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The Effects of Selenate, Molybdate and Chromate on the Triiodothyronine Induced Enzyme Activity and mRNA Level for Both Fatty Acid Synthase and Malic Enzyme

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THE EFFECTS OF SELENATE, MOLYBDATE AND CHROMATE ON THE
TRIIODOTHYRONINE INDUCED ENZYME ACTIVITY AND
mRNA LEVEL FOR BOTH FATTY ACID SYNTHASE
AND MALIC ENZYME

by

Yuan Zhu

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Western Michigan University
Kalamazoo, Michigan
December 1991

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Yuan Zhu, M.A.

Western Michigan University, 1991

In chick embryo hepatocytes in culture, triiodothyronine (T3) stimulates enzyme activity, mRNA level and transcription rate for both fatty acid synthase (FAS) and malic enzyme (ME). Recent evidence has demonstrated the effects of selenate, molybdate and chromate on various physiological processes. Little information, however, is available on the effects of these metal ions on the T3-regulated gene expression for FAS and ME.

In chick embryo hepatocytes incubated in a chemically defined medium, addition of sodium selenate (20 μ M) coincident with T3 almost completely inhibited the T3-induced stimulation of FAS and ME activity and accumulation of their respective mRNA's. Neither ammonium molybdate nor ammonium chromate has any effect on either the enzyme activity or mRNA level of FAS or ME under similar experimental conditions.

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Yuan Zhu

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INTRODUCTION

Fatty acids are synthesized from acetyl-CoA. In animals, liver is the primary tissue in which fatty acids are synthesized. The enzymes involved in this catalysis of fatty acids are acetyl-CoA carboxylase, fatty acid synthase and malic enzyme. Acetyl-CoA carboxylase catalyzes the first step of fatty acid synthesis, the carboxylation of acetyl-CoA to malonyl-CoA. Fatty acid synthase, a multi-enzyme complex, catalyzes the formation of long chain fatty acids using acetyl-CoA as a primer and malonyl-CoA as a source of 2 carbon units. Malic enzyme generates the NADPH necessary for fatty acid synthesis to occur.

One major mechanism for the control of fatty acid synthesis is a change in the concentration of active enzymes available for catalysis. Energy supply is an important element that dictates the amount of acetyl-CoA carboxylase, fatty acid synthase and malic enzyme. For example, the concentrations of fatty acid synthase, acetyl-CoA carboxylase and malic enzyme in liver are reduced by 10-, 5- and 50-fold after fasting (1). When the animal is allowed to eat again, the concentrations of fatty acid synthase, acetyl-CoA carboxylase, and malic enzyme rise dramatically.

Hormones play an important role in communicating the

energy supply of the whole animal to the liver. In the starved animal, glucagon is elevated (2, 3, 4), and in the fed animal, insulin and triiodothyronine (T3) concentrations are high. The changes that occur in fatty acid biosynthesis and the changes in the activities of fatty acid synthase and malic enzyme which occur *in vivo* during starvation or starvation followed by refeeding can be mimicked quantitatively in liver cells cultured in a chemically defined medium. Using chick embryo hepatocytes in culture, it was found that the activities of fatty acid synthase and malic enzyme fell slightly during a 3-day incubation in the absence of hormones (5). The addition of insulin to the cells in culture caused a small increase in fatty acid synthase and malic enzyme activities while T3 alone caused a 6- and 23-fold increase, respectively. Insulin plus T3 stimulated malic enzyme and fatty acid synthase activities 100- and 10-fold (6). When glucagon was added to these cells in culture with insulin and T3, this increase in activities was almost completely blocked (7). These changes in activities were shown to be due to changes in the concentrations of the proteins for fatty acid synthase and malic enzyme (5). The marked effects of T3 and glucagon on the concentrations of fatty acid synthase and malic enzyme were specific. Neither hormone affected the amount of total protein per plate or the concentration of soluble protein. Furthermore, the effects of T3

and glucagon on the activities of NADP isocitrate dehydrogenase and lactate dehydrogenase were very small and in the opposite direction to the effects of these hormones on fatty acid synthase and malic enzyme.

Similar to the observed effects by diet in the *in vivo* experiments, the T3-induced increases in enzyme synthesis for malic enzyme and fatty acid synthase in cells in culture are correlated with comparable increases in the level of their respective mRNAs (8, 9, 10, 11). Messenger RNA levels, in turn, are correlated with rates of transcription for the malic enzyme (12) and fatty acid synthase genes (13). Thus, the T3-induced accumulations of malic enzyme and fatty acid synthase are due primarily to regulation of transcription. The effects of T3 on abundance of acetyl-CoA carboxylase mRNA and transcription of its gene have not yet been reported.

The question then becomes what is the major mechanism by which insulin and T3 regulate the expression of fatty acid synthase and malic enzyme. Recent experiments aimed at understanding the regulation of metabolic pathways suggest a phosphorylation event may be involved.

Protein phosphorylation has been shown to play an important role in the regulation of several metabolic proteins. The major form of regulation of acetyl-CoA carboxylase *in vivo* appears to be protein phosphorylation. Purified acetyl-CoA carboxylase can be phosphorylated *in*

vitro by seven different protein kinases at up to seven serine residues which have now been defined by amino acid and cDNA sequencing. At least five of these sites are phosphorylated in isolated hepatocytes or adipocytes. Phosphorylation at ser-79 and/or ser-13200 is responsible for the direct regulation of enzyme activity. The AMP-activated protein kinase is the only kinase that can account for this phosphorylation, and therefore, appears to be the kinase most important for regulation of acetyl-CoA carboxylase *in vivo* (14).

Much evidence has also accumulated indicating that the activities of specific transcription factors are also regulated by phosphorylation. The first direct evidence for the regulation of transcription factor activity by phosphorylation came from studies of the heat-shock response in yeast (15). In all organisms studied, exposure to elevated temperatures and other types of physiological stress results in the elevated transcription of a small set of genes, the heat-shock genes. In eukaryotes, this response is mediated by the heat-shock transcription factor (HSF) and that activation of the ability of HSF to stimulate transcription *in vivo* involves HSF phosphorylation. Besides heat-shock factor, other transcriptional regulators have been shown to also be phosphorylated. The serum response factors, E4F and E2F, appear to be controlled by phosphorylation (16) as well as the mammalian transcription

factor, CREB, which mediates rapid transcriptional induction in response to high cAMP levels. Much of this regulation via phosphorylation appears to be mediated through either protein kinase A or C (17-21).

Phosphorylation has also recently been indicated to play a role in T3-regulated gene expression. T3 administration to thyroidectomized rats induces a significant increase in liver nucleolar protein kinase activity. This enhanced level of kinase activity seems to reflect a higher concentration and not the catalytic efficiency of an enzyme (22). If a nuclear protein kinase is responsible for adding phosphate to the nuclear proteins and if its activity depends on the presence of thyroid hormones, then phosphorylation should be decreased after thyroidectomy and administration of T3 should increase incorporation of $\alpha^{32}\text{P}$ from ATP into nuclear proteins. This appears to be the case since results showed that hepatic nuclei, isolated from thyroidectomized rats were phosphorylated 21% less than the nuclei of control. The phosphorylation was enhanced up to 128% of the control value after 24 hours of T3 treatment (23).

T3 also increases activity of nuclear protein kinases and phosphorylation of nuclear protein in glial cells. The results showed that T3 produces very selective effects on protein phosphorylation in primary glial cells in cultures from rat cerebral hemispheres (24). Other investigators

have also examined the effects of T3 on protein phosphorylation in different subcellular fractions of cultured neurons. Using a neuronal culture system, they did not find any effects of T3 outside the nucleus. In contrast, T3 increases the phosphorylation of 2 specific proteins in the nucleus. One of these proteins affected by T3 belongs to the histones, a class of proteins which plays an important role in the structure of the chromatin. It is believed that histone modification by phosphorylation, acetylation or other means affects histone-histone as well as histone-DNA interactions and may influence DNA transcription and replication through this mechanism (25).

T3 not only appears to affect phosphorylation but its actions may also be regulated by phosphorylation. T3 action is generally thought to be initiated by the binding of the hormone to a nuclear receptor (26). In target cells, T3 binds to high affinity, low capacity chromatin-associated sites. It is thought that this complex acts as a genetic regulatory element controlling gene expression (27). ATP in the submillimolar range and exogenous dephosphorylation are able to drastically reduce the binding activity of solubilized T3 receptors *in vitro* (28). ATP is the only mononucleotide with intracellular concentrations in the millimolar range (29, 30, 31). Thus, it can be speculated that in the living cell, ATP is able to interact with and modify the activity of the T3 nuclear receptor, at

least when it is not bound to chromatin [e.g., in the cytoplasm (32) or in the nucleoplasm (33)]. ATP is known to potentiate steroid receptor binding and transform the hormone-receptor complex to a DNA-binding form (34). The precise mechanism by which ATP acts in cells is not fully understood.

Another indication that T3 binding to its receptor is regulated by phosphorylation is from results showing that calf intestinal alkaline phosphatase in concentrations equivalent to those employed for other hormone receptor assays inhibits T3 binding activity (35, 36).

Recently, it was demonstrated that the proto-oncogene c-erb A binds T3 with high affinity and specificity, which suggests that the protein encoded for by c-erb A and the T3 receptor are physiologically related (37, 38). Sequence and multidomain structure similarities between c-erb A and the steroid receptors suggest that they have evolved from a common ancestral gene (38). Examination of the amino acid sequence of the human c-erb A-related proteins reveals several putative acceptor residues for phosphoryl groups (39).

The α -form of the T3 receptor (v-erb A α) is phosphorylated *in vivo* and *in vitro* at two separate sites (40); one site is a substrate for protein kinase A and C and the other by casein kinase II or an activity with similar properties. Protein kinase C and A are involved in complex

biochemical pathways that transduce growth and differentiation signals received at the membrane to the transcriptional machinery (41). The fact that both protein kinase C and protein kinase A enhance phosphorylation of the same tryptic peptide, suggests that phosphorylation of this region is important in the control of erb A protein function. In a series of experiments carried out with mutations, it was observed (42) that when the N-terminal phosphorylation site in v-erb A is not phosphorylated, the ability of v-erb A to inhibit differentiation and repress transcription of the genes for carbonic anhydrase II and band 3 is lost. Thus phosphorylation of the T3 receptor or other transcription factor(s) may be involved directly in this mechanism of action of T3.

From the evidence presented thus far, it appears phosphorylation is important in T3 action. Insulin is also involved in the regulation of fatty acid synthase and malic enzyme gene expression and phosphorylation is known to be important in its actions. Insulin action in target cells requires the transmembrane signaling activity of the insulin receptor. The insulin receptor is a protein kinase that undergoes tyrosine autophosphorylation immediately after insulin binding. Regulation of the intrinsic kinase activity at the receptor depends on the phosphorylation state of the β -subunit of the receptor. The tyrosine kinase activity of the insulin receptor is under positive and

negative control by the state of receptor phosphorylation. Autophosphorylation of the insulin receptor on tyrosine residues enhances tyrosine kinase activity. In contrast, activation of both protein kinase A and protein kinase C in intact cells leads to Ser/Thr phosphorylation of the insulin receptor which is associated with reduced tyrosine kinase activity and a decrease in insulin action. Until recently there has been no direct evidence to suggest that phosphorylation plays a role in the insulin and T3 regulation of malic enzyme and fatty acid synthase gene expression. H-8, H-7 and HA-1004 are isoquinoline sulfonamide derivatives that inhibit protein kinase activity via competitive inhibition of the ATP binding site. H-8 and other protein kinase inhibitors inhibited the T3-induced accumulation of fatty acid synthase and malic enzyme activities and their respective mRNAs but had no effect on the activities of 6-phosphogluconate dehydrogenase and isocitrate dehydrogenase, enzymes not induced by T3 in chick-embryo hepatocytes. H-8 also had no effect on the activities of malic enzyme, fatty acid synthase and acetyl-CoA carboxylase in hepatocytes not treated with T3. H-8 had no effect on the synthesis of soluble protein nor on the levels of mRNA for β -actin or glyceraldehyde-3-phosphate dehydrogenase or on the synthesis of total RNA in isolated nuclear receptor (43). These results concerning the inhibition by H-8 of the T3 induced expression of malic enzyme and fatty

acid synthase clearly indicate the involvement of a kinase in this regulation; however, identification of this kinase or the possible involvement of insulin in this kinase's action cannot be determined from the data accumulated thus far.

To investigate more fully the role of phosphorylation and possible identification of potential kinases or phosphatases involved in the insulin and T3 regulation of fatty acid synthase and malic enzyme, other factors which have been implicated in regulation by phosphorylation or regulation by insulin-mimetic action must be tested.

Evidence has accumulated to demonstrate that a variety of metals are also involved in phosphorylation events. Zinc and manganese are both implicated in the chemical phosphorylation of proteins in the presence of ATP (44). Vanadate and molybdate have been shown to affect phosphorylation of similar proteins in some cells in culture (45). Vanadate and selenate, phosphate analogues, inhibit enzymes involved in phosphate release (phosphatase) and transfer reactions (phosphoryl transferase activities) (46). Surprisingly, vanadate, selenate and chromate have induced rats (48). These insulin-mimetic actions are presumably brought about by affecting the phosphorylated state of the insulin receptor.

The objective of this research was therefore to investigate the effects of some of these metal ions (selenate,

molybdate and chromate) on the regulation of fatty acid synthase and malic enzyme by T3 and insulin.

EXPERIMENTAL PROCEDURE

Preparation and Maintenance of Isolated Cells

Unincubated embryonic eggs from white leghorn chickens obtained from Townline Poultry, Zeeland, MI, were incubated in an electric forced-draft incubator at $39.5^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ and 60% relative humidity. Chicks were killed by decapitation. The livers were removed rapidly and placed in the Modified Krebs-Ringer Bicarbonate Buffer (MKRBB) (4.8 mM NaCl, 120.0 mM KCl, 1.3 mM CaCl_2 , 1.2 mM MgSO_4 , 1.2 mM KH_2PO_4 , 2.5 mM NaHCO_3 ; pH 7.2, bubbled for one hour with 5% CO_2). Liver slices were prepared with a surgical blade. The slices from 10-12 livers were placed in a 250ml flask which contained 40ml of filter sterilized MKRBB with 0.5 mg/ml collagenase. The flasks were gassed with $\text{O}_2 + \text{CO}_2$ (95:5) for one minute and incubated at 39°C for 20 minutes in a shaking water bath (150 cycles per min). The flasks were gassed one or two more times in 20-minute intervals. The resulting suspension was chilled on ice and filtered through two layers of nylon mesh. The cells were collected by centrifugation (4°C) at 900 rpm for 5 minutes. The supernatant was discarded and the cells resuspended in 10-15 ml MKRBB and sedimented for 10-15 min on ice to separate red blood cells. The red blood cells were aspirated and

the cells were washed with 10 ml MKRBB and collected by centrifugation. After the wash, the cells were resuspended in Waymouth's Media 705/1 (1 ml packed cells per 9 ml media) containing penicillin (60 $\mu\text{g/ml}$) and streptomycin (100 $\mu\text{g/ml}$) and incubated in untreated, 60 mm Petri dishes (Falcon) at 39°C in an atmosphere of 95% air, 5% CO₂. A volume of 0.4 ml of the cell suspension (1.0-1.5 mg total protein, about 0.5×10^7 cells) was incubated with 5 mL Waymouth medium. After about 20 h of incubation the medium was changed, and hormones and (or) metals were added as indicated in the legends to Figures 1, 2, 3, 4, 5, and 6. Cells were harvested after the indicated periods of incubation.

Preparation of Homogenates and Assay of Enzyme Activity

The media were aspirated off and the cells on the plate were washed with 2 ml PBS (0.15 M NaCl, 15 mM Na₂HPO₄, 6.5 mM NaH₂PO₄). The cells were scraped into 1 ml KED buffer (0.1 M KPi, pH 7.0, 3 mM EDTA, 1 mM DTT), homogenized 20 times with Dounce homogenizer, and then transferred into a 1.5 ml eppendorf tube. The suspensions were centrifuged for 0.5 h at 4°C in a microfuge. The resulting aqueous supernatant fraction was employed for the assay of enzyme activity by spectrophotometric measurement of the rate of the change of NADPH adsorbance at 340 A and 22°C. The enzyme activity of fatty acid synthase was assayed by the

following method: A 1 ml cuvette containing 25 μ M acetyl-CoA, 100 μ M NADPH in KED buffer was mixed with a 100 μ l of cell supernatant. The reaction was initiated with 10 mM malonyl-CoA and change in absorbance at 340 nm was measured for 3 min. The enzyme activity of malic enzyme was assayed by the following method: A 1ml cuvette containing 85 μ M Tris, pH 7.4, 100 μ M EDTA, 236 mM MgCl_2 , 490 μ M NADP was mixed with 30 μ l of cell supernatant. The reaction was initiated by the addition of 10 mM malate and the change in absorbance at 340 nM was measured for 3 min. Isocitrate dehydrogenase was assayed similarly except the reaction was initiated with 10 mM isocitrate.

Isolation of RNA

The guanidinium-phenol-chloroform method was used to isolate RNA from cultured cells (49). Immediately after removal of the media, the cells from 2 plates were scraped into 1 ml of a solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7; 0.5% sarcosyl, 0.1 M 2-mercaptoethanol, and subsequently transferred to two 1.5 ml eppendorf tubes. The solution was brought to a final concentration of 0.2 M sodium acetate, pH 4. This aqueous layer was extracted with phenol/chloroform (1:1). Samples were centrifuged at 10,000g for 20 min at 4°C. After centrifugation, RNA that was present in the supernatant was precipitated by the addition of equal volumes of isopropa-

nol. Sedimentation at 10,000g for 20 min was again performed and the resulting RNA pellet was dissolved in a solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7; 0.5% Sarcosyl, 0.1 M 2-mercaptoethanol and transferred into a 1.5 ml eppendorf tube, and precipitated with 1 vol of isopropanol at -20°C for 1 h. After centrifugation for 10 min at 4°C the RNA pellet was resuspended in 75% ethanol, sedimented, dried and dissolved in diethyl pyrocarbonate (DEPC)-treated autoclaved H₂O. At this point the RNA preparation could be used for northern blot analysis.

Quantitation of mRNA Levels (Northern Blot Analysis)

The gel for RNA analysis was composed of 1% agarose in a MOPS (0.2 M MOPS {3-N-morpholino} propanesulfonic acid), 50 mM sodium acetate, 10 mM EDTA and 0.02 M formaldehyde buffer. The running buffer was composed of 0.2 M MOPS, 50 mM sodium acetate, and 10 mM EDTA. The RNA sample was loaded on the gel and the gel was electrophoresed at about 33 V at room temperature for 14 h. The gel was stained with ethidium bromide to visualize the RNA under UV light. The separated RNAs were transferred to "GeneScreen" (NEN/Dupont) membranes using a vacuum transfer apparatus (Pharmacia) as per the manufacturer's recommendations. The mRNAs were hybridized with ³²P-labeled DNA probes labeled by the multiprime DNA labelling method (Amersham).

Preparation of ^{32}P -labeled DNA for Hybridization

The ^{32}P -labeled DNA probe was synthesized by using Amersham multiprime DNA labelling systems. The gel-purified DNA fragment was denatured by heating to 95-100°C for two minutes in a boiling water bath, then chilled on ice. The labelling reaction was carried out as per the manufacturer's recommendation. The incubation occurred at room temperature; normally 3-5 h was sufficient but reactions could be left overnight. After the reaction was completed, the labelled DNA was purified by gel filtration (Sephadex G-50) chromatography. Before use, the probe was denatured in a boiling water bath.

Northern Gel Analysis of RNA (Hybridization)

Genescreen containing the separated RNAs was placed in a polyethylene bag. Sufficient pre-hybridization buffer (50% deionized formamide, 0.5 M NaHPO_4 , pH 7.2, 7% NaDodSO_4 , 1 mM EDTA) was added. The bag was heat-sealed and placed in 42°C water bath for 8-12 h. After this pre-hybridization period, the probes were added to the bag. The bag was re-sealed, incubated at 42°C for 8-24 h in a water bath. After the hybridization, the filter was washed at room temperature for 5 min with agitation in a solution containing 1 mM EDTA, 40 mM NaHPO_4 , pH 7.2, 5% NaDodSO_4 . Three additional washes were carried out at 42, 50 and 60°C for

15 min each. The filter was blotted dry and subjected to autoradiography at -70°C with Kodak XAR-5 film and an intensifying screen.

RESULTS

Enzyme Activities

In chick-embryo hepatocytes incubated in a chemically defined medium, the increases in the activities of malic enzyme (Figure 1) and fatty acid synthase (Figure 1) caused by the addition of T3 for 48 h were almost completely inhibited when sodium selenate was added for the same period of time as the T3. Enzyme activities were inhibited in a dose-dependent manner by sodium selenate. Fifty percent of maximal inhibition was achieved at 5-10 μM sodium selenate for both malic enzyme and fatty acid synthase. This inhibition by sodium selenate of the T3 induced activities of malic enzyme and fatty acid synthase was not changed if insulin was added to the cells in culture, therefore sodium selenate's effects were directly on the T3 response and in no way appeared to mimic insulin in this system.

Isocitrate dehydrogenase activity was inhibited slightly or unaffected by T3 in avian hepatocytes in culture. Selenate at up to 20 μM had no effect on the activity of cytoplasmic isocitrate dehydrogenase in cells treated with T3 (Figure 1). Thus, sodium selenate selectively inhibited the induction of these lipogenic enzyme activities and appeared to have no effect on total protein synthesis.

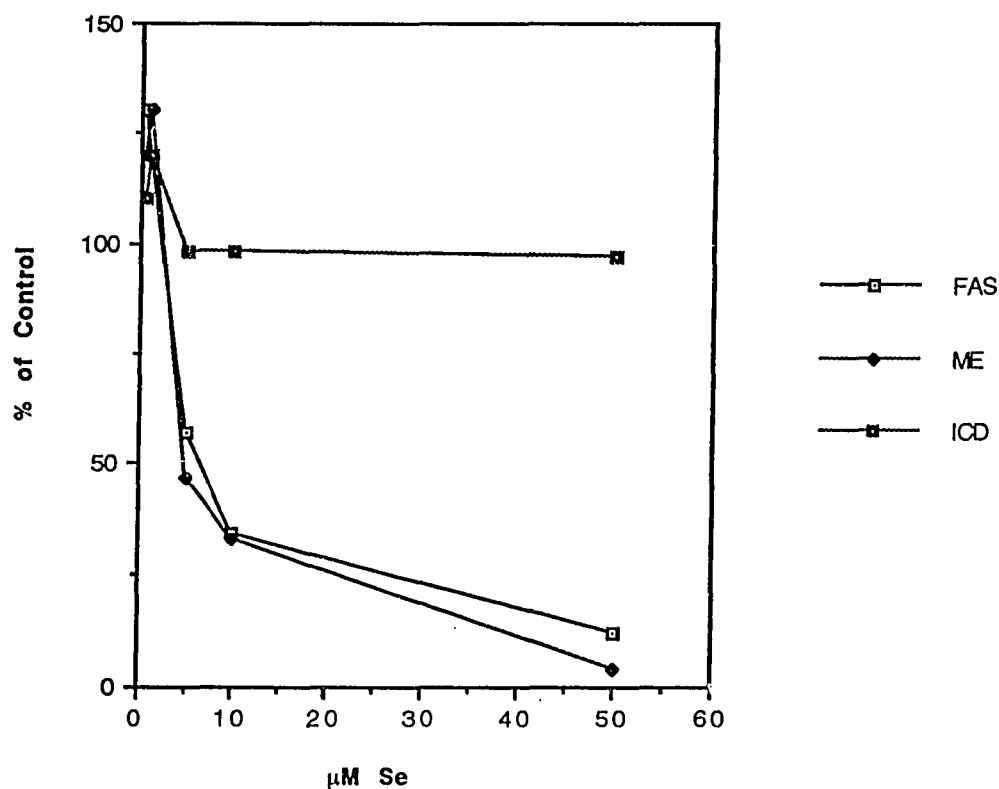


Figure 1. Inhibition of the T3-Induced Increase in Fatty Acid Synthase and Malic Enzyme Activity as a Function of the Concentration of Sodium Selenate.

Hepatocytes were incubated for 48 h in the presence of T3 (1 $\mu\text{g/ml}$) and either 0, 1, 5, 10, or 50 μM sodium selenate (Se). After harvest, the activities for fatty acid synthase (FAS), malic enzyme (ME) and isocitrate dehydrogenase (ICD) were determined. The results are expressed as a percentage of the value for cells incubated with T3. Similar results (data not shown) were obtained with cells incubated in the presence of insulin. The results are an average of 5 experiments.

Molybdate, another metal ion involved in regulation of protein phosphorylation, or chromate, another potential insulin-mimetic agent had no effect on malic enzyme or fatty acid synthase activities at concentrations similar to those of selenate and under the similar experimental conditions (Figure 2, 3).

Messenger RNA Levels

In chick-embryo hepatocytes in culture, the addition of T3 in the presence of insulin caused a 50-fold and 10-fold increase in the mRNA levels for malic enzyme and fatty acid synthase, respectively. mRNA abundance, therefore, increased in parallel with enzyme activity (1).

Sodium selenate caused a concentration dependent decrease in T3 induced accumulation for both malic enzyme and fatty acid synthase (Figure 4, 5). Sodium selenate (20 μ M), added coincidentally with T3, caused an almost 90% inhibition of the accumulation of malic enzyme mRNA at 24 and 48 h (Figure 4). Likewise, selenate caused the abundance of fatty acid synthase mRNA in T3-treated cells to decrease to undetectable levels within 24 h (Figure 5). The abundance of malic enzyme mRNA and fatty acid synthase mRNA was inhibited to about the same extent as enzyme activity at each concentration of inhibitor. Sodium selenate inhibited the mRNA accumulation in a similar fashion whether insulin was present or not. Thus, selenate inhibits

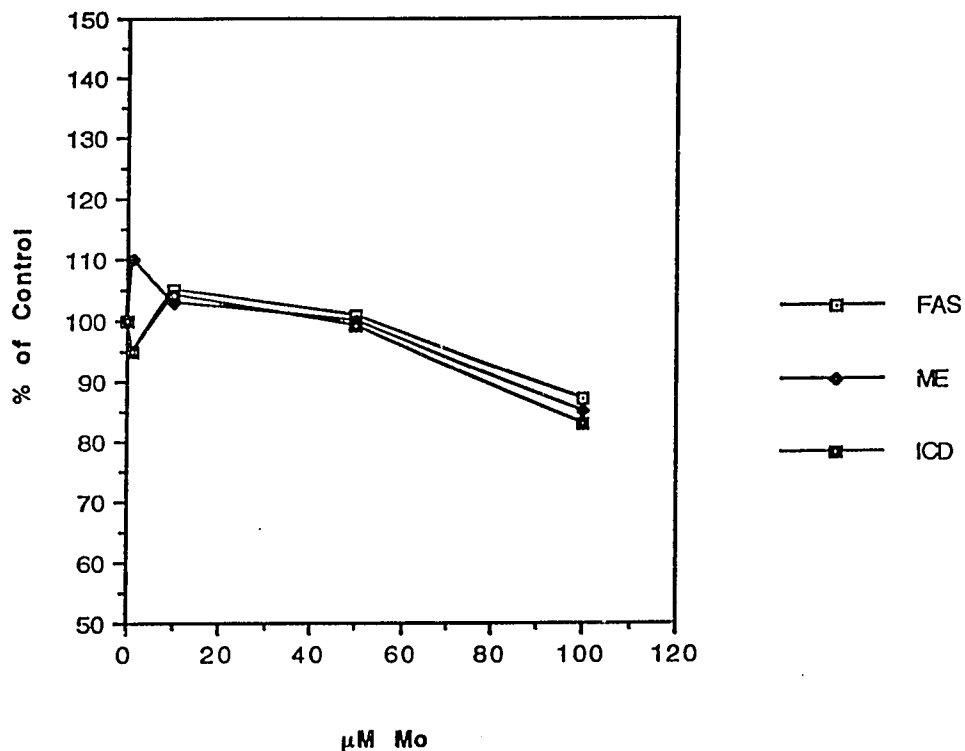


Figure 2. Effect of Ammonium Molybdate on the T3-Induced Increase in Fatty Acid Synthase and Malic Enzyme Activity.

Hepatocytes were incubated for 48 h in the presence of T3 ($1 \mu\text{g/ml}$) and either 0, 1, 10, 50, or 100 μM ammonium molybdate (Mo). After harvest, the activities for fatty acid synthase (FAS), malic enzyme (ME) and isocitrate dehydrogenase (ICD) were determined. The results are expressed as a percentage of the value for cells incubated with T3. Similar results (data not shown) were obtained with cells incubated in the presence of insulin. The results are an average of 5 experiments.

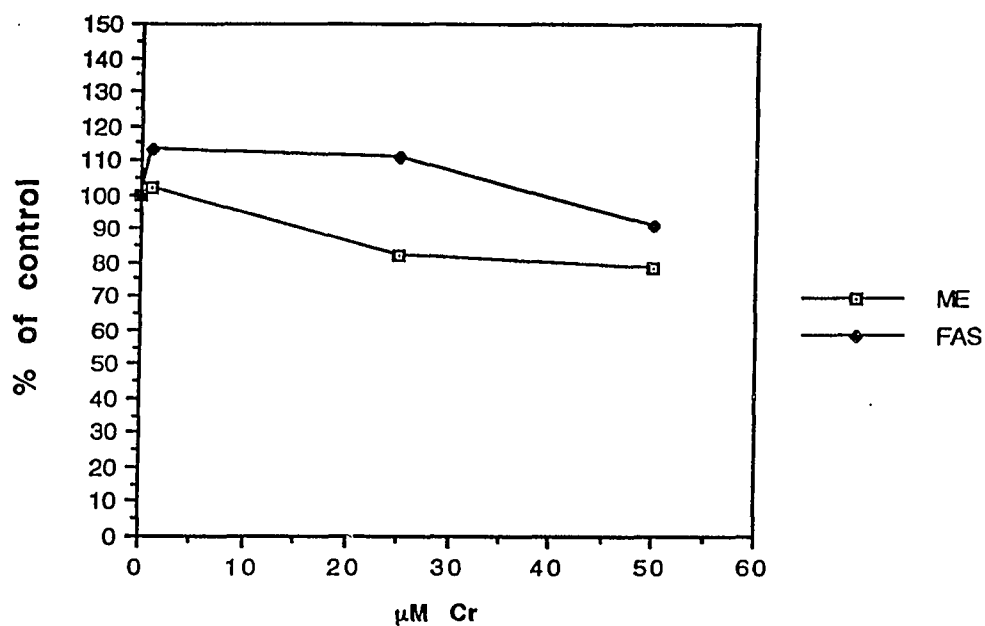


Figure 3. Effect of Ammonium Chromate on the T3-Induced Increase in Fatty Acid Synthase and Malic Enzyme Activity.

Hepatocytes were incubated for 24 h in the presence of T3 (1 $\mu\text{g/ml}$) and either 0, 1, 25 or 50 μM ammonium chromate (Cr). After harvest, the activities for fatty acid synthase (FAS), malic enzyme (ME) were determined. The results are expressed as a percentage of the value for cells incubated with T3. The results are a duplicate of one experiment.

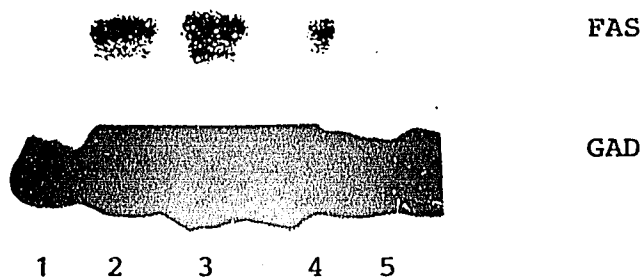
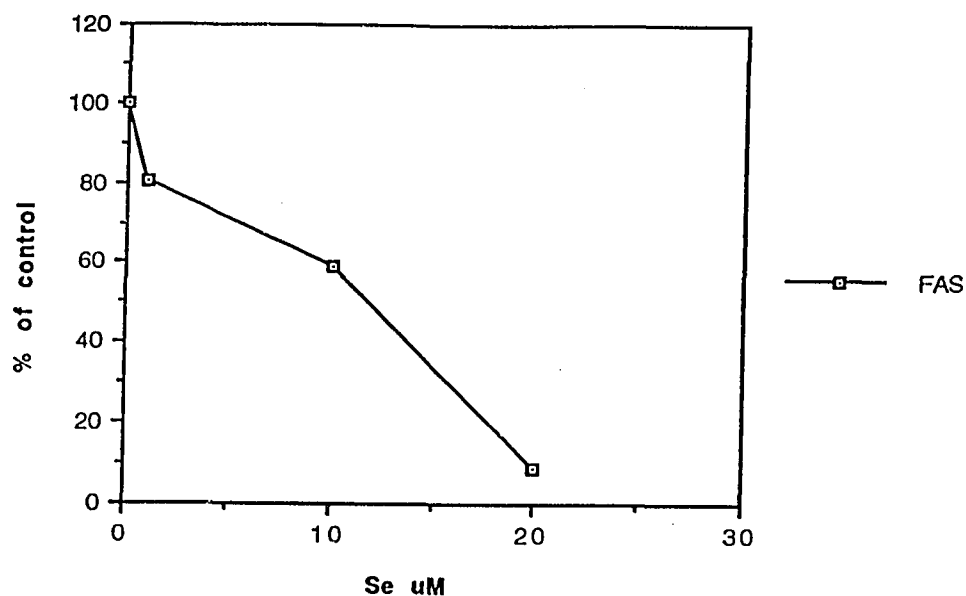


Figure 4. Effect of Sodium Selenate on the T3-Induced Increase in mRNA Level for Fatty Acid Synthase.

Hepatocytes were incubated for 24 h with either no additions (lane 1) or 1 $\mu\text{g/ml}$ T3 (lane 2) or T3 plus 1 (lane 3), 10 (lane 4), 20 (lane 5) μM sodium selenate. Total RNA was isolated and separated by gel electrophoresis. The RNA was transferred to "Genescreen" and hybridized to labeled cDNAs for fatty acid synthase (FAS) and glyceraldehyde-3-phosphate dehydrogenase (GAD). The relative intensity of the mRNA for FAS in each lane was measured by scanning densitometry. The results are representative of 10 experiments.

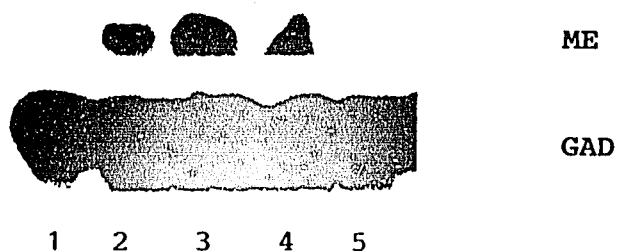
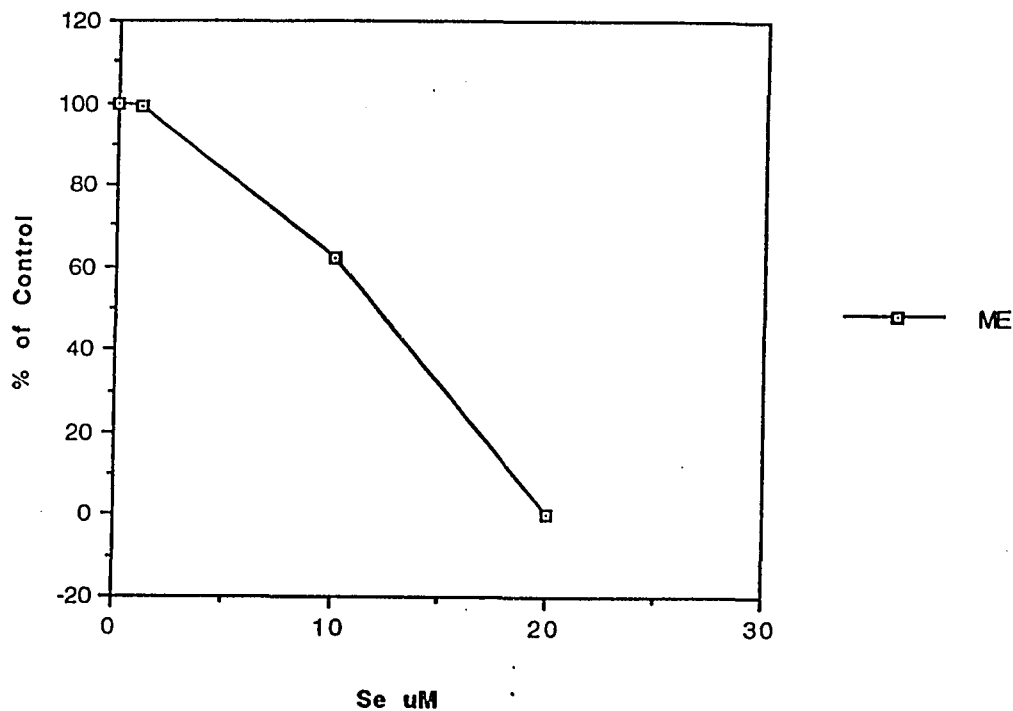


Figure 5. Effect of Sodium Selenate on the T3-Induced Increase in mRNA Level for Malic Enzyme.

Hepatocytes were incubated for 24 h with either no additions (lane 1) or 1 μ g/ml T3 (lane 2) or T3 plus 1 (lane 3), 10 (lane 4), 20 (lane 5) μ M sodium selenate. Total RNA was isolated and treated as described in Figure 4. The results are representative of 10 experiments.

the T3-increased accumulation of malic enzyme and fatty acid synthase mRNAs primarily at a pretranslational step.

Neither molybdate ($25\ \mu\text{M}$) nor chromate ($50\ \mu\text{M}$) had any effect on the T3 induced mRNA levels for both malic enzyme and fatty acid synthase (Figure 6). These results paralleled the results obtained on enzyme activities. The specificity of the effects of these ions was assessed by measuring recovery of the abundance of glyceraldehyde-3-phosphate dehydrogenase mRNA level. The effect of sodium selenate was selective because it had no effect on the level of glyceraldehyde-3-phosphate dehydrogenase mRNA in the cells in culture. Neither molybdate or chromate had any effect on glyceraldehyde-3-phosphate mRNA suggesting that these ions had no ill effects on these cells in culture.

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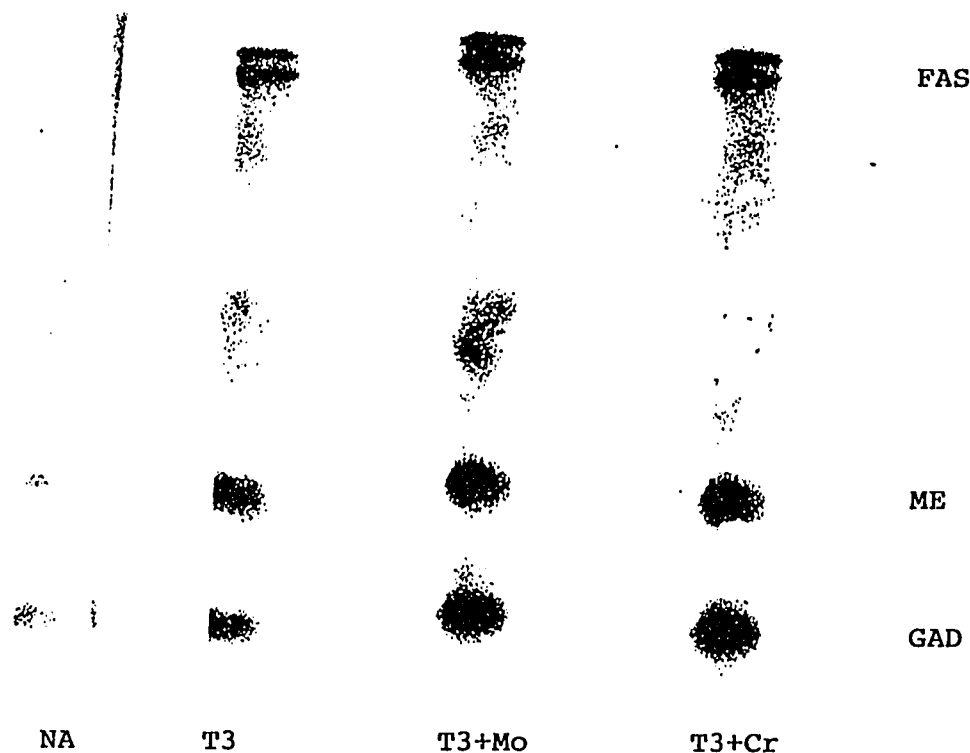


Figure 6. Effect of Ammonium Molybdate and Ammonium Chromate on the T3-Induced Increase in mRNA Levels for Fatty Acid Synthase and Malic Enzyme.

Hepatocytes were incubated for 24 h with either no additions (NA) or 1 $\mu\text{g/ml}$ T3 (T3) or T3 plus 25 μM ammonium molybdate (T3+Mo) or T3 plus 50 μM ammonium chromate (T3+Cr). Total RNA was isolated and hybridized as described in Figure 4. These results for molybdate are representative of 5 experiments and for chromate, 1 experiment.

DISCUSSION

The biosynthesis of fatty acids is regulated by the nutritional state of the animal. In general, the liver in fed animals has a high rate of fatty acid synthesis and a high level of lipogenic enzymes, whereas the liver in fasted animals has a low level of lipogenic enzyme activities (5). Chick-embryo hepatocytes in culture provide an excellent model system to study the dietary regulation of fatty acid synthesis. By adding hormones known to be elevated in the fed state (insulin and T3) or the starved state (glucagon), experiments can be designed to address the mechanism of regulation of fatty acid biosynthesis.

Insulin plus T3 causes a large increase in the rate of synthesis of malic enzyme and fatty acid synthase in chick embryo hepatocytes maintained in a chemically defined medium (6, 7). Glucagon inhibits this increased synthesis of malic enzyme and fatty acid synthase caused by insulin plus T3. Interestingly, insulin alone has no effect on enzyme accumulation, but it can amplify the stimulatory effect of T3. An increase in the abundance of full-length malic enzyme and fatty acid synthase mRNAs caused by T3 is correlated positively with the rate of enzyme synthesis under these conditions, and the increased mRNA levels, in turn, are correlated with a quantitatively comparable increase in

transcription of the genes for malic enzyme and fatty acid synthase. Regulation by insulin plus T3 is therefore transcriptional (12, 13). Glucagon or dibutyryl cyclic AMP inhibits the T3 induced transcription of the gene for malic enzyme but has little or no effect on transcription of the gene for fatty acid synthase (13).

Substantial evidence now exists to indicate that a phosphorylation event may be involved in the regulation of lipogenesis by these hormones. T3 stimulates protein kinase activity or protein phosphorylation in intact animals and in cells in culture. H-8, H-7 and HA1004, protein kinase inhibitors, block the ability of T3 to stimulate transcription of these lipogenic genes (43). In an attempt to elucidate how phosphorylation affects the T3 induction of lipogenesis, experiments involving other agents involved in phosphorylation were carried out.

Sodium selenate, a substance shown to be involved in various phosphorylation events, blocks the T3-induced increase in activity of malic enzyme and fatty acid synthase (Figure 1). The T3-induced accumulation in their respective mRNA's is also blocked by sodium selenate (Figure 4, 5). Recent evidence has demonstrated that selenate has an insulin-mimicking effect on various physiological processes (47). Thus, we expected to find that selenate would stimulate the T3-induced enzyme activity and mRNA accumulation for malic enzyme and fatty acid synthase. The results

obtained, however, indicate that if sodium selenate had insulin-mimicking effects in these cells in culture, its negative effects on T3 action are more potent. To fully investigate whether or not selenate has any insulin-mimicking effect in these cells in culture, one must study its effects on a gene solely regulated by insulin. Selenate's effects were specific. At concentrations of 20 μ M or less, selenate had little or no effect on enzyme activity of isocitrate dehydrogenase (Figure 1), or on the level of mRNA of glyceraldehyde-3-phosphate dehydrogenase (Figure 4, 5). From these results, it can be concluded that selenate was not toxic to the chick-embryo hepatocytes at the concentrations tested and that selenate selectively inhibited the T3-induced enzyme activity and mRNAs for these lipogenic proteins. The fact that selenate inhibits T3 induced lipogenesis is, however, quite interesting in lieu of the H-8 inhibition of T3 induced lipogenesis. Since H-8 is a protein kinase inhibitor and selenate is linked to an increase in phosphorylation of certain proteins either by increasing kinase activity or inhibiting phosphatase activity, and both these compounds have the same effect on T3 induced lipogenesis, it might be concluded that T3 action occurs via a mechanism involving phosphorylation/dephosphorylation. This cascade of events has been proposed recently for the control of a variety of other cellular processes (50) and could potentially explain the results

obtained in these experiments. Another anion, chromate, has also been shown to have insulin-mimicking action under certain condition. If its action on cellular processes are by mechanisms more similar to insulin than selenate, then the observed results should be an amplification of the T3 response. Chromate, however, had no effect on fatty acid synthase or malic enzyme activities or mRNA levels (Figure 3, 6). Chromate, at concentration up to 100 μ M, was unable to amplify the T3 effect as insulin does, nor did it inhibit as selenate does. Unfortunately, there is no convenient positive control that can be used to ensure that these cells have actually taken up the chromate and are utilizing it. By analogy to the other systems as well as the fact that other anions are taken up, we can conclude that chromate has no effect on the regulation of fatty acid biosynthesis in chick embryo hepatocytes in culture.

Molybdate, as well as a few other anions, has recently been shown to be involved in the regulation of certain protein tyrosine phosphatases (45). A low molecular mass protein tyrosine phosphatase (PTPase) from human placenta, PTPase 1B is inhibited by vanadate, molybdate and zinc ion. Vanadate and molybdate also inhibit a PTPase from human spleen, CD45, but zinc ion activates it (45). Therefore, the type of effect an ion may have is phosphorylase dependent. Preliminary studies carried out by others in the laboratory indicate that both vanadate and zinc inhibit T3

induced regulation of fatty acid biosynthesis. If molybdate inhibits this process also, the evidence would suggest the possible involvement of a PTPase similar to PTP 1B. Molybdate incubated in the presence of T3 had no effect on the T3 induced activities of malic enzyme or fatty acid synthase (Figure 2). Molybdate also had no effect on the T3-induced accumulations of the mRNAs for malic enzyme or fatty acid synthase (Figure 6). As in the case of chromate, there is no convenient positive control available and an assumption must be made with regard to the uptake of the molybdate.

The results of the experiments presented unfortunately have no clear conclusion. Previous results with the protein kinase inhibitors indicate a phosphorylation event is involved in the T3-induction of the expression of the genes for malic enzyme and fatty acid synthase. Metal ions have recently been indicated in the regulation of phosphorylation by the identification of either the presence or absence of certain phosphoproteins when the metal ions are present or by the identification of specific phosphatases that are affected by them. Selenate, chromate and molybdate are all ions that have been implicated to play roles in phosphorylation. In some physiological processes they may play similar roles and in others they may not. It is clear that the latter is true for these metals and their effects on the regulation of fatty acid biosynthesis by T3.

Selenate inhibits the T3-induced expression of the genes for malic enzyme and fatty acid synthase, while molybdate and chromate have no effect. If the action of selenate is modulated through a phosphorylation event, a likely point would be at the T3 receptor itself. If selenate somehow alters the phosphorylation state of the receptor so that the ligand-receptor-DNA complex cannot form, then T3 inducible expression cannot take place. Future work is needed to investigate this and other possibilities.

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