The Effect of Thyroxine on Ribonucleic Acid Synthesis by Premetamorphic Tadpole Liver Cell Suspensions*

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LOIS M. BLATT,$§ KI-HAN KIM,‡ and PHILIP P. COHEN
From the Department of Physiological Chemistry, University of Wisconsin, Madison, Wisconsin 53706

SUMMARY

Suspensions of tadpole liver cells have been developed as a model to further understanding of the biochemical differentiation of this tissue during thyroxine-induced metamorphosis. The cell population is homogeneous and stable; no cell division or DNA synthesis occurs in these isolated cells. RNA synthesis in cell suspensions is stimulated by thyroxine and triiodothyronine. The optimum concentration of both thyroxine and triiodothyronine for stimulation of RNA synthesis is about $10^{-10}$ M.

Rapidly labeled RNA isolated from thyroxine-treated and nontreated cell suspensions sediments in the region between 4 and 10 S. The base composition of RNA synthesized by liver cells treated in vitro with thyroxine differs significantly from that of untreated liver cells and from bulk tadpole liver RNA. The ratio of cytosine plus guanine to total bases of RNA from thyroxine-treated cells is similar to that of DNA from this species. Neither thyroxine nor triiodothyronine had any effect on the incorporation of amino acids into protein. Frog serum stimulates RNA and protein synthesis. Rana catesbeiana serum causes the aggregation of isolated liver cells.

EXPERIMENTAL PROCEDURES

Materials

Rana catesbeiana tadpoles and frogs were purchased from Lemberger Company, Oshkosh, Wisconsin. Tadpoles were removed from the 15°C stock tanks and kept at room temperature for 2 days before they were used in the preparation of liver cell suspensions. Thyroxine and triiodothyronine were obtained from Sigma; actinomycin D was a gift from Merck Sharp and Dohme. Radioisotopically labeled compounds were purchased from New England Nuclear and Schwarz BioResearch. Specific activity is reported with each individual experiment. Tricaine methanesulfonate (MS 222—Sandoz) was purchased from Sandoz Pharmaceuticals, Hanover, New Jersey. Antibiotics, sera, and complex but chemically defined media were obtained from the Grand Island Biological Company, Grand Island, New York. Medium 199 of Morgan, Morton, and Parker (8) was used routinely. Serum from Rana pipiens was obtained from Pel-Freez Biologicals, Inc., Rogers, Arkansas. R. catesbeiana serum was prepared in our laboratory.

Preparation of Cell Suspensions

Several procedures have been developed for the preparation of sterile, viable cell suspensions in good yield. All glassware, in-
solution (10) in a Packard Tri-Carb liquid scintillation counter.

Method A—Tadpoles were soaked in an Aureomycin solution (5 μg per ml) for at least 1 hour and then washed several times with tap water. The animals were anesthetized in a 1% solution of tricaine methanesulfonate. After the tadpoles were washed with 70% alcohol, the livers were removed and placed immediately in medium (Medium A) containing antibiotics. This medium consisted of Medium 199 of Morgan et al. (8) (diluted 1:1 with sterile double distilled water) containing the following antibiotics: penicillin, 200 units per ml; streptomycin, 200 μg per ml; neomycin, 100 μg per ml; Fungizone, 5 μg per ml; Mycostatin, 10 units per ml. The livers were washed at least four times with Medium A and then transferred to a flask containing calcium-free frog Ringer’s solution. To remove blood from the tissue, the livers were stirred with a large volume of Ringer’s solution for about 2 hours with several changes of medium.

The washed livers were drained and finely minced in a Petri dish with two scalpels. The minced tissue was dispersed in 0.25 m sucrose (containing 200 units per ml of penicillin and 200 μg per ml of streptomycin) with a loose fitting glass pestle in a Dounce-type homogenizer. This material was filtered through two layers of gauze, and the cells were collected by centrifugation at 300 rpm for 6 min in a clinical centrifuge. The cells were then washed at least four times with Medium A. To remove antibiotics, the cells were washed twice with Medium 199 containing no antibiotics. The cells were incubated at a cell density between 1 × 10⁶ and 3 × 10⁶ cells per ml in Medium 199 (diluted as described above) containing 20% serum. Cell count was measured with a hemocytometer.

Method B—The greatest risk of introducing microbial contamination exists during the mincing of livers in an open Petri dish and the dispersing of tissue in the Dounce homogenizer. These difficulties were overcome by combining the two steps with the use of a VirTis “45” homogenizer. In addition, the use of Ringer’s solution and sucrose was eliminated. The procedure described for Method A was followed in the other steps.

With either method, about 100 tadpoles could be processed to produce cell yields between 40 and 60%. Cell suspensions were incubated with rotary shaking at about 120 rpm in a New Brunswick incubator-shaker at 25°C.

Incorporation of ¹⁴C-Amino Acids

To test the ability of isolated cells to synthesize protein, they were incubated with either L-leucine-¹⁴C (0.2 Ci per mmole) or ¹⁴C-algal protein hydrolysate. Cells were harvested and washed with medium supplemented with 2% Casamino acids (Difco) at least four times, then washed three times with cold 10% trichloroacetic acid which was similarly supplemented with Casamino acids. Protein precipitates were further washed by the procedure of Stievelt’s (9), dissolved in 90% formic acid, and counted in Bray’s solution (10) in a Packard Tri-Carb liquid scintillation counter.

Preparation of RNA

RNA was prepared by the method of Schmidt and Thannhauser (11) with the modifications previously described (7). With this method, RNA synthesis by independent duplicates agreed within 10%.

RNA was isolated and purified as described by Nakagawa and Cohen (7). In order to minimize breakdown of RNA during the isolation procedure from liver cell suspensions, bentonite (20% of the original cell sample weight) was added to the homogenization buffer. Sucrose solutions were treated with bentonite prior to use in the density gradient experiments.

Zonal Sucrose Density Gradient Centrifugation

The procedure described by Nakagawa and Cohen (7) was followed, except that centrifugation was carried out at 50,000 rpm in a Spinco SW-65 swinging bucket rotor for 2.7 hours. Fractions of 10 drops each were collected and diluted with 1 ml of 0.1 m sodium acetate buffer, pH 5.1. The absorbance at 260 μm in a 1-ml cuvette (1-cm light path) was measured. For the determination of radioactivity, 0.5 ml of each sample was mixed with Bray’s solution (10) and counted in a Packard Tri-Carb liquid scintillation spectrometer.

Base Composition of RNA

The base composition of RNA synthesized by liver cells in suspension was determined after isolation and purification as described above. RNA was labeled by the addition of the four tritiated ribonucleosides (adenosine, cytidine, guanosine, and uridine) as a mixture to the cell incubation medium so that the medium contained equal specific activity of each nucleoside.

RNA was isolated from the cells and then hydrolyzed with LiOH by the method of Gebicki and Freed (12). The nucleotides were separated by electrophoresis on Whatman No. 4 paper in a flat bottom electrophoresis apparatus for 2 hours at 3 kv. The buffer used in the electrophoresis was 0.05 m ammonium formate, pH 3.25. The ultraviolet absorbing spots were cut out and counted in toluene scintillation fluid.

RESULTS

Tadpole Liver Cells in Suspension—Cells in fresh suspension appeared to be intact when examined by phase contrast microscopy. At a magnification of × 400, cell nuclei and nucleoli were clearly visible. However, cell preparations maintained in suspension for several days showed evidence of varying degrees of degenerative changes in the cytoplasm when examined after fixation and staining with hematoxylin and eosin. Electron micrographs of fixed cell preparations revealed that cytoplasmic organelles (particularly the mitochondria) had undergone varying degrees of alteration during suspension of the cells.

The effect of several defined media on the survival of isolated cells was tested (Table I). Cell survival and appearance were judged to be best in medium based on Medium 199. Cultures incubated with Medium 199 and either fetal calf, calf, or chicken serum did not vary significantly in their survival or ability to incorporate L-leucine-¹⁴C into protein. Until work with frog serum was initiated, the medium composed of Medium 199 with 20% fetal calf serum was used routinely.

The effect of antibiotics on the synthesis of RNA and protein by isolated liver cells was tested. The incorporation of L-leucine-¹⁴C (0.2 Ci per mmole) was not affected by the presence of neomycin or streptomycin. No difference in incorporation was found when a mixture of ¹⁴C-amino acids (alanine, aspartic acid, leucine, serine, and valine) was added to cell suspensions with or without antibiotics. The addition of antibiotics to the incubation me-
dium did not affect the incorporation of tritiated uridine (8 Ci per mmole). The total amount of RNA and DNA per sample was constant throughout the experiment and unaffected by the presence of the antibiotics. Although no significant difference in the over-all incorporation of precursors into RNA or protein was observed, these findings do not exclude the possibility that antibiotics cause other, more subtle effects on cell function (14, 15).

**Tests for Cell Division and DNA Synthesis** The study of biochemical differentiation of the liver of the metamorphosing tadpole is especially advantageous because the liver cells passed through metamorphosis without significant division, degeneration, or replacement (5). For studies of cells in vitro to be relevant to studies of the tissue in vivo, the cell population should be stable and nondividing. Although no marked changes in cell number were observed in cell suspensions, cell multiplication balancing cell death to maintain a nearly constant number of cells could account for this. Therefore, we tested for the presence of mitotic figures by the use of colchicine (16). Colchicine-treated cells revealed no mitotic figures when observed with the phase contrast microscope. The cell count remained relatively constant throughout the course of the experiment (Table II).

In agreement with the cytological evidence for lack of cell division in tadpole liver during metamorphosis, Paik, Metzenberg, and Cohen (17) have reported that the amount of liver DNA phosphate remained essentially constant during thyroxine-induced metamorphosis. To determine whether DNA was being synthesized in isolated liver cells, the incorporation of tritiated thymidine was studied. A cell suspension was divided into two portions, and thyroxine was added to one of them (2.5 X 10^-4 M). Thymidine methyl-

\[ ^3H \] was added to the cell suspensions (1 μCi per ml). Aliquots of both suspensions were taken on Day 0, 1, 2, 3, and 5 for the determination of DNA content and tritium incorporation. No DNA synthesis was detectable in either thyroxine-treated or untreated cells. This is consistent with the observation that no cell division occurred in these cell suspensions.

**Protein Synthesis**—A low level of incorporation of radioactive amino acids was observed during an incubation period of 4 days in the absence of frog serum (Fig. 2, Curve c). To determine whether the failure to maintain a significant rate of protein synthesis was the result of changes in the amino acid composition of the medium, the amino acid content of the medium was analyzed after incubation with cells to determine whether any changes had occurred. Arginine was the only amino acid which changed in a significant manner, showing a drop from 0.14 μmole per ml to 0.02 μmole per ml after 6 hours of incubation. Protein synthesis was not enhanced by the further addition of either citrulline or arginine (5 μmole per ml) to the medium. Thus, the low level of arginine does not appear to be a limiting factor in the ability of cell suspensions to synthesize protein.

Thyroxine, at concentrations from 10^-5 to 10^-10 M, had no significant effect on the incorporation of radioactive amino acids into protein. Because the effect of the different thyroid hormones varies considerably in some systems (18, 19) triiodothyronine was also tested. Neither hormone affected either protein synthesis or cell survival. This is consistent with the evidence of DeGroot and Cohen (20) that the levels of amino acid-activating enzymes in tadpole liver did not change during thyroxine-induced metamorphosis.

**Effect of Frog Serum**—Frog serum had several remarkable effects on tadpole liver cells in suspension. RNA synthesis was stimulated by serum from either *R. catesbeiana* or *R. pipiens*. The relationship between the concentration of frog serum in the incubation medium and the synthesis of RNA is shown in Fig. 1. Although it appears that the higher concentrations of frog serum are most favorable to RNA synthesis, they were not routinely used because other experiments indicated that high concentrations of frog serum (>15%) had an adverse effect on cell survival.

**Table I**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Cells surviving after incubation for 4 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 days</td>
</tr>
<tr>
<td>No. 1</td>
<td></td>
</tr>
<tr>
<td>199 (10x)</td>
<td>1.0</td>
</tr>
<tr>
<td>CEE</td>
<td>1.7</td>
</tr>
<tr>
<td>FCS</td>
<td>5.4</td>
</tr>
<tr>
<td>No. 2</td>
<td></td>
</tr>
<tr>
<td>NCTC 100</td>
<td>3.3</td>
</tr>
<tr>
<td>CEE</td>
<td>1.7</td>
</tr>
<tr>
<td>FCS</td>
<td>4.1</td>
</tr>
<tr>
<td>No. 3</td>
<td></td>
</tr>
<tr>
<td>Eagle's MEM</td>
<td>8.6</td>
</tr>
<tr>
<td>No. 4</td>
<td></td>
</tr>
<tr>
<td>GIB</td>
<td>3.5</td>
</tr>
<tr>
<td>CEE</td>
<td>2.0</td>
</tr>
<tr>
<td>FCS</td>
<td>0.2</td>
</tr>
<tr>
<td>H2O</td>
<td>3.5</td>
</tr>
</tbody>
</table>

*The abbreviations used are: CEE, chick embryo extract; FCS, fetal calf serum; MEM, minimum essentials medium.

**Table II**

A sample was taken from the original culture every day for 4 days (Samples 1 through 4). These samples were treated with colchicine (1 μg per ml) and the cells were examined daily under the phase contrast microscope for 4 days after the initiation of treatment. No mitotic figures were found.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cell count after colchicine treatment for</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 days</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>97</td>
</tr>
<tr>
<td>2</td>
<td>101</td>
</tr>
<tr>
<td>3</td>
<td>94</td>
</tr>
<tr>
<td>4</td>
<td>96</td>
</tr>
</tbody>
</table>

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suspensions with 0, 2, 5, 10, and 20% thromboplastin treated frog serum and another set with untreated frog serum were prepared. Fetal calf serum was added to bring the serum content of the medium to 20%. All samples containing frog serum aggregated into a single mass of cells within 60 min. The samples with the treated serum were identical in appearance with the other samples. The cell aggregate remained intact for more than 3 weeks of incubation with shaking.

After repeated freezing and thawing, *R. catesbeiana* serum lost the ability to clump tadpole liver cells. *R. pipiens* serum did not cause the aggregation of *R. catesbeiana* tadpole liver cells. Both of these sera stimulated the incorporation of $^{3}$H-amino acids into protein. This effect was independent of the ability of *R. catesbeiana* serum to aggregate cells. The effect of different concentrations of frog and fetal calf serum on protein synthesis is shown in Fig. 2. No significant changes in the incorporation of labeled amino acids occurred in Suspension c, which contained only fetal calf serum.

**Effect of Thyroxine on RNA Synthesis**—Maximum stimulation of RNA synthesis was observed when cells were treated with thyroxine at a concentration of $2.6 \times 10^{-7}$ M (see Fig. 3). At a concentration of $2.6 \times 10^{-7}$ M, radioactive thyroxine ($^{3}$H-$\alpha$-thyroxine) was bound to proteins in the incubation medium to the extent of 60%. Increasing the concentration of thyroxine to $2.6 \times 10^{-6}$ M resulted in a decreased incorporation of $^{3}$H-uridine into RNA. Similar results were obtained with triiodothyronine (see Fig. 3). The optimum concentration of this hormone with respect to RNA synthesis was also $2.6 \times 10^{-6}$ M. The magnitude of stimulation was similar to that obtained with thyroxine. The synthesis of RNA by thyroxine-treated and untreated cell suspension was inhibited by actinomycin D (2 $\mu$g per ml).

The capacity of the isolated cells to incorporate $^{3}$H-uridine into RNA was studied at various time intervals of thyroxine treatment. As shown in Fig. 4, the amount of $^{3}$H-uridine incorporated by samples taken from the untreated suspension was relatively constant throughout the experiment. In contrast, the thyroxine-treated cells incorporated more isotope into RNA and exhibited peaks of synthesis at 1 and 6 days after hormone treatment was initiated. When cells were incubated for 2 days, a similar pattern of RNA synthesis was obtained (see Fig. 4). RNA synthesis reached a peak after 24 hours of thyroxine treatment. A second period of increased RNA synthesis occurred between 3 and 5 days after the initiation of hormone treatment. A similar biphasic response has been observed with intact tadpoles (6). Because the tadpole population is heterogeneous with respect to age and condition, minor variations in this pattern would be expected with cell preparations from different batches of animals. However, in general, the pattern of RNA synthesis with time of hormone treatment was reproducible.

**Effect of Mg$^{++}$ Concentration on Cell Yield and Isolation of RNA**—RNA was prepared as described under "Experimental Procedures." The Mg$^{++}$ concentration in the incubation medium was found to be a critical factor in the protection of RNA in cell suspensions. Under optimum conditions, three peaks with sedimentation coefficients of 28, 18, and 5 S were obtained by zonal sucrose density gradient centrifugation. The sedimentation coefficients and relative proportions of RNA in the peaks correspond to those obtained from fresh tadpole liver preparations.

RNA profiles of cells prepared in Medium 199 at Mg$^{++}$ concentrations of 0.8, 10, 20, 50, and 80 mM are shown in Fig. 5. Concentrations of 2 and 4 mM Mg$^{++}$ gave RNA profiles and cell
Fig. 3. Effect of hormone concentration on RNA synthesis. In the experiment represented in A, cells were incubated with the following concentrations of thyroxine: a, $2.6 \times 10^{-6}$ M; b, $2.6 \times 10^{-7}$ M; c, $2.6 \times 10^{-8}$ M; d, $2.6 \times 10^{-9}$ M; e, control (no thyroxine). After a 24-hour preincubation, $^3$H-uridine (8 Ci per mmole) was added (1 µCi/2 ml of suspension). Aliquots of each cell suspension were taken at 1, 2, and 4 hours after the addition of the radioactive uridine. The radioactivity for $5 \times 10^6$ cells is reported. All values have been corrected for a zero-time sample. In B, the radioactivity for $5 \times 10^6$ cells after the 4-hour incubation is plotted against the concentration of thyroxine in the medium. The experiments represented in C and D are similar except that triiodothyronine was added to the cells at the concentrations described for thyroxine in A.

Fig. 4. The effect of thyroxine on RNA synthesis with time of treatment. Cells were suspended in incubation medium with $2.6 \times 10^{-7}$ M and without thyroxine. Aliquots were taken every 24 hours and incubated with $^3$H-uridine (25 mCi per mmole) for 8 hours. The radioactivity (counts per min) per $5 \times 10^6$ cells is shown. In the experiment represented in A, thyroxine was added to cells on the day of preparation (labeling medium, 1 µCi/5 ml). Thyroxine was added to cells after a 2-day preliminary incubation in the experiment represented in B (labeling medium, 2 µCi/5 ml).

Fig. 5. Effect of Mg$^{++}$ concentration on the zonal centrifugation pattern of RNA isolated from freshly prepared tadpole liver cell suspensions. Cells were prepared in medium adjusted to the following Mg$^{++}$ concentrations (millimolar): a, 0.8; b, 10; c, 20; d, 50; e, 80. RNA was isolated from each cell preparation as described under "Experimental Procedures."
with or without thyroxine for 24 hours. \(^3\H\)Uridine (8 Ci per mmole) was added to the control (Curve 1) and the thyroxine-treated (Curve 2) cell suspensions (1 \(\mu\)Ci/2 \(\mu\)l of suspension). After a 3-hour incubation the cells were harvested and RNA was prepared as described under "Experimental Procedures." Curve 3, \(A_{260}\) of RNA isolated from control cell suspension; Curve 4, \(A_{260}\) of RNA isolated from thyroxine-treated cell suspension. The experiment represented in B was similar except that the tadpoles used in the cell preparation had been immersed in a thyroxine solution (2.6 \(\times\) 10\(^{-8}\) M) for 2 days.

**TABLE III**

<table>
<thead>
<tr>
<th>Sample and experiment</th>
<th>Base composition</th>
<th>Ratio of cytosine to total bases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ura</td>
<td>Adenine</td>
</tr>
<tr>
<td>Control</td>
<td>12.5</td>
<td>18.4</td>
</tr>
<tr>
<td>I</td>
<td>13.7</td>
<td>21.3</td>
</tr>
<tr>
<td>Thyroxine</td>
<td>17.5</td>
<td>37.1</td>
</tr>
<tr>
<td>II</td>
<td>16.4</td>
<td>39.1</td>
</tr>
</tbody>
</table>

Fig. 6. Pattern of RNA synthesis in tadpole liver cell suspensions. In the experiment represented in A, cells were incubated with or without thyroxine for 24 hours. \(^3\H\)Uridine (8 Ci per mmole) was added to the control (Curve 1) and the thyroxine-treated (Curve 2) cell suspensions (1 \(\mu\)Ci/2 \(\mu\)l of suspension). After a 3-hour incubation the cells were harvested and RNA was prepared as described under "Experimental Procedures." Curve 3, \(A_{260}\) of RNA isolated from control cell suspension; Curve 4, \(A_{260}\) of RNA isolated from thyroxine-treated cell suspension. The experiment represented in B was similar except that the tadpoles used in the cell preparation had been immersed in a thyroxine solution (2.6 \(\times\) 10\(^{-8}\) M) for 2 days.

A system has been described in which the effects of thyroxine can be studied in isolated surviving cell suspensions. This system provides a direct approach to the problem of whether thyroxine per se is responsible for the initiation of biochemical differentiation in the tadpole liver. Biochemical differentiation in this system is uncomplicated by tissue interactions, growth, cell division, and cell selection. Although care must be taken when extending observations made with isolated cells, the results observed in this system are similar to many of the observations made with whole animals. The liver undergoes little if any cell multiplication, degeneration, or replacement during metamorphosis. Although special attention was directed to the search for dividing cells, none was found in any cell suspension preparation. Finamore and Frieden (21) reported that the amount of DNA decreases markedly during metamorphosis induced by triiodothyronine injection. However, Paik et al. (17) found no significant change in the amount of RNA and DNA in liver during metamorphosis induced by immersion of tadpoles in thyroxine solutions. In agreement with the latter finding, the DNA content of tadpole liver cell populations remains constant with or without thyroxine treatment.

Studies on the mode of hormone action indicate that pro-
nounced effects on the ability of the target tissue to synthesize RNA are exerted prior to enzyme induction (22, 23). Nakagawa, Kim, and Cohen (6) explored the relationship between RNA synthesis and the induction of carbamyl phosphate synthetase during thyroxine-induced metamorphosis. A rapidly labeled RNA fraction (6 to 10 S) which had a base ratio similar to that of tadpole liver DNA was found (7). These properties were considered to be consistent with an operational definition of messenger RNA (24).

The synthesis of RNA by tadpole liver cells in suspension is markedly influenced by the duration of thyroxine treatment. Rapidly labeled RNA synthesized in vitro sedimented between 4 and 10 S. The base ratio of RNA synthesized by isolated cells treated with the hormone is similar to that of tadpole liver DNA. This RNA has a lower S value than that synthesized in vivo which was characterized, operationally at least, as messenger. However, messenger RNAs from different animal tissues have revealed great molecular heterogeneity (24, 25). Further work is obviously necessary to characterize this RNA fraction more rigorously and to clarify its relationship to RNA synthesized in vivo. Also, the effect of hormone treatment on the pool size of RNA precursors should be examined to determine whether differences at this level are reflected in the base ratio determination. Indeed, the possibility that changes in the metabolism of nucleotides may play a role in the response to thyroid hormone has been discussed recently by Frieden (26).

Both thyroxine and triiodothyronine stimulated the synthesis of RNA by isolated tadpole liver cells, and the response is dependent on the hormone concentration. The optimum concentration of hormone for stimulation of RNA synthesis and the magnitude of the effect was about the same for both hormones. Many investigators have reported strikingly different potencies for these two hormones. Triiodothyronine has been reported to be from 10 to 300 times more active than thyroxine (18, 19). In tadpole liver cell suspensions the problems of absorption by the intact animal, metabolic alteration, and distribution of hormone to the target site encountered when dealing with whole animals or isolated organs are excluded.

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