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Thyroid Hormone Action In Cultured Hepatocytes

Elizabeth Jane Wilson

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THYROID HORMONE ACTION
IN CULTURED HEPATOCYTES

By

Elizabeth Jane Wilson

Department of Biochemistry

Submitted in partial fulfillment

of the requirements for the degree of

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Faculty of Graduate Studies

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TO THE MEMORY OF MY MOTHER

BÀRBARA ANN BANNIHR WILSON

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A B S T R A C T

The object of the research in this thesis was to demonstrate and characterize the actions of thyroid hormones at the cellular level. It was necessary to develop a cell culture system responsive to thyroid hormones. A method for the primary culturing of adult rat hepatocytes in serum-free medium is described. Culture surfaces were coated with a film of rat tail collagen which increased the longevity of the cells in culture. The addition of insulin or dexamethasone to the culture medium altered the morphology of the cells when compared to hepatocytes maintained in hormone-free medium. Insulin and dexamethasone, together, substantially improved the maintenance of morphology and longevity of the cells in culture. The addition of triiodothyronine, alone or with insulin and dexamethasone did not alter cell morphology or longevity.

Treatment of hepatocyte cultures with triiodothyronine in the presence of insulin and cortisol caused the concurrent inductions of mitochondrial α-glycerophosphate dehydrogenase and cytosolic malic enzyme which follow the same time course as the *in vivo* response to triiodothyronine. Hepatocytes isolated from thyroidectomized rats also respond to triiodothyronine with increased α-glycerophosphate dehydrogenase activity. Because hepatocyte cultures are only useful for short term studies (4 days), thyroid hormone actions on other enzymes, e.g. succinate dehydrogenase,

NADPH-cytochome c reductase and glucose-6-phosphatase, could

not be fully characterized.. The content of mitochondrial

cytochromes a($+$a$_3$), b and c were increased in hepatocytes by

3 days of triiodothyronine treatment. Thyroid hormone effects

on glycerolipid synthesis were observed within 1 or 2 days

of triiodothyronine treatment. The incorporation of choline

into microsomal phospholipids was decreased by triiodothyro-

nine. Triiodothyronine treatment increased glycerol

incorporation into triglycerides but not into phospholipids

of cultured hepatocytes.

Although increases in α-glycerophosphate dehydrogenase

and malic enzyme activities were observed when triiodothy-

ronine was added to the culture medium, in the presence of

insulin and cortisol or dexamethasone, the response was

significantly greater. Insulin and glucocorticoids did not

cause any marked increase in either enzyme in the absence of

triiodothyronine. Thyroxine also caused an increase in the

activities of both enzymes.

The potentiation of triiodothyronine-action varied

with the concentrations of insulin, dexamethasone or cort-

isol in the medium. In the presence of bovine serum

albumin to prevent metabolism of triiodothyronine, hepato-

cytes showed increased enzyme activity at concentrations as

low as 10^{-10}M.

The ability of different steroids to potentiate

triiodothyronine-action coincided with their ability to

induce tyrosine aminotransferase, a specific nuclear action

of glucocorticoids. The concentration of dexamethasone required for maximum potentiation of triiodothyronine-action $(10^{-7}M)$ also maximally induced tyrosine aminotransferase. These results suggest glucocorticoids may act at the nuclear level to potentiate triiodothyronine action in liver cells.

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NOMENCLATURE

ATP	adenosine triphosphate
BES	N,N-bis(2-hydroxyethyl)-2-amino ethane sulfonic acid
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis-(β-amino ethyl ether) N,N'-tetraacetic acid
FBS	fetal bovine serum
Hepes	N-(2-hydroxyethyl)-piperazine-N'-2-ethane sulfonic acid
mRNA	messenger RNA
NADP	nicotinamide adenine dinucleotide phosphate
$NADP^+$	NADP (oxidized form)
NADPH	NADP (reduced form)
$Na^+K^+ATPase$	$(Na^+ + K^+)$-dependant adenosine triphosphate phosphohydrolase
PBS^-	Dulbecco's phosphate buffered saline without Ca^{2+} or Mg^{2+}
RNA	ribonucleic acid
T_3	triiodothyronine; 3,3',5-triiodo-L-thyronine
T_4	thyroxine; 3,3',5,5'-tetraiodo-L-thyronine
TES	N-tris(hydroxymethyl)methyl-2-amino ethane sulfonic acid
Tris	tris(hydroxymethyl)aminomethane

CHAPTER 1. INTRODUCTION

The thyroid hormones, T_4 and T_3, are remarkable for their ability to affect the metabolism or development of virtually all tissues in the mammalian body. In spite of the fact that the changes observed in thyroid states, either naturally occurring or induced in laboratory animals have been studied for a long time, the cellular effects and mechanism of action of these hormones remain to be characterized clearly (Wolff and Wolff, 1964; Turakulov et al., 1975). One reason for the difficulty in examining the actions of thyroid hormone at the cellular level has been the lack of tissue culture systems for definitive studies (Samuels et al., 1973).

In the past few years and during the course of these studies, much new information and new concepts on the actions of thyroid hormone have been published. My research has sought to investigate what the direct actions of thyroid hormone are on cellular processes in a defined tissue culture system, primary cultured hepatocytes. Because of the rapid developments in both areas of research, hepatocyte culturing and thyroid hormone research, the focus of my studies evolved to take advantage of the recent information in the literature. In particular the involvement and interactions of other hormones on the cellular actions of T_3 were examined.

In the rest of this introduction I have discussed con-

cepts of thyroid hormone action emphasizing mammalian effects and included very recent developments, in order to provide the background into which the research in this thesis fits.

1.1.1 Mechanism of Action and Cellular Effects

For information on the history, synthesis, secretion and catabolism of the thyroid hormones, the reader is referred to the comprehensive text The Thyroid (Werner and Ingbar, 1978). T_4 is the predominant secretory product but its conversion to the more active T_3 largely determines the biological effects (Larsen and Frumess, 1977; Chopra et al., 1978; Silva and Larsen, 1978).

One of the earliest recognized actions of thyroid hormones was the calorigenic effect (Barker, 1964). Since mitochondria are the major site of oxygen consumption in the cell, they were centered upon by researchers as the most likely target of hormone action. Additions of large concentrations of T_4 (10^{-5}M) to isolated mitochondria results in uncoupling of oxidative phosphorylation which was assumed to explain increased oxygen consumption (see review by Turakulov et al., 1975). However, oxidative phosphorylation is tightly coupled in vivo irrespective of the thyroid status of the animal (Nishiki et al., 1978). In another report Primack et al. (1971) reported that thyroid hormones at concentrations about 10^{-5}M stimulated protein synthesis in isolated mitochondria. Again, this in vitro action has been shown to be unrelated to the in vivo actions

of thyroid hormones on the basis of the time course of
action and concentration requirements (Gordon et al.,1973).

Ismail-Beigi and Edelman (1971) proposed that increased
activity of $Na^+K^+ATPase$ by T_3-treatment in vivo is the ATP
utilizing process responsible for the calorigenic action.
De novo synthesis of $Na^+K^+ATPase$ is stimulated by thyroid
hormones (Lo and Lo, 1980). Sestoft (1980) disagrees that
$Na^+K^+ATPase$ plays a dominant role in thyroid hormone-induced
calorigenesis and reviews evidence that increased oxygen
consumption is due to futile cycling of free fatty acids
into triglycerides. Increased synthesis and oxidation of
fatty acids in the hyperthyroid state may also contribute
to thyroid hormone-induced calorigenesis (Fain, 1980).

It was realized in some instances (e.g. hepatic
mitochondrial α-glycerophosphate dehydrogenase and cytosolic
malic enzyme) that the activities of enzymes are increased
by thyroid hormones due to the induction of new enzyme pro-
tein (Tarentino et al., 1966; Murphy and Walker, 1974).
Since thyroid hormones influence growth and development, it
followed that the many actions of these hormones might be
explained by regulation of the synthesis of cellular con-
stituents. The time course of the various responses of
thyroidectomized rats to an injection of T_3 demonstrated
that the earliest observed effects included labelling of
nuclear RNA and increased activity of RNA polymerases,
suggesting control at the gene level (Tata, 1969). High
affinity, limited capacity T_3-binding sites were identified

in the nuclei of rat liver and kidney cells (Oppenheimer et
al., 1972). Considerable evidence has now accumulated that
supports the view that these sites are the true receptors
responsible for the initiation of thyroid hormone action
(Oppenheimer and Dillmann, 1978; Oppenheimer et al., 1979).
Other proposed mechanisms of action of thyroid hormones
involve stimulation of mRNA translation (Carter et al., 1976)
and incorporation of iodothyronines into proteins (Dratman
et al., 1970).

Thyroid hormones play an important role in mitochon-
drial biogenesis (reviewed by Wooten and Cascarano, 1980).
Most mitochondrial proteins are coded for by nuclear DNA
but some of the proteins increased by thyroid hormones such
as cytochrome b and subunits of cytochrome c oxidase (Roodyn
et al., 1965; Nishiki et al., 1978) are coded for by the
mitochondrial DNA (Milner, 1976; Tzagoloff et al., 1979).
The assembly of mitochondrial enzyme complexes is dependent
upon the cooperation between the mitochondrial genetic
system and the nuclear system (Freedman and Chan, 1978).
Thus, the ability of thyroid hormones to regulate mitochon-
drial biogenesis may yet be explained by actions at the
nuclear level regulating the mitochondrial genetic system.

High affinity, low capacity binding of thyroid hor-
mones to a lipoprotein of the inner mitochondrial membrane
isolated from rat liver has been reported (Sterling et al.,
1978). Direct activation of mitochondrial energy metabolism
via this putative receptor would explain rapid hormone

actions independent of protein synthesis (Sterling et al.,
1980). Corroborative evidence is so far lacking as other
researchers have been unable to find specific mitochondrial
T_3-binding sites in vivo or in vitro (Greif and Sloane,
1978).

Actions and developmental effects of thyroid hormones
that are explained by enzyme induction or increases in
protein synthesis are surveyed below. Different mechanisms
of stimulation of general protein synthesis have been pro-
posed to explain the role of thyroid hormones in brain
development (Sokoloff, 1977, Valcana and Eberhardt, 1977).
More specifically, thyroid hormones may regulate neurotubule
assembly by controlling the levels of a promoter of micro-
tubule polymerization (Fellows et al., 1979). Increased
levels of lysosomal proteases in hyperthyroidism or
thyrotoxicosis account for increased protein catabolism
(DeMartino and Goldberg, 1978). The levels of lipogenic
enzymes such as fatty acid synthetase, NADPH generating
enzymes and fatty acid elongation enzymes are increased by
thyroid hormones (Roncari and Murthy, 1975; Gnoni et al.,
1978; Mariash et al., 1980). The cholesterol synthesizing
enzyme, hydroxymethyl glutaryl-CoA reductase is induced by
thyroid hormones (Lakshmanan et al., 1975; reviewed by
Eberhardt et al., 1980). At the same time thyroid hormones
increase the levels of lysosomal acid lipases responsible
for lipid degradation (DeMartino and Goldberg, 1978; Coates
et al., 1979). Thyroid hormones regulate the expression of

the gene for growth hormone (Coulombe et al., 1978). Many in vivo effects of the thyroid hormones on growth and development are attributed to the stimulation of growth hormone production (Krane and Goldring, 1978a).

Another mechanism by which thyroid hormones act is by altering the response of certain tissues to catecholamines. The actions of thyroid hormones on the cardiovascular system include tachycardia, increased contractibility, glycogenolysis and lipolysis. These effects are mediated by an increased sensitivity to catecholamines caused by an increased number of β-adrenergic receptors (Kempson et al., 1978). Thyroid hormones increase receptor levels by rapid post-translational events and long term transcriptional-translational events (Kempson et al., 1978). In the case of bone resorption, thyroid hormones are thought to increase the response to catecholamines by inhibiting phosphodiesterases which degrade catecholamines' secondary messenger, cAMP (Krane and Goldring, 1978b).

The effects of thyroid hormones on carbohydrate metabolism are complex in that opposing metabolic pathways are activated. Baquer et al. (1976) suggest that increases in cAMP and cGMP phosphodiesterases which occur in hypothyroidism, depress the response to hormones such as epinephrine, glucagon and insulin which act via secondary messengers cAMP and cGMP. Thus, both anabolic and catabolic pathways are depressed in the absence of thyroid hormone.

In summary, evidence exists for multiple mechanisms

of action of the thyroid hormones including direct activation of cellular processes, modulation of the action of other hormones and control of gene expression.

1.1.2 Thyroid Hormone Action in Tissue Culture

Because of the involvement of other hormones, tissue interactions, and the complexity of metabolic regulation, in vivo studies are unable to define clearly the primary actions of the thyroid hormones. Tissue culture systems have the potential to solve many of the problems associated with in vivo studies since a homogeneous cell population can be studied in a defined environment. Much effort has been put into developing culture systems that retain differentiated properties and can respond to or are dependent upon hormones (MacIntyre, 1974). Finding cultured cells that will respond to the thyroid hormones has been particularly difficult.

Some actions of thyroid hormones exhibited in culture systems that require high concentrations (10^{-5}M) include growth inhibition of human cells from fetal lung and kidney (Leslie and Sinclair, 1959) and increased glucose and lactic acid production in chick embryo fibroblasts (Halevy and Avivi, 1960). These same actions of thyroid hormone have been described in human fibroblasts (Yoshizato et al., 1980). A heteroploid cell line of human kidney cells (T or T-1 cells) respond to T_4 or T_3 concentrations of 10^{-5}- 10^{-7}M with increased plating efficiency and changes in the

cell cycle, protein and nucleic acid synthesis (Siegel and Tobias, 1966; Burki and Tobias, 1970).

A cell line, GH_1, initiated from a rat pituitary gland tumour was the first cell system shown to respond to physiological levels of the thyroid hormones. T_4 and T_3 decrease the doubling time, increase glucose utilization, stimulate de novo synthesis of growth hormone and inhibit production of prolactin in GH_1 cultures (Samuels et al., 1973; Samuels and Shapiro, 1976). Other researchers have confirmed that thyroid hormones regulate the production of growth hormone in related rat pituitary cell lines, GH_3 (Ivarie et al., 1980) and GC cells (Martial et al., 1977). In all these cell lines, thyroid hormones induce the synthesis of pre-growth hormone mRNA by binding to specific nuclear receptors (Samuels, 1978; Latham et al., 1978).

In another rat pituitary tumour cell line $GH_3/C14$, thyroid hormones are required for in vivo estrogen-dependent growth but when the cells are cultured in vitro, thyroid hormones are directly mitogenic (Sorrentino et al., 1976; Kirkland et al., 1976).

Tissue culture systems originating from chick embryos have been very useful in the study of thyroid hormones. In chick embryo heart cells, T_3 (10^{-9}M) stimulates deoxyglucose uptake, an effect which is partly insensitive to transcription or translation inhibitors (Segal et al., 1977; Segal and Gordon, 1977). Lipogenesis in chick embryo liver cells in primary culture is regulated by T_3 (Goodridge et al.,

1974). Malic enzyme is induced by T_3, and insulin, which has only a modest effect alone, potentiates the response to T_3 (Goodridge and Adelman, 1976).

T_3 stimulates the uptake of 2-deoxyglucose and 3-O-methylglucose by a mechanism that does not require protein synthesis in isolated rat thymocytes (Segal and Ingbar, 1979).

Synthesis of myelin-associated lipids is regulated by thyroid hormones in cultures of dissociated brain cells from embryonic mice (Bhat et.al., 1979).

During the course of the studies in this thesis, a few reports on the action of T_3 in isolated or primary cultured rat hepatocytes have appeared. In isolated hepatocytes, the level of extractable lactate dehydrogenase is increased by three hormones: insulin, glucagon and T_3 (Suleiman and Vestling, 1979). Glucokinase is induced by insulin and dexamethasone in primary cultures from a euthyroid rat but cultures derived from a thyroidectomized rat must be preincubated with T_3 before such glucokinase inductions are observed (Spence and Pitot, 1979). A similar phenomenon occurs with the regulation of glutamine synthetase where T_3 reinforces stimulation by growth hormone and dexamethasone (Gebhardt and Mecke, 1979b). In another report, addition of T_3 to primary cultured hepatocytes from a thyroidectomized rat results in an increase in oxygen consumption, Na^+K^+ATPase and α-glycerophosphate dehydrogenase activities (Ismail-Beigi et al., 1979).

In summary, studies on the control of growth hormone
production in rat pituitary cell lines and induction of
malic enzyme in cultured chick hepatocytes have provided
much information on the nuclear initiation of thyroid hor-
mone action. In vitro studies with isolated cells have
given direct evidence that glucose uptake is stimulated by
extra-nuclear action. Other cell culture studies demonstrate
that other hormones may interact with thyroid hormones at
the cellular level.

The research presented in this thesis takes tissue
culture studies of thyroid hormone action a step forward
through the use of a completely defined system and critical
examination of hormone concentrations and interactions.
Each chapter includes its own introduction, methods, results
and discussion of a particular aspect of this problem.

CHAPTER 2.   PRIMARY CULTURING OF
ADULT RAT HEPATOCYTES

2.1   INTRODUCTION

Experiments with the rat pituitary cell line, GH_1, gave the first demonstration that cultured mammalian cells could respond to physiological levels of thyroid hormone in vitro (Samuels et al., 1973).   In rat pituitary cell lines, the transcription of the gene for growth hormone is regulated by specific binding of T_3 to nuclear receptors (Samuels and Shapiro, 1976; Seo et al., 1977).   These cell lines have been extensively studied in the past few years as a model system for hormonal control of gene expression (Samuels et al., 1977, 1979a, 1979b, 1980; Martial et al., 1977; Eberhardt et al., 1980; Ivarie et al., 1981).

The control of pituitary hormones in vivo and in vitro is a tissue-specific action of thyroid hormones.   In other tissues such as liver, kidney and heart, thyroid hormone has a more general effect increasing the activities of many enzymes (Wolff and Wolff, 1964).   In order to study such regulation of enzyme activity at the cellular level, a method of culturing adult rat hepatocytes was developed.   The reason for using primary cultures of liver cells were: 1) in comparative studies, responses of enzymes in liver are greater than in other tissues following the administration of thyroid hormones in vivo (Wolff and Wolff, 1964; Lee and Lardy, 1965); 2) perma-

nent cell lines established from liver or hepatomas retain only a few differentiated functions (Thompson et al., 1966; Potter, 1972; Bissell et al., 1978; Pitot and Sirica, 1980); 3) primary cultured hepatocytes carry out many liver-specific functions and are responsive to hormones (Bissell et al., 1973; Bonney et al., 1974; Michalopoulos and Pitot, 1975).

Liver parenchymal cells are functionally hetero-geneous (Jungermann and Sasse, 1978). Since primary cultures of rat hepatocytes are mass cultures, they are thought to be more representative of liver tissue than cell lines or long term cultures in which selection of certain cell populations has occurred (Laishes and Williams, 1976a; Bonney et al., 1974; Gerschenson and Thompson, 1975).

The isolation of adult rat hepatocytes by methods involving in vivo perfusion with collagenase has become routine (Berry and Friend, 1969; Fry et al., 1976; for reviews see Wagle, 1975 and Seglen, 1976). Freshly iso-lated hepatocytes have been extensively used for short term studies (Sundler and Akesson, 1975; LeCam and Freychett, 1977; Katz et al., 1979a; for review see Seglen, 1976). Primary cultures of rat hepatocytes are required if thyroid hormone action is to be studied since many in vivo actions involve a long lag time. A maximum response to a single dose requires 2-4 days (Tata, 1969).

The development of the method for primary culturing of adult rat hepatocytes used in these studies involved the

trial of numerous modifications of perfusion and culture
techniques. In this chapter the method is detailed and
certain critical steps are discussed. The properties of
hepatocytes in different culture conditions are described.

2.2   MATERIALS AND METHODS

2.2.1 In Vivo Perfusion and Hepatocyte Isolation

Male Sprague-Dawley or Wistar rats (200-300 g) were
obtained from Canadian Breeders (Montreal, Quebec). A rat
was anesthetized by an intraperitoneal injection of
Nembutal ®, 0.1 ml 6% solution per 100g body weight (Abbott
Laboratories, Montreal, Quebec). Clippers were used to re-
move hair from the abdomen and chest areas. Operative pro-
cedures are based on the standard procedures for in situ
liver perfusion (Ross, 1972) and were carried out in a
horizontal laminar flow hood under sterile conditions.

The anesthetized rat was placed on an operating plat-
form above the level of the roller pump (MHRE.100, Watson-
Marlow Ltd.) and a 39°C water bath containing the perfusion
media. Tubing was sterilized by running through 80%
ethanol and then sterile water. The surgical area was
wiped with alcohol. The abdomen was opened and the portal
vein exposed. Loose ligatures were placed, one around the
inferior vena cava above the renal vein and two around the
portal vein about 5 mm apart. The more distal ligature
around the portal vein was tied off. The portal vein was
nicked with scissors between the ligatures. A cannula of

polyethylene tubing was inserted into the nick and secured in place by the second ligature. Perfusion was started immediately with sterile EGTA-Washout Medium (39°C) containing 0.5 mM EGTA in Ca^{2+} and Mg^{2+} free Hanks' balanced salt solution buffered with 50 mM Hepes, pH 7.2, at 8 ml/min (William et al., 1977). Escape of the perfusate was allowed by nicking the inferior vena cava below the renal vein. The chest cavity was opened and the thoracic inferior vena cava was cannulated by puncturing the right atrium with a sharp piece of polyethylene tubing and the tubing secured in place by a ligature. The subhepatic inferior vena cava was tied off and flow directed through the outflow cannula in the thoracic inferior vena cava. The flow rate was increased to 30-40 ml/min and let run to waste until all blood was flushed from the liver.

Collagenase Perfusion Medium was prepared before the operation by adding collagenase (45 U/ml, Type IV, Sigma Chemical Co., St. Louis, Mo.) to L-15 medium (Flow Laboratories, Mississauga, Ontario) supplemented with 5.5 mM glucose, 5 mM Ca^{2+}, antibiotics (100 U/ml penicillin G and 100 µg/ml streptomycin sulfate) and 50 mM Hepes, pH 7.4. The Collagenase Perfusion Medium was sterilized by filtration (Nalgene®, 0.2µ) and placed in the 39°C water bath.

Perfusion of the liver with collagenase was begun at 30-40 ml/min and allowed to run to waste for approximately 1 min before recirculation was started. Over the 10-12 min

perfusion period the flow rate was decreased to 20 ml/min (Williams et al., 1977).

The liver was excised and placed in 40 ml of Collagenase Perfusion Medium ($4^{O}C$). Cells were dissociated by 'combing' the digested liver using sterile forceps and a dog hair comb (Laishes and Williams, 1976a). The cell suspension was filtered through gauze. Parenchymal cells were isolated from debris and other cell types by 4 centrifugations at 50-70 x g for 2 min each, resuspending each time in $4^{O}C$ Washing Medium (L-15 medium, 5.5 mM glucose, antibiotics, 10 mM Hepes, pH 7.4, and 1% fraction V BSA (Sigma) (Bissell et al., 1973).

Viability of the parenchymal cells in Washing Medium was determined by exclusion of trypan blue. Primary cultures were initiated by inoculating 10^{5} viable cells/0.2 ml warmed medium/cm^{2} culture area (Tanaka et al., 1978). Details of the culture media and conditions are described for each experiment. All hormones were purchased from the Sigma Chemical Co.

2.2.2 Preparation of Collagen-coated Tissue Culture Dishes

Two or three rat tails were dissected to give 0.5-0.8 g of collagen fibers (weighed 1/2 h after dissection). A collagen solution was prepared using the method of Michalopoulos and Pitot (1975). This solution was poured through gauze into 50 ml Falcon tubes and centrifuged for 10 min at 3000 x g maintaining sterile conditions. The supernantant was stored at $4^{O}C$.

Culture surfaces were collagen-coated as described by Michalopoulos and Pitot (1975). Tissue culture flasks (75 cm^2, Costar or Falcon), sterile plastic petri dishes (56.7 cm^2) or 4 well Linbro ® plates (28.3 cm^2.) received 1.0 ml of collagen solution per 25 cm^2. The plates were dried at 55°C for 24 h and rinsed with 0.1 M phosphate buffer containing 2 mg/l phenol red.

2.2.3 Determination of Cellular Protein or DNA of the Inoculum and Monolayers

The volume of the initial hepatocyte suspension used as inoculum was mixed with 2-3 volumes PBS⁻ (4°C). Cells were pelleted by centrifugation at 500 x g for 10 min and suspended in a known volume of PBS⁻.

In order to harvest the hepatocyte monolayers, the medium was aspirated and the monolayers were rinsed with PBS⁻ to remove unattached cells. Cellular material was scraped off the culture surface using a rubber policeman into PBS⁻ (4°C) and treated the same way as the inoculum sample.

An aliquot of cellular material was incubated for 30 min at 37°C with an equal volume of 2N NaOH and protein determined (Lowry et al., 1951). Sodium citrate was used instead of tartrate and crystallized albumin was the standard.

If DNA was to be determined, a 0.5N $HClO_4$ extract was prepared from an aliquot of the cellular material by

incubating at 70°C for 20 min. The DNA content was deter-
mined by the method of Burton (1956) using diphenylamine
reagent without H_2SO_4. Calf thymus DNA was used for
standards.

2.3. RESULTS

2.3.1 Effect of Dissociation Method on Initiating Cultures

The plating ability of the cells was examined follow-
ing two different methods of dissociation of the cells from
the digested liver. One method was that described in
Materials and Methods in which the excised liver was placed
in 40 ml collagenase perfusion medium (4°C). In the other
method the excised liver was placed in 40 ml of collagenase
medium at 37°C, broken up and incubated at 37°C for 15 min
(Bissell et al., 1973). Dissociated cells from either
method were then purified by low speed centrifugation.

The plating ability of the cells was determined
indirectly by measuring the number of viable and non-viable
cells that were unable to attach after 1.5 h and were thus
floating in the medium (Table 1). Fewer viable cells were
present in the medium after 1.5 h following dissociation by
the 4°C method than the 37°C preincubation. This indicates
that the ability of viable cells to attach was greater for
hepatocytes isolated by the 4°C method and visual observa-
tion of the tissue culture plates confirmed this. The
difference between methods cannot be accounted for by a
greater loss of viability over the 1.5 h period since the

TABLE 1

EFFECT OF DISSOCIATION METHOD ON ATTACHMENT OF VIABLE CELLS

A rat liver was perfused in situ as described in methods. The 4°C dissociation method was that described in Methods. The 37°C preincubation method involves breaking up the liver with a blunt instrument and incubating for 15 min at 37°C (Bissell et al, 1973). Following both methods the cells were centrifuged and inoculated into 5 ml L-15 medium, 5.5 mM glucose, antibiotics, 10 mM Hepes, pH 7.4, and 10% FBS in Linbro ® plates as described in methods. After 1.5 h an aliquot of medium was removed and the number of viable and total cells was determined. Results are expressed as the number of cells ÷ 10^6 per Linbro ® well.

	Inoculated		Unattached after 1.5 h (% of Inoculated)	
	Viable	Total	Viable	Total
4°C Dissociation	2.79	3.03	---	<0.6 (20)
	4.65	5.05	1.75 (38)	2.25 (44)
	7.44	8.09	3.09 (41)	4.11 (51)
37°C Preincubation for 15 min	2.21	2.76	0.81 (37)	1.08 (39)
	4.42	5.52	3.22 (73)	3.85 (70)
	6.63	8.29	5.37 (81)	6.72 (81)

total number of cells as well as the number of viable cells unattached was also less following 4°C dissociation. Following dissociation by 37°C preincubation, especially at higher inoculum numbers, it was observed that a large number of viable cells representing a substantial percentage of the inoculated number was unable to attach. Following both methods of dissociation, the percentage of viable cells unattached at 1.5 h increased when the number of cells inoculated was increased.

The purification of parenchymal cells by low speed centrifugation is shown in Figure 1. The initial cell suspension (A) contained large parenchymal cells, cellular debris and smaller non-parenchymal cells. During the centrifugation steps, the large parenchymal cells were pelleted while the non-parenchymal cells remained in the supernatant (B). Almost no non-parenchymal cells could be observed in the final parenchymal cell preparation (C).

2.3.2 Effects of Different Culture Conditions on Monolayer Survival

At the time these culture methods were developed, other workers had consistently reported that serum was especially important for attachment and culturing of hepatocytes (Bissell et al., 1973; Bonney, 1974; Laishes and Williams, 1976a). Following along the same lines, the survival of hepatocytes plated in medium containing 10% FBS and maintained after 2 h in medium containing 2.5% FBS, rat

FIGURE 1

INITIAL CELL SUSPENSIONS

Hepatocytes were isolated as described in Materials and Methods. A - Initial cell suspension prior to low speed centrifugation; B - Supernatant of the first centrifugation step; C - Final suspension of purified parenchymal cells. Size bar represents 0.1mm.
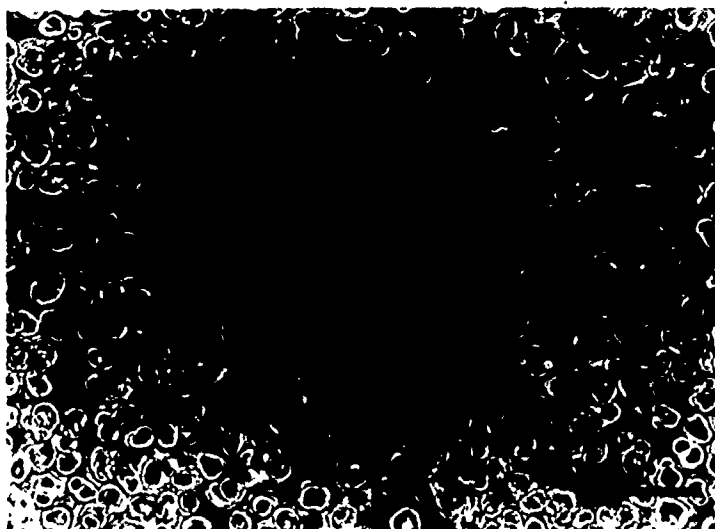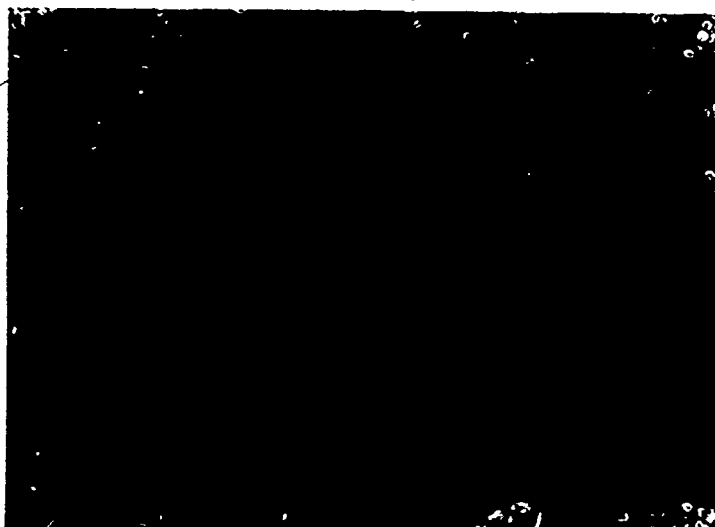
serum or heat inactivated rat serum for 3 days was examined (Fig. 2A). On the basis of cellular protein, approximately 70% of the inoculated hepatocytes were able to attach. During the period from 2 to 26 h, the parenchymal cells flattened and formed a partially confluent monolayer of epithelial-shaped cells. A substantial loss of cells (40% of inoculum) occurred. Between 26 and 50 h the monolayers appeared stable and little loss of cells occurred. After 50 h the monolayer ceased to exist as the hepatocytes aggregated into balls of cells which did not adhere to the growing surface. No difference in survival of the monolayer cultures as measured by cellular protein could be observed between hepatocytes maintained in medium containing the different sera.

Survival of hepatocytes plated and maintained in medium containing 2.5% rat serum and cultured on collagen-coated surfaces was examined (Fig. 2B). The plating and survival (measured as cellular DNA) of the monolayers over the first 50 h was the same as for the hepatocytes examined in Fig. 2A. Hepatocytes cultured on collagen substratum (Fig. 2B) survived better after 50 h. From 26 h to 122 h the monolayers contained epithelial-shaped cells and lost only 20% of inoculated cellular DNA.

The effects of further changes in medium composition were examined. Increasing the glucose concentration from 5.5 mM to 10 mM or adding 1 mM succinate did not affect plating or culture survival (Fig. 3A). When serum was

FIGURE 2

EFFECT OF SERUM AND COLLAGEN SUBSTRATUM

ON MAINTENANCE OF HEPATOCYTES IN CULTURE

Hepatocytes were isolated and plated as described in Materials and Methods. The culture medium consisted of L-15 medium, 5.5 mM glucose, antibiotics and 10 mM Hepes (pH 7.4) plus serum. A) Cells were plated in medium containing 10% FBS and maintained in medium containing 2.5% FBS (●—●) 2.5% rat serum (○—○) or 2.5% heat-inactivated rat serum (△—△). Results are expressed as the % of inoculated cellular protein. B) Tissue culture flasks were coated with a film of collagen as described in methods. Cells were plated and maintained in medium containing 2.5% rat serum. Results are expressed as the % of inoculated cellular DNA.

% of Inoculated Cellular Material

Time in Culture (h)

A

B
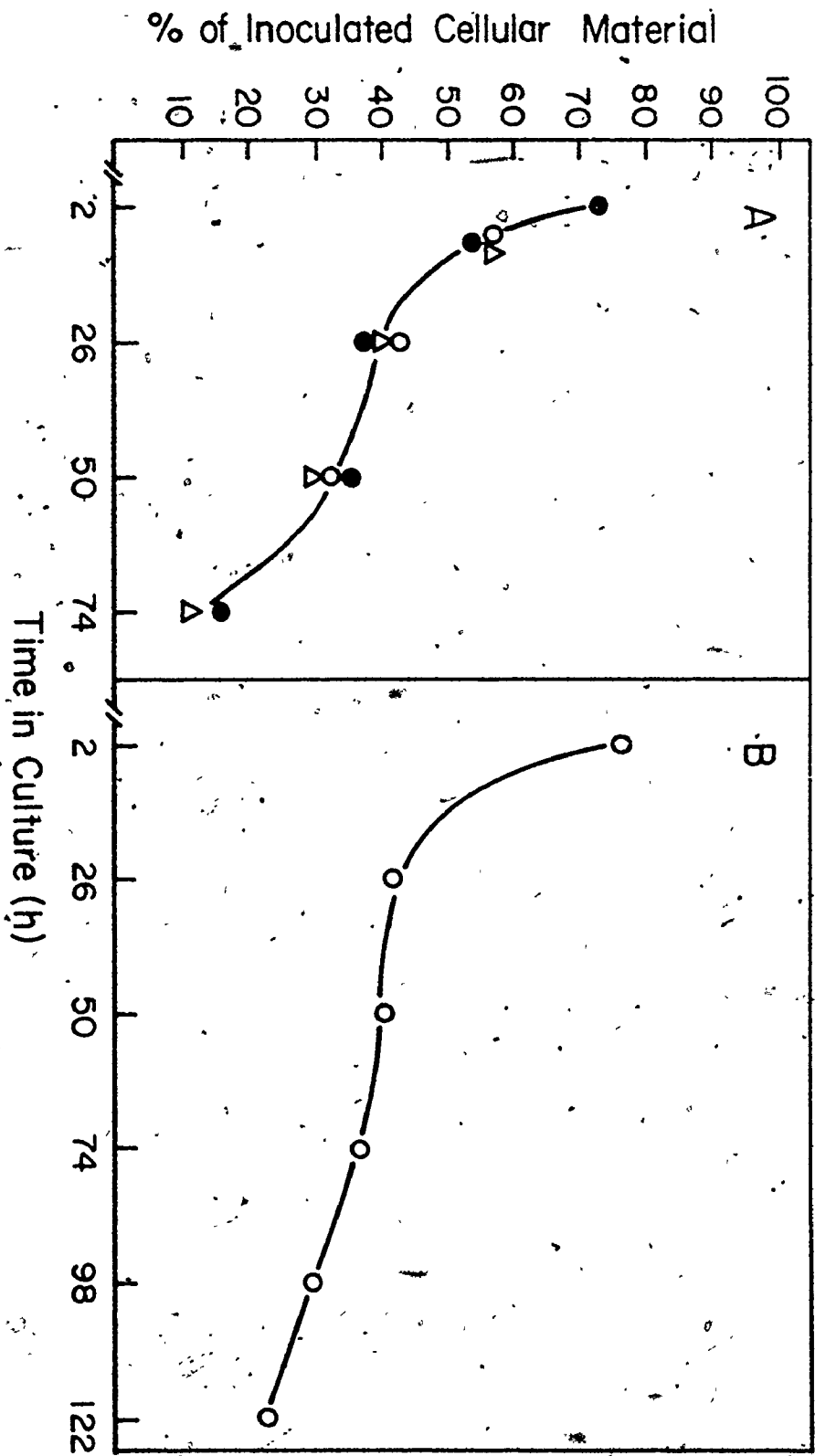
FIGURE 3

EFFECTS OF MEDIUM COMPOSITION AND HORMONES

ON MAINTENANCE OF HEPATOCYTES IN CULTURE

Hepatocytes were isolated and cultured on collagen-coated surfaces as described in Materials and Methods. The survival of hepatocytes in culture was determined by measuring the amount of cellular DNA attached to the culture plates expressed as a % of inoculated DNA. A) Hepatocytes were plated and maintained in L-15 medium, antibiotics, 20 mM Hepes, 10 mM TES and 10 mM BES, pH 7.4, with the following additions: 5.5 mM glucose and 2.5% rat serum (o—o); 10 mM glucose, 1 mM succinate and 2.5% rat serum (□—□); or 10 mM glucose and 1 mM succinate (●—●). B) Hepatocytes were plated in medium consisting of L-15 medium, 10 mM glucose, 1 mM succinate, antibiotics, 20 mM Hepes, 10 mM TES and 10 mM BES, pH 7.4. Control cultures were maintained in the medium used for plating (●—●). After 2h, test cultures received medium supplemented with 1.54×10^{-6}M T_3 (Δ—Δ), 10^{-6}M insulin and 10^{-7}M dexamethasone (■—■) or 10^{-6}M insulin, 10^{-7}M dexamethasone and 1.54×10^{-6}M T_3 (▲—▲).
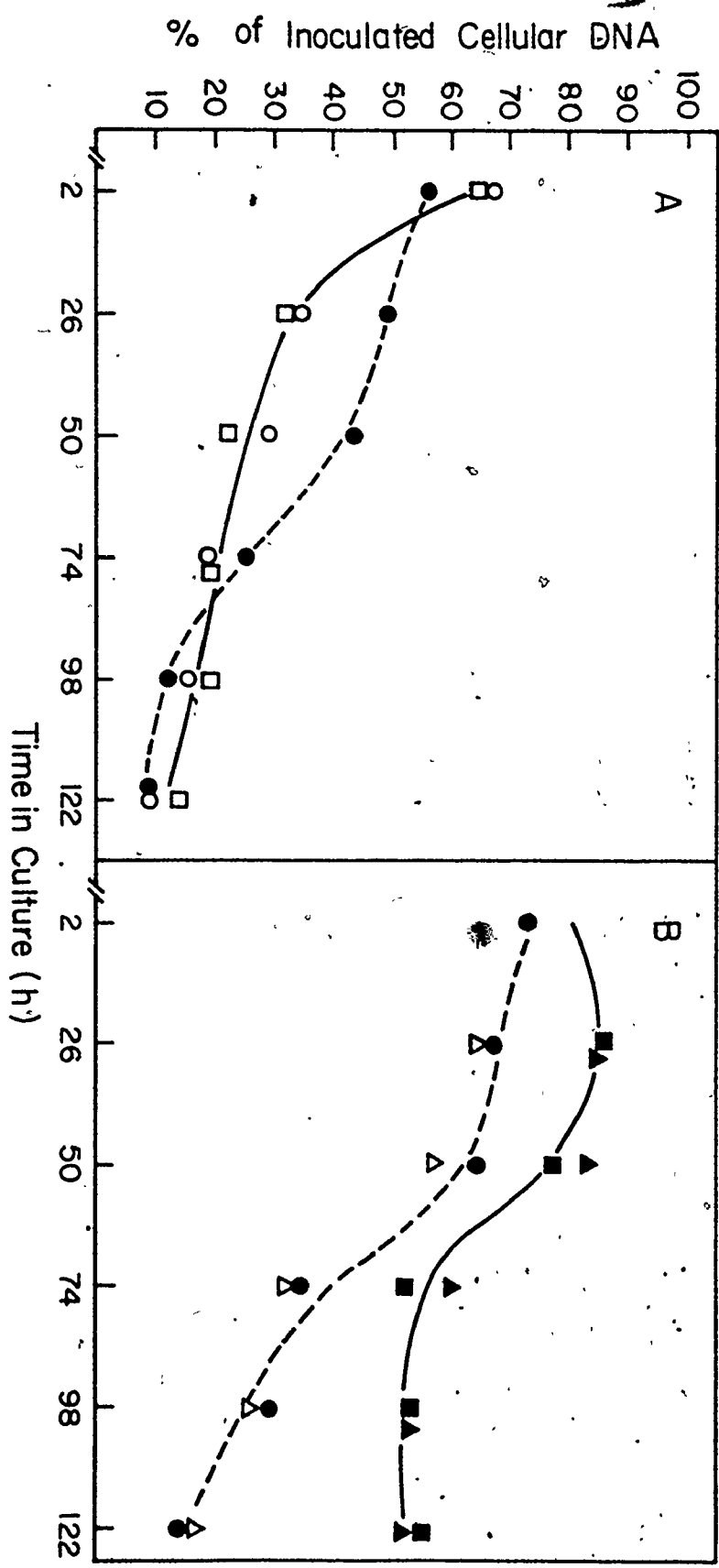
omitted from the medium the amount of cellular DNA (reflecting number of hepatocytes) which attached was decreased slightly. The shape of the survival curves of hepatocytes in serum-free versus serum-containing medium differed in two respects. First, over the first 50 h there was less loss of material from cultures maintained in serum-free medium. Secondly, between 50 and 74 h there was a decline of cellular material in the cultures not seen in serum-maintained hepatocytes such that after 74 h the survival of hepatocytes was similar in both culture conditions.

The effect of hormones on the maintenance of hepatocytes in culture was examined (Fig. 3B). Control cells were maintained in serum- and hormone-free medium used in Fig. 3A. The shapes of the survival curves for the same condition (Fig. 3A vs. Fig. 3B) were similar but the percentage of inoculated DNA in Fig. 3B was greater throughout the culture period. The addition of T_3 after 2 h neither improved nor impaired the ability of hepatocytes to remain in culture. Addition of insulin and dexamethasone after 2 h markedly improved the survival of hepatocytes in culture. Without more detailed experimentation, it is impossible to tell if the rise in percentage of inoculated DNA over the 2-26 h period was real or reflected experimental variability. Although hepatocytes maintained in insulin and dexamethasone underwent a decline in culture survival between 50 and 74 h; after 74 h almost no further loss of cellular material occurred. The addition of T_3 as well as insulin and

dexamethasone did not alter the survival curves.

2.3.3 Morphology of Cultured Hepatocytes

Phase contrast photography was used to follow the changes in cell morphology with time in culture (Fig. 4). During the first 24 h the hepatocytes flattened, forming into groups of polygonal cells. A partial monolayer formed as cells continued to flatten and groups of cells joined into cords. The polygonal shape of cells was retained throughout the culture period in cultures receiving medium supplemented with insulin and dexamethasone. Hepatocyte cultures maintained in hormone-free medium exhibited elongated cells with ragged edges by 74 h instead of the polygonal shaped cells observed up to 50 h.

The appearance of the hepatocytes in culture varied depending on the hormone or hormones present in the medium. Photographs of the cells after 74 h in culture illustrate the differences in morphology (Fig. 5). Briefly, hepatocytes maintained in hormone-free medium exhibited elongated shapes (A). Insulin supplementation resulted in cells with broader cytoplasm and denser monolayers, but the cells were somewhat elongated with ragged edges (B). As in the time course (Fig. 4), the polygonal shape of hepatocytes was observed in the insulin and dexamethasone condition (C). Dexamethasone supplementation resulted in sparse cultures with more rounded cells (D). In general, the additions of T_3 to the medium in the absence or presence of one or more

FIGURE 4

MORPHOLOGY OF HEPATOCYTES IN CULTURE

      Hepatocytes were isolated and cultured on collagen-coated surfaces as described in Materials and Methods. Cells were plated in the medium used in Fig. 3B containing No Hormones. After 2 h, cultures were maintained in the same medium or the medium supplemented with 10^{-6}M insulin and 10^{-7}M dexamethasone (Insulin + Dex.). Phase contrast photographs taken at 24 h intervals after plating show the morphological changes with time.

2 h

26 h

50 h

74 h

98 h
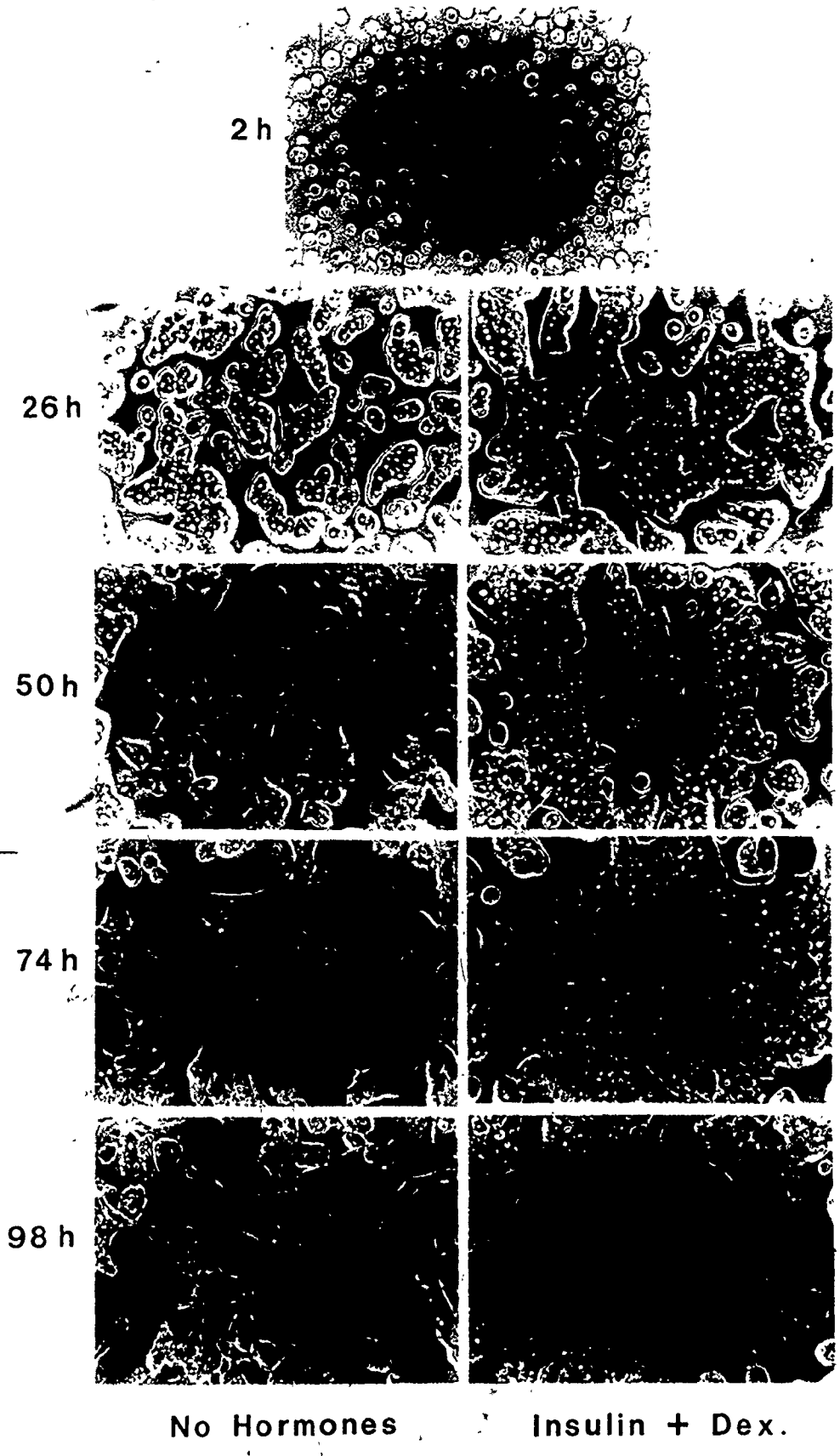
No Hormones   Insulin + Dex.

FIGURE 5

EFFECTS OF HORMONES ON CELL MORPHOLOGY

Isolation and plating procedures are described in Fig. 4. Phase contrast photographs show the appearance of hepatocytes maintained for 3 days in medium supplemented with different hormones after 2 h. A - no hormones; B - 10^{-6}M insulin; C - 10^{-6}M insulin and 10^{-7}M dexamethasone; D - 10^{-7}M dexamethasone. E, F, G, H are the same hormone conditions as A, B, C, D respectively plus 1.54×10^{-6}M T_3.
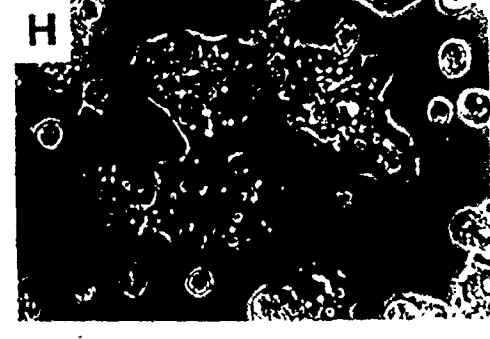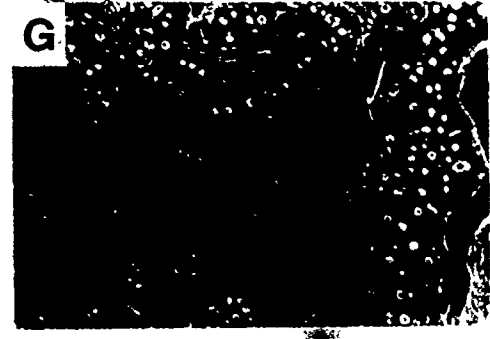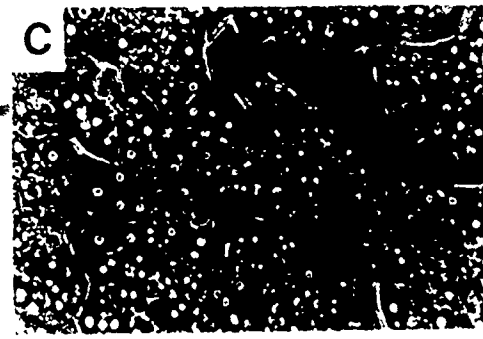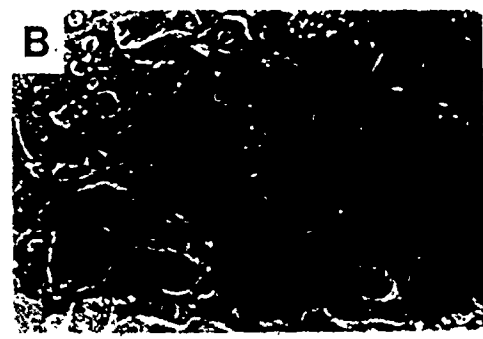
hormones (E,F,G,H) did not alter the morphology.
Occasionally, cultures with T_3 and dexamethasone contained
larger groups of cells with some polygonal appearance when
compared to the dexamethasone condition (D).

2.4   DISCUSSION

The liver perfusion method used in these studies was
patterned very closely after that of Williams et al. (1977).
The reason for the two-step procedure has been described in
detail by Seglen (1976). Although the perfusion buffers of
Bissell et al. (1973) were tried initially the use of EGTA
in the washout perfusion and culture medium supplemented
with extra Ca^{2+} for the collagenase perfusion were adopted
to improve yields of cells (Seglen, 1976; Marceau et al.,
1977).

Low speed centrifugation (50-70 x g) removed most of
the subcellular debris and non-parenchymal cells. This
type of purification of parenchymal liver cells has been
widely used and results in < 1% contamination by non-
parenchymal cells (Crisp and Pogson, 1972; Bissell et al.,
1973; Bonney et al., 1974; Michalopoulos and Pitot, 1975;
Seglen, 1976).

Hepatocytes dissociated at 4°C initiated cultures
better than cells dissociated by incubation at 37°C. The
latter method appeared to impair the ability of viable cells
to attach to the substratum confirming a similar observation
by Williams et al. (1977). This observation also pointed

out that viability determined by trypan blue dye exclusion does not reflect the attachment ability of cells. Therefore inoculating with the same number of viable cells does not ensure that the same number of hepatocytes will attach and initiate cultures. This may in part explain the variations in plating efficiency and maintenance of cells observed between experiments in which cultures were initiated with the same number of viable cells in the same medium. However, the shape of the survival curve between experiments remained the same for the same culture conditions.

Survival of hepatocytes in culture was determined by measuring cellular protein or DNA attached to the substratum. Attempts to quantitate hepatocyte survival by standard trypsinizing procedures were abandoned because of extensive cellular destruction and loss of viability. Parenchymal cells are known to be especially susceptible to destruction by proteases (Seglen and Fossa, 1978).

One of the goals in developing this culture method was to minimize serum supplementation in order to have a well-defined system for hormone studies. Serum was reduced to 2.5% and even omitted with only small losses in the amount of cellular material. Using homologous serum rather than FBS did not improve culture survival contrary to the report by Stenberg, Skett and Gustafsson (1978).

It was very important to develop a culture method that allowed examination of cellular functions for 3-4 days

because thyroid hormone action <u>in</u> <u>vivo</u> involves a long lag time (Tata, 1969; Oppenheimer and Dillmann, 1978). Hepatocytes cultured on collagen-coated surfaces did not undergo a rapid decline in survival between 50 and 74 h and could be cultured for 4-5 days.

Maintaining hepatocyte cultures in medium containing insulin and dexamethasone substantially improved survival of the cells agreeing with other reports (Tanaka <u>et</u> <u>al</u>., 1978; Michalopoulos and Pitot, 1975). Morphological studies agreed with the survival curves in that monolayers maintained in hormone-free medium appeared sparse. Cultures that were maintained in medium containing insulin and dexamethasone were denser and had retained the polygonal morphology. Contrary to the report by Laishes and Williams (1976b), dexamethasone alone did not appear to improve longevity and maintenance of morphology. Addition of T_3 to the medium did not cause any change in culture survival or morphology.

CHAPTER 3. TRIIODOTHYRONINE ACTIONS IN CULTURED HEPATOCYTES

3.1 INTRODUCTION

The number of enzymes whose activities are affected by thyroid hormone in vivo is very large (Pitt-Rivers and Tata, 1959; Wolff and Wolff, 1964). Some correlations between particular enzymes affected by thyroid hormone and the observed physiological actions have been made (Ismail-Beigi and Edelman, 1971; Goodridge, 1975; DeMartino and Goldberg, 1978; Eberhardt et al., 1980). In general the actions of thyroid hormone have been examined in in vivo experiments in which the influence of other factors such as hormones and tissue interactions cannot be ruled out or defined. It is the major advantage of tissue culture that direct effects of hormones can be studied on a single cell type. However, it follows that changes in liver due to T_3 in the whole animal need not be present in cultured hepatocytes since the culture system does not allow for indirect or secondary actions.

Reports in the literature have indicated that rat hepatocytes in culture retain the ability to respond to many hormones, insulin, glucagon, glucocorticoids, epidermal growth factor and estrogens (Richman et al., 1976; Michalopoulos et al., 1978; Geelen et al., 1978; Gebhardt and Mecke, 1979a; Schudt, 1979a,b). However, in primary cultured hepatocytes, T_3 was unable to stimulate DNA synthesis (Richman et al., 1976) or protein synthesis (Tanaka et al., 1978)

although both effects have been characterized in vivo
(Short et al., 1980; Tata, 1969). When maintained in cell
cultures, hepatocytes undergo some alterations in metabolic
character and gradually lose differentiated functions (Bon-
ney et al., 1974; Pitot and Sirica, 1980).

The primary task was to determine if cultured hepato-
cytes were capable of responses to T_3. This chapter des-
cribes the examination of different enzymes and cell para-
meters of cultured hepatocytes. Both normal and
thyroidectomized rats were used as sources of hepatocytes.
The relevance and consistency of the actions observed in
cultured hepatocytes with regard to the actions observed in
vivo has been considered.

3.2 MATERIALS AND METHODS

3.2.1 Cell Culture and Harvesting

Hepatocytes were isolated from adult male rats (200-
325 g, Spague-Dawley or Wistar) or adult thyroidectomized
rats (160-190 g, Spague-Dawley) as described in Materials
and Methods in Chapter 2. The thyroidectomy was performed
by Canadian Breeders on rats weighing 76-100 g. A weight
chart was kept and the thyroidectomy was considered success-
ful if the weight plateaued around 160 g. Non-thyroidectom-
ized rats of the same age weighed between 200 and 300 g.

Cells were plated for 2 h on collagen-coated tissue
culture flasks (75 cm^2) in medium without T_3. The basic
culture medium consisted of L-15 medium, 10 mM glucose 1 mM

succinate, antibiotics, 10^{-6}M insulin and 10^{-5}M cortisol unless otherwise stated. The buffering of the medium is described for each experiment. At 2 h, the medium was aspirated and control cultures received the control medium used for plating. Test cultures received the same medium supplemented with T_3 (1.54 x 10^{-6}M). Media were changed every 24 h thereafter.

Hepatocyte monolayers were harvested by 2 methods. In one method, monolayers were rinsed twice with PBS⁻ (4°C) and scraped off the culture surface with a rubber policeman into PBS⁻ (4°C). Cellular material was pelleted at 500 x g and suspended in a small volume (1.0 - 1.5 ml) of isolation medium STE (0.25 M sucrose, 50 mM Tris, 0.1 mM EDTA, pH 7.4). The second harvesting method involved rinsing the monolayers twice with PBS⁻ (37°C) and treating with 2 ml of sterile collagenase in PBS⁻ (130 U/ml) for approximately 4 min (37°C). Cells freed from the culture surface with collagenase were centrifuged at 122 x g or 500 x g for 10 min, resuspended in 5 ml PBS⁻ (4°C) and centrifuged at 500 x g for 10 min. The pellet was suspended in a small volume of STE as described above.

3.2.2 Cell Fractionation and Enzyme Assays

All steps were carried out at 4°C. The cellular material harvested and suspended in STE was homogenized using a Polytron® equipped with a PT 10 saw-tooth generator at a setting of 3 for 15-20 s (Lipton and McMurray, 1977). The

homogenate was centrifuged at 12,500 x g for 10 min. The supernatant was termed the post-mitochondrial supernatant which was either stored frozen (-20°C) or recentrifuged at 12,500 x g for 10 min before being stored frozen. The 12,500 x g pellet was suspended in STE (1.0 - 1.5 ml) and centrifuged at 500 x g for 10 min. Mitochondria were isolated from the supernatant by centrifuging at 5000 x g for 10 min, 10,000 x g for 1.5 min and washed once before being stored frozen.

Cell fractions from test cultures were always assayed and compared with cell fractions prepared from control cells harvested and fractionated at the same time as the test cultures. Usually each sample (post-mitochondrial supernatant or mitochondria) was assayed at two protein concentration and the specific activities were averaged, except in those cases where there was insufficient sample to allow for duplicate assays.

α-Glycerophosphate dehydrogenase (EC 1.1.99.5) and succinate dehydrogenase (EC 1.3.99.1) were assayed in the mitochondrial fraction by the method of Lee and Lardy (1965). The following enzymes were assyed in the post-mitochondrial supernatant by the methods referenced: NADPH-cytochrome c reductase (EC 3.2.3.5), Lipton and McMurray, 1977; glucose-6-phosphatase (EC 3.1.3.9), Baginski et al., 1974. The activity of cytosolic NADP-specific malic enzyme (EC 1.1.1.40) was assayed in the post-mitochondrial supernatant by a modification of the method of Hsu and Lardy (1969). The final

assay volume was 1.0 ml and contained 3 times the concentra-
tions of NADP$^+$ and L-malate. The reaction was started by
the addition of L-malate after an 8 min preincubation.

The protein content of the cell fractions was deter-
mined by a modification of the method of Lowry et al. (1951)
using sodium citrate instead of tartrate with crystallized
BSA (Sigma) as the standard. The presence of Tris in the
isolation medium, STE, resulted in a slight overestimation
of protein values. The protein values for the cell fractions
were corrected for Tris interference (Appendix 1).

3.2.3 Incorporation of Choline into Microsomal Phospholipids

Hepatocyte monolayers were labelled for 1-3 h with
5 ml of medium supplemented with 0.1 µ Ci/ml [methyl-^{14}C]
choline chloride (NEN Canada, Lachine, Que.). The monolay-
ers were harvested and cell fractions prepared as described
in previous sections. The post mitochondrial supernatant
was centrifuged at 105,000 x g for 1 h. The microsomal
pellet was suspended in 0.5 ml STE. The lipid extraction
procedure used was that described by McMurray and Rogers
(1973) using appropriate volumes in relation to the volume
of the sample. The lower phase was washed with theoretical
upper phase (chloroform:methanol:0.9% NaCl; 3:48:47) contain-
ing 1 mM choline chloride before 3 washes with theoretical
upper phase without choline chloride. The phosphorus con-
tent of the lipid extract was determined by the method of
Rouser et al. (1966) reading optical density at 797 nm. A

sample of the lipid extract was dried down, 5 ml ACS®
(Amersham, Oakville, Ont.) was added and cpm determined in a
liquid scintillation spectrometer (LS-3133T, Beckman®).

3.2.4 Incorporation of ^{14}C-Glycerol into Triglycerides and Phospholipids

Hepatocytes were labelled in hormone-free medium con-
taining 0.5 mM glycerol carrier and 1 µCi/ml [2-^{14}C]-glycerol.
Labelling was stopped by rinsing the monolayers with PBS⁻ with
5 mM glycerol prior to harvesting by collagenase treatment.
After harvesting all steps were carried out at 4°C. Hepato-
cytes were pelleted at 120 x $\underline{g}$, resuspended in PBS⁻ and
pelleted at 500 x $\underline{g}$. Lipids were extracted from a 0.5 ml
aliquot of whole cells by the method described for micro-
somal phospholipids except that the first wash of the lower
phase contained 1 mM or 5 mM glycerol instead of 1 mM choline
chloride.

An aliquot of the lipid extract was spotted on Whatman
LK5D plates and chromatographed in hexanes:ether:acetic
acid (60:40:1) with triolein, diolein (Serdary Research
Laboratories, London, Ont.) and phosphatidyl choline (Sigma)
as standards. The triglyceride and phospholipid spots were
scraped off the plates and counted in 0.5 ml H_2O and 5 ml
ACS®. Counts per min (cpm) were corrected for recovery
from the thin layer plates and expressed as cpm/mg cellular
protein. Recovery was generally 70-90%. The protein content
of aliquots of cellular material boiled for 5 min in 1 N NaOH

was determined as described previously.

3.2.5 Low Temperature Difference Spectra

Mitochondria was isolated as described previously and suspended in 50% glycerol. Low temperature difference spectra were obtained as described by Lipton and McMurray (1977). The light path was 1 mm.

3.3   RESULTS

3.3.1 Effect of Triiodothyronine on Enzyme Activities in Cultured Hepatocytes

The enzyme mitochondrial α-glycerophosphate dehydrogenase, is a sensitive indicator of thyroid hormone action in rat liver in vivo (Lee and Lardy, 1965; Larsen and Frumess, 1977; Oppenheimer and Dillmann, 1978). The microassay of Lee and Lardy (1965) provided a reliable means of measuring this enzyme in mitochondria isolated from hepatocyte mono-layers. The first indication that hepatocytes were responding to T_3 was observed in hepatocytes maintained in medium supplemented with 2.5% rat serum, 10^{-6}M insulin and 10^{-5}M cortisol. The substantial stimulation of α-glycerophosphate dehydrogenase activity is shown in Figure 6. The response was unchanged if serum was omitted or if growth hormone was added. The response to T_3 was slightly less in hepatocytes maintained in medium buffered with only 10 mM Hepes instead of 20 mM, suggesting that the lower pH reached in such medium during the culture period may have decreased the

FIGURE 6

INCREASE IN α-GLYCEROPHOSPHATE DEHYDROGENASE

ACTIVITY IN RESPONSE TO T_3

Hepatocytes were isolated, cultured and harvested as described in Materials and Methods. The basic culture medium was supplemented with 20 mM Hepes, pH 7.4. The variations of medium composition were the following: A, 2.5% rat serum, batch 1; B, 2.5% rat serum, batch 2; C, 10 mM Hepes instead of 20 mM with 2.5% rat serum, batch 1; D, no serum; E, 17 mU/ml (8.5 µg/ml) growth hormone (Sigma) with 2.5% rat serum, batch 1. The enzyme activities in hepatocytes maintained for 3 days in the different media conditions with (hatched bars) or without (open bars) T_3.
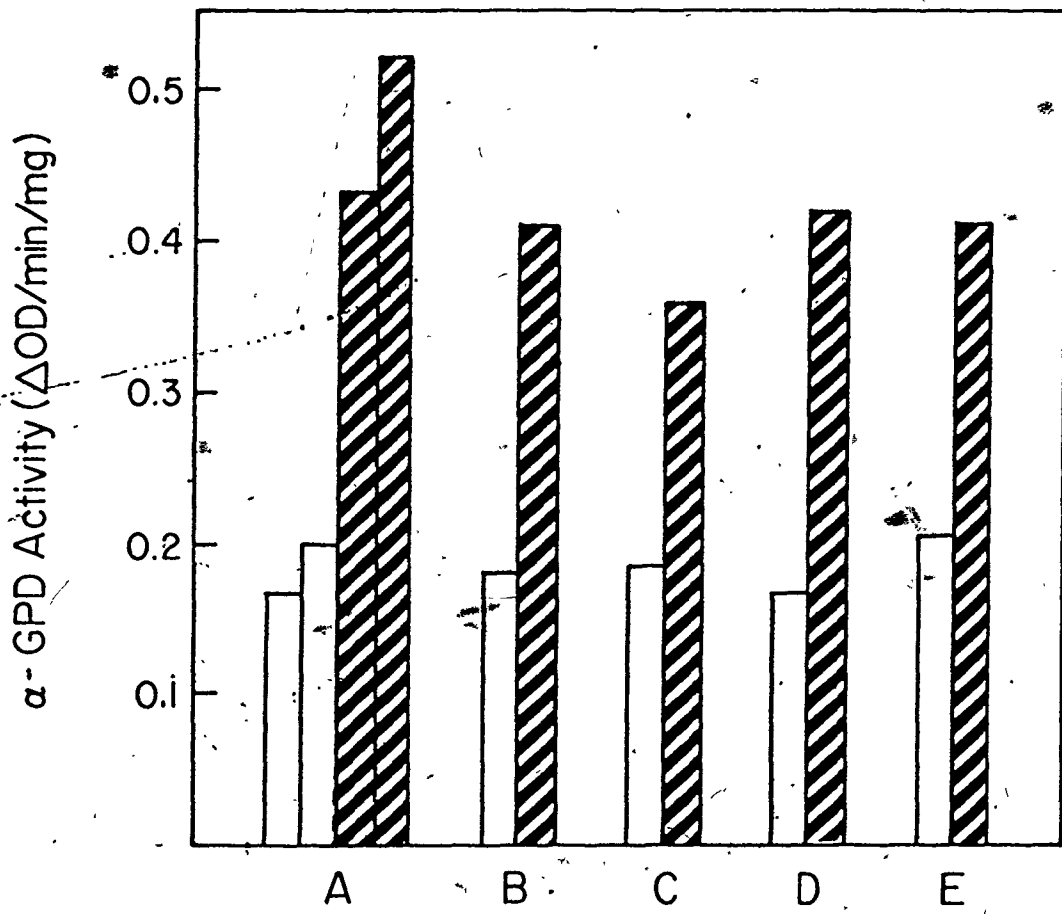
response.

The effects of T_3 on cultures of hepatocytes isolated from thyroidectomized rats were examined (Table 2). Enzyme activities in the control cells were very low. α-Glycerophosphate dehydrogenase activity was increased approximately 2.5 fold after 3 days in culture with T_3. In the presence of serum the increase in enzyme activity was larger.

In monolayers of hepatocytes obtained from a euthyroid rat, the activities of α-glycerophosphate dehydrogenase and succinate dehydrogenase were monitored over 4 days. Supplementation of the medium with T_3 caused a steady increase in α-glycerophosphate dehydrogenase activity with time (Fig. 7A). The activity of succinate dehydrogenase was not affected by T_3 (Fig. 7B).

The activities of certain non-mitochondrial enzymes were examined in hepatocytes over the same 4 day period. If non-mitochondrial enzymes were to be examined in the post-mitochondrial supernatant it was important to harvest by collagenase treatment. Scraping the cells off the plate with a rubber policeman resulted in a loss of cytoplasmic protein presumably due to breakage of the cells (Appendix 3). Activity of the cytosolic NADP malic enzyme increased in hepatocytes maintained in medium containing T_3 (Fig. 8A). In the absence of T_3 the activity of malic enzyme decreased slightly throughout the culture period similar to α-glycerophosphate dehydrogenase activity (Fig. 7A). The microsomal enzyme glucose-6-phosphatase decreased slightly during the

TABLE 2

α-GLYCEROPHOSPHATE DEHYDROGENASE ACTIVITY IN

CULTURED HEPATOCYTES FROM THYROIDECTOMIZED RATS

All procedures for isolation, culturing and harvesting of hepatocytes are described in Materials and Methods. The medium used was the basic medium with 20 mM Hepes ± 2.5% -serum from thyroidectomized rats (TRS), Expt. A or 10 mM Hepes, 10 mM BES, 10 mM TES, pH 7.4, Expt. B. T_3 at the concentrations indicated was added after 2 h. [3]Hepatocyte monolayers were harvested after 3 days in culture and α-glycerophosphate dehydrogenase activities measured. Results are expressed as ΔOD/min/mg mitochondrial protein (% of Control).

	Expt. A	Expt. B
Control	0.034 (100)	0.035 (100)
1.54×10^{-7} M T_3		0.056 (159)
1.54×10^{-6} M T_3	0.090 (264)	0.059 (169)
1.54×10^{-5} M T_3		0.085 (243)
2.5% TRS	0.050 (100)	
2.5% TRS + 1.54×10^{-6} M T_3	0.255 (509)	

FIGURE 7

α-GLYCEROPHOSPHATE DEHYDROGENASE AND SUCCINATE

DEHYDROGENASE ACTIVITIES IN CULTURED HEPATOYCTES

Hepatocytes were cultured and enzymes assayed as described in Materials and Methods. The medium was buffered with 20 mM Hepes, pH 7.4. Mitochondrial enzyme activities of hepatocytes maintained after 2 h in medium containing 1.54×10^{-6} M T_3 (▲—▲) or without T_3 (●—●) are shown. A - α-glycerophosphate dehydrogenase. B - succinate dehydrogenase.

A

B

Enzyme Activity (ΔOD/min/mg)

Time in Culture (h)
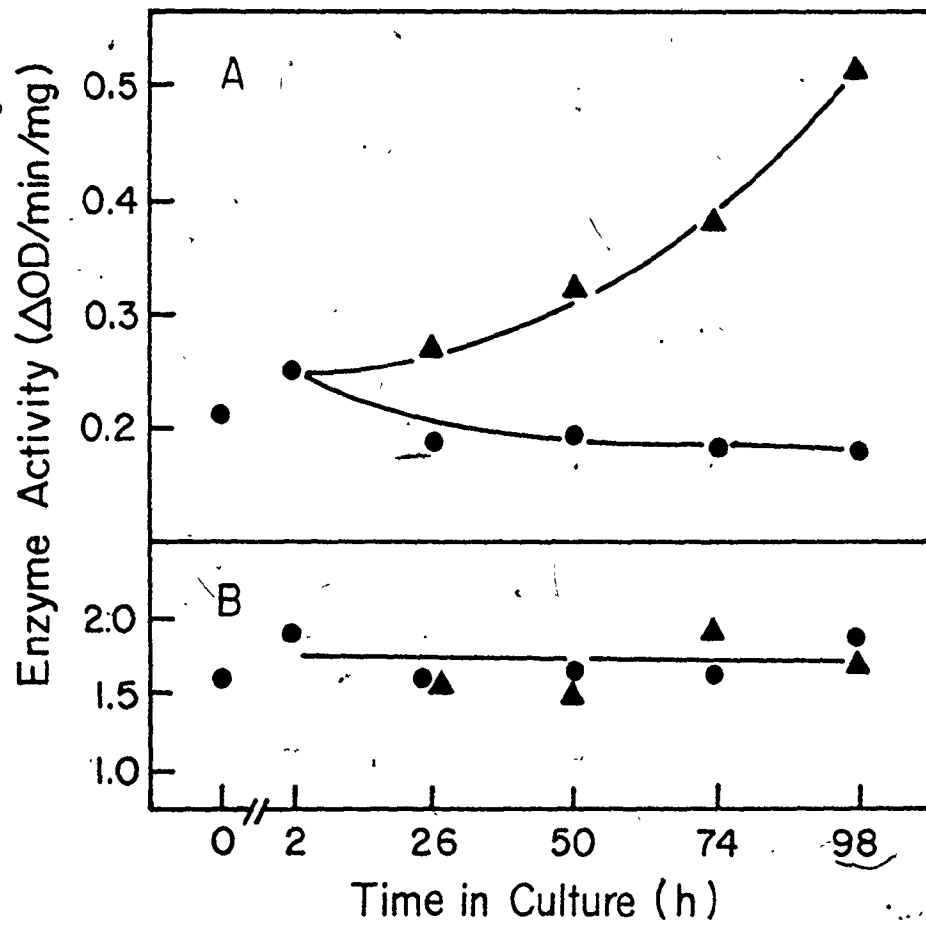
FIGURE 8

ENZYME ACTIVITIES IN THE POST-MITOCHONDRIAL

SUPERNATANT OF CULTURED HEPATOCYTES

Procedures for culturing hepatocytes are the same as in Figure 7. Enzyme activities were assayed as described in Materials and Methods. A - Malic enzyme (product measured was NADPH). B - glucose-6-phosphatase (phosphate). C - NADPH-cytochrome c reductase (reduced cytochrome c).
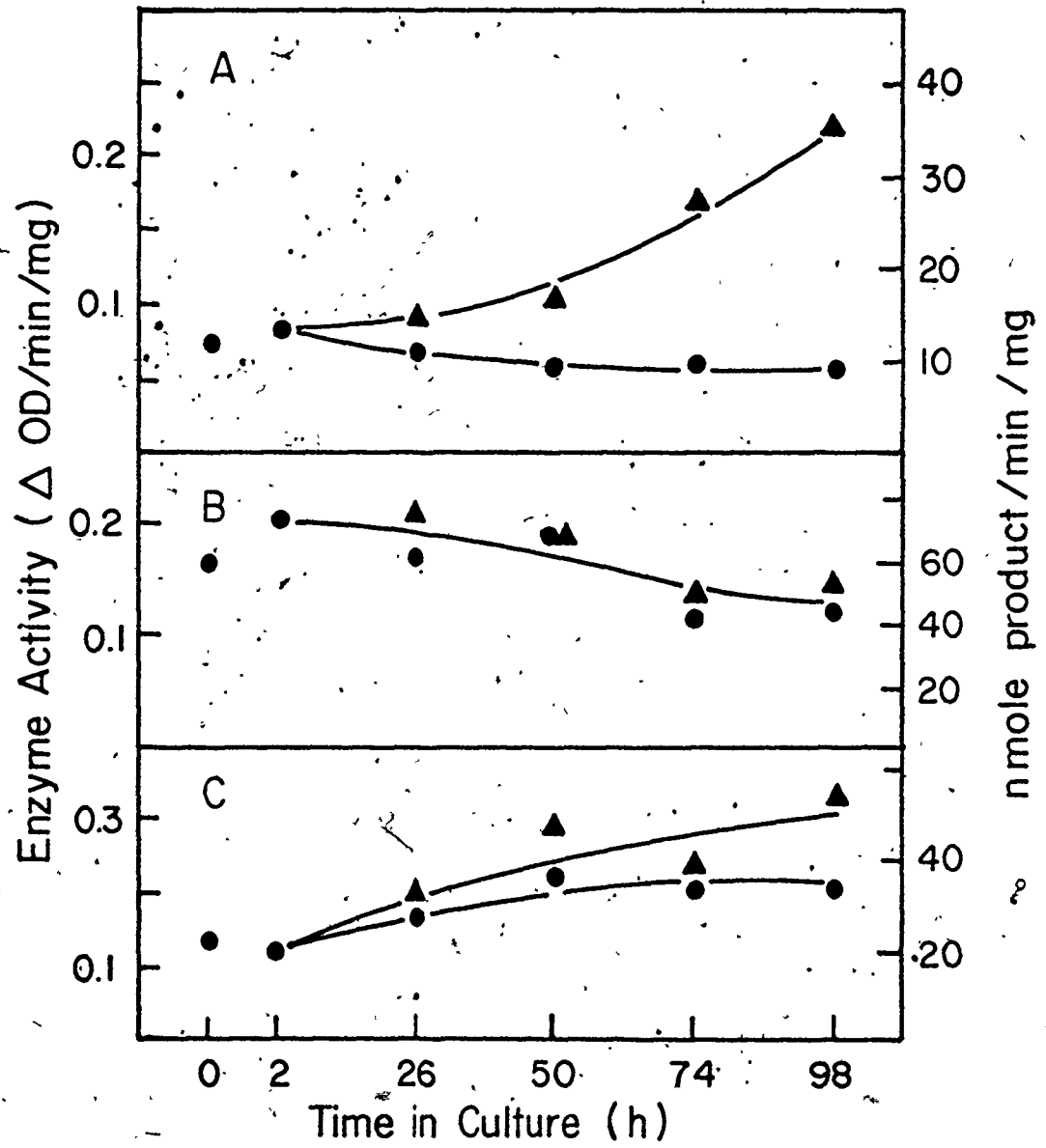
culture period and T_3 treatment of the hepatocytes did not
alter the enzyme's activity (Fig. 8B). T_3-treated hepato-
cytes had slightly greater NADPH-cytochrome c reductase
activity than non-treated cells (Fig. 8C). Both treated
and non-treated hepatocytes exhibited a small but gradual
increase in enzyme activity over the 4 day period.

The increases in α-glycerophosphate dehydrogenase and
malic enzyme were examined in more detail in another experi-
ment. The time courses were followed over a 4 day period.
Concurrent increases in the activities of these enzymes were
again observed (Fig. 9 and Fig. 10). By 74 h in culture the
activities in T_3-treated cells were about 2.5 times that of
cells maintained in insulin and cortisol medium. Enzyme
activities in T_3-treated cells were increased substantially
above the activity of freshly isolated hepatocytes (0 h time
points). Addition of 1μM cycloheximide after 50 h of cul-
ture caused little difference in α-glycerophosphate dehydro-
genase and malic enzyme activities in control cultures but
blocked any further increase in activities in T_3-treated
cultures.

3.3.2 Incorporation of Choline into Microsomal Phospholipids

The ability of hepatocytes to incorporate choline into
microsomal phospholipids after 26 or 50 h in culture was
examined. Hepatocytes were labelled for 1, 2 and/or 3 h with
[methyl-^{14}C]-choline. Monolayers maintained in medium con-
taining 1.54 x 10^{-6}M T_3 incorporated less ^{14}C-choline into

FIGURE 9

TIME COURSE OF α-GLYCEROPHOSPHATE

DEHYDROGENASE INDUCTION BY T_3

Hepatocytes were isolated, cultured and enzyme activities were assayed at the times indicated as described in Materials and Methods. Control cultures were maintained in insulin and cortisol (●—●). Test cultures received T_3 (1.54 x 10^{-6}M) (▲—▲). Media were buffered with 10 mM of each Hepes, TES and BES. Error bars represent ± the range between activities measured in 2 separately prepared samples. At 50 h, 1 μM cycloheximide was added to control cultures (○--○) and test cultures (△--△). Insets represent the same hormone supplementation conditions but media were buffered with 20 mM Hepes, 10 mM TES and 10 mM BES.

α GPD Activity (Δ OD/min/mg)

Time in Culture (h)
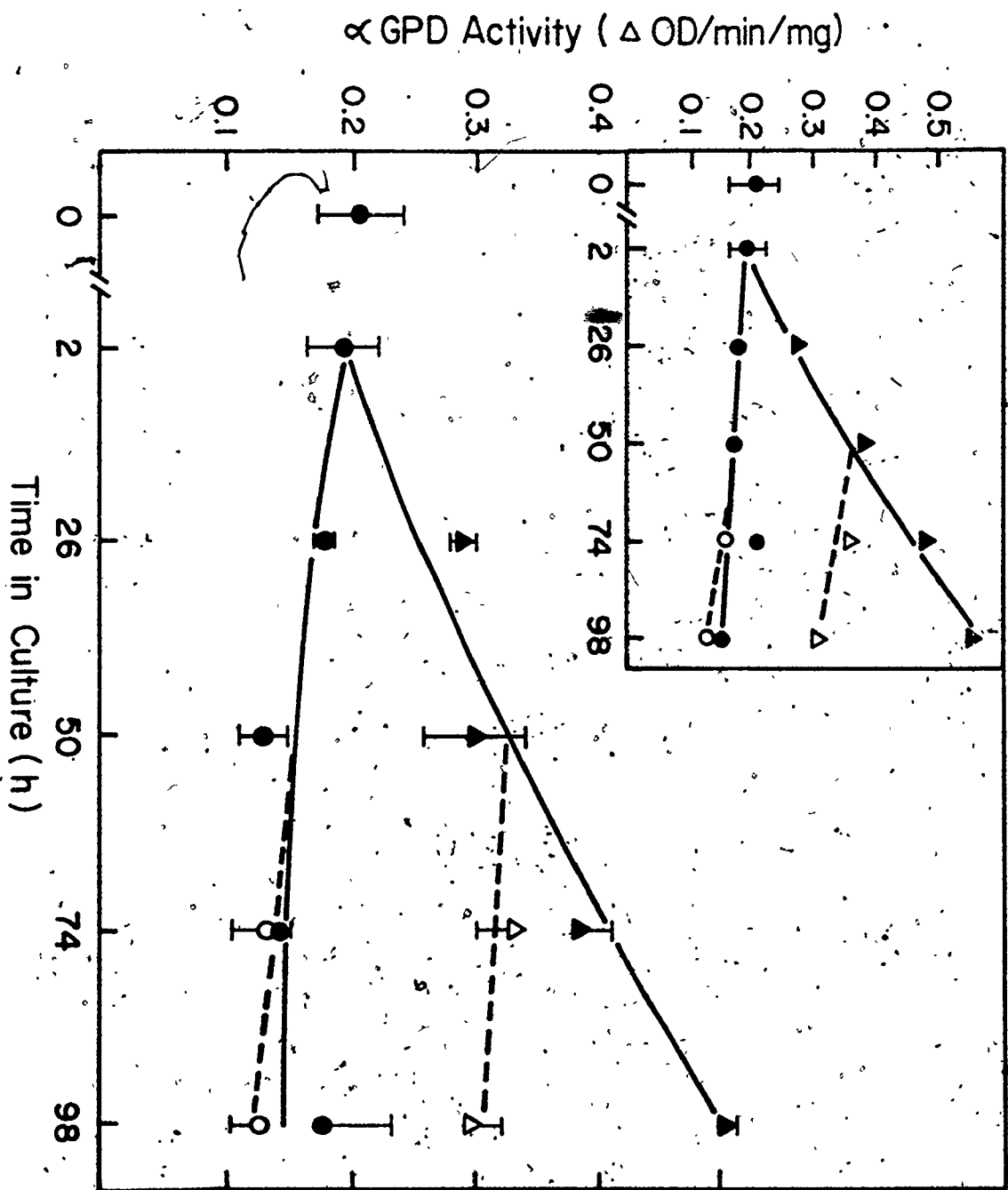
FIGURE 10

TIME COURSE OF MALIC ENZYME INDUCTION BY T_3

Experimental conditions are the same as those in Figure 9.

Malic Enzyme Activity (ΔOD/min/mg)

Time in Culture (h)
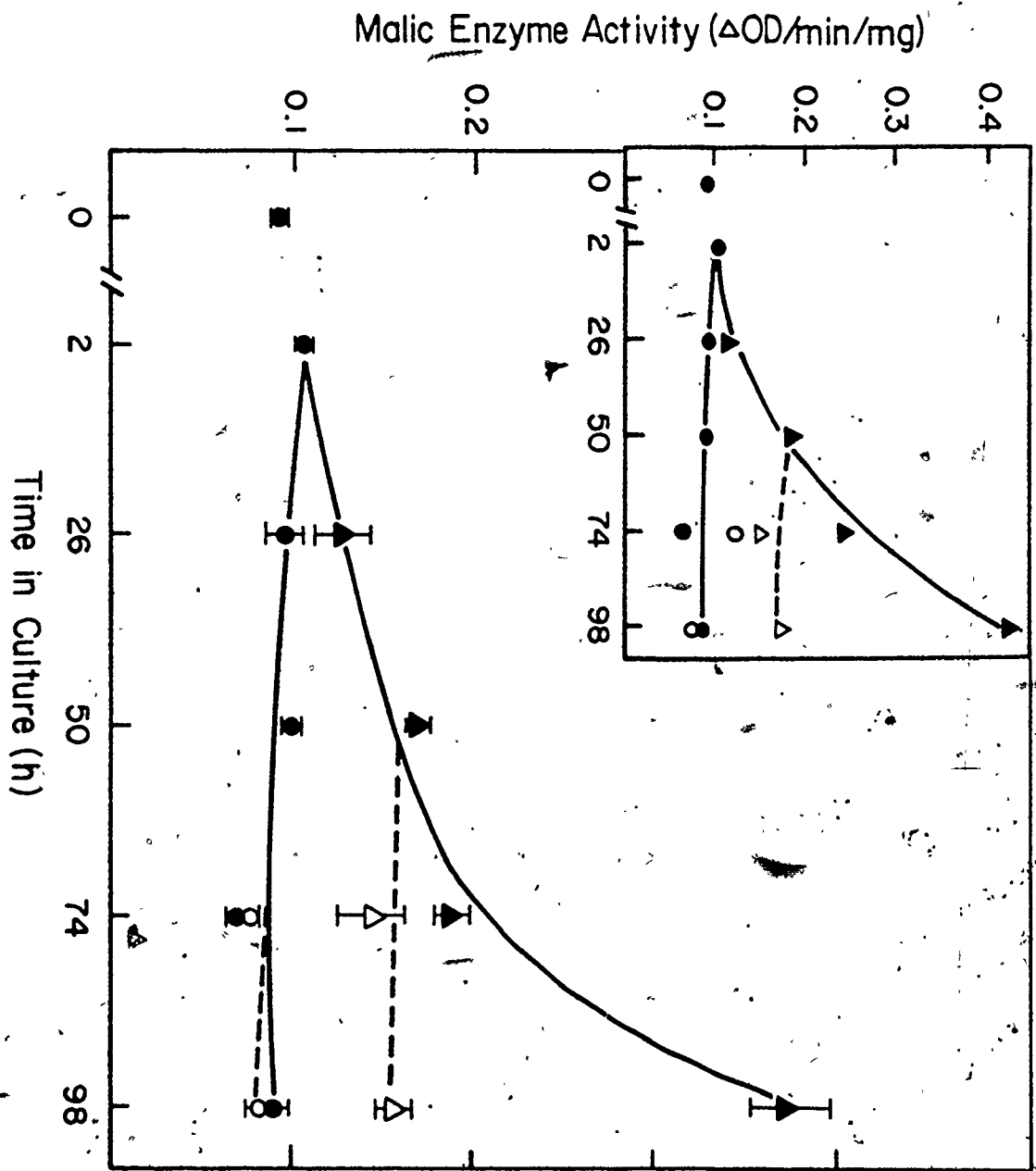
lipid than non-treated control cells (Fig. 11). The rate of incorporation of choline in both control and T_3-treated cells was less at 50 h than at 26 h.

3.3.3 Incorporation of Glycerol into Glycerolipids

Following 48 h of hormone treatment, the ability of hepatocytes to incorporate ^{14}C-glycerol into triglycerides and phospholipids was examined. Control cultures were maintained in hormone-free medium while test cultures received hormones at 2 h (Table 3). The incorporation of labelled glycerol into phospholipids was not affected by hormone treatment. Triglyceride labelling was increased by insulin and dexamethasone when compared to the control. T_3 alone appeared to increase incorporation only slightly if at all. However, in the presence of insulin and dexamethasone, T_3 increased the amount of incorporation into triglycerides above that of insulin- and dexamethasone-treated cells.

In another study hepatocytes were maintained in medium containing no hormones until 26 h. Hormone treatment was then begun and incorporation of glycerol into triglycerides and phospholipids was examined 48 h later after 74 h total time in culture. When compared to the control (no hormones condition), incorporation of glycerol into phospholipids was increased by insulin and dexamethasone treatment (Table 4). T_3 had no effect on the labelling of phospholipids. The effect of the different hormones on the incorporation into triglycerides was the same as that observed in Table 3. The

FIGURE 11

INCORPORATION OF ^{14}C-CHOLINE INTO

MICROSOMAL PHOSPHOLIPIDS

Hepatocytes from an euthyroid rat were isolated, cultured and microsomal phospholipids labelled as described in Materials and Methods. The media were buffered with 10 mM of each Hepes, TES and BES. Results are expressed as 10^3 cpm/μgP versus incubation time with ^{14}C-choline for T_3-treated hepatocytes (▲—▲) and control hepatocytes (●—●). 'A' represents cells cultured for 26 h before labelling. 'B' represents cells cultured for 50 h before labelling.

Incorporation (10^3 cpm/μg P)

Incubation (h)

A

B
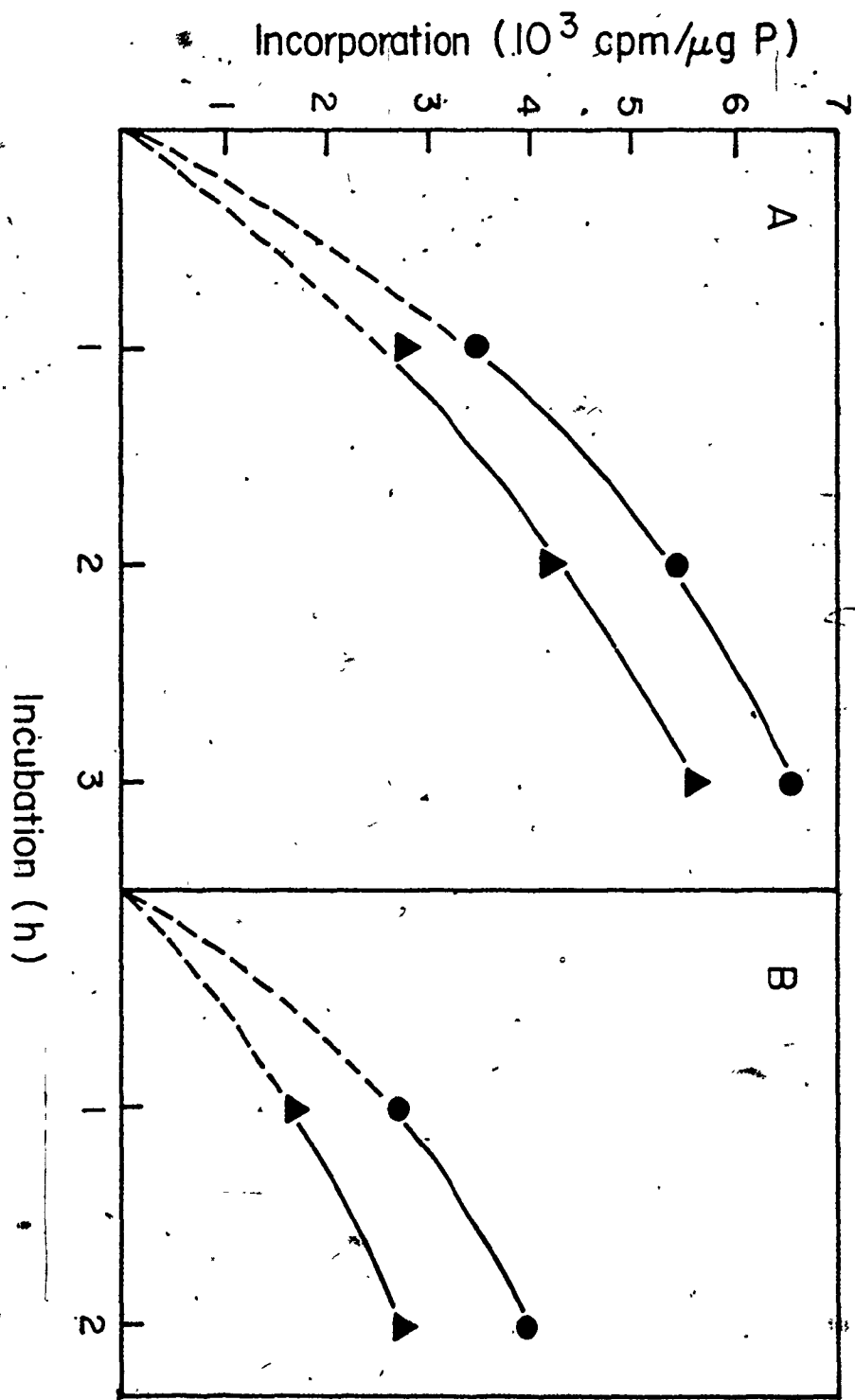
TABLE 3

INCORPORATION OF GLYCEROL INTO TRIGLYCERIDES AND

PHOSPHOLIPIDS AFTER 50 h IN CULTURE

Hepatocytes were cultured as detailed in Materials and Methods. The control medium designated NH (indicating that no hormones were present) consisted of L-15, 10 mM glucose, 1 mM succinate, antibiotics, 20 mM Hepes, 10 mM TES and 10 mM BES, pH 7.4. All cells were plated in control medium and at 2 h test cultures received medium supplemented with the following hormones: $+T_3$, 1.54 x 10^{-6}M T_3; I + D, 10^{-6}M insulin and 10^{-7}M dexamethasone; I + D + T_3 contained all three hormones at the given concentrations. ^{14}C-Glycerol incorporation was linear for at least one hour. Results are expressed as 10^3 cpm incorporated/mg cellular protein/h $\pm$ range (n = number of experiments).

	Triglycerides	Phospholipids	(n)
NH	6.3 $\pm$ 1.4	6.2 $\pm$ 2.2	(2)
+ T_3	7.1	6.3	(1)
I + D	12.3 $\pm$ 3.4	6.3 $\pm$ 3.2	(2)
I + D + T_3	19.1 $\pm$ 6.6	5.8 $\pm$ 3.2	(2)

TABLE 4

INCORPORATION OF GLYCEROL INTO TRIGLYCERIDES

AND PHOSPHOLIPIDS AFTER 74 h IN CULTURE

Experimental conditions were the same as detailed in Table 3 with the following change. Hepatocytes were plated and maintained in control medium until 26 h at which time test cultures received hormone supplemented medium. Cells were exposed to hormones for 2 days (26-74 h). Results are expressed as 10^3 cpm incorporated/mg cellular protein/hr. $\pm$ range for $n = 2$ or $\pm$ S.E. for $n = 3$. +CHI indicates 1 μM cycloheximide was present in the medium from 26 h on.

	Triglycerides	Phospholipids	(n)
NH	5.6 $\pm$ 0.9	5.6 $\pm$ 0.4	(3)
+ T_3	7.3 $\pm$ 3.2	5.7 $\pm$ 2.2	(2)
I + D	8.8 $\pm$ 0.6	7.0 $\pm$ 0.2	(3)
I + D + T_3	12.0 $\pm$ 1.6	7.5 $\pm$ 0.3	(3)
NH + CHI	2.6	4.1	(1)
I + D + CHI	2.7	4.1	(1)
I + D + T_3 + CHI	3.8	4.6	(1)

greatest incorporation was observed when all three hormones were present. In all hormone conditions the addition of cycloheximide markedly depressed the incorporation into triglycerides while depressing incorporation into phospholipids to a lesser extent. Cycloheximide could not be added at 2 h, since it impairs the ability of hepatocytes to form monolayers (personal observation). Incorporation of label into both triglycerides and phospholipids was marginally elevated in the presence of insulin, dexamethasone, T_3.

3.3.4 Cytochrome Spectra and Content

The low temperature (77°K) difference spectra of mitochondria isolated from hepatocytes cultured under different hormonal conditions is shown in Figure 12. The content of the different cytochromes was determined by calculating the ΔOD at paired wavelengths from the spectra (Chance, 1957; Kleitmann et al., 1973). In order to compare the contents between hormonal conditions, the ΔOD for each cytochrome of the control condition (No Hormones) was set at 100% (Table 5). Absolute and relative values for cytochromes were not calculated because at low temperature the intensification of cytochrome peaks varies and can distort the relative values obtained (Chance, 1957; Chance and Hagihara, 1961).

Except for cytochrome c, the contents of the other cytochromes were increased slightly (9-26%) in hepatocytes maintained in insulin and cortisol. The activity of α-glycerophosphate dehydrogenase was increased to the same extent in the insulin and cortisol condition. When T_3 was

FIGURE 12

LOW TEMPERATURE DIFFERENCE SPECTRA OF

MITOCHONDRIAL CYTOCHROMES

Hepatocytes were isolated, cultured and cytochrome spectra determined as described in Materials and Methods. The media were buffered with 20 mM Hepes, 10 mM TES and 10 mM BES.   Control hepatocytes were plated and maintained in medium containing no hormones. T_3 was added at 2 h to insulin and cortisol plated cells. Hepatocytes were cultured for 3 days with or without hormones. The protein concentrations of the mitochondrial samples before the addition of an equal volume of glycerol were, 3.49 mg/ml for the control (No Hormones), 3.35 mg/ml for Insulin + Cortisol and 3.84 mg/ml for Insulin + Cortisol + T_3. The bar indicates the optical density scale.
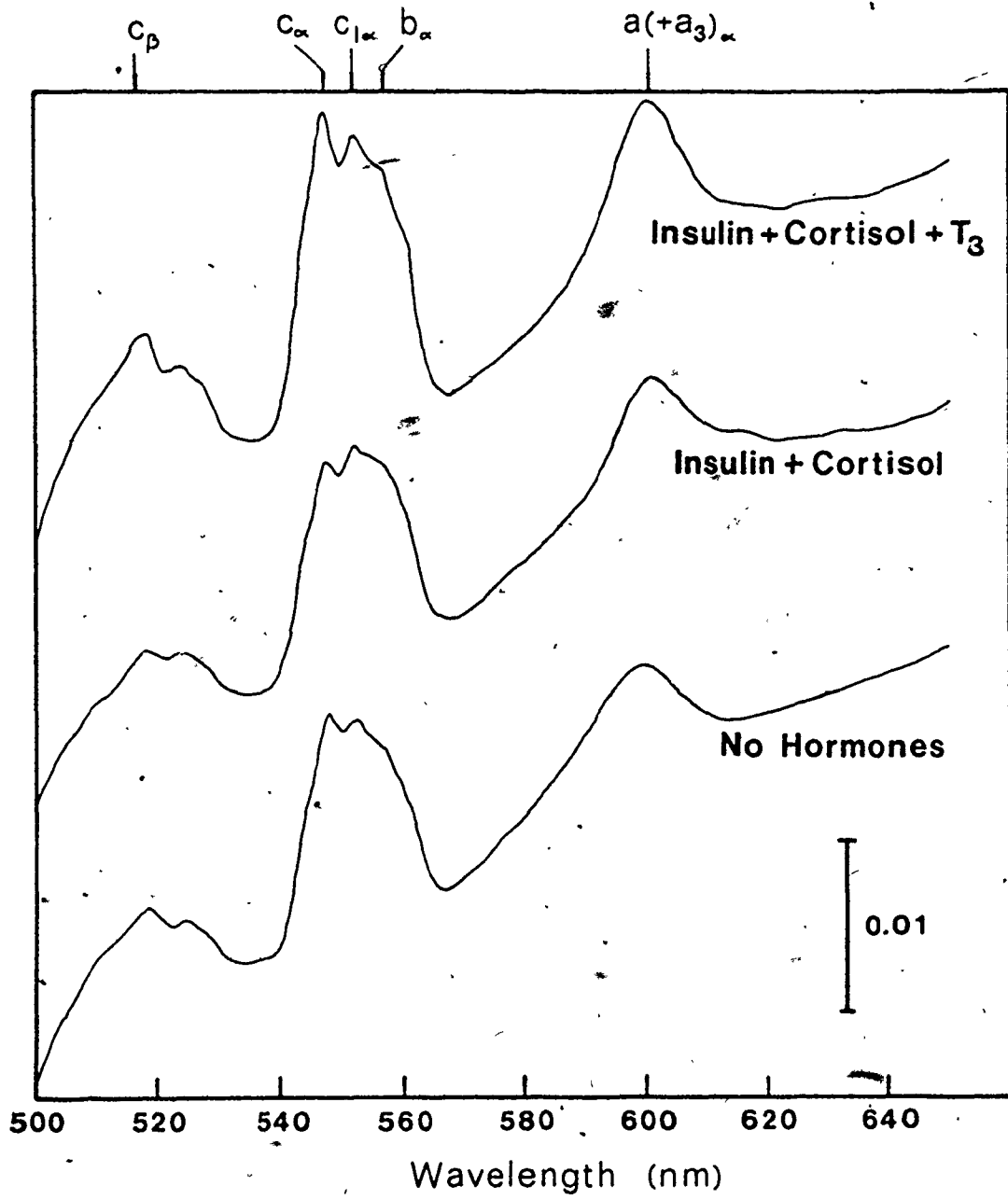
TABLE 5

CHANGES IN CYTOCHROME CONTENTS

AND ENZYME ACTIVITY

The cytochrome contents of hormone-treated hepatocytes relative to control cells were determined from the low temperature difference spectra (Fig. 12) as discussed in Results. The spectral differences were measured at the following wavelength pairs: $a(+a_3)$, 602 vs. 620 nm; b, 558 vs. 575 nm; c, 548 vs. 540 nm; c_1, 554 vs. 540 nm. The change in OD was divided by the protein concentration of the mitochondrial sample (Fig. 12). In this experiment α-glycerophosphate dehydrogenase (α-GPD) specific activity was determined.

	α-GPD	$a(+a_3)$	b	c	c_1
Control (No Hormones)	100	100	100	100	100
Insulin + Cortisol	121	124	126	98	109
Insulin + Cortisol + T_3	262	184	167	123	113

included in the medium with insulin and cortisol, there was
an increase cytochromes a$(+a_3)$, b and c but not c_1. The
spectral peaks for c, c_1 and b overlap. The increase in
cytochrome c caused by T_3 is very evident from the spectral
peaks (c_β and c_α , Fig. 12) and may be underestimated because
of the cross-interference of cytochrome peaks. As noted in
previous experiments, the activity of α-glycerophosphate
dehydrogenase was increased substantially by T_3.

3.4 DISCUSSION

α-Glycerophosphate dehydrogenase activity was increa-
sed substantially in cultured hepatocytes treated with T_3.
This stimulation of enzyme activity was not dependent on
growth hormone or serum factors. The involvement of insulin
and cortisol in thyroid hormone action in vitro is discussed
in detail in the following chapter.

The basal activity of α-glycerophosphate dehydrogen-
ase in cultured hepatocytes from thyroidectomized rats was
low as expected from in vivo reports (Lee and Lardy, 1965).
In the absence of serum, the increase in enzyme activity
relative to basal activity caused by T_3 treatment was about
2 - 2.5 fold, and attained values similar to those in hepa-
tocytes from an euthyroid rat. In vivo the increase in
α-glycerophosphate dehydrogenase activity was larger relative
to basal activity in hypothyroid rats but in terms of absolute
values was the same as for T_3-treated euthyroid rats (Oppen-
heimer et al., 1977). When hepatocytes from a thyroidectomized

rat were cultured with serum, the incremental increase in enzyme activity (0.2 OD units/min/mg) was similar to that observed in T_3-treatment of euthyroid hepatocyte monolayers. Serum factors may be important for this response in hepatocytes from a thyroidectomized rat but they do not seem to be required in euthyroid monolayers. Therefore, there were no advantages in using hypothyroid rats in these studies. In fact, there were a few disadvantages: 1) unless serum was used the incremental increase in enzyme activity was less than in hepatocytes from euthyroid rats, 2) the smaller thyroidectomized rats yielded fewer hepatocytes which did not seem to initiate cultures well and 3) the low basal level of enzyme activity was difficult to measure. Another reason for using hepatocytes from euthyroid animals was the report that hypothyroid rats exhibit a reduced response to T_3 with respect to malic enzyme induction (Oppenheimer et al., 1977).

Increases in the activities of hepatic mitochondrial α-glycerophosphate dehydrogenase and $NADP^+$ malic enzyme are the best characterized responses to T_3 in vivo (Tarentino et al., 1966; Oppenheimer and Dillmann, 1978; Lee and Lardy, 1965; Murphy and Walker, 1974). Increases in α-glycerophosphate dehydrogenase and malic enzyme in T_3-treated hepatocytes followed the same time course as occurs in vivo. The sensitivity of these increases in enzyme activities to the protein synthesis inhibitor, cycloheximide, was used as a check that the effect produced by T_3 in the cell cultures was similar to the effect in vivo involving an

induction of enzyme synthesis. Therefore hepatocytes in culture retain the ability to respond to T_3 in a characteristic and physiologically meaningful way.

In vivo the enzymes succinate dehydrogenase, NADPH-cytochrome c reductase and glucose-6-phosphatase have all been reported to respond to thyroid hormones with increased activities (Wolff and Wolff, 1964). However, the action of thyroid hormones on these enzymes is not as well defined as the actions on malic enzyme and α-glycerophosphate dehydrogenase. Succinate dehydrogenase activity shows relatively small (10-50%) increases even after 5 or 14 days of treatment with T_3 in vivo (Reith et al., 1973; Wooten and Cascarano, 1980). Lee and Lardy (1965) noted no significant decrease in succinate dehydrogenase activity in thyroidectomized rats and no increase in activity when such animals were treated with T_3. There are conflicting reports on thyroid hormone effects on glucose-6-phosphatase. Decreases in activity have been reported (Winnick, 1970; Paul and Dhar, 1980) as well as increases in activity of 28% after 8 days or 132% after 13 days of thyroid hormone treatment (Colton et al., 1972; Batterbee, 1974). These studies in hepatocyte monolayers were done over a 3 day period unlike the 7-14 day in vivo studies and changes in enzyme activity of 10-30% would be within the amount of variation observed between samples in an experiment (e.g., see Fig. 6). Thus, the inability to demonstrate changes in enzyme activity of succinate dehydrogenase and glucose-6-phosphatase in cultured hepatocytes treated with T_3 is not inconsistent

with *in vivo* studies.

When euthyroid rats received daily injections of thyroxine it requires more than 8 days for a maximum increase in NADPH-cytochrome c reductase (Phillips and Langdon, 1956). Based on the preliminary study of the enzyme in T_3-treated hepatocytes, a gradual but small increase in activity was observed. This agrees with *in vivo* data in which the increase in NADPH-cytochrome c reductase activity is 20-40% after 3 or 4 days of T_3-treatment (Phillips and Langdon, 1956).

The regulation of lipogenic enzymes involved in fatty acid synthesis by thyroid hormones has been well characterized *in vivo* in the rat (Diamont et al., 1972; Roncari and Murthy, 1975; Mariash et al., 1980; Sestoft, 1980; Oppenheimer et al., 1981) and *in vitro* in chick hepatocytes (Goodridge et al., 1974; Goodridge, 1975). Much less is known about the hormonal regulation of glycerolipid synthesis in liver (Van Golde and Van den Bergh, 1977). T_4 increased the activity of hepatic phospholipid synthesizing enzymes in hypothyroid chicks but not in normal chicks (Lyman et al., 1976). Thyroid hormones increase incorporation of ^{32}P-phosphate into mitochondrial phospholipids (Nelson and Cornatzer, 1965) and microsomal phospholipids (Tata, 1970) in rat liver *in vivo*. In other studies, T_4 did not alter phospholipid synthesis in rat liver (Glenny and Brindley, 1978) or in cultured rat lung cells (Post et al., 1980). Tata (1970) reported that choline incorporation into microsomal phospholipids in thyroidectomized rats is increased

by T_3. In contrast to this, the present studies showed
that T_3 caused a decrease in choline incorporation into
microsomal phospholipids in cultured hepatocytes from an
euthyroid rat. Such differences will only be explained by
further investigators both in vivo and in vitro.

Insulin does not significantly affect the incorporation
of glycerol into triglycerides or phospholipids in cultured
rat hepatocytes (Geelen et al., 1978). In vivo work has
indicated that cortisol increases the incorporation of gly-
cerol into triglycerides (Glenny and Brindley, 1978).
Dexamethasone, a synthetic glucocorticoid, may be responsible
for the increase in incorporation of glycerol observed in
hepatocytes cultured for 2 days with insulin and dexamethasone.
Treatment of hepatocytes with T_3 in the presence of insulin
and dexamethasone appeared to increase glycerol incorporation
into triglycerides. Some in vivo studies support this find-
ing. Thyroid hormones increase the acylation of glycerol-
phosphate in rat liver (Rohcari and Murthy, 1975) and in
rabbit heart (Kako and Liu, 1974). Glenny and Brindley
(1978) reported increases in glycerol incorporation into
triglycerides and a decrease in diglyceride following treat-
ment of rats with T_4. Stimulation of the activity of
diacylglycerol acyltransferase (EC 2.3.1.20) (Young and
Lynen, 1969) may be responsible for these effects observed
in vivo and in vitro.

By using low temperature difference spectra, increases
in cytochromes a($+a_3$), b and c were detected in cultured

hepatocytes treated with T_3. These results agree well with *in vivo* studies on thyroid hormone-stimulated increases in mitochondrial cytochrome content in rat liver and heart (Roodyn *et al.*, 1965; Kadenbach, 1966; Schafer *and* Nagel, 1968; Booth and Holloszy, 1975; Jakovcic *et al.*, 1978; Nishiki *et al.*, 1978). When hepatocytes were treated with insulin and cortisol but without T_3, the content of cytochromes a $(+a_3)$, b and c_1 increased 9-26% but cytochrome c did not. The lower amount of cytochrome c present in this condition when compared to the other cytochromes may reflect the decrease in cytochrome c content caused by insulin *in vivo* (Schafer and Nagel, 1968).

CHAPTER 4.   REGULATION OF α-GLYCEROPHOSPHATE DEHYDROGENASE
            AND MALIC ENZYME BY THYROID HORMONES, INSULIN
            AND GLUCOCORTICOIDS

4.1.   INTRODUCTION

Insulin and glucocorticoids are used extensively as
hepatotrophic agents for maintenance of primary cultures of
adult rat hepatocytes (Michalopoulos and Pitot, 1975;
Laishes and Williams, 1976a,b; Tanaka et al., 1978;
Jeejeebhoy, et al., 1980). It was evident in the first
trial experiments that increases in α-glycerophosphate de-
hydrogenase and malic enzyme activities due to T_3 treatment
were greater in hepatocytes cultured in medium which was
also supplemented with insulin and cortisol. Insulin is
reported to potentiate the action of T_3 in chick hepatocytes
in culture (Goodridge and Adelman, 1976). The possibility
that both insulin and glucocorticoids are involved in the
response of mammalian liver to thyroid hormones has not been
previously described in vivo or in vitro. Therefore, it was
of interest to characterize the role of insulin and cortisol
in potentiating T_3 action in this culture system for rat
hepatocytes.

This chapter examines the changes in enzyme activities
in hepatocytes cultured in medium supplemented with differ-
ent combinations of T_3, insulin and cortisol, and the con-
centration dependence of these hormone effects.

4.2   MATERIALS AND METHODS

4.2.1 Cell Culture and Enzyme Assays

The procedures used to isolate and culture hepatocytes from adult male Wistar rats (200-300 g) are described in the Materials and Methods section of Chapter 2. Hepatocyte cultures were initiated on collagen-coated tissue culture flasks. The basic culture medium consisted of L-15 medium (Flow), 10 mM glucose, 1 mM succinate, antibiotics and was buffered with 20 mM Hepes, 10 mM TES and 10 mM BES, pH 7.4. Hepatocytes were plated in basic culture medium unless otherwise stated. The additions of hormones after 2 h is described for each experiment.

Hepatocytes were cultured for 3 days. For each experiment, cells were harvested at 74 h or 70 and 74 h if duplicate samples were to be prepared. Hepatocyte monolayers were harvested by the collagenase-treatment method described in Chapter 3. Cell fractions were prepared in STE or SE (0.25M sucrose, 0.1 mM EDTA) isolation medium (pH 7.4).

α-Glycerophosphate dehydrogenase and malic enzyme activities were assayed in cell fractions prepared from test and control cultures as described in Chapter 3.

4.2.2 Extraction of Cortisol from Medium and its Concentration Determination

Medium (20 ml) was extracted 3 times with ether: chloroform (4:1). This extract was dried down, spotted on

Whatman LK5D thin layer plates and chromatographed in chloroform:ethanol:water (87:13:1) (Bailey, 1968). The cortisol spot was scraped off and eluted once with methanol and 2 times with ether. The extract was taken to dryness and redissolved in dichloromethane. A gas chromatograph (Hewlett-Packard 5830A) equipped with an SE 30 column was used to determine the amount of cortisol in the extract.

4.2.3 Equilibrium Dialysis

Dialysis membranes were prepared by washing in 50 mM Tris-HCl, pH 8.0, followed by boiling in 25 mM $NaHCO_3$, water, 1 mM EDTA, pH 7.0 and water (Lo and Sanwal, 1975). The binding of T_3 to BSA (fraction V, fatty acid-free, Sigma) in culture medium supplemented with 1% BSA was determined by equilibium dialysis against medium containing $[U-^{125}I]-T_3$ (NEN Canada, Lachine, Que.). Dialysis chambers contained 0.1 ml and the procedure was carried out at 4°C.

4.3 RESULTS

4.3.1 Requirement for Insulin and Cortisol for a Maximum Response to T_3

The role of insulin and cortisol in the induction of α-glycerophosphate dehydrogenase and malic enzyme was examined in detail by combining the results of numerous experiments and determining the significance between treatment groups. The effects of medium supplementation with different combinations of T_3, insulin and cortisol are shown in

Table 6 for α-glycerophosphate dehydrogenase and in Table 7 for malic enzyme. α-Glycerophosphate dehydrogenase and malic enzyme activities were significantly increased in all T_3-supplemented conditions when compared to the corresponding non-T_3-supplemented condition, eg. insulin + T_3 versus insulin. The maximum induction of both enzymes by T_3 occurred in the presence of insulin and cortisol and was significantly greater than that by T_3 alone (p <.0005). The response to insulin and T_3 was significantly lower than with the insulin, cortisol and T_3-supplemented condition (p <.0005). Similarly if insulin was omitted, the response to cortisol and T_3 was significantly lower (p <.0005 for α-glycerophosphate dehydrogenase, p <.025 for malic enzyme).

4.3.2 Concentration Curves for the Response to T_3 and T_4

The activities of α-glycerophosphate dehydrogenase and malic enzyme were measured in hepatocytes maintained for 3 days in varying concentrations of T_3 (Fig. 13). Maximum induction occurred at a medium concentration of 1.54×10^{-6} M T_3.

The effectiveness of T_4 as an inducer of α-glycerophosphate dehydrogenase and malic enzyme in cultured hepatocytes was tested (Fig. 13). T_4 appears to be as active as T_3 in inducing both enzymes with maximum induction occurring in the same concentration range. However, a decrease in enzyme activities was observed at concentrations greater than 10^{-6} M.

TABLE 6

EFFECTS OF HORMONES ON α-GLYCEROPHOSPHATE

DEHYDROGENASE SPECIFIC ACTIVITY OF HEPATOCYTES

AFTER 3 DAYS IN CULTURE

Cell culture and assay procedures are described in Materials and Methods. The hormone concentrations used are the following: $1.54 \times 10^{-6}M$ T_3, $10^{-6}M$ insulin and $10^{-5}M$ cortisol. Results are expressed as a % of the activity of control cultures $\pm$ S.E. of n individual samples. The p values were determined by a t-test on the % differences between treatment groups as indicated.

	Hormone Addition	n	% Activity	
I	None (control)		100†	
II	T_3	11	162 ± 20**	$p < .0005$
III	Insulin + Cortisol	16	108 ± 10	
IV	Insulin + Cortisol + T_3	16	257 ± 44	$p < .0005$
V	Insulin	13	95 ± 11	
VI	Insulin + T_3	13	161 ± 22**	$p < .0005$
VII	Cortisol	7	63 ± 15	
VIII	Cortisol + T_3	5	137 ± 25**	$p < .005$

** $p < .0005$ when compared to IV

† The average specific activity ± S.E. for the control was 0.140 ± 0.019 ΔOD/min/mg (n = 20).

TABLE 7

EFFECT OF HORMONES ON MALIC ENZYME SPECIFIC

ACTIVITY OF HEPATOCYTES AFTER 3 DAYS IN CULTURE

Experimental conditions are the same as in Table 6. Results are expressed as a % of the control cultures enzyme specific activity $\pm$ S.E. of n individual samples.

	Hormone Addition	n.	% Activity	
I	None (Control)		100†	$p<.0005$
II	T_3	13	136 $\pm$ 16**	
III	Insulin + Cortisol	16	117 $\pm$ 13	$p<.0005$
IV	Insulin + Cortisol + T_3	16	264 $\pm$ 52	
V	Insulin	13	117 $\pm$ 12	$p<.005$
VI	Insulin + T_3	13	160 $\pm$ 39**	
VII	Cortisol	5	77 $\pm$ 19	$p<.01$
VIII	Cortisol + T_3	5	155 $\pm$ 39*	

* $p<.025$ and ** $p<.0005$ when compared to IV

† The average specific activity $\pm$ S.E. for the control was 0.093 $\pm$ 0.019 ΔOD/min/mg (n = 20).

FIGURE 13

EFFECT OF VARYING CONCENTRATIONS OF T_3

AND T_4 ON THE INDUCTION OF α-GLYCEROPHOSPHATE

DEHYDROGENASE (α-GPD) AND MALIC ENZYME

Hepatocytes were isolated and cultured for 70 or 74 h as described in Materials and Methods. Control cultures were plated and maintained in medium containing no hormones. After 2 h in culture test cultures received medium containing $10^{-6}M$ insulin, $10^{-5}M$ cortisol and the concentrations indicated of T_3 (•-•). In another experiment the responses to different concentrations of T_4 (o-o) were investigated in the same manner. Results represent the enzyme activities expressed as % activity of hepatocytes maintained in medium containing no hormones $\pm$ range between samples prepared at 70 h versus duplicate samples prepared at 74 h.
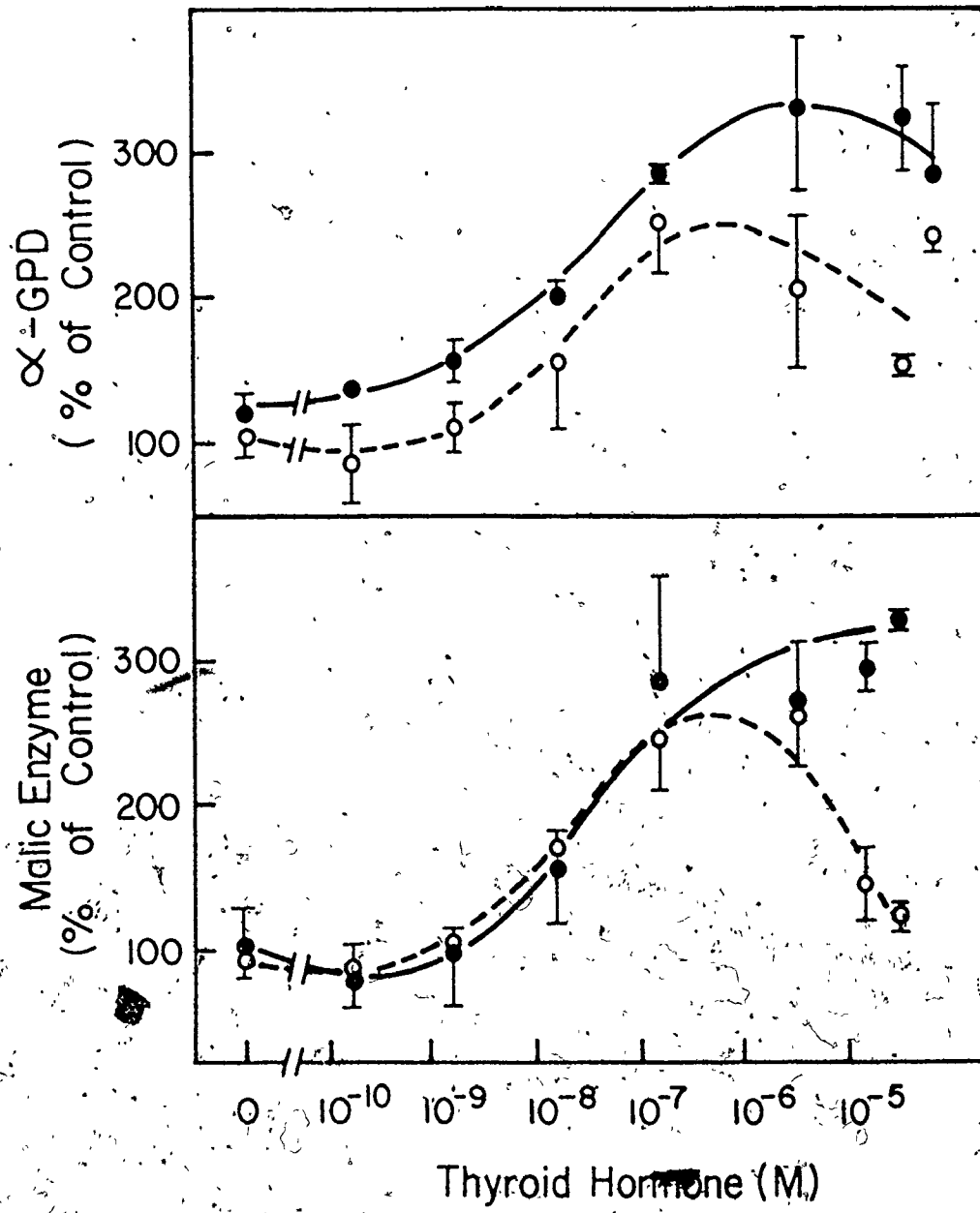
4.3.3 Effect of Varying Insulin or Cortisol Concentration

The role of insulin in enzyme induction was investi-
gated by varying the concentration of insulin in the culture
medium while cortisol and T_3 concentrations were kept con-
stant. The ability of insulin to potentiate T_3 action varies
with the concentration of insulin in the medium (Fig. 14).
No increase in enzyme activity is observed until 10^{-6} M insulin
is added, and a maximum potentiation occurs at about 10^{-5} M.

Similarly, the role of cortisol in cultured hepato-
cytes was studied by varying its concentration while insulin
and T_3 concentrations were kept constant (Fig. 15). Insulin-
treated cells were used as controls for this experiment. At
10^{-7} M cortisol, no change in α-glycerophosphate dehydrogenase
activity over insulin- and T_3-treated hepatocytes can be
observed, and only a slight change if any can be observed
for malic enzyme. With increasing concentrations of corti-
sol, the α-glycerophosphate dehydrogenase and malic enzyme
induction by T_3 was increased.

Hepatocytes in culture retain the ability to metab-
olize the hormones, insulin (Terris and Steiner, 1975;
Duckworth et al., 1981) and T_3 (Ismail-Beigi et al., 1979)
and cell lines derived from the liver of young rats retain
the pathways of steroid metabolism (Chessebeuf et al., 1975).
The level of cortisol in the culture medium over the first
24 h culture period was examined and found to have decreased
rapidly (Table 8).
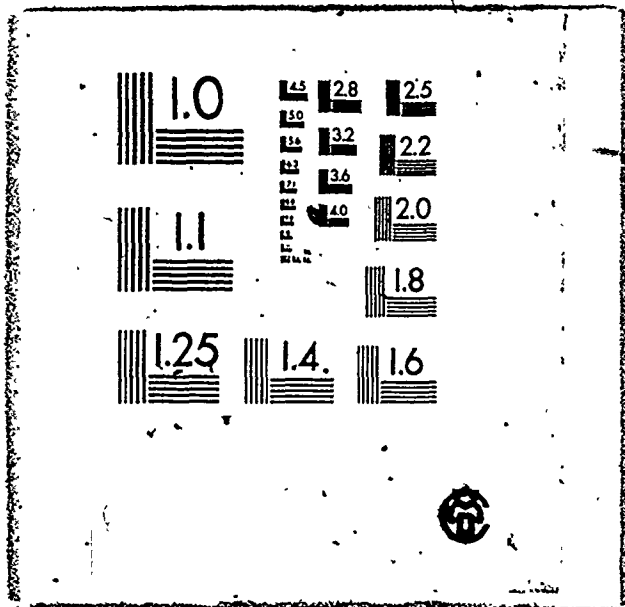
FIGURE 14

EFFECT OF VARYING INSULIN CONCENTRATION

Hepatocytes were isolated, cultured and enzymes were assayed as described in Materials and Methods. Control cultures were maintained in hormone free medium. Test cultures received media containing 10^{-5}M cortisol and 1.54 x 10^{-6}M T_3 plus the concentrations of insulin indicated. Results are expressed as a % of the activity in control cultures ± range between samples prepared after 70 h in culture versus duplicate samples at 74 h. α-Glycerophosphate dehydrogenase (α-GPD) and malic enzyme activities are shown.
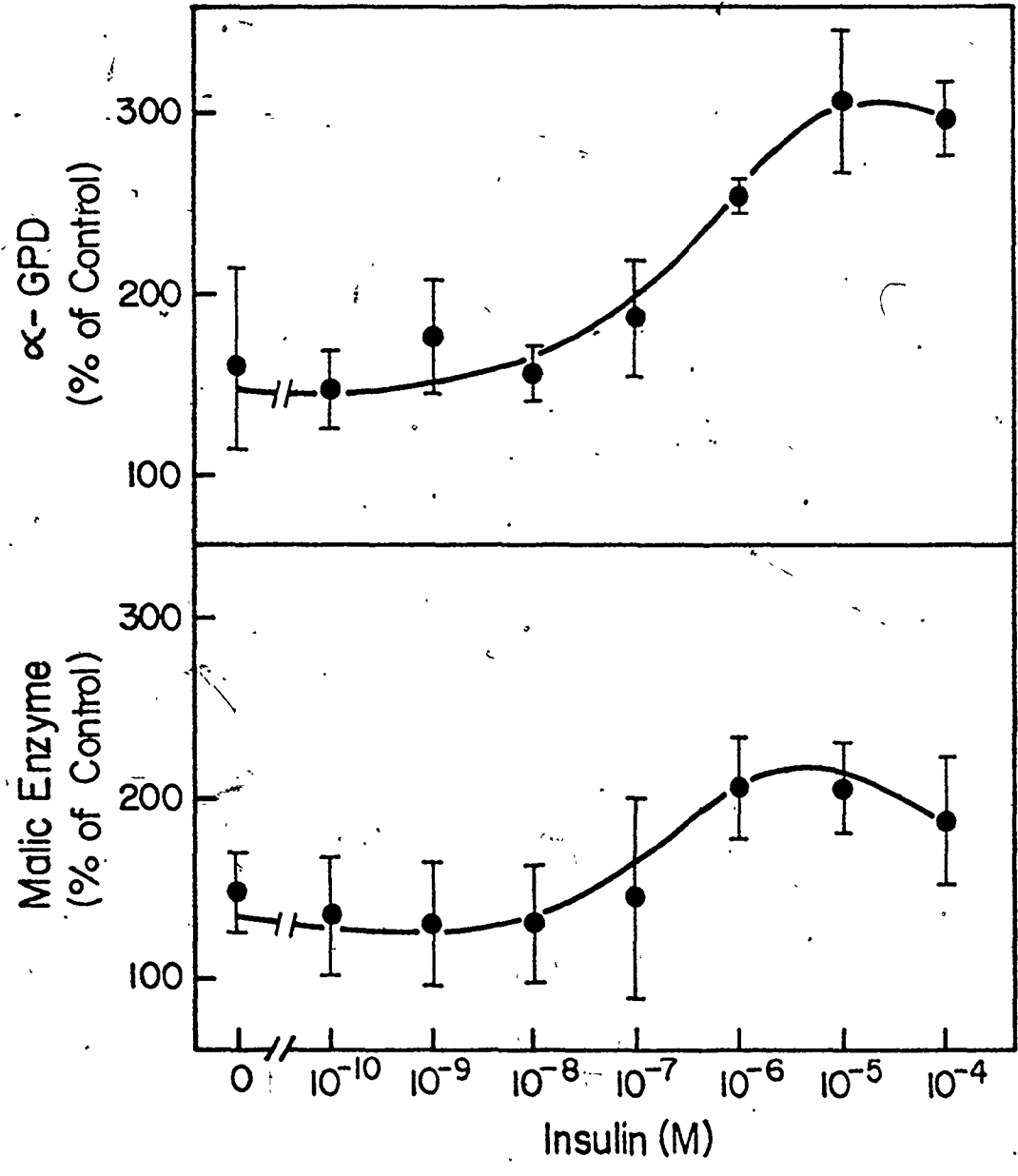
FIGURE 15

EFFECT OF CORTISOL CONCENTRATION ON

T_3 INDUCTION OF α-GLYCEROPHOSPHATE

DEHYDROGENASE AND MALIC ENZYME

Hepatocytes were isolated, cultured and enzymes assayed as described in Materials and Methods. Control cultures were maintained in insulin-supplemented medium. All test cultures were maintained in media containing $10^{-6}M$ insulin and 1.54 x $10^{-6}M$ T_3 with the concentrations of cortisol indicated. Results are expressed as a % of the activity in control cultures $\pm$ S.E., n = 4 except for the $10^{-6}M$ cortisol condition for α-glycerophosphate dehydrogenase where n = 3. The open bars represent α-glycerophosphate dehydrogenase and the hatched bars represent malic enzyme.
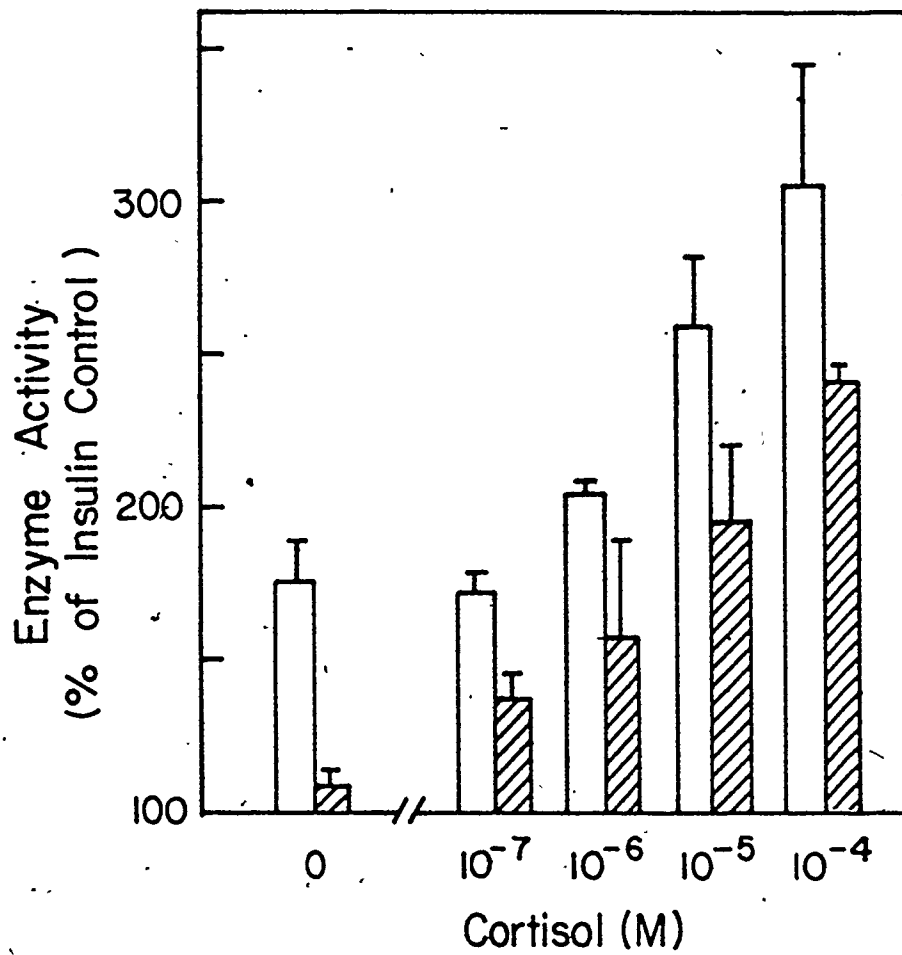
TABLE 8

DECREASE IN MEDIUM CORTISOL CONCENTRATION WITH TIME

The concentration of cortisol was determined as described in methods. Hepatocytes were plated in hormone-free medium. After 2 h, the medium was changed to medium containing 10^{-6}M insulin, 10^{-5}M cortisol and 1.54×10^{-6}M T_3. Cortisol was extracted from the medium after incubation with hepatocyte monolayers at the times indicated. Results are expressed as a % of the cortisol extracted from medium not exposed to cultured cells.

Incubation Time	% Cortisol Remaining*
0 h	92.0
0.5 h	53.5
2 h	9.6
24 h	not detectable

* The half-life of cortisol calculated from these data was approximately 37 min.

4.3.4 Effect of Dexamethasone on T_3 Action

In the absence of T_3, dexamethasone did not increase the activity of either α-glycerophosphate dehydrogenase or malic enzyme (Fig. 16). The response to a constant level of T_3 (1.54×10^{-6}M) varied with the concentration of dexamethasone. Maximum induction of α-glycerophosphate dehydrogenase (3-fold) and malic enzyme (4.5-fold) occurred at a dexamethasone concentration of 10^{-7}M. Dexamethasone at a concentration of 10^{-9}M has the same ability to potentiate T_3 action as 10^{-5}M cortisol.

4.3.5 Response to T_3 in the Presence of BSA

Dexamethasone (10^{-7}M) and insulin (10^{-6}M) were included in the medium to potentiate T_3 action and aid in detecting a response to low levels of T_3. The medium was supplemented with 1% BSA (fraction V, fatty acid-free) which bound 94% (Appendix 4) of the T_3 added to medium as measured by equilibrium dialysis. The BSA provided a sink of hormone unavailable for metabolism (Goodridge, 1975). At free hormone concentrations of $\cdot10^{-10}$ to 10^{-9}M measurable induction of α-glycerophosphate dehydrogenase and malic enzyme occurred (Fig. 17).

4.4 DISCUSSION

Although the addition of T_3 alone was capable of significantly inducing α-glycerophosphate dehydrogenase (62% increase) and malic enzyme (36%), a maximum induction by T_3

FIGURE 16

POTENTIATION OF T_3 ACTION BY DEXAMETHASONE

Hepatocytes were isolated, cultured, harvested and enzyme activities were assayed as described in Materials and Methods. Control cultures were maintained in medium containing no hormones. Test cultures received media containing 10^{-6}M insulin ($\bullet$—$\bullet$) or 10^{-6}M insulin and 1.54×10^{-6}M T_3 ($\blacktriangle$—$\blacktriangle$) and the concentration of dexamethasone indicated. The specific activities of α-glycerophosphate dehydrogenase (α-GPD) and malic enzyme are expressed as a % of the activity of control cultures $\pm$ range between samples prepared at 70 h and 74 h. The enzyme activities in hepatocytes maintained in medium containing 10^{-6}M insulin, 1.54×10^{-6}M T_3 and 10^{-5}M cortisol is shown ($\blacksquare$).
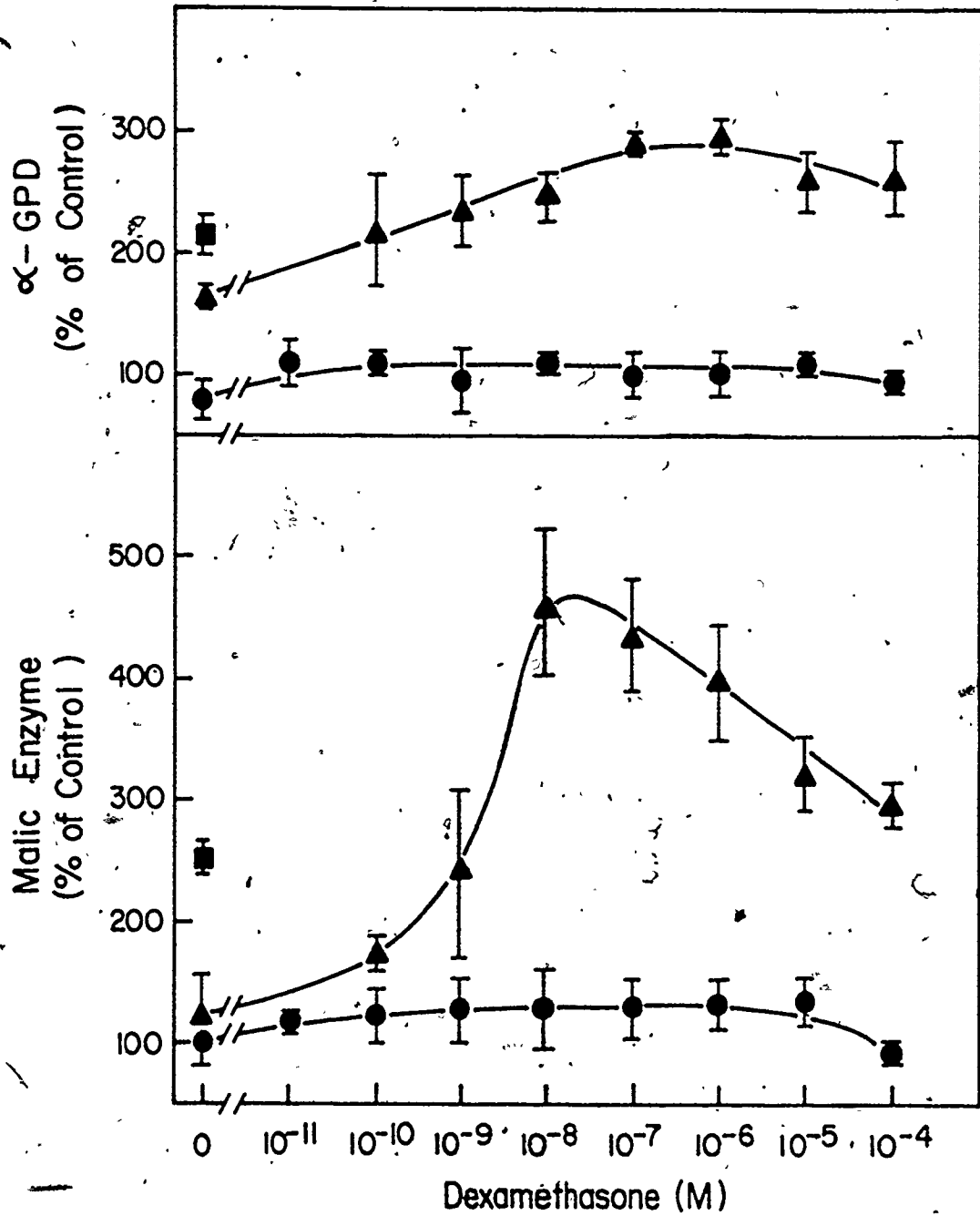
FIGURE 17

RESPONSE TO T_3 IN THE PRESENCE OF 1% BSA

Hepatocytes were isolated as described in Materials and Methods. Following the initial 2 h culture period in medium containing no hormones or BSA, test media were added containing $10^{-6}M$ insulin, $10^{-7}M$ dexamethasone, 1% BSA and the concentrations of T_3 indicated. Hepatocytes were harvested after 70 h and 74 h in culture. α-Glycerophosphate dehydrogenase (α-GPD) and malic enzyme activities were assayed as described in Materials and Methods. Results are expressed as a percentage of the activity of control cultures maintained in medium containing no hormones or BSA $\pm$ the range between 70 h and 74 h samples.
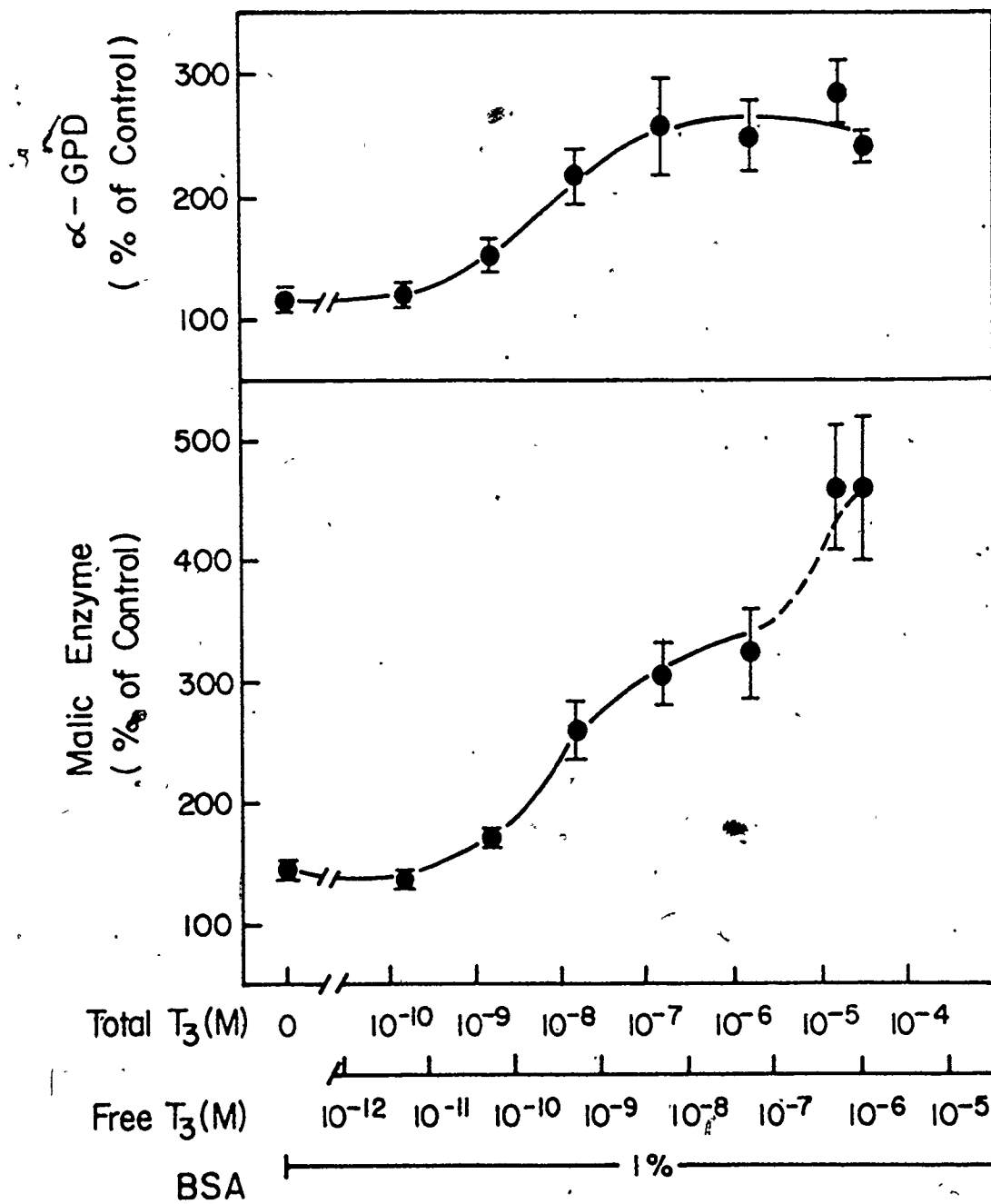
occurred only in the presence of insulin and cortisol. The omission of either insulin or cortisol resulted in a significant loss of enzyme induction. Ismail-Beigi et al. (1979) reported that increases in α-glycerophosphate dehydrogenase in cultured hepatocytes from thyroidectomized rats were unaffected by the omission of insulin, corticosterone and serum. The level of insulin they were using was 4 mU/ml (2.9×10^{-8}M) which from our insulin concentration curve would not potentiate T_3 action in normal hepatocytes. Rather high concentrations of around 10^{-6}M insulin were required to see stimulation of the T_3 response, but it should be remembered that cultured hepatocytes internalize and degrade insulin (Terris and Steiner, 1975; Duckworth et al., 1981).

Corticosterone is the major glucocorticoid of the rat but in bioassays it is less active than cortisol (Schulster et al., 1976). A concentration of 10^{-6}M cortisol was the lowest concentration at which potentiation of T_3 induction of α-glycerophosphate dehydrogenase occurred. Thus, the level of corticosterone (10^{-6}M) used by Ismail-Beigi et al. (1979) may be too low to potentiate T_3 action in their cell cultures.

The requirement for high concentrations of cortisol (10^{-4} - 10^{-6}M when compared to free physiological levels of about 10^{-8}M (Ballard, 1979)) is likely due to metabolism of the hormone by hepatocytes over the culture period. The concentration of cortisol in the medium decreased rapidly with a half-life of 37 min. Dexamethasone, a potent non-

metabolizable synthetic glucocorticoid, was able to potentiate T_3 induction of α-glycerophosphate dehydrogenase and malic enzyme at concentrations as low as $10^{-10} - 10^{-9}$ M.

The production of growth hormone in the rat pituitary cell line, GH_1, has been shown to be under the control of T_3 and cortisol (Martial et al., 1977; Shapiro et al., 1978). In both systems, GH_1 or cultured hepatocytes, cortisol alone cannot induce the production of growth hormone (Samuels et al., 1979) or α-glycerophosphate dehydrogenase and malic enzyme respectively but rather potentiates the action of T_3.

Adult rat hepatocytes exhibited insulin potentiation of T_3 induction of malic enzyme as reported earlier for chick hepatocytes (Goodridge, 1975). Although not reported for chick hepatocytes, the induction of mitochondrial α-glycerophosphate dehydrogenase in response to T_3 was also potentiated by insulin in rat heptocytes in culture.

Liver is the major organ of thyroid hormone catabolism and cultured rat hepatocytes break down T_3 rapidly (Ismail-Beigi, et al., 1979). Consequently, in the experiment without BSA supplementation, the concentration of 10^{-6} M T_3 required to obtain maximum induction of α-glycerophosphate dehydrogenase and malic enzyme in cultured hepatocytes represents the concentration that saturated the receptors over the 24 h period between media changes (Oppenheimer et al., 1977).

Most of the action of T_4 probably comes from its conversion to T_3 (Silva and Larsen, 1978; Oppenheimer, et al., 1979). Although it can bind to nuclear receptors and thereby exert

some intrinsic effects, T_4 has a lower affinity than T_3 (Oppenheimer et al., 1976). Rat hepatocytes in primary culture retain the liver's ability to deiodinate T_4 to T_3 (Krenning et al., 1980). Presumably both conversion of T_4 to T_3 and T_4 binding directly to nuclear receptors are involved and could account for the similarity between T_3 and T_4 concentration curves.

Hepatocytes in culture for 3 days showed an increase in α-glycerophosphate dehydrogenase and malic enzyme at concentrations of T_3 between 10^{-10} and 10^{-9}M, whereas without BSA to prevent rapid degradation, 10^{-8}M was required. The addition of BSA may influence the response in ways other than binding T_3 such as binding dexamethasone (Ballard, 1979) and altering the uptake of T_3 by hepatocytes (Krenning et al., 1979). The inductions of hepatic α-glycerophosphate dehydrogenase and malic enzyme require a high degree of saturation of nuclear receptors by T_3 (Oppenheimer and Dillmann, 1978). Thus, although the correlation with stimulating physiological free levels of T_3, 10^{-11} - 10^{-10}M, is not exact, the response to concentrations of 10^{-10} and 10^{-9}M represents a reasonable degree of sensitivity of cultured hepatocytes.

CHAPTER 5. GLUCOCORTICOID ACTION AND STEROID

SPECIFICITY OF THE POTENTIATION OF

T_3 ACTION

5.1   INTRODUCTION

Interactions between hormones in the regulation of enzyme activities in cultured hepatocytes have recently been described; in most instances glucocorticoids are involved (Michalopoulos et al., 1978; Gebhardt and Mecke, 1979a,b; Schudt, 1979 a,b, 1980a,b). The role of glucocorticoids in hormonal control of enzyme activity is sometimes described as "permissive" since the glucocorticoids often have no action on their own but are required for or potentiate the action of another hormone (Gebhardt and Mecke, 1979a,b; Granner, 1979; Katz et al., 1979b; Redshaw, 1980). These permissive actions of glucocorticoids in vitro have not been characterized with respect to steroid specificity.

From the results presented in Chapter 4, it is clear that cortisol and dexamethasone potentiated the response of rat hepatocytes to T_3. In this chapter the ability of different steroids to potentiate T_3 action is compared with their ability to induce tyrosine aminotransferase (EC 2.6.1.5), a typical and specific action of glucocorticoids (Thompson, 1979).

The effect of glucagon on the response of hepatocytes to T_3 and dexamethasone was examined because interactions between these hormones have been reported (Goodridge, 1978;

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Michalopoulos et al., 1978; Dillmann and Oppenheimer, 1979;
Ernest and Feigelson, 1979; Thompson, 1979).

5.2 MATERIALS AND METHODS

The procedures used to isolate and culture hepatocytes
from adult male Wistar rats (200-300 g) are detailed in
Materials and Methods, Chapter 4. Hepatocytes were plated
in basic culture medium consisting of L-15 medium, 10 mM
glucose, 1 mM succinate, antibiotics, 20 mM Hepes, 10 mM TES
and 10 mM BES. The addition of different hormones to the
medium after 2 h of culture is described for each experiment.
Hepatocyte monolayers were harvested after 3 days in culture
by collagenase treatment (Materials and Methods, Chapter 4).
The activities of α-glycerophosphate dehydrogenase and malic
enzyme were determined as described previously. Tyrosine
aminotransferase (EC 2.6.1.5) was assayed in the post-
mitochondrial supernatant by the method of Granner and
Tomkins (1975).

5.3 RESULTS

5.3.1 Comparison of the Glucocorticoid Action of Steroids
and Their Potentiation of T_3 Action

The effect of different concentrations of dexamethasone
on tyrosine aminotransferase activity was measured (Fig. 18).
Insulin and T_3-treatment neither increased nor decreased
tyrosine aminotransferase activity in the absence of dexame-
thasone. Tyrosine aminotransferase activity was increased,

FIGURE 18

EFFECT OF VARYING DEXAMETHASONE CONCENTRATION

ON TYROSINE AMINOTRANSFERASE

Procedures for culturing hepatocytes and assaying tyrosine aminotransferase are described in Materials and Methods. Hepatocytes were cultured for 3 days with 10^{-6}M insulin, 1.54×10^{-6}M T_3 and the concentration of dexamethasone indicated. Results are expressed as % activity of control cells maintained in medium containing no hormones $\pm$ range between 70 and 74 h samples.

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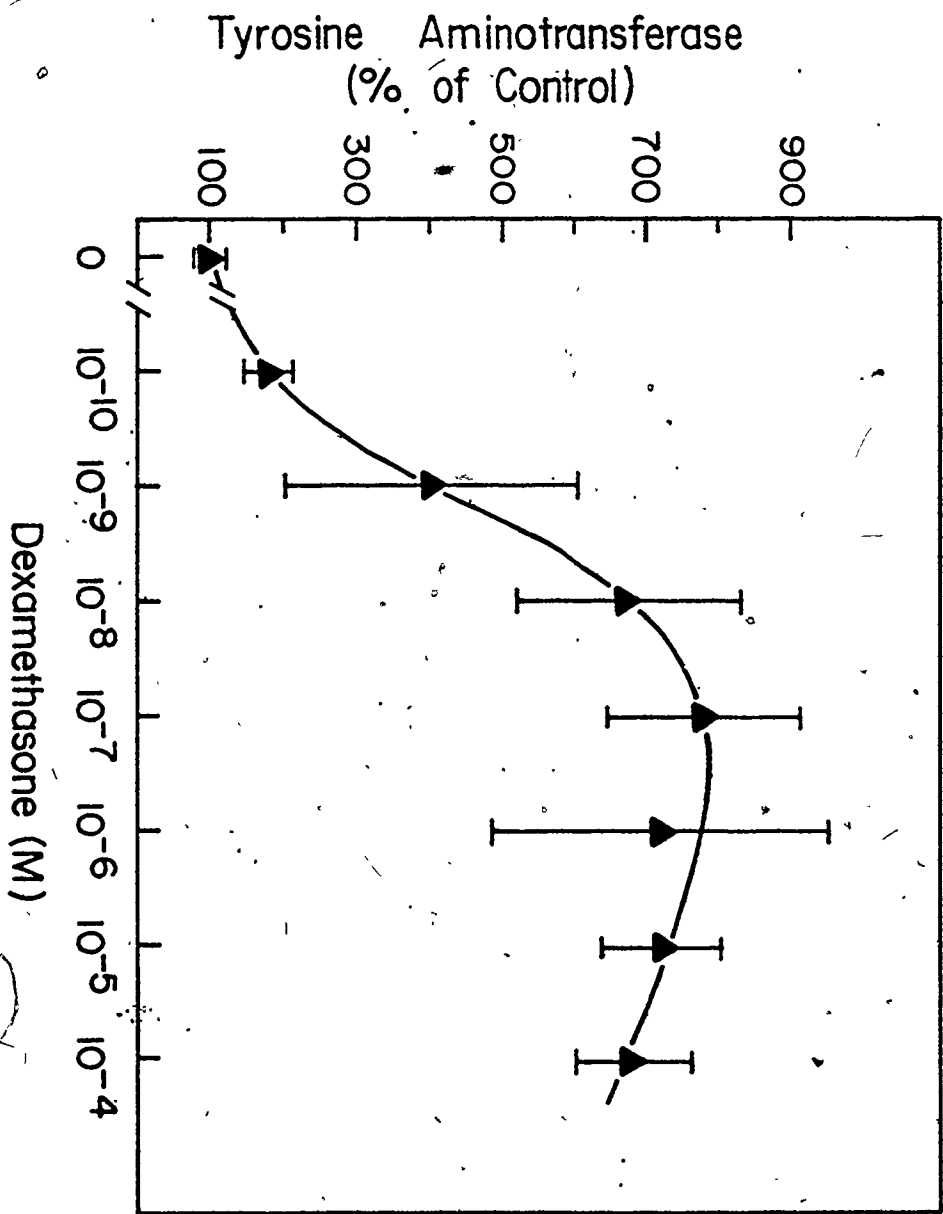
by a concentration of dexamethasone as low as 10^{-9}M and possibly 10^{-10}M. The maximum increase in enzyme activity was 7-8 times control values and occurred with 10^{-7}M dexamethasone. The activity of tyrosine aminotransferase decreased slightly in hepatocytes maintained in higher concentrations (10^{-6} - 10^{-4}M) of dexamethasone.

In order to examine the steroid specificity, test cultures received medium supplemented with insulin, T_3 and different steroids after the 2 h plating interval. These steroids were added at 10^{-5}M since at this concentration cortisol markedly increased T_3 action (Fig. 15, Chapter 4). From previous experiments it was determined that maximum potentiation of T_3 action (Fig. 16, Chapter 4) and maximum increase in tyrosine aminotransferase activity (above) occurred at a concentration of 10^{-7}M dexamethasone (a synthetic and non-metabolizable glucocorticoid). Therefore, the ability of steroids to potentiate T_3 action or to increase tyrosine aminotransferase activity was compared with the maximum effect caused by 10^{-7}M dexamethasone (set at 100%). The results of 3 experiments (2 samples/experiment) are combined and presented in Table 9. Cortisol, corticosterone and aldosterone significantly increased the activities of malic enzyme and tyrosine aminotransferase above the insulin and T_3 condition. Deoxycorticosterone and testosterone slightly increased malic enzyme activity (not significant for deoxycorticosterone but low n value) but had no effect on tyrosine aminotransferase activity. Chol-

TABLE 9

EFFECT OF DIFFERENT STEROIDS ON THE ACTIVITIES

OF MALIC ENZYME AND TYROSINE AMINOTRANSFERASE

The activities of malic enzyme and tyrosine amino-transferae (TAT) were determined in post-mitochondrial supernatant fractions from hepatocytes cultured for 3 days with $10^{-6}M$ insulin, $1.54 \times 10^{-6}M$ T_3 and the different steroids indicated. Results are expressed as a % of maximum glucocorticoid action (i.e. the increase in enzyme activities caused by $10^{-7}M$ dexamethasone above the activity observed in hepatocytes cultured with insulin and T_3) $\pm$ S.E.. All steroids were added to the culture medium at a concentration of $10^{-5}M$. The specific activity of malic enzyme in the control condition (insulin + T_3) was 0.116 $\pm$ 0.020 $\Delta OD/min/mg$. In the presence of insulin, dexamethasone and T_3 the specific activity was 0.345 $\pm$ 0.087 $\Delta OD/min/mg$. Control (insulin + T_3) specific activity of tyrosine amino-transferase was 0.253 $\pm$ 0.080 $\Delta OD/min/mg$ (13 $\pm$ 4 milliunits/mg). Specific activity in the presence of insulin, dexamethasone and T_3 was 2.362 $\pm$ 0.963 $\Delta OD/min/mg$ (119 $\pm$ 48 milliunits/mg). The p values were determined by a t-test on % increases. p< **.0.25, ***.01, +.005, ++.0005.

Steroid	(n)	Malic Enzyme	TAT
Cortisol	(6)	40 $\pm$ 3^{+++}	43 $\pm$ 7^{+}
Corticosterone	(6)	39 $\pm$ 11***	18 $\pm$ 4^{+}
Aldosterone	(6)	38 $\pm$ 4^{++}	21 $\pm$ 1^{++}
Deoxycorticosterone	(4)	9 $\pm$ 5	-1 $\pm$ 2
Testosterone	(6)	10 $\pm$ 4**	3 $\pm$ 2
Cholesterol	(4)	-4 $\pm$ 5	-1 $\pm$ 2
17β-Estradiol	(4)	2 $\pm$ 3	4 $\pm$ 2

esterol and estradiol had no effect on either enzyme.

5.3.2 Effects of Dexamethasone, Insulin, T_3 and Glucagon on Enzyme Activity

The activities of α-glycerophosphate dehydrogenase (Table 10), malic enzyme (Table 11) and tyrosine aminotransferase (Table 12) were examined (in 4 experiments, 2 samples/experiment) in hepatocytes cultured for 3 days with different combinations of insulin, dexamethasone, T_3 and glucagon. Although the number of samples was 8 or less, statistical analyses by $\underline{t}$-tests on % differences were done to aid in the interpretation of the results. The effects of the different hormones on α-glycerophosphate dehydrogenase and malic enzyme activities were similar to those observed in Tables 6 and 7 (Chapter 4) when cortisol was used instead of dexamethasone. Glucagon alone had no effect on either enzyme. In the presence of insulin, dexamethasone and T_3, glucagon did not alter α-glycerophosphate dehydrogenase activity but appeared to decrease malic enzyme activity although with variation between samples the decrease was not significant.

Tyrosine aminotransferase was not increased by insulin or T_3, either alone or together (Table 12), which agrees with the results in Figure 18. In every condition in which dexamethasone was added to the medium, tyrosine aminotransferase activity was significantly increased (6-12 fold). Glucagon alone increased tyrosine aminotransferase activity 2 fold (which was significant). When glucagon was added

TABLE 10

EFFECT OF DEXAMETHASONE, INSULIN, T_3 AND

GLUCAGON ON α-GLYCEROPHOSPHATE DEHYDROGENASE

Procedures for culturing hepatocytes and assaying enzyme activity are described in Materials and Methods. Hepatocytes were cultured for 3 days with the hormones indicated at the following concentrations: insulin, 10^{-6}M; T_3, 1.54 x 10^{-6}M; dexamethasone (Dex.), 10^{-7}M; glucagon, 10^{-6}M. Enzyme specific activity is expressed as a % of the enzyme specific activity in control cultures ± S.E. The p values were determined by a t-test on the % differences between test and control cultures. p *.05, **.025, ***.01, +.005, ++.0005

Hormone Addition	n	% Activity
None (Control)	8	100†
Insulin	6	102 ± 9
T_3	4	156 ± 14**
Dex.	3	75 ± 31
Glucagon	5	105 ± 10
Insulin + Dex.	6	116 ± 11
Dex. + T_3	4	139 ± 23
Insulin + T_3	6	177 ± 14+
Insulin + Dex. + T_3	8	258 ± 13++
Insulin + Dex. + T_3 + Glucagon	6	271 ± 10++

n.s.

† Specific Activity was 0.117 ± 0.015 ΔOD/min/mg.

TABLE 11

EFFECT OF DEXAMETHASONE, INSULIN, T_3 AND

GLUCAGON ON MALIC ENZYME

Details of the experimental conditions, hormone concentrations and enzyme assays are given in Table 10. Results are expressed as % of the enzyme specific activity of the control ± S.E. The p values were determined by a t-test and are listed in Table 10.

Hormone Addition	n	% Activity
None (Control)	8	100†
Insulin	6	115 ± 9
T_3	4	144 ± 19*
Dex.	4	58 ± 2
Glucagon	6	117 ± 2
Insulin + Dex.	6	143 ± 12***
Dex. + T_3	4	141 ± 40
Insulin + T_3	6	159 ± 12$^+$
Insulin + Dex. + T_3	8	374 ± 43^{++}
Insulin + Dex. + T_3 + Glucagon	6	298 ± 24^{++}

n.s.

† Specific Activity was 0.095 ± 0.025 ΔOD/min/mg.

TABLE 12

EFFECT OF DEXAMETHASONE, INSULIN, T_3 AND GLUCAGON ON TYROSINE AMINOTRANSFERASE

Details of the experimental conditions, hormone concentrations and enzyme assays are given in Table 10. Results are expressed as % of the enzyme specific activity of the control $\pm$ S.E. The p values were determined by a t-test and are listed in Table 10.

Hormone Addition	n	% Activity
None (Control)	8	100†
Insulin	6	106 $\pm$ 6
T_3	4	114 $\pm$ 6
Dex.	4	621 $\pm$ 148***
Glucagon	6	213 $\pm$ 34**
Insulin + Dex.	6	915 $\pm$ 98++
Dex. + T_3	4	985 $\pm$ 193***
Insulin + T_3	6	122 $\pm$ 10
Insulin + Dex. + T_3	8	795 $\pm$ 80++
Insulin + Dex. + T_3 + Glucagon	6	1189 $\pm$ 115++

† Specific Activity was 0.228 ± 0.080 $\Delta OD/min/mg$.

with insulin, dexamethasone and T_3, the increase in enzyme activity was 12 fold compared to 8 fold without glucagon. This increase by glucagon was significant.

5.4   DISCUSSION

The induction of tyrosine aminotransferase by gluco-corticoids has been extensively studied in vivo and in vitro in HTC cells and in isolated or cultured hepatocytes (Lin and Knox, 1957; Tanaka et al., 1978; Ernest and Feigelson, 1979; Granner, 1979; Thompson, 1979; Ivarie et al., 1980). Unlike the T_3 induction of α-glycerophosphate dehydrogenase and malic enzyme, the increase of tyrosine aminotransferase by dexamethasone treatment of cultured hepatocytes reached a maximum within 1 day, then gradually decreased (Appendix 5) which agrees with the report by Ivarie et al. (1980). In order to compare the effects of dexamethasone and other steroids on tyrosine aminotransferase and T_3 action, a 3 day treatment period was used throughout these studies.

The increases in tyrosine aminotransferase activity caused by different concentrations of dexamethasone parall-eled the increases in α-glycerophosphate dehydrogenase and malic enzyme activities caused by dexamethasone in the presence of insulin and T_3 (see Chapter 4). Concentrations of 10^{-10} - 10^{-9} M were required to observe a response and the maximum effect occurred at 10^{-7} M dexamethasone.

Only the steroids (cortisol, corticosterone and aldo-sterone) with known glucocorticoid action (Schulster et al.,

1976) were able to potentiate T_3 action substantially and to increase tyrosine aminotransferase activity. Deoxycorticosterone is a mineralocorticoid which by bioassays has no glucocorticoid action (Schulster et al., 1976). In HTC cells, deoxycorticosterone is a poor inducer of tyrosine aminotransferase requiring sustained high concentrations (Samuels and Tomkins, 1970). One distinction between these culture systems is that HTC cells cannot metabolize steroids (Samuels and Tomkins, 1970) but primary cultured hepatocytes can (see Chapter 4). Presumably deoxycorticosterone was being metabolized by the hepatocytes and was not maintained at a concentration sufficient to increase tyrosine aminotransferase. The activity of malic enzyme in the presence of deoxycorticosterone and testosterone appeared slightly elevated whereas tyrosine aminotransferase activity was definitely not affected. The reason for this is unclear and may reflect independent actions of these hormones in the culture system not related to any glucocorticoid action.

The interaction of glucocorticoids and T_3 in GH_1 and GH_3 cells has been extensively studied (Martial et al., 1977; Shapiro et al., 1978; Samuels et al., 1979; Ivarie et al., 1980, 1981). These hormones are thought to interact at a pre-translational level (Shapiro et al., 1978). Recent studies in the GH_3 cells show that T_3 alone has as large an effect as dexamethasone and T_3 if the cells were pretreated with dexamethasone (Ivarie et al., 1980). Ivarie et al. (1980) suggest that dexamethasone is controlling the syn-

thesis of a regulator which then acts in combination with T_3.

These studies in cultured hepatocytes have shown a remarkable similarity in steroid specificity and concentration requirements of glucocorticoid action and steroid hormone potentiation of T_3 action. Since the glucocorticoid action examined (increases in tyrosine aminotransferase activity) is known to occur at the nuclear level (Higgins et al., 1979), this suggests that glucocorticoids may be acting at the nuclear level to potentiate T_3 action in cultured hepatocytes by a mechanism comparable to their action in rat pituitary cell lines. However, only further experimentation in cultured hepatocytes will reveal whether the glucocorticoid effects are direct or involve the expression of a regulator of T_3 action.

The changes in the activities of α-glycerophosphate dehydrogenase and malic enzyme in medium supplemented with different combinations of insulin, dexamethasone and T_3 are very similar to those observed in Chapter 4 when cortisol was used in place of dexamethasone. Glucagon alone did not affect the activities of α-glycerophosphate dehydrogenase or malic enzyme but did increase significantly tyrosine aminotransferase activity in agreement with the report by Michalopoulos et al. (1978). Ernest and Feigelson (1979) reported that dexamethasone and either glucagon or dibutyryl cAMP must be present to induce tyrosine aminotransferase in isolated rat liver cells. In these studies and studies by Michalopoulos et al. (1978) either a glucocorticoid

(dexamethasone, these studies; cortisol, Michalopoulos et al., 1978) or glucagon could increase tyrosine aminotransferase activity independently. An effect of insulin on tyrosine aminotransferase could not be demonstrated in primary cultured hepatocytes although an increase in enzyme activity due to decreased degradation of the enzyme has been described in HTC cells (Gelehrter, 1979). In the presence of both dexamethasone and glucagon, the activity of tyrosine aminotransferase was significantly increased above the level of each hormone alone. The synergism of glucagon and glucocorticoids on tyrosine aminotransferase in primary cultured hepatocytes has also been described by Michalopoulos et al. (1978).

The effect of glucagon on T_3 action has been extensively studied in cultured chick hepatocytes (Goodridge and Adelman, 1976; Goodridge, 1978; Siddiqui et al., 1981). Glucagon rapidly blocks induction of malic enzyme by T_3 by inhibiting the production of translatable mRNA (Siddiqui et al., 1981). In these studies with primary cultured rat hepatocytes, glucagon appeared to decrease malic enzyme activity, although the effect was not statistically significant. α-Glycerophosphate dehydrogenase activity was unaffected by glucagon which is consistent with in vivo results (Dillmann and Oppenheimer, 1979). The elevation of glucagon by starvation is thought to prevent the induction of malic enzyme by T_3 in starved rats or chicks (Dillmann and Oppenheimer, 1979; Siddiqui et al., 1981). Malic enzyme and other lipogenic enzymes are increased by T_3 and high carbohydrate diets

in vivo (Oppenheimer et al., 1981). The inability of gluca-gon to block T_3 induction of malic enzyme can not be explained at present. Further work in the cultured rat hepatocyte system with respect to hormone levels, glucagon metabolism and culture conditions (such as serum and glucose levels) need to be investigated before an explanation can be put forward.

CHAPTER 6.   DISCUSSION

6.1.1 The Use of Cultured Hepatocytes for Hormone Studies

In the past few years, primary cultures of rat hepato-
cytes have been used extensively to study the actions of
many different hormones (Geelen et al., 1978; Michalopoulos
et al., 1978; Gebhardt and Mecke, 1979 a,b; Schudt, 1980a,b).
In many instances, serum and/or hormones are added to improve
the maintenance and longevity of hepatocyte cultures (Bonney
et al., 1974; Michalopoulos et al., 1978, Gebhardt and Mecke,
1979b).   Some workers omit serum and hormones following the
plating of the cells (Spence et al., 1979; Spence and Pitot,
1979; Schudt, 1980a) but the possible effects of this initial
exposure on the metabolism of hepatocytes throughout the
culture period has not been considered.   The culture method
used in the studies in this thesis is novel because hepato-
cytes from normal adult rats were routinely plated and
maintained in serum- and hormone-free medium.   Only recently
has another hormone- and serum-free culture method for rat
hepatocytes been described (Yamada et al., 1980).

The studies in Chapter 2 on the morphology and longev-
ity of hepatocytes cultured with different hormones generally
agree with reports by other workers (Michalopoulos and
Pitot, 1975; Laishes and Williams, 1976a; Tanaka et al.,
1978).   It is clear that insulin and/or dexamethasone
markedly alter the properties of these cells in culture.
Consistently, it was noted in these studies that enzyme

activities and other cell parameters (e.g. cytochrome content) were elevated (10-20%) in cultures maintained in medium supplemented with insulin and dexamethasone above the values obtained for hepatocytes maintained in hormone-free medium. Some of these observations may be related to improved maintenance of cells in culture and general hepatotrophic effects of insulin rather than specific actions of these hormones. However T_3 did not alter the survival or morphology of these cells in culture. Therefore, the effects of T_3 on cultured hepatocytes represent specific actions of the hormone and can not be explained by overt changes in longevity or maintenance of these cells in culture.

6.1.2 Actions of T_3 in Cultured Hepatocytes

At the time this research was begun, information on the mechanism of action and direct cellular effects of T_3 was limited. Studies over the past 5 years, in vivo in the rat (for a review see Oppenheimer, 1979), in vitro in chick hepatocytes (Goodridge and Adelman, 1976; Goodridge, 1978; Siddiqui et al., 1981) and in vitro in rat pituitary cell lines (for a review see Eberhardt et al., 1980) have considerably expanded current knowledge on the control of gene expression by T_3. Because T_3 actions in cultured mammalian liver cells had not been reported when these studies were initiated, the first goal of this research was to demonstrate specific and biologically relevant effects of thyroid hormones in vitro. The inductions of α-glycerophosphate

dehydrogenase and malic enzyme in liver in vivo have been well characterized and are the only actions in liver that have been linked directly to the binding of T_3 to specific nuclear receptors (Oppenheimer and Dillmann, 1978). The concurrent inductions of α-glycerophosphate dehydrogenase and malic enzyme by T_3 treatment of hepatocyte monolayers follow the same time course as occurs in vivo. Therefore, the first objective of these studies, i.e. the development of a culture system which could respond to T_3 as the liver does in vivo was achieved.

The short life-span of these cells in culture prevented extensive examination of certain T_3 actions which are known to be quantitatively less than increases in α-glycerophosphate dehydrogenase or malic enzyme and require greater than one week to observe a maximum response (e.g. succinate dehydrogenase or NADPH-cytochrome c reductase). However, another in vivo action of thyroid hormones, increased cytochrome content, was observed in cultured hepatocytes thus showing this action to be a direct cellular effect of T_3. Some effects such as changes in the synthesis of glycerolipids could be observed after 24 and 50 h of treatment with T_3. In contrast to the in vivo report by Tata (1970), T_3 caused a decrease in choline incorporation in vitro. This in vitro response is definitely a direct action of T_3 on a single cell type. Possibly the in vivo response requires accessory factors or tissue conditions that one not duplicated during the primary culturing of hepatocytes.

6.1.3 Potentiation of T_3 Action by Insulin and Glucocorticoids

By using increases in α-glycerophosphate dehydrogenase and malic enzyme activities, it was possible to characterize the effects of other hormones on the response to T_3. Insulin has been reported to enhance the induction of malic enzyme by T_3 in chick hepatocytes (Goodridge and Adelman, 1976). In the past few years the effect of glucocorticoids on the induction of growth hormone by T_3 in rat pituitary cell lines has been extensively examined (reviewed by Eberhardt et al., 1980). It was a unique finding of the studies in this thesis that both insulin and glucocorticoids potentiate the action of T_3 in mammalian liver cells. A report by Ismail-Beigi et al. (1979) concluded that there was no effect of corticosterone, insulin or serum on increases in activity of α-glycerophosphate dehydrogenase caused by T_3 treatment of hepatocytes from a thyroidectomized rat. The hormone concentrations required and hormone metabolism character-ized in this thesis plus other reports in the literature suggested reasons for the negative results obtained by Ismail-Beigi et al. (1979) which were discussed in detail in Chapter 4.

The ability of cultured hepatocytes to respond to many hormones has opened up an area of research into the interactions of hormones. During the course of this work, many reports on the direct and permissive actions of gluco-corticoids have appeared. In this thesis, the potentiation

of T_3 action by steroids was characterized with respect to hormone concentration and steroid specificity. The results demonstrated that the potentiation of T_3 action was specific for glucocorticoids and suggest a close parallel to the effect of glucocorticoids on T_3 action in cultured rat pituitary cells (observed by other workers, reviewed by Eberhardt et al., 1980).

In a broad sense these studies emphasize the point that the actions of hormones can not be considered in isolation even at the cellular level. Although my research has dealt with the effects of other hormones (insulin and glucocorticoids) on the actions of thyroid hormones, there is evidence that thyroid hormones alter the response of liver cells to other hormones. For example, the gene for α_{2u} globulin is under multi-hormonal control in rat liver (Kurtz and Feigelson, 1978). In euthyroid rats, androgens and growth hormone increase the synthesis of α_{2u} globulin but in thyroidectomized rats this response is lacking. Insulin increases glucokinase and ATP-citrate lyase and dexamethasone increases glucokinase in primary cultures of hepatocytes from a euthyroid rat but not in cultures from a thyroidectomized rat (Spence et al., 1979; Spence and Pitot, 1979). Pretreatment of hepatocyte monolayers from thyroidectomized rats with T_3 restored the responses to insulin and dexamethasone. Thus the imbalance (deficiency or excess) of one hormone can drastically affect, either impair or accentuate, the primary actions of another

hormone.

The research in this thesis is the first demonstration that both insulin and glucocorticoids potentiate the actions of thyroid hormones in mammalian liver. The physiological significance and molecular mechanisms of these hormone interactions are interesting questions raised by this finding. Because the culture system for rat hepatocytes developed for these studies is completely defined and retains the ability to respond characteristically to thyroid and glucocorticoid hormones, it is an ideal system for future investigations on the role and molecular mechanism of these hormone interactions.

APPENDIX 1

CORRECTION OF PROTEIN VALUES FOR THE INTERFERENCE

OF TRIS IN THE LOWRY ASSAY

The effect of Tris on protein values measured by a modified Lowry assay was determined as follows. By adding STE to known quantities of BSA, the apparent μg protein value was determined by comparison to a standard curve of BSA without STE. For each level of STE, the apparent μg protein values for each actual μg protein value were averaged from 5 different assays done in duplicate and the standard error was always <0.9 μg protein. Appendix 2 shows a graph of the values for 10, 25 and 40 μl of STE. The apparent vs. actual μg data was subjected to linear regression analysis. In all cases the correlation coefficient was 1.000. From the equations for these lines (apparent μg = a x actual μg + b), the protein values for the cell fractions were corrected for Tris interference. Sucrose and EDTA(SE) in the absence of Tris did not affect the assay at the concentrations used. It was determined that the addition of Tris to cell fractions prepared in the absence of Tris caused the same amount of interference as with BSA.

STE		CONSTANTS	
μl	mM*	a	b
10	2.5	1.006	1.513
20	5	0.986	3,894
25	6.25	0.976	4,846
30	7.5	0.962	6.278
40	10	0.940	8.136

* This is the concentration in the sample volume. The concentration in the complete reaction mixture is 1/12 this value.

APPENDIX 2

GRAPH OF APPARENT µg PROTEIN VS. ACTUAL µg PROTEIN

The apparent g values measured in the presence of Tris were obtained as described in Appendix 1. The apparent vs. actual µg lines are shown for protein measured in the presence of STE, 10 µl (●—●), 25 µl (○—○) and 40 µl (□—□). Data for 20 and 30 µl are not shown. The influence of Tris on protein values was greatest for low protein concentrations. Cell fractions generally contained 1 mg/ml protein which results in about a 15% overestimation of protein in the presence of Tris.
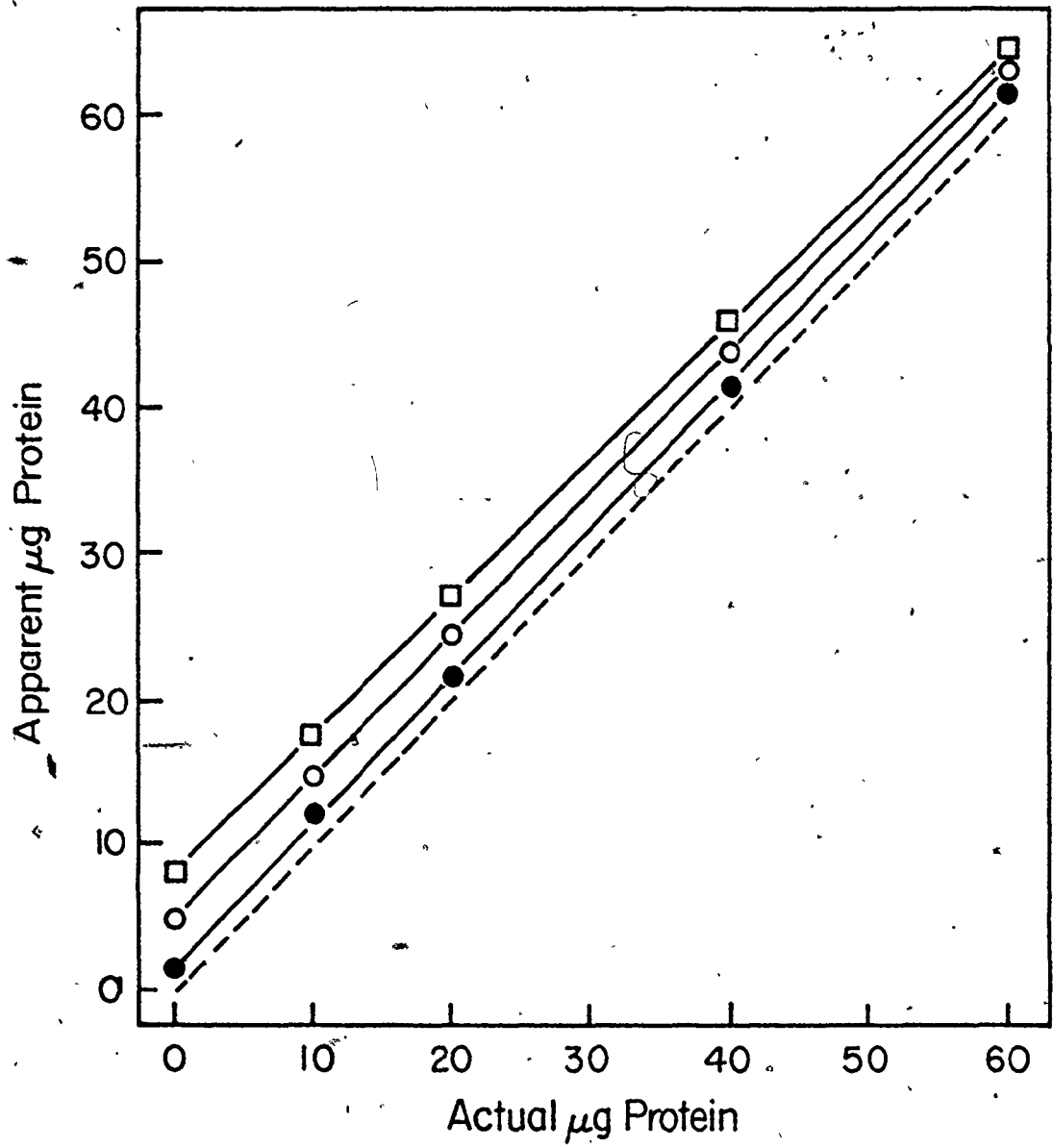
APPENDIX 3

EFFECTS OF HARVESTING METHOD ON PROTEIN

CONCENTRATION IN CELL FRACTIONS

Hepatocytes were isolated from a thyroidectomized rat and cultured for 3 days as described in Materials and Methods (Chapter 3). Cells from one flask were harvested by scraping with a rubber policeman. Cells from a second plate were harvested by collagenase treatment. Both methods are described in Materials and Methods (Chapter 3). Volumes of the mitochondria and post-mitochondrial superna- tant (PMS) fractions were the same.

Harvesting Method	Protein Concentration (mg/ml)	
	Mitochondria	PMS
Collagenase treatment	1.03	1.12
Scraped	1.15	0.42

APPENDIX 4

EQUILIBRIUM DIALYSIS

Equilibrium dialysis was performed as described in Materials and Methods (Chapter 4). A volume (100 µl) of basic medium supplemented with 1% BSA, 10^{-7}M dexamethasone, 10^{-6}M insulin and the indicated concentration of T_3 was placed in the test chamber and dialyzed against medium (100 µl) containing 10^{-7}M dexamethasone, 10^{-6}M insulin and approximately 8000 cpm ^{125}I-T_3. Following equilibrium of radio-activity the cpm in each chamber were determined by liquid scintillation counting of aliquots from both chambers. Results are the average of duplicates and are expressed as the % of T_3 bound in the test chamber. The average % of T_3 bound in the medium calculated from this data is 94%.
a) When the concentration of T_3 in test and control chambers was 1.54×10^{-6}M the % of T_3 bound was the same. b) The % of T_3 bound by BSA containing medium used to culture hepato-cytes for 24 h was determined.

Concentration of T_3 (M)	% of T_3 Bound
1.54×10^{-10}	93.8
1.54×10^{-9}	94.1
1.54×10^{-8}	94.1
1.54×10^{-7}	94.4
1.54×10^{-6}	93.9
1.54×10^{-5}	93.5
3.08×10^{-5}	93.6
1.54×10^{-6} a)	92.6
1.54×10^{-6} b)	92.4

APPENDIX 5

INCREASE IN TYROSINE AMINOTRANSFERASE

ACTIVITY BY DEXAMETHASONE

Procedures for culturing hepatocytes and measuring tyrosine aminotransferase activity are described in Materials and Methods (Chapter 4). After the 2 h plating interval, all cultures received medium containing 10^{-6}M insulin, 1.54 x 10^{-6}M T$_3$ and 10^{-7}M dexamethasone. Enzyme activities were determined at the times indicated after the addition of hormones.

Time of Treatment (h)	Tyrosine Aminotransferase Specific Activity (milliunits/mg)
0	14
4	81
8	114
24	164
48	112
72	108
	83

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