ease by Rebel *et al.* (*3*). They postulated the presence of a papovavirus, but 20 serums from our patients with Paget's disease showed no increase in papovavirus antibody titers (*4*). Rebel *et al.* could not find similar nuclear inclusions in biopsies from patients with a variety of other bone diseases. Since two laboratories now have identified these inclusions in all cases of Paget's disease studied (41 patients) and not in any (72 patients) without Paget's disease, it is probable that they are not normally found in osteoclasts. The only other instance of similar intranuclear microfilaments observed in bone cells has been reported by Welsh and Meyer in two patients with giant cell tumors which contain innumerable osteoclasts (*5*).

The significance of the nuclear inclusions we have observed in the osteoclasts of patients with Paget's disease is not known. Since nuclear bodies of several types have been found in many different human and animal cells, it is possible that the inclusions are an indication of some unidentified pathologic process. Bouteille *et al.* (*6*) have concluded that "simple" nuclear bodies may be nuclear organelles associated with cellular hyperactivity. In Paget's disease, there is great cellular activity, and we did observe "simple" and "complex" nuclear bodies in osteoclasts and other bone cells. However, the nuclear inclusions found only in the osteoclasts of Paget's disease and giant cell tumor do not resemble any of the nuclear bodies described by Bouteille *et al.* (*6*).

The possibility that the pagetic inclusions are a handling or processing artifact is remote, since all the bone biopsies were handled in an identical manner and none were found in the nonpagetic specimens. The inclusions are unlike prenecrotic nuclear changes (*7*), although some of the osteoclasts and their nuclei appeared to be degenerating. In nine patients with renal osteodystrophy in whom osteoclastic degeneration was noted this was not accompanied by the nuclear inclusions seen in pagetic patients. Since mitoses were not observed in any of the pagetic osteoclasts, it seems unlikely that the microfilaments are associated with the mitotic spindle which contains microfilaments of 28 nm in diameter (*8*). Neither are the nuclear inclusions pseudonuclei, as can be seen by observing the nuclear remnants of the ring-shaped nuclei with the enclosed microfilaments. Likewise, the nuclear inclusions of multiple sclerosis or lupus erythematosis are morphologically distinct from those seen in Paget's disease (*9*).

The ultrastructural findings in the osteoclasts of patients with Paget's disease resemble those that have been reported in nerve cells from patients with subacute sclerosing panencephalitis (*10*). The clinical course of this central nervous system disorder is not unlike that of Paget's disease in which localized lesions may remain latent over many years. Evidence exists which implicates a measles-like virus as the etiologic agent responsible for the disorder (*11*). The typical inclusions that we have observed are intranuclear, the same size, tubular, hexagonally packed, and sometimes found in single strands. The primary difference is that they appear "stiffer," with less undulations than measles nucleocapsids.

In addition to the morphology, two other features suggest a viral presence in the osteoclasts. The presence of degenerating nuclei and the apparent release of the microfilaments into the cell cytoplasm is a sequence of events known to occur with viral infections. The formation of multinucleated cells occurs after measles infection, and, although osteoclasts normally contain multiple nuclei, in Paget's disease there may be increased numbers of nuclei. Since neither budding nor particles typical of mature virions were seen, proof of a viral nature of these microfilaments will await the isolation of an agent or identification of a viral antigen.

Although the origin and significance of the nuclear inclusions in Paget's disease remain to be determined, the finding of characteristic nuclear inclusions in pagetic osteoclasts suggests that these will be important markers in studies of the etiology of Paget's disease.

BARBARA G. MILLS
Department of Physiology, School of Dentistry, University of Southern California, Los Angeles 90007
FREDERICK R. SINGER
Department of Medicine, School of Medicine, University of Southern California, Los Angeles 90033

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Thyroid Hormones: Effect of Physiological Concentrations on Cultured Cardiac Cells

Abstract. *Cultured cardiac cells prepared from newborn rat heart will respond in vitro to physiological concentrations of* L*-triiodothyronine. The cells are grown in a culture medium that contains hypothyroid calf serum. A dose response relationship of* L*-triiodothyronine indicates that this system may be a useful model for elucidation of the mechanism of thyroid hormone effects on the heart.*

Thyroid hormones have a variety of biological effects in numerous organ systems. In spite of extensive studies in vivo (*1*), the mechanism of action of the thyroid hormones on the heart remains to be defined. By using cultures of GH_1 cells (*2*), a clone derived from a rat pituitary tumor, it has been shown that L-thyroxine (T4) and L-triiodothyronine (T3) modulate the rate of prolactin and growth hormone production as well as the rate of GH_1 cell replication (*3*, *4*). Because the cellular receptors for thyroid hormones, which appear to mediate these biologic responses, are localized in the cell nucleus (*5*, *6*), cultured GH_1 cells are useful for studying the molecular aspects of thyroid hormone action with particular regard to control of pituitary function.

Although there have been several studies of the effects of thyroid hormone on isolated cardiac tissue (*7*), no myocardial system responsive to physiological concentrations of thyroid hormones in vitro has been described. We have now developed such a system.

Myocardial cells were prepared as described by Harary and Farley (*8*). The cells were derived from the ventricular tissue of Sprague-Dawley rats aged 1 to 3 days. The cardiac tissue was excised and blood clots were rinsed off with Hanks balanced salt solution (BSS) (Gibco). The tissue was minced into small fragments of approximately 1 to 2 mm in diameter. These fragments were digested with 0.12 percent of trypsin (Difco, 1:250) in Mg^{2+}- and Ca^{2+}-free Hanks BSS buffer in a shaking water bath at 37°C. The dispersed cells were removed by aspiration every 15 to 20 minutes and then washed twice by centrifugation with 10 ml of buffer solution to remove trypsin. The cells were pooled, suspended in Ham's F-10 medium (Gibco) supplemented with 10 percent fetal calf serum (FCS), and inoculated into plastic T-flasks (75 cm^2, Falcon) for 1 to 2 hours. During this time, the dispersed fibroblasts selectively attached to the plastic surface (*9*). The myocardial cells, which require longer incubation for attachment, remained in the culture medium. The myocardial cells were then inoculated into separate T-flasks or a multiple well plate (Linbro) for long-term culture studies; densities ranged from 200,000 to 400,000 cells per square centimeter. The cells were incubated at 37°C (95 percent air, 5 percent CO_2) and after 24 hours were observed to contract spontaneously. Subsequently, the cells organized into groups of cell colonies which could also contract spontaneously in culture for up to 2 months. The cultured cells appeared morphologically uniform when examined by light microscopy; when examined with the electron microscope they contained contractile elements similar to those in rat heart. Myocardial cells in culture, in exhibiting rhythmic contractions, and retaining their morphologic as well as physiologic properties, are thus similar to myocardial cells in vivo.

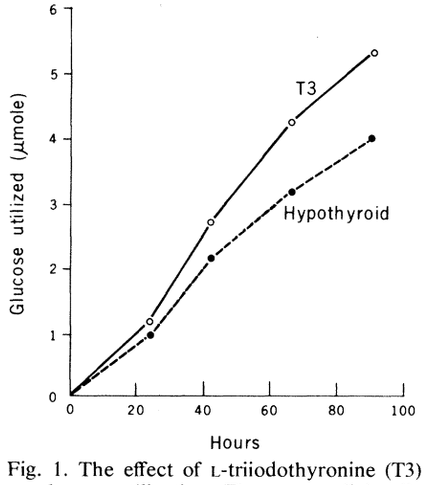

Fig. 1. The effect of L-triiodothyronine (T3) on glucose utilization. The myocardial cells were inoculated into a multiple well plate (Linbro), the cell densities ranging from 200,000 to 400,000 cells per square centimeter. The cells were incubated for 24 hours with hypothyroid medium to deplete them of thyroid hormone; this medium was then replaced with either hypothyroid medium or medium containing a final concentration of $1 \times 10^{-8}M$ T3. Samples (50 μl) of medium were taken for glucose determination every 24 hours. The rate of glucose utilization was calculated by correcting for the volume of the medium and for the micromoles of glucose removed as a result of the sampling technique. Total cell protein and DNA were not altered in the T3-treated cultures. Each point represents the mean of three cell cultures; none of the points in any of the three determinations varied more than 10 percent from the mean.

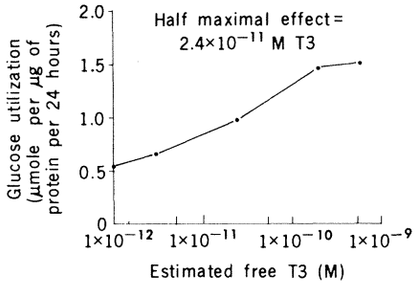

Fig. 2. Relation of glucose utilization to free hormone concentration. The cardiac cells were inoculated into a multiple well plate as described in Fig. 1, and were then incubated with various concentrations of T3. Glucose determinations were made after 60 hours of incubation. To make comparisons between experiments, we expressed the results as micromoles of glucose utilized per microgram of cell protein per 24 hours.

For studies on the effect of thyroid hormone, the dispersed myocardial cells were first grown for 3 to 7 days in a medium containing 10 percent FCS (Gibco). This medium was then replaced with Ham's F-10 medium supplemented with 10 percent hypothyroid calf serum, in which the cells remained for 24 hours in order to deplete them of thyroid hormone. This stage was of particular importance because commercial preparations of serum contain substantial amounts of T4 and T3 (*3*). The rate of glucose utilization served as a measure of the metabolic response of the myocardial cell culture system. The glucose concentration of the medium was determined with *o*-toluidine reagent (Dow). Portions of the medium from the culture grown on the Linbro plate were taken for analysis every 24 hours. The rate of glucose utilization was calculated by correcting for the volume of the medium and for the micromoles of glucose removed as a result of the sampling techniques. The iodothyronines do not influence the glucose determination with the *o*-toluidine method. Cell growth was quantitated by determining: (i) the content of DNA by the method of Burton (*10*), (ii) cell protein by the method of Lowry *et al.* (*11*), and (iii) the rate of radioactive thymidine incorporation. The free hormone fraction in the medium was 0.105 of the total T3 concentration, as estimated by comparing the biological response and the nuclear receptor occupancy in GH_1 cells in serum-free and serum-containing media (*12*).

Figure 1 illustrates the effect of T3 on the kinetics of the induced increase in glucose utilization rate in myocardial cells. After a lag period of 20 to 40 hours, the rate of glucose utilization was significantly enhanced in the cultures treated with T3 (total concentration, $1 \times 10^{-8}M$). Because this increase in glucose utilization was not observed earlier during incubation, the effect of T3 probably reflects the induction of a metabolic change rather than a direct activation by thyroid hormone of enzyme systems concerned with glucose metabolism.

This effect of T3 on glucose utilization appears to be independent of cell replication. We found that T3 had no effect on the rate of [^{3}H]thymidine incorporation into DNA in myocardial cells, up to 82 hours of incubation. In addition, T3 did not affect cell growth, as reflected by the total DNA or the cell protein content of each culture during the course of in-

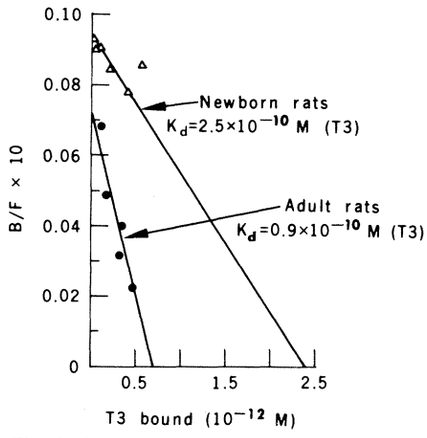

Fig. 3. Scatchard (*6, 14*) analysis of nuclear binding of ^{125}I-labeled T3. Isolated heart nuclei were prepared by homogenizing cardiac tissue from adult and newborn rat ventricles in 0.25*M* sucrose, 20 m*M* tris (*p*H 7.5), and 2 m*M* of β-mercaptoethanol at 4°C. The homogenates were centrifuged at 600*g*. The supernatant and upper layer of the pellet were aspirated after centrifugation. The nuclear pellet was washed with the same buffer containing 0.5 percent Triton X-100 and the nuclear binding of ^{125}I-labeled T3 was determined as described (*6, 14*). The binding at each hormone concentration represents the mean of three determinations; none of the determinations varied more than 5 percent from the mean (*B/F*, ratio of bound to free T3).

cubation. Figure 2 shows the effects of various T3 concentrations on the rate of glucose metabolism in myocardial cells after 40 to 60 hours of incubation. The maximum increase in the rate of glucose utilization was approximately twice the rate in cultures containing hypothyroid calf serum and a half-maximal biologic effect was observed at an estimated free T3 concentration of $2.4 \times 10^{-11} M$.

Nuclear binding of thyroid hormone in cardiac tissue in vivo has been reported (*13*), but such binding has not been related to specific myocardial responses. We compared the affinity constant of T3 for isolated nuclei in vitro obtained from newborn and adult rat hearts, using methods previously described (*6, 14*). Figure 3 shows that K_d was $2.5 \times 10^{-10} M$ for nuclei from newborn rat heart and $0.9 \times 10^{-10} M$ for nuclei from adult rat heart. At these concentrations, with the T3 occupying 50 percent of the nuclear binding sites, the results were virtually identical to those previously reported for isolated rat liver and GH_1 cell nuclei (*6*). With isolated nuclei in vitro, the K_d values were approximately five times the values obtained with intact cells (*6*). Although the exact mechanism of the effect of T3 on glucose utilization is not known, the similarities in the K_d values for T3 binding to isolated nuclei from newborn rat heart and from GH_1 cells in vitro, and the similar biologic responses in the two systems support the concept that the effect of T3 on cultured myocardial cells is regulated at the nuclear level (*3, 12*). This concept is also supported by the kinetic studies illustrated in Fig. 1 in which the effect of T3 is consistent with a time-dependent induced change in the system rather than an immediate response to thyroid hormone.

JIR S. TSAI
AMY CHEN

Endocrine Division, Department of Medicine, New York University School of Medicine, New York 10016

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Trypanosomiasis: An Approach to Chemotherapy by the Inhibition of Carbohydrate Catabolism

Abstract. *When the infected mammalian host of* Trypanosoma brucei brucei *is injected with a solution of the iron chelator salicyl hydroxamic acid and glycerol, the aerobic and anaerobic glucose catabolism of the parasite is blocked and the parasite is rapidly destroyed.*

The protozoans *Trypanosoma brucei rhodesiense* and *T. brucei gambiense* are the causative agents of African human sleeping sickness, and the closely related *T. brucei brucei* is one of several tyrpanosomes that cause a similar disease in cattle. All three parasites are transmitted by the tsetse fly vector to a mammalian host (*1*). No new effective drugs against these diseases have been developed in recent years, and resistance of the parasites to most of the drugs now in use has been reported (*2*). We are able to block both aerobic and anaerobic pathways of adenosine triphosphate (ATP) production in these parasites by the simultaneous administration of salicyl hydroxamic acid (SHAM) and glycerol to the host. Because the production of energy in the form of ATP is entirely dependent on carbohydrate metabolism (*3*), the parasites are immediately immobilized and almost entirely cleared from the blood within 5 minutes.

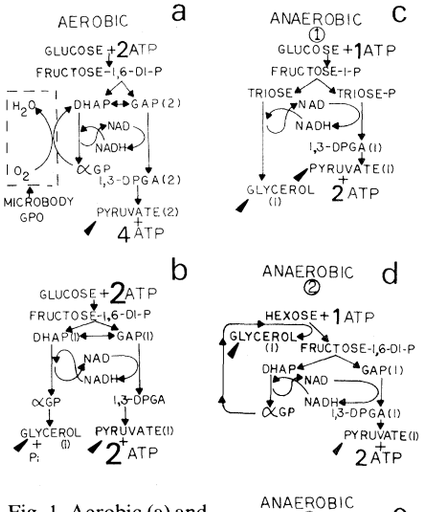

Fig. 1. Aerobic (a) and possible anaerobic (c through e) metabolic pathways in *T. b. brucei*. The pathway illustrated in (b) would generate no net ATP. Metabolic end products are marked by arrowheads. Abbreviations: ATP, adenosine triphosphate; fructose-1,6-di-P, fructose-1,6-diphosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde-3-phosphate; NAD, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; αGP, L-α-glycerophosphate; 1,3-DPGA, 1,3-diphosphoglyceric acid; GPO, L-α-glycerophosphate oxidase; fructose-1,6-di-P, fructose-1,6-diphosphate; Pi, inorganic phosphate; fructose-1-P, fructose-1-phosphate; GA, glyceraldehyde.

Carbohydrate metabolism of the trypanosome in the insect vector is complete—that is, it includes glycolysis, a tricarboxylic acid (TCA) cycle, and reoxidation of coenzymes by cytochromes in the kinetoplast-mitochondrion (*4*). In contrast, only glycolysis occurs in the long slender (LS) form, the principal stage of the parasite in the bloodstream of the mammal. The other stage in mammals, the short stumpy (SS) form, develops in the bloodstream from the LS form. The SS forms are believed to be preadapted to the insect vector and have a partial TCA cycle but no functional cytochromes (*3*). It is the LS forms that divide in the mammal and probably account for the pathology of the disease. Evidence indicates that the SS forms are at an end point in the mammal and do not reproduce unless taken up by the vector (*5*). Parasitemias in mammals infected with these organisms differ in their ratios of LS forms to SS forms, the ratios varying with the strain of the trypanosome and the stage of infection, even in cloned populations of parasites. To diminish this variation and to concentrate on the LS form, we used a clone of *T. brucei brucei* that was isolated from EATRO (East African Trypanosomiasis Research Organization) strain 110 and made monomorphic (LS form only) by 30 rapid pas-