionally dark rings were evident in the region of the intersegmental membranes. These animals survived for 12–24 hr. However, the physiologically relevant responses could be distinguished quite easily from the necrotic patches.

An important indication that BrdUrd inhibition of tanning was not a consequence of generalized purine toxicity was based on the observation than large amounts of thymidine reversed BrdUrd inhibition (Table 3). Thymidine negated all the deleterious effects of BrdUrd but had no effect on pupariation when injected alone. BrdUrd–thymidine-injected larvae retracted, contracted, shrank and tanned normally and at the correct time, survived, and developed. In one experiment, of the 15 larvae injected with this combination, 12 formed pupae, and 10 developed into flies inside the puparia; 2 flies managed to emerge as adults. These data support the assumption that BrdUrd can be used as a diagnostic probe and that it inhibits a process concerned with the transcription of DNA. Uridine did not reverse BrdUrd inhibition.

DISCUSSION

Several authors have described the fluxes of ecdysone levels and dopa decarboxylase activity in different fly species prior to pupariation. Shaaya and Sekeris (12) found a remarkable coincidence between hormonal levels and enzymatic activity during the course of metamorphosis in *Calliphora erythrocephala*. Both activities started to rise at the beginning of the critical period for ecdysone release, about 12 hr before pupariation, and reached a sharp peak at about the time tanning began.

Bioassays for ecdysone in *Lucilia cuprina* (13) and radioimmunoassays for ecdysone in *Drosophila melanogaster* (14) are in better agreement with the Shaaya and Sekeris (12) data. From the above, it is clear that changes in ecdysone levels and appearance of dopa decarboxylase occur in all species several hours before pupariation. In contrast, release of the pupariation factors PTF and ARF has a more intimate temporal relationship with the pupariation process itself.

In most cyclorrhaphous Diptera, no known morphological or behavioral markers are available that correspond to the endocrinological status of the animal prior to pupariation. In *S. bullata*, reddening around the spiracles heralds pupariation, which invariably ensues 3–4 hr after beginning of the red-spiracle stage. In Fig. 1, it is clearly shown that the red-spiracle stage separated early and late events in the pupariation process.

Table 4. Effects of PTF, cAMP, and catecholamines on tanning in the presence of transcriptional and translational inhibitors

Inhibitors	Reversal of tanning inhibition		
	PTF	cAMP	Catecholamines
Transcriptional	−	+	+
Translational	−	−	+

The early events were increases in the levels of ecdysone and dopa decarboxylase activity, and the late events were release of the pupariation factors ARF and PTF.

Hence, we can conclude that PTF activity does not have anything to do with the synthesis of dopa decarboxylase, because dopa decarboxylase reaches a maximum titer prior to the release of PTF. Regulation of *N*-acetyldopamine synthesis of PTF in the white puparium must be at some other step in the metabolic pathway. We suggest that some component of the complex enzymatic interactions associated with the hydroxylation of tyrosine to dopa must be the step regulated by PTF. This suggestion is based on the following observations: (*i*) Dihydric phenols such as dopa, dopamine, and *N*-acetyldopamine mimic the action of PTF, and they accelerated tanning of the red-spiracle larva. Tyrosine is ineffective. (*ii*) The same substances (*including dopa*) will reverse the inhibitory effects of the protein synthesis inhibitors in the PTF bioassay. Tyrosine, once again, is ineffective (see below).

The results with inhibitors of RNA and protein synthesis can be summarized as follows: (*i*) all the inhibitors used inhibited tanning completely, (*ii*) PTF did not reverse the inhibitory effects on tanning of any of the inhibitors, (*iii*) cAMP reversed the deleterious effects of only the transcriptional inhibitors (BrdUrd and actinomycin D) but was entirely ineffective in the presence of translational inhibitors, (*iv*) tanning was initiated by all the dihydric catecholamines (including dopa) in the presence of the inhibitors, and (*v*) thymidine provided good protection against all the harmful effects of BrdUrd, indicating that the effect of BrdUrd was concerned specifically with some aspect of DNA transcription. The results are summarized in Table 4.

The results presented in this paper are consistent with the following suggestions: (*i*) the entire enzymatic machinery required for the transformation of dopa to the tanning substrate is intact in the red-spiracle larvae because dopa will initiate tanning in the presence of all the inhibitors tested.

(*ii*) The PTF effect is mimicked by cAMP, and cAMP is thus presumed to mediate in the action of this hormone (see also ref. 6). However, it is also clear that not all of the PTF effect is mediated through the cAMP channel, because cAMP will reverse the effects of the transcriptional inhibitors but *not* the translational inhibitors, whereas both classes of inhibitors inhibit PTF-dependent tanning in the target tissue. Thus, we postulate that, in the nucleus of the target tissue, PTF accelerates transcription of a particular mRNA required for tanning and that cAMP is concerned with activation of some processes dependent on the gene product derived by transcription from the PTF-activated gene (see Fig. 2).

(*iii*) It would seem that release and/or synthesis of the pupariation factors from/in the CNS is inhibited by both transcriptional and translational inhibitors because neither PTF nor ARF could be detected in the hemolymph of inhibitor-treated larvae.

(*iv*) Protein synthesis in the target tissue is also required for expression of the PTF response because injected PTF did not elicit any tanning in the presence of the inhibitors. Natori and Ohtaki (2) stated that protein(s) required for complete tanning

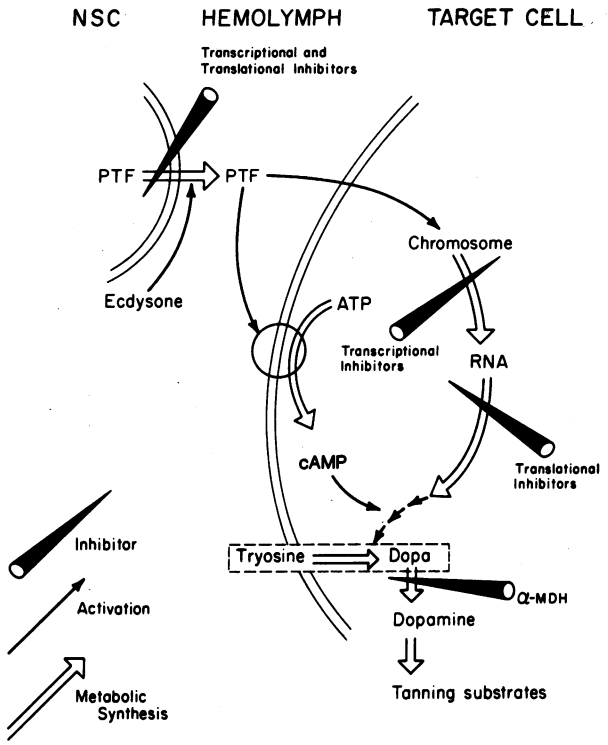

FIG. 2. Hypothetical interrelationships between ecdysone, PTF, and cAMP in the regulation of puparial tanning. NSC = neurosecretory cells. PTF = puparium tanning factor. α-MDH, DL-α-methyl-α-hydrazion-β-(3,4-dihydrophenyl)propionic acid monohydrate.

are synthesized late in the pupariation process. This conclusion is diametrically opposed to Ohtaki's earlier (19) findings. We are uncertain which protein is synthesized but feel that anything concerned with activation of the hemolymph protyrosinase is an unlikely condidate. Rapid conversion of protyrosinase to active tyrosinase occurs at about the time of the red-spiracle stage, some 4 hr prior to the release of PTF from the CNS (unpublished data). Thus, PTF is clearly not associated with the conversion of the hemolymph protyrosinase to tyrosinase. We have not yet investigated the intracellular tyrosinase isozymes. The characteristically rapid tanning response during pupariation is due to simultaneous acceleration and amplification of several PTF-dependent steps in the tanning sequence (Fig. 2). First, PTF stimulates a transcriptional process in the nucleus of the target cell that results in an enhanced rate of synthesis of a specific mRNA. It is because of low levels of this mRNA and its gene product that the initial stages of a tanning response can be seen in larvae injected with cAMP and transcriptional inhibitors. However, if translation is blocked, no gene products with cAMP sensitivity are made.

The second PTF-dependent process appears to be a cAMP-mediated acceleration of one of the steps associated with the hydroxylation of tyrosine to dopa. We do not know what this step might be.

Studies (21) on the hormonal control of tanning of the fly puparium in the 1930s ultimately led to the discovery of the steroid hormone ecdysone (7). It was then, and is even now, widely believed that ecdysone is *the* general tanning hormone in insects. However, in the 1960s several authors showed that tanning in the adult fly is controlled by a proteinaceous hormone bursicon (22). It subsequently emerged that almost all tanning in insects is controlled by bursicon and that a role of ecdysone in tanning is an exception, applying so far only to very aberrant processes such as pupariation. Work in this laboratory in the 1970s made it clear that, even in pupariation, ecdysone does not act directly on pupariation but through at least two other hormones of neurosecretory origin, ARF and PTF, the latter specifically functioning in tanning through the second messenger, cAMP (5,6,11).

PTF and bursicon are not one and the same hormone, despite similarities of their actions on tyrosine metabolism and the implication of cAMP in the action of both. Hemolymph from newly emerged flies rich in bursicon has no PTF activity, and hemolymph from orange puparia rich in PTF has no bursicon activity. PTF-dependent tanning is inhibited by inhibitors of RNA and protein synthesis, whereas bursicon-dependent tanning is not (23).

Release of both ARF and PTF from the CNS is entirely dependent on ecdysone (1, 11). No similar evidence exists linking the appearance of bursicon to ecdysone. However, it would be premature to exclude such a possibility because release of ecdysone invariably precedes release of bursicon in the ontogeny of insects. Ecdysone thus has a dual role in puparium formation of flies: (*i*) derepression of at least one gene coding for synthesis of enzymes in the *N*-acetyldopamine pathway, occurring concomitantly with ecdysone release, and (*ii*) release of ARF and PTF during pupariation about 15 hr later.

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