Table 2. Effect of cytosine arabinoside and hydroxyurea on the incorporation of [^{3}H]thymidine into DNA in erythroleukemic cells. Log phase T3C12 cells, (1×10^5)/ml, were incubated in the presence or absence of the indicated antibiotic for 15 minutes. [^{3}H]Thymidine was added to each culture to a specific activity of 1 μc/ml, and the incubation was continued for 1 hour. Portions of cells were taken at zero time (immediately after addition of [^{3}H]thymidine) and at the conclusion of the labeling period. The cells were then centrifuged and washed in phosphate-buffered saline. The pellet was then washed with 5 percent trichloroacetic acid (TCA), centrifuged, and dissolved in 0.5*N* KOH. After incubation for 30 minutes at 37°C, the samples were neutralized, and TCA was added to 10 percent. The precipitate was collected on Millipore filters and the radioactivity was counted by liquid scintillation.

Addition	[^{3}H]Thymidine incorporated (count/min per 10^3 cells)
None	5509
Ara-C, 1 μg/ml	710
Ara-C, 2 μg/ml	588
Hydroxyurea, 0.1 m*M*	652
Hydroxyurea, 0.2 m*M*	632
Zero time control	680

of either or both of these effects, or a generalized toxic effect on the erythroleukemic cells.

Our recent observation that butyric acid induces erythroid differentiation at concentrations less than a hundredth of that required for dimethyl sulfoxide (*6*) has allowed us to overcome these serious experimental difficulties. Butyric acid is less toxic to cultured cells during prolonged incubation and, in contrast to dimethyl sulfoxide, effectively induces stationary phase cells to undergo erythroid differentiation (Table 1).

As a further test of the requirement for DNA synthesis and cell division, 1β-D-arabinofuranosylcytosine (ara-C) and an unrelated inhibitor of DNA synthesis, hydroxyurea, were added to cultures of control and butyric acid–induced cells. Both antibiotics were added at concentrations that profoundly inhibited DNA synthesis (Table 2). No further cell division was observed. In both cases, in vitro differentiation, as measured by the appearance of benzidine-positive (containing hemoglobin) cells, occurred as efficiently as in control cultures (Table 3). That the percent of benzidine-positive cells provides a quantitative estimate of the extent of erythrodifferentiation has been established by our previous studies (*7*), which showed that the percent of positive cells is directly correlated with the globin messenger RNA accumulated by erythroleukemic cells and the percent of cells containing oxyhemoglobin, as determined by microspectrophotometry. The degree of benzidine staining of individual cells observed in these studies was no different in control cultures and those treated with antibiotics.

Table 3. Effect of cytosine arabinoside and hydroxyurea on butyric acid–induced erythrodifferentiation. Cells were grown, and butyric acid and antibiotics (where indicated) were added at day zero; cells were assayed for benzidine staining as described in the legend of Table 1.

Addition	Benzidine-positive cells (%) at butyric acid concentration:	
	None	1 m*M*
None	0	38
Ara-C, 1 μg/ml	0	39
Hydroxyurea, 0.1 m*M*	0	34

It can be argued that a small portion or a specific region of the genome is being replicated in the presence of either ara-C or hydroxyurea and this possibility cannot be ruled out entirely. Nevertheless, the two antibiotics have different mechanisms of action (*8*) and both quickly inhibit [^{3}H]thymidine incorporation as well as cell division in treated cells. In addition, the ability of nondividing, stationary phase cells to undergo erythroid differentiation in the presence of butyric acid lends additional support to the conclusion that neither division nor DNA synthesis is required for the expression of hemoglobin genes under these conditions.

Our results seem to eliminate the complicating assumptions that were needed to explain the regulated expression of globin genes that required mitosis or, at least, gene replication in erythroleukemic cells. Thus the globin system becomes formally more analogous to those prokaryotic operons in which control operates on nonreplicating DNA.

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9. We thank the members of the Department of Biological Chemistry of the Hebrew University of Jerusalem for hospitality, interest, and helpful advice. We also thank R. Ampel for technical assistance.

18 April 1975; revised 14 July 1975

Prostaglandin $F_{2\alpha}$ Production by the Brain During Estrogen-Induced Secretion of Luteinizing Hormone

Abstract. *The arteriovenous difference in the concentration of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) across the brain of the anestrous sheep was measured before and during the induction of luteinizing hormone secretion with 17β-estradiol. The results indicate that (i) the brain in vivo is a significant source of $PGF_{2\alpha}$, (ii) the release of $PGF_{2\alpha}$ from the brain occurs in pulses with a circhoral rhythm, and (iii) the process through which estrogen exerts its negative and positive feedback effects on luteinizing hormone secretion may involve amplitude modulation of $PGF_{2\alpha}$ output from the brain.*

Although the role of estrogen in the regulation of luteinizing hormone (LH) secretion by negative and positive feedback actions is firmly established (*1*), the underlying mechanisms are not fully understood. Recent reports have shown that prostaglandins (PG's) can elicit release of LH from the anterior pituitary gland in vitro (*2*) and in vivo (*3, 4*). Our studies have led us to suggest that PG's of central origin, acting at a site (or sites) proximal to the anterior pituitary, may play a role in the neuroendocrine mechanism which translates an increase in the circulating level of estrogen into a signal for the release of LH (*4, 5*). We now extend this evidence by demonstrating that the brain releases prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in circhoral pulses which undergo pronounced modulations during the induction of LH secretion by the intravenous infusion of 17β-estradiol (17β-E_2).

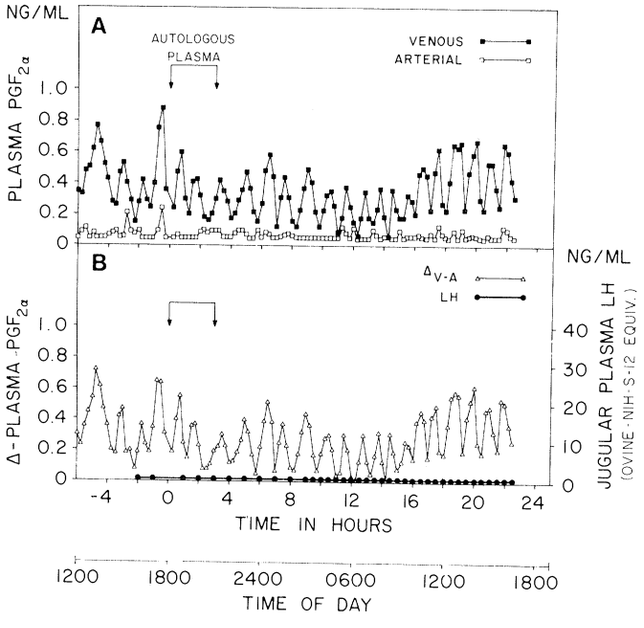
Anestrous Merino sheep were used as experimental subjects because, in response to the administration of 17β-E_2, these animals exhibit a very pronounced release of LH (*5, 6*) after a short latent period (8 to 12 hours). Eighteen hours before each experiment was begun, the left carotid artery and left jugular vein, exteriorized in a loop

of skin, were cannulated. Each subject was given 8000 international units of heparin as an anticoagulant 10 minutes before the start of each experiment and at 4-hour intervals thereafter. Samples of blood were withdrawn simultaneously from the left carotid artery (2 ml) and left jugular vein (4 ml) and transferred immediately to heparinized tubes on ice. After the blood was sampled at intervals of 15 minutes for a control period of 6 hours, 17β-E_2 dissolved in autologous plasma (0.67 μg/ml) was infused into the right jugular vein at a rate of 2 μg/hour for 3 hours by means of a constant infusion pump (Harvard Apparatus). Samples were taken at the same intervals as before for 24 hours after the infusion was begun. In control experiments the same procedure was followed with autologous plasma alone.

As blood samples accumulated, plasma was separated after centrifugation at 4°C and stored at −20°C prior to the determination of the concentrations of LH and $PGF_{2\alpha}$. Jugular venous levels of LH were determined by a double antibody radioimmunoassay (*7*) with specific antiserum against ovine LH. The reference standard was NIH-LH-S-12. The $PGF_{2\alpha}$ was measured directly in unextracted arterial and venous plasma; we used the radioimmunoassay procedure described by Van Orden and Farley (*8*), with an antibody prepared according to the method of Stylos *et al.* (*9*). The standard curve and cross-reactivity data were similar to those reported by Van Orden and Farley, but the 13,14-dihydro-15-keto and the 13,14-dihydro metabolites of $PGF_{2\alpha}$ were also tested. Only the latter compound showed slight cross-reactivity (1.5 percent). When standard $PGF_{2\alpha}$ (0.05 to 0.5 ng/ml) was added to arterial and venous plasma that was known to contain undetectable amounts of endogenous $PGF_{2\alpha}$, average recovery (± S.E.M.) was 110 ± 17 percent (arterial, N = 6) and 108 ± 14 percent (venous, N = 5).

Under control conditions (Fig. 1), venous $PGF_{2\alpha}$ was higher than arterial $PGF_{2\alpha}$ with few exceptions. Most striking was the occurrence of remarkably regular oscillations in the arteriovenous difference (Δ_{V-A}) for $PGF_{2\alpha}$. These pulses exhibited an apparent period of 69.9 ± 3.3 minutes (mean ± S.E.M. for control data from four subjects) and an average height of 0.52 ± 0.03 ng/ml. The mean Δ_{V-A} was 0.29 ± 0.01 ng/ml ($P < .001$), which suggests that the brain is a significant source of $PGF_{2\alpha}$ in anestrous ewes (*10*).

During the course of the infusion of 17β-E_2 (Fig. 2), the pulses of $PGF_{2\alpha}$ became suppressed. The average pulse height in the 6-hour control period was significantly higher ($P < .001$) than the average pulse height in the first 6 hours after the infusion of estrogen. However, this suppressive phase was transient and, during the LH surge, the peaks of $PGF_{2\alpha}$ were again significantly higher ($P < .001$) than they had been during the 6-hour period that followed the infusion of estrogen. Since mean values, peak values, and the frequency of the pulses of $PGF_{2\alpha}$ were essentially the same during the LH surge as during the control period, the reappearance of the pulses during the LH surge appeared to be



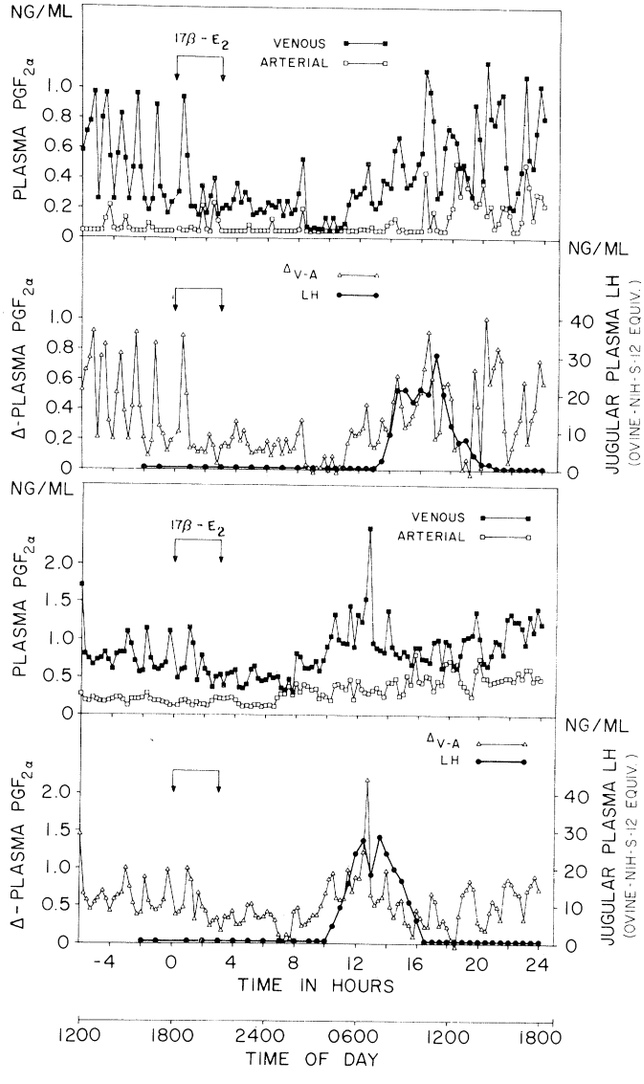


Fig. 1 (above). Experimental control. Samples of jugular venous and carotid arterial blood were withdrawn every 15 minutes from an anestrous ewe. At time zero, autologous plasma was infused as a vehicle control for 3 hours. (A) Concentration of $PGF_{2\alpha}$ (ng/ml) in jugular venous and carotid arterial blood plasma. (B) Arteriovenous difference (Δ_{V-A}) in $PGF_{2\alpha}$ and (superimposed) the concentration in jugular venous plasma of LH (ng/ml) measured by radioimmunoassay suitable for the detection of major peaks of LH. Regular pulses of $PGF_{2\alpha}$ release from the brain were observed with a periodicity of 69.9 ± 3.3 minutes. Fig. 2 (right). Effect of an intravenous infusion of 17β-E_2 dissolved in autologous plasma on the concentration of $PGF_{2\alpha}$ and LH in jugular and carotid plasma in two anestrous ewes. Compared to the first 6 hours after the infusion of 17β-E_2 ended, the average pulse height of $PGF_{2\alpha}$ was significantly higher ($P < .001$) during both the 6-hour control period and the period of the LH surge. The pulses of $PGF_{2\alpha}$ were transiently suppressed by 17β-E_2 but appeared to return just before the LH surge. The marker bounded by the arrows indicates 17β-E_2 at 2 μg/hour.

a rebound phenomenon without substantial "overshoot." In each case, however, at least one pulse of $PGF_{2\alpha}$ preceded the first detectable elevation in the circulating LH. Further experiments will be required to establish the latter relationship statistically.

In the two animals that received only autologous plasma, there was no statistically significant alteration in the frequency or pulse height of the peaks of $PGF_{2\alpha}$. In one of the control experiments (not shown), plasma LH increased slightly (to 12 ng/ml) for a brief period, beginning about 4 hours after the infusion of autologous plasma ended.

Since cells are not thought to store preformed PG's in significant quantities (*11*), the Δ_{V-A} for $PGF_{2\alpha}$ was taken to represent net synthesis of $PGF_{2\alpha}$ by the brain. Using Δ_{V-A} as an index, our report shows that (i) synthesis of $PGF_{2\alpha}$ by the brain can be monitored with relative ease under physiological conditions in the conscious, resting animal; (ii) the release of $PGF_{2\alpha}$ by the brain occurs in pulses whose frequency approximates the circhoral rhythm of LH release in ovariectomized ewes (*12*); and (iii) after the infusion of 17β-E_2, the pulses of $PGF_{2\alpha}$ first decline and later return to control levels coincident with the LH surge.

These pulses might conceivably come from nonneural elements such as the cerebrovascular system. At present, it seems more likely that they arise from the brain itself because (i) central nervous tissue is known to synthesize PG's at several loci (*13*) and (ii) neurogenic stimuli can release PG's from the brain and spinal cord (*13*). Moreover, the system that produces the pulses of $PGF_{2\alpha}$ is apparently sensitive to estrogen, a property it shares with many central neurons. Because the system is estrogen-sensitive, measurement and comparison of the relative suppressive effects of intra-arterial and intraventricular infusions of 17β-E_2 on the pulses of $PGF_{2\alpha}$ might indicate whether the pulses arise from blood vessels or from neurons.

Since current evidence suggests that LH release is associated with elevated PG's, the initial decline in Δ_{V-A} for $PGF_{2\alpha}$ might at first sight seem paradoxical. However, in addition to its positive feedback actions, estrogen has well-known negative feedback effects on the secretion of LH. In particular, 17β-E_2 reduces the basal level of LH in the blood of cycling ewes (*14*) and eliminates the circhoral pulses of LH observed in ovariectomized ewes (*15*). Since the rate at which 17β-E_2 reduces LH in these instances approximates the rate at which we observed it to suppress Δ_{V-A} for $PGF_{2\alpha}$ in anestrous ewes, the declining pulses of $PGF_{2\alpha}$ may well be a manifestation of the negative feedback action of estrogen on LH release.

Similarly, the recurrence of large-amplitude pulses of $PGF_{2\alpha}$ immediately before the actual surge of LH may play a role in the positive feedback action of 17β-E_2. This conclusion is consistent with the finding that plasma LH increases shortly after intracarotid infusion of $PGF_{2\alpha}$ (*4*) and with the related observation that 17β-E_2 cannot induce LH release in sheep undergoing treatment with indomethacin (*5*).

Although these points are compatible with the hypothesis that PG's of central origin play a role in mediating the negative and positive feedback effects of estrogen on LH secretion, they do not prove that either the pulsatile pattern of $PGF_{2\alpha}$ synthesis or its modulation over periods of time are necessary conditions for the regulation of LH secretion. It is possible that these phenomena may reflect as yet unknown events in neuroendocrine control processes. Assessment of the significance of the pulses of $PGF_{2\alpha}$ and their apparent modulation by 17β-E_2 must therefore await further investigation.

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16. Supported by PHS research grants HD-04411 and HD-08129 and by the Ford Foundation. We thank M. E. Glew, C. Bennett, and L. F. Underwood for skilled technical assistance. We thank Dr. G. D. Niswender and Dr. H. Esber for antiserum against ovine LH.

21 March 1975; revised 23 May 1975

Cyclic AMP-Dependent Protein Kinase: Pivotal Role in Regulation of Enzyme Induction and Growth

Abstract. *Dibutyryl cyclic adenosine 3',5'-monophosphate (cyclic AMP) produces phosphodiesterase induction, growth arrest, and cytolysis in S49 lymphoma cells. The striking parallelism between protein kinase activity that is dependent on cytosol cyclic AMP and cellular responses to dibutyryl cyclic AMP in wild-type cells and three classes of clones resistant to cyclic AMP indicates that protein kinase mediates cyclic AMP regulation of growth and enzyme induction in S49 cells.*

The ways in which cyclic adenosine 3',5'-monophosphate (cyclic AMP) exerts its diverse regulatory effects in eukaryotic cells are still unknown. Investigations of enzymes in cell-free systems have established that cyclic AMP regulates lipolysis and glycogen metabolism by activating cyclic AMP-dependent protein kinase or kinases (*1*). Similar cyclic AMP-dependent kinases are ubiquitous in mammalian cells (*2*), implying that they are involved in other cyclic AMP-mediated processes as well, such as regulation of cell growth and differentiation.

Enzymologic approaches have not revealed these other roles of cyclic AMP-dependent protein kinase. We now report the results of a genetic approach for investigating the role of cyclic AMP-dependent protein kinase in cell regulation. Cultured S49