*ADENOSINE 3′,5′-MONOPHOSPHATE IN RAT UTERUS: ACUTE ELEVATION BY ESTROGEN**

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These laboratories have been engaged in a continuing effort to identify and characterize very early manifestations of the presumed "primary" molecular interaction between selected hormones and their sensitive targets.[1–6] In the course of these studies, it has been demonstrated that the site-specific release of sequestered amines, accompanied by hyperemia and enhanced vascular permeability, precedes to a highly significant degree any hitherto reported influence of estrogen upon uterine metabolism. The present report describes a possible biochemical link between these acute effects and ultimate metabolic stimulation.

Adenosine 3′,5′-monophosphate (cyclic AMP) possesses significant estrogen-like influences,[5, 7–9] and is a recognized propagator of the acute metabolic actions of a growing number of hormones and other biocatalytic substances including certain amines.[10] Investigation of the responsiveness to estrogen of the uterine adenyl cyclase system therefore seemed indicated. Upon direct analysis of the intact uterus for endogenous cyclic AMP by adaptation[11] of the highly sensitive method devised for brain by Breckenridge,[12] it was found that uterine cyclic AMP was depleted after ovariectomy.[13] These observations have now been extended to determine whether net synthesis of this important metabolic agent was acutely responsive to alterations in prevailing estrogen level. The present report demonstrates that *within 15 seconds* after intravenous administration of physiological doses of estradiol-17β to the ovariectomized rat, uterine cyclic AMP is approximately doubled. The reaction is hormone-specific and exhibits tissue selectivity.

Materials and Methods.—Female Sprague-Dawley rats, 6 weeks old and weighing about 150 gm, were ovariectomized and maintained in a carefully controlled, low-steroid environment for approximately 3 weeks. On the day of experiment, they were matched by body wt into control and experimental groups. Under light sodium pentobarbital ("Nembutal") anesthesia, they were given estrogen in saline[14] or control vehicle by saphenous vein. At precise intervals after beginning the injection, uteri were rapidly excised and immediately plunged into liquid nitrogen. Three uteri from similarly treated animals constituted a pooled sample which was weighed in the frozen state and then pulverized in a stainless steel percussion mortar under liquid nitrogen. The frozen pellet, weighing approximately 300 mg, was homogenized without prior thawing in 8 volumes of ice-cold 5.6% trichloroacetic acid (TCA), and centrifuged at 0°C for 30 min at 3000 rpm. The supernate was promptly frozen at −86°C. Although no apparent alteration of cyclic AMP occurred under these conditions up to 90 days, most analyses were carried out within 2–3 weeks.

The Breckenridge microanalytical procedure,[12] though technically demanding, offers distinct advantages in the direct analysis of the endogenous cyclic AMP content of those intact tissues to which it can be proved[11] applicable, i.e., high sensitivity, precision, and linearity over a useful range of concentrations. It is patterned after the general principles adapted[15] for the fluorometric analysis of reduced coenzymes as metabolic indicators. In the present instance,[12] these are formed at a rate proportional to adenylate concentration, but the yield may be amplified several thousand-fold as a result of cyclic recurrence of coupled enzymatic steps.[15]

Enzymes used in the present investigation were obtained commercially as indicated,[12] with the exception of highly specific 3′,5′-cyclic mononucleotide phosphodiesterase.[16] The latter was gen-

erously contributed by Dr. R. W. Butcher and stored at −86°C in small lots, thawed briefly to ice temperature just before use, and promptly refrozen without demonstrable loss of activity.

Minor modifications were introduced for the present purposes[11] into the original[12] procedure. Other than changes in enzyme ratios to yield the desired cycling rate (approximately 1800 in the present experiments), and certain differences in sizes of aliquots removed at successive steps, alterations included use of a boiled phosphodiesterase control for the tissue "blank," and a 1:10 dilution of the originally specified glucose-6-P dehydrogenase concentration in step *f*. The latter change was instituted in order to overcome the effects of a persistently troublesome contaminant occurring in some lots of this enzyme which tended to enhance the fluorescence of the reduced coenzyme, presumably by altering the binding constant (cf. ref. 17).

All samples were analyzed in duplicate or triplicate. Cyclic adenylate standards[18] at two or more concentration levels, as well as TCA blanks, were invariably carried through the entire assay procedure in parallel with the unknown tissue extracts, notwithstanding the fact that in prior experiments virtually complete recovery of tritiated cyclic AMP added at this stage had been achieved. There was, moreover, no detectable loss of adenylate at the phosphodiesterase step, for 5′-AMP added at step *c* was stoichiometrically equivalent to 3′,5′-AMP added either at step *a* or *c*. The efficacy of removal of 5′-AMP added in the initial phosphohydrolytic step was complete.

In confirmation of Breckenridge,[12] sensitivity, linearity, and reproducibility were found to be satisfactory, once standardization of conditions was achieved.[11] Replication of the data within a single experiment was almost invariably excellent. Limitations occasionally encountered were attributable to inadequacies in glassware preparation.[19]

Results.—Effects of ovariectomy: Table 1 illustrates the dramatic dependence of uterine cyclic AMP content upon prevailing estrogen levels. The concentration of cyclic adenylate in the uterus of the intact, untreated rat sacrificed without regard for stage of the estrous cycle was similar in magnitude to the endogenous level of this nucleotide in those very few intact rat tissues for which values exist in the literature.[12, 20, 21] This figure was reduced about 50 per cent three weeks following ovariectomy. The differences in concentration were somewhat magnified when expressed on the more meaningful dry wt basis (Table 1) or when related to protein content (not shown). Total uterine cyclic AMP content of the intact group on a body weight basis was approximately seven times that observed in the absence of ovarian function.

Influence of estrogen: Since there are innumerable data in the literature on modifications of uterine composition which occur in parallel to organ involution after ovariectomy, the above observations alone have only limited significance. However, acute estrogen treatment had a marked effect upon uterine cyclic AMP levels in ovariectomized animals. Table 1 reveals that within 15 seconds of the intravenous administration of 1 μg/100 gm body wt of estradiol-17β, uterine cyclic AMP was elevated to concentrations indistinguishable from those seen in the intact animals. Attempts to make earlier measurements were not undertaken. Uterine 3′,5′-AMP was unaltered by administration of the control vehicle alone (Table 1). Control and experimental tissue blanks were indistinguishable.

The sharp increase in the cyclic nucleotide elicited by estrogen under these conditions was maintained essentially undiminished up to 5 minutes. It may be noted from Figure 1, however, that 3′,5′-AMP levels were restored to initial values within 60 minutes of estrogen administration.

Specificity and selectivity of the response to estrogen: The physiological relevance of these observations seems indicated. The abrupt onset of the uterine cyclic AMP response to estrogen coincides with other early evidences of estrogenic stimulation, including liberation of sequestered histamine and the accompanying expansion of

TABLE 1

ACUTE ELEVATION BY ESTRADIOL-17β[a] OF UTERINE CYCLIC 3′,5′-AMP IN THE RAT

Group	No. observations[b]	Interval to tissue sampling (min)	3′,5′-AMP[c] μMoles/kg fresh wt uterus	3′,5′-AMP[c] μMoles/kg dry wt uterus	3′,5′-AMP[c] μMoles/100 gm body wt[d]
Intact, untreated[e]	5	—	1.83 ± 0.28	10.01 ± 1.65	3.6×10^{-4}
Ovariectomized					
Untreated	6	—	1.05 ± 0.12[g]	4.93 ± 0.54[h]	4.9×10^{-5} [h]
Vehicle control[f]	3	0.25	0.91 ± 0.13	4.25 ± 0.70	
	4	0.50	1.06 ± 0.21	5.25 ± 1.10	
	2	2.0	1.06 [1.26; 0.85]	4.98 [6.28; 3.68]	
	2	5.0	1.14 [1.08; 1.19]	4.90 [4.67; 5.13]	
	Avg, 0.25—5 min, inclusive		*1.04 ± 0.09*	*4.87 ± 0.45*	
Estradiol-17β[a]	2	0.25	2.38 [2.20; 2.56]	10.10 [9.55; 10.64]	
	6	0.50	2.14 ± 0.16[h]	10.24 ± 0.59[h]	
	2	2.0	2.00 [2.11; 1.89]	9.33 [10.50; 8.15]	
	2	5.0	2.08 [2.00; 2.15]	8.96 [9.27; 8.65]	
	Avg, 0.25—5 min, inclusive		*2.14 ± 0.03*[h]	*9.85 ± 0.38*[h]	

[a] Estradiol-17β, 1 μg/100 gm body wt, was administered intravenously at zero time as the sodium salt prepared as indicated in the text.
[b] Uteri from three ovariectomized rats treated successively as indicated were pooled in the frozen state for further processing as described in the text. Pooling was not required for the intact series.
[c] To be multiplied by correction factor of 0.95 for conversion to absolute values, as indicated.[18] Concentrations shown are means and their standard errors; where two observations are indicated, individual values are given.
[d] Based on average uterine weights in similar series.
[e] Sampled without regard to stage of the estrous cycle.
[f] Received the blank vehicle[14] at zero time.
[g] $P < 0.05$ for difference between this value and corresponding control.
[h] $P < 0.01$ for difference between this value and corresponding control.

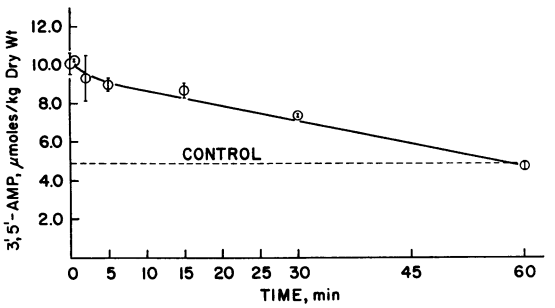

FIG. 1.—Time-course of estradiol effect on uterine cyclic 3',5'-AMP. Estradiol-17β, 1μ g/100 gm body wt, was administered intravaneously at zero time as the sodium salt.[14] Control animals received the vehicle alone. Sample pools, as indicated in Table 1, were collected under "Nembutal" anesthesia at various intervals after intravenous injection. The latter procedure was performed under "Nembutal" as indicated in text except for the 60-min groups in which ether was used. The latter did not interfere with the 30-sec response to estradiol in separate control experiments (not shown). Values for cyclic AMP, to be corrected as indicated,[18] are represented for estradiol-treated groups by the points, each composed of average of duplicates whose deviations are shown, except for the 0.5-min group which represents the mean of six samples and the corresponding S.E.M. Contributing to control values represented by the horizontal line are one sample taken at 30 min, and two at 60 min after I.V. injection. It is evident that these do not differ from the control levels at earlier periods (Table 1).

the microcirculation and associated alterations in membrane functions. Moreover, uterine cyclic AMP concentration is responsive to estrogen levels of a physiological order. As little as 0.06 μg of estradiol-17β per 100 gm body wt was capable of evoking a clear increase over the control value of uterine 3',5'-AMP within 30 seconds (Fig. 2). This response was submaximal. Figure 3 demonstrates that the potent estrogen diethylstilbestrol was also effective in promoting a rise in uterine cyclic AMP, whereas estradiol-17α, in a dose more than 40 times greater than the apparent MED of its estrogenically active β-epimer, was inert.

In a limited number of experiments, estradiol-17β was itself incapable of eliciting any change in the cyclic AMP concentration of diaphragm, an organ which does not accumulate the hormone under physiological circumstances.[22]

It was considered possible, but unlikely, that an artifact arising directly from the presence of minute quantities of estrogen per se in the uterine extracts could augment the activity of the specific phosphodiesterase used in step *c* of the assay and yield the effects seen. This possibility seems remote on the basis of the ease of extraction with ether[12] of such traces of residual estrogen as might appear in the protein-free TCA supernates of the organs. Moreover, phosphodiesterase was used well above its limiting concentration. In addition, simultaneous analyses of

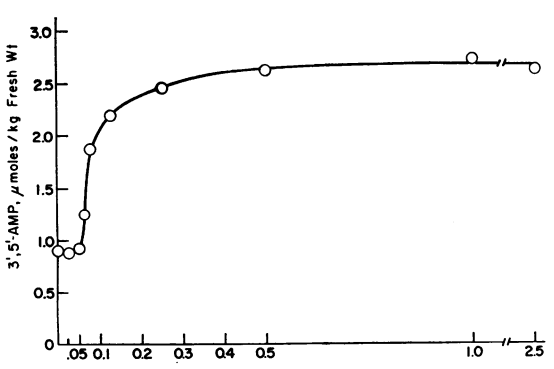

FIG. 2.—Response of uterine cyclic 3',5'-AMP in the ovariectomized rat to varying doses of estradiol-17β. Estrogen solutions were injected intravenously as indicated in text and Table 1. Sampling was carried out at 30 sec thereafter. Each point represents analysis of a single sample pooled from three uteri.

The minimum effective dose, which was not established within this experiment because of the sharp inflection point, lies below 0.06 μg/100 gm body wt. A maximal response is apparently achieved with 0.5 μg/100 gm.

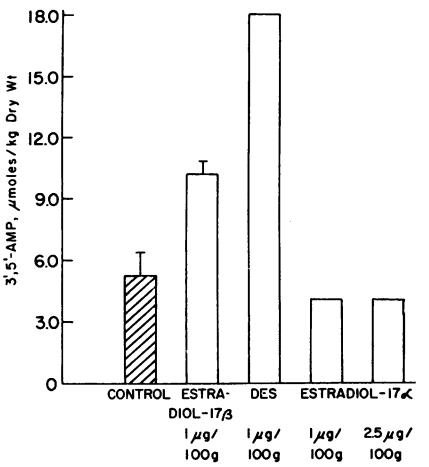

FIG. 3.—Hormone specificity of the uterine cyclic 3',5'-AMP response in the ovariectomized rat. Observations were carried out at 30 sec. The cross-hatched bar represents the mean value from four sets of vehicle control-injected animals. The estradiol-17β group is composed of six observations. Variation, where indicated, is S.E.M. All other data are individual values. DES refers to diethylstilbestrol.

varying aliquots of unknown extracts, arising from either control or experimental samples, yielded analytical values in appropriate proportion. A further evidence of reliability of the observed values was the additivity of aliquots of either control or experimental sample extracts and standard cyclic AMP, which came within 94–98 per cent of calculated values.[11] Thus, augmentation within the assay per se, rather than effect of estrogen at the *in vivo* equilibrium between formation and destruction of cyclic AMP, while not ruled out, is believed to be rendered unlikely by these control studies.

Discussion.—The concentration of tissue cyclic AMP at any given moment appears to be the resultant of appropriately counterbalanced processes of synthesis by adenyl cyclase and degradation by a relatively specific[10, 16] 3',5'-cyclic mononucleotide phosphodiesterase. In the uterus this dynamic equilibrium is shifted toward phosphodiesterase activity by removal of circulating estrogen.

Nevertheless, the uterine adenyl cyclase system appears to be in a highly poised state, for, notwithstanding a relatively long period of hormonal deprivation, it is capable of virtually instantaneous responsiveness to estrogen, yielding a full complement of cyclic AMP by 15 seconds after the start of intravenous administration of the steroid. This abrupt change, reminiscent of the rapid effect of epinephrine on cardiac 3',5'-AMP,[21] which is evident in isolated perfused preparations by 2–3 seconds, precedes or coincides with the onset of explosive histamine discharge from uterine binding-sites under estrogen influence.[5] Fifteen seconds encompass two or more total circulation times in the rat.[23] The time period during which estrogen effects an approximately 100 per cent elevation of uterine cyclic AMP in the ovariectomized rat is conconcurrent with organ-selective hormone binding by what may or may not be "receptor" protein.[24, 25] Not far behind, by two minutes, are such evidences of modified transport function as accentuated uptake from the circulation of radioactive precursors to protein[26] and RNA[27] synthesis. Indeed, provision of excess substrate may per se underlie the induction phenomenon which, as had been predicted,[3] is highly responsive to this means of regulation.[28, 29] Moreover, in related investigations it has been shown that incubation of surviving uterine segments of untreated ovariectomized rats in the presence of mM amounts of cyclic AMP for only 5 min is sufficient to elicit a significant increase in accumulation of

C^{14}-leucine or -lysine.[8] Thus, although elevation of endogenous cyclic AMP due to estrogen subsides within 60 min despite the apparent continued presence[24] of the hormone, the metabolic "triggering" effects of the nucleotide[5, 8] are presumably achieved well before the end of this time. Limitation of uterine ATP reserve does not seem a likely mechanism for restraint upon continued elevation of cyclic AMP. Not only is the uterine ATP concentration several orders of magnitude higher[30] than cyclic product, but the frequently cited[31] 50 per cent "decline"[32] in uterine ATP purportedly elicited by estrogen over a 4-hr period is more apparent[33] than real. The latter was expressed[32] on the basis of fresh weight which is known to double in the interval indicated.

Numerous clues to the mechanism of the acute increase of uterine cyclic AMP elicited by estrogen already exist, but do not yet appear to lend themselves to unequivocal interpretation. The abruptness of onset, specificity of estrogen structure, effectiveness at low systemic doses, and the organ selectivity suggest a highly specific interaction with virtually no induction period. "Release" of preformed cyclic AMP from sequestered form is excluded in the present experimental design, by virtue of established completeness of extraction and by parallel controls at all stages of the investigation. Hence some form of interaction[34] of the hormone with the membrane-bound[35] adenyl cyclase system resulting in conformational activation may be postulated. By analogy with the known stimulatory effects of amines on the adenyl cyclase system,[10, 34, 36] and in consideration of the parallel discharge of intrinsic histamine,[2, 4, 5] serotonin,[6] and epinephrine[37] during acute hormonal responses of various target organs, including uterus,[2, 5, 37] however, it is possible that uterine cyclic AMP elevation occurs as a secondary consequence to site-specific amine liberation by estrogen. The possibility that an amine may function in this capacity as a "secondary" messenger[10] while the cyclic nucleotide itself occupies a tertiary role[5, 38] in metabolic regulation is rendered the more likely by the fact that isolated preparations of organs in which smooth muscle is a prominent component, including uterus, respond to epinephrine with cyclic AMP augmentation.[34, 39] Uterine cyclic nucleotide levels following the initial surge due to estrogen, therefore, would be subject to dual restriction by action of the specific phosphodiesterase[16] as well as by the rapid dissipation[5] of unsequestered activating amines. That the latter may be discharged from site-specific binding in discrete units, yielding a graded estrogen response, is suggested by previous data.[5] It is evident from the present observations that submaximal increases in cyclic AMP may also occur at extremely low estrogen does.s Diethylstilbestrol, which appears at least as effective as estradiol-17β in acute histamine discharge[5] and by various other criteria,[40] had a striking effect on uterine cyclic AMP in the present investigation.

Modification of the ionic environment by estrogen[1, 5, 33] with indirect cytostructural consequences may be still another mechanism by which adenyl cyclase activation[10] or phosphodiesterase inhibition[41] may be achieved. Less likely, though not excluded in the present experiments, is the possibility that trace substances, including divalent cations (cf. ref. 33) which may affect the assay itself, may already be present in uterine extracts in greater concentration than in those of control tissues by as little as 15 sec after hormone treatment, by virtue of metabolic stimulation, altered transport, or through increased blood content. It has not yet been determined whether the cyclic AMP level or the hyperemia[5] responds first.

The unique triggering event in the regulatory influence of estrogen on uterine biochemical differentiation is as yet unknown. The present findings provide the first evidence that estrogen may be added to the growing list of hormones which, directly or indirectly, interact with the adenyl cyclase system. Any attempt, however, to link cyclic AMP to the chain of metabolic events resulting from the application of estrogen to the castrate uterus *in vivo* must meet certain minimum requirements, including demonstration of ultra-acute onset, organ-selectivity, sensitivity to doses of a physiological order and to estrogenic compounds, whether endogenous or synthetic, in accordance with their established degree of effectiveness. These criteria have been met, within the framework of the present assay, indicating that such a sequence may indeed occur under normal conditions of estrogen stimulation. This does not prove, however, a causal relationship. The full implications of these findings in the larger context of mechanism of estrogen action remain to be evaluated.

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(*a*) Following removal of trichloroacetic acid, hydrolysis of preformed noncyclic adenylates with apyrase and phosphatase is carried out on an aliquot.

(*b*) The phosphohydrolytic enzymes are degraded with pepsin, followed by

(*c*) Transformation of 3′,5′-AMP to 5′-AMP by means of a specific phosphodiesterase.

(*d*) Stoichiometric conversion of 5′-AMP to ATP is achieved in the presence of adenylate myokinase and pyruvate kinase.

(*e*) Coupled cyclic reactions catalyzed by hexokinase and pyruvate kinase are then utilized to produce glucose-6-P.

(*f*) The hexose phosphate formed is then oxidized by glucose-6-P-dehydrogenase in the presence of TPN^+. Consumption of the substrate is reflected in the production of TPNH which is measured fluorometrically in the linear range.

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