Title
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Permalink
https://escholarship.org/uc/item/96x959tw

Journal
The Journal of general physiology, 73(3)

ISSN
0022-1295

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Publication Date
1979-03-01

DOI
10.1085/jgp.73.3.369

Peer reviewed
Thyroid Thermogenesis in Adult Rat Hepatocytes in Primary Monolayer Culture

Direct Action of Thyroid Hormone In Vitro

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ABSTRACT We have studied the effect of 3,5,3'-triiodothyronine (T₃) on the respiration of adult rat hepatocytes in primary monolayer culture prepared from hypothyroid rat liver. After addition of T₃ 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 T₃, in parallel with the change in oxygen consumption, whereas the activity of Mg-dependent ATPase was unaffected. These responses to T₃ were dose dependent over similar concentration ranges, the half-maximal response for each occurring at ca 8 × 10⁻⁷ 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 T₃ was sufficient to elicit a significant thermogenic response, measured at a time (48 h later) when T₃ was no longer present in the medium. The response to T₃ 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 T₃ 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 T₃ 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
thermogenesis. It was reported previously that stimulation of oxygen consumption \( (Q_{O_2}) \) 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 \( Q_{O_2} \) (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 \( Q_{O_2} \) 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 \( Q_{O_2} \) 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.
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morbidity (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 T₃, 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 T₃ 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% NaClO₄ 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 ± 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. 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 × 10⁶ 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, 10⁻⁴ 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 T₃. 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. T₃ was dissolved in 5 × 10⁻⁴ M NaOH, and aliquots of the stock solution were frozen at −20°C. Before use, T₃ 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 (Qₒ₂) 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 Qₒ₂ 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

Somjen, D., F. Ismail-Beigi, and I. S. Edelman. Manuscript submitted for publication.
[\(Q_{O_2}(t)\)] was estimated from cultures incubated in the presence or absence of \(10^{-3}\) M ouabain in parallel plates, in duplicate. The initial measurements of \(Q_{O_2}\) (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 \(Q_{O_2}\) 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 \(Q_{O_2}\), 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-dicarboxymethylaminoethoxy)ethane (EGTA), scraped from the plates in 2.0 ml of the above solution and sonicated in an ice bath four times at 5 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 \(10^{-5}\) 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, Dickenson & 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 \(Q_{O_2}\) 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 \(Q_{O_2}\) 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 T₃-exposed and control cultures was maintained, without further increase, which suggested that the effect of T₃ at 4 d was maximal. Cultures exposed to T₃ 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 T₃ on protein content or the protein/DNA ratio of the cultures were inconsequential.

![Figure 1](image_url)

**Figure 1.** Time-course of effect of T₃ on QO₂ of primary hepatocyte monolayer cultures. Cultures were prepared from hypothyroid rats (HYPO) and incubated in the absence (●) or the presence (○) of 2 x 10⁻⁷ M T₃. T₃ 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 T₃ was similar to that observed in liver slices prepared from rats treated with T₃ 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 T₃ up to 2 x 10⁻⁷ M, was unaltered and ranged in all cultures from 9 to 10 nmol/mg protein. In
addition, T₃ 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 T₃, the specific activity of NaK-ATPase showed a time-dependent rise, regardless of whether expressed 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 T₃ are comparable to values obtained in crude liver homogenates of hypothyroid rats or rats injected with T₃ (Ismail-Beigi and Edelman, 1971, 1974). The activity of Mg-ATPase was unaffected by addition of T₃ 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 T₃ 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 T₃. The time-course of the response of these two enzyme activities is strikingly similar to that of cellular respiration.

![Figure 2](image-url)
Relationship between Concentration and Effect of T₃ on Qₒ₂, NaK-ATPase, and GPD of Hepatocyte Monolayer Cultures

The relationship of Qₒ₂, NaK-ATPase, and GPD in cultured hepatocytes was further explored in studies of the T₃ dose-response curves for each activity in an individual batch of cells (Fig. 4). For these experiments, the response to T₃ 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 Qₒ₂, NaK-ATPase, and GPD were nearly identical. For all three parameters of thyroid effect, 4 × 10⁻¹¹ M T₃ produced no change, whereas 4 × 10⁻⁸ M T₃ produced 90-100% of the maximal response. Half-maximal response occurred at ~8 × 10⁻¹¹ M.

![Graph showing time-course of effect of T₃ on mitochondrial α-GPD activity of primary hepatocyte monolayer cultures. Conditions were the same as those in Fig. 1.](image)

**Figure 3.** Time-course of effect of T₃ 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 T₃

More than ~90% of the increase in Qₒ₂ of hypothyroid rat liver slices induced by injection of T₃ 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 Qₒ₂ of hypothyroid and T₃-treated hepatocyte monolayers is summarized in Table I. In control cultures from hypothyroid rats, ouabain-sensitive respiration represented ~15 and 11% of the Qₒ₂ expressed per milligram protein and microgram DNA, respectively. In cultures exposed to 3 × 10⁻⁸ M T₃, ouabain inhibited ~90% of the increase in Qₒ₂. Thus, the ouabain-sensitive respiratory response to T₃ in hepatocyte cultures is similar to that of liver slices prepared from rats given T₃ in vivo.
Conditions Necessary for the Thermogenic Response of Hepatocyte Cultures to T₃

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 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 T₃, 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 T₃. The data summarized in Table II indicate that T₃ stimulated cellular respiration under all of the

![Graph showing the relationship between concentration and effect of T₃ on QO₂ (O), NaK-ATPase (●), and mitochondrial α-GPD (Δ) activity of primary hepatocyte monolayer cultures. Cells were exposed to various concentrations of T₃ 4-6 h after plating and daily thereafter. Measurements were carried out 3 d after initial exposure to T₃. 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.](image-url)
conditions tested. Thus, the thermogenic effect of T₃ 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 Qₒ₂ 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 T₃ by Cultured Hepatocytes and Their Response to Single or Multiple Exposure to T₃**

Because the liver is known to catabolize thyroid hormones in vivo (Taurog et al., 1952; Pitt-Rivers and Tata, 1959), utilization of T₃ by hepatocytes in culture was examined. T₃ (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, T₃ incubated with the hepatocyte monolayers diminished exponentially with an initial t½ of ~20 min and a later t½ of ~3.5 h. The concentration of T₃ 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 T₃ indicated that the disappearance of T₃ from culture media was due to metabolism of the hormone rather than uptake and binding of T₃ by the cells.³

These data suggest that the cells were exposed to stimulatory concentrations of T₃ for only a few hours after addition of the hormone to the medium. This would imply that a single short exposure to T₃ may be sufficient to elicit a thermogenic response. As a test of this possibility, hepatocytes were exposed to T₃ (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.
Ta-free medium or repeatedly over 48 h, and the respiratory response was determined at 48 h. Repeated exposure to T3 was obtained by renewing the medium (+T3) at 4, 20, and 44 h. Brief contact with T3 elicited an 18.6 ± 1.3% increase in QO₂ and repeated exposure, an increase of 25.8 ± 3.0% (single/repeated = 0.72 ± 1.5, n = 3). The absolute QO₂ in control plates subjected to the same changes in medium was 24 ± 2 μl/h·mg protein.

**DISCUSSION**

These findings demonstrate that T3 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

| Insulin and corticosterone | Serum | QO₂ | %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/v) 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. T3 (3 x 10⁻¹⁰ M) was added 4-6 h after cell plating and renewed with daily changes of the medium. QO₂ (μl O₂/h·mg protein) was measured 3 d after addition of T3. * P < 0.05 for T3-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 T3 added in vitro. T3 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 T3 in culture (Table II). The effective concentration of T3 appeared to be within the physiological range, with the half-maximal increase in QO₂ occurring at a nominal concentration of 8 x 10⁻¹⁰ M. The precise concentration of metabolically active “free” T3 is unknown under these culture conditions. Because the culture medium contained only 2% serum, the unbound fraction of T3 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 T3 level of 1.5 x 10⁻⁶ M produces >95% occupancy of “specific T3 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 T₃ in many ways mimicked the response assayed in liver slices and various cell fractions after administration of T₃ to hypothyroid rats. Previous studies with tissue slices from thyroid-treated animals have shown a close correlation between stimulation of Q₀₂ 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 T₃ dose-response curves for Q₀₂, NaK-ATPase, and GPD were nearly identical. These findings in intact hepatocytes confirm previous results with liver slices (Ismail-Beigi and Edelman, 1971, 1974). In addition, in both preparations (liver slices prepared from animals treated with T₃ in vivo and hepatocytes incubated in T₃ in vitro) the T₃-dependent increase in Q₀₂ (t) was about 90% (or more) of the increase in Q₀₂ (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 Q₀₂ 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

![Figure 5](image-url)
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 (bovine) 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 $QO_2$ or in the associated enzyme activities are moderately delayed in the hepatocyte system. The protracted time-course suggests that the status of nuclear T3 receptors in culture and the relationship between nuclear occupancy and physiological response should be examined. Similarly, the absolute magnitude of $QO_2$ 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 artifically low values for $QO_2$ by tissue slices. Moreover, in slices, the observed $QO_2$ 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 $QO_2$ 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 $QO_2$ 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.
T₃ 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 T₃ from the culture (Fig. 5) is an index of a similar rapid disappearance of T₃ 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.

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