The Effect of Estradiol-17β on the Activity of Carbamoyl Phosphate:L-Aspartate Carbamoyl Transferase in the Uteri of Immature Rats*

GEORGE C. TREMBLAY† AND SYDNEY A. THAYER

From the Department of Biochemistry, St. Louis University School of Medicine, St. Louis 4, Missouri

(Received for publication, May 4, 1964)

Previous investigations have indicated that, in bacteria, the biosynthesis of the pyrimidine nucleotides of ribonucleic acid, the biosynthesis of the pyrimidine nucleotides of deoxyribonucleic acid, and the biosynthesis of the pyrimidine nucleotides of phospholipids proceeds as shown in Scheme 1 (1, 2). Whether this is the major pathway for pyrimidine biosynthesis in mammalian tissue is not clear (3-5). The following study was undertaken to investigate the possibility of a relationship between growth, the synthesis of RNA, and the activity of carbamoyl phosphate:L-aspartate carbamoyl transferase in the uterus of the immature rat stimulated by estradiol-17β.

EXPERIMENTAL PROCEDURE

Chemicals—All chemicals except carbamoyl phosphate-14C were obtained commercially. The latter was synthesized from KClN0-14C according to the method of Jones, Spector, and Lipmann (11). The purity of the carbamoyl phosphate was determined by the differential phosphate analysis of Lowry and Lopez (12). The recrystallized product was 90 to 95% pure and had a specific activity of 1.7 × 10^5 d.p.m. per µmole. The dry carbamoyl phosphate was stored in a vacuum over P2O5 at 4°C and a solution of carbamoyl phosphate was prepared immediately before use.

Enzyme Preparation—Female rats of the St. Louis University colony, 20 to 22 days old, were treated by subcutaneous injection at 12-hour intervals for periods up to 84 hours with an aqueous ethanol solution of estradiol-17β. The animals were killed by decapitation at various periods up to 90 hours after the first injection of the hormone. The amount of hormone administered at each injection was such that the total dose at each period of assay was 10 µg. Control animals were treated by injection with the solvent. When the rat was killed, the uterus was quickly excised and frozen by dropping it into a vessel partially submerged in acetone and Dry Ice. The pooled uteri were then lyophylized with a VirTis Freeze-Mobile. The freeze-dried uteri were homogenized in a ground glass conical tissue grinder at 5°C in 1.5% potassium chloride solution to give a 6% w/v suspension. The homogenate was centrifuged at 15,000 × g for 45 minutes in a refrigerated Spinco model L...
centrifuge, and the supernatant liquid was used as the source of enzyme.

Estimation of Enzymatic Activity—Each complete incubation mixture, performed in triplicate, contained: diethanolamine buffer (pH 9.2), 50 μmoles; potassium L-aspartate, 5 μmoles; 15,000 × g supernatant liquid, 0.20 ml; carbamoyl phosphate-14C, 5 μmoles; total volume, 0.55 ml. The incubation period was 15 minutes at 37.5°C, and the reaction was stopped by the addition of 0.28 ml of a 1 N HCl solution.

Procedure 1—The determination of enzymatic activity was accomplished according to the method of Lowenstein and Cohen (13). This assay procedure is based on the observation that the carbamoyl carbon of carbamoyl phosphate is acid-labile while that of carbamoylaspartate is acid-stable.

Aliquots of the acidified incubation mixture were plated and dried under an infrared heat lamp for 20 minutes. The acid-stable 14C product was counted with a Geiger tube for the time required to give a maximum standard error of 2% of the net activity. An incubation mixture without enzyme was used to determine nonenzymatically synthesized product, and an incubation mixture without aspartate was used to determine acid-stable 14C product other than carbamoylaspartate. The sum of the radioactivity of the blanks and background was subtracted from the radioactivity determined in the complete incubation mixture to give the activity of the enzyme expressed as counts per minute per mg of protein. Protein was estimated by the trichloroacetic acid turbidity method (14).

Procedure 2—A combustion technique allowing the quantitative trapping of CO2 as an amin carbamate was developed to determine the specific activity of the enzyme. An aliquot of the acidified incubation mixture was placed on black absorbent paper (Arthur H. Thomas Company, No. 6471-Q25) which was inserted in a platinum carrier. The sample was baked to dryness under a heat lamp. Redistilled β-phenylethylamine (5.00 ml) was pipetted into a 500-ml Thomas-Ogg combustion flask, and the flask and contents were cooled for at least 10 minutes in an ice bath. The flask was then flushed with oxygen, and the platinum carrier containing the dried sample was suspended on a ball-joint stopper and the stopper clamped into the combustion flask (Arthur H. Thomas Company, No. 6471-P10). The dried material was ignited by focusing the infrared projection lamp of the Thomas-Ogg Safety Igniter (Arthur H. Thomas Company, No. 6472-B) on the platinum sample carrier. The combustion flask was allowed to stand for 1 hour to ensure complete trapping of the CO2 produced from combustion. Absolute methanol was added to give a total volume of 10.0 ml, and aliquots of the resulting solution were counted in a Packard Tri-Carb liquid scintillation spectrometer. The counting efficiency was determined by the addition of an internal 14C standard, a 3-ml aliquot counting at 47% efficiency when mixed with 15 ml of organic scintillation phosphor (2.5 diphenyloxazole, 3 g; p bie 2′ (5′ phenyloxazolyl)benzene, 300 mg; toluene to make 1 liter). In recovery experiments with 2683 d.p.m. of chenodeoxycholic acid-24-14C, six combustions resulted in an average of 98.8 ± 1.1% recovery.

The uteri of two groups of 20 animals per group were assayed for the activity of carbamoyl phosphate:L-aspartate carbamoyl transferase by this method and by the counting method employing the Geiger tube. Both groups were killed at the 18th hour after the first injection of estradiol-17β, one group receiving the hormone, and the other group undergoing concurrent ad-
again at the 12th hour and killed at the 18th hour. A minimum of three groups with 18 to 38 animals per group was used in each series of experiments. In one series the animals were treated by injection with the hormone only. In a second series puromycin dihydrochloride (2 mg) in a buffered solution made isotopic with sodium chloride was given intraperitoneally by injection every 4 hours for a total of 5 injections (10 mg), the first injection of the antibiotic preceding the first administration of the hormone by 15 minutes. In a third series actinomycin D in 0.9% sodium chloride solution was administered intraperitoneally ½ hour before each injection of the hormone, 67 μg and 33 μg being given in the first and second injections, respectively.

![Graph 1](https://via.placeholder.com/150)

**Fig. 1.** The relationship of the activity of carbamoyl phosphate: L-aspartate carbamoyl transferase to protein concentration. The activity of the enzyme is expressed in counts per minute of acid-stable 14C product per mg of protein after incubation at 37.5˚C for 15 minutes under the conditions used for the estimation of enzymatic activity by Procedure 1.

![Graph 2](https://via.placeholder.com/150)

**Fig. 2.** The activity of carbamoyl phosphate: L-aspartate carbamoyl transferase in the uteri of immature rats after stimulation by estradiol-17β (10 μg). The area within each point includes the standard error of the mean. Three to eight determinations were made at each point by using 13 to 26 pooled uteri per determination.

![Graph 3](https://via.placeholder.com/150)

**Fig. 3.** The rate of synthesis of uterine nucleic acids and the rate of uterine growth induced by estradiol-17β (10 μg). The area within each point includes the standard error of the mean. Three to eight determinations were made at each point by using 13 to 26 pooled uteri per determination.

**Table I**

<table>
<thead>
<tr>
<th>Series</th>
<th>Enzymatic activity</th>
<th>RNA per uterus</th>
<th>DNA per uterus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Series 1</td>
<td>Estradiol-17β (10 μg)</td>
<td>228 ± 7</td>
<td>308 ± 11</td>
</tr>
<tr>
<td></td>
<td>Control (no treatment)</td>
<td>117 ± 4</td>
<td>141 ± 6</td>
</tr>
<tr>
<td>Series 2</td>
<td>Puromycin (10 mg) + estradiol-17β (10 μg)</td>
<td>152 ± 14</td>
<td>225 ± 11</td>
</tr>
<tr>
<td></td>
<td>Inhibition of response to estradiol-17β</td>
<td>68.5%</td>
<td>49.7%</td>
</tr>
<tr>
<td>Series 3</td>
<td>Actinomycin D (100 μg) + estradiol-17β (10 μg)</td>
<td>156 ± 3</td>
<td>145 ± 4</td>
</tr>
<tr>
<td></td>
<td>Inhibition of response to estradiol-17β</td>
<td>0.4%</td>
<td>98%</td>
</tr>
</tbody>
</table>

*The animals were killed 18 hours after the first injection of the hormone. The enzymatic activities in Series 1 differ from the control and the 18th hour values in Fig. 2 as a result of the use of a different Geiger tube. The ratio of the control to the 18th hour values remains constant.

The activity of carbamoyl phosphate: L-aspartate carbamoyl transferase and the content of nucleic acids were determined as in the preceding experiments. The results are tabulated in Table I.

Concurrent treatment with estradiol-17β and puromycin resulted in a 69% inhibition of the rise in the enzymatic activity observed with estradiol-17β treatment only. The rise in the content of RNA per uterus accompanying treatment with estradiol-17β was inhibited 50% by concurrent treatment with puromycin.

In Series 3, treatment with actinomycin D resulted in a similar
(65%) inhibition of the response of the activity of the enzyme to estradiol-17β. A complete inhibition by actinomycin D of the synthesis of RNA due to the administration of the hormone was observed. Neither puromycin nor actinomycin D altered the enzymatic activity when administered to control animals. Actinomycin D caused a slight decrease in the content of RNA per uterus in control animals.

**DISCUSSION**

The rise in the content of RNA per uterus induced by treatment with estradiol-17β appears to follow an increase in the activity of carbamoyl phosphate:l-aspartate carbamoyl transferase. The peak of the activity of the enzyme (Fig. 2) is reached approximately 20 hours before the maximum content of RNA per uterus (Fig. 3). The decrease of the activity of the enzyme to control level is accompanied by a plateau in the rise of the uterine content of RNA. These observations suggest that the activity of the enzyme is controlled by some regulatory mechanism and that the rise in the content of RNA in the uterus is dependent upon an increase in the activity of the enzyme. The inhibition by puromycin of the synthesis of RNA is of similar magnitude to the inhibition of the increase in the enzymatic activity. This observation suggests further the dependence of the rise in the content of RNA in the uterus on an increased activity of carbamoyl phosphate:l-aspartate carbamoyl transferase. Since puromycin is an inhibitor of the synthesis of protein (19, 20), the inhibition of the rise in the activity of the enzyme by puromycin indicates that the enzyme is synthesized de novo as a result of the action of the hormone.

Noteboom and Gorski (21) have shown that estradiol-17β causes a rise in the activity of RNA polymerase in the uteri of immature rats 2 hours after treatment with the hormone. Puromycin blocked the rise in the activity of RNA polymerase and the effect of estrogen on the synthesis of RNA. These experiments with puromycin indicate that estrogen brings about the synthesis of some protein necessary for increased synthesis of RNA. The question that arises is whether the increase in the activity of carbamoyl phosphate:l-aspartate carbamoyl transferase is dependent on the synthesis of new RNA or vice-versa.

The concurrent administration of estradiol-17β and actinomycin D resulted in a 65% inhibition of the rise in the activity of the enzyme and total inhibition of the synthesis of RNA. The action of actinomycin D is the blocking of the synthesis of RNA, by inhibiting the activity of DNA-dependent RNA polymerase (22, 23). These results may be interpreted as an indication that the synthesis of the enzyme induced by estradiol-17β is a secondary response to the hormone. This secondary response may be required for the rapid accumulation of RNA but it appears to be dependent upon the new synthesis of messenger RNA. The possibility remains that the block of the activity of RNA polymerase by actinomycin D causes the accumulation of pyrimidine nucleotides resulting in feedback inhibition of carbamoyl phosphate:l-aspartate carbamoyl transferase.

**SUMMARY**

The changes induced by the administration of estradiol-17β in the activity of carbamoyl phosphate:l-aspartate carbamoyl transferase in the uteri of immature rats and in the content of ribonucleic acid and deoxyribonucleic acid per uterus were followed over a 90-hour period. A rise in the enzymatic activity was observed to be commensurate with a rise in uterine content of RNA. Data are presented suggesting the dependence of the accumulation of RNA on an increase in the activity of the enzyme.

Inhibition by puromycin of the rise in the enzymatic activity indicates that the enzyme is synthesized de novo as a result of the action of the hormone.

Inhibition by actinomycin D of the action of the hormone on the activity of the enzyme suggests that the increased enzymatic activity is dependent on the new synthesis of messenger RNA.

A method is presented for the combustion and liquid scintillation counting of 14C biological materials with the Thomas-Ogg Safety Igniter and combustion apparatus.

**Acknowledgments**—Actinomycin D was a gift for which the authors are gratefully indebted to Mr. Horace D. Brown of the Merck Sharp and Dohme Research Laboratories, Division of Merck and Company, Inc., Rahway, New Jersey.

Grateful acknowledgment is extended to the Misses Corinne Dewes and Margaret Caltagirone for their assistance in caring for the animals.

**REFERENCES**

The Effect of Estradiol-17β on the Activity of Carbamoyl Phosphate:l-Aspartate Carbamoyl Transferase in the Uteri of Immature Rats
George C. Tremblay and Sidney A. Thayer