Unfortunately the position of the cyclopropane ring cannot be determined directly by mass spectrometry (Pohl *et al.*, 1963; Wood and Reiser, 1965; Christie and Holman, 1966). The exact location of this group will be investigated in the near future according to McCloskey and Law (1967).

Gas Chromatography. Relative retention times on the stationary phases PEGA and Apiezon L of components A, B, and C have already been given in our first paper (Oudejans *et al.*, 1971).

The presence of cyclopropane fatty acids in female specimens of *G. tumuliporus* is a unique feature because until now this type of fatty acids has been reported only in bacteria, plants, and protozoa.

Moreover, the number of carbon atoms of the most abundant component (18 carbon atoms) is different from the cyclopropane fatty acid structures published already, which are all odd numbered (review by Christie, 1970). Both the high amount of these fatty acids and their radioactivities after injection of [1-^{14}C]acetate indicate that these substances play an important role in the metabolism of this millipede, which is discussed in another paper (van der Horst *et al.*, 1971).

Acknowledgments

The authors are indebted to Professor Dr. D. I. Zandee for his interest and stimulating discussions and to Dr. W. Heerma for recording the mass spectra.

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Effects of Estradiol on Uterine Ribonucleic Acid Metabolism. I. *In Vitro* Uptake and Incorporation of Ribonucleic Acid Precursors*

Theodore W. Munns and Philip A. Katzman†

ABSTRACT: The *in vitro* uptake and incorporation of [5-^{3}H]-uridine, [^{32}P]P_i, and L-[*methyl*-^{3}H]methionine were monitored at intervals during a 2-hr incubation to assess RNA synthesis in uteri of immature rats at various times following intraperitoneal injection of 17β-estradiol. With short *in vitro* pulses, uptake and incorporation of uridine were most prominent during the first few hours after estrogen treatment; with P_i, increases were not evident even at 12 hr; while with methionine the increases were progressive throughout this period. During longer *in vitro* pulses the rate of incorporation of uridine fell to control levels and even lower, particularly, after longer periods of hormone treatment; that of P_i was somewhat enhanced; while that of methionine increased linearly throughout the period of incubation. These data are interpreted on the basis of the effects of estradiol on parameters influencing the specific activities of the precursor pools in question, as well as on the rate of synthesis of RNA.

While the marked accumulation of uterine RNA accompanying the estrogen response has been well documented, the use of a variety of isotopic precursors, as well as labeling systems, to monitor the rate of uterine RNA synthesis has yielded discordant results. For example, Gorski and Nicollete (1963), using a 60-min pulse *in vivo*, observed significantly increased [^{32}P]P_i incorporation into RNA of uterine subcellular fractions during the initial hours of the estrogen response. In an analogous *in vitro* labeling system, increased incorporation was not detected. On the other hand, Billing *et al.* (1969b), utilizing an *in vivo* system in which the radioactivity associated with the adenine nucleotide pool was stabilized, reported that incorporation of this isotope into uterine RNA increased only slightly during the initial phase of the response and did not become substantial until after 5 hr. In a somewhat different type of study, Hamilton *et al.* (1968) found that during a 10-min pulse *in vivo*, [5-^{3}H]uridine incorporation into uterine nuclear RNA was maximal 20 min after estrogen administration.

While it is recognized that the extent of incorporation of an

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TABLE I: Selective Hydrolysis of RNA in the Perchloric Acid Insoluble Fraction of Uterine Homogenates.[a]

Isotope	Perchloric Acid Insoluble (10^{-3} dpm/uterus) Total	Hydrolyzable	% Hydrolyzed
[5-^{3}H]Uridine	20.5	20.2	98.5
L-[*methyl*-^{3}H]Methionine	226.2	28.2	12.5
L-[*carboxyl*-^{14}C]Methionine	36.2	0.66	1.8
L-[^{14}C]Leucine	162.0	3.1	1.9

[a] Duplicate groups of 5 uteri from untreated immature rats were incubated in the presence of each isotope for 30 min. The homogenates were treated to prepare perchloric acid insoluble fractions which were selectively hydrolyzed and from the hydrolysates, aliquots withdrawn for radioactivity determinations (described in Experimental Procedures). Values are based on duplicate determinations of each fraction of each group.

isotope into RNA is a function not only of the rate of RNA synthesis, but also of the specific activity associated with its nucleotide precursor(s) at the time of incorporation, all too often, measurement of only the incorporation under a given set of labeling conditions has been taken to constitute a valid means of assessing the rate of RNA synthesis. With regard to the uterus it is expected that estrogen treatment will affect the specific activities of various RNA precursors due to increased vascularity (Noall and Allen, 1961; Szego, 1965), increased precursor permeability (Means and Hamilton, 1966; Billing *et al.*, 1969a; Greenman, 1971), and fluctuating nucleotide pool sizes (Mueller *et al.*, 1958; Oliver and Kellie, 1970). Unfortunately, few investigations have attempted to cope with these problems. A notable exception is the *in vivo* study of Billing *et al.* (1969b) in which injected radioactive adenosine was allowed to equilibrate with the uterine adenine nucleotide pool prior to and for a limited period after the administration of estradiol into immature rats.

We have attempted to assess the effect of estradiol or uterine RNA synthesis by employing L-[*methyl*-^{3}H]methionine in an *in vitro* system in which effects due to vascularity, and perhaps permeability, are minimized, and in which the precursor bypasses the difficulty associated with fluctuating nucleotide pools. Borek and coworkers (Fleissner and Borek, 1962, 1963; Mandel and Borek, 1963; Srinivasan and Borek, 1964) have shown that the methyl group of methionine, by way of *S*-adenosylmethionine and specific RNA methylases, is transferred to recently synthesized RNA molecules (reviews by Borek, 1963; Starr and Sells, 1969).

In this investigation we have examined the behavior of L-[*methyl*-^{3}H]methionine with respect to both its rate of uptake and incorporation into RNA. Comparative studies with [5-^{3}H]uridine and [^{32}P]P_i were conducted under identical labeling conditions.

Experimental Procedures

Treatment of Animals. Carworth CFE immature female rats, 22–24 days old and weighing 40–50 g, were injected intraperitoneally with a single dose of 5 μg of 17β-estradiol (Sigma Chemical Co.) in 0.25 ml of aqueous 1% ethanol. At the prescribed times thereafter, the animals were killed (cervical dislocation) and within 1 min the whole uterus was removed free of fat and connective tissue and collected in ice-cold isotonic saline. Groups of from 3 to 5 uteri were gently blotted and weighed prior to *in vitro* incubation.

Incubation Conditions. Each group of tissues was placed in a 50-ml beaker containing 5 ml of Eagle's HeLa medium (Difco 565) which had been preequilibrated at 37° under an atmosphere of 95% O_2–5% CO_2 and supplemented with glutamine (1.0 mM) and one of the following isotopic precursors (obtained from Schwarz BioResearch); carrier-free [^{32}P]P_i, [5-^{3}H]uridine (20 Ci/mmole), L-[*methyl*-^{3}H]methionine (2.5 Ci/mmole), L-[*carboxyl*-^{14}C]methionine (15 mCi/mmole), and L-[^{14}C]leucine (316 mCi/mmole). Quantities of isotope ranged from 10 to 100 μCi per incubation flask. At the end of incubation the tissues were immediately rinsed in ice-cold isotonic saline and processed at once or stored at −80°.

Determination of Isotope Uptake. All procedures were conducted at 0–5° unless otherwise stated. The tissues were homogenized in 1 to 2 ml of distilled H_2O, using a conical ground-glass tissue grinder (Duall) and an equal volume of 0.5 N perchloric acid. The resulting suspension was mixed (at 10-min intervals for 30 min) and centrifuged at 800*g* for 5 min. The radioactivity in the supernatant (perchloric acid soluble fraction) was a measure of the uptake and accumulation of unincorporated isotope and was determined by placing small aliquots of the supernatant into scintillation vials to which 10 ml of aqueous scintillator was added (180 g of naphthalene + 5 g of 2,5-diphenyloxazole + 0.3 g of 1,4-bis[2-(5-phenyloxazolyl)]-benzene in 350 ml of toluene + 350 ml of dioxane + 210 ml of absolute ethanol). All radioactivity determinations in the present study were conducted with a Packard Tri-Carb liquid scintillation spectrometer. Efficiencies ranged from 20 to 70% (depending on the isotope and fraction employed) and were determined by internal standardization.

Determination of Isotope Incorporation. The pellet obtained from the perchloric acid treated homogenates, after several washings with perchloric acid and centrifugation, was used to determine the extent of incorporation of precursor into protein and/or RNA.

To determine the incorporation of [^{32}P]P_i into RNA, the pellet was further washed sequentially with 95% ethanol, chloroform–ethanol (2:1), and ether and then hydrolyzed with 0.3 N KOH at 40° for 60 min. After cooling (4°) and acidifying with an equal volume of cold 0.8 N perchloric acid, the suspension was centrifuged and the radioactivity in the supernatant (hydrolyzed RNA) determined as previously described. Alkaline hydrolysis was used occasionally to determine the incorporation of [5-^{3}H]uridine into RNA, although the hydrolysis with PCA (described below) was routinely employed.

In determining the incorporation of [5-^{3}H]uridine (RNA), L-[*carboxyl*-^{14}C]methionine and L-[^{14}C]leucine (protein), and L-[*methyl*-^{3}H]methionine (RNA and protein), the RNA in the perchloric acid insoluble pellet was selectively hydrolyzed in 0.5 N perchloric acid at 70° for 15 min. After cooling and vigorous mixing to obtain a uniform suspension of the hydrolyzed RNA and insoluble protein, aliquots were transferred to scintillation vials and neutralized with 3 N KOH, and 1 ml of NCS solubilizer was added (Nuclear-Chicago). The vials were capped and after standing at room temperature until digestion was complete (24–48 hr), 10 ml of aqueous scintillator was added. Determination of the radioactivity in these samples indicated the extent of incorporation into RNA and/or protein depending upon the isotope employed.

TABLE II: Effect of Estradiol (E_2) on Uterine Accumulation of DNA, RNA, Protein, and Wet Weight.[a]

E_2 Pretreatment *in Vivo* (hr)	Amount/Uterus			
	DNA (μg)	RNA (μg)	Protein (mg)	Wet Wt (mg)
0 (control)	258 ± 36	124 ± 12	2.56 ± 0.2	27.8 ± 5.8
12	271 ± 32	205 ± 17	3.33 ± 0.4	44.5 ± 7.1
24	282 ± 37	371 ± 43	3.95 ± 0.5	62.6 ± 8.3
48	423 ± 45	469 ± 40	5.82 ± 0.5	72.5 ± 11.9
72	348 ± 38	248 ± 32	4.63 ± 0.4	48.3 ± 8.0

[a] Triplicate groups of immature rats (3–5/group) were sacrificed at the times indicated after an intraperitoneal injection of 5 μg of estradiol. The excised uteri were weighed and processed for DNA, RNA, and protein content as described in Experimental Procedures. The average of duplicate determinations for each group was employed in calculating the standard deviation.

The remainder of the hydrolysate was centrifuged and the radioactivity in aliquots of the supernatant containing the hydrolyzed RNA (perchloric acid insoluble hydrolyzable fraction) was used to determine the amount of isotope incorporated into RNA.

Assessment of the Selective Hydrolysis. The extent to which the RNA in the perchloric acid insoluble fractions is selectively hydrolyzed by heating at 70° for 15 min in 0.5 N perchloric acid was determined by measuring the radioactivity which was released from the perchloric acid insoluble fraction prepared from uteri after incubation with [5-^{3}H]uridine. The extent of "protein hydrolysis" was measured by the radioactivity released from the perchloric acid insoluble fractions of uteri which had been incubated with L-[^{14}C]leucine or L-[*carboxyl*-^{14}C]methionine. In either case, the radioactivity which becomes perchloric acid soluble by this treatment represents the "perchloric acid insoluble hydrolyzable fraction."

Under the prescribed conditions (Table I), hydrolysis of RNA was essentially complete (>98% of the radioactivity of the incorporated [5-^{3}H]uridine became perchloric acid soluble), whereas, that of protein was very small (<2% of the radioactivity derived from either of the ^{14}C-labeled amino acids was released). In other experiments in which hydrolysis of the [5-^{3}H]uridine-labeled perchloric acid insoluble material was accomplished by treatment with 0.3 N KOH or 0.5 N perchloric acid, the same amounts of radioactivity were released into the perchloric acid soluble material.

The significant quantity (12.5%) of radioactivity which was liberated from the L-[*methyl*-^{3}H]methionine-labeled perchloric acid insoluble fraction by hydrolysis with perchloric acid was thus attributed to, and taken as a measure of, the incorporation of labeled-methyl groups into RNA. Although the data in Table I were obtained with perchloric acid insoluble fractions from unprimed uterine tissue which had been pulse-labeled *in vitro* for 30 min, in similar studies with estrogen-primed uteri incubated with L-[^{14}C]leucine for as long as 2 hr, "protein hydrolysis" in no instance exceeded 2%. Furthermore, since extending the period of perchloric acid hydrolysis to 45 min did not significantly increase "protein hydrolysis" (<2.5%), it is likely that a sizable portion of this readily released radioactivity which is referred to here as "hydrolyzed protein" actually represents labeled aminoacylated tRNA.

Results which we have obtained (Munns and Katzman, 1971) under the *in vitro* labeling conditions described here, support the findings of Brown and Attardi (1965) and Craddock (1969) that the extent of incorporation of L-[*methyl*-^{3}H]-methionine into nucleic acids *via* "1-carbon" metabolism and into DNA by direct methylation was insignificant with respect to the amount incorporated into RNA by methylation.

Chemical Determinations. The perchloric acid insoluble fraction was used for both DNA and RNA determinations; DNA was determined by the method of Burton (1959) after acid hydrolysis, and RNA by the methods of Fleck and Monro (1962) and/or Ceriotti (1955) after alkaline hydrolysis. Suitable aliquots from homogenates of uterine tissues, homogenized in distilled H_2O, were used for determining protein (Lowry *et al.*, 1951).

Results and Interpretations

Effect of Estradiol on the Accumulation of RNA, DNA, and Protein in the Uterus of the Immature Rat. The effects of estradiol on the uterus of the intact immature rat were assessed by determining the DNA, RNA, protein, and wet weight of uteri excised from animals at various times, up to 72 hr, after a single intraperitoneal injection of estradiol. The accumulation of these macromolecules measured the *net* effect of estrogen-induced synthesis throughout a defined period of stimulation.

The data obtained from these initial studies (Table II and Figure 1) are in general agreement with those reported by others

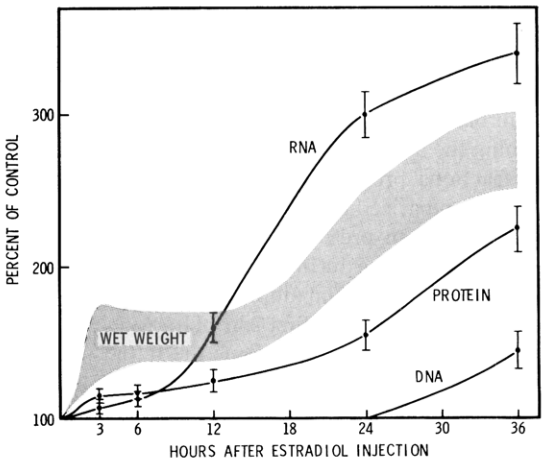

FIGURE 1: The effect of estradiol on wet weight and accumulated DNA, RNA, and protein of uteri during the initial 36 hr of the response. The shaded area represents the range of uterine wet weights observed in at least five experiments (3–5 uteri/group). Animals were processed as described in Table II; all values are expressed in terms of per cent of control mean.

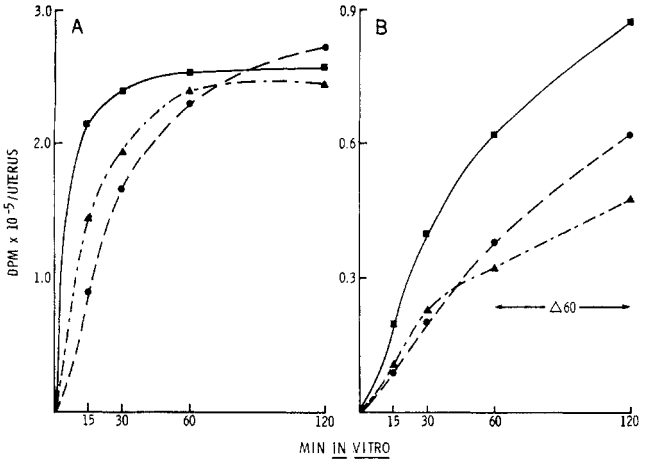

FIGURE 2: [5-^{3}H]Uridine uptake into the perchloric acid soluble fraction (A) and incorporation into the RNA (B) of control and estrogen-pretreated uteri during a 2-hr *in vitro* incubation (see Table III). ●, control; ■, 30-min estrogen-pretreated; ▲, 360-min estrogen-pretreated.

(Mueller *et al.*, 1958; Hamilton *et al.*, 1967), that accumulation of DNA, RNA, and protein, as well as wet weight, was maximal approximately 48 hr after hormone injection. Measurements at more frequent intervals during the initial 36 hr of the response (Figure 1) indicated that uterine wet weight increased dramatically during the first 6 hr (water-imbibition phase) with no further change until after 12 hr when a second increase began that lasted until the 48th hr and which paralleled the accumulation of protein. The *rate* of accumulation of RNA was greatest during the interval between 6 and 24 hr of the response and for protein it occurred during the 12- to 36-hr interval. No accumulation of DNA was evident in the first 24 hr.

Since the RNA accumulation profile (Figure 1), reflects primarily the synthesis of "stable" RNA (rRNA and tRNA), it may, or may not, be representative of the effect of estradiol on the rate of *total* RNA synthesis. With the above limitation in mind, the rate of uterine RNA synthesis was monitored with three different radioactive precursors. The same labeling system was employed for each precursor which consisted of incubating whole surviving uteri, previously primed with estradiol for prescribed periods of time *in vivo*, for short periods of time *in vitro*.

In Vitro Uptake and Incorporation of [5-^{3}H]Uridine. The uptake of uridine into control and estradiol-pretreated uteri was monitored throught a 2-hr *in vitro* incubation period by determining the amount of radioactivity in the perchloric acid soluble fractions prepared from whole homogenate of the tissues. As summarized in Table III and illustrated (in part) in Figure 2A, estrogen pretreatment significantly enhanced the uptake of [5-^{3}H]uridine during short periods (15 and 30 min) of incubation, being maximum (approximately 200% of controls) in uterine tissues during the first 2 hr of hormone action. However, with successively longer pulses the initial differences in perchloric acid soluble radioactivity between experimental and control tissues progressively decreased, becoming indistinguishable from control values when a 60- or 120-min pulse was employed.

Although the mechanism of this early estrogen-promoted uptake is unknown, it coincides with the H_2O-imbibition phase of the response. Recently, Oliver (1971) has suggested that a facilitated diffusion of nucleosides occurs in the estrogen-primed uterus, both *in vivo* and *in vitro*, as a result of the increased tissue volume (H_2O imbibition). Since the size of the uridine nucleotide pool remains unaltered during the first few hours of the response (Oliver and Kellie, 1970), the initial increases in [5-^{3}H]uridine uptake would temporarily increase the specific activity of the uridine nucleotide pool which, in turn, would increase the incorporation of isotope into RNA.

TABLE III: Effect of Estradiol on the *in Vitro* Rates of [5-^{3}H]-Uridine Uptake into the Uterine Perchloric Acid Soluble Fraction and Incorporation into RNA.[a]

Estradiol *in Vivo* (min)	Incubation *in Vitro* (min)	10^{-3} Dpm/Uterus (% Control) Perchloric Acid Soluble	Perchloric Acid Insoluble
0 (control)	15	91 (100)	9.2 (100)
	30	168 (100)	20.5 (100)
	60	231 (100)	38.3 (100)
	120	272 (100)	62.7 (100)
	120–60 (Δ60)	41 (100)	24.4 (100)
10	15	142 (156)	13.6 (148)
	30	209 (124)	28.6 (139)
	60	253 (110)	41.0 (107)
	120	278 (102)	65.8 (105)
	120–60 (Δ60)	25 (61)	24.8 (102)
20	15	167 (184)	15.1 (164)
	30	213 (127)	29.8 (145)
	60	256 (111)	44.2 (115)
	120	283 (104)	71.5 (114)
	120–60 (Δ60)	27 (66)	27.3 (112)
30	15	214 (235)	19.6 (213)
	30	240 (143)	39.6 (192)
	60	252 (109)	62.4 (163)
	120	257 (94)	88.3 (141)
	120–60 (Δ60)	5 (12)	25.9 (106)
60	15	211 (232)	16.9 (184)
	30	236 (140)	32.7 (160)
	60	256 (111)	50.2 (131)
	120	262 (96)	72.6 (104)
	120–60 (Δ60)	6 (15)	22.4 (92)
120	15	218 (240)	13.5 (147)
	30	225 (134)	25.8 (126)
	60	246 (106)	38.6 (101)
	120	260 (96)	52.7 (84)
	120–60 (Δ60)	14 (34)	14.1 (58)
360	15	144 (158)	10.5 (114)
	30	194 (115)	23.3 (114)
	60	241 (104)	32.2 (84)
	120	246 (90)	48.3 (77)
	120–60 (Δ60)	5 (12)	16.1 (66)

[a] The uteri from triplicate groups of immature animals (3/group) were removed at intervals after estrogen administration *in vivo* and incubated in the presence of [5-^{3}H]-uridine for prescribed periods. Determination of radioactivity in the above fractions is described in experimental procedures. Results are expressed as dpm/uterus and represent the average of duplicate determinations of each of the triplicate groups. Standard deviation (not shown) was less than ±12% for all values listed.

TABLE IV: Effect of Estradiol on the *in Vitro* Rates of Uptake of [^{32}P]P_i into the Uterine Perchloric Acid Soluble Fraction and Incorporation into RNA.[a]

Estradiol *in Vivo* (min)	Incubation *in Vitro* (min)	10^{-3} Dpm/Uterus (% Control) Perchloric Acid Soluble	Perchloric Acid Insol (RNA)
0 (control)	15	105 (100)	0.8 (100)
	30	182 (100)	1.9 (100)
	60	263 (100)	5.1 (100)
	120	396 (100)	13.9 (100)
	120–60 (Δ60)	133 (100)	8.8 (100)
60	15	120 (114)	0.85 (106)
	30	195 (107)	1.9 (100)
	60	257 (98)	4.8 (94)
	120	408 (103)	15.7 (113)
	120–60 (Δ60)	151 (114)	10.9 (124)
360	15	122 (116)	0.95 (119)
	30	201 (110)	2.2 (116)
	60	307 (117)	6.6 (129)
	120	462 (117)	20.8 (150)
	120–60 (Δ60)	155 (117)	14.2 (161)
720	15	117 (111)	0.93 (116)
	30	194 (107)	2.2 (116)
	60	310 (118)	7.3 (143)
	120	502 (127)	23.3 (168)
	120–60 (Δ60)	192 (144)	16.0 (182)

[a] Experimental procedure identical with that used in Table III except RNA hydrolyzed with KOH as described under methods. Standard deviation (not shown) was less than ±10% for all values listed.

Since the transitory changes detected in the incorporation of [5-^{3}H]uridine into RNA (perchloric acid insoluble fraction) were directly related to the changes in perchloric acid soluble radioactivity (see Table III), the increased incorporation of [5-^{3}H]uridine in uterine tissue observed with short *in vitro* pulses during the early phase of the estrogen response reflects, primarily, an enhanced uptake (facilitated diffusion) of isotope rather than a net increase in RNA synthesis. Similarly increased incorporation of other nucleoside labels has been reported to occur during short-term *in vivo* pulses (Hamilton *et al.*, 1968; Billing *et al.*, 1969a; Greenman, 1971).

During longer *in vitro* pulses (60 and 120 min) the rate of incorporation of [5-^{3}H]uridine into estrogen-pretreated tissues was significantly reduced. This occurred at a time in the incubation period when there was no apparent difference in the perchloric acid soluble radioactivity between control and estrogen-pretreated tissues. Incorporation below control levels was most evident during the last 60 min of a 2-hr incubation (Δ60 values, Table III) in those tissues pretreated with hormone for extended periods of time. These results reflect a second difficulty associated with the use of nucleoside labels to monitor RNA synthesis in tissues undergoing changes in nucleotide pool size.

Prior to being incorporated into RNA, [5-^{3}H]uridine has to pass through an estrogen-induced, expanded uridine nucleotide pool (Mueller *et al.*, 1958; Oliver and Kellie, 1970). Although this expansion is not immediately evident, during longer estrogen-pretreatment periods (6 and 12 hr) it is apparently sufficient to dilute the specific activity of uridine nucleotide precursors and thus mask the increase in uterine RNA synthesis which is indicated by RNA accumulation (Figure 1).

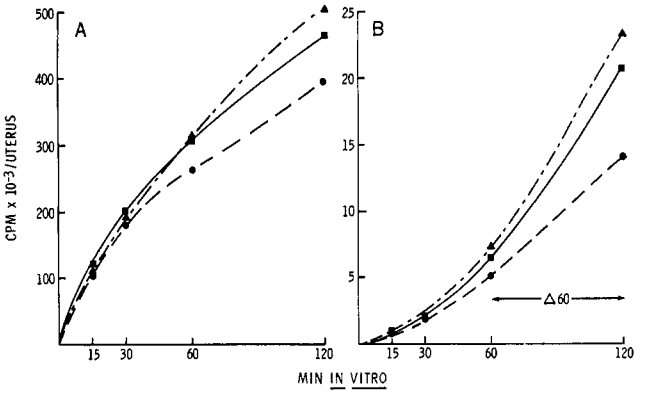

FIGURE 3: [^{32}P]P_i uptake into the perchloric acid soluble fraction (A) and incorporation into the RNA (B) of control and estrogen-pretreated uteri during a 2-hr *in vitro* incubation (see Table IV). ●, control; ■, 360-min estrogen-pretreated; ▲, 720-min estrogen pretreatment.

In Vitro Uptake of [^{32}P]P_i *and Incorporation into RNA.* Unlike [5-^{3}H]uridine, the uptake of [^{32}P]P_i into uterine perchloric acid soluble fractions was not appreciably influenced by estrogen pretreatment (Table IV and Figure 3A). This was also true of [^{32}P]P_i incorporation (Figure 3B) into RNA during the first 60 min of *in vitro* incubation, confirming and extending the findings of Gorski and Nicolette (1963). However, the rate of incorporation was significantly increased during a 2-hr pulse in those tissues pretreated with hormone for 6 and 12 hr, corresponding to the period in the response when RNA accumulation becomes evident (Figure 1). The incorporation of [^{32}P]P_i into the RNA of these tissues was most apparent during the last 60 min of a 2-hr incubation (Δ60 values, Table IV).

Assuming that the major pathway for the incorporation of [^{32}P]P_i into RNA involves [^{32}P]P_α nucleotide formation *via* purine and pyrimidine biosynthesis (P_β- and P_γ-labeled nucleotides are not incorporated into RNA), equilibration of the uterine [^{32}P]P_α nucleotide and P_i pools would be dependent upon nucleotide-pool turnover. The data in Figure 3B indicate that although the specific activity of the [^{32}P]P_α nucleotides, as reflected by their incorporation into RNA, continually increases during a 2-hr *in vitro* incubation, equilibration of these pools was not complete. If equilibration was complete, the specific activity of the [^{32}P]P_α nucleotide pool would be constant and be reflected by a linear rate of incorporation of [^{32}P]P_i into RNA. Fluctuations in the specific activities of uterine nucleotides would be expected prior to equilibration since the observed differences in nucleotide pool sizes accompanying the estrogen response (Mueller *et al.*, 1958; Oliver and Kellie, 1970) reflect differential rates of nucleotide synthesis and utilization.

In Vitro Uptake and Incorporation of L-[*methyl*-^{3}H]*Methionine.* In contrast to the results obtained with [5-^{3}H]uridine and [^{32}P]P_i, the duration of *in vitro* incubation had little or no effect on the rates of incorporation of L-[*methyl*-^{3}H]methionine into RNA or protein (Table V, Figure 4B and 4C).

The initial uptake of L-[*methyl*-^{3}H]methionine into the

TABLE V: Effect of Estradiol on the *in Vitro* Rates of Uptake of L-[*methyl*-^{3}H]Methionine into the Uterine Perchloric Acid Soluble and Insoluble (Protein and RNA) Fractions.[a]

Estradiol *in Vivo* (min)	Incubation *in Vitro* (min)	10^{-3} Dpm/Uterus (% Control)		
		Perchloric Acid Soluble	Perchloric Acid Insoluble	
			Protein	RNA
0 (control)	15	148 (100)	63.1 (100)	11.7 (100)
	30	288 (100)	198.0 (100)	28.2 (100)
	60	391 (100)	459.0 (100)	63.0 (100)
	120	405 (100)	982.0 (100)	133.0 (100)
	120–60 (Δ60)	14	523.0 (100)	70.0 (100)
30	15	194 (131)	73.2 (116)	14.7 (126)
	30	383 (133)	234.0 (118)	34.3 (122)
	60	432 (110)	577.0 (126)	75.3 (120)
	120	458 (113)	1220.0 (124)	156.0 (117)
	120–60 (Δ60)	26	643.0 (123)	80.7 (115)
120	15	195 (132)	102.0 (162)	20.2 (173)
	30	402 (140)	271.0 (137)	47.7 (169)
	60	450 (115)	634.0 (138)	103.0 (163)
	120	496 (122)	1360.0 (138)	218.0 (164)
	120–60 (Δ60)	46	726.0 (139)	115.0 (164)
360	15	219 (148)	175.0 (278)	38.4 (328)
	30	425 (148)	474.0 (239)	92.0 (326)
	60	542 (139)	1020.0 (222)	198.0 (314)
	120	590 (146)	2170.0 (221)	402.0 (302)
	120–60 (Δ60)	48	1150.0 (220)	204.0 (291)

[a] Processing of uterine tissues and determination of radioactivity as described in Table III. Results are expressed in dpm/uterus, protein values representing the difference between total perchloric acid insoluble radioactivity (RNA + protein) and perchloric acid insoluble hydrolyzable radioactivity (RNA). Each value represents the average of duplicate determinations from three separate experiments (3 uteri/experiment), with the standard deviation less than ±10% in each case.

perchloric acid soluble fractions of control and experimental tissues was rapid and linear during the first 15 and 30 min of incubation, being somewhat greater in the estrogen-pretreated tissues. Perchloric acid soluble radioactivity continued to increase at a reduced rate during longer incubations, although most of the label was rapidly incorporated into both protein and RNA. The small increases in perchloric acid soluble radioactivity observed during the last 60 min of a 2-hr incubation may, in part, be attributed to an increasing accumulation of radioactive metabolites.

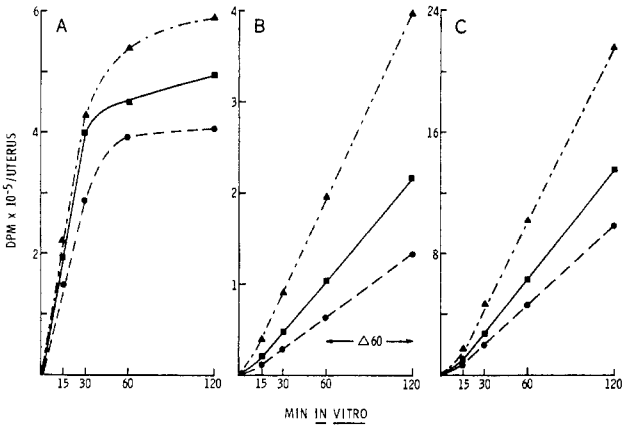

FIGURE 4: L-[*methyl*-^{3}H]Methionine uptake into the perchloric acid soluble fraction (A) and incorporation into the RNA (B) and protein (C) of control and estrogen-pretreated uteri during a 2-hr *in vitro* incubation (see Table V). ●, control; ■, 120-min estrogen-pretreated; ▲, 360-min estrogen-pretreated.

The linear incorporation of isotope into RNA (Figure 4B) and protein (Figure 4C) suggests that the turnover of both the endogenous methionine and *S*-adenosylmethionine pools was extremely rapid. Additional evidence for this rapid turnover was provided by the marked differences between isotope uptake and incorporation during the last 60 min of a 2-hr incubation (Δ60 values, Table V). During this period the incorporation of isotope (RNA and protein) continued to increase at a linear rate in the absence of significant changes in the perchloric acid soluble radioactivity, indicating that a steady state had been attained with regard to isotope entry and incorporation. These results indicate that within a relatively short time after the initiation of incubation, the specific activities of methionine and *S*-adenosylmethionine in all tissues employed became constant and equivalent to that of the methionine in the medium. Thus, the linear incorporation of L-[*methyl*-^{3}H]methionine into the RNA and protein of control and estrogen-pretreated tissues was considered to be representative of the rates of methylation (RNA) and synthesis (protein).

Figure 5 illustrates the rate of incorporation (% of controls) of L-[*methyl*-^{3}H]methionine into uterine RNA during the initial 24 hr of the estrogen response as determined after a 2-hr *in vitro* pulse. Methylation of RNA increased steadily

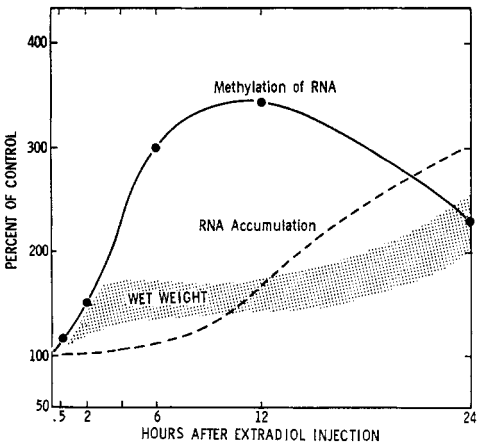

FIGURE 5: Comparison of the profiles of (1) rate of L-[*methyl*-^{3}H]-methionine incorporation into RNA, (2) RNA accumulation, and (3) wet weight of uterine itssues during the initial 24 hr of the estrogen response.

and became maximal after 12 hr (340% of controls). Although it slowly declined thereafter, it was still 240% of control levels at 24 hr. This methylation profile approximates that of RNA accumulation as estimated by the *slope* of the RNA accumulation profile (Figure 5).

General Discussion

Figure 6 summarizes the data regarding the incorporation of each precursor with respect to the effects of duration of hormone pretreatment *in vivo* and duration of radioactive pulses *in vitro*. Under the labeling conditions employed, the incorporation patterns of these precursors differed from one another during the first 12 hr of the estrogen response. In addition, incorporation patterns of both [5-^{3}H]uridine and [^{32}P]P_i changed with longer *in vitro* pulses, whereas those of L-[*methyl*-^{3}H]methionine remained constant. Thus, it is apparent from Figure 6 that the indiscriminate use of radioactive precursors as well as of pulse periods to reflect the increased synthesis of uterine RNA accompanying the estrogen response may result in erroneous conclusions.

The sharp increases in [5-^{3}H]uridine incorporation, observed during the early phase of the estrogen response, when short pulses were employed, were attributed to a preferential uptake of isotope rather than increased RNA synthesis. During the later periods in the response (6 and 12 hr), when RNA accumulation was becoming increasingly evident, the inability of both [5-^{3}H]uridine and [^{32}P]P_i to reflect increases in RNA synthesis was attributed to the expansion of uterine nucleotide pools (Mueller *et al.*, 1958; Oliver and Kellie, 1970). Since the metabolic pathways of both precursors pass through these pools, their specific activities become diluted in estrogen-treated tissues prior to incorporation.

The initial dilution of the specific activity of [^{32}P]P_i-labeled precursors that occurred primarily in 6- and 12-hr estrogen-pretreated tissues during short *in vitro* pulses was gradually overcome during longer pulses (as reflected by increasing rates of incorporation of [^{32}P]P_i into RNA, Figure 3), presumably as a result of equilibration of [^{32}P]P_α nucleotide and P_i pools due to nucleotide pool turnover. On the other hand, the specific activity of [5-^{3}H]uridine precursors continued to be diluted during longer pulses, especially in those tissues pretreated with hormone for longer periods (as reflected by decreasing rates of incorporation of [5-^{3}H]uridine into RNA, Figure 2). This dilution was attributed to the differential rates of synthesis of uridine nucleotides, *i.e.*, synthesis *via* unlabeled pyrimidine precursors being greater than that *via* phosphorylation of [5-^{3}H]uridine. In this regard, Tremblay and Thayer (1964) demonstrated that increased aspartate transcarbamylase activity was commensurate with RNA accumulation during the estrogen response.

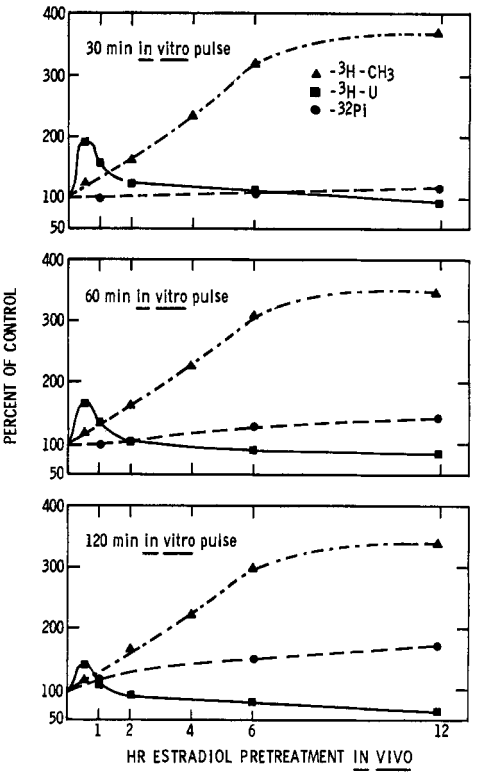

FIGURE 6: The effect of duration of *in vitro* pulse on the incorporation of [5-^{3}H]uridine (^{3}H-U), [^{32}P]P_i ($^{32}P_i$), and L-[*methyl*-^{3}H]-methionine (^{3}H-CH_3) into uterine RNA during the initial 12 hr of the estrogen respose. Triplicate groups of uteri (3/group) from immature rats previously primed with 5 μg of estradiol for prescribed periods (up to 12 hr) were incubated *in vitro* in the presence of the above isotopes for 30, 60, and 120 min. The extent of incorporation of each isotope into RNA was determined as described in Experimental Procedures and represents the average of duplicate determinations of each of the triplicate groups. Results are expressed in terms of per cent of control incorporation.

In contrast to the behavior of the above precursors, the use of L-[*methyl*-^{3}H]methionine to monitor RNA synthesis *in vitro* presents several distinct advantages. First, this precursor bypasses fluctuating nucleotide pools enroute to incorporation (methylation); secondly, since methionine is not synthesized endogenously, its supply is controlled by the concentration in the incubation medium; and finally, as a result of its rapid uptake and turnover, a constant specific activity of both methionine and *S*-adenosylmethionine pools appears to be attained rapidly in both control and estrogen-treated tissues. These characteristics are reflected in the linear rates of incorporation of L-[*methyl*-^{3}H]methionine into both RNA and protein (Figures 4B and 4C), and in the excellent agreement between the *in vitro* rate of isotope incorporation into RNA and *in vivo* RNA accumulation (Figure 5).

Of primary importance to this study, is the question whether or not the rates of methylation and synthesis of methylated RNA species can be considered to be equivalent. A number

of studies have presented evidence which shows that methylation of rRNA takes place in the nucleolus at the time of transcription or immediately thereafter (Burdon, 1966; Greenberg and Penman, 1966; Zimmerman and Holler, 1967). Other investigations have suggested that precursor-tRNAs have only a transitory existence prior to becoming methylated (Burdon *et al.*, 1967; Bernhardt and Darnell, 1969). Our own studies with actinomycin D (Munns and Katzman, 1971) support the above conclusions, indicating that the interim between synthesis and methylation of both tRNA and rRNA is exceedingly short.

Since mRNAs appear to be unmethylated (Brown and Attardi, 1965; Moore, 1966), and since tRNA and rRNA account for the bulk of total RNA as well as of methylated RNA populations (Starr and Sells, 1969), we have assumed that the increased incorporation of L-[*methyl*-^{3}H]methionine into the uterine RNA during the estrogen response (Figure 5) represents the increasing rates of synthesis of methylated RNAs, primarily tRNA and rRNA.

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