JCI The Journal of Clinical Investigation

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J Clin Invest. 1984;74(6):2254-2262. https://doi.org/10.1172/JCI111652.

Research Article

To elucidate the regulatory mechanism of ontogenetic development of iodothyronine-5'-deiodinase in the fetal and neonatal period, fetal mouse liver of the 19th day of gestation, in which no iodothyronine-5'-deiodinating activity was detectable, was cultured in Dulbecco-Vogt medium supplemented with 10% thyroid hormone-depleted fetal calf serum, insulin, hydrocortisone, and thyroid hormones. Iodothyronine-5'-deiodinating activity of the homogenate was assessed by the amount of iodide released from outer-ring-labeled reverse T3 and expressed as picomoles of 127I- per milligram of protein per minute. The enzyme activity was induced in a dose-dependent manner; optimal concentrations for insulin, hydrocortisone, and thyroxine were 1 microgram/ml, 0.4 microgram/ml, and 10(-6) M, respectively. Without supplementation of either hydrocortisone or thyroxine, no 5'-deiodination was detected. The enzyme activity was observed after 3 d of culture, peaked at days 14-20, and then gradually decreased. Lineweaver-Burk analysis revealed that the increase in activity was primarily due to an increase in Vmax (day 3, 0.2 pmol/mg protein per min; day 20, 2.5 pmol/mg protein per min). Half maximal thyroxine (T4) and triiodothyronine (T3) concentrations were 1 X 10(-7) M (free T4: 4 X 10(-10) M), and 2 X 10(-9) M (free T3: 5.0 X 10(-11) M), respectively, whereas reverse T3 did not elicit any activity at 10(-8)-10(-6) M. These results suggest that ontogenetic development of iodothyronine-5'-deiodinase in the liver of the fetal and […]

Ontogenesis of Iodothyronine-5'-Deiodinase

Induction of 5'-Deiodinating Activity by Insulin, Glucocorticoid, and Thyroxine in Cultured Fetal Mouse Liver

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Abstract. To elucidate the regulatory mechanism of ontogenetic development of iodothyronine-5'-deiodinase in the fetal and neonatal period, fetal mouse liver of the 19th day of gestation, in which no iodothyronine-5'-deiodinating activity was detectable, was cultured in Dulbecco-Vogt medium supplemented with 10% thyroid hormone-depleted fetal calf serum, insulin, hydrocortisone, and thyroid hormones. Iodothyronine-5'-deiodinating activity of the homogenate was assessed by the amount of iodide released from outer-ring-labeled reverse T_3 and expressed as picomoles of $^{127}I^-$ per milligram of protein per minute. The enzyme activity was induced in a dose-dependent manner; optimal concentrations for insulin, hydrocortisone, and thyroxine were 1 μg/ml, 0.4 μg/ml, and 10^{-6} M, respectively. Without supplementation of either hydrocortisone or thyroxine, no 5'-deiodination was detected. The enzyme activity was observed after 3 d of culture, peaked at days 14–20, and then gradually decreased. Lineweaver-Burk analysis revealed that the increase in activity was primarily due to an increase in V_{max} (day 3, 0.2 pmol/mg protein per min; day 20, 2.5 pmol/mg protein per min). Half maximal thyroxine (T_4) and triiodothyronine (T_3) concentrations were 1×10^{-7} M (free T_4: 4×10^{-10} M), and 2×10^{-9} M (free T_3: 5.0×10^{-11} M), respectively, whereas reverse T_3 did not elicit any activity at 10^{-8}–10^{-6} M.

These results suggest that ontogenetic development of iodothyronine-5'-deiodinase in the liver of the fetal and neonatal mouse is induced by physiological concentrations of glucocorticoid and thyroid hormones, and that insulin plays a permissive role in enhancing T_3 formation from T_4 in the liver.

Introduction

Recently, the ontogenesis of thyroid hormone metabolism in peripheral tissues was extensively studied in man, sheep, chick, and especially rodents (1–5). In contrast to newborn of humans and sheep, in which hypothalamic-pituitary-thyroid function is already mature and the sera contain adult thyroxine (T_4)[1] levels, the rat (and probably mouse) is delivered in the hypothyroid state. The serum T_4 concentration of newborn of rats is very low (1.6 μg/dl) and triiodothyronine (T_3) is hardly detectable (~5 ng/dl) (1–3). Active thyroid hormone (T_3) then increases in parallel with serum T_4. In fetal liver of mice (6) and rats (3), iodothyronine-5'-deiodinating activity is hardly detectable. However, in the neonatal period, there occurs a progressive increase in the enzyme activity, which peaks at 2–3 wk after birth in the case of mice (6). In parallel with the increase in enzyme activity, serum T_3 increases progressively during the first month to a peak concentration of 60–90 ng/dl (2, 3). The increase in T_3 production in the peripheral tissues, especially in the liver, seems to be advantageous for

A part of this work was presented at the International Symposium for "Peripheral Metabolism of Thyroxine: Biochemical Background and Clinical Meaning," 15–18 May 1983, at the Reisensburg Castle, Ulm, Germany.

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Received for publication 10 February 1984 and in revised form 23 August 1984.

J. Clin. Invest.

0021-9738/84/12/2254/09 $1.00
Volume 74, December 1984, 2254–2262

1. *Abbreviations used in this paper:* DTT, dithiothreitol; DV-T_4(−)FCS, Dulbecco-Vogt medium supplemented with nonessential amino acids, penicillin, streptomycin sulphate, and 10% resin-treated fetal calf serum; GSSG, glutathione disulfide; rT_3, 3,3',5'-triiodo-L-thyronine; 3,3'-T_2, 3,3'-diiodo-L-thyronine; T_3, 3,3',5-triiodo-L-thyronine; T_4, thyroxine; T-GSH, total glutathione.

the survival of newborn mammals. However, the factors controlling this maturation process are not yet known.

On the assumption that iodothyronine-5'-deiodinase is regulated by certain hormones, we developed an organ culture system of fetal mouse liver. We found that the ontogenetic development of iodothyronine-5'-diodinase, which converts T_4 to T_3 and reverse T_3 (rT_3) to 3,3'-T_2 (7, 8), is induced by glucocorticoid and thyroid hormones at their physiological concentrations and that insulin plays a permissive role in inducing the enzyme activity.

Methods

[3',5'-^{125}I]triiodo-L-thyronine (^{125}I-rT_3) with a specific activity of about 1 mCi/μg was obtained from New England Nuclear Corp. (Boston, MA). [3'-^{125}I]triiodo-L-thyronine (^{125}I-T_3) and [3',5'-^{125}I]thyroxine (^{125}I-T_4) with a specific activity of 1.5 mCi/μg was prepared (9) and purified (10) as described elsewhere. Nonradioactive thyroxine (T_4), 3,3',5-triiodo-L-thyronine (T_3), and hydrocortisone hemisuccinate were purchased from Sigma Chemical Co. (St. Louis, MO) and rT_3 from Calbiochem-Behring Corp. (San Diego, CA). Our T_4 was contaminated by 0.8% T_3 (determined by radioimmunoassay), and used without purification. AG-1X-10 anion exchange resin (chloride form, minus 400 mesh) and Dowex 50W-X4 cation exchange resin (200–400 mesh) were obtained from Bio-Rad Laboratories (Richmond, CA). Fetal calf serum and nonessential amino acids were obtained from Gibco Laboratories (Grand Island, NY) and other amino acids and vitamins for tissue culture from Wako Pure Chemicals, Ltd. (Tokyo, Japan). Dithiothreitol (DTT) was purchased from Nakarai Chemicals, Ltd. (Kyoto, Japan). Silicon rubber tubing (inner diam, 2 mm; outer diam, 3 mm) was obtained from Imamura Co., Ltd. (Bunkyo-ku, Tokyo, Japan), and sterile membrane filters (0.45 μm, 47 mm, HAWG 047) were from Millipore Corp. (Bedford, MA).

Resin treatment of fetal calf serum. To remove iodothyronines in fetal calf serum, it was treated with AG-1X-10 resin according to the method of Samuels et al. (11). T_4, T_3, and cortisol concentrations in the original fetal calf serum were 8 μg/dl, 130 ng/dl, and 1.0 μg/dl, respectively. In one experiment, ^{125}I-T_4 or ^{125}I-T_3 was added to the fetal calf serum and the serum was treated with resin. The serum was precipitated by TCA and radioactivity in the precipitate was counted. Approximately 93% of T_4 and 96% of T_3 were depleted by the resin treatment.

Culture conditions of fetal mouse liver. Pregnant mice on the 19th day of gestation were sacrificed by dislocation of the neck, and the fetuses were removed from the placenta through an abdominal incision. They were immediately decapitated and placed in a culture dish (60 mm) kept on ice. Then, a traverse abdominal incision was made and the fetal liver was removed with forceps. Combined livers from 20–24 fetuses from two pregnant mice were then cut into explants of <1 mm^3, which were placed on a filter membrane that had been immersed in Dulbecco-Vogt medium. Usually, 10–20 pieces of liver were placed on a membrane. The membrane with liver pieces was transferred to a culture flask (60 × 20 mm) containing 12 ml of Dulbecco-Vogt medium supplemented with 1% nonessential amino acids, penicillin (100 U/ml), streptomycin sulphate (100 μg/ml), 10% resin-treated fetal calf serum (DV-T_4(−)FCS), and hormones (insulin, hydrocortisone hemisuccinate, and thyroid hormones) as specified in each experiment. To culture the liver pieces floating on the surface on the medium, the filter membrane was placed on two floating loops with diameters of 30 and 40 mm. These floating loops were made of silicon tubing. A polyethylene shaft (2.5 × 8 mm) cut from a disposable micropipet tip (Exel tips for 20 μl, Shoei Co., Tokyo) was inserted into the tube to make a loop, which had been sterilized by autoclaving. Pieces of fetal liver were cultured at 37°C under 5% CO_2 and 95% air for 9 d unless otherwise specified. Every 3 d, two thirds of the medium was replaced with fresh medium.

At the end of incubation, the membrane was washed with serum-free Dulbecco-Vogt medium; then, the membrane surface was scraped with a sharp-edged spatula and hepatocytes that proliferated on the membrane were transferred to a Potter-Elvehjem homogenizer. They were homogenized in 0.4 ml of ice-cold 0.25 M sucrose solution containing 10 mM Tris–HCl buffer (pH 7.0) with a motor-driven teflon pestle at 3,000 rpm for 30 s. A 50-μl aliquot of homogenate was taken for measurement of protein by the method of Lowry et al. (12). The homogenate was kept at 0–4°C when deiodinating activity was measured on the same day. Otherwise, it was frozen at −80°C until assayed. The enzyme activity was stable for at least 1 wk under this condition.

Assay for iodothyronine-5'-deiodinating activity. The enzyme activity was assessed as described elsewhere with slight modifications (13). In brief, hepatocyte homogenates (10–100 μg of protein) were incubated in 200 μl of sodium phosphate buffer (100 mM, pH 7.0) containing 10 mM DTT, 1.3 mM EDTA, and 10^{-7} M [3',5'-^{125}I]rT_3. After 15-min incubation under air, the reaction was stopped by adding 0.1 ml human serum followed by 1 ml of cold 10% TCA. After centrifugation at 3,000 rpm for 20 min, the supernatant was applied on Dowex 50W-X4 cation exchange resin columns (200–400 mesh, 0.7 × 1.0 cm) equilibrated with 8 N acetic acid. Columns were washed twice with 1 ml of 8 N acetic acid. ^{125}I released from outer-ring deiodination of rT_3 was collected in 3.2 ml eluate and counted in a gamma-spectrometer. In each assay, three or four tubes without hepatocyte homogenate were processed exactly as the test samples, and the radioactivity obtained, representing nonspecific deiodination, was subtracted from that of test samples. Usually, nonspecific deiodination was 2–3% of the total. Since monodeiodination from the 3'- and 5'- positions of rT_3 occurs equally, the quantity of $^{125}I^-$ released was multiplied by two and iodothyronine-5'-deiodinating activity was expressed as picomoles of $^{127}I^-$ per milligram of protein per minute (13). Generally, the sensitivity of the assay was <0.08 pmol of $^{127}I^-$/mg of protein per min.

In another experiment, ^{125}I-rT_3 metabolites were analyzed by thin layer chromatography. In brief, the reaction was stopped after 30-min incubation by adding 10 μg of rT_3. The reaction mixture was immediately acidified with 0.1 ml 4 N HCl, and the acidified sample was extracted twice with 2 ml of *n*-butanol. Then, 4 ml of chloroform was added to the combined *n*-butanol and mixed well. The turbid mixture was extracted with 2 and 1 ml of 2 N NH_4OH, successively. The combined extract was lyophilized and rT_3 metabolites were analyzed by thin layer chromatography with the solvent system of *n*-butanol saturated with 2 M NH_4OH (13).

To investigate whether T_3 is produced from T_4 in cultured fetal liver, hepatocyte homogenate (~0.5 mg) was incubated in 200 μl of sodium phosphate buffer (100 mM, pH 7.0) containing 10 mM DTT, 1.3 mM EDTA, and 1 μg/ml T_4. After 60-min incubation at 37°C under air, the reaction was stopped by adding 0.8 ml ice-cold ethanol, and stored at −20°C for 1 h. After centrifugation at 3,000 rpm for 30 min, the supernatant (40 μl) was analyzed using the radioimmunoassay kit for T_3 (Eiken Co., Tokyo). Each assay included tubes (zero incubation tubes) that were handled exactly as described except that the homogenate was added immediately before extraction with ethanol.

The amounts of T_3 in the zero incubation tubes were subtracted from the T_3 in test samples. The results were expressed as nanograms of T_3 generated per 200 nanograms T_3 per milligram protein per hour.

Measurement of total glutathione (T-GSH) and glutathione disulfide (GSSG) levels in cultured fetal liver. In some experiments, T-GSH and GSSG were measured enzymatically (14) as described elsewhere (15, 16). In brief, hepatocytes cultured as described above were washed, scraped from the membrane, and homogenized in 400 μl of ice-cold 40 mM HCl solution in a Potter-Elvehjem homogenizer. Homogenates were transferred to another tube in which 40 μl of 50% TCA was added. After 30-min centrifugation at 3,000 rpm, the supernatant was kept frozen at −80°C until assayed for T-GSH. For GSSG assay, hepatocytes were homogenized in 400 μl of 100 mM *N*-ethylmaleimide. The homogenates were transferred to another tube, in which 40 μl of 50% TCA was added. The supernatant obtained as described above was also kept at −80°C until assayed. T-GSH and GSSG levels were expressed as micrograms per milligrams of protein.

Iodothyronine-5'-deiodinating activity of the liver of fetal, neonatal, and adult mouse. Liver of fetal mice of the 19th day of gestation, neonatal mice 3 d after birth, and adult mice were weighed and homogenized (1:20, wt/vol) in 0.25 M sucrose solution containing 10 mM Tris-HCl buffer (pH 7.0). The enzyme activity in the homogenate was determined as described above.

Measurement of free T_4 and T_3 in the medium. Free T_3 and T_4 concentrations in DV-T_4(−)FCS medium supplemented with insulin (1 μg/ml), hydrocortisone hemisuccinate (0.1 μg/ml), and various concentrations of thyroid hormones were assessed according to the method of Uchimura et al. (17).

Results

Effects of insulin, hydrocortisone, and T_4 on iodothyronine-5'-deiodinating activity. In preliminary experiments, pieces of fetal mouse liver were cultured in DV-T_4(−)FCS medium without addition of hormones. No 5'-deiodinating activity was detected even if the liver was cultured for up to 4 wk. Therefore, insulin (1 μg/ml), hydrocortisone hemisuccinate (1 μg/ml), and/or T_4 (10^{-7} M) were added to the DV-T_4(−)FCS medium in various combinations. As shown in Table I, neither insulin, hydrocortisone, or T_4 alone could induce the enzyme activity. However, when fetal mouse livers were cultured for 9 d in the DV-T_4(−)FCS medium supplemented with T_4 together with hydrocortisone, iodothyronine-5'-deiodinating activity was clearly demonstrated. Increased enzyme activity was demonstrated when the DV-T_4(−)FCS medium was further supplemented with insulin. However, supplementation of the DV-T_4(−)FCS medium either with insulin plus T_4 or insulin plus hydrocortisone could not induce the enzyme activity, suggesting that the fetal and neonatal development of iodothyronine-5'-deiodinase is regulated mainly by steroid and thyroid hormones.

Effects of T_4 concentration on iodothyronine-5'-deiodinating activity. The dose dependence of T_4-stimulated iodothyronine-5'-deiodinating activity in fetal liver cells cultured for 9 d in the DV-T_4(−)FCS medium supplemented with insulin (1 μg/ml) and hydrocortisone hemisuccinate (0.1 and 1.0 μg/ml) is shown in Fig. 1. At the higher hydrocortisone hemisuccinate concentration (1.0 μg/ml), T_4 at 10^{-8} M elicited a questionable enzyme activity; in three of five experiments, little enzyme activity was detected. The maximum iodothyronine-5'-deiodinating activity was observed at 10^{-7}–10^{-6} M T_4. At 10^{-5} M T_4, however, the 5'-deiodinating activity was distinctly lower.

Table I. Effects of Insulin (I), Hydrocortisone (HC), and T_4 on Iodothyronine-5'-Deiodinating Activity

Hormones added to the medium	*n*	Enzyme activity
I	4	ND
HC	3	ND
T_4	3	ND
I + HC	10	ND
I + T_4	3	ND
HC + T_4	3	0.35±0.17
I + HC + T_4	11	0.89±0.32

HC, hydrocortisone; I, insulin.
Fetal liver explants were cultured in DV-T_4(−)FCS medium supplemented with insulin (1 μg/ml), hydrocortisone hemisuccinate (1 μg/ml), and thyroxine (10^{-7} M) at various combinations for 9 d. Then, liver explants were homogenized and iodothyronine-5'-deiodinating activity in the homogenate was determined. The enzyme activity was expressed as picomoles of $^{127}I^-$ per milligram of protein per minute released from outer ring deiodination of rT_3. Data are the mean±SD of 3 to 11 experiments. ND, not detectable (<0.08 pmol of $^{127}I^-$/mg·protein per min).

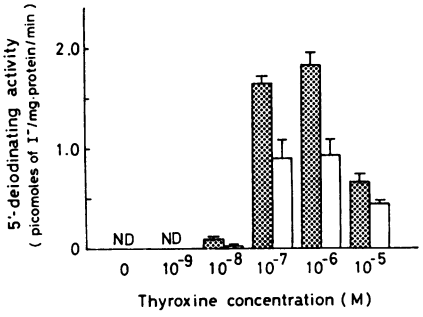

Figure 1. Effects of thyroxine on iodothyronine-5'-deiodinating activity. Pieces of fetal mouse liver of the 19th day of gestation in which no thyroxine-5'-deiodinating activity was demonstrated were cultured in DV-T_4(−)FCS medium supplemented with insulin (1 μg/ml), hydrocortisone hemisuccinate (0.1 μg/ml or 1 μg/ml), and various concentration of T_4. After 9-d culture, the liver was homogenized and the enzyme activity was determined as described in Methods. Iodothyronine-5'-deiodinating activity was expressed by picomoles of I^- released from the outer ring of rT_3 per milligram of protein per minute. Dotted and white columns indicate that the livers were cultured at 0.1 and 1 μg/ml hydrocortisone hemisuccinate, respectively. The data are the mean±SEM of five experiments. Note that no enzyme activity was detected (ND) when the livers were cultured in the medium containing $<10^{-8}$ M T_4.

At 0.1 μg/ml hydrocortisone hemisuccinate, T_4 elicited a slight but significant iodothyronine-5'-deiodinating activity at 10^{-8} M T_4. The activity increased steeply between 10^{-8} and 10^{-7} M and peaked at 10^{-6} M, but also distinctly decreased at 10^{-5} M. At all T_4 concentrations, the enzyme activity was greater in liver cells cultured in the DV-T_4(−)FCS medium supplemented with physiological concentration of hydrocortisone hemisuccinate (0.1 μg/ml).

Effects of hydrocortisone concentrations on iodothyronine-5'-diodinating activity. As expected from the above data, there was a dose-dependent induction of iodothyronine-5'-deiodinating activity by glucocorticoid (Fig. 2). When hydrocortisone was not added to the culture medium, no enzyme activity was demonstrated even if the medium was supplemented with insulin (1 μg/ml) and T_4 (10^{-7} M). The addition of hydrocortisone hemisuccinate (0.01 μg/ml) induced a minimal enzyme activity, which increased to 1.6 pmol of $^{127}I^-$/mg protein per min at 0.1 μg/ml, and peaked at 0.4 μg/ml (2.2 pmol of $^{125}I^-$/mg protein per min). At a supraphysiological hydrocortisone hemisuccinate concentration (1–10 μg/ml), however, the enzyme activity decreased.

Effects of insulin concentrations on iodothyronine-5'-deiodinating activity. Iodothyronine-5'-deiodinating activity was demonstrated in fetal liver cells cultured for 9 d in the DV-T_4(−)FCS medium supplemented with hydrocortisone hemisuccinate (0.1 μg/ml) and T_4 (10^{-7} M), even though insulin was not added (Table I, Fig. 3). The enzyme activity further increased when insulin was added to the medium; its optimal concentration was observed at about 1 μg/ml (Fig. 3).

Effects of T_4, T_3, and rT_3 on the induction of iodothyronine-5'-deiodinating activity. As shown in Fig. 4, T_3 was much more effective than T_4 in inducing iodothyronine-5'-deiodinating activity in fetal liver explants cultured for 9 d in the DV-T_4(−)FCS medium supplemented with insulin (1 μg/ml) and hydrocortisone hemisuccinate (0.1 μg/ml). While the minimum T_4 concentration for induction was 10^{-8} M, T_3 was effective at 10^{-10} M. Maximal enzyme activity (2.0 pmol of $^{127}I^-$/mg of protein per min) was attained at 10^{-7} M T_3 compared with 10^{-6} M T_4. At 10^{-6} M T_3, the enzyme activity decreased to 60% of the maximum level in two other experiments (data not shown). In contrast to T_3 and T_4, rT_3 was completely inactive at concentrations from 10^{-8} to 10^{-6} M (Fig. 4).

The approximate T_4 and T_3 concentrations for inducing

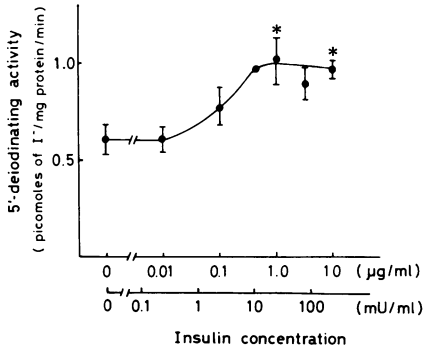

Figure 3. Effects of insulin on iodothyronine-5'-deiodinating activity. Fetal mouse liver on the 19th day of gestation was cultured in DV-T_4(−)FCS medium supplemented with T_4 (10^{-7} M), hydrocortisone hemisuccinate (0.1 μg/ml), and various concentration of insulin. After 9-d culture, liver was homogenized and iodothyronine-5'-deiodinating activity was determined. The data are the mean±SEM of three experiments. $^*P < 0.05$.

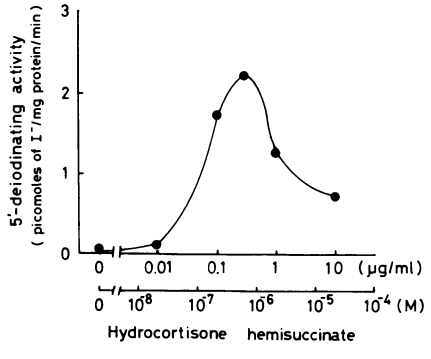

Figure 2. Effects of hydrocortisone concentration on iodothyronine-5'-deiodinating activity. Fetal mouse liver on the 19th day of gestation was cultured in DV-T_4(−)FCS medium supplemented with insulin (1 μg/ml), T_4 (10^{-7} M), and various concentration of hydrocortisone hemisuccinate. After 9-d culture, liver was homogenized and iodothyronine-5'-deiodinating activity was determined. Three experiments were done and data from one representative experiment are shown. Note that no enzyme activity was determined in the absence of glucocorticoid.

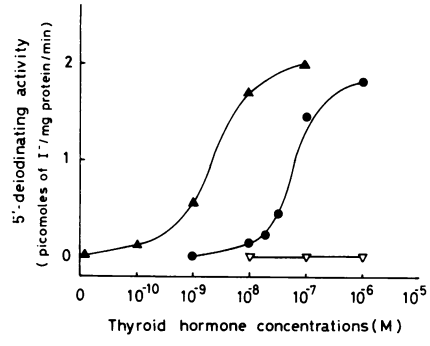

Figure 4. Effects of T_4, T_3, and rT_3 on iodothyronine-5'-deiodinating activity. Fetal mouse liver on the 19th day of gestation was cultured in DV-T_4(−)FCS medium supplemented with insulin (1 μg/ml), hydrocortisone hemisuccinate (0.1 μg/ml), and various concentrations of thyroid hormones. After 9-d culture, liver was homogenized and iodothyronine-5'-deiodinating activity was determined. Total thyroid hormone concentrations in the medium are indicated in the abscissa. Representative data from three experiments are shown. ▲, T_3; ●, T_4; ▽, rT_3.

half-maximal 5'-deiodinating activity were 7×10^{-8} and 2×10^{-9} M, respectively. The corresponding free T_4 and T_3 concentration determined by equilibrium dialysis were 2.8×10^{-10} and 5.0×10^{-11} M, respectively, suggesting that T_3 is five to six times more active than T_4.

Time course of induction of iodothyronine-5'-deiodinating activity by T_4. To investigate the time course of induction of iodothyronine-5'-deiodinating activity by T_4 in fetal liver cells, they were cultured in the DV-T_4(−)FCS medium supplemented with insulin (1 μg/ml), hydrocortisone hemisuccinate (0.1 μg/ml), and with or without T_4 (10^{-7} M). As shown in Fig. 5, the enzyme activity was distinctly detected on day 3, gradually increased thereafter, and peaked at day 15–20. The enzyme activity was barely detected on culture day 1 and decreased by 4 wk in other experiments (data not shown). Lineweaver-Burk analysis revealed that the increase in enzyme activity was primarily due to an increase in maximum velocity (V_{max}) rather than alteration of the Michaelis constant (K_m) (Fig. 6). V_{max} increased from 0.20 pmol of $^{127}I^-$/mg protein per min (day 3) to 2.5 pmol $^{127}I^-$/mg protein per min (day 20), whereas K_m for the outer-ring deiodination of rT_3 was not altered throughout the culture period (7.7×10^{-8} M). These K_m values are comparable with those obtained from adult mouse liver homogenate, suggesting that the iodothyronine-5'-deiodinase induced in vitro under the present experimental condition reflects the 5'-deiodinase of adult mouse liver.

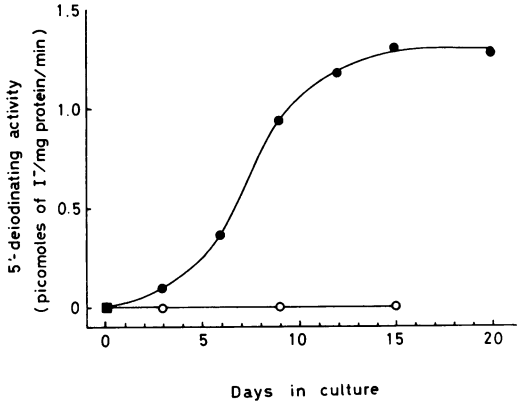

Figure 5. Time course of iodothyronine-5'-deiodinating activity induced by hormones in cultured fetal liver. Fetal mouse liver on the 19th day of gestation was cultured in DV-T_4(−)FCS medium supplemented with insulin (1 μg/ml), hydrocortisone hemisuccinate (0.1 μg/ml), and in the presence (—●—) and absence (—○—) of T_4 (10^{-7} M). After 3–20 d in culture, liver was homogenized and iodothyronine-5'-deiodinating activity was determined. Note that no enzyme was detectable in the fetal liver on the 19th day of gestation (■) and that no enzyme activity was induced when the fetal liver was cultured in the absence of T_4 for up to 15 d. In other experiments, no enzyme activity was detected for up to 25 d in the liver cultured in the absence of T_4 in the medium. Representative data from three experiments are shown.

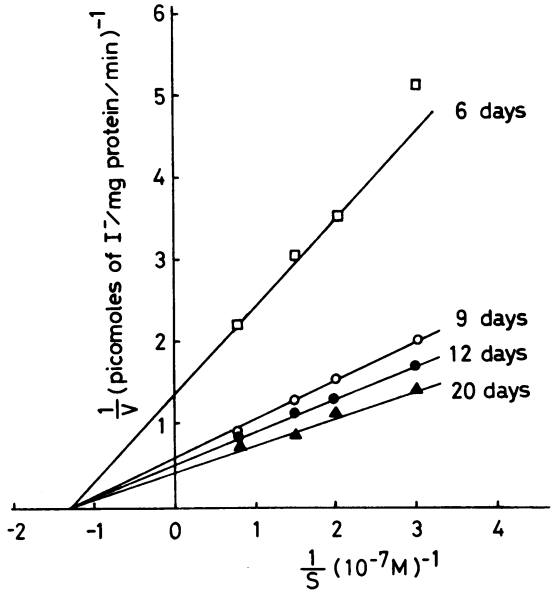

Figure 6. Lineweaver-Burk analysis of iodothyronine-5'-deiodinase. Fetal liver on the 19th day of gestation was cultured in DV-T_4(−)FCS medium supplemented with insulin (1 μg/ml), hydrocortisone hemisuccinate (0.1 μg/ml), and T_4 (10^{-7} M). After 3–20 d in culture, liver was homogenized and iodothyronine-5'-deiodinating activity was determined. Note the progressive increase in V_{max} with no change of K_m (7.7×10^{-8} M). V_{max} and K_m values of liver homogenate cultured for 3 d are 0.20 pmol of I^-/mg of protein per min and 8.0×10^{-8} M, respectively (data not shown in the figure). Data are means of duplicate determinations.

Characterization of iodothyronine-5'-deiodinating activity induced in cultured fetal liver of mouse. Iodothyronine-5'-deiodinating activity in homogenates of fetal liver cultured for 9 d at nearly optimal hormone concentrations (insulin, 1.0 μg/ml; hydrocortisone hemisuccinate, 0.4 μg/ml; and T_4, 10^{-7} M) were characterized. First, the deiodinating activity was linear with protein concentrations over the range in the assay (20–200 μg/tube). When 90 μg of protein was used, the reaction was linear at least for 30 min. Propylthiouracil (10^{-6} M) inhibited 5'-deiodinating activity uncompetitively in the assay condition. When ^{125}I-rT_3 metabolites were extracted and analyzed by thin layer chromatography, only three bands corresponding to iodide (~10%), 3,3'-T_2 (~11%), and rT_3 (79%) were demonstrated.

Furthermore, when the homogenate was incubated with T_4 for 1 h, a distinct amount of T_3 was produced from T_4 (0.46±0.16 ng T_3/mg protein per h, mean±SD, $n = 4$), whereas the homogenates of fetal liver cultured for 9 d with insulin (1 μg/ml) and hydrocortisone hemisuccinate (0.4 μg/ml), but without T_4 synthesized no detectable amount of T_3. These findings suggest that iodothyronine-5'-deiodinase induced in cultured fetal liver by hormones accepts not only rT_3 but also T_4 and converts them to 3,3'-T_2 and T_3, respectively.

T-GSH and GSSG concentrations in the cultured fetal liver. Glutathione levels were investigated in fetal liver cells cultured for 9 d in the DV-T_4(−)FCS medium supplemented with insulin (1 μg/ml), hydrocortisone hemisuccinate (1 μg/ml), and various T_4 concentrations (0–10^{-5} M). Irrespective of T_4 concentration, T-GSH concentration was ~6 μg/mg of protein. GSSG comprised ~2% of total glutathione and was not influenced by thyroid hormone levels in the culture medium (Table II*A*). T-GSH concentration also was not dependent on T_4 concentration even though fetal liver was cultured at optimal hormone concentrations (insulin, 1.0 μg/ml; hydrocortisone hemisuccinate, 0.1 μg/ml) (Table II*B*). There was no correlation between GSSG concentration and iodothyronine-5′-deiodinating activity (Table II).

Iodothyronine-5′-deiodinating activity in the liver of fetal, neonatal, and adult mice. As reported previously (6), little or no iodothyronine-5′-deiodinating activity was detected in the fetal mouse liver on the 19th day of gestation. The enzyme activity gradually increased in the neonatal period (6). Lineweaver-Burk analysis revealed that V_{max} of the enzyme activity increased from 2.45±1.87 (SD, n = 6, determined in four experiments) in the liver of neonatal mice (3 d after delivery) to 32.2±5.4 (SD, n = 5, determined in four experiments) pmol I^-/mg protein per min in the adult. However, no significant difference in K_m was observed between the neonatal liver ($6.6±2.4 \times 10^{-8}$ M, mean±SD, n = 6) and the adult liver ($7.2±2.9 \times 10^{-8}$ M, mean±SD, n = 5) ($P > 0.1$), indicating that the increase in enzyme activity in the extrauterine life is primarily due to an increase in the enzyme capacity.

Table II. T-GSH and GSSG Levels in Cultured Fetal Liver of Mouse

T_4 concentration	*A* T-GSH (n = 4)	GSSG (n = 2)	GSSG/T-GSH (n = 2)	*B* T-GSH (n = 2)
M	*μg/mg protein*	*μg/mg protein*	*%*	
0	5.7±1.7	0.11	1.8	5.1
10^{-9}	7.2±1.5	0.075	1.7	—
10^{-8}	5.8±1.5	0.13	1.9	5.1
10^{-7}	6.0±0.8	0.11	1.5	4.7
10^{-6}	7.2±1.1	0.21	2.4	4.6
10^{-5}	7.3±1.0	0.15	1.8	5.3

Fetal liver of mouse on the 19th day of gestation was cultured in DV-T_4(−)FCS medium supplemented with insulin (1 μg/ml), hydrocortisone hemisuccinate (1.0 μg/ml [*A*] or 0.1 μg/ml [*B*]), and T_4 (0–10^{-5} M). After culturing for 9 d, liver was homogenized and T-GSH and GSSG were measured. Results are the mean±SEM of four (T-GSH[*A*]) and the mean of two (GSSG and T-GSH[*B*]) experiments. Note that, in contrast to iodothyronine-5′-deiodinating activity, glutathione concentration is not dependent on T_4 concentration in the medium.

Discussion

A number of recent studies revealed that thyroid hormone metabolism in the peripheral tissues is regulated multifactorially, e.g., by hormones such as glucocorticoids, insulin, glucagon, and by putative cytosol cofactors (18–24). Therefore, it is quite reasonable to speculate that ontogenetic development of iodothyronine-5′-deiodinase, which converts T_4 to T_3 and rT_3 to 3,3′-T_2 (7, 8), is also under multihormonal regulation. To elucidate the regulatory mechanism of this ontogenetic development, we have developed an organ culture system using fetal mouse liver of the 19th day of gestation, in which no iodothyronine-5′-deiodinating activity was detected (6). We found that iodothyronine-5′-deiodinase is induced in the fetal liver by the synergistic action of glucocorticoid and thyroid hormones at their physiological concentrations attained in the sera of the fetal or neonatal period.

Although at pharmacological doses, glucocorticoid decreases serum T_3 levels in the adult human (18) and animals by decreasing iodothyronine-5′-deiodinating activity in the peripheral tissues such as the liver (7, 20), a physiological dose of glucocorticoids does the opposite particularly in the fetus (1, 5). Thomas et al. (25) showed in prematurely delivered sheep that infusion of cortisol simulating the prepartum plasma corticosteroid surge was accompanied by an increase in fetal plasma T_3 concentration, which was ascribed to an increase in the T_3-generating capacity of the fetal liver (26). In this respect, the present findings that minimum and optimum hydrocortisone concentrations to induce iodothyronine-5′-deiodinating activity are on the order of 0.01 and 0.4 μg/ml, respectively, are physiologically very feasible, since these corticosteroid levels are those attainable in the perinatal period of humans and rodents (27, 28). A supraphysiological concentration of glucocorticoid, however, inhibits the enzyme activity in accordance with clinical observations in human subjects (18) (Fig. 2). Our present report directly demonstrates that iodothyronine-5′-deiodinase should be added to the list of corticosteroid-inducible enzymes in the fetal liver (29). Glucocorticoids, however, cannot induce 5′-deiodinase activity in the absence of thyroid hormone in the culture medium. A similar phenomenon was observed in cultured GH, cells in which glucocorticoid and thyroxine synergistically increase GH secretion by stimulating the synthesis of mRNA for GH (30).

In the presence of glucocorticoid, thyroxine caused a dose-dependent increase in iodothyronine-5′-deiodinating activity in cultured fetal liver of the mouse. Stimulation by T_4 of conversion of T_4 to T_3 was previously reported by Kaplan and Utiger (31) and Balsam et al. (32) in the liver of normal, thyroidectomized, and hypophysectomized adult rats. Our present data, however, shows directly that a physiological concentration of T_4 is also capable of inducing iodothyronine-5′-deiodinating activity in the fetal liver. The minimum T_4 and T_3 concentrations in medium containing 10% of fetal calf serum to induce the enzyme activity were ~10^{-8} M (free T_4, 3×10^{-11} M) and 10^{-10} M (free T_3, 3×10^{-12} M), respectively.

These free T_3 and T_4 concentrations are attained in the serum of neonatal rodents; free T_4 concentration in rat serum increases from 0.79 ng/dl ($\sim 10^{-11}$ M) at birth to 2.5 ng/dl (3.2×10^{-11} M) at 14–22 d (1).

On the basis of free thyroid hormone concentrations, half-maximal enzyme activity was induced by 5.0×10^{-11} M T_3 and 2.8×10^{-10} T_4. These values are comparable with those observed in GH_1 cells (33), rat hepatocytes (34), and especially in chick liver embryo cells in which free T_3 concentrations of 4×10^{-11} M exerted 50% of maximum effect in inducing the synthesis of malic enzyme (35). Since little or no T_3 could be formed from T_4 at the beginning of the organ culture, hormonal activity should be attributed to T_4 itself. It is the free thyroid hormones that are biologically active (36). Therefore, T_4 may have one-sixth the intrinsic biological activity of T_3. However, as the T_4 used was contaminated with 0.8% T_3, half maximal concentration of T_4 (7×10^{-8} M) contains 5.6×10^{-10} M T_3, which is able to induce a significant enzyme activity (0.3 pmol of $^{127}I^-$/mg · protein per min). Therefore, the dose response curve of T_4 shifts to the right, giving rise to the real half maximal T_4 concentration of 1×10^{-7} M. Since the corresponding free T_4 concentration is $\sim 4 \times 10^{-10}$ M, T_3 is eight times more active than T_4 in inducing the enzyme activity. The apparent equilibrium dissociation constants (K_d) of thyroid hormones for nuclear receptors of rat liver varies widely, dependent on the study procedures. Oppenheimer et al. (37) reported K_d for T_3 of 2.1×10^{-12} M and for T_4 of 4.5×10^{-11} M from in vivo studies. High K_d values for T_3 were reported in isolated rat liver nuclei (2.1×10^{-10} M) (38). The estimated K_d values for T_3 and T_4 in intact GH_1 cells are 2.9×10^{-11} and 2.6×10^{-10} M, respectively (39), which are virtually identical with the free T_3 and T_4 concentrations in the medium required to elicit 50% of the maximal stimulation of iodothyronine-5′-deiodinating activity in fetal liver. These findings suggest that T_3 induction of iodothyronine-5′-deiodinase is mediated by control of regulatory events at the level of the hepatocyte nucleus.

Since fetal calf serum contains about 10^{-7} M T_4 and 2–3 $\times 10^{-11}$ M free T_4, the total T_4 level in the medium containing 10% fetal calf serum is 10^{-8} M. However, the free T_4 concentration is largely independent of serum dilution over this range (40). Therefore, without having used the thyroid hormone-depleted serum, we could not have developed such a sensitive culture system for thyroid hormone. These findings were emphasized by Samuels et al. (38) who first reported a cell culture system responsive to physiological concentrations of thyroid hormones (33).

In addition to thyroid hormone and glucocorticoid, insulin further stimulated iodothyronine-5′-deiodinating activity in the presence of both hormones. However, when either thyroid hormone or hydrocortisone is not present in the culture medium, insulin could not induce the enzyme activity. These findings suggest that insulin plays a permissive role in enhancing the enzyme activity induced by thyroid and glucocorticoid hormones. Stimulation by insulin of T_3 production from T_4 or thyroxine-5′-deiodination was reported in diabetic rat liver (20) and rat hepatocytes in monolayer culture (23). Furthermore, a bolus injection of insulin to normal subjects (22) and patients with diabetic ketoacidosis (41) caused a gradual increase in serum T_3 concentration. Whether this stimulatory action of insulin resides in the stimulation of synthesis of iodothyronine-5′-deiodinase, putative cytosol cofactor, or other effects remain to be elucidated.

T-GSH concentrations in the cultured fetal liver (6 μg/mg of protein) were lower than our previous data found in cultured hepatocytes of adult rats (15, 16). This is partly due to an intrinsic low glutathione level in the fetal liver (42), but a more likely explanation may be that types of cells (i.e., fibroblasts) that contain lower intracellular glutathione levels than hepatocytes (43) might have replicated more rapidly in the liver explants. It should be noted that the medium used in the present experiment was the usual Dulbecco-Vogt medium, and not the arginine-free, ornithine-supplemented medium that selectively favors the growth of hepatocytes since they contain enzymes for the urea cycle (44). A lack of correlation between T-GSH concentration and the enzyme activity in the present experiment confirms our previous observation that a putative sulfhydryl reductant in the cytosol, mainly glutathione, does not modulate the enzyme activity (15). Furthermore, the GSSG level, which may modulate thyroxine-5′-deiodination under certain pharmacological condition (16), also is unrelated to the enzyme activity in the organ culture.

The time course of the in vitro induction of iodothyronine-5′-deiodinating activity in fetal liver reflects that found in the fetal and neonatal mouse (6). The increase in enzyme activity in vitro as well as in vivo is primarily due to an increase in V_{max}, suggesting an increase in the enzyme capacity, whereas an alteration of K_m was not observed. It should also be pointed out that the K_m values for outer ring deiodination of rT_3 in the cultured fetal liver is identical to that of adult liver of mouse (7×10^{-8} M) and rat (6.5×10^{-8} M) (7, 45). Taking these observations together, we assume that fetal liver of mouse cultured under the present experimental conditions in the presence of thyroid and glucocorticoid hormones have differentiated enough to synthesize iodothyronine-5′-deiodinase as newborn mice do in their neonatal period. Although the V_{max} obtained in the present experiments (~ 3 pmol of $^{127}I^-$/mg of protein per min) is less than that of adult mouse liver (~ 30 pmol/mg of protein per min), this may be due to the growth of nonparenchymal cells that contain less enzyme activity. We presume that a similar mechanism is involved in the ontogenetic development of iodothyronine-5′-deiodinase in the prenatal and postnatal period of newborn sheep and humans.

Acknowledgments

The authors are greatly indebted to Dr. Jacob Robbins of the National Institutes of Health, Bethesda, MD, for advice in the preparation of the manuscript, and Mrs. K. Matsumoto for typing the manuscript.

This work was supported by a research grant from the Foundation for Growth Science, Shinjuku-ku, Tokyo, Japan.

References

1. Fisher, D. A., J. H. Dussault, J. Sack, and I. J. Chopra. 1977. Ontogenesis of hypothalamic-pituitary-thyroid function and metabolism in man, sheep and rat. *Recent Prog. Horm. Res.* 33:59–116.

2. Dubois, J. D., and J. H. Dussault. 1977. Ontogenesis of thyroid function in the neonatal rat. Thyroxine (T_4) and triiodothyronine (T_3) production rates. *Endocrinology.* 101:435–441.

3. Harris, A. R. C., S. L. Fang, J. Prosky, L. E. Braverman, and A. G. Vagenakis. 1978. Decreased outer ring monodeiodination of thyroxine and reverse triiodothyronine in the fetal and neonatal rat. *Endocrinology.* 103:2216–2222.

4. Borges, M., J. LaBourene, and S. H. Ingbar. 1980. Changes in hepatic iodothyronine metabolism during ontogeny of the chick embryo. *Endocrinology.* 107:1751–1761.

5. Thomas, A. L., and P. W. Nathanielsz. 1983. The fetal thyroid. *In* Fetal Endocrinology and Metabolism. Martin, L., and V. H. T. James, editors. Academic Press, New York. 5:97–116.

6. Sato, K., and J. Robbins. 1984. Does glutathione regulate thyroxine deiodinase activity in cells? *Horm. Metab. Res.* In press.

7. Kaplan, M. M., and R. D. Utiger. 1978. Iodothyronine metabolism in rat liver homogenate. *J. Clin. Invest.* 61:459–471.

8. Eisenstein, Z., A. Balsam, J. R. Garber, and S. H. Ingbar. 1980. A study of the properties of the enzyme in rat liver that deiodinates 3,3',5'-triiodothyronine to 3,3'-diiodothyronine. *Endocrinology.* 107:530–537.

9. Kochupillai, N., and R. S. Yalow. 1978. Preparation, purification, and stability of high specific activity ^{125}I-labeled thyronines. *Endocrinology.* 102:128–135.

10. Sato, K., and H. Cahnmann. 1980. Synthesis of [3,5-^{125}I]triiodo-L-thyronine of high specific activity. *Anal. Biochem.* 102:237–242.

11. Samuels, H. H., F. Stanley, and J. Casanova. 1979. Depletion of L-3,5,3'-triiodothyronine and L-thyroxine in euthyroid calf serum for use in cell culture studies of the action of thyroid hormone. *Endocrinology.* 105:80–85.

12. Lowry, O. H., N. J. Rosebough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.

13. Sato, K., and J. Robbins. 1980. Thyroid hormone metabolism in cultured monkey hepatocarcinoma cells. Monodeiodination activity in relation to cell growth. *J. Biol. Chem.* 255:7347–7352.

14. Tietze, F. 1969. Enzymatic method for glutathione determination of nanogram amounts of total and oxidized glutathione. Application to mammalian blood and other tissues. *Anal. Biochem.* 27:502–522.

15. Sato, K., and J. Robbins. 1981. Gutathione deficiency induced by cystine and/or methionine deprivation does not affect thyroid hormone deiodination in cultured rat hepatocytes and monkey hepatocarcinoma cells. *Endocrinology.* 109:844–852.

16. Sato, K., H. Mimura, K. Wakai, T. Tsushima, and K. Shizume. 1983. Modulating effect of glutathione disulfide on thyroxine-5'-deiodination by rat hepatocytes in primary culture: effect of glucose. *Endocrinology.* 113:878–886.

17. Uchimura, H., S. Nagataki, T. Tabuchi, M. Mizuno, and S. H. Ingbar. 1976. Measurements of free thyroxine in diluted and undiluted sera. *J. Clin. Endocrinol. Metab.* 42:561–566.

18. Chopra, I. J., D. E. Williams, J. Orgiazzi, and D. H. Solomon. 1975. Opposite effects of dexamethasone on serum concentrations of 3,3',5'-triiodothyronine (reverse T_3) and 3,3',5-triiodothyronine (T_3). *J. Clin. Endocrinol. Metab.* 41:911–920.

19. Chopra, I. J. 1978. Sulfhydryl groups and the monodeiodination of thyroxine to triiodothyronine. *Science (Wash. DC).* 199:904–906.

20. Balsam, A., and S. H. Ingbar. 1978. The influence of fasting, diabetes, and several pharmacological agents on the pathways of thyroxine metabolism in rat liver. *J. Clin. Invest.* 62:415–424.

21. Harris, A. R. C., S. L. Fang, L. Hinerfeld, L. Braverman, and A. G. Vagenakis. 1979. The role of sulfhydryl groups on the impaired hepatic 3,3',5-triiodothyronine generation from thyroxine in the hypothyroid, starved, fetal, and neonatal rodent. *J. Clin. Invest.* 63:516–524.

22. Tevaawerk, G. J. M., C. J. Hurst, P. Ursik, and L. Reese. 1979. Effect of insulin-induced hypoglycemia on the serum concentrations of thyroxine, triiodothyronine, and reverse triiodothyronine. *Can. Med. Assoc. J.* 121:1090–1093.

23. Sato, K., and J. Robbins. 1981. Thyroid hormone metabolism in primary cultured rat hepatocytes. Effects of glucose, glucagon, and insulin. *J. Clin. Invest.* 68:475–483.

24. Kabadi, U., and B. N. Premachandra. 1983. Effect of glucagon on thyroid hormone levels in euthyroid subjects. Program of the 65th Annual Meeting of the Endocrine Society, San Antonio, TX. 211 (Abstract #521).

25. Thomas, A. L., E. J. Krane, and P. W. Nathanielsz. 1978. Changes in the fetal thyroid axis after induction of premature parturition by low dose continuous intravascular cortisol infusion to the fetal sheep at 130 days of gestation. *Endocrinology.* 103:17–23.

26. Wu, S. Y., A. H. Klein, I. J. Chopra, and D. A. Fisher. 1978. Alterations in tissue thyroxine-5'-monodeiodinating activity in perinatal period. *Endocrinology.* 103:235–239.

27. Murphy, B. E. P., and C. T. L. Branchaud. 1983. Fetal metabolism of cortisol. *In* Fetal Endocrinology and Metabolism. Martin, L., and V. H. T. James, editors. Academic Press, New York. 5:197–229.

28. Martin, C. E., M. H. Clark, P. E. Hartman, and I. F. Cook. 1977. Relationship between fetal corticosteroids, maternal progesterone and parturition in the rat. *Acta Endocrinol.* 84:167–176.

29. Liggins, C. C. 1976. Adrenocortical-related maturational events in the fetus. *Amer. J. Obstet. Gynecol.* 126:931–941.

30. Shapiro, L. E., H. H. Samuels, and B. M. Yaffe. 1978. Thyroid and glucocorticoid hormones synergistically control growth hormone mRNA in cultured GH_1 cells. *Proc. Nat. Acad. Sci. USA.* 75:45–49.

31. Kaplan, M. M., and R. D. Utiger. 1978. Iodothyronine metabolism in liver and kidney homogenates from hyperthyroid and hypothyroid rats. *Endocrinology.* 103:156–161.

32. Balsam, A., F. Sexton, and S. H. Ingbar. 1978. The effect of thyroidectomy, hypophysectomy, and hormone replacement on the formation of triiodothyronine from thyroxine in rat liver and kidney. *Endocrinology.* 103:1759–1767.

33. Samuels, H. H., J. S. Tsai, and R. Cintron. 1973. Thyroid hormone action: a cell-culture system responsive to physiological concentrations of thyroid hormones. *Science (Wash. DC).* 181:1253–1256.

34. Mariash, C. N., C. R. McSwigan, H. C. Towle, H. L. Schwartz, and J. H. Oppenheimer. 1981. Glucose and triiodothyronine both induce malic enzyme in the rat hepatocyte culture: evidence that triiodothyronine multiplies a primary glucose-generated signal. *J. Clin. Invest.* 68:1485–1490.

35. Goodridge, A. G., and T. G. Adelman. 1976. Regulation of malic enzyme synthesis by insulin, triiodothyronine, and glucagon in liver cells in culture. *J. Biol. Chem.* 251:3027–3032.

36. Robbins, J., and J. E. Rall. 1957. The interaction of thyroid hormone and protein in biological fluids. *Recent Prog. Horm. Res.* 13:161–208.

37. Oppenheimer, J. H., H. L. Schwartz, D. Koerner, and M. I. Surks. 1974. Limited binding capacity sites for L-triiodothyronine in rat liver nuclei. Nuclear cytoplasmic interaction, binding constants, and cross-reactivity with L-thyroxine. *J. Clin. Invest.* 53:768–777.

38. Samuels, H. H. 1978. In vitro studies on thyroid hormone receptors. *In* Receptors and Hormone Action III. L. Birnbauer and B. W. O'Malley, editors. Academic Press, New York. 3:35–74.

39. Samuels, H. H., and J. S. Tsai. 1973. Thyroid hormone action in cell culture: demonstration of nuclear receptors in intact cells and isolated nuclei. *Proc. Natl. Acad. Sci. USA.* 70:3488–3492.

40. Oppenheimer, J. H., and M. I. Surks. 1964. Determination of free thyroxine in human serum: a theoretical and experimental analysis. *J. Clin. Endocrinol.* 24:785–793.

41. Madsbad, S., P. Lauerberg, J. Weeke, H. Ørskov, O. K. Faber, C. Binder, T. Krarup, and L. Regeur. 1981. Very early change in circulating T_3 and rT_3 in diabetic patients. *Acta Med. Scand.* 209:385–387.

42. Lambert, G. H., and S. S. Thorgeirsson. 1976. Glutathione in the developing mouse liver I: developmental curve and depletion after acetaminophen treatment. *Biochem. Pharmacol.* 25:1777–1781.

43. Schulman, J. D., J. A. Schneider, K. H. Bardley, and J. E. Seegmiller. 1972. Cystine, cysteine, and glutathione metabolism in normal and cystinoic fibroblasts in vitro, and in cultured normal amniotic fluid cells. *Clin. Chim. Acta.* 37:53–58.

44. Leffert, H. L., and D. Paul. 1972. Studies on primary cultures of differentiated fetal liver cells. *J. Cell. Biol.* 52:559–568.

45. Chopra, I. J., S. Y. Wu, Y. Nakamura, and D. H. Solomon. 1978. Monodeiodination of 3,5,3′-triiodothyronine and 3,3′,5′-triiodothyronine to 3,3′-diiodothyronine in vitro. *Endocrinology.* 102:1099–1106.