and centrifuged to remove the virus particles, was injected into the host larva. The host containing the dying parasitoids was not able to pupate and eventually died. However, the toxic factor obtained from the centrifuged hemolymph of virus-infected larvae had no detectable adverse effect upon unparasitized armyworm larvae when inoculated into the hemocoel.

The toxic factor responsible for the failure of the parasitoids to develop seems to be proteinaceous. This factor could be precipitated from the supernatant of the centrifuged hemolymph from virus-infected larvae with 50 percent ammonium sulfate. Ten percent trichloroacetic acid, on the other hand, destroyed its activity. The factor retained its toxic activity when kept frozen or at 4°C for 3 months. It was heat labile and lost its activity when heated above 50°C in a water bath for 10 minutes. The toxic factor was lost after an initial passage through a host larva, and this indicated that no multiplication of the toxic factor occurred. Possibly there was a breakdown or a dilution of the factor below the toxic concentration.

As far as I am aware, the present observation of a toxic factor within a virus-infected host has not been reported for invertebrates infected with viruses. However, toxins have been reported from viruses infecting mammals (*3*, *4*). From the results of the present study, I propose the hypothesis that the granulosis virus, in its interaction with the host, produces a toxic factor that affects the parasitoid but not the host.

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References and Notes

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Steroid Hormones: Effects on Adenyl Cyclase Activity and Adenosine 3′,5′-Monophosphate in Target Tissues

Abstract. *The adenyl cyclases of chick oviduct and rat prostate were not stimulated by estrogen and testosterone, respectively, suggesting that growth and differentiation of these target tissues are not mediated by adenosine 3′,5′-monophosphate. Estrogen acutely activated adenyl cyclase in the castrate rat uterus, but this was prevented by administration of* DL-*propranolol, suggesting that the effect was mediated by catecholamines. Progesterone produced a delayed stimulation of oviduct adenyl cyclase preceding and concomitant with the induction of synthesis of avidin.*

The role of cyclic adenosine 3′,5′-monophosphate (AMP) in the mediation of the effects of steroid hormones is unclear, there being only one report of an increase in tissue 3′,5′-AMP after a steroid was administered (*1*). To clarify the role of 3′,5′-AMP in the stimulation of protein synthesis by steroid hormones (*2*), the effects on the adenyl cyclase system were investigated relative to (i) estrogen and progesterone in the chick oviduct, (ii) testosterone in the ventral prostate of hypophysectomized rats, and (iii) estrogen in the castrate rat uterus.

In our studies, adenyl cyclase activity was determined by a modification (*3*) of the method of Krishna *et al.* (*4*), with the addition of 100 to 150 μg of protein of the membrane fraction, obtained by sedimentation of homogenates at 2200*g*, to the reaction mixture in 25 μl of 0.05*M* tris-HCl (*p*H 7.4) buffer with 0.026*M* theophylline, 0.04 percent bovine plasma albumin, 0.013*M* $MgCl_2$, 0.014*M* mercaptoethanol, and 20 percent glycerol. The concentration of adenosine triphosphate (ATP) in the reaction mixture was 1.22 mmole/liter. Incubations were carried out for 20 minutes at 37°C. Adenyl cyclase activity varied among control groups by as much as 20 percent.

Tissue 3′,5′-AMP was assayed by the radiophosphate exchange method (*5*). With this assay, a linear standard curve was invariably obtained between 0.02 and 0.24 nmole of 3′,5′-AMP. Final recoveries varied between 20 and 35 percent. Protein was determined by the method of Lowry *et al.* (*6*).

Table 1 summarizes some of the effects of diethylstilbestrol (DES) and progesterone on adenyl cyclase activity and tissue 3′,5′-AMP concentrations in the chick oviduct. Given intravenously, DES did not acutely activate adenyl cyclase from 30 seconds to 120 minutes. In previous studies, DES given subcutaneously also failed to activate adenyl cyclase from 1 to 18 days (*7*), with a consistent fall in oviduct adenyl cyclase activity after DES was administered for several days. As we have found that DES caused no direct effect on adenyl cyclase activity in vitro, the decreased activity seen at day 5 may reflect (i) altered adenyl cyclase activity in the new cell populations which develop during prolonged estrogen administration,

Table 1. Effect of diethylstilbestrol and progesterone on adenyl cyclase activity and cyclic 3′, 5′-AMP concentrations in the chick oviduct. Each value represents the mean ± 1 S.D. of triplicate or quadruplicate determinations of results of a single representative experiment. Chicks were killed by cervical fracture; the oviducts were excised and frozen in liquid nitrogen within 10 seconds. Oviducts from Rhode Island Red chick oviducts (50 per group) were used for cyclic 3′, 5′-AMP determinations. For adenyl cyclase determinations, 12 oviducts per group were allowed to thaw at 4°C, minced, and then homogenized with 20 strokes of a motorized Teflon pestle; activity was measured as described. Diethylstilbestrol (5 mg in oil) was administered subcutaneously (s.c.) daily. Progesterone (5 mg) was given as a single subcutaneous injection in oil. For the intravenous administration (i.v.), DES or progesterone (6.5 μg per 100 g of body weight) were given in 0.1 ml of a 90 percent normal saline, with 10 percent ethanol carrier; control groups received carrier alone. Animals were killed at precise intervals after hormone was administered. All experiments were repeated at least three times with similar results, different animal groups being used. Adenyl cyclase activity is expressed as the numbers of picomoles of 3′, 5′-AMP accumulated per milligram of protein per 20 minutes.

Route	Interval after administration	Adenyl cyclase activity [pmole mg^{-1} $(20\ min)^{-1}$]	Tissue 3′, 5′-AMP (nmole/g, wet weight)
		Control	
		307 ± 17	1.28 ± 0.12
		Diethylstilbestrol	
i.v.	0.5, 2, 5, 10, 60, and 120 min	307 ± 17	
s.c.	1 day	308 ± 15.5	1.15 ± 0.20
s.c.	5 days	136 ± 10.5	1.28 ± 0.14
		Progesterone	
i.v.	0.5; 2, 5, 10, 60, and 120 min	307 ± 17	
s.c.	3 hours	473 ± 62	
s.c.	6 hours	785 ± 34	2.96 ± 0.65
s.c.	10 hours	926 ± 40	
s.c.	24 hours	1055 ± 150	2.73 ± 0.40
s.c.	48 hours	761 ± 68	

(ii) indirect inhibition of the enzyme, or (iii) changing ratios of DNA to protein. Thus, adenyl cyclase activation does not appear to be a prerequisite for the marked stimulation of oviduct growth, differentiation, and protein synthesis caused by estrogen.

In contrast to the lack of stimulation with estrogen, progesterone caused a delayed and progressive activation of chick oviduct adenyl cyclase first noted at 3 hours and reaching 340 percent of control by 24 hours (Table 1). Intravenous administration of progesterone did not activate adenyl cyclase from 30 seconds to 120 minutes. The activation of adenyl cyclase by progesterone is specific for oviduct because progesterone did not stimulate lung or liver cyclase (*7*). When 3′,5′-AMP content of the chick oviduct was measured, no increase was noted after DES was administered. However, tissue 3′,5′-AMP concentrations were significantly elevated at 6 and 24 hours after progesterone was administered. Since synthesis of avidin, a specific protein induced in the oviduct by progesterone, is not measurable until 10 hours after administration of progesterone (*8*), this delayed adenyl cyclase activation by progesterone does precede the induction of synthesis of this protein.

Despite numerous technical modifications, neither DES nor progesterone activated oviduct particulate (2200*g* fraction) adenyl cyclase during in vitro incubations. These manipulations included a variation of *p*H (6.6, 7.4, 8.4), time of incubation (2 minutes to 2 hours), concentration of hormone, methods of membrane preparation, addition of various solvents and solubilizers (propylene glycol, ethanol, Tween 80, and deoxycholate), addition of ATP regenerating systems, incubation with whole tissue homogenates, and use of the water-soluble estrogen Estradurin.

Solubility is an unlikely explanation for the failure of progesterone to stimulate adenyl cyclase in vitro, since in identical solution it caused avidin synthesis in vitro. Various progesterone analogs and metabolites and other steroids, including testosterone, 17-methyl-nortestosterone, 17-ethyl-19-nortestosterone, Δ^4-pregnene-20α-ol-3-one, and Δ^5-pregnene-20α-ol-3-one, did not stimulate oviduct particulate adenyl cyclase activity. This failure to demonstrate in vitro activation of adenyl cyclase suggests that 3′,5′-AMP does not act initially as a second messenger in this tissue. It is unclear whether the increased concentrations of tissue 3′,5′-AMP act to facilitate the effects of progesterone.

Table 2. Effect of testosterone propionate on adenyl cyclase activity in the ventral rat prostate. Each value represents the average ± 1 S.D. of quadruplicate determinations from a single experiment with the particulate fraction resulting from centrifugation of a homogenate of the ventral prostates from ten rats. Sprague-Dawley rats were hypophysectomized at 20 days of age and kept on a high protein diet for 3 weeks. Testosterone propionate in sesame oil (2 mg in 0.2 ml) was given subcutaneously, the animals were killed by skull fracture at precise intervals, and the ventral prostate was excised and frozen immediately on Dry Ice. After being allowed to thaw at 4°C, tissue was homogenized with ten strokes of a Teflon homogenizer. For in vitro experiments, testosterone propionate (10 μg, in 1 μl of ethanol) was added to the incubation mixture. Ethanol alone was used in the control group. The particulate fraction from the centrifuged homogenate from ten different prostates was used for each group. Experimental results were confirmed in a second, identical experiment, except for no change at 3 and 12 hours. The variations between groups are within variations seen between control groups.

Group	Adenyl cyclase activity [pmole mg^{-1} (20 min)$^{-1}$]
In vivo	
Control	864 ± 30
5 Minutes	816 ± 16
30 Minutes	646 ± 9
4 Hours	914 ± 54
24 Hours	788 ± 57
In vitro	
Control	953 ± 40
Testosterone (1)	1090 ± 51
Testosterone (2)	836 ± 42

Table 3. Effect of diethylstilbestrol administration on adenyl cyclase activity in the castrate rat uterus. Each value represents the average ± 1 S.D. of triplicate or quadruplicate determinations with uterine tissue from five rats. Sprague-Dawley rats were oophorectomized at 6 weeks of age. Four weeks later, DES (5 μg per 100 g of body weight) was administered intravenously in 0.1 ml of 85 percent normal saline (with 15 percent ethanol carrier), controls receiving carrier alone. DL-propranolol (Ayerst) (0.05 mg per 100 g of body weight) was given intraperitoneally 20 minutes before DES was administered. Animals were killed by skull fracture at precise intervals; the uterus was removed, weighed, and immediately frozen on Dry Ice. Tissue was allowed to thaw at 4°C; it was then finely minced and homogenized with 40 strokes of a Teflon homogenizer. These results were confirmed in a second experiment, with an additional group receiving D-propranolol (Ayerst), (0.05 mg per 100 g of body weight) intraperitoneally 20 minutes before DES was administered.

Group	Adenyl cyclase activity [pmole mg^{-1} (20 min)$^{-1}$]
Control	587.5 ± 130
DL-Propranolol	552.3 ± 52
DES (5 minutes)	1050 ± 80
DES (5 minutes) + DL-propranolol	585 ± 28
DES (2 hours)	551.3 ± 66
DES (2 hours) + DL-propranolol	580*

* Average of two determinations, which differed by less than 5 percent.

The second model system studied was that of testosterone in the ventral rat prostate. Testosterone propionate causes an increase in prostate weight and protein synthesis (*2*), with enhancement of RNA synthesis and RNA polymerase activity within 2 hours (*9*). Thus, if cyclic 3′,5′-AMP were related to the action of testosterone, early activation of adenyl cyclase might be expected. However, testosterone propionate given to hypophysectomized rats did not activate ventral prostate adenyl cyclase either early or later (Table 2). Similarly, testosterone did not stimulate particulate preparations (2200*g*) of rat prostate in vitro.

In order to clarify the mechanism by which intravenous DES or 17β-estradiol caused an early increase in concentration of 3′,5′-AMP in the uterus of the castrate rat, as reported by Szego and Davis (*1*), the effect of DES on adenyl cyclase was studied in this tissue (Table 3). Intravenous administration of DES consistently activated adenyl cyclase at 5 minutes, but 2 hours after estrogen was administered adenyl cyclase activity returned to that in controls. Prior administration of the β-adrenergic blocking agent, DL-propranolol, abolished this activation, although DL-propranolol itself had no effect on uterine adenyl cyclase. Since DL-propranolol has been reported to have membrane effects other than those on β-adrenergic receptors (*10*), we tested the effect of D-propranolol, which has similar membrane effects but only 1/10 to 1/40 of the beta-blocking effects of DL-propranolol (*11*). When given at the same dosage (0.05 mg per 100 g of body weight) D-propranolol, in contrast to DL-propranolol, did not prevent the early activation of adenyl cyclase mediated by estrogen. Thus, the β-adrenergic blocking effects of DL-propranolol, rather than its membrane effects, were probably responsible for the abolition of cyclase activation by estrogen.

These data, then, suggest that the early increase in concentrations of tissue cyclic 3′,5′-AMP after estrogen administration may be mediated by catecholamines and may not be an absolute prerequisite for estrogen-stimulated protein synthesis. Estrogen has been reported to change the concentrations of epinephrine in this tissue (*12*), and it has been found that epinephrine causes activation of uterine adenyl cyclase (*13*).

There have been few previous data to implicate the adenyl cyclase system in relation to the effects of steroid hor-

mones. By analogy only, it has been suggested that the effect of aldosterone in the toad bladder (*14*) and cortisone in HeLa and strain L cells in culture (*15*) may be mediated by 3′,5′-AMP. Hechter *et al.* (*16*) showed that many biosynthetic processes in the rat uterus could be caused by 3′,5′-AMP, but that other nucleotides had similar effects. It also seems unlikely that an increased 3′,5′-AMP concentration is an absolute prerequisite for L-tyrosine-2-oxoglutarate aminotransferase (TAT) induction by glucocorticoids, as the effects of the N^6-2′-*O*-dibutyryl 3′,5′-AMP derivative and hydrocortisone on TAT were additive (*17*), and dexamethasone produced a 10- to 15-fold increase in TAT activity in cultured hepatoma tissue in the absence of detectable cell concentrations of adenyl cyclase (*18*).

In the tissue models described, in which effects of steroid hormones on growth or differentiation have been well characterized, the data suggest that these effects are not mediated by cyclic 3′,5′-AMP. Progesterone, however, caused a delayed and progressive activation of adenyl cyclase and an increase in concentration of tissue 3′,5′-AMP in the chick oviduct. The relation of this observation to the mechanism of progesterone action and the synthesis of avidin is not clear at present.

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Inside-Out Red Cell Membrane Vesicles: Preparation and Purification

Abstract. *Plasma membranes purified from human red cells were converted into small vesicles by disruption in alkaline buffer of low ionic strength. Most of these vesicles were inside-out. The presence of divalent cations prevented this inversion. The inside-out vesicles were separated from right-side-out vesicles by centrifugation to equilibrium in dextran density gradients.*

The plasma membrane deals with two distinctly different compartments, separates them, and mediates between them. Investigation of the biochemical specialization across this membrane has been limited by the inaccessibility of its inner surface to direct examination. We have approached this problem by promoting the budding of red cell plasma membrane ghosts into their cytoplasmic space, thereby generating inside-out vesicles whose outer faces are the cytoplasmic aspects of the parent membranes. Conversely, normally oriented vesicles are formed when a surface membrane buds into the extracellular space. We now present methods for the preparation and purification of inside-out and right-side-out red cell membrane vesicles suitable for the direct comparative analysis of the membrane's two faces.

Ethylenediaminetetraacetate (EDTA) was added to a concentration 0.010*M* to freshly drawn human blood. The red cells were washed three times with cold 0.15*M* NaCl in 0.005*M* sodium phosphate buffer, *p*H 8.0. Each milliliter of packed red cells was lysed by resuspension in 40 ml of cold 0.005*M* phosphate buffer, *p*H 8.0. The plasma membranes were centrifuged at 20,000*g* for 10 minutes to form pellets, which were washed twice more with the same buffer. The resulting pellets were white and were comprised of intact membrane ghosts.

Each pellet was resuspended in 25 ml of cold $5 \times 10^{-4}M$ sodium phosphate buffer, *p*H 8.0, for 1 hour or more, and then sedimented at 10^5g for 30 minutes. As judged by phase contrast light microscopy and thin-section electron microscopy the membranes appeared to be budding spontaneously into the ghost interior (endocytosis), leading to an accumulation of many small vesicles within each parent ghost. Gentle homogenization by pestle or by passage through a 27-gauge hypodermic needle reduced the residual ghost membrane to small vesicles and liberated the entrapped vesicles. The prior addition of divalent cations, such as $1 \times 10^{-4}M$ $MgSO_4$, to the $5 \times 10^{-4}M$ phosphate buffer stabilized the ghosts against spontaneous vesiculation. Homogenization of these stabilized ghosts caused them to vesiculate, primarily by budding into the extracellular space (exocytosis).

Right-side-out and inside-out vesicles were separated on linear gradients (density 1.01 to 1.07 g/cm^3) of Dextran-110 (Pharmacia Fine Chemicals), containing 5×10^{-4} phosphate buffer, *p*H 8.0, and $1 \times 10^{-4}M$ $MgSO_4$ (added to stabilize the vesicles). Homogenates were layered on the gradients and centrifuged to equilibrium at 10^5g for 16 hours. Roughly 90 percent of the membrane protein was recovered in three zones (Fig. 1): (i) a bottom band at the density of intact ghosts, 1.050 to 1.065; (ii) a top band at a density of approximately 1.01; and (iii) a scant zone spreading diffusely between 1.020 and 1.035. [The middle zone becomes enriched at the expense

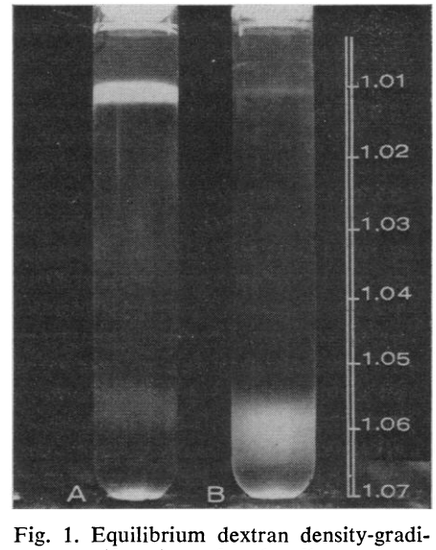

Fig. 1. Equilibrium dextran density-gradient centrifugation of red cell membrane vesicles homogenized in the absence (A) and presence (B) of $1 \times 10^{-4}M$ $MgSO_4$. The scale indicates the approximate density distribution of the gradient.