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End-Organ Effects of Thyroid Hormones: Subcellular Interactions in Cultured Cells

Abstract. *Both actinomycin D and puromycin suppress the formation of colonies by cultured human kidney epithelial cells (T-1), but inactivation by puromycin is partially reversed with thyroid hormones. Uptake by the cells of* L-*thyroxine labeled with iodine-125, 60 to 80 percent of which is nuclear, is depressed by actinomycin and enhanced by puromycin. Genome and possibly nuclear membrane are implicated as initiating loci.*

While it is increasingly evident that many hormones act at the cellular level in producing peripheral effects, the subcellular sites that are involved require identification and the responsible mechanisms elucidation. In pursuing our earlier findings of the end-organ action of thyroid hormones on human leukocytes in vitro (*1*), we thought that cultured mammalian cells would be a particularly suitable test system. Although tissue-culture studies that deal with the mode of action of thyroid hormones were conducted from inception of the technique until three decades ago (*2*), no such applications appear to have been made of modern monolayer cell cultures. We found that several cultured mammalian cell lines simulate in vivo behavior in their response to pathophysiological concentrations ($1.78 \times 10^{-5}M$ to $1.78 \times 10^{-7}M$) of thyroid hormones, and we inferred that these effects involved modifications in protein and nucleic acid metabolism, with the nucleus participating as an initial locus (*3*). Studies were undertaken with the antibiotics puromycin and actinomycin D and with I^{125}-L-thyroxine to characterize these actions more precisely.

Human kidney epithelial cells (T-1) (*4*) were grown in a water-saturated mixture of CO_2 (5 percent) and air (95 percent) at 37°C in Eagle's minimum essential medium (*5*) supplemented with 10 percent fetal bovine serum. Plating efficiency (PE) was determined by seeding 300 cells, in the logarithmic phase of growth, that had been dispersed with trypsin into 100-mm plastic petri dishes; after 2 weeks of incubation the colonies that were made visible by staining with methylene blue were scored. As has been reported (*3*), the PE for this cell line, which ranged between 40 and 70 percent, could be enhanced substantially by addition of thyroid hormones L-thyroxine (T_4) or 3,5,3′-L-triiodothyronine (T_3). The modifications in PE produced by either puromycin or actinomycin in hormone-treated and untreated cells were compared. Exposure of the experimental cells to either antibiotic was started 1 week after seeding to permit unimpaired establishment of colonies.

Suppression of colony-formation by puromycin depends not only upon concentration of the antibiotic and the duration of exposure but also upon the presence and titer of thyroid hormones. Treatment for 2 to 4 hours with concentrations of puromycin below or equal to 2.5 μg/ml produced no inhibition of PE. When 5 μg/ml of puromycin was used PE was depressed almost linearly with duration of exposure from the 4th to the 15th hour, reaching zero by 24 hours (Fig. 1). For hormone-treated cells, however, there was a marked inactivation of the effect of puromycin on PE. Thus, the PE for cells incubated in $4.45 \times 10^{-6}M$ of T_4 and exposed to 5 μg/ml of the antibiotic for 9 hours was 37 percent greater than for cells grown without hormone; extending the exposure to 15 hours resulted in a relative rise of 67 percent in PE. Similar reversals of inhibition of colony-formation by puromycin occurred with other concentrations of hormone; moreover, these effects were independent of the sequence in which hormone and antibiotic were introduced.

Formation of colonies was obliterated by immersing the cells in concentrations of puromycin exceeding 0.5 μg/ml during the entire 2nd week. For this period of exposure, 0.25 μg/ml of puromycin caused a fall in PE to 16 percent from 75.5 percent for the controls, but the addition of $1.78 \times 10^{-7}M$ of T_3 produced twice as many colonies (PE, 37.6 percent). Figure 2 illustrates the variation of PE with the concentration of T_3 for cells grown in 0.15 μg/ml of puromycin.

Such reversal by thyroid hormones could not be demonstrated for cells treated with actinomycin. No colonies developed from cells incubated for the entire 2nd week in 0.005 to 0.5 μg/ml of this antibiotic. The formation of colonies began between 0.005 and 0.0005 μg/ml, but the suppression caused by actinomycin was uninfluenced by thyroid hormones. Similarly a shorter exposure to the antibiotic (3 hours) re-

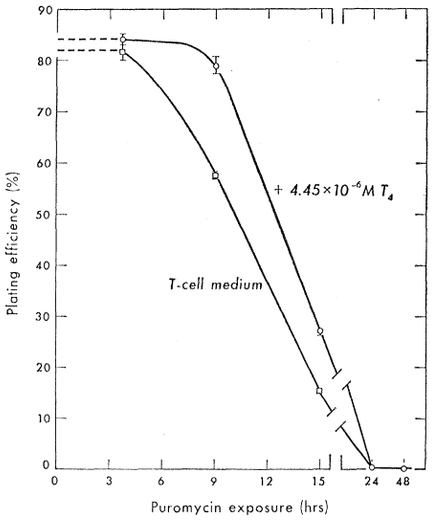

Fig. 1. Effect of $4.45 \times 10^{-6}M$ of L-thyroxine (T_4) on T-1 cells exposed to puromycin (5 μg/ml). Points plotted represent the means of triplicate dishes with standard deviation indicated by the length of the bars.

sulted in no alteration in colony development due to the presence of hormones.

Radioautographic studies of the distribution pattern of I^{125}-L-thyroxine (*6*) together with modifications produced by the antibiotics we used implicate the nucleus as a center for hormonal localization and action. For the preparation of radioautographs, microscope slides were placed in petri dishes overgrown by 2×10^5 cells for 2 to 3 days before labeling, and then dipped into melted Kodak NTB-2 emulsion. Iodine-125 (half-life, 60 days) is effectively a weak beta emitter (*7*), hence radioautographs can be obtained that have resolutions similar to those attainable with H^3. In four such studies, 60 to 80 percent of the photographic grains over cells were associated with nuclei. Iodine-125 was frequently localized in the perinuclear zone; nucleoli were not appreciably tagged (Fig. 3*a*). Pretreatment with puromycin elicited significant enhancement of I^{125} uptake throughout the cell, with perinuclear labeling again noted. Exposure to actinomycin greatly depressed radioiodine uptake, particularly by the nucleus (Fig. 3*b*). For the study illustrated, radioautographs assayed after treatment with 0.05 μg/ml of actinomycin for 8 hours yielded a mean grain count per cell of 4.1 compared with 10.3 for the controls; the corresponding grain count per nucleus was 1.2 and 7.2, respectively. After exposure to puromycin (5 μg/ml for 8 hours) the uptake results were 12.7 grains per cell and 8.7 grains per nucleus. These figures, corrected for background, were derived from scoring 50 cells on each duplicate slide.

Our experiments with cultured cells, together with those reported previously, imply interactions at several subcellular levels. After exposure to labeled thyroxine, uptake by nuclei of I^{125} is correlated with colony development. Plating efficiency, a parameter for expressing colony-formation, has proved to be highly specific and sensitive to thyroid hormonal activity (*3*). Unlike the effects of puromycin, nuclear uptake of I^{125} and PE are both profoundly and irreversibly depressed by actinomycin. Since actinomycin presumably interferes with the template function requisite for synthesis of messenger RNA by inhibiting DNA-dependent polymerase (*8*), it seems plausible to assume that the tagged hormone interacts, as the radioautographs suggest, with either the genome or the nuclear membrane, or with both. On the other hand, the block in colony-formation produced by puromycin can be partially overcome by thyroid hormones. Although puromycin impairs incorporation of amino acids into protein at the level of the ribosome (*9*), it is unsettled whether the hormones effect reversal directly or through the nucleus. Exposure to puromycin prior to labeling with I^{125}-L-thyroxine actually augments radioiodine uptake, so that a feedback mechanism may be operative. Additional experiments are required, however, with other tagged compounds and analogs to establish these views more firmly. The thyroid-puromycin antagonism has an interesting in vivo counterpart in the observation made by Weiss and Sokoloff of the reversal, produced with puromycin, of the hypermetabolism induced by thyroxine in rats (*10*).

Among these several participating loci, evidence does exist for favoring the nucleus as the initial site of hormonal action (*3*). Thus, prior protracted exposure to thyroid hormones promoted RNA and protein synthesis in these cells, which was demonstrable by radioautography after the cells were labeled with tritiated precursors. However, the increased production of nuclear RNA, particularly that associating with nucleoli, was manifested within minutes in contrast to hours required for the enhancement of incorporation of amino acids. A related structural sequela, the appearance of supernumerary nucleoli, whose frequency distribution was dependent on the dose, was observed within several hours of hormone introduction. Unpublished determinations of the duration of latency for several cellular responses to thyroid hormones, including enhancement of protein synthesis, revealed that the rise

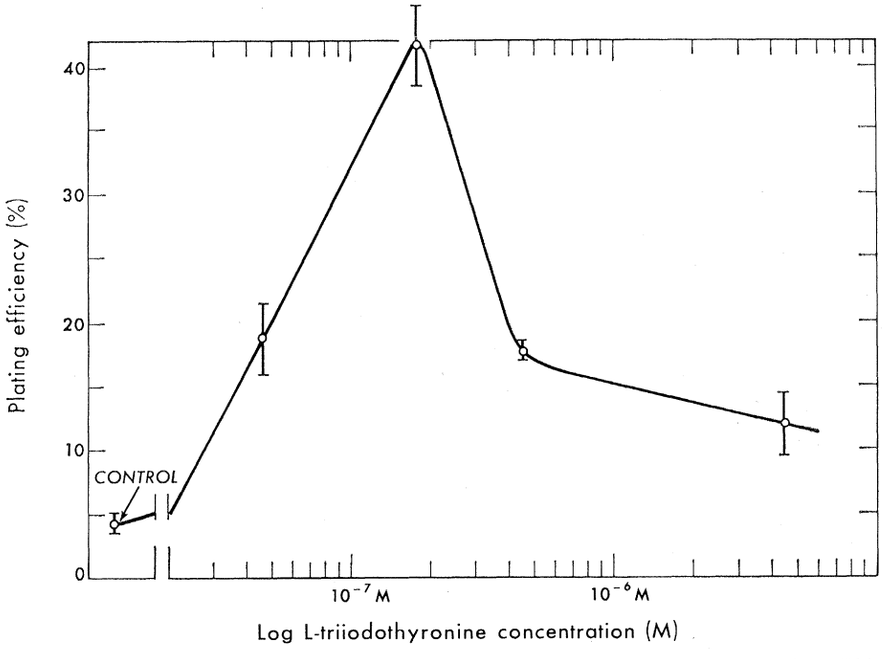

Fig. 2. Variation of plating efficiency with concentration of L-triiodothyronine (T_3) for T-1 cells exposed to 0.15 μg/ml of puromycin for the entire 2nd week after seeding. Each point is the mean of triplicate dishes; length of bars indicates standard deviation. The point labeled "control" designates plating efficiency for puromycin-exposed cells not treated with T_3.

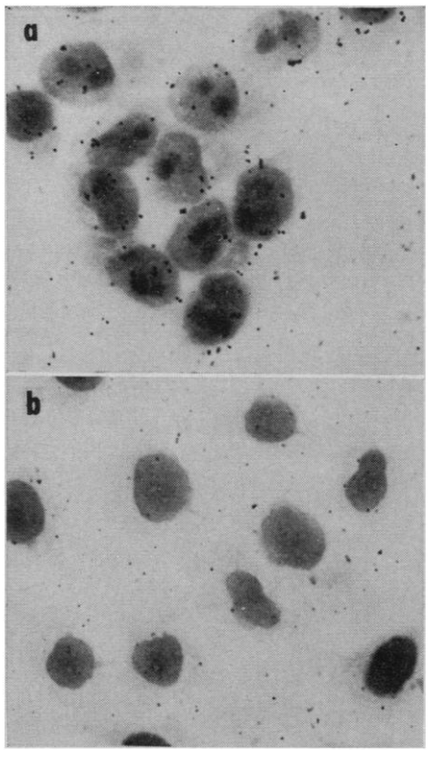

Fig. 3. Radioautographs of T-1 cells labeled with I^{125}-L-thyroxine (2 μc/ml) for 30 minutes and exposed for 3 days ($\times$ 900). (*a*) Cells pretreated 30 minutes with 4.45 $\times$ $10^{-6}M$ of L-thyroxine. Note high localization by nuclei, especially by perinuclear zone. (*b*) Cells pretreated with actinomycin D (0.05 μg/ml) for 8 hours. Virtually no grains appear over nuclei.

in production of nuclear RNA was the first evoked in this way and was detectable after treating for a fraction of an hour. These findings are consonant with those of Widnell and Tata (*11*), which indicate that exogenous thyroid hormones in vivo affected RNA polymerase activity prior to the augmentation of protein synthesis. Nevertheless, the possibility of direct action by these hormones on protein production independent of RNA messenger intervention must be entertained, especially in view of such findings with cell-free rat liver preparations made by Sokoloff, Francis, and Campbell (*12*), who used 6.5 $\times$ $10^{-5}M$ to 6.5 $\times$ $10^{-4}M$ L-thyroxine. Since these superphysiological and toxic concentrations of thyroxine were ineffective or inhibitory in influencing proliferation and plating efficiency of T-1 cells (*3*), the relation of their observations to in vivo response patterns should be explored further. Similar reservations may hold for the more recent studies of the inability of L-thyroxine to alter the DNA melting profile (*13*).

The loci within peripheral cells which concentrate thyroid hormones have not yet been detected, despite intensive search. In a detailed and painstaking study, Tata, Ernster, and Suranyi (*14*) determined the subcellular distribution in liver and skeletal muscle of endogenously labeled thyroid hormone as well as of I^{131}-labeled T_4 and T_3 added to tissue homogenates; no specific subcellular sites of localization were found. It would seem that hunting with radioautography for such centers has the overriding merit of analysis being conducted on intact cells, particularly when coupled to a cultured homogeneous population capable of hormonal response.

While it appears that thyroid hormones mediate their effects by way of the nucleus and participating messenger RNA, the precise mechanism for this interaction remains to be established. The I^{125} radioautographs suggest the genetic matter and the nuclear envelope as possible primary foci of thyroid hormonal activity. If the latter is involved, a conceivable hormonal role would be regulation of the transfer of synthesized messenger RNA and of its precursors across the nuclear-cytoplasmic interface. Interaction of hormone and DNA could be the mode by which the milieu triggers genetically predetermined latent behavior and could account for the differential specificity of cellular response (*15*). Distinguishing among these and other possible mechanisms must await attack by higher resolution techniques, perhaps by electron microscope radioautography of these cultured cells. In any event, to the older ideas of hormonal action, namely, control of enzymatic action (*16*) and regulation of membrane permeability (*17*), must now be added hormonal control of genic expression (*18*).

EDWARD SIEGEL*
CORNELIUS A. TOBIAS
Donner Laboratory, University of California, Berkeley 94720

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* Present address: Division of Nuclear Medicine, Department of Radiology, Stanford University School of Medicine, Palo Alto, California 94304.

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Tetrodotoxin Does Not Block Excitation from Inside the Nerve Membrane

Abstract. *Tetrodotoxin does not block the action potential or membrane sodium current when internally perfused through the giant axon of a squid at much higher concentrations than those required for blocking by external application. It is suggested that the gate for the sodium channel is located on the exterior surface of the axon, because tetrodotoxin is not lipid soluble.*

Tetrodotoxin, the active component of the puffer fish poison, has now become a very popular and important tool for electrophysiological studies of excitable tissues. This is primarily because of its unique ability, at very low concentrations, to selectively block the voltage-dependent mechanism for increase in sodium conductance, the mechanism that is responsible for the excitation in axons. The action is quite distinct from that of procaine or cocaine, which block the mechanism for increase in potassium conductance as well (*1*). The selective blocking action of tetrodotoxin was first suggested in frog muscle fibers (*2*), and then definitely confirmed by voltage-clamp experiments with giant axons of lobsters