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*PROTEIN SYNTHESIS BY RETICULOCYTE RIBOSOMES, I. INHIBITION OF POLYURIDYLIC ACID-INDUCED RIBOSOMAL PROTEIN SYNTHESIS BY CHLORAMPHENICOL**

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Communicated by John H. Dingle, May 31, 1963

Chloramphenicol is known to inhibit microbial protein synthesis both in intact cells[1-4] and in cell-free systems.[5, 6] Similar inhibition of protein synthesis has not, however, been demonstrable in analogous mammalian systems.[7-9] Matthaei and Nirenberg observed inhibition of protein synthesis by *E. coli* ribosomes with 0.3 μmoles of chloramphenicol per ml reaction mixture.[5] However, von Ehrenstein and Lipmann were unable to obtain an appreciable inhibition in mammalian cell-free systems with 0.01 molar amounts of chloramphenicol.[7] Similarly, inhibition of amino acid incorporation into intact reticulocytes *in vitro* has been noted only with amounts of chloramphenicol which greatly exceed pharmacologic concentrations.[9]

Although mammalian ribosomes do not appear to be affected by chloramphenicol, there is considerable evidence that chloramphenicol can exert deleterious effects in man. Cells of the hematopoietic system appear to be particularly susceptible as manifested by the occasional development of anemia, leukopenia, thrombocytopenia, or aplastic anemia during chloramphenicol therapy.[10, 11] Although the mechanism of toxicity is unknown, certain clinical observations suggest that chloramphenicol is most likely to affect primitive or immature cells during a stimulatory or maturation phase. Thus, Saidi *et al.*[12] observed that patients with pernicious anemia in relapse who were receiving chloramphenicol at the time when B_{12} therapy was initiated, failed to manifest the reticulocyte response seen as a result of this type of therapy. Furthermore, the characteristic reticulocyte response did not occur in these patients until after chloramphenicol was discontinued. In such patients normoblastic maturation of the megaloblastic marrow occurred despite the inhibition of reticulocytosis, indicating that purine and pyrimidine biosynthesis was unaffected. This is analogous to the sequence of events in bacteria where chloramphenicol inhibits protein synthesis without inhibiting DNA or RNA synthesis.[1]

These observations suggest that megaloblasts may be uniquely susceptible to chloramphenicol inhibition, whereas cells at a later stage of maturation remain un-

affected. Presumably the initial steps of maturation would require the synthesis of ribosomal template RNA capable of directing the synthesis of hemoglobin and other proteins necessary for cell development. Marks *et al.*[13] demonstrated that little or no new formation of ribosomal RNA occurs in reticulocytes capable of protein synthesis. The possibility therefore exists that chloramphenicol may exert an inhibitory effect before or during the deposition of "messenger" or template RNA in primitive hematopoietic cells and that once this template has been formed on the ribosome, the cell is no longer susceptible to chloramphenicol inhibition.

Although the intact reticulocyte has no mechanism for the synthesis of new template RNA, it is possible to stimulate protein synthesis by reticulocyte ribosomes in cell-free systems with polyuridylic acid (poly U), a synthetic polyribonucleotide which functions as template or "messenger" RNA in directing the incorporation of phenylalanine into polypeptide chains.[14, 15] Since this essentially represents the deposition of new template RNA on the ribosome, the possibility of inhibiting poly U-induced ribosomal protein synthesis by chloramphenicol was investigated. Under the conditions of the experiments to be reported, inhibition of protein synthesis by ribosomes was obtained with as little as 0.001 μmole of chloramphenicol per ml reaction mixture. This inhibition appeared to be due to an effect of chloramphenicol on uridylic acid residues of poly U, resulting in either inactivation of the template or in preventing its deposition on the ribosome. It is not possible to state from these experiments whether chloramphenicol can affect all "messenger" RNA in a similar manner or whether it has a specific affinity for uridylic acid residues of poly U.

Material and Methods.—The cell-free systems used in these studies were modifications of those employed by Matthaei and Nirenberg[5] and Allen and Schweet[8] and consisted essentially of rabbit reticulocyte ribosomes, supernatant factors obtained from either reticulocytes or *E. coli*, an energy generating system, amino acids, and buffer in a final volume of 1 ml.

Preparation of ribosomes: Reticulocytosis was produced in New Zealand rabbits with phenylhydrazine by the method of Borsook *et al.*,[16] and ribosomes were prepared by a modification of the method of Allen and Schweet.[8] Blood with a reticulocyte count of 85–95% was obtained by cardiac puncture and washed with 0.13 *M* NaCl, 0.005 *M* KCl and 0.0075 *M* $MgCl_2$. The packed cells were lysed with 4 volumes of 0.002 *M* $MgCl_2$, stirred at 4°C for 10 min, after which 1 vol. of 1.5 *M* sucrose containing 0.15 *M* KCl was added. The mixture was stirred for 20 min and centrifuged at 15,000 × *g* for 30 min at 4°C. The sediment was discarded, and the supernate was centrifuged for 2 hr at 105,000 × *g* in a preparative ultracentrifuge at 4°C. The supernatant fluid was subsequently used to prepare pH 5 enzyme.[17] The ribosomal pellet was rinsed and resuspended in 0.002 *M* $MgCl_2$, 0.0175 *M* $KHCO_3$, and 0.25 *M* sucrose to the original volume, and then centrifuged at 4°C for 1 hr at 105,000 × *g*. This supernatant was discarded and the pellet rinsed with 0.25 *M* sucrose. The ribosomal pellet was then resuspended in $^1/_2$ the original volume of packed cells by gentle homogenization with 0.25 *M* sucrose in a loose fitting glass homogenizer. The ribosomal suspension was centrifuged at 15,000 × *g* for 5 min, the sediment discarded, and aliquots of the supernate were stored at −70°C. This preparation retained activity for at least 4 weeks.

Preparation of supernatant factors: The pH 5 enzyme was prepared from reticulocytes by a modification of the method of Keller and Zamecnik,[17] using the supernatant obtained after centrifuging the lysed reticulocytes for 2 hr at 105,000 × *g*. This supernate contained large amounts of hemoglobin, and the procedure to be described removed all but traces of hemoglobin and other interfering substances. Three volumes of 0.9 *M* sucrose, 0.004 *M* $MgCl_2$, and 0.025 *M* KCl (Medium B of Keller and Zamecnik) were added to the reticulocyte supernate and the pH adjusted to 5.2 by slowly adding 1 *N* acetic acid. All solutions were kept at 4°C during this and subsequent preparative procedures. The precipitate was recovered by centrifugation at 10,000 × *g* for 15 min, washed, and then resuspended by homogenization with 40–50 ml of Medium B.

The protein pellet was again collected by centrifugation at 30,000 × *g* for 10 min. The pellet was rinsed with 0.35 *M* sucrose, 0.035 *M* $KHCO_3$, 0.004 *M* $MgCl_2$, and 0.025 *M* KCl (Medium A of Keller and Zamecnik) and then dissolved in this same solution by homogenization. The final volume was adjusted to half the original packed cell volume. Insoluble protein was removed by centrifugation at 30,000 × *g* for 10 min. The supernatant solution containing approximately 4–5 mg of protein per ml was stored in aliquots at −70°C and retained activity for more than 4 weeks.

A supernatant fraction (S100) was obtained from *E. coli* K12 λ harvested in early log phase, by the methods of Matthaei and Nirenberg.[5] Aliquots were stored at −70°C and retained activity for more than 4 weeks.

Protein determinations were made by the Folin-phenol method of Lowry,[18] and RNA content was estimated by the orcinol method,[19] using d-ribose as a standard. The protein concentrations in the pH 5 enzyme preparations ranged between 2.9 and 4.0 mg per ml, and in the S100 fractions ranged between 3.85 and 4.4 mg per ml. The pH 5 enzyme contained 0.25 mg of RNA per ml as compared to 0.30 mg per ml in the S100 fraction. The S100 fraction contained the equivalent of 2.5 μgm per ml of RNAase activity,[20] whereas no nuclease activity was demonstrable in the pH 5 enzyme fraction.

Reaction mixtures and assay techniques: The components of the reaction mixtures were essentially those employed by Matthaei and Nirenberg.[5] Each reaction mixture contained 100 μmoles of Tris (hydroxymethyl) aminomethane, pH 7.8; 10 μmoles magnesium acetate; 50 μmoles KCl; 6 μmoles mercaptoethanol; 0.05 μmoles of 20 L-amino acids;[9] 0.01–0.025 μmoles U-C^{14}-L-phenylalanine (600,000–1,200,000 c/m); 0.025 μmoles GTP; 0.025 μmoles CTP; 0.025 μmoles UTP; 5 μmoles phosphoenolpyruvate (PEP); 1 μmole K_2 ATP; 20.0 mg of pyruvate kinase; 0.2–0.25 mg of ribosomal protein, and 0.2–0.4 mg of either pH 5 or S100 protein. When C^{14}-L-leucine incorporation was investigated, C^{12}-L-leucine was omitted from the amino acid mixture, and 0.002 μmoles of C^{14}-L-leucine (125,000 c/m) were added to the reaction. All components were added at 4°C, and the final volume adjusted to 1.0 ml with water. The reaction mixtures were incubated at 37°C for 1 hr, and the reaction was terminated by the addition of 2 ml of 5% trichloroacetic acid (TCA). The protein precipitate was washed with 2 ml of 5% TCA and then extracted with 5% TCA at 90°C for 20 min. The precipitate was again washed with 5% TCA, washed once with ethanol-ether (3:1), and then with anhydrous ether. The residue was dried, dissolved in 1.3 ml of concentrated formic acid, and 1.0 ml aliquots were counted in a liquid scintillation counter (Nuclear-Chicago).

Materials: 2-Phosphoenolpyruvic acid (crystalline tricyclohexylammonium salt) and pyruvate kinase (muscle) A grade, specific activity 168–254 E.U./mg protein were obtained from either California Corporation for Biochemical Research or Sigma Chemical Company. ATP (potassium salt) was obtained from Nutritional Biochemical Corporation. GTP, UTP, and CTP were obtained from California Corporation for Biochemical Research. Uniformly labeled C^{14}-L-phenylalanine, specific activity 50 mc per mM, was obtained from Nuclear-Chicago. U-C^{14}-L-leucine, specific activity 110 mc per mM, was obtained from Schwarz BioResearch, Inc. Crystalline chloramphenicol was supplied through the courtesy of Parke-Davis & Co. Polyuridylic acid (potassium salt) was furnished initially by Dr. Oliver Jones of the National Institutes of Health and was subsequently obtained from Miles Chemical Co., Elkhart, Indiana.

Results.—The requirements for protein synthesis by reticulocyte ribosomes in the system employed were identical with those previously reported by others using similar reaction mixtures.[5–7] Omission of any of the essential components such as the energy generating system, supernatant factors, amino acid mixture, or ribosomes resulted in absent or markedly reduced incorporation of C^{14}-L-leucine into TCA precipitable protein. Protein formation was directly proportional to the amount of ribosomes added and was also influenced by the amount of supernatant factor added. Chloramphenicol, in a concentration of 2 μmoles per ml of reaction mixture, did not appreciably inhibit the incorporation of C^{14}-L-leucine, the maximum inhibition being less than 5 per cent.

Poly U stimulation of C^{14}-L-phenylalanine incorporation: Poly U stimulated the

incorporation of C^{14}-L-phenylalanine in the ribosomal system both with *E. coli* supernate (S100) and with reticulocyte pH 5 enzyme as a source of sRNA, amino acid activating enzyme, and transfer enzyme. The pH 5 enzyme was 10 to 25 times more effective per mg of protein than the S100 supernate in promoting the formation of polyphenylalanine in the presence of poly U (Fig. 1). Similar results were obtained with both freshly prepared or frozen pH 5 and S100 fractions. When both supernatant factors were used in the reaction mixture, the amount of C^{14}-L-phenylalanine incorporated was not additive and was less than the mean value (Fig. 1). Maximum amounts of phenylalanine incorporation were obtained with 25–50 μg of poly U with either pH 5 enzyme or S100 supernate.

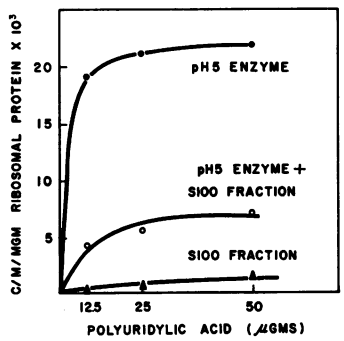

FIG. 1.—Comparative effects of pH 5 enzyme and S100 supernatant factor on ribosomal protein formation stimulated by poly U. Components of the reaction mixture are described in *Methods*. The S100 fraction contained 0.19 mg of protein, and the pH 5 enzyme contained 0.4 mg of protein. 0.02 μM of C^{14}-L-phenylalanine ~ 1,200,000 c/m was present in each reaction mixture.

Although the reticulocyte pH 5 enzyme was more effective than the *E. coli* S100 supernate in the C^{14}-L-phenylalanine-poly U system, their relative effectiveness in promoting the incorporation of C^{14}-L-leucine into TCA precipitable protein was reversed, the S100 fraction being 2 to 3 times as effective as the pH 5 enzyme.

Inhibition of poly U stimulation by chloramphenicol: Chloramphenicol inhibited the formation of C^{14}-labeled polyphenylalanine induced by poly U. At the completion of reaction, the percentage of inhibition by a given concentration of chloramphenicol was inversely proportional to the amount of poly U used to stimulate phenylalanine incorporation, the most marked inhibition being evident with small amounts of poly U.

Partial inhibition of the stimulation obtained with 12.5–25 μg of poly U was observed with 0.5 μmoles of chloramphenicol per ml when S100 was used as the supernatant factor (Fig. 2). Maximal inhibition was obtained with 2–3 μmoles of chloramphenicol, the most marked inhibition being observed with 12.5 μg of poly U which was the smallest amount tested in the experiments with the S100 fraction (Table 1).

TABLE 1

INHIBITION BY CHLORAMPHENICOL OF POLY U EFFECT ON RETICULOCYTE RIBOSOMES

Amount of poly U	Supernatant factor	Incorporation of C^{14}-Phenylalanine (% of control) Chloramphenicol 0.005μM	Chloramphenicol 0.5μM	Chloramphenicol 1.0μM
50 μg	S100	100	81	76
25 "	S100	100	82	72
12.5 "	S100	100	75	31
5.0 "	pH 5 Enz.	94	75	76
2.5 "	pH 5 Enz.	84	35	40
1.0 "	pH 5 Enz.	52	6	0

The sensitivity of the system to chloramphenicol inhibition was greater when pH 5 enzyme was used instead of S100 as the supernatant factor. The most effective inhibition with chloramphenicol was obtained in reaction mixtures containing pH 5 enzyme and 1 μg of poly U. In such reaction mixtures an inhibitory effect

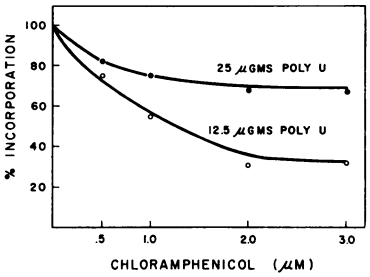

FIG. 2.—The effect of chloramphenicol on polyphenylalanine incorporation stimulated by poly U using S100 supernatant factor. Components of the reaction mixture are stated in *Methods*. Each assay contained 0.38 mg of S100 protein and 0.01 μM of C^{14}-phenylalanine ~ 600,000 c/m. The inhibitory effect of chloramphenicol obtained with 12.5 μg of poly U was compared with that obtained when 25 μg of poly U was used to stimulate the ribosomes. Greater inhibition was obtained with equivalent amounts of chloramphenicol when 12.5 μg of poly U were used.

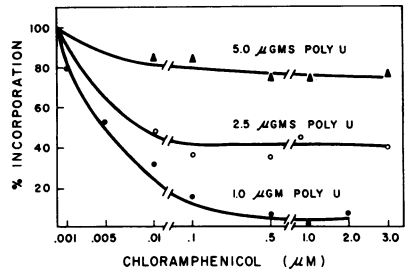

FIG. 3.—The effect of chloramphenicol on phenylalanine incorporation stimulated by poly U using reticulocyte pH 5 enzyme as a supernatant factor. Components of the reaction mixture are stated in *Methods*. Each assay contained pH 5 enzyme (0.4 mg of protein) and 0.02 μM of C^{14}-phenylalanine ~ 1,200,000 c/m. The inhibitory effect of chloramphenicol was determined in relation to the stimulation obtained with 1.0, 2.5, and 5.0 μg of poly U. The most effective inhibition was obtained when 1.0 μg of poly U was used. An inhibitory effect was detectable with as little as 0.001 μM of chloramphenicol in relation to 1.0 μg poly U (0.003 μM of uridylic acid residues).

was detectable with as little as 0.001 μM of chloramphenicol per ml, and 100 per cent inhibition was obtainable with 1.0 μM of chloramphenicol per ml (Table 1, Fig. 3). No inhibition was obtained with less than .001 μM of chloramphenicol per ml. Chloramphenicol inhibition was less effective when the ribosomes were stimulated with 2.5 μg or 5.0 μg of poly U.

Inhibition of the ribosomal protein formation induced by poly U was dependent upon the presence of chloramphenicol in the reaction mixture before the poly U could react with ribosomes and phenylalanine. No inhibition could be obtained if poly U was incubated with ribosomes in the presence of phenylalanine for 5 min prior to adding chloramphenicol (Table 2). This lack of chloramphenicol effect occurred despite the fact that no protein formation took place during this 5 min incubation period.[24] The maximum inhibitory effect occurred when both poly U and chloramphenicol were present in the reaction mixture prior to the addition of ribosomes.

TABLE 2

THE EFFECT OF CHLORAMPHENICOL ON RIBOSOMES PREINCUBATED WITH POLY U

Complete system:	Net C/M/mg ribosomal protein	% Incorporation
+ 1 μg poly U	125	100
+ μg poly U + 1 μM chloramphenicol (no preincubation)	0	0
+ 1 μg poly U + 1 μM chloramphenicol after 5 min incubation	155	120
+ 2.5 μg poly U	450	100
+ 2.5 μg poly U + 1 μM chloramphenicol (no preincubation)	315	70
+ 2.5 μg poly U + 1 μM chloramphenicol after 5 min incubation	490	108
+ 5.0 μg poly U	1.550	100
+ 5.0 μg poly U + 1 μM chloramphenicol (no preincubation)	1,150	74
+ 5.0 μg poly U + 1 μM chloramphenicol after 5 min incubation	1,625	105

Discussion.—These results indicate that, under certain conditions, chloramphenicol can exert an inhibitory effect on protein synthesis by reticulocyte ribosomes. Inhibition occurred only in the presence of added "messenger" or template RNA and could be readily overcome by adding an excess of poly U. Matthaei and Nirenberg also observed chloramphenicol inhibition of poly U stimulation, but their studies are carried out on *bacterial* ribosomes.[6] Since chloramphenicol readily inhibits microbial ribosomal protein synthesis, it is not possible to attribute the inhibition observed in such a system to an effect of chloramphenicol on poly U. In contrast, the reticulocyte ribosome is known to be unaffected by chloramphenicol under ordinary circumstances, and the inhibition observed in the present studies can be attributed to an effect of chloramphenicol on poly U. Chloramphenicol did not inhibit phenylalanine incorporation stimulated by poly U after it was incubated with ribosomes for a brief interval, during which time the poly U presumably becomes attached to the ribosome and interacts with activated sRNA-phenylalanine. Chloramphenicol may therefore either prevent the deposition of poly U on ribosomes or combine with or inactivate the uridylic acid residues before they can react with the activated sRNA-phenylalanine. It is not known from these experiments whether chloramphenicol can exert a similar inhibitory effect on any new "messenger" or template RNA, or whether its effect is specific for poly U. Preliminary results indicate that chloramphenicol can inhibit viral and other RNA-directed ribosomal protein synthesis.[21] It remains to be determined whether chloramphenicol inhibition is specifically related to an effect on uridylic acid residues of poly U or whether it can inhibit other ribonucleotides. The ability of chloramphenicol to inhibit various types of "messenger" or template RNA is currently being investigated.

The incorporation of phenylalanine induced by 1 μg of poly U (0.003 μmoles of uridylic acid residues per ml) was partially inhibited by as little as 0.001 μmole of chloramphenicol per ml. This suggests that an inhibitory effect may become manifest when chloramphenicol combines with or inactivates an average of 1 of 3 uridylic acid residues of poly U at random. Since the code for phenylalanine is presumably a triplet of uridylic acid, inactivation of any one of three consecutive uridylic acid residues could cause decreased formation of polyphenylalanine. This would account for the decreased inhibition observed with 2.5 μg (0.0075 μM uridylic acid residues) and 5.0 μg (0.015 μM uridylic acid residues) of poly U per ml when exposed to comparable amounts of chloramphenicol, as well as the lack of an inhibitory effect when excessive amounts of poly U are added to the system. The attainment of a limiting value of inhibition when 2–3 μM of chloramphenicol (Figs. 2 and 3) were used in the reaction mixtures may be due to saturation of available binding sites. Another factor which would contribute to the plateau is that the same amount of inhibition of polyphenylalanine formation would be expected whether 1, 2, or 3 consecutive uridylic acid residues of poly U were inactivated. Presumably, inhibition should be observed even with large amounts of poly U if sufficient chloramphenicol were present to combine in a random fashion with a critical number of uridylic acid residues. Such studies were not possible because of the limited solubility of crystalline chloramphenicol.

The marked differences in polyphenylalanine formation observed with S100 and pH 5 enzyme supernatant factors may be due to the presence of ribonuclease in

the former causing degradation of poly U. No ribonuclease activity was detected in the pH 5 enzyme fraction. Accordingly, the assay with pH 5 enzyme is a more reliable index of the inhibitory effect of chloramphenicol relative to poly U.

The ability of chloramphenicol to prevent the incorporation of amino acids by synthetic "messenger" RNA would appear to correlate with clinical observations on the inhibitory effect of the drug during therapy of megaloblastic anemias, namely, during that stage of development when the primitive erythrocyte is rapidly synthesizing messenger RNA. It is also possible to explain the occurrence of hematologic toxicity following chloramphenicol administration under other circumstances.[25]

These experiments do not elucidate the fundamental difference in the sensitivity of bacterial and mammalian ribosomes to chloramphenicol. Although it has been assumed that the mechanism of protein synthesis is similar in all cells, there may be a fundamental difference in the microbial ribosomes as suggested by von Ehrenstein and Lipmann.[7] Conceivably reactive sites on microbial template RNA may be more accessible for combination with chloramphenicol.

Summary.—An inhibitory effect of chloramphenicol on protein synthesis by rabbit reticulocyte ribosomes is described. This inhibition occurred only in the presence of added "messenger" or template RNA. The possibility that chloramphenicol may inactivate template RNA is discussed.

We wish to thank Dr. Lazarus Astrachan and Dr. Melvin D. Schoenberg for valuable advice, suggestions, and assistance in performing these experiments and to express our appreciation to Dr. Oliver Jones of the National Institutes of Health for supplying us with the initial samples of polyuridylic acid. We also wish to acknowledge the technical assistance of E. Ceasar Moss.

* Supported by research grants H-3952 and C-4944 from the U.S. Public Health Service.

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[25] Such postulation requires the assumption that 0.001 μM of chloramphenicol in a cell-free system is equivalent to toxic levels found in the serum (0.05 to 0.1 μM per ml)[22, 23] and that the amount of "messenger" RNA present in maturing hematopoietic cells is less than the equivalent of 1 μg of poly U. Although these are reasonable assumptions, it is quite probable that chloramphenicol has other toxic effects on cellular metabolism as well.

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*THE NATURE OF THE RNA RESPONSE TO ESTRADIOL ADMINISTRATION BY THE UTERUS OF THE RAT**

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Communicated by C. J. Watson, May 31, 1963

Over the past several years abundant evidence has accrued to indicate that the administration of sex hormones results in an increase both in the content[2–6] and in the rate of synthesis[7] of ribonucleic acid (RNA) in the accessory sex tissues. Furthermore, although evidence has been presented to the contrary,[8] several kinds of studies have indicated that the enhancement of protein synthesis by these hormones is secondary to the increased rate of RNA synthesis. First, the biochemical site of action of testosterone on protein synthesis in rat seminal vesicle and of estradiol on protein synthesis in hen oviduct has been localized to the RNA-rich microsomes.[9–11] Second, inhibition of protein synthesis does not interfere with the estrogen-induced acceleration of RNA synthesis.[12] Finally, the addition of synthetic polyribonucleotides has been shown to accelerate the synthesis of protein in homogenates of control but not of testosterone-treated prostate.[13]

The implication that the acceleration of protein synthesis following hormone administration is secondary to enhanced RNA synthesis does not of itself, however, explain the mechanism by which protein synthesis is accelerated. At least three different types of RNA are involved in protein synthesis—soluble or transfer RNA, ribosomal structural RNA, and messenger or template RNA. That transfer RNA is not rate-limiting for protein synthesis in these hormone-responsive tissues has been suggested by two types of experiments—measurements of transfer C^{14}-RNA-amino acids in slices of rat seminal vesicle,[9] and the demonstration that no soluble factors appear to be rate-limiting in protein synthesis by homogenates of hen oviduct.[10, 11] Furthermore, the studies of Liao and Williams-Ashman indicate that template RNA is rate-limiting for protein synthesis in the prostate of the castrated rat.[13]

The present communication describes studies on the sequence and nature of the RNA response to estradiol administration by the immature rat uterus. Evidence is presented that the administration of estradiol initially enhances the synthesis of transfer RNA and of template RNA, and that enhanced synthesis of ribosomal structural RNA is a late effect.