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3′:5′-Cyclic AMP and hormonal control of puparium formation in the fleshfly *Sarcophaga bullata*

(fly puparium formation/phenolic tanning/ecdysone action/tanning hormones)

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ABSTRACT **Injection of 3′:5′-cyclic AMP (cAMP) into larvae of the fly *Sarcophaga bullata* 3–4 hr before the beginning of puparium formation (red-spiracle stage) greatly accelerates the onset of tanning without affecting initiation of puparium formation (anterior retraction). Accelerated tanning resembles real tanning in two important respects: the solubility of cuticular proteins becomes reduced and [*U*-^{14}C]tyrosine is incorporated into the cuticle. Of a number of cAMP analogues tested, 3′:5′-cyclic GMP, 2′:3′-cyclic AMP, and 5′-AMP were inactive, dibutyryl-3′:5′-cAMP had only slight activity, and cyclic IMP and deoxy-3′:5′-cAMP showed some activity. Theophylline enhanced the effect of small doses of cAMP or of blood, diluted 1:8, active in the puparium tanning factor. Injection of dopa, dopamine, acetyldopamine, or epinephrine, but not of tyrosine, had an accelerating effect similar to that of cAMP. The tanning-inhibiting effect of DL-α-methyl-α-hydrazino-β-(3,4-dihydroxyphenyl)propionic acid monohydrate is reversed by dopamine or epinephrine, but not by tyrosine, dopa, or cAMP. Evidence is presented to indicate that the responses to cAMP are not artifacts but reflect actual biochemical events during tanning.**

At the end of the larval period in cyclorrhaphous flies the larval cuticle contracts into a barrel-like structure, the puparium, inside of which the pupa is formed. Formation of the puparium (*pupariation*) is a complicated process involving the following main stages: *retraction* of the anterior end of the larva into the body, gradual shortening of the body by muscular *contraction*, and smoothening or *shrinkage* of the cuticle, until the *white puparium* stage is reached; then a gradual darkening and hardening caused by *phenolic tanning* (1, 2). This whole process—from retraction to onset of tanning—takes 30–60 min, depending on species and temperature.

Over 40 years ago Fraenkel found that pupariation in flies is induced by a hormone now called ecdysone (3). Zdarek and Fraenkel recognized that the processes leading to puparium formation and tanning can be greatly accelerated by injection of extracts from the central nervous system or of hemolymph taken from larvae in the process of pupariation (4). This led to the discovery of two separate hormones named anterior retraction factor (ARF) and puparium tanning factor (PTF). Both are proteinaceous neurosecretions. It has been postulated that ARF and PTF migrate from the brain to the periphery of the larva through axons, and that release of these factors into the hemolymph is initiated by ecdysone (5, 6).

During a systematic investigation of the effects of many physiologically active compounds, we discovered that tanning was greatly accelerated by 3′:5′-cyclic AMP (cAMP), and similarly by dihydroxyphenolic precursors or analogues of the tanning substrate, *N*-acetyldopamine (7).

The scheme of hormonal control of tanning of the puparium by ecdysone was developed largely by Karlson and Sekeris (8). According to their postulate, one of the main effects of ecdysone is that it initiates the transcription of a gene coding for the synthesis of dopa decarboxylase (aromatic L-amino-acid decarboxylase, EC 4.1.1.28), a pivotal enzyme in the metabolic pathway leading from tyrosine to the formation of *N*-acetyldopamine. However, this important ecdysone-dependent synthesis is initiated about 8 hr before development of sensitivity of the third instar larva to the pupariation factors and cAMP (7).

Thus, with the realization that other regulatory substances, such as ARF, PTF, and cAMP, are concerned with initiation of tanning, it is clear that Karlson's simple hypothesis needs revision and amplification. The present paper deals with the effects of cAMP and various phenolic precursors on tanning. A hypothesis based on these newer finding for hormonal regulation of tanning will be presented in a subsequent paper (9).

MATERIALS AND METHODS

The fleshfly, *Sarcophaga bullata* Parker, was used throughout this investigation. The methods of selecting donors and hosts of appropriate age for injection are similar to those described in previous publications (4–6). Host larvae were immobilized on ice and injected with the aid of finely drawn-out glass pipettes with a tip diameter of about 40 μm, injecting usually 5 μl per larva. In most fly species the time between emptying of the crop and initiation of pupariation cannot be predicted by simple inspection of the mature, crop-empty larva. In the genus *Sarcophaga*, however, the cuticle between and around the posterior spiracles begins to tan and assume a reddish hue 3–4 hr before formation of the white puparium. This is a reliable method for recognizing the age of the larva at this stage, and *red-spiracle* larvae were usually selected for injection. In some experiments Ohtaki's (10) water treatment was used to get larvae of approximately known physiological age. Fully grown larvae do not pupariate in a very moist environment, presumably because the release of ecdysone is inhibited. When returned to dry conditions they pupariate after 20–30 hr.

We are mostly concerned with acceleration of tanning caused by injection of particular substances or preparations. Hormonal activity is expressed as the time between injection and initiation of retraction and/or tanning in treated animals compared to the time required by controls. This relationship is expressed as a percentage. The *critical time* for the accumulation of sufficient ecdysone in the hemolymph to induce pupariation is 12–16 hr before pupariation in *S. bullata*. Injections that accelerated pupariation were therefore made long after the release of ecdysone into the hemolymph. "Active hemolymph," containing ARF and PTF, was drawn from orange puparia 30–60 min after beginning of tanning when the titers of these hormones reach their maxima.

cAMP and its analogues and derivatives, as well as theophylline, were purchased from Sigma Chemical Co., St. Louis,

Abbreviations: cAMP, 3′:5′-cyclic AMP; PTF, puparium tanning factor; ARF, anterior retraction factor; αMDH, DL-α-methyl-α-hydrazino-β-(3,4-dihydrophenyl)propionic acid monohydrate.

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Table 1. Effect of injection of cAMP into red-spiracle larvae of *S. bullata* on the onset of retraction and tanning*

Injections (5 μl per larva)	Average time (min ± SE)	% Control
	Anterior retraction	
Control	180.0 ± 8.5 (15)	100.0
10 μg cAMP	185.0 ± 7.2 (12)	103.0
25 μg cAMP	160.7 ± 5.1 (14)	89.3
50 μg cAMP	162.9 ± 4.7 (14)	90.4
	Tanning	
Control	216.0 ± 8.0 (15)	100.0
10 μg cAMP	137.5 ± 8.6 (12)	63.7†
25 μg cAMP	91.1 ± 4.6 (14)	42.2†
50 μg cAMP	77.1 ± 4.1 (14)	35.7†

* Results are expressed as average time from injection to retraction or tanning, standard errors of these times, and percentage of the time in the uninjected controls. Numbers of animals injected are in parentheses.
† $0.001 > P$.

MO. [*U*-^{14}C]Tyrosine was purchased from Radiochemical Centre, Amersham. DL-α-Methyl-α-hydrazino-β-(3,4-dihydroxyphenyl)propionic acid monohydrate (αMDH), a specific inhibitor of dopa decarboxylase, was a gift from Merck, Sharp, and Dohme.

RESULTS

Acceleration of tanning by cAMP

Injection of cAMP greatly accelerates the onset of tanning without affecting the time of initiation of anterior retraction (Table 1). An interesting consequence of precocious tanning is that it starts while the larvae are still crawling. Precocious hardening and darkening impedes the normal retraction process, as well as subsequent contraction and shrinkage of the cuticle. This produces elongated puparia with a rough and folded larva-like surface; these irregularities are expressed to different degrees. Incomplete anterior retraction occurs at the time it would have occurred normally. Similar puparia are produced by larvae injected with PTF (5, 6) or certain catecholamines (see below).

Larvae injected with cAMP yield normal adult flies, as long as the puparial surface is not too irregular. Despite such irregularities, the larvae inside the malformed puparia invariably complete the pupal molt. In very irregular puparia death usually occurs during metamorphosis. Occasionally flies develop fully but do not manage to emerge.

The possibility that the very fast tanning response induced by cAMP masks acceleration of retraction was tested. It was ruled out by injecting the tanning inhibitor, αMDH, simultaneously with cAMP. In the absence of tanning these larvae formed normal-looking puparia, but retraction was not accelerated. Thus, cAMP does not accelerate retraction, contraction, or shrinkage, only tanning.

Fifty micrograms of cAMP per larva, the dose used in most of the experiments, had a consistently maximal effect on tanning. Fig. 1 represents the results on acceleration of pupariation caused by the injection of 5, 10, 25, or 50 μg. The accelerations on all the samples tested, except for the three with results over 90%, were statistically different from the controls. The data suggest equal and stronger responses at 25 and 50 μg per larva, but the group means are not significantly different from that at 10 μg, based on analysis of variance, and subsequent fitting of linear trend. However, the response to doses as large as 50 μg varied considerably (between 35.7 and 71.3% in Fig. 1).

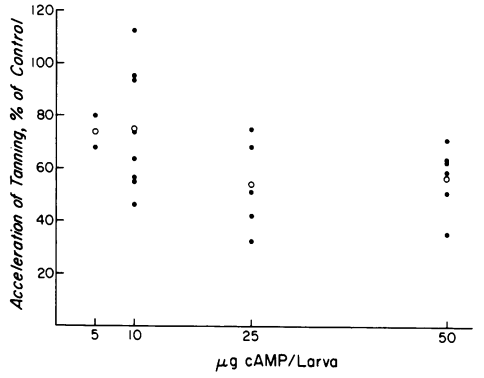

FIG. 1. Acceleration of tanning, caused by the injection of 5, 10, 25, or 50 μg of cAMP per larva, expressed as a percentage of the time in noninjected or water-injected controls. Each solid point represents the mean of an individual experiment, performed on 10–15 larvae. The open circles represent the means of these individual experiments.

The response of larvae at the pre-red-spiracle stage to cAMP was also tested. Water-treated larvae were injected with 50 μg of cAMP 5, 10, or 15 hr after removal from the water. There were no consistent accelerating effects in any of those groups, although the 15-hr group, which was near the red-spiracle stage, showed a slight but not significantly different acceleration in a few individuals. Thus, acceleration of tanning by cAMP does not occur before the red-spiracle stage is reached. This result also shows that injected cAMP is either metabolized or excreted before the larvae have reached the age where it would have become effective.

Effect of cAMP on solubility of cuticular proteins

We have assumed that acceleration of tanning by cAMP, "active blood," and catecholamines is similar to the processes occurring during normal development of the puparium. During normal tanning the water solubility of the cuticular proteins decreases markedly (11). In order to establish the essential identity of accelerated and normal tanning, we determined the effect of cAMP on the solubility of cuticular proteins. Estimates of "hot-water-soluble protein" content of the third larval instar cuticle were made at the following stages: (*i*) late red-spiracle stage; (*ii*) during retraction of the anterior segments; (*iii*) 2 hr after initiation of normal tanning; and (*iv*) 2 hr after initiation of cAMP-accelerated tanning (i.e., about the time when retraction occurs in the noninjected controls). Larvae were placed in 80° water for a few minutes until the proteins coagulated. They were cut longitudinally, and all adhering tissues were scraped off the cuticle under water at room temperature (11). Cuticles cleaned in this way are entirely transparent. They were dried at 100° for 1 hr, weighed, extracted with boiling water for 1 hr, dried as above, and weighed again. The difference in weight is a measure of hot-water-soluble protein in the cuticle (11).

Table 2 gives the results of three different sets of determinations, with each individual determination made in duplicate. The amount of water-soluble material in the cuticles of larvae after 2 hr of cAMP-accelerated tanning is consistently lower than in late red-spiracle or just retracted larvae, but, except for one determination, not as low as in puparia after 2 hr of natural tanning. Differences in the three sets may be due to differences in individual lots and to differences inherent in the cleaning and extraction procedures. However, each set deals with a particular batch, and we are concerned here with relative values applying to each individual batch. Since about 50% of the cuticle consists of insoluble chitin (11), the relative reduction in solubility of proteins is twice that measured in the whole cuticles.

Table 2. Soluble protein in the cuticle of larvae where tanning was accelerated by cAMP

Groups	% Soluble protein I	II	III
Late red-spiracle larvae	15.2	26.1	26.9
	17.0	23.8	26.1
Just retracted larvae	20.0	28.2	27.9
	18.0	21.8	26.5
Puparia after 2 hr tanning	11.3	16.7	17.8
	10.0	19.2	20.0
After 2 hr accelerated tanning	14.8	18.5	24.0
	12.8	19.2	24.5

Three different sets of determinations were made. Each analysis was made in duplicate on pooled cuticles from 10 larvae that had been cleaned and submitted to boiling water for 1 hr.

Effects of cAMP analogues on tanning and retraction

Table 3 summarizes the results of experiments on the effects of several cAMP analogues on tanning of pupariating larvae. cAMP did not accelerate anterior retraction (Table 1) and, as anticipated, none of the analogues tested did so. However, the effects of the cAMP analogues on tanning were interesting because some compounds that are biologically inactive in other systems showed some cAMP-like activity. 3′:5′-cGMP, 2′:3′-cAMP, and 5′-AMP were inactive at all concentrations tested. Dibutyryl-3′:5′-cAMP had only slight activity at 100 μg per larva. Dibutyryl-cAMP did not stimulate tanning of the adult fly at the one low concentration used (12).

Oxidative deamination of cAMP to 3′:5′-cIMP reduces activity to some extent. cIMP (50 μg) produced an effect quantitatively equivalent to that obtained with 10 μg or less of cAMP. cIMP was the only nucleotide, other than cAMP, that stimulated tanning and hormonally regulated cell death in newly emerged adult flies (12).

Table 3. Effect of injection of various analogues of cAMP on acceleration of tanning in *S. bullata**

Compound injected	Tanning time (min ±SE)	% Control	*P*
5′-AMP			
50 μg	215.0 ± 6.5 (12)	109.1	N.S.†
100 μg	231.4 ± 6.2 (14)	100.1	N.S.
2′:3′-cAMP			
50 μg	210.0 ± 24.0 (10)	88.2	N.S.
100 μg	235.3 ± 11.2 (16)	98.9	N.S.
200 μg	266.3 ± 15.7 (16)	119.9	N.S.
Dibutyryl-3′:5′-cAMP			
50 μg	231.2 ± 4.3 (12)	106.0	N.S.
100 μg	181.3 ± 7.5 (12)	83.1	<0.01
200 μg	199.5 ± 16.4 (10)	82.7	<0.05
Deoxy-3′:5′-cAMP			
50 μg	142.0 ± 10.4 (15)	77.2	<0.01
100 μg	132.2 ± 5.3 (16)	55.4	<0.001
100 μg	146.7 ± 10.2 (9)	69.4	<0.001
200 μg	160.3 ± 9.8 (16)	75.9	<0.001
3′:5′-cGMP			
50 μg	215.0 ± 8.3 (9)	100.2	N.S.
100 μg	186.4 ± 7.3 (7)	86.9	<0.05
3′:5′-cIMP			N.S.
50 μg	153.8 ± 10.9 (8)	67.3	<0.001
100 μg	197.1 ± 16.5 (7)	86.4	N.S.
200 μg	127.5 ± 4.5 (16)	64.7	<0.001

* Explanation as in Table 1.
† N.S., not significant.

Table 4. Effect of simultaneous injection of theophylline and diluted active blood (1:8)*, or of theophylline and cAMP, on acceleration of tanning†

Compound injected	Average time (min ±SE)	% Control	*P*
Control‡	245.0 ± 6.2 (15)	—	
5 μl H_2O + 5 μl 1:8 blood	225.0 ± 12.9 (14)	91.8	
50 μg theophylline + 5 μl 1:8 blood	162.9 ± 11.2 (14)	66.5	<0.01
Control‡	218.0 ± 6.2 (15)	—	
5 μl H_2O + 5 μl 1:8 blood	216.9 ± 14.5 (13)	99.5	
50 μg theophylline + 5 μl 1:8 blood	158.6 ± 15.1 (14)	72.7	<0.02
Control‡	220.0 ± 16.2 (13)	—	
10 μg cAMP	248.1 ± 31.7 (8)	112.9	
50 μg theophylline	222.5 ± 13.8 (12)	101.8	
10 μg cAMP + 50 μg theophylline	124.5 ± 9.5 (10)	56.6	<0.001

* Blood was taken from several animals 2 hr after tanning, and pooled. All dilutions were made from this pool.
† Explanation as in Table 1.
‡ Controls were not injected.

Stimulation of tanning with deoxy-3′:5′-cAMP was entirely unexpected. Seligman and Doy (12) were unable to show any effect of this nucleotide on bursicon-dependent cell death of wing hypodermal cells. This analogue is a poor activator of bovine brain protein kinase (13) but is almost as good an activator of lipolysis as cAMP [G. Michal (1971), cited in ref. 13, p. 322]. However, most of the cAMP derivatives were more active in the lipolysis test system than cAMP itself.

Effects of theophylline

Theophylline may stimulate hormonally dependent processes mediated by cAMP by inhibiting phosphodiesterase-catalyzed hydrolysis of cAMP.

Injection of theophylline in doses up to 200 μg per larva did not accelerate tanning. However, it did markedly inhibit anterior retraction, contraction, and shrinkage. It is possible that these inhibitory effects are nonspecific pharmacological responses to theophylline.

The effect of theophylline was then tried in the presence of relatively small doses of cAMP (5 and 10 μg per larva). We expected that theophylline might enhance a near threshold effect of cAMP. The results were equivocal. Theophylline accelerated tanning in cases where cAMP alone had no effect (Table 4), but not in other cases where cAMP alone had accelerated tanning. This is hardly evidence for the normal participation of cAMP in the tanning process, since the theophylline might have exerted its action by protecting the small injected dose of cAMP.

Theophylline was also tested in combination with small doses of active hemolymph (containing ARF and PTF). We first ascertained that blood diluted 1:4 still showed considerable activities of ARF and PTF, while a dilution 1:8 reduced activity to a level indistinguishable from the controls. [This result was largely, though not entirely, in agreement with one reported earlier (Table 3 of ref. 5), where a 1:8 dilution of ARF was still very active whereas a 1:4 dilution of PTF was inactive.] Si-

Table 5. Incorporation of [^{14}C]tyrosine into cuticles during normal and cAMP-accelerated tanning, measured after 2 hr of tanning

	n	dpm per cuticle ± SE	dpm/mg of cuticle ± SE
Control*	9	4313 ± 289	698 ± 58
Tanned (normal)†	9	15797 ± 1212	2269 ± 167
Tanned (cAMP)‡	9	17390 ± 1396	2352 ± 163

* Three hours after injection of [^{14}C]tyrosine, before tanning had begun.
† After 2 hr of tanning, 5–6 hr after injection of [^{14}C]tyrosine.
‡ After 2 hr of tanning, 3–3½ hr after injection of [^{14}C]tyrosine + cAMP.

multaneous injection of theophylline and of hemolymph diluted 1:8 accelerated tanning very significantly (Table 4). This experiment is strong evidence for a normal role of cAMP in tanning.

Effect of cAMP on incorporation of [^{14}C]tyrosine into tanning puparia

To compare the rates of incorporation of [^{14}C]tyrosine into the tanning puparium during cAMP-accelerated tanning with normal tanning, we injected [^{14}C]tyrosine (0.05 mCi per larva; 483 mCi/mmol) into red-spiracle larvae with or without cAMP. The larvae injected with [^{14}C]tyrosine alone served as controls. Larvae injected with cAMP began to tan 1–1½ hr after initiation of the experiment and were killed 2 hr later (i.e., 3 to 4½ hr after being injected). One set of controls was taken at the same time. These controls had not yet initiated the tanning process. Another control set consisted of larvae killed 2 hr after normal tanning had begun (i.e., 5–6 hr after injection).

The larvae were killed by heating to 80° in water. The cuticles were cleaned, oven dried, weighed, and oxidized individually in a Packard model 305 Tri-carb sample oxidizer (14). The CO_2 thus obtained was collected in scintillation fluid and ^{14}C was measured (with 16% efficiency). The results (Table 5) show that incorporation of tyrosine was about the same in normal and accelerated tanning. Incorporation of radioactivity into tanned samples was three to four times greater than into untanned samples.

Table 6. Accelerating effect of various catecholamines on tanning of puparium of *S. bullata**

Substances injected per larva	Tanning time (min ± SE)	% Control	*P*
Tyrosine, 60 μg†	163 ± 6 (15)	109	<0.4
Dopa, 20 μg‡	122 ± 19 (14)	60	<0.05
40 μg	60 ± 5 (13)	34	<0.001
80 μg	57 ± 5 (10)	37	<0.001
Dopamine, 20 μg§	170 ± 8 (13)	97	<0.5
50 μg	147 ± 19 (13)	64	<0.01
100 μg	100 ± 7 (10)	44	<0.001
Acetyldopamine, 1 μg§	198 ± 8 (14)	88	<0.05
10 μg	182 ± 9 (12)	81	<0.01
50 μg	108 ± 10 (12)	48	<0.001
100 μg	89 ± 9 (14)	39	<0.001
Epinephrine, 20 μg¶	175 ± 8 (15)	89	<0.4
50 μg	106 ± 9 (10)	54	<0.001

* Expressed as a percentage of the period between injecting and tanning in the controls injected with water or the HCl or NaOH solution in which the substances in question had been dissolved.
† Dissolved in 0.1 M NaOH.
‡ Dissolved in 25 mM HCl.
§ Dissolved in water.
¶ Dissolved in 0.1 M HCl.

Table 7. Inhibitory effect of αMDH on tanning and its reversal by certain catecholamines*

Injections (5 μl per larva)	Tanning time (min ± SE)	% Control
50 μg cAMP	95 ± 5 (14)	45
5 μg αMDH	Never tan	—
50 μg cAMP + 5 μg αMDH	Never tan	—
50 μg tyrosine	204 ± 7 (12)	93
5 μg αMDH	Never tan	—
50 μg tyrosine + 5 μg αMDH	Never tan	—
40 μg dopa	76 ± 6 (18)	34
5 μg αMDH	Never tan	—
40 μg dopa + 5 μg αMDH	Never tan	—
100 μg dopamine	79 ± 6 (12)	35
5 μg αMDH	Never tan	—
100 μg dopamine + 5 μg αMDH	91 ± 7 (11)	40
50 μg epinephrine	124 ± 11 (14)	55
5 μg αMDH	Never tan	—
50 μg epinephrine + 5 μg αMDH	127 ± 12 (17)	56

* Explanation as in Table 1; solvents as in Table 7. None of the injected catecholamines showed an effect on retraction.

Tanning accelerated by catecholamines

It is now generally accepted that tanning of the puparium is preceded by the following transformations of phenolic compounds: tyrosine → dopa → dopamine → acetyldopamine (8). Injection of these compounds (except tyrosine) caused accelerated tanning, closely resembling the effect of cAMP (Table 6). The larvae started tanning while still moving, and developed irregular puparia. Retraction of the anterior segments was not accelerated.

The effects of catecholamines was also tested in combination with the dopa-decarboxylase inhibitor, αMDH. Dopamine reversed αMDH inhibition of tanning, as expected, but dopa did not (Table 7). Dopamine-induced tanning in the presence of αMDH is not normal. It is comparable to the first 2 hr of normal tanning, when only the outer layer of the cuticle becomes dark, stopping short of full tanning. Similarly, epinephrine accelerated tanning and also reversed αMDH inhibition (Table 7). Substrate quantities of epinephrine were required in the experiments. Therefore, we assume that the injected epinephrine is a tanning agent, not a hormonal stimulator of tanning.

cAMP, like dopa, did not reverse αMDH-inhibited tanning. This suggests that cAMP accelerates a step in the synthesis of the tanning substrate prior to the formation of dopa.

DISCUSSION

Several authors have suggested that cAMP is concerned with the regulation of tanning and other processes initiated by the insect hormone, ecdysone. These reports are contrary to current views on the mode of action of steroid hormones, as it is generally assumed that transmission of these hormonal stimuli does *not* depend on an adenylate cyclase system (15).

However, at least two endocrinological signals other than ecdysone, ARF and PTF, are required for completion of the

pupariation process. Both these factors are proteins (5, 6). cAMP mediation of the action of a protein hormone is in accord with endocrinological dogma, but cAMP is not necessarily involved in every physiological response to protein hormones. Thus, cAMP mimics the action of PTF but is entirely inactive in the bioassay for ARF (Table 1). It might be that protein hormones like PTF or the protein factor described by Kambysellis and Williams (16) are necessary intermediates between ecdysone and initiation of a cAMP-dependent process. Thus, the physiological regulation of tanning of the puparium by ecdysone is more complex than the elegant model proposed by Karlson and Sekeris (8), according to which the main effect of ecdysone is that it initiates *directly* the transcription of a gene coding for the synthesis of dopa decarboxylase.

A role for cAMP in tanning processes has been demonstrated in three other instances. All three differ in some important respects, and thus comparison with cAMP involvement in the mode of action of PTF needs to be evaluated carefully.

Fuchs and Schlaeger (17) demonstrated that dibutyryl-cAMP enhanced ecdysone-dependent synthesis of dopa decarboxylase in the ovaries of female mosquitoes that had not been fed blood. They conclude that cAMP is not acting as a second message for ecdysone, since the effect of the steroid hormone is not mimicked by the cyclic nucleotide. An important difference between the results with the mosquito and the results presented in this paper is that dopa decarboxylase is clearly not the regulated step in the biosynthesis of the tanning substrates in the post-red-spiracle larva (9). It could be that in the mosquito the synergistic interaction between ecdysone and cAMP is due to stimulation of ecdysone synthesis by cAMP, as has been demonstrated to occur in isolated prothoracic glands (18).

von Knorre *et al.* (19) prevented tanning in newly emerged adult blowflies, *Calliphora erythrocephala*, by applying a neck ligature, and then induced tanning by injecting cAMP. They did not consider their evidence good enough to prove a bursicon-cAMP relationship because none of their pharmacological tests substantiated this hypothesis. However, similar experiments with another adult fly, *Lucilia cuprina*, suggest that cAMP mediates at least two responses initiated by bursicon, cell death of the wing hypodermis and tanning (12).

The work of Vandenberg and Mills (20) on the regulation of tanning in the cockroach by bursicon is superficially similar to the results enumerated in Table 5. However, they used dibutyryl-cAMP, not cAMP, to initiate darkening of ligated thoraces and acceleration of tyrosine uptake by the cuticle. Dibutyryl-cAMP had relatively low activity in the PTF assay (Table 3).

It would be desirable to correlate the effects of injected cAMP with actual changes of cAMP content or adenylate cyclase activities in the target tissue concerned, a single layer of epidermal cells sandwiched between the cuticle and layers of muscle. Measurement of cAMP in the epidermal cells alone requires a technique that we have not so far been able to devise. The onset of pupariation in *Drosophila* is marked by an increase in cAMP levels in extracts of the entire organism, which occurs 6 hr after ecdysone is released (21). It is impossible to decide whether and to what extent such changes in cAMP level, occurring in a whole organism at a time of rapid morphogenetic changes, can be attributed to metabolism of cAMP in a minute part of the body specifically concerned with tanning.

We have used several procedures and arguments to show that the response to cAMP is not a pharmacological artifact, but reflects actual biochemical changes in cells concerned with synthesis of the tanning agents:

(*i*) Tanning is accelerated by injected cAMP only after the beginning of the red-spiracle stage, 3–4 hr before pupariation. Younger larvae do not react. Thus, responsiveness to cAMP is restricted to a short period during development of the puparium.

(*ii*) Of the few morphogenetic processes occurring during pupariation, only tanning is accelerated by cAMP. Retraction of the anterior segments, contraction, and shrinkage of the cuticle do not appear to be cAMP-dependent processes.

(*iii*) Of the analogues tested, 3′:5′-cAMP was by far the most potent nucleotide in the PTF assay.

(*iv*) cAMP-accelerated tanning and normal tanning resemble each other in two important respects: Cuticular protein solubility decreases at a similar rate under both conditions (Table 2), and the incorporation of [^{14}C]tyrosine into the cuticle has similar kinetics in normal tanning and cAMP-accelerated tanning (Table 5).

(*v*) The effects of cAMP on tanning of the puparium closely resemble the effects produced by dopa and dopamine, precursors of the tanning substrate (Table 6).

(*vi*) The interaction between blood diluted 1:8 and the phosphodiesterase inhibitor, theophylline, is synergistic. Each of the two components of the mixture was inactive by itself, but the two injected together evoked a very strong tanning response (Table 4).

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