Thyroid Thermogenesis in Adult Rat Hepatocytes in Primary Monolayer Culture

Direct Action of Thyroid Hormone In Vitro

FARAMARZ ISMAIL-BEIGI, D. MONTGOMERY BISSELL, and ISIDORE S. EDELMAN

From the Cardiovascular Research Institute, the Liver Center of the Department of Medicine, and the Department of Biochemistry and Biophysics of the University of California, San Francisco, California 94143. Dr. Ismail-Beigi's present address is the Department of Medicine, Pahlavi University School of Medicine, Shiraz, Iran; he worked on the present material during sabbatical leave granted by Pahlavi University, spent at the University of California, San Francisco.

ABSTRACT We have studied the effect of 3,5,3'-triiodothyronine (T_3) on the respiration of adult rat hepatocytes in primary monolayer culture prepared from hypothyroid rat liver. After addition of T_3 to the culture medium at a concentration of 2×10^{-7} M, oxygen consumption of the cultured cells increased detectably at 24 h and was maximal at 72–96 h, relative to control cultures (38.0 ± 1.8 vs. 25.0 ± 1.5 μl/h · mg protein). The thyroid-responsive enzymes, $Na^+ + K^+$-activated adenosine triphosphatase (NaK-ATPase) and α-glycerophosphate dehydrogenase (GPD), each exhibited increased activity in response to T_3, in parallel with the change in oxygen consumption, whereas the activity of Mg-dependent ATPase was unaffected. These responses to T_3 were dose dependent over similar concentration ranges, the half-maximal response for each occurring at ca 8×10^{-10} M. In thyroid-treated cells, the observed increase in respiration was almost completely (90%) inhibited after addition of ouabain (10^{-3} M) to the culture medium. It was found also that a 4-h exposure of the cultured hepatocytes to T_3 was sufficient to elicit a significant thermogenic response, measured at a time (48 h later) when T_3 was no longer present in the medium. The response to T_3 occurred in fully defined culture medium and was independent of the presence or absence of hypothyroid rat serum, corticosterone, or insulin, and cellular ATP was unaffected by T_3 in concentrations up to 2×10^{-7} M. The findings document that adult rat hepatocytes in primary monolayer culture respond directly to thyroid hormone; the increases in respiration and NaK-ATPase activity elicited by T_3 were cotemporal and apparently coordinate.

INTRODUCTION

Administration of thyroid hormone in vivo elicits a thermogenic response (measured as increased oxygen consumption) which can be ascribed to its action on specific target tissues (Barker, 1951). Among these tissues is the liver, which has been utilized extensively in studies of the biochemical basis of thyroid

Volume 73 March 1979 369–383

thermogenesis. It was reported previously that stimulation of oxygen consumption (Qo_2) by 3,5,3'-triiodothyronine (T_3) was closely associated with increased activity of Na^+ + K^+-activated adenosine triphosphatase (NaK-ATPase), suggesting that the thyroid-related change in energy expenditure is attributable to increased transmembrane active sodium transport (Ismail-Beigi and Edelman, 1970, 1971, 1974; Israel et al., 1973; Edelman and Ismail-Beigi, 1974; Asano et al., 1976; Rahimifar and Ismail-Beigi, 1977). Consistent with this hypothesis was the finding that the increases in Qo_2 (elicited by injection of T_3 in vivo and assayed in vitro, in hepatic slices and diaphragm segments) were inhibited 50–90% by addition of ouabain to the media. The relevance of these findings to thyroid thermogenesis in vivo has been questioned recently (Folke and Sestoft, 1977). Some of these issues will be discussed below. Regardless of the merits of the arguments, however, ouabain-sensitive respiration provides an additional criterion of whether isolated cells challenged with T_3 exhibit the same characteristics as freshly prepared liver slices from rats given T_3 in vivo (Ismail-Beigi and Edelman, 1971, 1974; Asano et al., 1976).

The existence of high affinity nuclear binding sites for T_3 in responsive cells implies direct effects of thyroid hormone on target tissues (Oppenheimer et al., 1972; Samuels and Tsai, 1973). Moreover, T_3 induces the synthesis of specific proteins (e.g., growth hormone) in cultured pituitary tumor cells (Martial et al., 1977). A direct effect of thyroid hormone on Qo_2 or NaK-ATPase in target tissues, however, has not been established previously. Moreover, after injection of thyroid hormone in vivo, it was unclear as to which liver cell type (parenchymal or nonparenchymal) was responsible for the increased Qo_2 of liver slices.

A direct approach to these problems requires isolated cells. To date, however, neither liver-derived cells nor other cell types have demonstrated a thermogenic response to thyroid hormone added in vitro. Tsai and Chen (1976) recently reported that fetal rat cardiac cells in culture exhibited increased glucose consumption after addition of T_3 to the culture medium. This response, however, need not signify an effect on cellular respiration, because a correlation between these two processes has not been demonstrated. With regard to thyroid thermogenesis in isolated hepatocytes, the study requires a cell system that is viable for relatively long periods of time. Responses to T_3 in vivo appear 12–18 h after administration of the hormone and rise to a peak at 48–72 h (Tata and Widnell, 1966; Ismail-Beigi and Edelman, 1974). In previous attempts, liver-derived tumor cells in permanent culture did not exhibit a thermogenic response to thyroid hormone,[1] and the use of liver slices, isolated perfused liver, or hepatocytes in suspension is excluded by the relatively brief viability of these preparations.

Recently, however, an isolated hepatocyte system has been described that combines the stability of cell culture with the differentiated features of the intact liver: adult rat hepatocytes in primary monolayer culture (Bissell et al., 1973). By this technique, hepatic parenchymal cells—separated cleanly from nonparenchymal elements—are established in nonproliferating primary culture and maintained in a viable state for several days, as judged both by ultrastructural

[1] Ismail-Beigi, F., and I. S. Edelman. Unpublished observations.

morphology (Chapman et al., 1973) and by the preservation of numerous specific hepatocellular functions (Bissell et al., 1973; Bonney, 1974). In the present study, we examine the effect of T_3, added to the incubation medium, on total and ouabain-inhibitable respiration, on NaK-ATPase and on mitochondrial α-glycerophosphate dehydrogenase (L-glycerol-3-phosphate oxidase) (GPD). The findings in this culture system establish that the effect of T_3 on hepatic parenchymal cells is direct and involves coordinate increases in these parameters of thyroid thermogenesis.

METHODS

Preparation and Maintenance of Hepatocyte Cultures

Male Sprague-Dawley rats (150–180 g) were placed on iodide-deficient diet (Remington, iodide content <3 ppm; United States Biochemical Corp., Cleveland, Ohio) and drinking water containing 0.5% $NaClO_4$ to induce hypothyroidism (Alexander and Wolff, 1966). After 3 wk of the above regimen, rat serum thyroxine was 1.1 ± 0.1 μg/100 ml (normal $= 5.1 \pm 0.2$) ($n = 8$ pairs of rats). Although this degree of hypothyroidism is less profound than that of thyroidectomized rats, the respiratory rate of hepatic and renal cortical slices was reduced significantly.[2] Perchlorate was deleted from the diet 2 d before the actual experiments. Pooled hypothyroid rat serum was prepared from groups of male rats placed on the above dietary regimen.

Primary hepatocyte monolayer cultures were prepared by previously described procedures (Bissell et al., 1973), except that the starting material was resting—rather than regenerated—rat liver. Approximately 4×10^6 isolated hepatocytes were plated in 60-mm plastic Petri plates coated with 20 μg solubilized rat tail collagen (Bissell et al., 1978). Less than 1% of the plated cells was derived from the reticuloendothelial system (Bissell et al., 1973). The culture medium consisted of the amino acid mixture of medium 199 (Microbiological Associates, Inc., Bethesda, Md.) (Morgan et al., 1950), with Hanks' buffer salts, supplemented with 2.0% (vol/vol) hypothyroid rat serum, 10^{-6} M corticosterone, 4 mU/ml crystalline insulin, Eagle's MEM-vitamins, and 50 mg/liter ascorbic acid. Routinely, the medium was changed 4–6 h after plating and replaced with fresh medium with or without added T_3. Subsequent medium changes were carried out at 24-h intervals. The monolayers were studied for 4–5 d. The protein and DNA content per plate decreased slowly over this period, presumably as a result of cell loss with medium changes, so that by the 4th d ~50% of the initial cell content remained in the plates. T_3 was dissolved in 5×10^{-4} M NaOH, and aliquots of the stock solution were frozen at −20°C. Before use, T_3 solutions were diluted in the complete culture medium. These were stable as assessed by radioimmunoassay for at least a week at 4°C.

Measurement of Oxygen Consumption by Hepatocyte Monolayers

The rate of oxygen consumption (Qo_2) was determined by standard manometric techniques with a Gilson (Gilson Medical Electronics, Inc., Middleton, Wis.) apparatus (Umbreit et al., 1957). For assay of intact monolayer cultures, large respiratory flasks (~80 ml vol) were fabricated to accommodate 60-mm Petri plates, obviating the need to scrape the hepatocyte monolayers off the plates, which disrupts the culture and causes damage to individual cells. The Qo_2 determinations were made ~2 h after the daily renewal of medium. Before each determination, the complete medium was replaced with 2.0 ml of medium without sodium bicarbonate (pH 7.4). Ouabain-inhibitable respiration

[2] Somjen, D., F. Ismail-Beigi, and I. S. Edelman. Manuscript submitted for publication.

[Qo_2(t)] was estimated from cultures incubated in the presence or absence of 10^{-3} M ouabain in parallel plates, in duplicate. The initial measurements of Qo_2 (30-min interval) were made after preincubation in the respirometer for 30 min at 37°C, and the incubations were carried out for 2 h (Ismail-Beigi and Edelman, 1970). All determinations of Qo_2 were made at least in duplicate. Results were expressed as microliters o_2 consumed per hour per milligram total cellular protein (or per microgram DNA).

After measurement of Qo_2, monolayers were rinsed once with 2.0 ml of 0.25 M sucrose solution (pH 7.0) containing 10 mM Tris and 1.25 mM 1,2-bis-(2-dicarboxymethylamino-ethoxy)ethane (EGTA), scraped from the plates in 2.0 ml of the above solution and sonicated in an ice bath four times at 3 W/s for 5-s intervals interrupted by 15-s cooling periods (Microtip, model W140D, Branson Sonic Power Co., Danbury, Conn.). Sonicates were assayed directly for enzyme activities as indicated below, or precipitated at 4°C with trichloroacetic acid (10% wt/vol final concentration) for protein and DNA determinations. To minimize experimental variation, each longitudinal study (day 1 to day 5) was carried out with cultures derived from a single rat liver.

Analytical Methods

NaK-ATPase (E.C.3.6.1.3.) and Mg^{2+}-dependent ATPase were assayed in aliquots of the sonicate by previously described methods with the use of 10^{-3} M ouabain (Ismail-Beigi and Edelman, 1971). Mitochondrial GPD (E.C.1.1.99.5) was determined in the absence of NAD^+ by the method of Fried et al. (1961). All enzyme assays were performed in triplicate.

Measurement of T_3 in serum or in culture media was performed by the immunoassay of Gharib and co-workers (1971). ATP content of hepatocyte monolayers was assayed as previously described (Bissell et al., 1973). Protein was measured by the method of Lowry et al. (1951) and DNA as described by Burton (1956). All assays were carried out in duplicate.

Statistical Calculations

The data are presented as the mean ± SEM and were evaluated for significance by the unpaired Student's *t* test (Snedecor and Cochran, 1967), except where otherwise noted. A P value of <0.05 was considered statistically significant.

Materials

Na-L,T_3 (95% pure) and ouabain were obtained from Calbiochem (San Diego, Calif.; Na-ATP, EGTA, type IV collagenase, and amino acids from Sigma Chemical Co. (St. Louis, Mo.); Tris base from Schwarz/Mann Division, Becton, Dickinson & Co., (Orangeburg, N.Y.). Other reagents were analytical grade and were obtained from Mallinckrodt Inc. (St. Louis, Mo.).

RESULTS

Effects of T_3 on Respiration of Cultured Hepatocytes

The respiration of intact cultures was examined 4–6 h after cell plating and at 24-h intervals thereafter. The plates assayed for Qo_2 at 4–6 h provided the "time-zero" measurements, and the remaining plates were treated with T_3 (2×10^{-7} M) or the diluent at this time. Although the Qo_2 of hypothyroid cells rose slightly over a period of 4 d (Fig. 1), addition of T_3 to the culture medium stimulated respiration to levels significantly greater than those of control cultures. The difference in respiratory rate was statistically significant at 2 d and further increased at 3–4 d. In two experiments (not shown), the study was extended to 6 d, and the difference in respiration between T_3-exposed and control cultures was maintained, without further increase, which suggested that the effect of T_3 at 4 d was maximal. Cultures exposed to T_3 at 24 h postplating also exhibited a lag period and a time-course of response comparable to that shown in Fig. 1 (data not shown). Although these data have been expressed per total cellular protein, essentially identical curves were obtained when the results were expressed per microgram DNA, indicating that possible effects of T_3 on protein content or the protein/DNA ratio of the cultures were inconsequential.

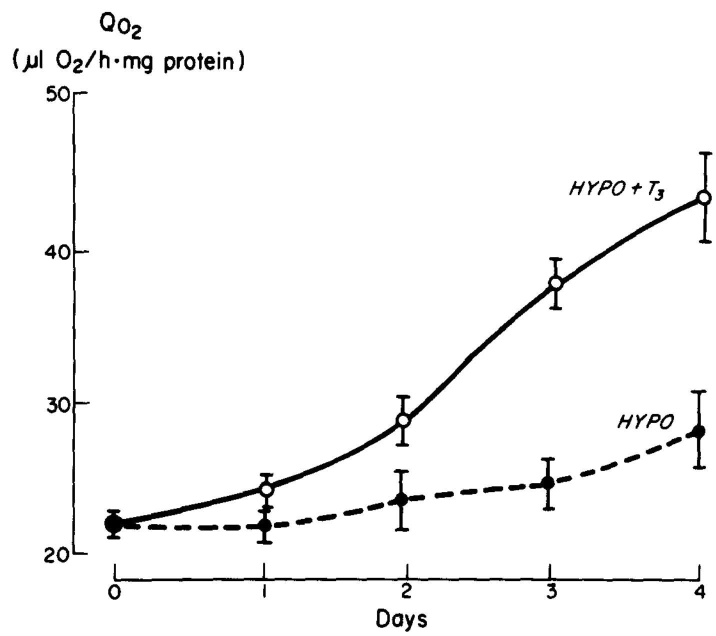

FIGURE 1. Time-course of effect of T_3 on Q_{O_2} of primary hepatocyte monolayer cultures. Cultures were prepared from hypothyroid rats (HYPO) and incubated in the absence (●) or the presence (○) of 2×10^{-7} M T_3. T_3 was added to the monolayers 4–6 h after plating and daily thereafter. Mean ± SE, $n = 4$, all determinations in duplicate.

During the 6 d of culture, the protein/DNA ratio remained constant; the protein and DNA contents declined in parallel as a consequence of some (~10%) loss of cells with daily changes of the medium.

Although the time-course of the response to T_3 was similar to that observed in liver slices prepared from rats treated with T_3 in vivo (Ismail-Beigi and Edelman, 1974), the increase in respiration could also have represented a toxic effect of this hormone, e.g., uncoupling of mitochondrial oxidative phosphorylation (Lardy and Feldott, 1951; Martius and Hess, 1951). If this were the case, the cells should exhibit decreased concentrations of ATP. However, total cellular ATP, in cells exposed for 3 d to concentrations of T_3 up to 2×10^{-7} M, was unaltered and ranged in all cultures from 9 to 10 nmol/mg protein. In addition, T_3 had no detectable effects on hepatocyte morphology evaluated in unstained preparations by phase microscopy.

Stimulation of Thyroid-Sensitive Enzyme Activities in Cultured Hepatocytes

NaK-ATPase and GPD activities were measured in parallel with cellular respiration. As shown in Fig. 2, the specific activity of NaK-ATPase in sonicates of monolayers obtained from hypothyroid rats fell slightly in the 1st d and remained relatively stable thereafter. In the presence of T_3, the specific activity of NaK-ATPase showed a time-dependent rise, regardless of whether expressed

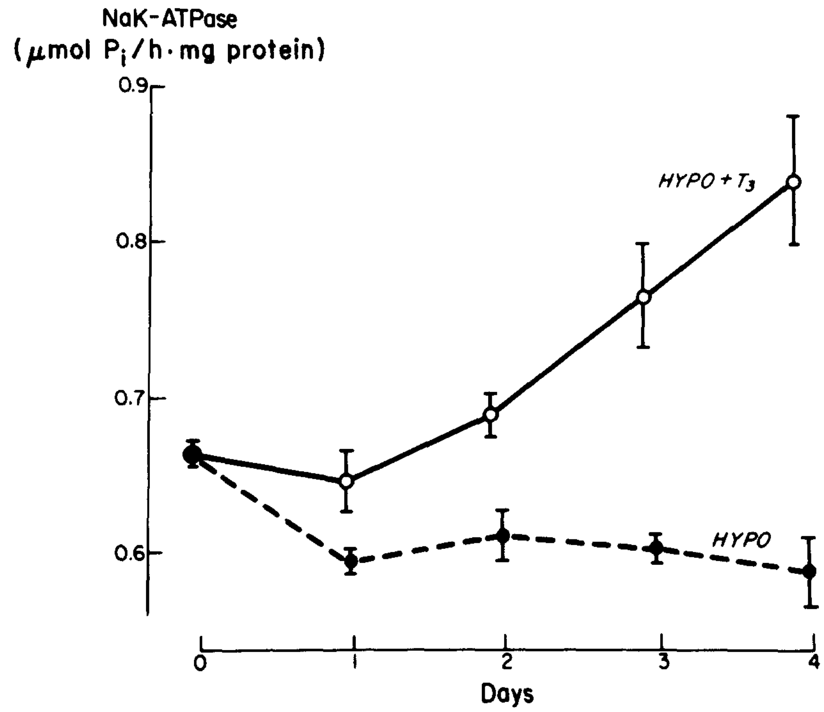

FIGURE 2. Time-course of effect of T_3 on NaK-ATPase activity of primary hepatocyte monolayer cultures. Conditions were the same as those in Fig. 1.

per milligram protein or per microgram DNA. Both the absolute value of NaK-ATPase activity of the hepatocytes and the magnitude of the response to T_3 are comparable to values obtained in crude liver homogenates of hypothyroid rats or rats injected with T_3 (Ismail-Beigi and Edelman, 1971, 1974). The activity of Mg-ATPase was unaffected by addition of T_3 to the culture medium.

Because thyroid hormone treatment in vivo is known to increase the specific activity of GPD in rat liver mitochondria (Lee et al., 1959, Lee and Lardy, 1965; Oppenheimer et al., 1977), we examined the effect of T_3 on this activity in culture, and the results are shown in Fig. 3. As with NaK-ATPase, GPD activity was stimulated by exposure of the cells to T_3. The time-course of the response of these two enzyme activities is strikingly similar to that of cellular respiration.

Relationship between Concentration and Effect of T_3 on Qo_2, NaK-ATPase, and GPD of Hepatocyte Monolayer Cultures

The relationship of Qo_2, NaK-ATPase, and GPD in cultured hepatocytes was further explored in studies of the T_3 dose-response curves for each activity in an individual batch of cells (Fig. 4). For these experiments, the response to T_3 was assessed 3 d after addition of the hormone to the culture medium and is expressed as a percent of the maximal response achieved (set at 100%). The dose-response curves for Qo_2, NaK-ATPase, and GPD were nearly identical. For all three parameters of thyroid effect, 4×10^{-11} M T_3 produced no change, whereas 4×10^{-8} M T_3 produced 90-100% of the maximal response. Half-maximal response occurred at $\sim 8 \times 10^{-11}$ M.

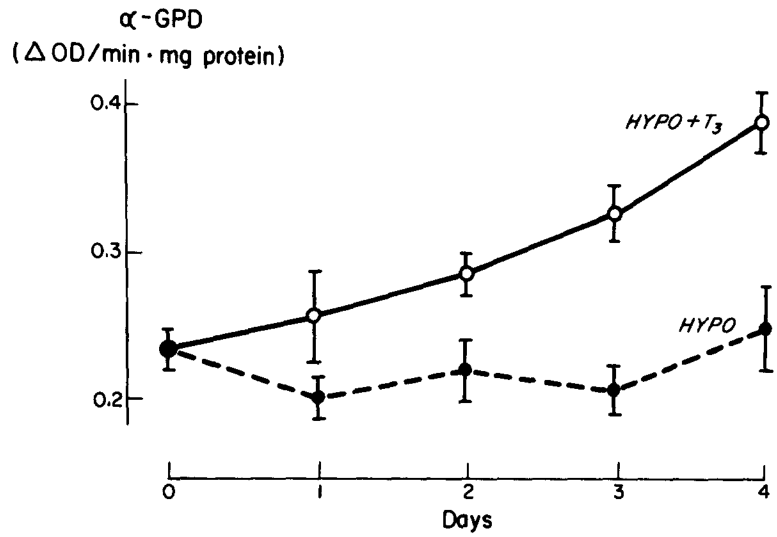

FIGURE 3. Time-course of effect of T_3 on mitochondrial α-GPD activity of primary hepatocyte monolayer cultures. Conditions were the same as those in Fig. 1.

Effect of Ouabain on the Respiratory Response to T_3

More than ~90% of the increase in Qo_2 of hypothyroid rat liver slices induced by injection of T_3 in vivo is inhibited by ouabain (Ismail-Beigi and Edelman, 1970, 1971; Israel et al., 1973). Preliminary studies demonstrated that the effect of ouabain on cellular respiration of hepatocytes in primary culture was maximal at 10^{-3} M, in agreement with previous observations (Ismail-Beigi and Edelman, 1971). The effect of ouabain on Qo_2 of hypothyroid and T_3-treated hepatocyte monolayers is summarized in Table I. In control cultures from hypothyroid rats, ouabain-sensitive respiration represented ~15 and 11% of the Qo_2 expressed per milligram protein and microgram DNA, respectively. In cultures exposed to 3×10^{-8} M T_3, ouabain inhibited ~90% of the increase in Qo_2. Thus, the ouabain-sensitive respiratory response to T_3 in hepatocyte cultures is similar to that of liver slices prepared from rats given T_3 in vivo.

Conditions Necessary for the Thermogenic Response of Hepatocyte Cultures to T_3

The complete medium for these studies contained insulin, corticosterone, and hypothyroid rat serum. Serum was necessary for efficient plating of hepatocytes prepared from resting rat liver (Bissell et al., 1973; Bonney, 1974; Bissell, 1976), and the hormones appeared to prolong the viability of the monolayers in this

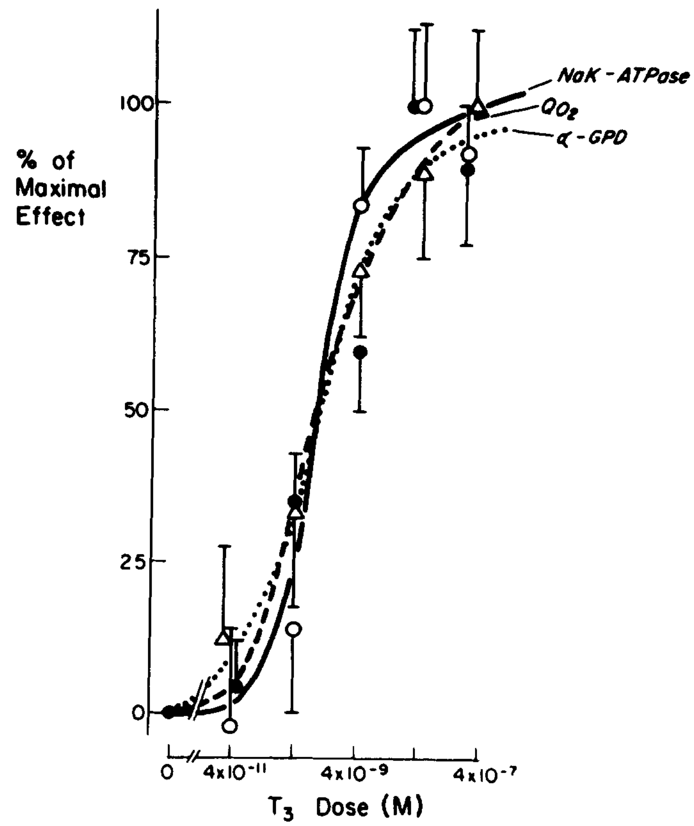

FIGURE 4. Relationship between concentration and effect of T_3 on Qo_2 (●) NaK-ATPase (○), and mitochondrial α-GPD (△) activity of primary hepatocyte monolayer cultures. Cells were exposed to various concentrations of T_3 4–6 h after plating and daily thereafter. Measurements were carried out 3 d after initial exposure to T_3. The maximum response in each parameter (calculated per microgram DNA) was taken as 100%, and the data are plotted as a percentage of the maximal effect. Mean ± SE, $n = 4$, all determinations in duplicate.

type of culture (unpublished observations). However, once the cells were established in culture (4–6 h postplating), serum could be deleted without significant detachment of cells from the plate. To examine the role of these additives in response to T_3, cells were cultured in the absence of insulin and corticosterone, or deprived of serum 4–6 h after plating, and the response to thyroid hormone was determined 3 d after addition of T_3. The data summarized in Table II indicate that T_3 stimulated cellular respiration under all of the conditions tested. Thus, the thermogenic effect of T_3 in the cultured hepatocytes is independent of these hormones and hypothyroid rat serum.

Culture morphology was altered with deletion of insulin and corticosterone from the medium, in that the cells appeared more attenuated and less granular than in complete medium. Concurrently, basal respiration of cells cultured under these conditions rose, relative to that of cells in the complete medium (Table II). Selective deletion of serum demonstrated that the change in Qo_2 was due to the absence of insulin and corticosterone rather than to the presence of serum. These alterations suggest a role for insulin and (or) corticosterone in controlling the basal respiration of differentiated hepatocytes.

Utilization of T_3 by Cultured Hepatocytes and Their Response to Single or Multiple Exposure to T_3

Because the liver is known to catabolize thyroid hormones in vivo (Taurog et al., 1952; Pitt-Rivers and Tata, 1959), utilization of T_3 by hepatocytes in culture

TABLE I

EFFECT OF OUABAIN ON Qo_2 OF PRIMARY RAT HEPATOCYTE CULTURES ± T_3*

	$-T_3$	$+T_3$	Δ	$\Delta Qo_2(t)/\Delta Qo_2 \times 100$
Control	21.8±1.1	28.8±3.0‡	7.0±1.6	
+Ouabain	18.6±2.0	19.9±2.3	1.3±0.7	
Qo_2(t)	3.2±0.3	8.9±1.2‡	5.7±0.9	
(per mg protein)				81±12%
Control	1.06±0.18	1.50±0.30	0.44±0.12	
+Ouabain	0.93±0.16	0.97±0.21	0.04±0.02	
Qo_2(t)	0.13±0.01	0.53±0.13‡	0.40±0.11	
(per μg DNA)				91±3%

* T_3 (3×10^{-8} M) was added 4 h after plating and daily thereafter; Qo_2 (ul O_2/h. × mg protein) was measured 3 d after the initial exposure to T_3 in the presence and absence of 10^{-3} M ouabain. Qo_2(t), ouabain-inhibitable respiration.

‡ $P < 0.05$ for T_3 vs. control values, mean ± SE, $n = 4$, all determinations in triplicate.

was examined. T_3 (3×10^{-8} M) added to complete medium in cell-free culture plates was stable for at least 6 h at 37°C (Fig. 5). By contrast, T_3 incubated with the hepatocyte monolayers diminished exponentially with an initial $t^1/_2$ of ~20 min and a later $t^1/_2$ of ~3.5 h. The concentration of T_3 in the medium 1 and 6 h after addition to the monolayers had diminished to about 42 and 12%, respectively, of the initial concentration. Measurement of cell-associated T_3 indicated that the disappearance of T_3 from culture media was due to metabolism of the hormone rather than uptake and binding of T_3 by the cells.[3]

These data suggest that the cells were exposed to stimulatory concentrations of T_3 for only a few hours after addition of the hormone to the medium. This would imply that a single short exposure to T_3 may be sufficient to elicit a thermogenic response. As a test of this possibility, hepatocytes were exposed to T_3 (3×10^{-8} M) for either a single period of 4 h terminated with a change to the

[3] Gavin, L. A., D. M. Bissell, M. E. Hammond, and R. R. Cavalieri. Unpublished observations.

T_3-free medium or repeatedly over 48 h, and the respiratory response was determined at 48 h. Repeated exposure to T_3 was obtained by renewing the medium ($+T_3$) at 4, 20, and 44 h. Brief contact with T_3 elicited an 18.6 ± 1.3% increase in Qo_2 and repeated exposure, an increase of 25.8 ± 3.0% (single/repeated = 0.72 ± 1.5, $n = 3$). The absolute Qo_2 in control plates subjected to the same changes in medium was 24 ± 2 μl/h·mg protein.

DISCUSSION

These findings demonstrate that T_3 stimulates increased respiration of adult rat hepatocytes in primary monolayer culture. As such, they constitute the first report of a thermogenic effect of thyroid hormone in an isolated cell system. Hypothyroid rats were used as the donors for the hepatocytes and the serum, in order to minimize endogenous thyroid hormone effects and to maximize the

TABLE II

EFFECT OF T_3 ON Qo_2 OF PRIMARY RAT HEPATOCYTE CULTURES IN VARIOUS MEDIA*

		Qo_2		
Insulin and corticoserone	Serum	$-T_3$	$+T_3$	%Δ
+	+	27.4±3.5	33.9±3.0	+25‡
−	+	47.8±8.2	62.6±9.5	+32‡
+	−	26.9±3.0	32.7±4.5	+19‡
−	−	40.0±8.0	52.4±7.5	+31‡

* Insulin (4 mU/ml), corticosterone (10^{-6} M), and hypothyroid rat serum (2%, v_0/v_0) were included in the culture medium, as indicated. All cultures were initiated in the presence of serum. For studies under serum-free conditions, serum was removed with a medium change at 4–6 h after cell plating. T_3 (3×10^{-8} M) was added 4–6 h after cell plating and renewed with daily changes of the medium. Qo_2 (μl O_2/h × mg protein) was measured 3 d after addition of T_3.
‡ $P < 0.05$ for T_3-treated plates with respect to control plates from the same batch of cells (paired t test), mean ± SE, $n = 3$, all determinations in duplicate.

response to T_3 added in vitro. T_3 appeared to exert its effect in the absence of other hormones or serum factors. Although the standard culture conditions included corticosterone, insulin, and hypothyroid rat serum, these supplements were not required for the thermogenic effect of T_3 in culture (Table II). The effective concentration of T_3 appeared to be within the physiological range, with the half-maximal increase in Qo_2 occurring at a nominal concentration of 8×10^{-10} M. The precise concentration of metabolically active "free" T_3 is unknown under these culture conditions. Because the culture medium contained only 2% serum, the unbound fraction of T_3 presumably is greater than in vivo. On the other hand, the culture medium was renewed only at 24-h intervals. Thus, with degradation, the average concentration of hormone over a 24-h period was considerably less than the nominal (initial value). Previous studies in vivo have suggested that a serum T_3 level of 1.5×10^{-8} M produces > 95% occupancy of "specific T_3 nuclear receptors" (Oppenheimer et al., 1972; Samuels and Tsai, 1973; DeGroot and Strausser, 1974) and near-maximal responses of hepatic GPD and malic enzyme activity (Oppenheimer et al., 1977).

The response of the cultured cells to T_3 in many ways mimicked the response assayed in liver slices and various cell fractions after administration of T_3 to hypothyroid rats. Previous studies with tissue slices from thyroid-treated animals have shown a close correlation between stimulation of Q_{O_2} and of NaK-ATPase activity, the latter appearing to involve de novo synthesis of the enzyme in rat kidney (Lo et al., 1976; Lo and Edelman, 1976). In liver, the increase in NaK-ATPase may reflect increased transport of sodium both at the plasma membrane of the cell and at the canalicular membrane, the latter process mediating, in part, the formation of bile (Layden and Boyer, 1976). Similarly, the respiratory response of the hepatocytes in culture was associated with parallel increases in NaK-ATPase and GPD activity, and the T_3 dose-response curves for Q_{O_2}, NaK-ATPase, and GPD were nearly identical. These findings in intact hepatocytes

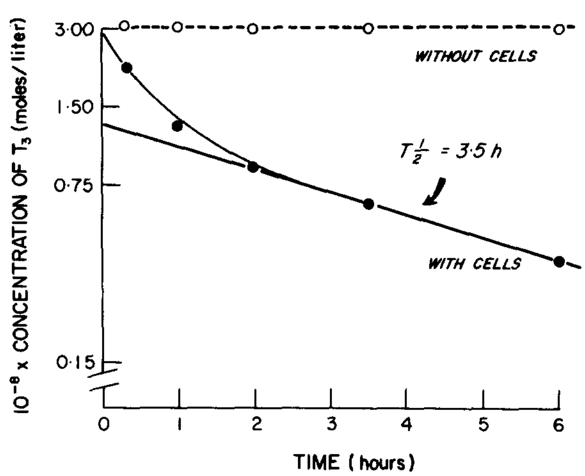

FIGURE 5. Disappearance of T_3 from the medium of hepatocyte cultures. Complete culture medium with T_3 was added to plates that either contained (●) or did not contain (○) hepatocyte monolayers. At the time points shown, the concentration of T_3 in the medium was determined by radioimmunoassay. Each point represents the mean of duplicate plates.

confirm previous results with liver slices (Ismail-Beigi and Edelman, 1971, 1974). In addition, in both preparations (liver slices prepared from animals treated with T_3 in vivo and hepatocytes incubated in T_3 in vitro) the T_3-dependent increase in Q_{O_2} (t) was about 90% (or more) of the increase in Q_{O_2} (Ismail-Beigi and Edelman, 1970, 1971). The physiological significance of the results obtained with liver slices (and now with primary hepatocyte cultures) was called into question recently by Folke and Sestoft (1977), on the basis of experiments with the isolated rat liver, perfused with beef erythrocytes. In their preparation, ouabain-inhibitable respiration constituted a negligible fraction of total Q_{O_2} during the first 12 min of exposure to the inhibitor. In addition, they estimated that active Na^+ and K^+ fluxes account for only 5–6% of the observed rate of hepatic oxygen consumption. The nature of the discrepancies between the findings of Folke and Sestoft (1977) and our present and previous studies is speculative. Cultured hepatocytes and the perfused liver differ in several important respects: The hepatocyte cultures consist almost solely of parenchymal cells (Bissell et al., 1973), but the perfused liver is heterogeneous with respect to cell type (circulating erythrocytes, vascular wall elements, and reticuloendothelial cells, in addition to hepatocytes). In addition, early effects of ouabain on the heterologous (beef) erythrocytes and on the vasculature may dominate the respiratory patterns. These issues are complex and not directly germane to the primary aim of the present study, i.e., the establishment of a cell system thermogenically responsive to thyroid hormone, in vitro. However, a detailed analysis of the relevance of the findings in isolated systems, e.g., liver perfusion, liver slices, diaphragm, intestinal epithelium, and kidney tubules, to thyroid thermogenesis in vivo is now in preparation.[4]

The response of the cultured hepatocytes may be distinguished in some respects from that of liver slices prepared from animals in various thyroid states. Specifically, the onset and peak change in Q_{O_2} or in the associated enzyme activities are moderately delayed in the hepatocyte system. The protracted time-course suggests that the status of nuclear T_3 receptors in culture and the relationship between nuclear occupancy and physiological response should be examined. Similarly, the absolute magnitude of Q_{O_2} in control cultures is almost three times that of hypothyroid rat liver slices (Ismail-Beigi and Edelman, 1971). This difference may reflect damage to a proportion of the cells during preparation of tissue slices. Also, oxygenation of the innermost cells of a slice may be relatively poor—these combining to produce artifactually low values for Q_{O_2} by tissue slices. Moreover, in slices, the observed Q_{O_2} reflects contributions from both parenchymal and nonparenchymal cell types. On the other hand, hepatocytes in culture may require increased energy expenditure for elaboration of surface constituents or cellular macromolecules associated with metabolic adaptation and/or attachment of cells to the culture plates (Bissell, 1976). Consistent with the latter postulate is the finding that, whereas the ouabain-inhibitable component of Q_{O_2} is comparable to that of liver slices (Ismail-Beigi and Edelman, 1971, 1974), ouabain-insensitive respiration is elevated. Finally, basal respiration was enhanced after removal of insulin and corticosterone from the culture medium (Table II) in association with a definite change in culture morphology. Although we have not characterized the ouabain sensitivity of cells cultured in the absence of the supplementary hormones, the associated morphologic changes suggest that the increase in Q_{O_2} may reflect an increase in energy expenditure required for adaptation of the cells to a new set of environmental conditions. Alternatively, partial uncoupling of oxidative phosphorylation may supervene in the absence of insulin and corticosterone in the medium.

The hepatocyte monolayer system should prove valuable for analyzing the nature and sequence of biochemical events mediating expression of thyroid thermogenesis. The response to thyroid hormone in vivo is characterized by an absolute lag period of 12–16 h, followed by a gradual increase in respiratory rates to a peak 48–72 h after administration of the hormone (Ismail-Beigi and Edelman, 1974; Tata and Widnell, 1966). The response of the cells in culture to

[4] Edelman, I. S., and T. J. Smith. Personal communication.

T_3 involves a similar delay. This period apparently is required for processing the hormonal signal and may involve events both at the transcriptional and at the translational level (Tata and Widnell, 1966; Tata, 1968; Kurtz et al., 1976). With regard to these events, it was unknown whether stimulatory levels of thyroid hormone are required only initially or must be sustained for expression of the thermogenic response. In the present studies, a single 4-h exposure stimulated 75% of the full thermogenic response when measured 48 h after removal of the hormone from the culture. If the rapid disappearance of T_3 from the culture (Fig. 5) is an index of a similar rapid disappearance of T_3 from intracellular receptors (as noted previously in vivo [Oppenheimer et al., 1974]), the observed peak of the response may have been obtained when receptor occupancy was virtually nil. These data suggest that thyroid hormone is required only for initiating a thermogenic effect and that its expression proceeds in the absence of the hormone.

The authors are grateful to Mrs. Yar Fen Teng and Miss Jennifer D'Artel-Ellis for technical assistance.

This study was supported from U.S. Public Health Service grants from the National Heart, Lung, and Blood Institute (HL-06285), General Medical Sciences (GM-21042), and National Institute of Arthritis, Metabolic, and Digestive Diseases (AM-11275 and P50 AM-18520), and by the Walter C. Pew Fund for Gastrointestinal Research. Dr. Bissell is the recipient of U.S. Public Health Service Research Career Development Award GM-00149.

A preliminary report of this work was presented at the 69th Annual Meeting of the American Society for Clinical Investigation, May 1977 (*Clin. Res.* **25**:464A).

Received for publication 28 June 1978.

REFERENCES

ALEXANDER, W. D., and J. WOLFF. 1966. Thyroidal iodide transport. VIII. Relation between transport, goitrogenic and autogoitrogenic properties of certain anions. *Endocrinology.* **78**:584–590.

ASANO, Y., U. A. LIBERMAN, and I. S. EDELMAN. 1976. Thyroid thermogenesis. Relationships between Na^+-dependent respiration and $Na^+ + K^+$-adenosine triphosphatase activity in rat skeletal muscle. *J. Clin. Invest.* **57**:368–379.

BARKER, S. B. 1951. Mechanism of action of thyroid hormone. *Physiol. Rev.* **31**:205–243.

BISSELL, D. M. 1976. Study of hepatocyte function in cell culture. *In* Progress in Liver Diseases. Vol. V. H. Popper and F. Schaffner, editors. Grune & Stratton Inc., New York. 69–82.

BISSELL, D. M., L. E. HAMMAKER, and U. A. MEYER. 1973. Parenchymal cells from adult rat liver in non-proliferating monolayer culture. I. Functional studies. *J. Cell Biol.* **59**: 722–734.

BISSELL, D. M., G. A. LEVINE, and M. J. BISSELL. 1978. Glucose metabolism by adult hepatocytes in primary culture and by cell lines from rat liver. *Am. J. Physiol.* **234**: C122–C130.

BONNEY, R. J. 1974. Adult liver parenchymal cells in primary culture: characteristics and cell recognition standards. *In Vitro.* **10**:130–142.

BURTON, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62**:315–323.

CHAPMAN, G. S., A. L. JONES, U. A. MEYER, and D. M. BISSELL. 1973. Parenchymal cells from adult rat liver in nonproliferating monolayer culture. II. Ultrastructural studies. *J. Cell Biol.* **59:**735–747.

DEGROOT, L. J., and J. L. STRAUSSER. 1974. Binding of T_3 in rat liver nuclei. *Endocrinology.* **95:**74–83.

EDELMAN, I. S., and F. ISMAIL-BEIGI. 1974. Thyroid thermogenesis and active sodium transport. *Recent Prog. Horm. Res.* **30:**235–257.

FOLKE, M., and L. SESTOFT. 1977. Thyroid calorigenesis in isolated perfused rat liver: minor role of active sodium-potassium transport. *J. Physiol. (Lond.).* **269:**407–419.

FRIED, G. H., N. GREENBERG, and W. ANTOPOL. 1961. Determination of alpha-glycerophosphate oxidation by tetrazolium. *Proc. Soc. Exp. Biol. Med.* **107:**523–525.

GHARIB, H., R. J. RYAN, W. E. MAYBERRY, and T. HOCKERT. 1971. Radioimmunoassay for triiodothyronine (T_3). I. Affinity and specificity of the antibody for T_3. *J. Clin. Endocrinol. Metab.* **33:**509–516.

ISMAIL-BEIGI, F., and I. S. EDELMAN. 1970. Mechanism of thyroid calorigenesis: role of active sodium transport. *Proc. Natl. Acad. Sci. U.S.A.* **67:**1071–1078.

ISMAIL-BEIGI, F., and I. S. EDELMAN. 1971. The mechanism of the calorigenic action of thyroid hormone: stimulation of $Na^{+}+K^{+}$-activated adenosinetriphosphatase activity. *J. Gen. Physiol.* **57:**710–722.

ISMAIL-BEIGI, F., and I. S. EDELMAN. 1974. Time-course of the effect of thyroid hormone on respiration and $Na^{+}+K^{+}$-ATPase activity in rat liver. *Proc. Soc. Exp. Biol. Med.* **146:**983–988.

ISRAEL, W., L. VIDELA, and A. MACDONALD. 1973. Metabolic alterations produced in the liver by chronic ethanol administration. Comparison between the effects produced by ethanol and by thyroid hormones. *Biochem. J.* **134:**523–529.

KURTZ, D. T., A. E. SIPPEL, and P. FEIGELSON. 1976. Effect of thyroid hormones on the level of the hepatic mRNA for $\alpha 2_{\mu}$globulin. *Biochemistry.* **15:**1031–1036.

LARDY, H., and G. FELDOTT. 1951. Metabolic effects of thyroxine in vitro. *Ann. N. Y. Acad. Sci.* **54:**636–644.

LAYDEN, T. J., and J. L. BOYER. 1976. The effect of thyroid hormone on bile salt-independent bile flow and Na^{+},K^{+}-ATPase activity in liver plasma membranes enriched in bile canaliculi. *J. Clin. Invest.* **57:**1009–1018.

LEE, Y-P., and H. A. LARDY. 1965. Influence of thyroid hormones on L-α-glycerophosphate dehydrogenases and other dehydrogenases in various organs of the rat. *J. Biol. Chem.* **240:**1427–1436.

LEE, Y-P., A. E. TAKEMORI, and H. A. LARDY. 1959. Enhanced oxidation of alpha-glycerophosphate dehydrogenase by mitochondria of thyroid fed rats. *J. Biol. Chem.* **234:**3051–3054.

LO, C-S., T. R. AUGUST, U. A. LIBERMAN, and I. S. EDELMAN. 1976. Dependence of renal ($Na^{+}+K^{+}$)-adenosine triphosphatase activity on thyroid status. *J. Biol. Chem.* **251:** 7826–7833.

LO, C-S., and I. S. EDELMAN. 1976. Effect of triiodothyronine on synthesis and degradation of renal cortical ($Na^{+}+K^{+}$)-adenosine triphosphatase. *J. Biol. Chem.* **251:** 7834–7840.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* **193:**265–275.

MARTIAL, J. A., J. D. BAXTER, H. M. GOODMAN, and P. H. SEEBURG. 1977. Regulation of growth hormone messenger RNA by thyroid and glucocorticoid hormones. *Proc. Natl. Acad. Sci. USA.* **74:**1816–1820.

MARTIUS, C., and B. HESS. 1951. The mode of action of thyroxine. *Arch. Biochem. Biophys.* **33**:486–487.

MORGAN, J. F., H. J. MORTON, and R. C. PARKER. 1950. Nutrition of animal cells in tissue culture. I. Initial studies on a synthetic medium. *Proc. Soc. Exp. Biol. Med.* **73**:1–8.

OPPENHEIMER, J. H., D. KOERNER, H. L. SCHWARTZ, and M. I. SURKS. 1972. Specific nuclear triiodothyronine binding sites in rat liver and kidney. *J. Clin. Endocrinol. Metab.* **35**:330–333.

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 interrelation, binding constants, and cross-reactivity with L-thyroxine. *J. Clin. Invest.* **53**:768–777.

OPPENHEIMER, J. H., E. SILVA, H. L. SCHWARTZ, and M. I. SURKS. 1977. Stimulation of hepatic mitochondrial α-glycerophosphate dehydrogenase and malic enzyme by L-triiodothyronine. Characteristics of the responses with specific nuclear thyroid hormone binding sites fully saturated. *J. Clin. Invest.* **59**:517–527.

PITT-RIVERS, R., and J. R. TATA. 1959. The Thyroid Hormones. Pergamon Press Ltd., London. 1–247.

RAHIMIFAR, M., and F. ISMAIL-BEIGI. 1977. Lack of thyroid hormone effect on activation energy of NaK-ATPase. *Mol. Cell. Endocr.* **6**:327–331.

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.

SNEDECOR, G. W., and W. G. COCHRAN. 1967. *Statistical Methods.* The Iowa University Press, Ames. 6th Edition. 1–593.

TATA, J. R. 1968. Coordinated formation of membranes and biosynthetic activity during growth and development. *In* Regulatory Function of Biological Membranes. BBA Library Series, Vol. 11. J. Jarnefelt, editor. North-Holland Publishing Co., Amsterdam. 222–235.

TATA, J. R., and C. C. WIDNELL. 1966. Ribonucleic acid synthesis during the early action of thyroid hormones. *Biochem. J.* **98**:604–620.

TAUROG, A., F. N. BRIGGS, and I. L. CHAIKOFF. 1952. I^{131}-Labeled L-thyroxine. II. Nature of the excretion product in bile. *J. Biol. Chem.* **194**:655–668.

TSAI, J. S., and A. CHEN. 1976. Thyroid hormones: effect of physiological concentrations on cultured cardiac cells. *Science (Wash. D.C.).* **194**:202–204.

UMBREIT, W. W., R. H. BURRIS, and J. F. STAUFFER. 1957. Manometric Techniques. Burgess Publishing Company, Minneapolis, Minn. 3rd edition. 333.