ENZYME DEVELOPMENT IN HUMAN AND RAT FETAL LIVER

by

LAWRENCE THOMAS KIRBY

B.Sc.F., University of Toronto, 1963

M.Sc., University of Victoria, 1970

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Department of Medical Genetics

The University of British Columbia
Vancouver 8, Canada

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ABSTRACT

A culture system has been established whereby it is possible to study the spontaneous or induced change in enzyme levels in human and rat fetal liver segments. The system is short term, i.e. of approximately 24 hours duration, and can be established with a minimum of facilities.

As gestational age increased from 10 to 21 weeks, there was approximately a 14 fold decrease in the cytosol level of tyrosine transaminase (E.C.2.6.1.5, TTA) in the fetal human liver and kidney. There was a 3 fold decrease in liver cytosol phosphoenolpyruvate carboxykinase (E.C.4.1.1.32, PEPck) between 10 and 13 weeks gestational age; this enzyme level then remained relatively constant. The ratio of liver or kidney cytosol to mitochondrial PEPck was 25 to 1 in the tenth week of gestation and decreased to 6 to 1 by the eighteenth week. Thus, with increasing gestational age the mitochondrial enzyme appeared to increase in significance. The levels of kidney and liver cytosol pyruvate kinase (E.C.2.7.1.40, PK) and glucose-6-phosphate dehydrogenase (E.C.1.1.1.49, G6PD) did not significantly change from 10 to 21 weeks of gestation.

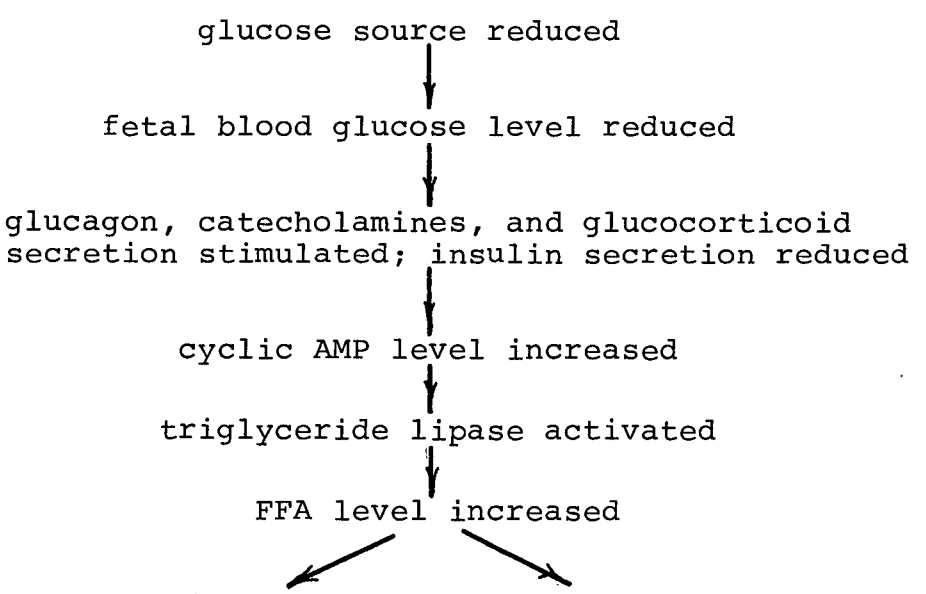
Comparisons between the fetal liver enzymes TTA, PEPck, PK, and G6PD in the human, pig, and rat indicated that the levels are similar for all except PEPck; this enzyme was approximately 7 times greater in pig than in human or rat.

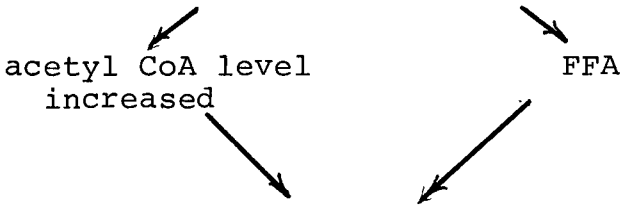
It was possible to increase the level of the cytosol enzymes PEPck and to a lesser extent TTA in fetal human liver as

it was in fetal rat liver. PEPck was increased *in vitro* approximately 4.5 fold and TTA by about 30%. The importance of the induction of these enzymes to fetal gluconeogenesis is stressed.

PEPck was increased 33 fold in the liver from a 12.5 week human fetus which had been exposed to the glucocorticoid prednisolone *in utero*. The importance of this induction to the gluconeogenic process is noted; also, the importance of the possible side effects of therapeutic agents given to a mother on enzymes in the fetus is stressed.

Free fatty acids (FFA) or acetyl CoA caused increased levels of PEPck in human fetal liver. Also, from work with inhibitors of FFA oxidation, viz. deoxycarnitine and citral, there was an indication that the inducing effect of N^6,O^2-dibutyryl cyclic adenosine 3',5'-monophosphate (dcAMP) on fetal rat liver cytosol PEPck and TTA could be partially blocked with these agents. One mechanism of induction of the gluconeogenic process, at least in the human, appears to be as follows:





induction (activation) of the gluconeogenic enzymes pyruvate carboxylase (E.C.6.4.1.1, PC) and PEPck, and induction (release) of TTA

FFA and/or acetyl CoA may, therefore, be involved with an increase in the level of TTA and PEPck at birth when the external glucose source for the fetus is terminated.

Lastly, it was possible to release TTA from the microsomal cell fraction of human and rat fetal liver with dcAMP, acetyl CoA, and oleic acid. This released enzyme was approximately 10% of the quantity normally found in human liver cytosol and approximately 20% of that found in rat liver cytosol. The suggestion is made that a reserve of TTA is available for rapid release; this is later complemented by de novo enzyme synthesis. This process may be very important at birth when a rapid dramatic increase in the level of TTA occurs.

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Nadˇa Hahnová: Pˇredpokládám ˇze nové kulturní perspektivy, které jsem u Vás poznal, budu pamatovat déle nežˇ metody analyzy enzymů. Dˇekuji Vám za Vaší pomoc, výuku a pˇrátelství.

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ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
cAMP	cyclic adenosine 3', 5'-monophosphate
CoA	coenzyme A
dcAMP	N^6,O^2-dibutyryl adenosine 3', 5'-monophosphate
EBSS	Earle's balanced salt solution
EDTA	ethylenediaminetetraacetate
FFA	free fatty acid(s)
Fl,6DP	fructose-1,6-diphosphate
Fl,6DPase	fructose-1,6-diphosphatase
F6P	fructose-6-phosphate
G1P	glucose-1-phosphate
G6P	glucose-6-phosphate
G6PD	glucose-6-phosphate dehydrogenase
G6Pase	glucose-6-phosphatase
GSH	glutathione
HBSS	Hanks' balanced salt solution
IDP	inosine 5'-diphosphate
ITP	inosine 5'-triphosphate
LDH	lactate dehydrogenase
LHBSS	Lorne's Hanks' balanced salt solution
MDH	malate dehydrogenase
MEM	Eagle's minimum essential medium
mRNA	messenger ribonucleic acid
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate

ABBREVIATIONS (continued)

NADPH	reduced nicotinamide adenine dinucleotide phosphate
OAA	oxaloacetate
PC	pyruvate carboxylase
PEP	phosphoenolpyruvate
PEPck	phosphoenolpyruvate carboxykinase
PK	pyruvate kinase
PLP	pyridoxal-5-phosphate
POPOP	bis(O-methylstyryl)-benzene
POP	2,5-diphenyloxazole
6PGD	6-phosphogluconate dehydrogenase
TCA	trichloroacetic acid
TEA	triethanolamine
TG	triglyceride
Tris	tris(hydroxymethyl)aminomethane
TTA	tyrosine transaminase
UDPG	uridine diphosphate glucose

INTRODUCTION

The main source of energy for the human and rat during fetal life is considered to be glucose received via the placenta from the mother's circulation (1). Glucose molecules, unlike proteins and triglycerides, readily cross the placental barrier and changes in the mother's blood glucose level are reflected in the fetal level (1). The fetus can, however, regulate its own blood glucose level at least to some degree, in that a decrease in the maternal glucose supply is often not reflected to the same degree in the fetus (2). At term, the maternal source of nutrient is suddenly terminated and the newborn must rely, at least for the first few hours, on its own endogenous reserves. Later, even when an external source of nutrient is available, in the form of the mother's milk which has a relatively high fat content and little carbohydrate, the neonate must adopt a new system of food processing to ensure its supply of utilisable energy.

A source of glucose is mandatory for both the human and rat (1,3). The red blood cells appear to have an absolute requirement for glucose as does the brain (3,4). Some researchers, however, question the need of the brain for glucose since it has been found that this organ is able to metabolise ketone bodies to a large degree if glucose is not available (3,5).

In the newborn and the adult, the sugar supply need not be exogenous since two main mechanisms are available for glucose production. First, glycogen is readily convertible to glucose. Glycogen is probably the initial source of glucose after birth (6). Second, metabolic mechanisms exist whereby the carbon skeletons of many compounds including amino acids, lactate, and glycerol can be used to build glucose molecules (see Figure 1). This process, termed gluconeogenesis, was mainly elucidated by Krebs and his coworkers (7). The main gluconeogenic organs in the mammal are the liver and kidneys (3,8).

The glycogenolytic and gluconeogenic processes either do not function or function only at an extremely low level in the fetus. A normal characteristic of birth is the dramatic increase in these processes. This is related to rapid changes in enzyme activities at term (1,6,9).

Many enzymes, e.g. PEPck*, barely detectable in the fetus may increase up to 20 fold at term (see Figure 2). Other enzymes, e.g. PK and G6PD, decrease in activity after delivery. An increase in the blood level of FFA and a decrease in glucose content accompany these changes. This is illustrated for the rat and human in Figures 3 and 4. It has been reported that the blood levels of epinephrine, cortisone, and glucagon increase at term; considerable evidence has also been accumulated indicating that these hormones are involved in the sudden appearance of some enzyme systems at this time (9).

*See the list of abbreviations page xiv for this and all subsequent abbreviated forms.

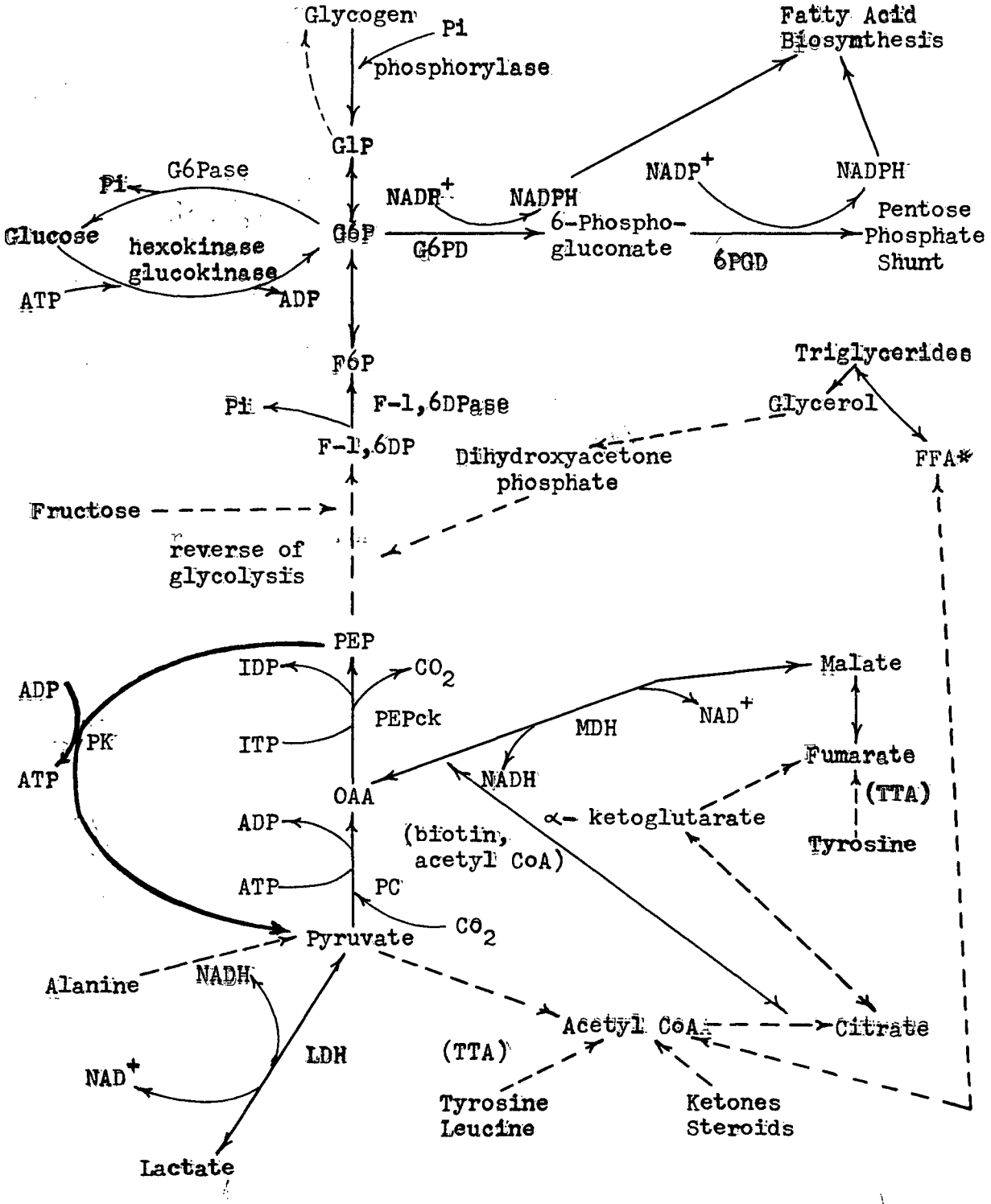


FIGURE 1: Some important biochemical pathways discussed in this thesis. Compiled from information in Harper (95). Broken lines (---) indicate reactions not shown.

* Carbon skeletons of FFA do not enter into glucose formation.

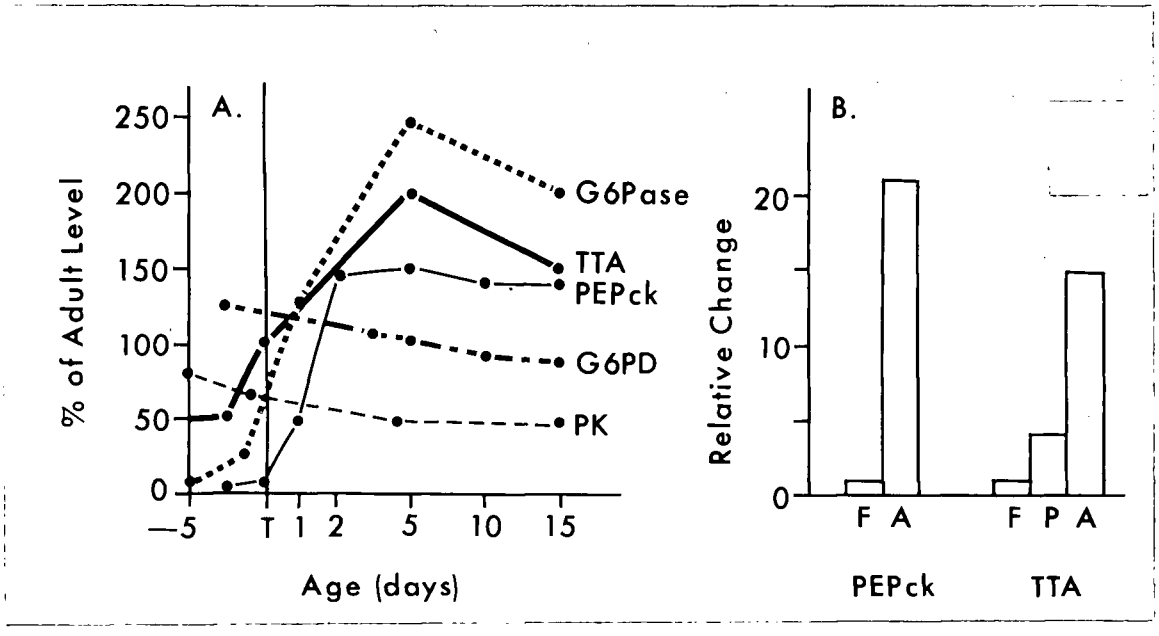


FIGURE 2: A. Change in the level of some rat liver cytosol enzymes with age. Compiled from Philippidis et al. (12), Walker (13), Stave (14), and this thesis study. T, term.

B. Change in the level of human liver cytosol PEPck and TTA with age. Compiled from Kretchmer et al. (15), Diesterhaft et al. (16), and this thesis study. Ordinate: Arbitrary units. F,fetus; P, postnatal; A, adult.

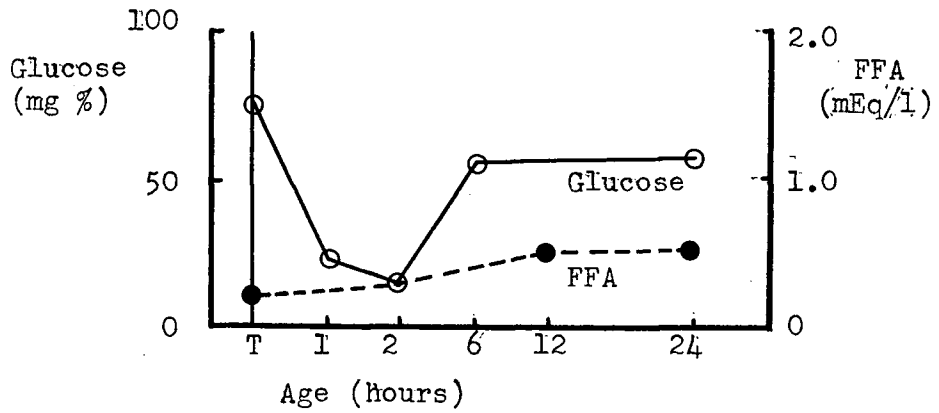


FIGURE 3: Change in the level of blood glucose and FFA with age in the rat. The glucose curve is modified from Cake et al. (6) and the FFA curve from Hahn and Koldovsky (5). T, term.

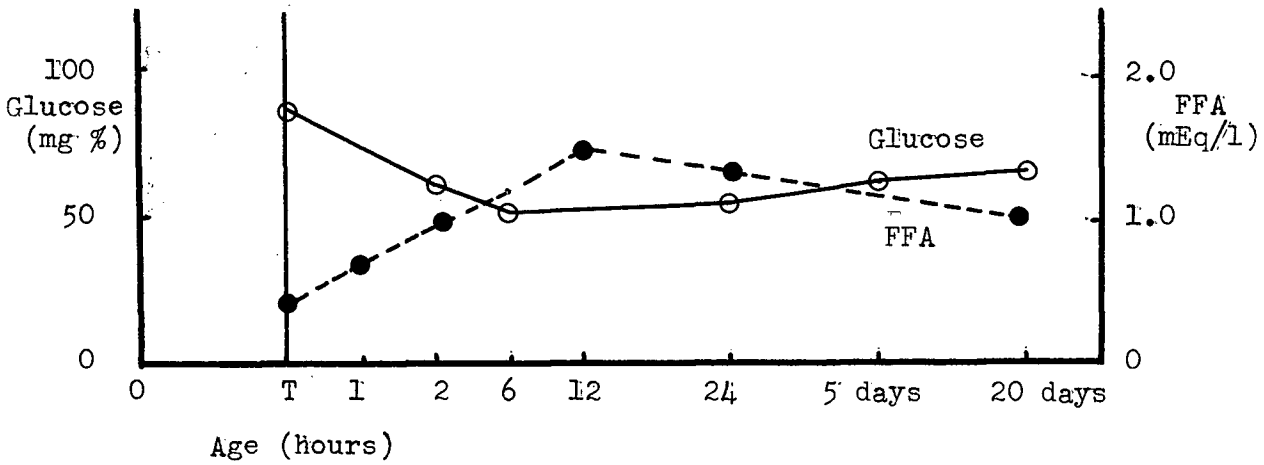


FIGURE 4: Change in the level of blood glucose and FFA with age in the human. The glucose curve is modified from Hahn and Koldovsky (5), and the FFA curve from Mignant (17). T, term.

Mechanisms Underlying the Changed Metabolic State of the Newborn

The question arises as to what causes the increase in blood FFA level, glycogenolysis, and gluconeogenesis at birth. Both glucagon and epinephrine activate glycogenolysis and lipolysis; glucagon also affects gluconeogenesis (10). The actions of these hormones appear to be mediated by cAMP in many instances. Depending on the system being studied, enzyme activation, release, de novo synthesis, and/or reduced degradation seem to be involved. The phosphorylase system in glycogenolysis in skeletal muscle is an example of enzyme activation. cAMP is intimately involved in this proess as can be seen in Figure 5.

De novo enzyme synthesis, also involving cAMP, has been intensively studied in the lac operon in E. coli. The main enzyme involved is β-galactosidase which catalyses the conversion of lactose to glucose plus galactose. The operon consists of 3 structural genes, viz. z, y, and a (z codes for β-galactosidase synthesis); and, 3 regulatory genes, viz. i, p, and o. The i gene codes for a repressor protein which binds to the operator gene o ; when the repressor is active, i.e. it is attached at o, then transcription of lac mRNA is prevented. The promotor site (p) is thought to be the binding site for RNA polymerase to the DNA and thus the starting point for the synthesis of lac mRNA. The rate at which lac mRNA is transcribed resides at least partly in the promotor site since this would determine the rate at which the polymerase attaches to the DNA (18,19).

The action of cAMP is thought to be as follows: A protein has been isolated and named CR protein (cAMP receptor protein); cAMP can reversibly bind to this unit. When cAMP is available,

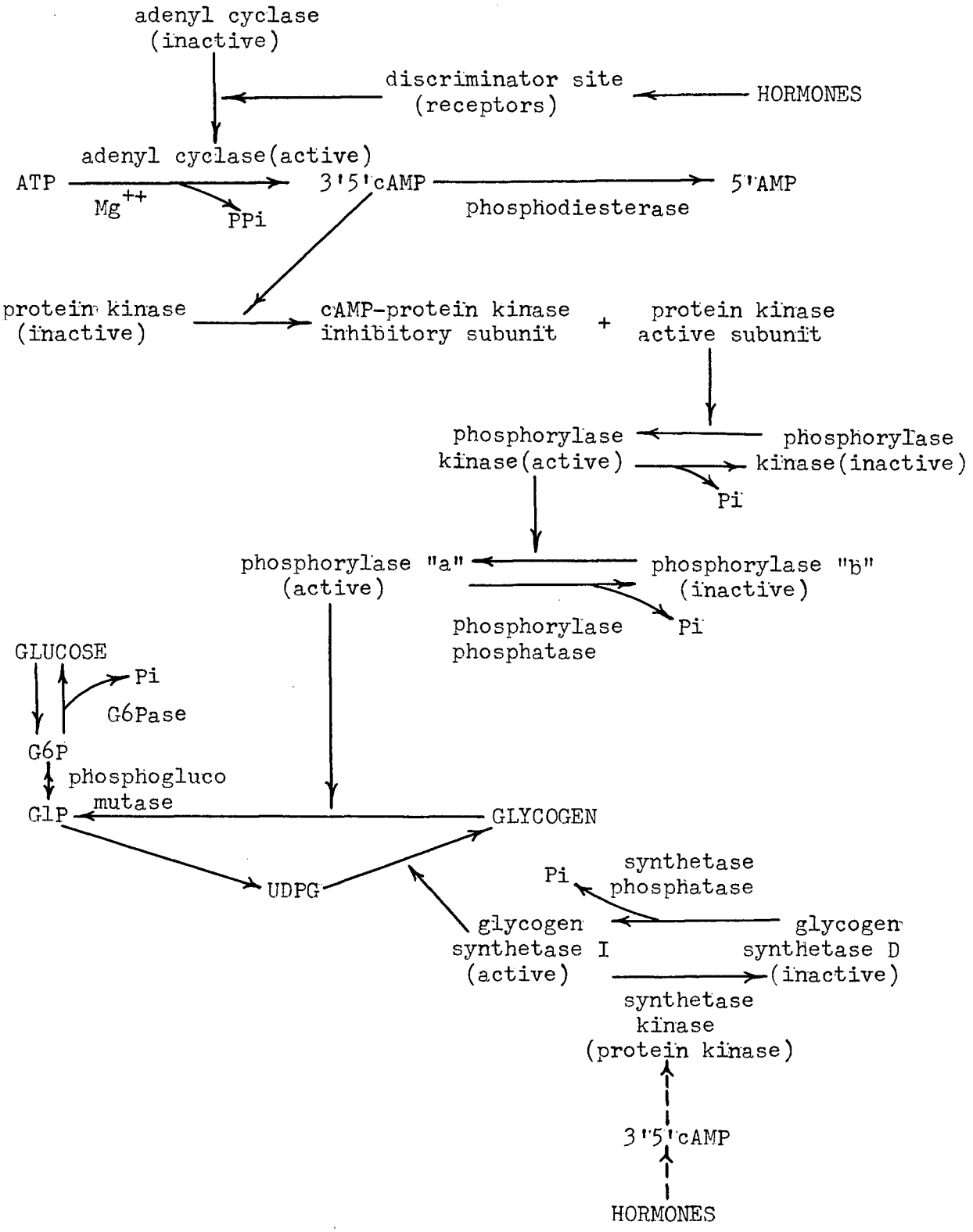


FIGURE 5: Proposed mechanism of action of cAMP in glycogenolysis in skeletal muscle. Compiled from Exton et al. (22), Robison et al. (10), and Drummond (23).

it will bind to the CR protein and cause an increased rate of lac mRNA transcription. This occurs in the absence of glucose in the cell, i.e. with low glucose there is a relatively high cAMP level. The evidence for this comes from both cellular (20) and cell-free systems (21). One thought is that cAMP stimulates the synthesis of lac mRNA by increasing the frequency of initiation of the lac mRNA chains; thus, it acts with CR protein at the p site. Part of the above information is shown diagramatically in Figure 6.

The action of cAMP in the production of tryptophanase in E. coli appears to be different from that of β-galactosidase. It has been suggested that the site of action is at the translation level thus increasing the rate of polypeptide chain elongation; the mechanism is not understood (20).

As a generalisation, de novo synthesis must occur in all organisms as they develop. The synthesis of new protein can be determined by the use of inhibitors, e.g. actinomycin D or cycloheximide, and immunochemical-isotopic techniques. A problem, however, still exists in determining where synthesis is initiated, i.e. whether transcription or translation is enhanced. Preformed mRNA templates may exist and their life span may be many weeks as indicated by results from studies with enucleate Acetabularia (a unicellular alga) (24).

The mechanism of action of cAMP in the activation and/or induction of many hepatic enzymes, e.g. TTA, PEPck, and G6Pase, is poorly understood. Perhaps a few words should be stated regarding induction. In this thesis study we usually did not know

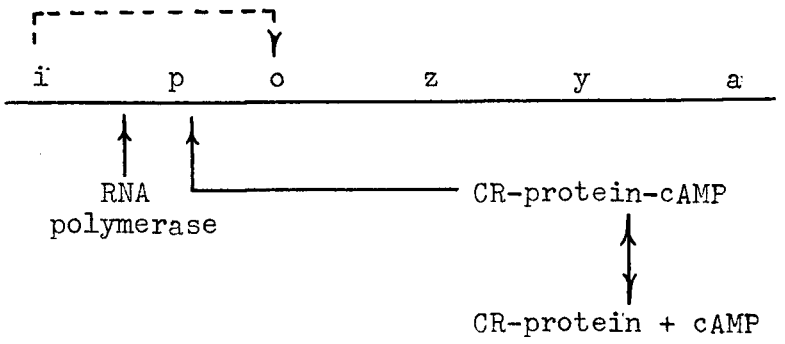


FIGURE 6: Proposed mechanism of action of cAMP on the lac operon. Compiled from Pastan and Perlman (21) and DeCrombrugghe (20). See the text for a detailed explanation.

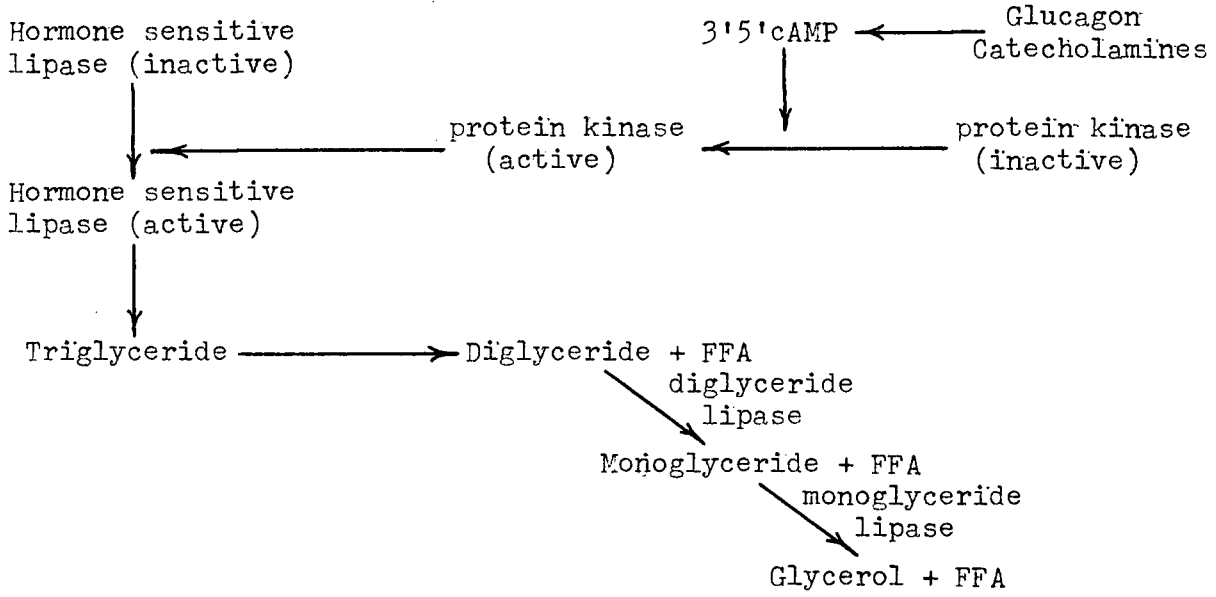


FIGURE 7: Proposed mechanism of action of cAMP in the release of FFA from adipose tissue. Compiled from Robison et al. (10) and Huttunen and Steinberg (28).

if there was de novo enzyme synthesis, reduced enzyme degradation, or if the enzyme was preformed (or at least partly so) and simply required some type of stimulation to become active. Strictly speaking, the term induction, applies to the stimulation of the synthesis of a given enzyme in response to a specific inducer (25). This presumably is to be interpreted as synthesis starting with the production of mRNA templates. The term is, in reality, used rather loosely by many workers to describe an increase in assayable enzyme which may have been caused by known or unknown factors. Perhaps the problem has developed since another suitable word has not been coined. When reading this thesis one should beware of the word induction and not interpret its meaning too rigidly.

The processes as outlined for phosphorylase and β-galactosidase may be involved in hepatic induction of some enzymes. Langan (26) has suggested that the phosphorylation of histones may be the key, since cAMP stimulates this. He found that glucagon stimulated phosphorylation by a factor of 25 in the liver of rats injected with this hormone. Hydrocortisone, however, had no influence (27). The thought is that the phosphorylated proteins are less effective repressors of DNA activity.

The question of enzyme release is a little more difficult; however, there are definite indications that this does occur at least in mammalian systems. Work by Chuah and Oliver (29) and experiments undertaken in this laboratory with TTA and the microsome-polysome cell fraction tend to point very definitely to enzyme release. This will be discussed in detail later in the thesis.

Lastly, a reduced rate of protein degradation will result in increased enzyme levels relative to controls where normal enzyme turnover is occurring. This has been demonstrated in the "superinduction" phenomenon with TTA when high levels (5 µg per ml) of actionmycin D were used in cell cultures (30).

Glucagon Action

The action of glucagon, a polypeptide hormone produced in the alpha cells of the islets of Langerhans in the pancreas, is known to be mediated by cAMP in many instances (10,31). The influence of glucagon on the liver is dramatic in this respect. It stimulates glycogenolysis, gluconeogenesis, and there is evidence that it stimulates lipolysis (10). The mechanism of action of glucagon in the activation of adenyl cyclase in glycogenolysis was outlined in Figure 5. The mode of action in gluconeogenesis is unknown.

A number of studies have been undertaken concerning the action of glucagon on the conversion of inactive hormone sensitive lipase (TG lipase) to the active form in adipose tissue. It appears that there is a protein kinase activation similar to that in skeletal muscle, which is mediated by cAMP (28). Figure 7 outlines the mechanism favored. The FFA undergo β-oxidation in the mitochondria thus producing mainly acetyl CoA and energy (see Figure 8). The acetyl CoA, as well as entering the citric acid cycle, activates the pyruvate carboxylase reaction in gluconeogenesis (32).

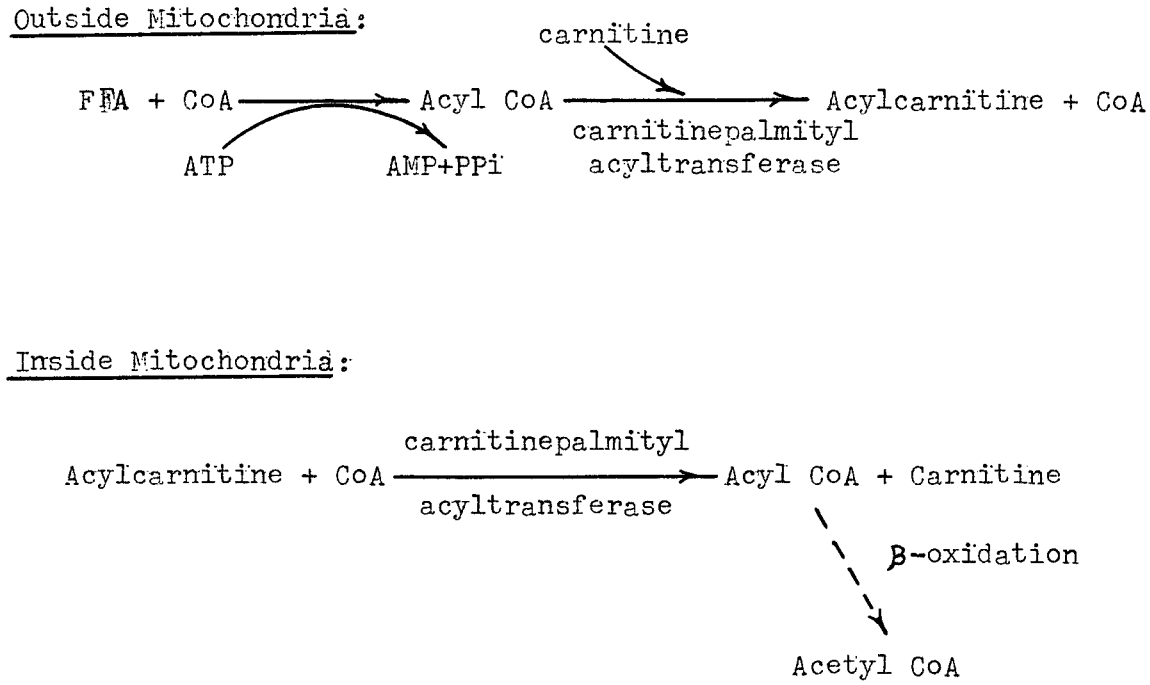


FIGURE 8: Proposed mechanism of action in the transfer of FFA into mitochondria. Modified from Harper (11). The broken line indicates reactions not shown.

Enzyme Induction In Liver

Rat material has been used in most of the studies with liver tissue; therefore, the following review will be concerned almost exclusively with this animal. The main enzymes to be discussed are TTA and PEPck.

It has been fairly well established that hepatic TTA activity can be increased by glucocorticoids, catecholamines, glucagon, and cAMP. (Note that dcAMP is often used in place of cAMP in experiments since it penetrates cell membranes more efficiently and is not degraded by phosphodiesterase (10). PEPck activity can be increased by catecholamines, glucagon, and cAMP; it is, however, only slightly affected by glucocorticoids and is repressed by insulin (33). It has been concluded, from work with enzyme inhibitors and immunochemical-isotopic analyses, that all of the inducing agents stimulate de novo synthesis of TTA. The same is suggested for PEPck; however, the detailed immunochemical techniques have not been used with this enzyme except in the natural increase at birth where Philippidis et al. (12) recently reported that there was extensive new synthesis. These authors also concluded that there was reduced degradation at the time of birth.

Sereni and Sereni (34) have reported that there is spontaneous induction of TTA in fetal rat liver cultures. This increase may be up to 5 times the basal level during a 72 hour culture period. The authors found that actinomycin D, at 2 µg per ml, would inhibit the induction by about 75% from 12 hours to 72 hours of culture and by only 35% from the start of the culture to 48 hours.

They concluded that their results were consistent with the hypothesis that synthesis of TTA is repressed during gestation.

Many exceptions to the above generalisations have been reported depending on the stage of development of the liver and the conditions under which the experiments were undertaken. Greengard (35) has found that epinephrine and glucagon injected into fetal rats are ineffective in inducing TTA until 2 days before term; however, dcAMP will induce this enzyme 4 days prior to term. Sereni et al. (36) indicate that hydrocortisone will not cause an increase in the level of TTA in the fetus in vivo; whereas, it will in the adult. In adrenalectomised adults the influence of hydrocortisone on TTA induction is greater after starvation or an injection of glucagon. Wicks (37) has induced TTA with hydrocortisone in tissue cultures of fetal livers.

Philippidis and Ballard (38) reported that in liver slices in vitro, glucagon stimulated the production of PEPck (about 10 fold) and increased the incorporation of pyruvate (C^{14}) into glycogen. Injection of fetuses with glucagon has the same effect; however, there is no detectable incorporation of pyruvate into glycogen.

A number of interesting studies have been undertaken by Wicks and his coworkers concerning the induction of TTA by cAMP and cortisol in fetal liver tissue cultures (39). If cAMP is added together with actinomycin D to a culture, there is enzyme induction for about 2 hours; thereafter, the enzyme level starts to decrease. If cortisol is added together with actinomycin D there is no induction. With cortisone there is a distinctive time course

of action in that there is a time lag in the induction of TTA relative to induction by cAMP or glucagon (40). After TTA has been induced with cAMP, removal of the nucleotide leads to an immediate decrease in enzyme level. However, after TTA has been induced by cortisol and if the steroid is then removed there is a time lag of about 2 hours before the enzyme begins to decrease. The same type of experiment with PEPck and cAMP gave rise to similar results but the enzyme decrease was not as rapid. If cAMP and cortisol are combined, the resultant TTA increase is greater than additive. It is also known that glucagon is synergistic with cortisol and also with insulin. Insulin and cAMP have less than an additive effect (38). Wicks has formulated his results into the following model: Cortisol increases the number of TTA templates and thus acts at the level of transcription; whereas, cAMP (formed by the action of glucagon or catecholamines on adenyl cyclase) acts at the translation level. The synthesis of this enzyme is, therefore, regulated by two distinct but sequential steps (33).

The TTA mRNA template half life is 1 to 2 hours; thus, cAMP is able to stimulate pre-existing template even if actinomycin D is present (33). The PEPck mRNA template half life according to Wicks is about 5 hours; this would account for the more gradual decrease in enzyme level after the removal of cAMP from the culture medium. The half life of TTA is approximately 4 hours (37) and that of PEPck approximately 13 hours (12).

Cycloheximide, an inhibitor of protein synthesis at the translation level, is effective in blocking to a large degree the effects of the inducing agents previously noted (40,41).

The action of this antibiotic is almost immediate.

Chuah and Oliver (29) have used an approach involving the isolation of the cell microsomal fraction from livers of 1 and 2 day old rats. They have succeeded in stimulating the release of TTA from this cell fraction with cAMP and, indeed, from the polysomes bound to it. This 3 fold increase in enzyme was not affected by cycloheximide. Three factors in these experiments should be noted: Firstly, the authors had no success with fetal or adult animals; secondly, only neonates in which TTA could be induced with epinephrine would respond; and thirdly, they had no success in releasing PEPck in the 3 to 6 hour old rats which were tested. The authors conclude that cAMP is involved in the release of TTA from "preloaded" polysomes. They indicate that it is also possible that cAMP activates the enzyme; however, release is still involved.

A further complication, and indeed a controversy, has now developed with the apparent confirmation by Iwasaki and Pitot (42) that TTA has 4 isozymic forms. Form I is ubiquitous; whereas, forms II, III, and IV are found only in the liver. Only form I is present in fetal liver. Forms II and III appear on the first day of birth and form IV at the time of weaning. At birth, the ratio of I:II:III:IV is 2:9:1:0; whereas, at 28 days of age it is approximately 1.5:1:2.5:1. The total enzyme increased by a factor of 4 from the fetal to the adult stage; the quantity of form I remained relatively constant. Cortisone, glucagon, cAMP, and epinephrine appear to induce form II in the adrenalectomised adults tested. Wicks (33) after having studied several parameters such as heat stability, Km's, immunological reactivity,

and migration of enzyme during polyacryamide gel electrophoresis has found no evidence that TTA exists in isozymic forms - thus the controversy.

The process of birth appears to be the trigger for the change in the level of many enzymes. TTA, PEPck, and G6Pase are examples of enzymes evoked by premature delivery. If the gestational period is prolonged, these enzymes will remain at their reduced levels until birth (9,43,44). A postnatal injection of glucose will inhibit the increase in the level of these enzymes (35,43,45); an injection of insulin will largely inhibit the increase in the level of PEPck (46). Glucagon injected with the glucose will nullify the action of glucose on PEPck according to Cake et al. (6). Actinomycin D injected at birth will inhibit the action of glucagon or cAMP given at the same time and will inhibit the normal increase in the level of TTA (35). Cake et al. (6) reported that actinomycin D allowed only about a 33% increase in the PEPck level relative to the control at birth; the same result is found even if glucagon is injected with the antibiotic. These authors also suggest that actinomycin D does not affect liver phosphorylase since the glycogen level continues to decrease as in normal rats. The glycogen reserves in the fetal rat liver at term are more than twice as great as those found at any future time. Even with this supply, hypoglycemia occurs for at least a 5 hour period after birth. If the normal increase in PEPck is blocked at this time, then hypoglycemia remains (6). It is known that both fetal decapitation and maternal adrenalectomy are required to prevent fetal glycogen formation (9); premature

glycogen formation can be stimulated by hydrocortisone (9).

Greengard (9) reports that the ability of the fetus to secrete glucagon and epinephrine is present about one day before birth. Also, there is over a 3 fold increase in the blood level of corticosterone within the first 5 hours after birth (43). The above information has resulted in the formation of the hypothesis that hypoglycemia and, therefore, the secretion of hormones may be key factors in the rapid production of some enzymes at term (9). Hydrocortisone, even though it has been found to be ineffective in the stimulation of enzyme production in the fetus, is thought to be necessary for the inductive events at birth since adrenalectomy prevents the postnatal increase in TTA (36).

Exton and his coworkers (22,31) hypothesise that the hepatic actions of glucagon and epinephrine are mediated by an increase in the tissue level of active cAMP. A large portion of the cAMP would be inactive under basal conditions due perhaps to binding. In perfused adult livers, glucagon caused a 60 fold increase in cAMP content within 4 minutes; epinephrine leads to a doubling in the same period. (The rats had been fed ad libitum). The same level of glucose production was achieved with either agent, thus leading the authors to conclude that only a slight increase in cAMP is in fact required for enzyme activation. The authors note that insulin inhibits the action of glucagon on glycogenolysis, gluconeogenesis, glycogen synthesis, and ketogenesis; they hypothesise that this is due to a reduction in the content of active cAMP. The reduction could be a result of adenyl cyclase inhibition, activation of phosphodiesterase, or

some other means (31). This system may be very efficient at birth when, during hypoglycemia, glucagon is secreted and insulin secretion is suppressed. During a period of hyperglycemia, insulin secretion is stimulated and glucagon secretion suppressed; thus the body fuels, viz. fats, carbohydrates, and proteins are controlled. If there are low insulin levels, for example as in diabetes, then both the glucose and FFA blood levels are elevated. PEPck levels are also relatively high in those with diabetes (47).

A controversy has developed over the past 5 years as to the involvement of FFA in the control of gluconeogenesis. Exton et al. (48), in agreement with Williamson et al. (49) and Söling et al. (50), have reported that high concentrations (4 μmoles per minute) of unbound oleate stimulate gluconeogenesis from lactate or pyruvate by 30 to 100%. All of these workers used perfused adult rat livers. Exton and his coworkers, however, question the physiological significance of the results since in vivo fatty acids are tightly bound to albumin and are at lower concentrations than those used in the experiments. Glucagon in these experiments approximately doubled the rate of gluconeogenesis; when infused together with oleate an additive affect was noted. Carnitine, with or without fatty acid, had no significant influence. In Exton's experiments, oleate bound to albumin had no influence on the glucose level when lactate (C^{14}) was used in the perfusing medium. As was noted previously, the unbound form of oleate was effective in stimulating gluconeogenesis and in stimulating ketogenesis 5 fold.

Williamson et al.(49) hypothesise that the stimulation of gluconeogenesis by glucagon is mediated by an increased availability

and oxidation of FFA. They believe that it is not the FFA as such which are important but probably one of the oxidation products, viz. acetyl CoA. Acetyl CoA is a factor required in the PC reaction. These authors stress, however, that a number of other enzyme steps must be controlled by unknown mechanisms. Williamson et al. (49,51) also used (+) decanoylcarnitine, an inhibitor of carnitine dependent FFA oxidation. The effects of glucagon on gluconeogenesis are inhibited about 50% by this agent. There are reports of in vivo experiments with rats and dogs where FFA were injected into the animals and changes in glucose levels measured (48,52,53). The results of these experiments are conflicting; however, a decrease in glucose is the general observation.

Weber et al. (54,55) using an adult rat liver cell-free system have found that FFA, alanine, and acetyl CoA inhibit PK. The inhibition of this enzyme appears to be important if gluconeogenesis is to proceed since the activity ratio of PEPck plus PC to PK is approximately .04 (55). This indicates that the process of glycolysis is much more pronounced than gluconeogenesis under normal conditions. Weber and his coworkers also observed that other glycolytic enzymes were inhibited by acetyl CoA and FFA.

According to Hahn and Koldovsky (5) fatty acid oxidation in fetal human liver does exist, but the rate is low. In the rat, there is negligible fatty acid oxidation in fetal liver; however, the rate increases many fold within a few hours after birth when an increase in the blood level of FFA occurs. Augenfeld and

Fritz (56) reported that the level of acylcarnitine transferase, an enzyme involved in fatty acid oxidation, is low in fetal rat liver but increases rapidly at birth.

There is little doubt that the main control of gluconeogenesis involves the PC or the PEPck reaction. Exton and Park (57) have reported, for adult perfused rat liver, that glucagon, catecholamines, and cAMP all stimulate gluconeogenesis from pyruvate or lactate but not from fructose or dihydroxyacetone. Both PC and PEPck levels increase rapidly at birth, and if blocked hypoglycemia persists (6). Remembering that PEPck, at least in the postnatal rat, is mainly in the cytosol; whereas, PC is mainly in the mitochondria (58,59), the following possibilities according to Exton et al. (60) may be considered regarding the mechanism of action of hormones such as glucagon on gluconeogenesis: a) activation or de novo synthesis of PC, b) activation or de novo synthesis of PEPck, c) stimulation of pyruvate uptake by the mitochondria, and d) stimulation of precursor efflux from the mitochondria. None of these reactions are affected in cell-free systems by any of the known inducing agents; however, as noted by Exton et al. (60) the action may be indirect as with phosphorylase.

Glucose

If glucose is injected into rats at term, the normal increase in FFA content in the blood and the increase in enzyme levels, as previously noted, are prevented. Intravenous infusions of glucose given to newborn infants prevent the FFA increase (1,5).

Other interesting experiments have also been undertaken, both *in vivo* and *in vitro*, to investigate glucose and enzyme induction. In rats, an intragastric administration of glucose given simultaneously with an injection of glucagon, inhibited the induction of TTA by 60% compared with controls given only glucagon. The rats had been maintained on a protein free diet for 5 days and had been fasted overnight. Glucose had no influence on the increase in the liver content of cAMP. This nucleotide increased approximately 100 fold within 20 minutes of the glucagon injection, and the TTA level increased about 8 fold within 4 hours. The authors concluded that glucose repression in mammalian liver appears to be independent of the cAMP level (61). This is in contrast to catabolite repression in microorganisms such as *E. coli* where increased glucose causes a decrease in cAMP and reduced β-galactosidase formation (20).

A report on experiments with rat hepatoma cells (Reuber H-35) in culture indicates that TTA is almost doubled in medium lacking D-glucose but with an excess of L-tyrosine in contrast to medium with normal glucose and excess L-tyrosine. When dcAMP was added to the former system, the TTA level did not change; however, when it was added to the latter system the enzyme doubled. If D-glucose is replaced by the L form, then the system responds as if no glucose had been added, i.e. TTA doubles. Cortisol had almost the same influence as dcAMP; these agents were additive. Grossman *et al.* (62) reported that both cycloheximide and actinomycin D would prevent the TTA increase in the glucose plus dcAMP experiment. The authors conclude that a deficiency of

D-glucose induces TTA in rat hepatoma cells in a similar way to β-galactosidase induction in E. coli (62,63).

G6PD

Since a number of G6PD assays were undertaken on tissues studied in this thesis, some comments regarding this enzyme are required. In embryonic tissues, it is generally assumed that the pentose phosphate pathway is more important in glucose metabolism than it is in the adult. This pathway is important for the synthesis of ribose precursors of nucleic acids and for NADPH production required for lipogenesis (64). Rudak et al. (65) found recently that dcAMP and glucagon prevent the induction of rat liver G6PD which occurs when rats are fed a high carbohydrate diet.

Cell Cultures

This section outlines the main findings to the present time regarding TTA induction in cell cultures.

Butcher et al. (66) reported that dcAMP approximately doubled the TTA level in Reuber (H-35) hepatoma cells. Insulin increased the level by almost 75%. Cycloheximide prevented the enzyme increase with dcAMP; no results were given for insulin. Actinomycin D at 0.2 µg per ml reduced the induction by dcAMP by 50%; however, at 5 µg per ml actinomycin D caused TTA to increase by approximately 50% in 6 hours and increased the induction by dcAMP by about 50%. The induction by insulin was not affected with 0.2 µg per ml of actinomycin D. The authors concluded that the inducers act at a post-transcriptional event (66). dcAMP appears to have no influence on TTA in the hepatoma

cell line designated HTC (67); however, insulin (68) as well as hydrocortisone (69) will induce an increase in the level of TTA in these cells. No induction of TTA could be obtained with glucagon in the H-35 line; the cAMP level was also not affected by the addition of glucagon. Lee and Kenny (30) found that hydrocortisone elevated the level of TTA by at least a factor of 4 in the H-35 line. From their work with actinomycin D, they concluded that the action of the steroid is at the transcription level thus resulting in increased mRNA. These authors reported that there was inhibition of TTA induction with 0.2 µg per ml of actinomycin D; however, at 5 µg per ml there was an increase in the enzyme level. This "superinduction" was, in fact, due to reduced enzyme degradation as determined by immunochemical-isotopic techniques. Tomkins et al. (69) from studies with HTC cells had concluded that the increase, when 5 µg per ml of actinomycin D were added to the cultures, was due to the blocking of a repressor that limits synthesis at the translation level. They hypothesised that mammalian gene expression may be controlled by repressor elements at the translation level of mRNA. Lee and Kenny stress that agents such as glucagon, epinephrine, and insulin, which certainly appear to act via cAMP but have a mechanism of action different from hydrocortisone, may exert their influence at the translation level (30). These authors (70) also found that L-leucine, but not the D form, increased TTA 8 fold; approximately half of this was due to reduced enzyme degradation. L-leucine with insulin or hydrocortisone gave additive results thus implying that these agents act by different mechanisms.

An experiment was undertaken by Thompson and Gelehrter (71) in which they fused two cell lines, viz. HTC which is TTA inducible and BRL-62 which has little or no TTA activity and cannot be induced by steroids. The authors reported that the resulting heterokaryons after 24 hours of fusion had no detectable TTA activity and they were unable to induce the enzyme. The authors also reported that the heterokaryons were as viable as the individual cells and that there was no loss of chromosomes.

Human Liver Enzyme Induction

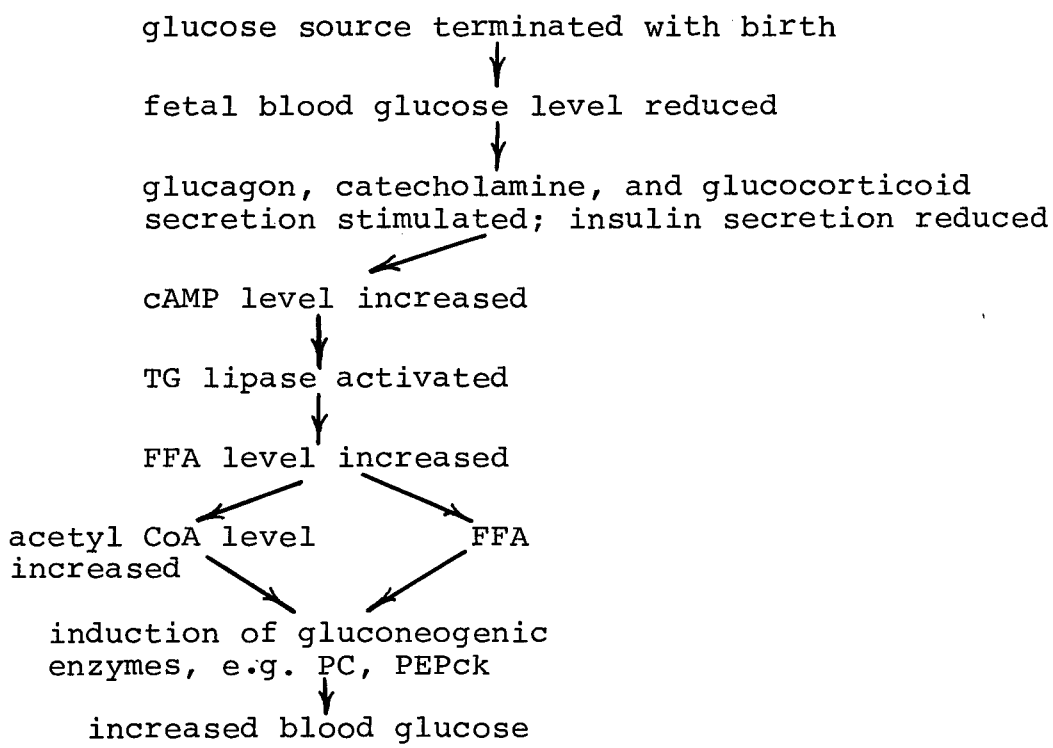
Very little is known concerning enzyme induction in human liver (1,72). Räihä et al. (72) have undertaken a limited number of experiments with fetal liver in culture with the objective of studying TTA induction. They were unable to induce this enzyme, in livers from fetuses of 14 to 24 weeks gestational age, with any of the agents tested, viz. hydrocortisone, triamcinolone, insulin, glucagon, cAMP, and dcAMP. They did, however, obtain a 4 fold enzyme increase using triamcinolone in a 28 week fetus. Villee et al. (73) reported that in liver slices, from approximately 18 week old fetuses, there was a 4 fold increase in glycolysis and a 10 fold decrease in lipogenesis under anaerobic compared to aerobic conditions. Hahn and Vavrouskova (74) reported that human fetal liver is capable of oxidising fatty acids as early as the tenth week of gestation; however, the ability is low compared with the neonate. Hahn (75) also has determined that the activity of acylcarnitine transferase, an enzyme involved with FFA oxidation, can be measured in fetuses 10 to 20 weeks gestational age. The findings concerning FFA oxidation are important when one considers the induction of an

enzyme such as PEPck by products of the oxidation.

Objectives

The objectives of this thesis study were three fold. First, we wished to determine if FFA or their oxidation products are involved in the induction of enzymes, mainly TTA and PEPck, in fetal liver. Second, we wished to determine if it is possible to induce enzymes in fetal human liver as it is in rat tissue. Third, we wished to collect as much basic enzyme data as possible on the changes in enzyme levels in the human fetal liver and kidney during gestation.

The rationale of the FFA hypothesis is outlined as follows:



Reasons For The Study

The reasons for this study are briefly summarised as follows:

1) The mechanisms by which hormones (and birth) stimulate enzymes, such as PEPck and TTA, to increase in level are not known.
2) Almost nothing is known concerning the induction of enzymes in fetal human liver.
3) Very little is known about changes in the levels of enzymes in the human liver and kidney during gestation.

It is important to have some knowledge of the development of the enzymes of the gluconeogenic pathway for example, since a defect in these could be important in pathological forms of hypoglycemia in the neonate. The importance of the ability to artificially induce enzymes is, therefore, seen in that metabolic adjustments could then be facilitated. As a specific example, one can consider infants who have recurrent hypoglycemia. Hypoglycemia is usually treated by giving glucose either orally or intravenously. There is a problem with this, however, as has been demonstrated in the newborn rat. An injection of glucose will alleviate hypoglycemia; however, it also suppresses the normal induction of PEPck together with a number of other enzymes (6). Gluconeogenesis is, therefore, greatly reduced. With cases of recurrent hypoglycemia, perhaps the injection of an agent such as glucagon, which is known to stimulate gluconeogenesis, would at least partially overcome the problem.

One last factor should perhaps be considered in this section, viz. the caution that one must use when attempting to apply data obtained from one animal species to another species. The majority of liver enzyme induction studies have been undertaken with rat tissue; hopefully, much of the data obtained will also

apply to man. There is no way, however, that one can be sure to what degree the data are applicable unless tests are undertaken with human material. For this reason we felt that it was very important to use human tissue when possible.

Experimental Approach

A number of approaches can be used in the study of enzyme induction in liver tissue. Greengard has concentrated mainly on in vivo injection of fetuses, Wicks on fetal tissue cultures, Weber on cell free systems, and Williamson and Exton on adult liver perfusion experiments. As well as tissue cultures, cell cultures are now being extensively studied. There are advantages to each system and the specific objectives of the project as well as the facilities available must be considered before making a decision as to the method(s) to be used.

In vivo injections make it possible to study the organism as a unit; however, there is the disadvantage of all of the complexities inherent in a multicellular animal. The results observed may be far removed from the initial reaction involved.

Tissue cultures, interpreted here as meaning the incubation of pieces of tissue for no more than 48 hours in a defined medium at 37°C, enable us to maintain the integrity of at least small pieces of organ under relatively well defined conditions. The disadvantages are that the tissue is placed in a foreign environment and damage certainly occurs when preparing the tissue pieces.

Cells in culture are far removed from their original natural location. There is the advantage that it is possible

to develop relatively pure cell lines and to maintain these for many months; however, these cells may bear little relationship to the original tissue from which they were derived. Cell fibroblasts, for example, are probably not found as such in the animal; thus, care must be taken when attempting to relate results obtained with these cells to their original source (76).

Cell-free systems allow us to work, in many instances, with a chemically defined mixture. Thus, the action of individual molecules can be studied. The advantages of this are that the complexities of the cell component interactions have been eliminated. The disadvantage of this approach is the great distance it is removed from the natural environment. Another problem is the great difficulty in obtaining pure cell components which are usually required as part of the system.

Lastly, perfusion experiments have all the advantages of an intact isolated system. They have the disadvantages of being relatively short term, i.e. a few hours at the most, and are relatively difficult to adapt to small friable organs such as fetal rat liver.

<u>In vitro</u> systems, in the form of tissue cultures and microsomal suspensions, were used in the studies described in this thesis with the main emphasis on tissue cultures.

MATERIALS AND METHODS

Chemicals

All chemicals used in this study, except those listed in Appendix I were obtained from Fischer Scientific in Vancouver.

Tissue Preparation

Source:

The human liver and kidney samples were from fetuses of gestational age 7 to 21 weeks. Age determinations were made mainly from crown-rump measurements (see Appendix II). All were products of therapeutic abortions and had no obvious abnormalities.

Wistar rats (Rattus rattus) were used as a source of fetal and postnatal liver. The fetuses ranged in weight from 0.4 to 5.75 gm or in gestational age from minus 5 days to term (see Appendix III).

The 5 day old pig was obtained from the Faculty of Agriculture University of British Columbia and the fetal pigs from a sow slaughtered 15 minutes previously at International Packers in Vancouver.

Removal:

The liver and kidneys were removed from the human fetuses within 10 minutes after the hysterotomy. The livers from the rat fetuses were removed within 15 minutes after decapitation of the mother animal. Samples to be considered as fresh, i.e. not cultured, were immediately placed in a beaker on ice. Excess fluid was removed, the tissue weighed, and 0.15 M KCl-.001 M EDTA pH 7.4 added in the ratio 0.5 ml buffer to 100 mg of tissue.

Material to be cultured was placed directly in culture medium at 37°C. The livers from all rat fetuses of a litter were pooled. The tissue was cut into pieces approximately 1.5 mm per side in preparation for incubation.

Cell Fractionation:

The tissue fresh or cultured with buffer, was homogenised in a glass homogeniser with a teflon pestle for 1 minute. The homogenate was transferred to centrifuge tubes. All materials were kept on ice.

a) Materials for TTA, PEPck, PK, and G6PD assays - The homogenate was centrifuged at 100,000 x g for 40 minutes in an ICE centrifuge at 1 to 4°C. The supernatant was decanted, placed in test tubes, frozen, and normally used within 72 hours to determine any or all of the above enzymes.

b) Material for mitochondrial assays - The homogenate was centrifuged as in (a) above and the resulting supernatant completely removed. The pellet was again homogenised in a quantity of buffer equal to the original volume. The homogenate was centrifuged for 10 minutes at 600 x g and the pellet discarded. The supernatant was centrifuged for 15 minutes at 8,000 x g and the resulting supernatant discarded. The mitochondrial pellet was then resuspended in buffer at the ratio of 100 mg original tissue to 0.1 ml buffer. The suspension was subjected to freezing and thawing 3 times prior to centrifugation at 30,000 x g for 10 minutes. The resulting supernatant was used to determine the mitochondrial PEPck activity.

c) Material for G6Pase assay - The homogenate was centrifuged for 10 minutes at 600 x g and the supernatant decanted and stored frozen as in (a) until used.

d) Material for microsomal TTA assay - The homogenate was centrifuged for 20 minutes at 15,000 x g. The supernatant was decanted and centrifuged at 105,000 x g for 60 minutes. The resulting supernatant was completely removed and the pellet resuspended in one-fifth of the original volume of buffer. The suspension was then divided into test tubes, so that 200 λ of material would be available for each TTA assay, i.e. 100 λ for the sample and 100 λ for the control.

In Vitro Techniques

Tissue Culture:

The definition of tissue culture based on the recommendations of the Committee on Terminology, Tissue Culture Association 1967 as outlined by Priest (77) is as follows:

> "Animal tissue culture concerns the study of cells, tissues, and organs, explanted from animals and maintained or grown in vitro for more than 24 hours."

The system to be described can, therefore, be termed tissue culture.

Four different media were tested, viz. Eagle's MEM with HBSS, Eagle's MEM with EBSS, HBBS, and LHBSS. The details of these are given in Appendix IV. In all, except Eagle's MEM with EBSS, 70 mg of $NaHCO_3$, in the form of a sterile commercial solution #4103 from Abbott Laboratories, were added per 100 ml of medium. Unless indicated otherwise, Eagle's MEM + HBSS was used in the experiments. Also, 100 units of penicillin and 100 μg

of streptomycin were added per ml of medium.

Sterile disposable 250 ml tissue culture flasks #3024 from Falcon Plastics were usually used; some 30 ml flasks #3012 were also tested.

Approximately 150 mg of tissue, in the form of pieces 1.5 mm per side, were cultured in a 250 ml flask with 2.5 ml of medium. The tissue was incubated for 5 to 42 hours (usually 24 hours) at 37°C. Inducers or inhibitors were added by usually dissolving them in 25 λ of the medium used; the pH was adjusted when necessary. The inducers or inhibitors were added for the last 5 hours of incubation unless otherwise noted. Unless indicated otherwise, the following concentrations were used:

dcAMP 9×10^{-4}M

glucagon 10^{-4}M

oleic acid 10^{-4}M

dl-carnitine 5×10^{-5}M

acetyl CoA 8×10^{-4}M

acetyl carnitine 9×10^{-4}M

linolenate 10^{-4}M

*citral (Na bisulfite) 3×10^{-5}M

*deoxycarnitine 3×10^{-4}M

*cycloheximide 3×10^{-5}M

*actinomycin D 8×10^{-5}M

See Appendix V for an outline of the action of those agents marked with an asterisk (*).

All of the procedures previously described were under sterile conditions.

At the completion of the incubation period, the pH of the medium in each flask was determined. The tissue was removed, placed in a beaker on ice, and treated as described under Tissue Preparation.

Petri Dish Incubation:

This technique was used to study changes in the TTA level in fetal liver at various time intervals during a maximum of 4 hours of incubation. The tissue was cut into pieces as previously described and placed in culture medium in Petri dishes. The dishes were then placed in a water bath at 37°C. At fixed time intervals, tissue was removed from the dishes, placed in beakers on ice, and prepared for enzyme assay.

Microsomal incubation:

This technique was used for the microsomal suspensions in the determination of TTA levels. The test tubes with the suspensions were placed in a water bath at 37°C. At zero time various inducers as follows were added: dcAMP 9×10^{-4}M; oleic acid 10^{-4}M; or acetyl CoA 5×10^{-4}M. After 45 minutes the tubes were placed on ice and then frozen for at least 36 hours. TTA was determined by the normal procedure.

Liver Composition

The diverse cell population of the fetal liver and the changes which occur during development, create problems for the researcher. The concentration of hematopoietic cells in rat liver decreases from 50 to 25% during the last 5 days of gestation; the number of these cells is insignificant by 5 days after birth (78). When comparing enzyme levels at various gestational ages, and when comparing prenatal and postnatal changes, one must remember that the relative quantity of parenchymatous liver tissue varies with these different stages. The number of hepatocytes, however, remains relatively constant on a unit volume basis (79).

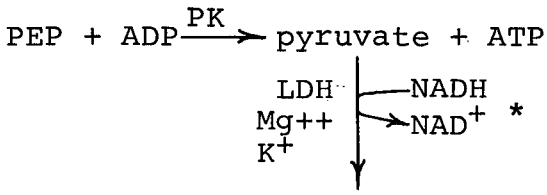
Slides and Photos

Slides were made of various samples of fetal liver to determine if any changes in histological appearance had occurred due to culturing. The tissues were fixed in either 10% formalin, with 2.0 mg calcium acetate per ml, or Bouin's fluid then processed on a Technicon tissue processor according to the method of Culling (80). Sections were cut at 5 to 7 microns and stained with hematoxylin-eosin. Permanent mounts were made.

Photos were taken on Kodak high speed Ektachrome film with a Reichert Nr.48.456 camera mounted on a Reichert Nr.322-323 microscope.

PK Assay (EC 2.7.1.40)

The assay is described by Bucher et al. (81).



*Reaction which is recorded.

Materials:

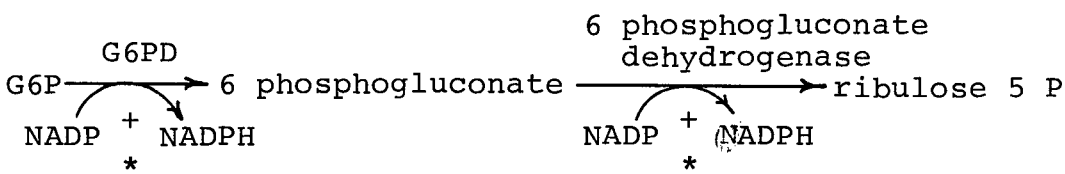
The following materials are given in mg per ml (the values in brackets are molar concentrations): TEA 93 (5×10^{-1}); pH 7.6; EDTA 20.8 (5×10^{-2}); $MgSO_4$ 19.7 (8×10^{-2}); KCl 56 (7.5×10^{-1}); ADP 5.6 (1.1×10^{-2}); NADH 1.0 (1.3×10^{-3}); PEP 2.5 (1.1×10^{-2}). The solvent is glass distilled water. TEA, EDTA, $MgSO_4$, and KCl are stock solutions stored frozen. For fresh tissue 5 λ of enzyme sample were used and for cultured tissue 10 λ. All materials are kept on ice.

Procedure:

The quantities of material are per sample. 0.1 ml of each of TEA, EDTA, $MgSO_4$, KCl, ADP, and NADH, plus 5 λ LDH was added to a cuvette. A quantity of water was added to the cuvette so that the total volume including enzyme and PEP was 1.0 ml. The enzyme was added. The cuvette was placed in the Unicam SP800 spectrophotometer at 340 mμ and 30°C; water was used as a blank. When no reaction was recorded then the reaction was started with 0.1 ml PEP. The change in absorbance was measured over a 1 minute time interval.

G6PD Assay (EC 1.1.1.49)

The assay is described by Bucher et al. (81).



*Reactions which are recorded.

It is noted that the reaction as measured accounts for both of the reactions indicated above.

Materials:

The following materials are given in mg per ml (the values in brackets are molar concentrations): TEA 93 (5×10^{-1}); pH 7.6; EDTA 20.8 (5×10^{-1}); $NADP^+$ 2.3 (3×10^{-3}); G6P 6.9 (1.8×10^{-1}). The solvent is glass distilled water. TEA and EDTA are stock solutions stored frozen. For fresh tissue 25 λ of enzyme sample were used and for cultured 50 λ. All materials are kept on ice.

Procedure:

The quantities of material are per sample. 0.1 ml of each of TEA, EDTA, and $NADP^+$ was added to a cuvette. A quantity of water was added to the cuvette so that the total volume including enzyme and G6P was 1.0 ml. The enzyme was added. The cuvette was placed in the Unicam SP800 spectrophotometer and 340 mμ and 30°C; water was used as a blank. When no reaction was recorded then the reaction was started with 0.1 ml G6P. The change in absorbance was measured over a 1 minute interval.

TTA Assay (EC 2.6.1.5)

The assay is described by Diamondstone (82).

$$\text{L-tyrosine} + \underset{\text{acid}}{\alpha\text{-ketoglutaric}} \underset{\text{(PLP)}}{\overset{\text{TTA}}{\rightleftharpoons}} \underset{\text{acid}}{\text{L-glutamic}} + \underset{\text{pyruvic acid}}{\text{p-hydroxyphenyl}}$$

$$\underset{\text{pyruvic acid}}{\text{p-hydroxyphenyl}} + \text{NaOH} \longrightarrow \text{p-hydroxybenzaldehyde}^*$$

*measure at 331 mμ

Materials:

The following are stock solutions which are stored frozen: KH_2PO_4 2.72 gm per ml water (2×10^{-2}M); α-ketoglutaric acid 43.8 mg per ml KH_2PO_4 solution (3×10^{-1}M) pH 7.3; PLP 0.3 mg per ml KH_2PO_4 solution (1.5×10^{-3}M). NaOH, 10 N.

Usually 100 λ of enzyme are used, the material having been stored frozen for at least 36 hours. For each assay two tubes, viz. 1 sample tube and 1 control, are required (100 λ of enzyme each).

Fresh L-tyrosine solution is used. This consists of 1.24 mg of L-tyrosine per ml KH_2PO_4 solution (6.8×10^{-3}M); the pH is adjusted to 7.3.

Procedure:

0.8 ml of the L-tyrosine solution was added to each tube, sample and control, containing the enzyme. 25 λ PLP were added to each tube. The tubes were placed in a water bath at 37°C for about 30 minutes. To start the reaction 25 λ α-ketoglutaric acid were added to the sample and the control tubes. Immediately 50 λ NaOH were added to the control tube. After 20 minutes, 50 λ NaOH were added to the sample tube and both the sample and control tubes were placed at room temperature. After 30 minutes the absorbance was measured at 331 mμ using water as a reference. The difference between each sample and its control was determined in terms of absorbance units.

G6Pase Assay (EC 3.1.3.9)

The assay is described by Harper (83).

$$G6P + H_2O \xrightarrow{G6Pase} \text{glucose} + Pi$$

Materials:

The following materials are required: G6P 30.4 mg per ml water (1.1×10^{-1}M); 10% TCA; acid-molybdate solution (see Appendix I); Fiske-Subbarow reducer (see Appendix I). Use 25 λ of enzyme solution for fresh tissue and 100 λ for cultured. For each assay, 1 sample and 1 control tube is required.

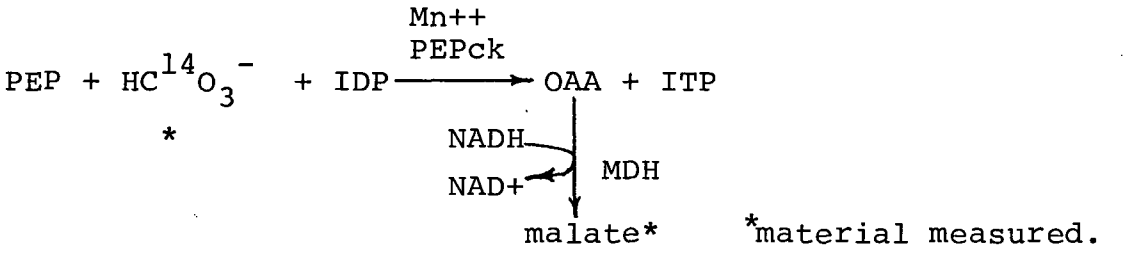
Procedure:

The enzyme was added to each sample tube and each control tube and the tubes placed in a water bath at 37°C for 5 minutes. To start the reaction 100 λ G6P were added to the sample tube and 100 λ of water to the control. Also 100 λ G6P were added to a

"blank" tube which had buffer in place of the enzyme. After 30 minutes 1.0 ml TCA was added to each tube to stop the reaction. The tubes were centrifuged until the protein precipitate formed a pellet. The resulting supernatant was decanted into clean tubes and to each, 0.5 ml of acid molybdate solution plus 1.5 ml water were added. To start the next reaction, 100 λ Fiske-Subbarow reducer were added to each tube. After 25 minutes the absorbance was measured at 660 mμ.

PEPck Assay (EC 4.1.1.32) Method I

The assay is described by Ballard and Hanson (58).



Materials:

The following are stock solutions, made with water, which are stored frozen: Imidazole-HCl buffer 6.8 gm per 100 ml (1.0M); pH 6.4; $MnCl_2 4H_2O$ 400 mg / 100 ml (2×10^{-2}M); $NaHCO_3$ 4.2 gm per 100 ml (5×10^{-1}M).

The following solutions are made fresh: GSH 15 mg per 5 ml (9.8×10^{-3}M); IDP 13 mg per 2.5 ml (1.2×10^{-2}M); NADH 100 mg per 5 ml (2.6×10^{-2}M); PEP 60 mg per 5 ml (5.1×10^{-2}M); MDH 10 λ per 50 sample plus control tubes. Two vials of C^{14} bicarbonate (50 μC SP Act., 33.3 μC per μM, in .083 ml H_2O) per 50 sample plus control tubes. TCA 10%.

Use 100 λ of enzyme solution for fresh tissue and 200 λ for cultured. Each assay requires 1 sample tube and 1 control.

All of the above materials are kept on ice.

The scintillation fluid consists of the following: naphthalene 25 gm; 1,4-dioxane 425 ml; PPO 3.5 gm; POPOP 100 mg.

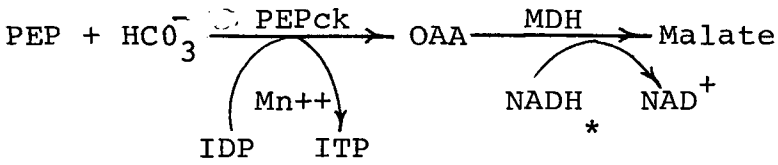
A tank of CO_2 is also required.

Procedure:

This outline is for a 50 tube assay, i.e. 25 sample and 25 control tubes. 5 ml each of imidazole-HCl, $MnCl_2$, $NaHCO_3$, GSH, NADH, and PEP plus 10 λ MDH were mixed. The pH of the mixture was approximately 7. Two vials of C^{14} bicarbonate were added. 10 λ of the mixture were added to each of at least 5 scintillation bottles filled with the scintillation fluid. These were used as standards. A quantity of water was added to each sample and control tube so that the final volume, after the addition of all components, was 1.5 ml. 0.6 ml of the mixture was added to each tube. The enzyme solution was added to the tubes; the tubes were then removed from the ice and placed in a water bath at 37°C. The reaction was started by adding 100 λ of IDP to each sample tube. After 20 minutes the reactions were stopped by adding 0.5 ml TCA to each sample and each control. The tubes were centrifuged until the protein precipitates formed pellets, then the resulting supernatants were decanted into liquid scintillation vials. The solutions were bubbled with CO_2 for about 1 minute, then 15 ml of scintillation fluid were added to each vial.

PEPck Assay (EC 4.1.1.32) Method II

The assay is described by Swiatek et al. (84).



*Reaction which is recorded.

Materials:

The following materials are given in mg per ml (the values in brackets are molar concentrations): Tris buffer 60.5 (5×10^{-1}); $MnCl_2$ 5.0 (4×10^{-2}); $NaHCO_3$ 8.4 (1×10^{-1}); IDP 6.0 (1.4×10^{-2}); NADH 1.0 (1.3×10^{-3}); PEP 16.0 (6.8×10^{-1}). The solvent is glass distilled water. Tris, $MnCl_2$, and $NaHCO_3$ are stored as frozen solutions. Use approximately 50 λ of enzyme solution for fresh tissue and 100 λ for cultured. All materials are kept on ice.

Procedure:

The quantities of material are per sample. 0.1 ml of each of Tris, $MnCl_2$, $NaHCO_3$, NADH, and PEP plus approximately 1 λ MDH were added to a cuvette. A quantity of water was added to the cuvette so that the total volume including enzyme and IDP was 1.0 ml. The enzyme was added. The cuvette was placed in the Unicam SP800 spectrophotometer at 340 mμ and 30°C; water was used as a blank. When no reaction was recorded then the reaction was started with 0.1 ml IDP. The change in absorbance was measured over a 1 minute time interval.

Protein Assay

The assay is described by Lowry et al. (85).

Materials:

Solution A - 10 gm Na_2CO_3 (anhydrous) (1.9×10^{-1}M) plus 2 gm NaOH (1×10^{-1}M) per 500 ml .

Solution B - 1.25 gm NaK-tartarate per 20 ml (2.2×10^{-2}M).

Solution C - 500 mg Cu $SO_4 \cdot 5H_2O$ per 20 ml (1×10^{-1}M).

The solvent used for the above solutions is distilled water.

Folin's solution - dilute using 1 part Folins to 1 part water.

Albumin - The stock solution, which is stored frozen, consists of 500 mg albumin in a total volume of 10 ml. For use, take 100 λ of the stock solution and add water to a total volume of exactly 10 ml. Use 50 λ of this solution for the standard.

It should be noted that solutions A, B, and C are all stock solutions and can be stored at room temperature.

Procedure:

A ratio of 50 ml A to 0.8 ml B to 0.2 ml C was used, all freshly mixed. The quantity of protein sample depended on the tissue used. For fresh liver 5 λ of supernatant were used and for cultured 10 λ . For fresh kidney 10 λ of supernatant were used. 25 λ of liver mitochondrial solution and 50 λ of kidney solution were required.

3 ml of the A+B+C mixture were added to each sample tube, each standard, and the blank. After 15 minutes 0.3 ml of diluted Folin's solution were added to each tube. After approximately 30 minutes the absorbance was measured at 545 mμ. Appropriate dilutions were made with water if the absorbance was too great.

RESULTS

Tissue Culture

A number of experiments were undertaken before a satisfactory culture system was developed. Initially, 30 ml flasks were used with 2.5 ml of medium; the tissue was, therefore, completely submerged. This system was unsatisfactory since it was not possible to induce TTA and, indeed, there was a dramatic decrease in all enzymes assayed compared to the fresh tissue. Next, a decision was made to use much less medium, i.e. just enough to wet the lower surface of the tissue. This was satisfactory for a culture of 5 hours or less, or for 24 hours if only very small quantities of tissue were used, e.g. 25 mg or less. With larger quantities of material, the pH of the medium decreased from the normal 7.4 to the 6.8 range; again, there was a loss of enzyme and we were unable to induce any enzymes.

Two hundred and fifty ml flasks were then tested with 2.5 ml of medium and approximately 150 mg of the fetal liver tissue. There was a dramatic spontaneous increase in the level of TTA in rat liver and we were able to induce this enzyme, as well as PEPck, with dcAMP and glucagon. In human tissue, a spontaneous increase in the level of PEPck occurred and we were able to induce this enzyme with a number of chemical agents.

Our system, therefore, certainly appeared to be viable at least for the 24 hour period tested. One problem was puzzling, viz. there was a loss of protein during the culture period.

The longer the culture period the greater the loss. When the medium was observed under the microscope many red blood cells were noted. This could account for at least part of the protein decrease. Also, there may be dilution of the protein due to the diffusion of culture medium into the incubating tissue. Lastly, slides of the tissue made at different periods of culture time indicate some cell autolysis. This can be seen by comparing fresh tissue with tissue cultured for 42 hours, (see Figures 9 and 12). The 24 hour tissue (see Figure 10) has a healthy appearance,i.e. the nuclei are definite and well formed and the cell membranes in general still retain their integrity. There has been a loss of red cells and there are a few areas where the parenchymatous cells have autolysed. Because of the technique of cutting the liver into small pieces and placing the material in a relatively foreign environment, some damage can be expected. The 42 hour cultured liver (see Figure 12), in contrast to the 24 hour cultured material, is in a state of degeneration. The 36 hour material (see Figure 11) has a state of appearance intermediate between the 24 and 42 hour tissue. Widespread autolysis can be seen in the 42 hour tissue. Many of the cell nuclei are pycnotic and many cell membranes have lost their original definite shape. It should be noted that a number of the parenchymatous cells still appear viable; the blood forming tissue appears to be the most affected.

A number of different types of media were tested as is shown in Table I. The specific medium used does not appear to make any substantial difference with respect to the levels of the enzymes studied. There certainly are some differences, e.g. between LHBSS

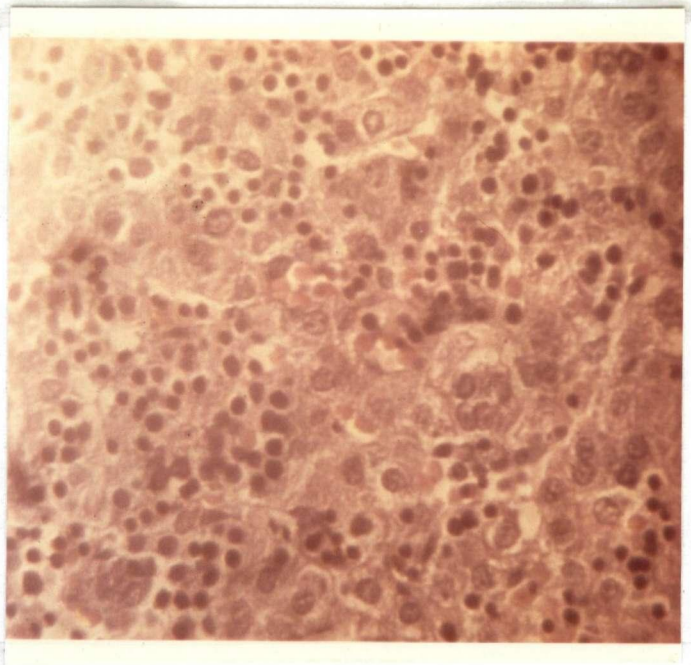


FIGURE 9: Section of fresh human fetal liver 16 weeks gestational age. Hematoxylin-Eosin stain. Magnification 450 x.

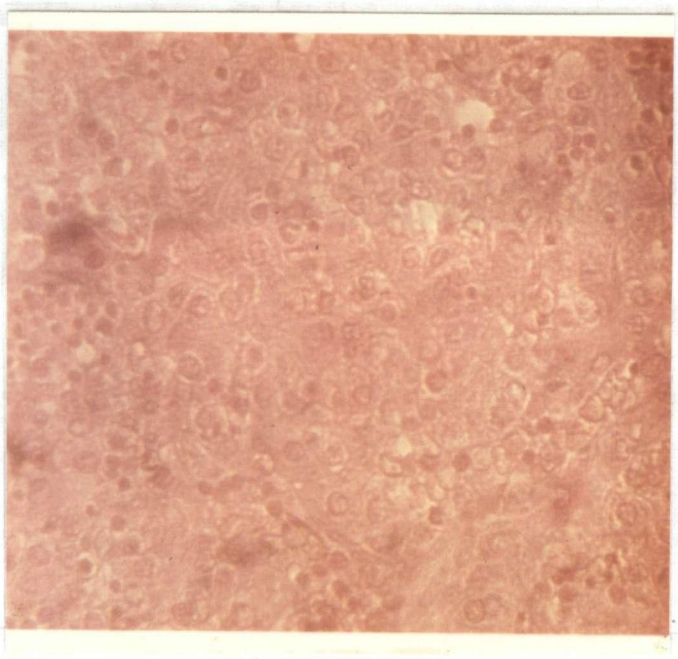


FIGURE 10: Section of 24 hour cultured human fetal liver 16 weeks gestational age. Hematoxylin-Eosin stain. Magnification 450 x.

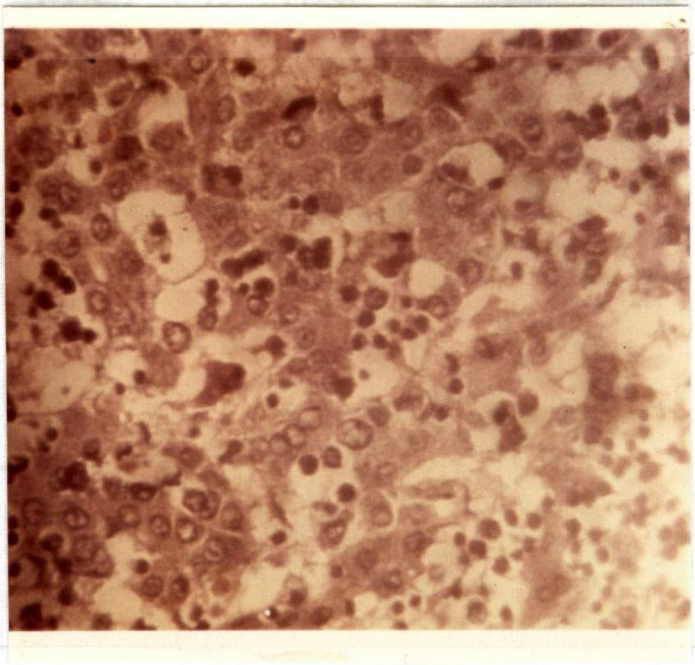


FIGURE 11: Section of 36 hours cultured human fetal liver 16 weeks gestational age. Hematoxylin-Eosin stain. Magnification 450 x. Note the loss of cell contents relative to the two previous figures.

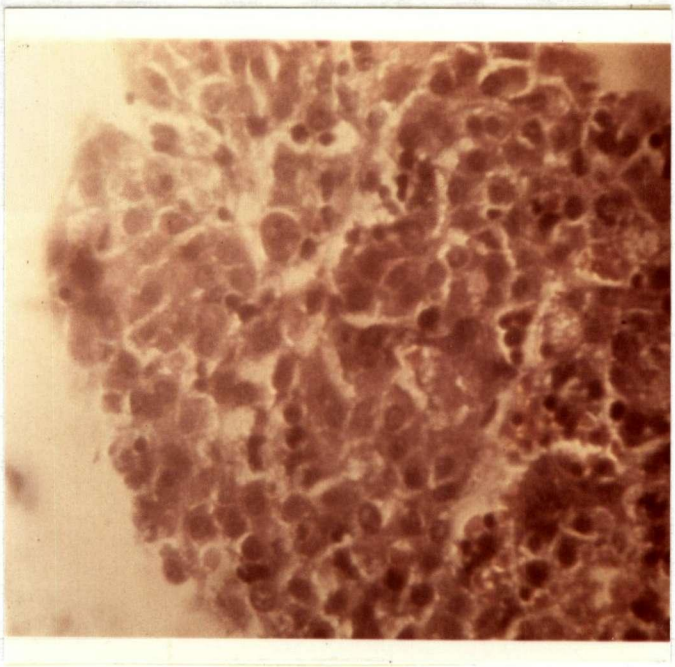


FIGURE 12: Section of 42 hour cultured human fetal liver 16 weeks gestational age. Hematoxylin-Eosin stain. Magnification 450 x. Note the widespread cell autolysis; many nuclei are pycnotic and the tissue in general has lost its integrity.

TABLE I

Comparison of Culture Media Influence on Cytosol Enzyme Changes During 24 Hours of Incubation

Human Fetal Liver Fetus Age (wks)	Sample	*MEM+HBSS % ch.C/F	MEM+HBSS+$NaCH0_3$** % ch.C/F	HBSS+$NaHCO_3$ % ch.C/F	LHBSS+$NaHCO_3$ % ch.C/F
13.1	Protein	-71		-72	-73
	TTA	-43		-53	-48
	G6PD	-63		-61	-71
15.1	Protein	-28	-26		
	TTA	-33	+12		
	PEPck	+11	+155		
	PK	-2	-5		
	G6PD	-17	-21		
16.7	Protein	-48	-46		
	TTA	+14	+83		
	PEPck	+44	+31		
	PK	-30	-27		
	G6PD	-3	-7		
18.4	Protein		-37		-48
	TTA		+47		+14
	PEPck		+131		+133
	PK		+8		+3
	G6PD		-24		-30

TABLE I (CONT'D)

Rat Fetal Liver Fetus Wt. (gm)	Sample	*MEM+HBSS % ch.C/F	MEM+HBSS+$NaCH0_3$** % ch.C/F	HBSS+$NaHC0_3$ % ch.C/F	LHBSS+$NaHCO_3$ % ch.C/F
1.39	Protein		-67	-65	
	TTA		+84	+78	
	G6PD			+2	
2.02	Protein		-63	-65	-64
	TTA		+170	+173	+222
3.08	Protein		-63	-69	-60
	TTA		+229	+228	+314
	G6PD		+73	+108	+119
5.57	Protein		-82	-83	
(2 hr. culture)	TTA		+430	+396	

* % ch. C/F = % change 24 hour cultured tissue/fresh tissue

** $NaHC0_3$ = 70 mg $NaHC0_3$ added/100 ml medium.

and MEM + HBSS; however, they are not dramatic. One factor of concern was the decrease in pH even in the large flasks. This was remedied by the addition of twice the normal quantity of $NaHCO_3$ to the medium. The pH was then maintained at 7.35 ± .25 which is quite acceptable according to Paul (76). Wicks (37) and Räihä et al. (72) doubled the bicarbonate and glucose content in their cultures. The medium from a number of our experiments was tested for glucose level with Tes Tape; we observed that even after 42 hours of culture substantial levels of glucose remained.

One test was undertaken with the 250 ml flasks placed upright thus submerging the tissue. The results of this experiment are shown in Table II. Submerged tissue in the 250 ml flasks remained much healthier than tissue in the 30 ml flasks; however, submerged tissue is not as viable as that under the standard conditions, i.e. with only a portion of the material covered with culture medium.

TABLE II

Comparison of Cytosol Enzyme Levels in Submerged and Non-Submerged (Normal) Tissues

Fetal Rat Liver Cultures			% Change Control/Fresh		% Change Glucagon/Control	
Fetus Wt. (gm)	Hours of Culture	Sample	Normal	Submerged	Normal	Submerged
3.35	5	Protein	-45	-53		
		TTA	+482	+283	+5	+5
1.45	42	Protein	-84	-93		
		TTA	+2300	+585	+27	-4
		G6PD	+86	-24	-19	-20
Human Fetal Liver Cultures						
Fetus Age (wks)						
16.1	24	Protein	-45	-56		
		TTA	-41	-47		

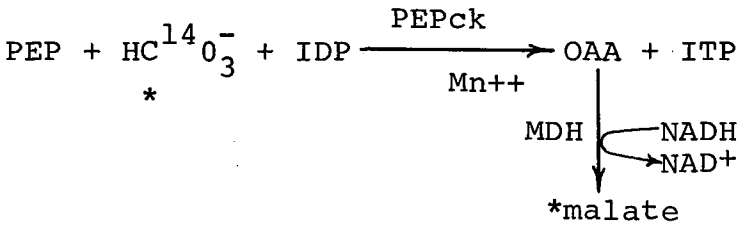
The submerged tissues were completely covered with MEM+HBSS; the normal tissues were approximately one-third submerged in culture medium.

PEPck Assay

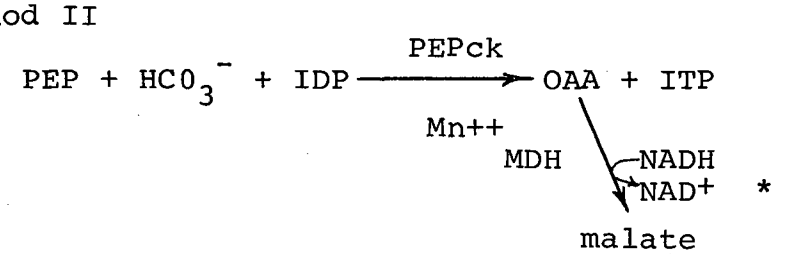
The enzyme assays used in this thesis presented very few problems with the exception of PEPck. The method outlined by Ballard and Hanson (58), Method I, is based on the incorporation of radioactive carbon (C^{14}) into malate. The method according to Swiatek et al. (84), Method II, is based on the absorbance change at 340 mμ due to the conversion of NADH to NAD^+. The third, Method III, as outlined by Berndt and Ulbrich (86), also involves a NADH to NAD^+ conversion.

The reactions involved in these techniques are as follows:

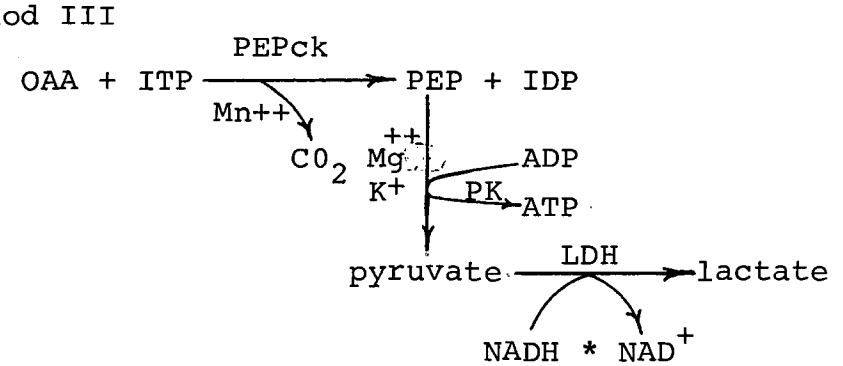
Method I



Method II



Method III



The key reaction or material measured in the respective assays is indicated with an asterisk (*).

Method II was initially used in this thesis study. In contrast to the results of Ballard and Hanson (58), who reported PEPck levels approximately 25 fold greater in postnatal rat liver than in fetal liver at pH 7, we obtained values approximately 10% higher in postnatal tissue than in fetal tissue at this pH (see Figure 13). Our fetal liver values were approximately 20 times greater than those of Ballard and Hanson. We continued both human and rat liver experiments since the results appeared to be satisfactory. Fetal and postnatal pig livers were then compared. Tildon et al. (87) had reported that the fetal levels were two-thirds of the postnatal levels. We obtained the same relative results; however, our absolute values were approximately 5 times lower than those of Tildon et al. We were able to repeat the work of Ballard and Hanson using their assay method; also, the same relative values for the pig liver were obtained using this radioactive technique.

Method III was then used; we found that the results were similar to those of Ballard and Hanson. Curves of pH were drawn (see Figure 14) using the 3 different methods. It appeared that different enzymes were being measured. We found that no bicarbonate was required using Method II; however, a source of bicarbonate is required for the PEPck reaction. It was also noted that in fetal liver the level of PK is approximately 50 times greater than that of PEPck. The solution to the problem became obvious when we observed that IDP would replace ADP to the extent of approximately 75% as a substrate for PK. This also had been observed by Davidson (88).

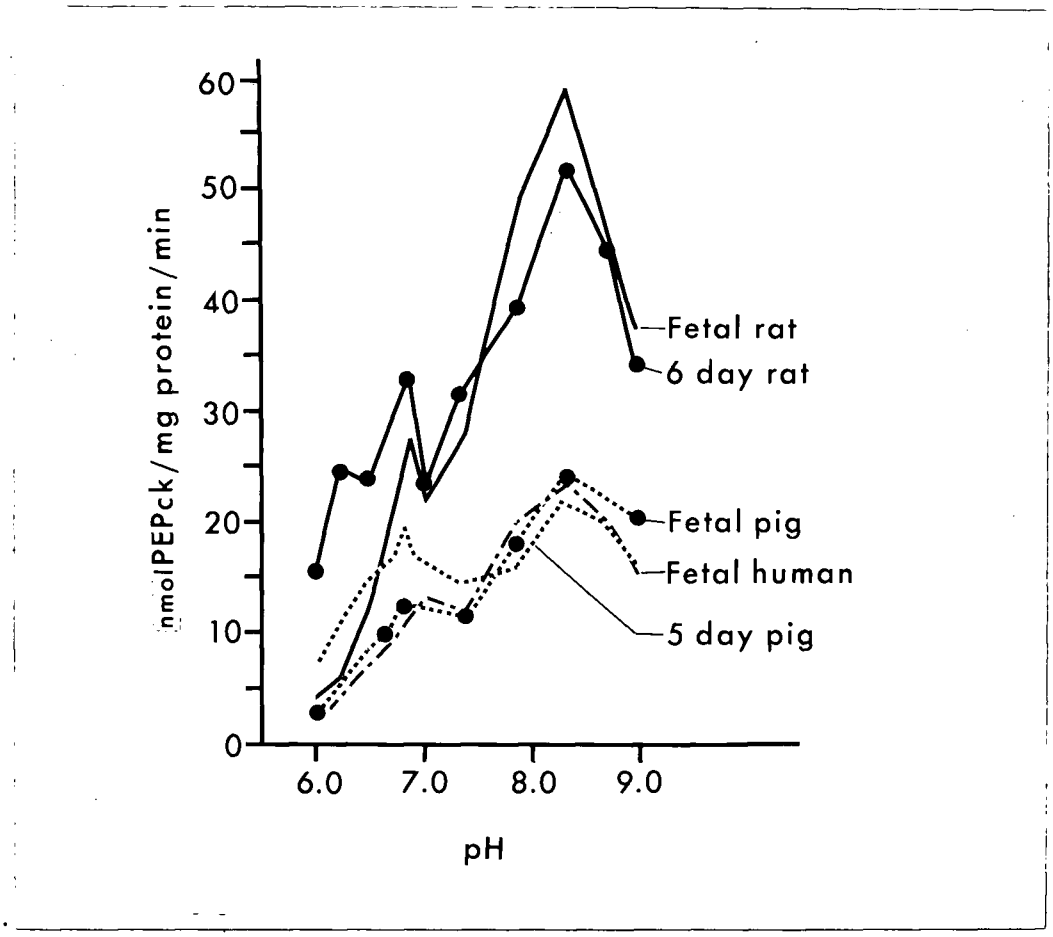


FIGURE 13: Comparison of PEPck levels in liver cytosol using assay Method II. Details of Method II are given in the text.

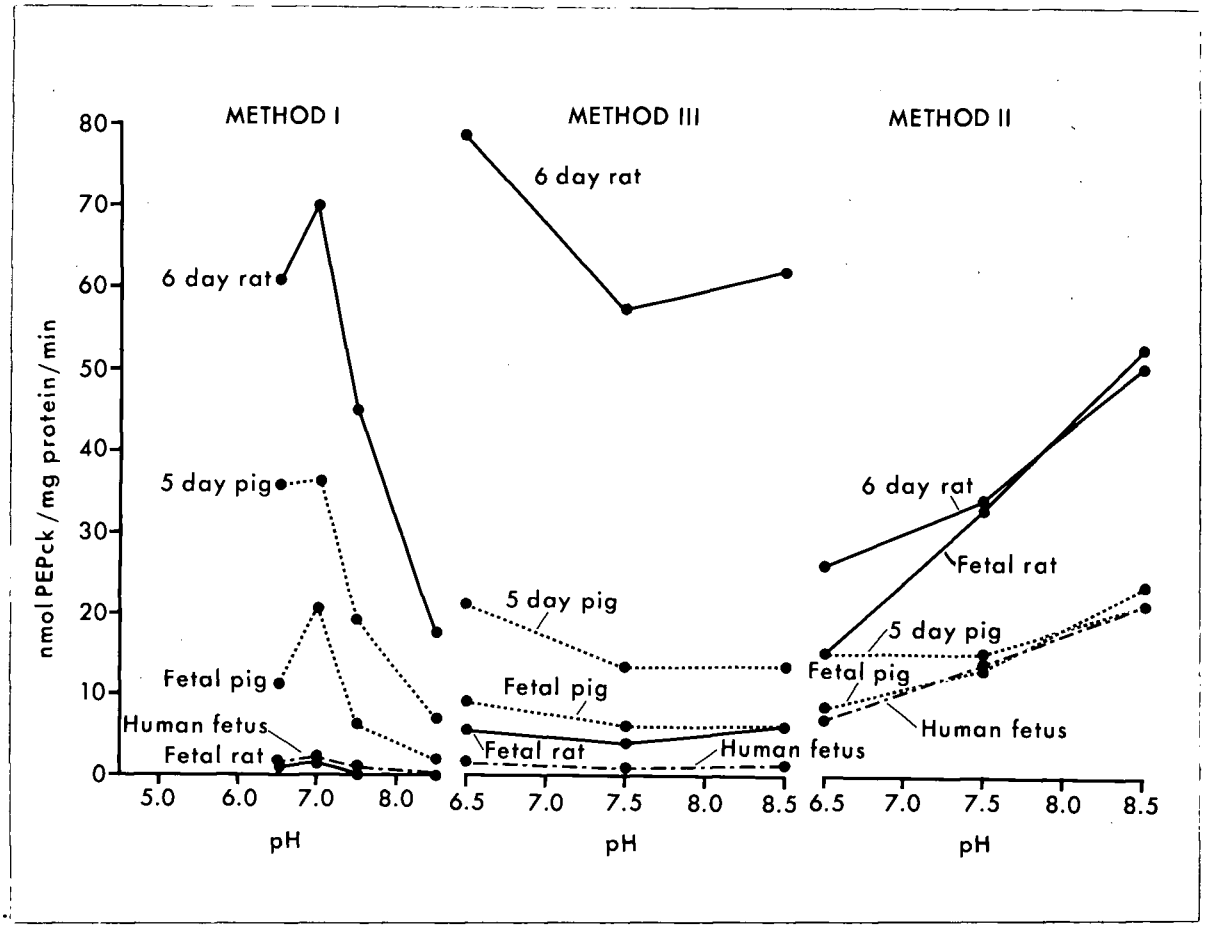


FIGURE 14: Comparison of assay Methods I, II, and III for PEPck in liver cytosol. Details of the methods are given in the text.

The components necessary for the PK reaction except LDH and K^+ were contained in the PEPck mixture of Method II. The enzyme supernatant must, therefore, contain a sufficient quantity of LDH and K^+ for the PK reaction to proceed.

Human Fetal Liver and Kidney Enzyme Levels

Very little data on enzyme development in the liver and kidney are available for the developing human fetus. This information is necessary before meaningful studies on induced enzyme changes can be undertaken. The data are also important to facilitate a comparison with animals such as rats which have been intensively studied.

Protein: First, the protein levels in the liver and kidney cytosol and mitochondria had to be determined since all of the enzyme assays are based on protein as the standard. These levels are outlined in Figure 15. The mitochondrial solution is 5 times more concentrated than the cytosol; it should be noted that the mitochondrial solution was centrifuged at 30,000 x g for 10 minutes to remove the cristae. The liver cytosol contains approximately 25 times more protein than does the mitochondrial solution and the kidney cytosol about 35 times more protein than the corresponding mitochondrial solution.

There appears to be a slight increase in kidney cytosol protein with increasing gestational age. The liver cytosol protein peaks between 13 and 14 weeks of age. The liver cytosol contains about twice as much protein as does the kidney; the same is true for the mitochondrial solutions.

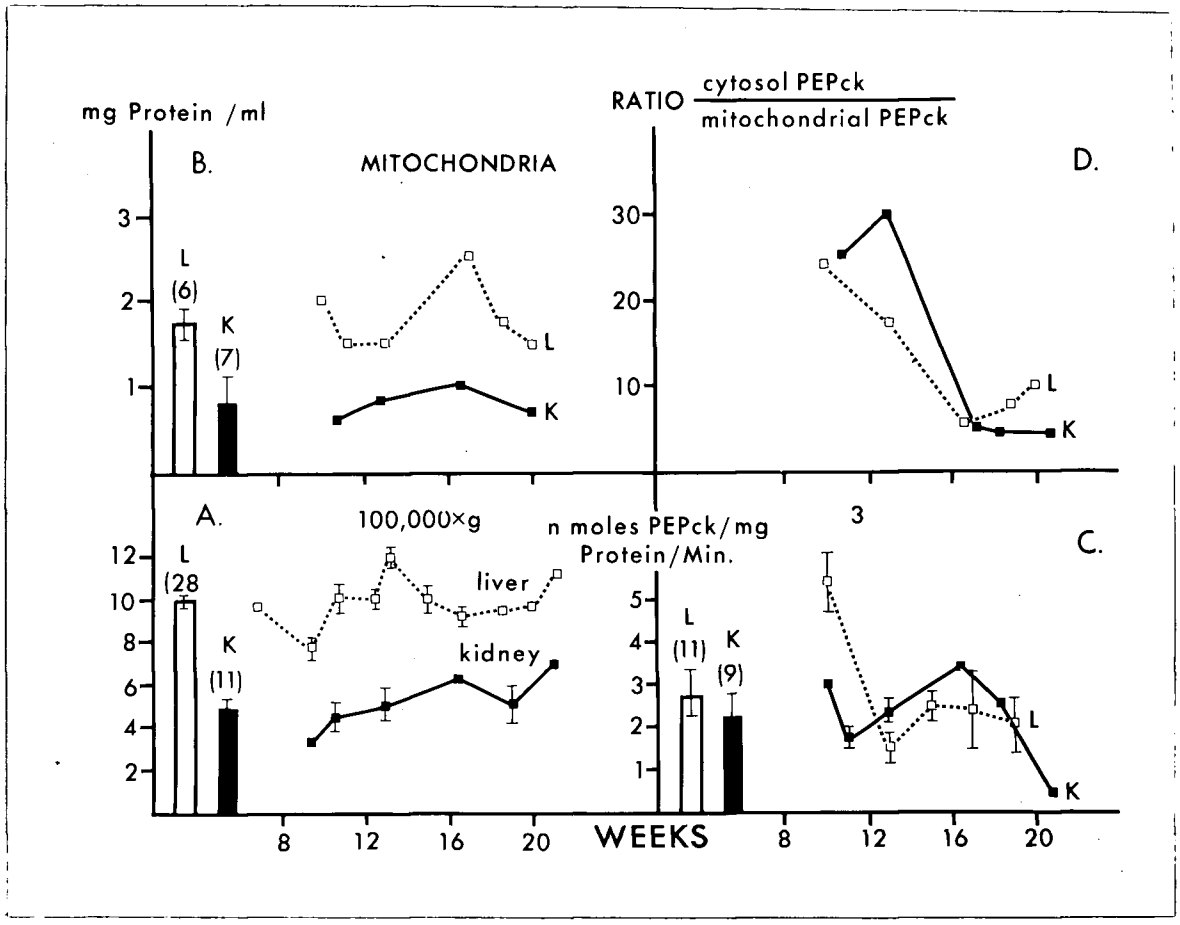


FIGURE 15: Change in the human liver and kidney cytosol (A) and mitochondrial (B) protein level during fetal development. Change in the cytosol PEPck level (C) and change in the ratio of cytosol to mitochondrial PEPck (D). L, liver; K, kidney. The columns show Mean + SEM values for all fetuses. The figures in parentheses show the number of fetuses used. Abscissa: Gestational age in weeks.

TTA: The cytosol TTA levels are outlined in Figure 16. Note the dramatic decrease of TTA with increasing age. There was a decrease by a factor of 13 from 9 weeks to 21 weeks for the liver and a decrease by a factor of 15 from 11 weeks to 21 weeks for the kidney. At about 11 weeks the liver and kidney contain approximately the same quantity of TTA on a protein basis; whereas, at 18 weeks the liver has 5 times more of this enzyme than does the kidney.

No postnatal material was available for assay; however, Kretchmer et al. (15) had determined the TTA activity in the liver of fetuses (approximately 27 weeks), newborns (approximately 1 day), and adults. The activity ratios were 1:4:15.

PEPck: The cytosol and mitochondrial PEPck levels are outlined in Figures 15 and 17. There is a rapid decrease in PEPck in the liver cytosol between 10 and 12 weeks of gestation; the level then remains relatively constant. The liver and kidney cytosol and mitochondrial levels on a protein basis are similar.

The cytosol contains a much greater quantity of PEPck than does the mitochondrial solution. On a protein basis the liver mitochondrial solution contains approximately 3 times more PEPck than the cytosol and the kidney mitochondrial solution about 5 times more; however, on a wet weight basis the liver mitochondrial solution has at least 25 times less protein than the cytosol and the kidney 35 times less. Also, there is a definite decrease in the ratio of cytosol to mitochondrial enzyme, on a wet weight basis, with increasing gestational age for both the liver and kidney. The ratio decreases from 25:1 in the tenth week to about

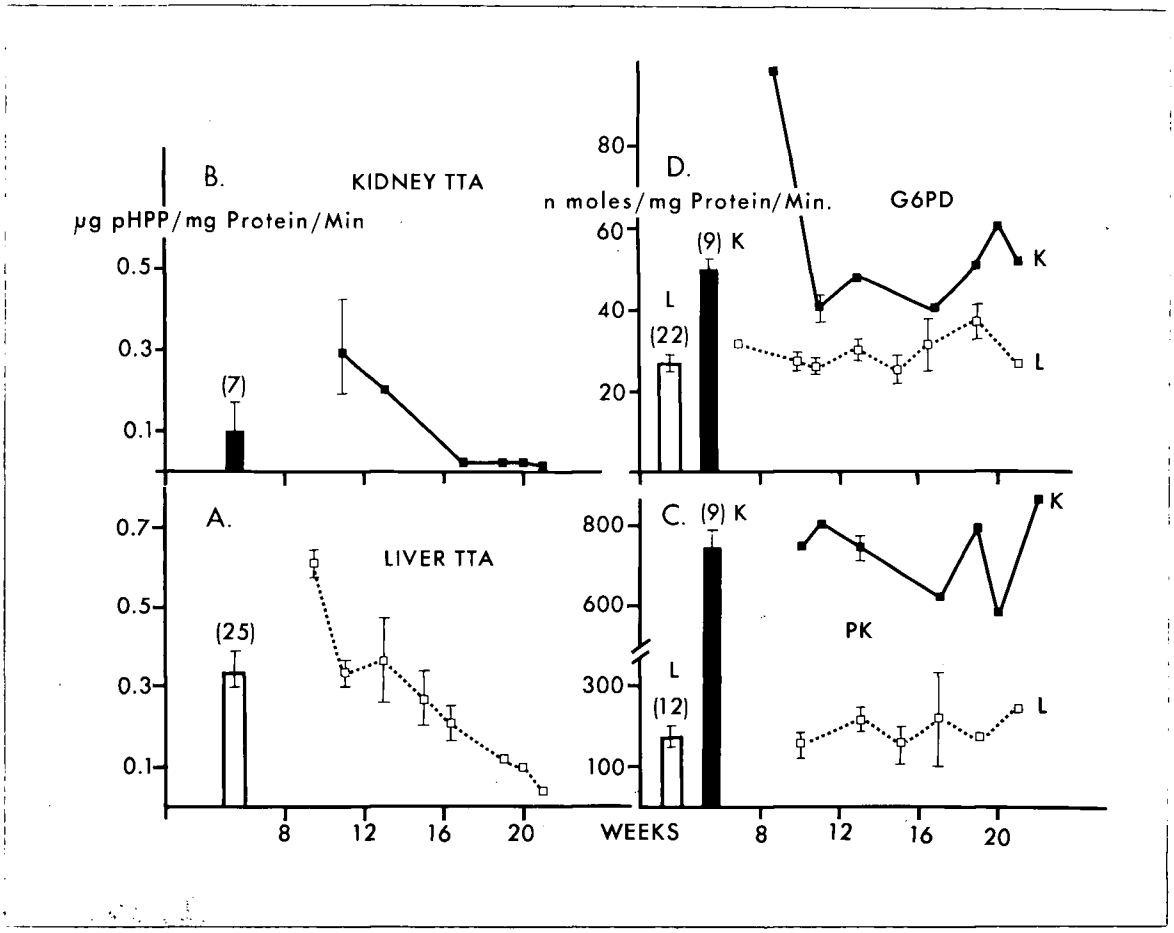


FIGURE 16: Change in the human liver and kidney cytosol level of TTA (A,B), PK (C), and G6PD (D) during fetal development. L, liver; K, kidney. The columns show Mean ∓ SEM values for all fetuses. The figures in parentheses show the number of fetuses used. Abscissa: Gestational age in weeks.

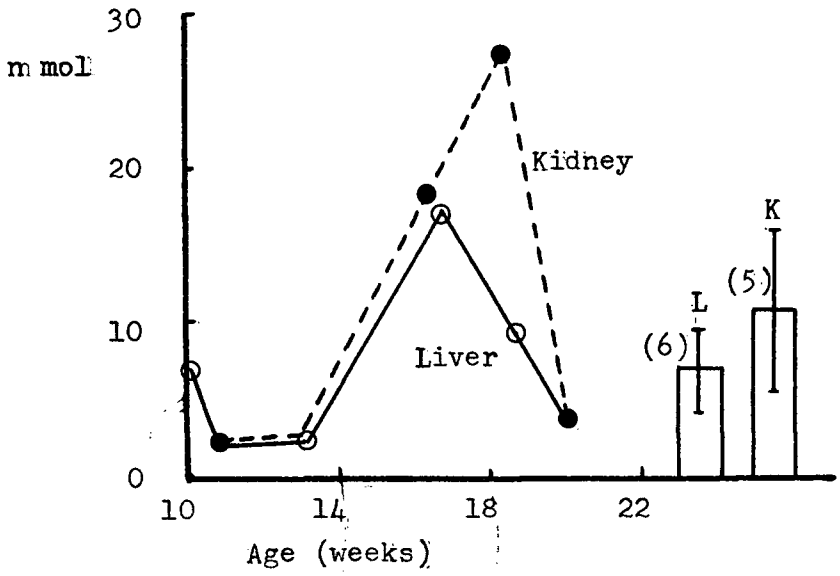


FIGURE 17: Change in the mitochondrial PEPck level during fetal development in the human liver (L) and kidney (K). The columns show Means ∓ SEM for all fetuses. The figures in parentheses show the number of fetuses used. Abscissa: Gestational age in weeks. Ordinate: n moles PEPck/mg protein/min.

6:1 in the eighteenth week. This change in the liver appears to be mainly due to decreased cytosol enzyme; whereas, in the kidney there is an increase in the mitochondrial enzyme. Diesterhaft (16) reports values of 60 nmoles PEP per mg protein per minute for PEPck activity in the cytosol and 120 for the mitochondria in adult man. The ratio of cytosol to mitochondrial activity is about 1:1 according to this report. Using both Diesterhaft's data and data collected in this laboratory, it is seen that there is an increase by a factor of approximately 22 in the PEPck level in the cytosol and by a factor of 17 in the mitochondria from the fetal to the adult stage.

PK: There is 4 times more PK per unit of protein in the kidney than in the liver cytosol. No significant change in level was noted with increasing age (see Figure 16).

G6PD; The G6PD levels in the liver and kidney cytosol are shown in Figure 16. The kidney has twice the quantity of this enzyme on a protein basis or about the same quantity per unit of wet weight. The liver G6PD level appears to peak at an early gestational age, i.e. prior to 9 weeks, with a lesser peak at 19 to 20 weeks of gestation. The latter peak has been reported by Mino et al. (89). Their data indicate that this enzyme increases by approximately 75% from 16 to 21 weeks then decreases by a factor of 17 from the peak level to term.

Enzyme Levels in the Liver from a Human Fetus Exposed to Prednisolone In Utero

The liver cytosol levels of PEPck, TTA, PK, and G6PD from a 12.5 week fetus, the mother of whom had been given 20 mg per day of

prednisolone for 8 days prior to hysterotomy, were measured to determine if there were any differences in the levels of these enzymes from those found in normal fetuses of the same approximate age. The following differences were noted: The PK level decreased by 30% and G6PD by 36%. The TTA level increased by 47% and PEPck increased by a factor of 33. As can be seen from Figure 15, the two normal fetuses of 13 weeks gestation with which the comparison was made have relatively low PEPck levels.

Rat Liver Enzyme Levels

The enzyme values as determined in this work are very similar to those reported in the literature (9,12,14,35,90). These data have been recorded to facilitate a comparison with tissue culture enzyme levels and with induced enzyme changes.

Protein: There is approximately a 45% increase in cytosol protein from the fetal to the postnatal stage (see Figure 18).

TTA: The increase in TTA between the fetal and postnatal stage is about 7 fold. The level of this enzyme increases very rapidly at term (see Figure 18). The neonate which had been born 15 minutes previously has an enzyme level approximately 3 times greater than the average fetus.

PEPck: There is a dramatic increase in this enzyme at birth as can be seen in Figure 19. PEPck before birth has a mean of 3.2; whereas, after birth the mean level is 54.

PK: This enzyme appears to decrease near term then increases at term and decreases again during the first two weeks of birth (see Figure 19).

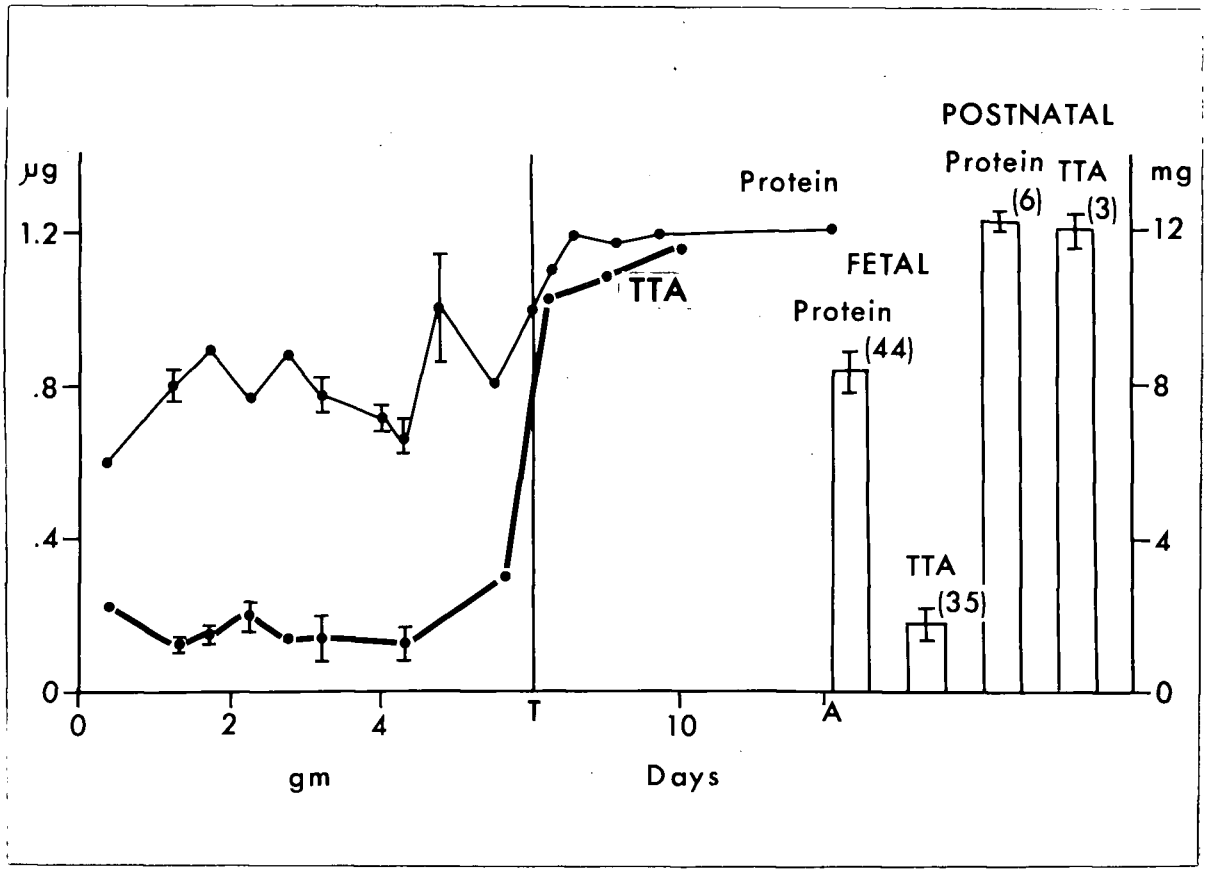


FIGURE 18: Change in the cytosol protein and TTA levels with age in rat liver. The columns show Means ∓ SEM values for all animals. The figures in parentheses show the number of animals used. Abscissa: Weight in gm (fetal) or age in days (postnatal). Left ordinate: TTA µg pHPP/mg protein/min. Right ordinate: Protein (mg/ml). T, term; A, adult.

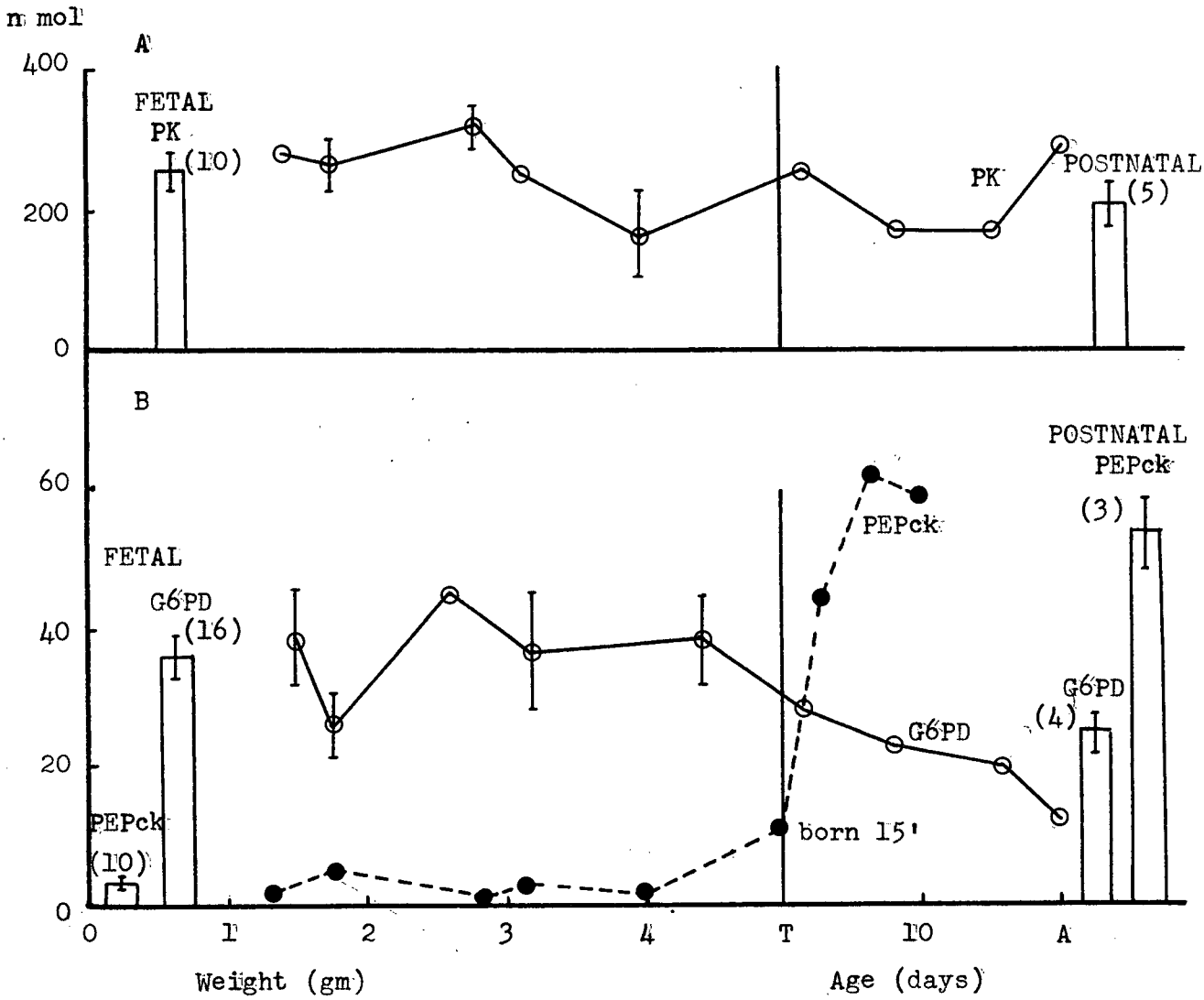


FIGURE 19: Change in the level of cytosol PK (A), and PEPck and G6PD (B) with age in rat liver. The columns show Mean ± SEM values for all animals. The figures in parentheses show the number of animals used. Ordinate: n moles/mg protein/min. Abscissa: Weight in gm (fetal) or age in days (postnatal). T, term; A, adult.

G6PD: The level of this enzyme gradually decreases after birth as is seen in Figure 19.

Pig Liver Enzyme Levels

Data for pig liver are useful to have as a comparison with the human and rat material. Most of the data reported for the pig are from studies by Tildon et al. (87), and Swiatek (91). Swiatek has calculated that the cytosol to mitochondrial ratio for PEPck in the adult is 4:1. Our data indicate that there is approximately a 65% increase in the cytosol enzyme level from the fetal stage to 5 days postnatally. Tildon et al. (87) have reported a similar increase. During the same period, the cytosol protein level increases by about 25%.

Comparison of Human, Rat and Pig Liver Enzyme Levels

These data are outlined to reinforce the caution that one must use when attempting to apply data obtained from one animal species to another species. The information is considered in detail in the Discussion.

Table III outlines the cytosol to mitochondrial PEPck ratios at different stages of development. Note the progressive decrease in the ratio in the human. The mean values for protein, TTA, PEPck, PK, and G6PD are shown in Figure 20.

In Vitro Changes in Enzyme Levels in Human Fetal Liver

The only data to our knowledge on the change in enzyme levels in fetal human liver in culture are those of Räihä et al. (72). They reported an approximate 40% increase in TTA during 40 hours of culture for fetuses 14 to 24 weeks gestational age.

TABLE III

Comparison of the Ratios of Cytosol to Mitochondrial PEPck Levels in Human, Pig, and Rat Liver

Sample	Ratio	Source
Human (adult)	1:1	Diesterhaft et al. (16)
Human (fetus) 18 weeks gestation	5:1	Thesis Work
10 weeks gestation	25:1	Thesis Work
Pig (adult)	4:1	Swiatek et al.(84)
Rat (adult)	25:1	Ballard and Hanson (58)
Rat (fetus)	1:4	Ballard and Hanson (58)

The ratios are on a wet weight basis.

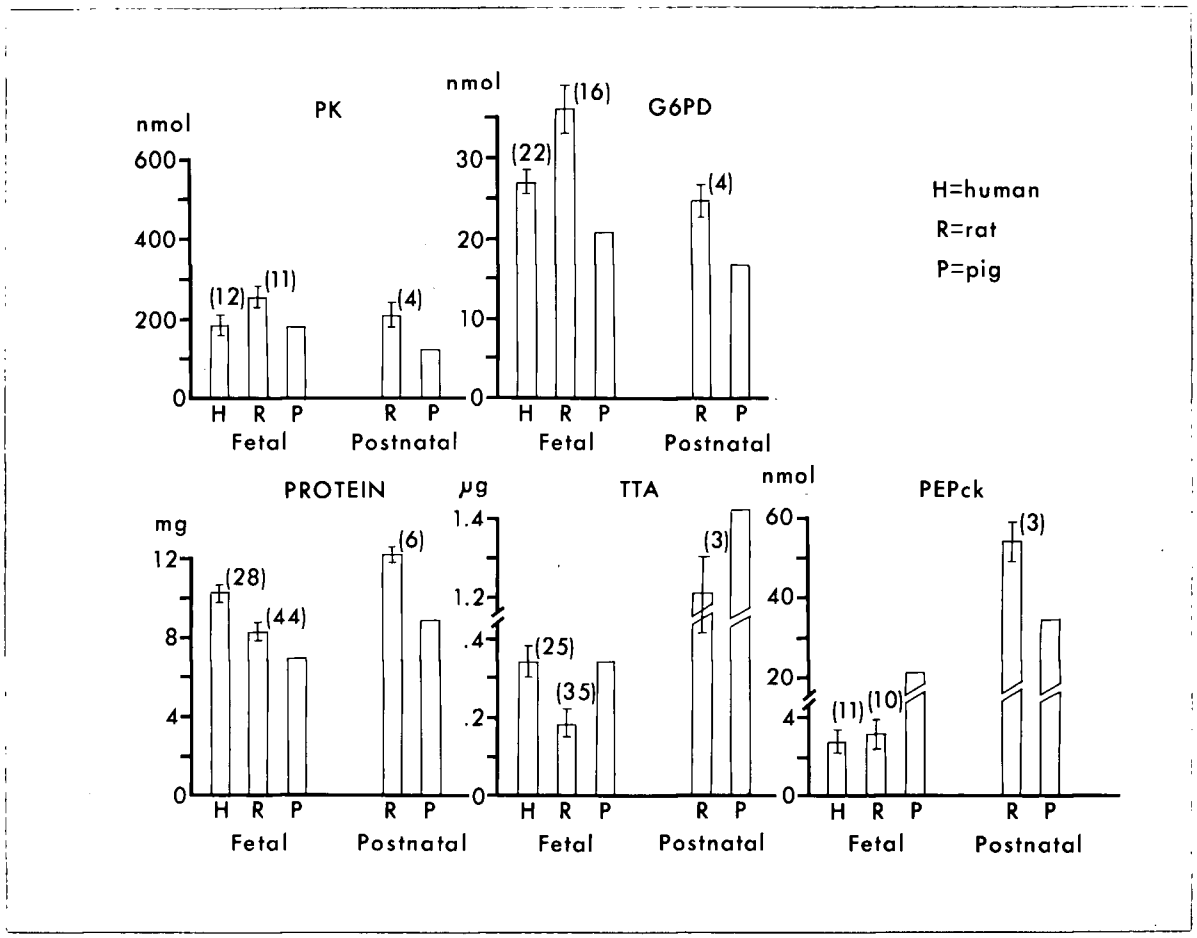


FIGURE 20: Comparison of cytosol enzyme levels in fetal and postnatal liver of the human, pig, and rat. Note the break in the ordinate. The columns show Means ∓ SEM values. The figures in parentheses show the number of animals used. H, human; R, rat; P, pig. The ordinate units for PK, PEPck, and G6PD are n moles/mg protein/min; for TTA are µg pHPP/mg protein/min; and for protein are mg/ml.

Tissue Cultures:

Spontaneous Changes - Under culture conditions, some liver enzymes increase and others decrease in level. Figure 21 outlines the changes in protein, TTA, PEPck, PK, and G6PD during 24 hours of incubation.

There is an average decrease of 43% (Significant at $p < .01$) in the protein level. The protein content consistently decreases; however, the degree is rather variable. The TTA level decreases by an average of 30% (Significant at $p < .01$) in livers obtained from fetuses 9 to 16.5 weeks of gestational age; after that age there is an average increase of 54% (Significant at $p < .01$). The PEPck level increased by an average of 172% (Significant at $p < .01$). Both PK and G6PD decrease in culture. PK decreases by an average of 13% (Significant at $p < .05$) and G6PD by 39% (Significant at $p < .01$).

One experiment was undertaken with a 5 hour incubation period and another with a 40 hour period. The results of these studies are given in Table IV. In the 5 hour experiment there was only a 3% decrease in the protein level. The TTA level decreased by 5% and G6PD by 15%. Forty hours of incubation resulted in a 77% decrease in the level of protein, a decrease in G6PD so great that no reading could be recorded, and a 152% increase in TTA.

Induced Changes - The following compounds were tested with the objective of studying the enzyme response: dcAMP, glucagon, acetyl CoA, oleic acid, linolenate, and carnitine. Different concentrations were tested and additions were made after different periods of culture. Tables V to VIII inclusive outline part of the

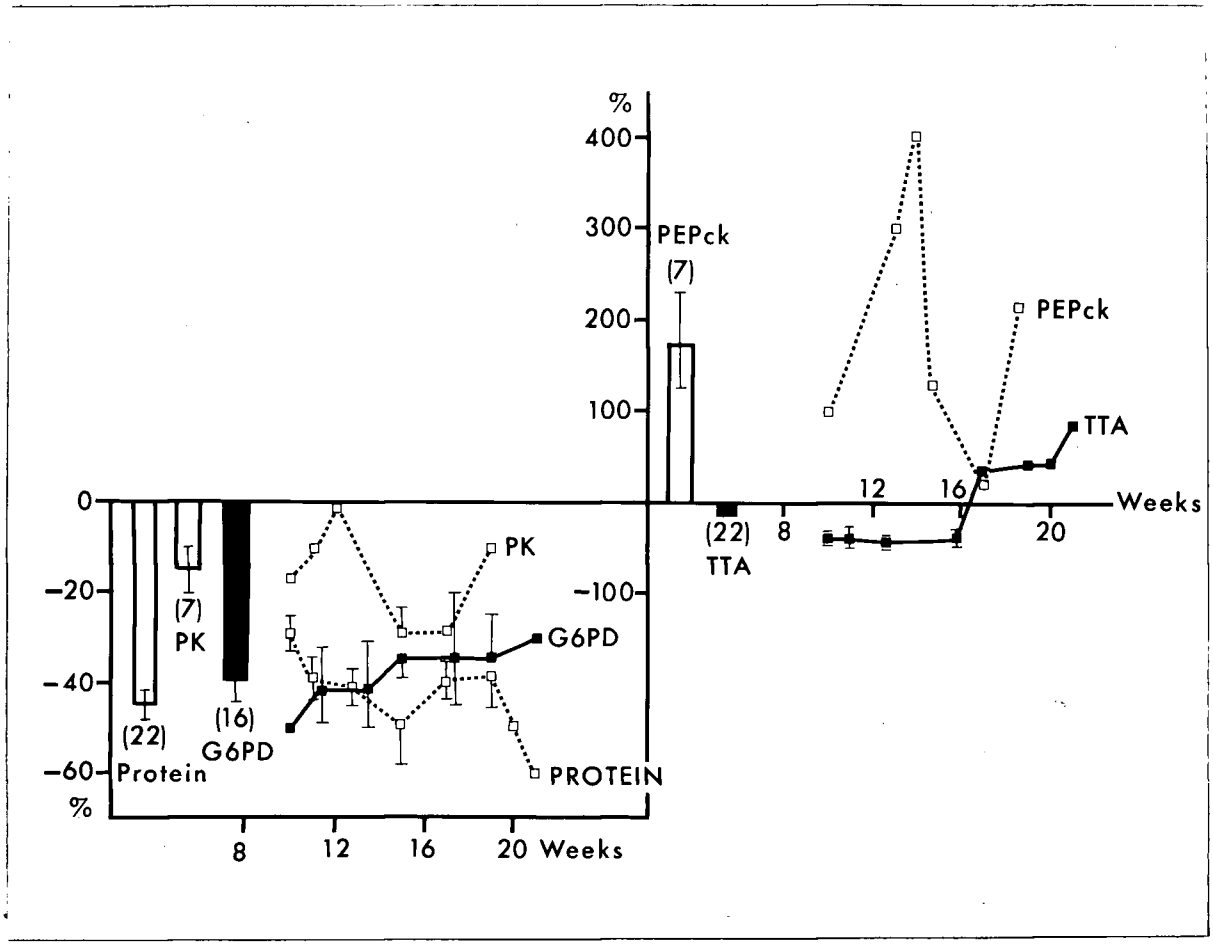


FIGURE 21: Percentage change in the level of human fetal liver cytosol PEPck, TTA, G6PD, PK, and protein after 24 hours of incubation. The changes are based on comparisons with fresh tissue (not incubated). The columns show Mean ∓ SEM values for all fetuses used. The figures in parentheses show the number of fetuses used. Abscissa: Gestational age in weeks.

TABLE IV

Comparison of Five and Forty Hour Incubation Periods Relative to Spontaneous Changes in Cytosol Enzyme Levels in Human Fetal Liver in Culture

Fetus Age (wks)	Hours of Incubation	Sample	% Change Incubated/Fresh
15.4	5	Protein	-3
		TTA	-5
		G6PD	-15
14.5	40	Protein	-77
		TTA	+152
		G6PD	no reading

TABLE V

Comparison of Five and Forty Hour Incubation Periods Relative to Induced Changes in Cytosol Enzyme Levels in Human Fetal Liver in Culture

Fetus Age (wks)	Hours of Incubation	Sample	% Change dcAMP/Control	% Change Oleic + Carnitine/Control
15.4	5	Protein	0	0
		TTA	0	-3
		G6PD	+21	+20
14.5	40	Protein	0	0
		TTA	-23	-22

The agents with the molar concentrations indicated were added for the last 5 hours of incubation. dcAMP (9×10^{-4}M); oleic acid (10^{-4}M); carnitine (5×10^{-5}M).

TABLE VI

Concentration of dcAMP and Change in the Level of Cytosol TTA in Human Fetal Liver in Culture

Fetus Age (wks)	Sample	% Change dcAMP/Control			
		10^{-4}M	9 x 10^{-4}M	5 x 10^{-3}M	10^{-2}M
14.6	TTA	3	13	28	
16.1	TTA		3	25	3

The incubation time was 24 hours with dcAMP added for the last 5 hours.

TABLE VII

Concentration of Oleic Acid and Cytosol Enzyme Change in Human Fetal Liver in Culture

Fetus Age (wks)	Sample	% Change Oleic Acid/Control 10^{-4}M	5×10^{-4}M	5×10^{-3}M	10^{-3}M
14.6	TTA		0	-1	-7
	TTA		+ 10(+car)	-13(+car)	-10(+car)
20.0	TTA	-2			-7
	TTA				-10(+car)
	G6PD	0			+30
	G6PD				+6(+car)

+car = plus the addition of 5×10^{-5}M carnitine.

The incubation time was 24 hours with oleic acid added for the last 5 hours.

TABLE VIII

Influence of Glucagon on the Level of Cytosol TTA in Human Fetal Liver Cultures

Fetus Age (wks)	% Change 10^{-4}M glucagon/control	% Change 5 x 10^{-4}M glucagon/control
10.7		25
14.5	-1	
16.1	-8	33

The incubation time was 24 hours with glucagon added for the last 5 hours.

data obtained.

The optimum concentration of dcAMP appears to be approximately 5×10^{-3}M for TTA induction. Glucagon at a concentration of 10^{-4}M decreases the level of TTA: however, a concentration of 5×10^{-4}M increases the level. The influence on G6PD is variable at both concentrations. The action of linolenate is variable for all enzymes. There is usually a decrease in TTA with all levels of oleic acid tested.

Details of the action of dcAMP with gestational age are given in Figure 22. The level of TTA increases by an average of 30% and PEPck by 66% (both are significant at $p < .01$). There is no significant change in PK or G6PD. The actions of oleic acid plus carnitine are given in Figure 23. There is no significant change in the level of TTA, PK, or G6PD. There is, however, a consistent increase in the level of PEPck; the average is 19% (Significant at $p < .05$). Figure 24 outlines the action of acetyl CoA. The influence on TTA, PK, and G6PD is negligible. The level of PEPck is consistently increased by an average of 38% (Significant at $p < .05$).

Petri Dish Incubation:

This was undertaken to observe how rapidly the enzyme changes as noted in the 24 hour cultures occurred. It is seen from Figure 25 that during 160 minutes of incubation, the protein content decreases by 16% and PK by 35%. During the same period, the TTA level increases by 2%, G6PD by 16%, and PEPck by 39%. It is important to note that the mother of this fetus had received prednisolone as previously described.

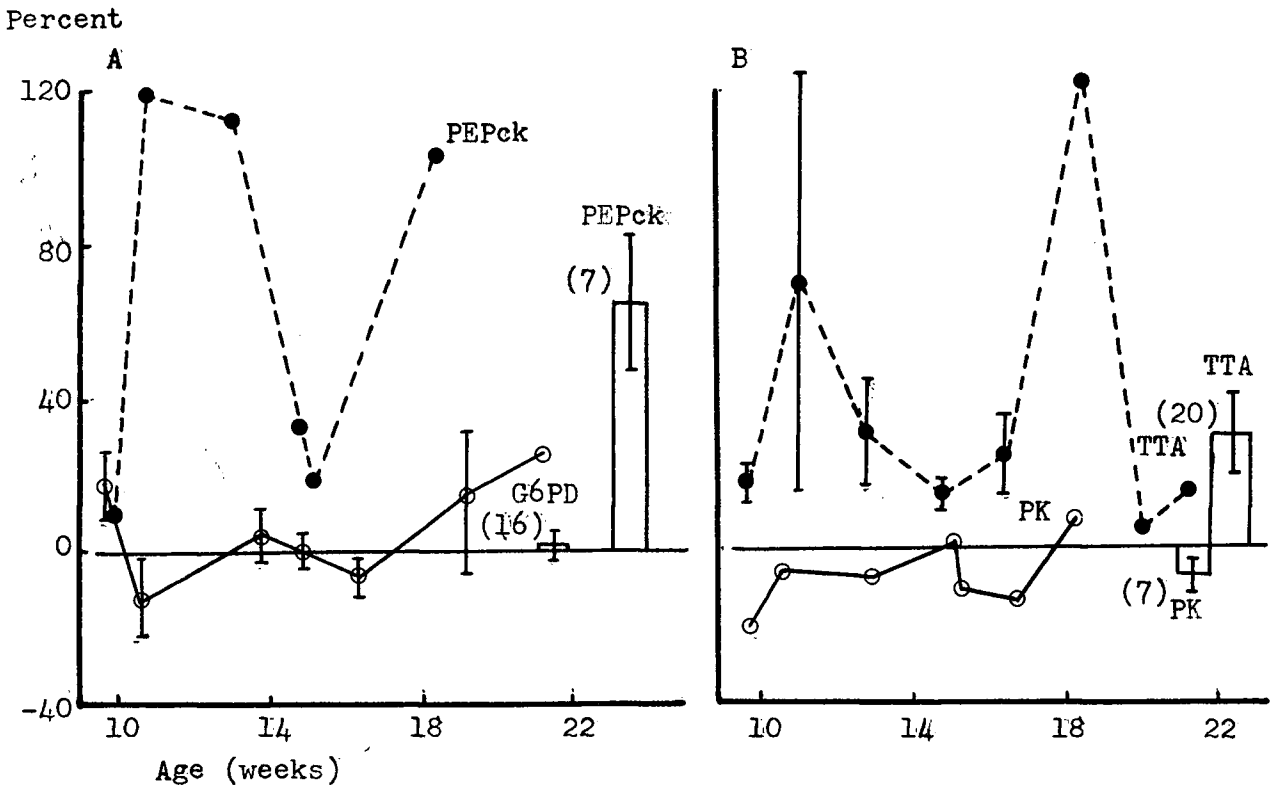


FIGURE 22: Percentage change in the level of human fetal liver cytosol PEPck and G6PD (A); and TTA andPK (B) due to the addition of dcAMP (9 x 10^{-4}M) for the last 5 hours of a 24 hour incubation period. The columns show the Mean ∓ SEM values for all fetuses. The figures in parentheses show the number of fetuses used. Abscissa: Gestational age in weeks.

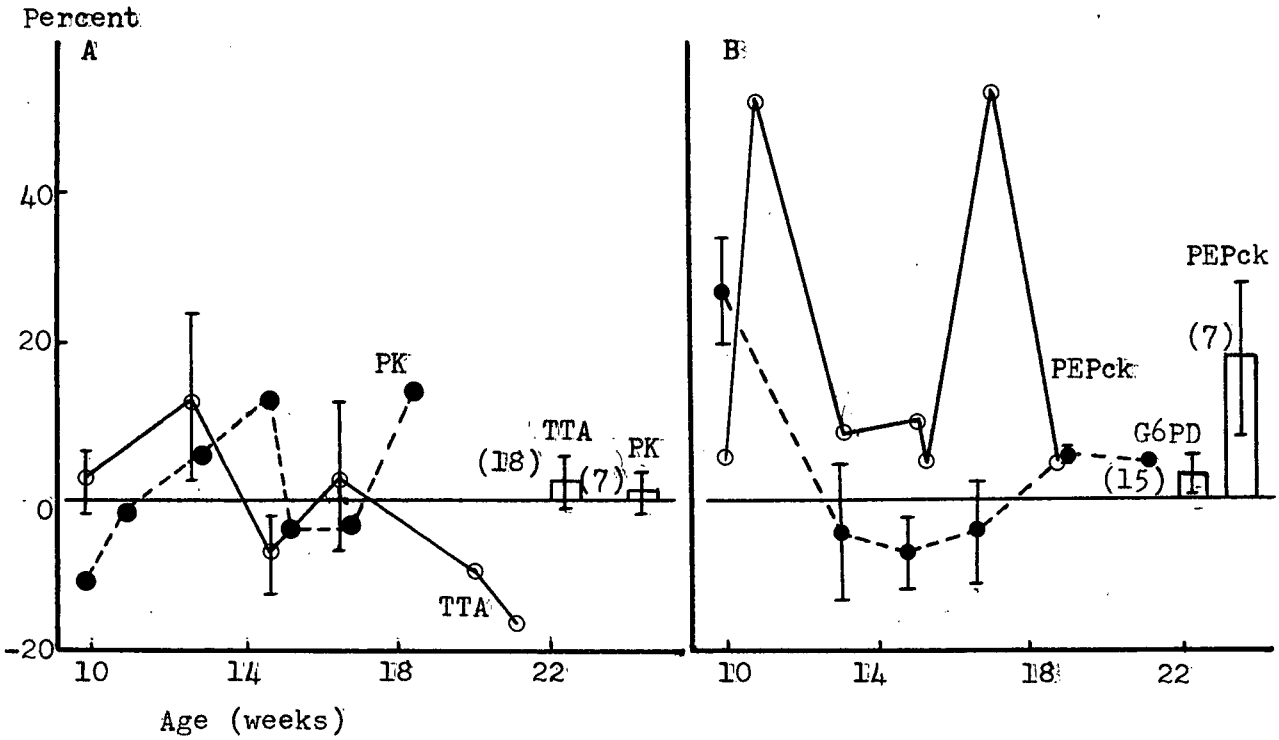


FIGURE 23: Percentage change in the level of human fetal liver cytosol TTA and PK (A); and PEPck and G6PD (B) due to the addition of oleic acid (10^{-4}M) plus carnitine (5×10^{-5}M) for the last 5 hours of a 24 hour incubation period. The columns show the Mean $\mp$ SEM values for all fetuses. The figures in parentheses show the number of fetuses used. Abscissa: Gestational age in weeks.

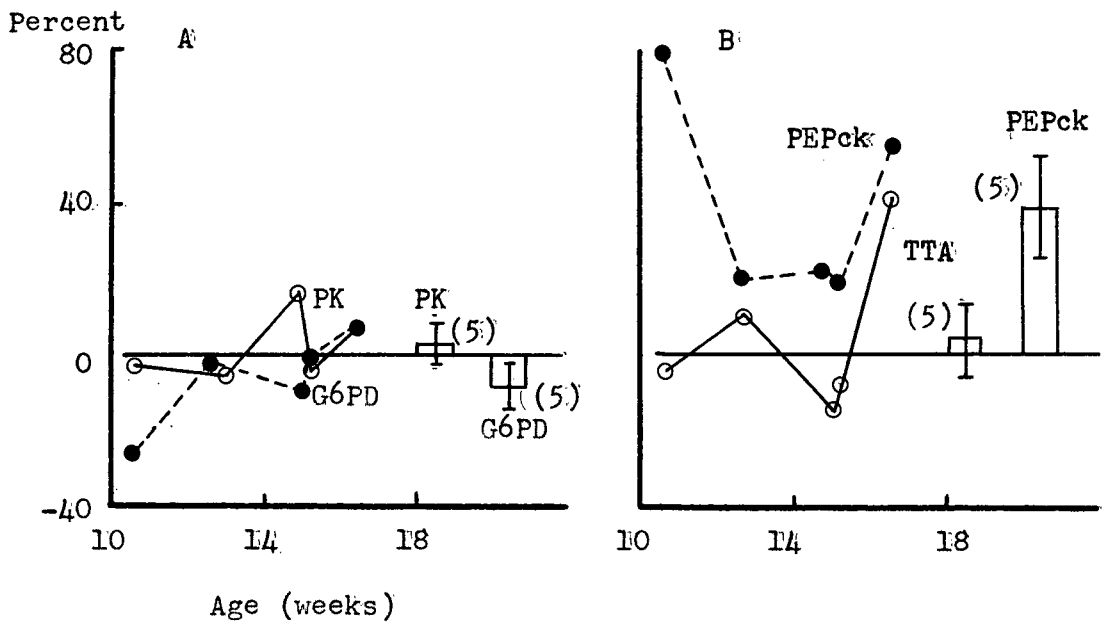


FIGURE 24: Percentage change in the level of human fetal liver PK and G6PD (A); and TTA and PEPck (B) due to the addition of acetyl CoA (8×10^{-4}M) for the last 5 hours of a 24 hour incubation period. The columns show the Mean $\pm$ SEM values for all fetuses. The figures in parentheses show the number of fetuses used. Abscissa: Gestational age in weeks.

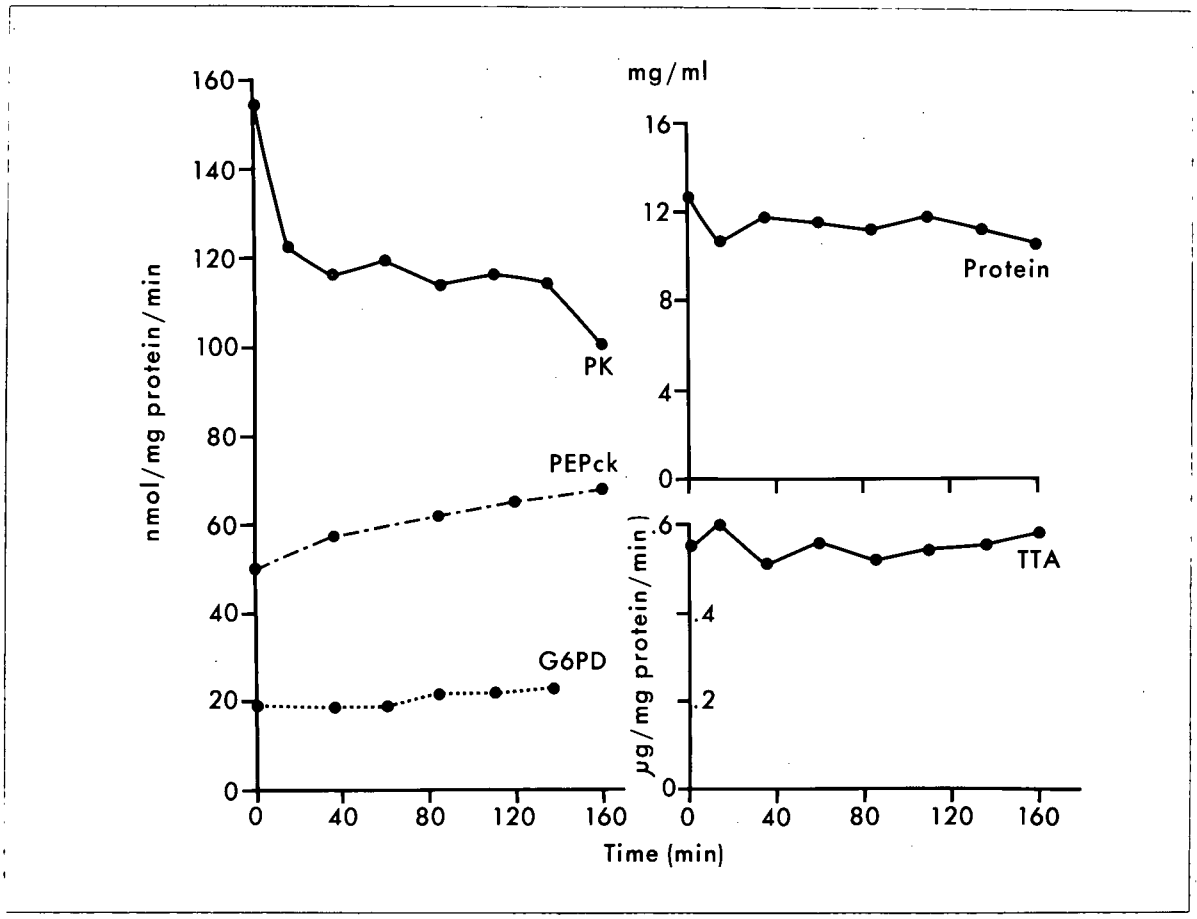


FIGURE 25: Influence of time of incubation on changes in the level of cytosol enzymes in the liver from a 12.5 week human fetus. The mother had been given prednisolone 20 mg/day for 8 days prior to the hysterotomy.

Microsomal Incubation:

The rapid rate by which the assayable level of TTA can be increased is outlined in these experiments. Figure 26 outlines the influence of acetyl CoA, dcAMP, and oleic acid on the level of TTA in the microsomal suspensions incubated for 45 minutes. There is an average increase of 245% with dcAMP, of 95% with oleic acid, and an increase of 275% (for one sample) with acetyl CoA (dcAMP is significant at $p < .01$ and oleic acid at $p < .05$).

When the ratio of cytosol enzyme to that released from the microsomes by dcAMP is calculated it is seen that there is an average of 10 times more in the 105,000 x g supernatant than was released.

In Vitro Changes in Enzyme Levels in Rat Fetal Liver

The main objectives with these experiments, as with the analogous human fetal liver studies, were to study the influence of FFA or their oxidation products on changes in liver enzyme levels, and to study the change in enzyme levels due to culturing. Glucagon and dcAMP were also tested to ensure that we could repeat results reported in the literature.

Tissue Cultures:

Spontaneous Changes - When fetal rat liver is maintained in culture the levels of some enzymes increase and others decrease. Figure 27 outlines the changes in protein, TTA, and G6PD content; Figure 28 outlines the changes in PEPck and PK contents. The protein levels consistently decrease; the longer the culture period the greater is the decrease. There is a dramatic increase

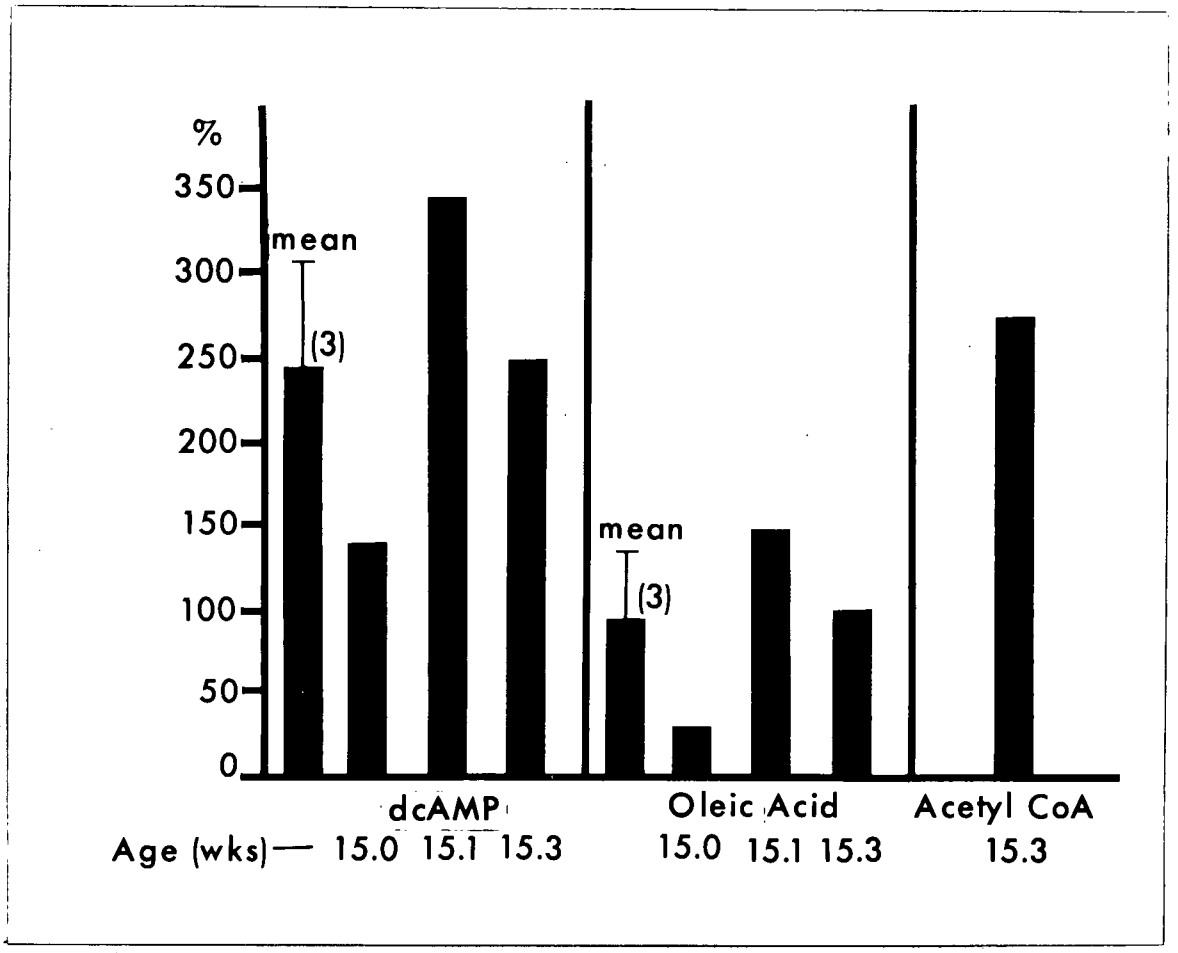


FIGURE 26: Percentage increase in the release of TTA from the human fetal liver microsomal cell fraction after 45 minutes incubation with dcAMP (9×10^{-4}M), oleic acid (10^{-4}M), or acetyl CoA (8×10^{-4}M). Age, gestational age in weeks.

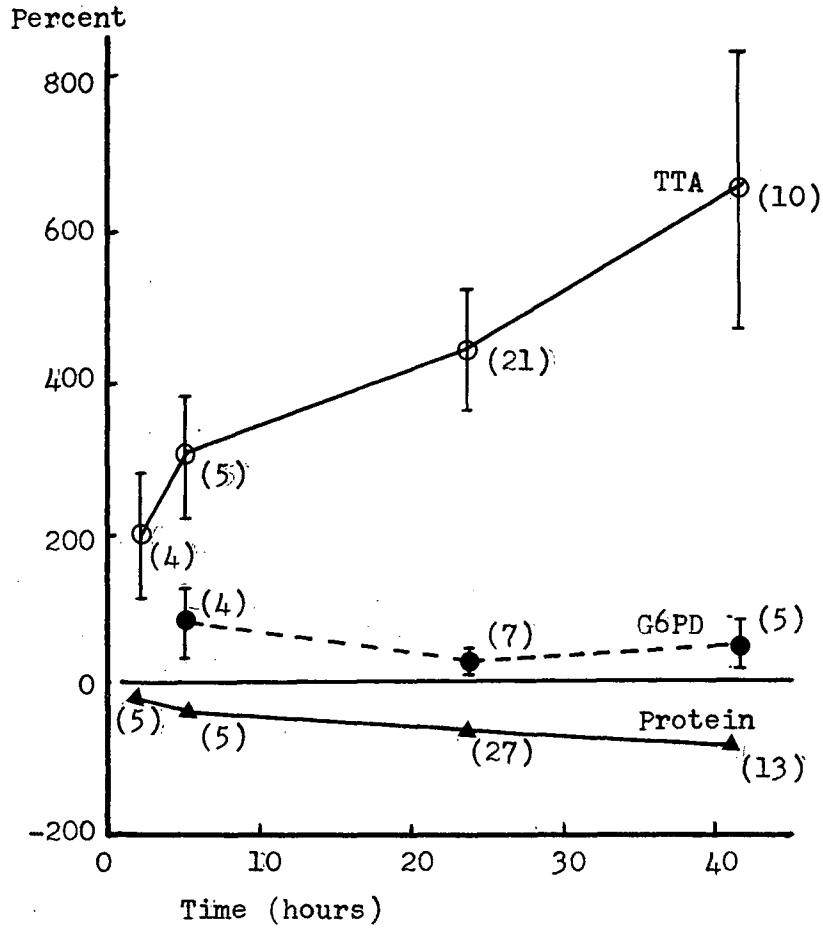


FIGURE 27: Percentage change in the cytosol protein, TTA, and G6PD levels in fetal rat liver after different periods of time in culture. The changes are based on comparisons with fresh tissue levels. Each point is the Mean ∓ SEM (vertical lines) of the number of fetuses shown in parentheses. Abscissa: Time of incubation in hours.

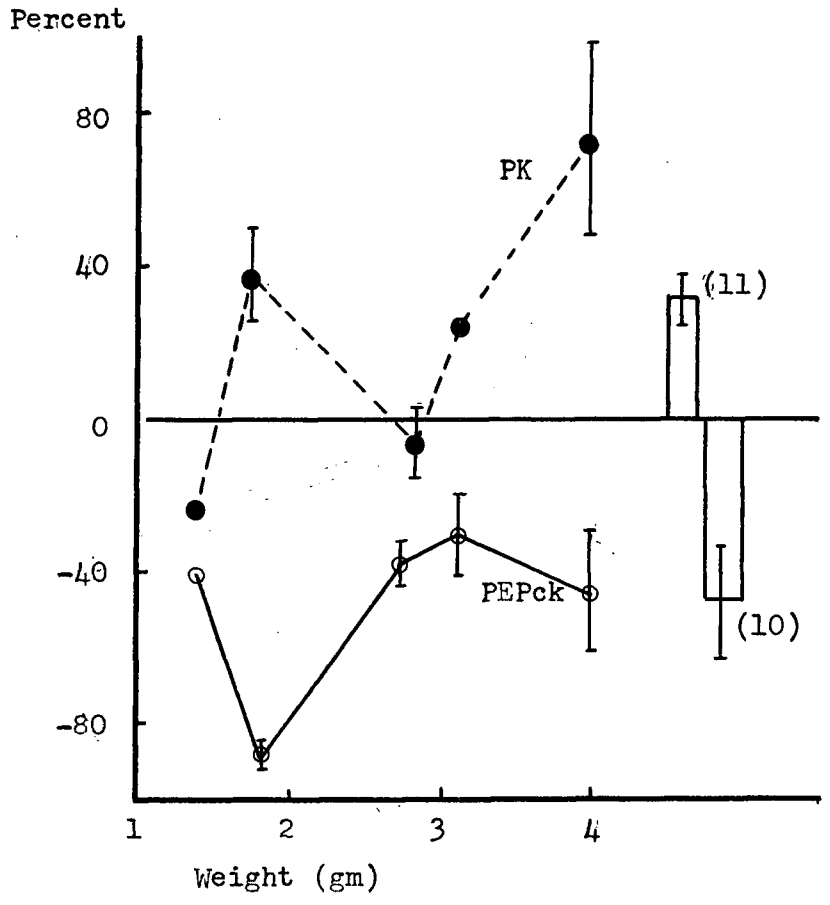


FIGURE 28: Percentage change in the cytosol PK and PEPck levels in rat fetal liver after 24 hours of incubation. The changes are based on comparisons with fresh tissue (not incubated). The columns show Mean ∓ SEM values for all fetuses. The figures in parentheses show the number of fetuses used. Abscissa: Fetus weight in gm.

in TTA; after 24 hours of incubation the TTA level increases by almost 450% (Significant at $p < .05$). After only 2 hours of culture this enzyme has increased by an average of 180% (Significant at $p < .05$). The PEPck level on the contrary decreases under culture conditions; the average decrease is 53% (Significant at $p < .01$). PK is somewhat variable in that a decrease in level occurred in some cultures and an increase in others. The average was an increase of approximately 32% (Significant at $p < .05$). The level of G6PD increased in culture. After 24 hours the increase was approximately 27%; however, this was not significant at $p < .05$.

Induced Changes - The following agents were used to test if changes in enzyme levels could be evoked: dcAMP, glucagon, acetyl CoA, acetyl carnitine, oleic acid, linolenate, and carnitine. Different concentrations of oleic acid and carnitine were tested and additions were made after various periods of culture time. Tables IX to XII outline part of the data obtained. Also, see Figures 29,30 and 31.

Oleic acid, regardless of the concentration used, usually decreases the level of TTA, PEPck, PK, G6PD, and G6Pase, i.e. all of the enzymes assayed. The addition of carnitine, at 3×10^{-4} or 10^{-3}M makes no significant difference. Linolenate lowers the level of TTA. Studies were not undertaken on the effectiveness of dcAMP or glucagon since these had been considered in detail by Wicks (37,40).

The influence of the various agents on the level of TTA can be seen in Figure 29. dcAMP increased TTA by only 6% for

TABLE IX

Influence of dcAMP on the Change in the Level of Cytosol TTA in Fetal Rat Liver Cultures

Fetus Wt. (gms)	Hours of Culture	% Change Control/Fresh	% Change dcAMP/Control
.34	24	183	15
.36	24	494	38
1.39	24	85	379
1.72	24	250	97
1.87	24	740	97
1.87	42	396	417
2.41	5	114	6
2.78	42	--	355
3.08	24	208	124
3.09	42	495	710
3.14	24	837	742
	24	465	65
4.00	24	-3	4
4.52	42	503	7
4.82	24	37	21
5.57	42	428	29

dcAMP (9×10^{-4}M) was added for the last 5 hours of the incubation period.

TABLE X

Influence of Acetyl CoA and Acetyl Carnitine on Cytosol Enzyme Changes in Fetal Rat Liver Cultures

	TTA % Change		PEPck % Change		PK % Change		G6PD % Change	
Fetus Wt.(gm)	*ACoA/C	AC/C	ACoA/C	AC/C	ACoA/C	AC/C	ACoA/C	AC/C
1.36	+2		+3		+10			
1.60		+23		-40		-17		
1.72	+118		0		+7		+17	
2.75		+53				+31		
2.86		+16				0		0
3.08	-55	-47			+6	+6		
3.98	-5		-12		-8			
Mean	15.0	11.2	-4.5		3.7	5.0		
∓ S.E.M.	∓ 36.6	∓ 21.0	∓ 8.8		∓ 4.0	∓ 9.9		

The incubation time was usually 24 hours with acetyl CoA (8×10^{-4}M) or acetyl carnitine (9×10^{-4}M) added for the last 5 hours.

*ACoA/C = acetyl CoA/control; AC/C = acetyl carnitine/control

TABLE XI

Concentration of Oleic Acid and Cytosol Enzyme Changes In Fetal Rat Liver Cultures

	% Change Oleic Acid/Control		
Sample	10^{-4}M	5 x 10^{-4}M	10^{-3}M
TTA	-17.80 ∓ 5.58(5)	18.25 ∓ 44.37(4)	4.50 ∓ 9.92(4)
G6PD	-14.00 ∓ 5.18(8)	-7.33 ∓ 1.34(3)	-18.00 ∓ 7.99(5)
G6Pase	- 6.78 ∓ 4.22(9)	-7.50 ∓ 8.00(4)	- 6.20 ∓ 6.64(5)

The incubation period was usually 24 hours with oleic acid added for the last 5 hours. The values are Means ∓ S.E.M. with the figures in parentheses showing the number of fetuses used. The fetuses were 1 to 3 days prior to term.

TABLE XII

Oleic Acid Vs Carnitine Vs Oleic Acid Plus Carnitine and Cytosol Enzyme Changes in Fetal Rat Liver Cultures

	% Change Test Agent/Control		
Sample	10^{-4}M Oleic Acid	$3x10^{-4}$M Carnitine	Oleic + Carnitine
TTA	-17.80 ∓ 5.58(5)	1.33 ∓ 5.95(3)	-9.18 ∓ 7.28(11)
PEPck	-51.00 ∓ 3.49(4)	--	-44.14 ∓ 8.64(7)
PK	0.00 ∓ 2.00(3)	--	7.33 ∓ 5.34(10)
G6PD	-14.00 ∓ 5.18(8)	-1.00 ∓ 5.69(3)	-4.60 ∓ 6.33(5)
G6Pase	-6.78 ∓ 4.22(9)	-8.71 ∓ 2.34(7)	-28.00 ∓ 5.92(9)

The incubation period was usually 24 hours with oleic acid or carnitine added for the last 5 hours. The values are Means ∓ S.E.M. with the figures in parentheses showing the number of fetuses used. The fetuses were 1 to 3 days prior to term.

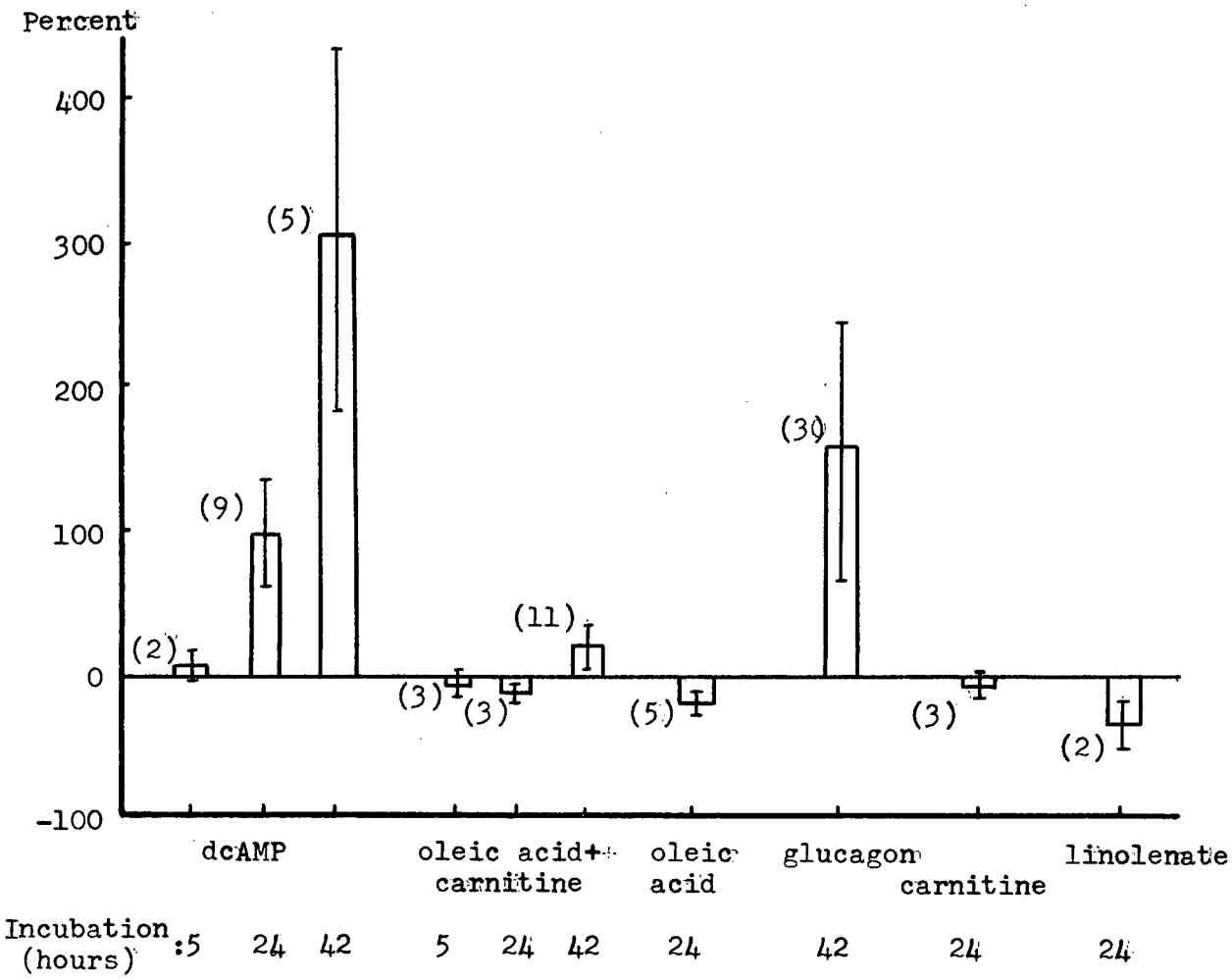


FIGURE 29: Percentage change in the cytosol TTA level in rat fetal liver (1 to 3 days prior to term) due to the addition of dcAMP (9×10^{-4}M), oleic (10^{-4}M), carnitine (5×10^{-5}M), or linolenate (10^{-4}M) for the last 5 hours of the incubation periods indicated. The columns show Means $\mp$ SEM (vertical lines) with the number of fetuses in parentheses.

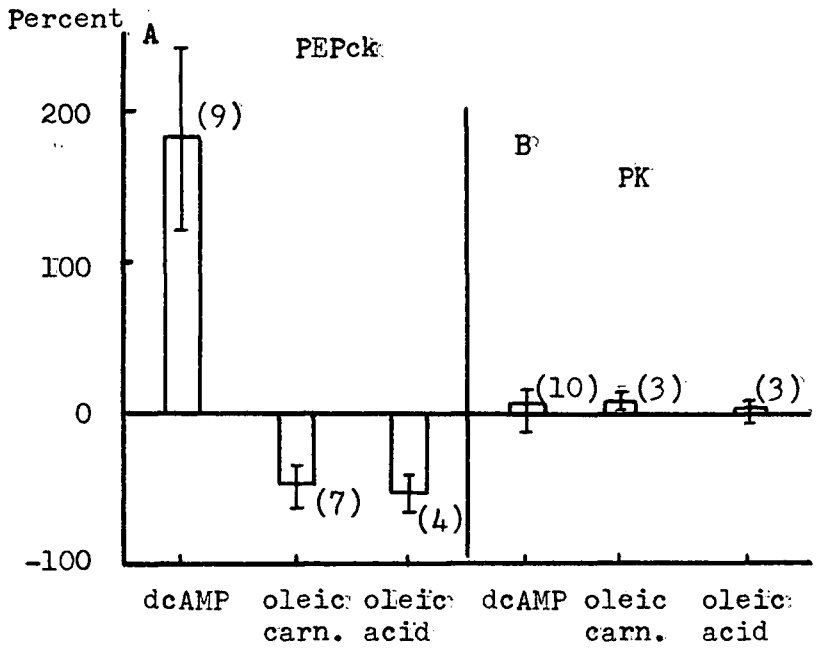


FIGURE 30: Percentage change in the level of cytosol PEPck (A) and PK (B) in rat fetal liver (1 to 3 days prior to term) due to the addition of dcAMP (9×10^{-4}M), oleic acid (10^{-4}M), or oleic acid plus carnitine (5×10^{-5}M) for the last 5 hours of a 24 hour incubation period. The columns show the Means $\pm$ SEM (vertical lines) with the number of fetuses in parentheses.
Oleic carn., oleic acid plus carnitine.

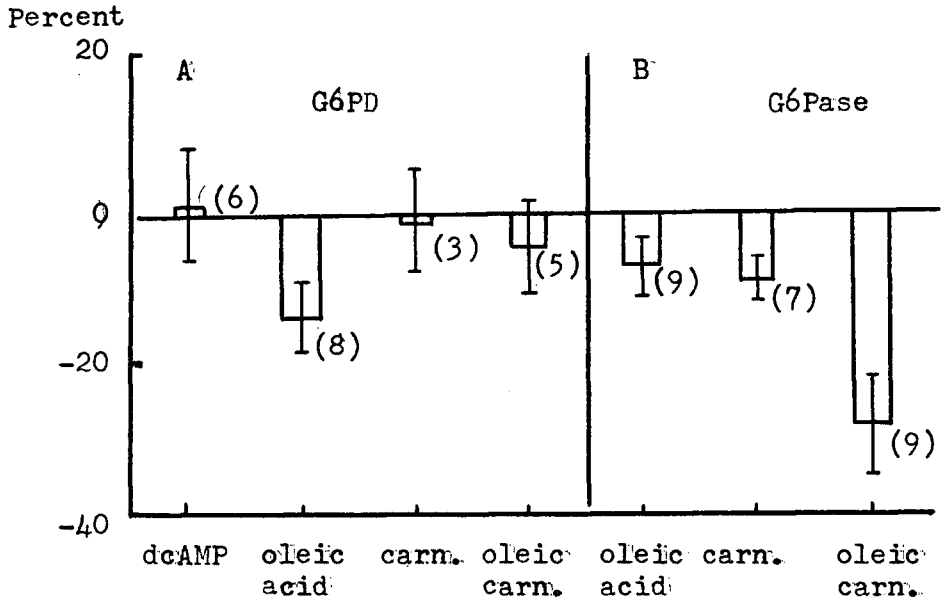


FIGURE 31: Percentage change in the level of cytosol G6PD (A) and G6Pase (B) in rat fetal liver (1 to 3 days prior to term) due to the addition of dcAMP (9×10^{-4}M), oleic acid (10^{-4}M), carnitine (5×10^{-5}M), or oleic acid plus carnitine for the last 5 hours of a 24 hour incubation period. The columns show the Means $\mp$ SEM with the number of fetuses used in parentheses.
Oleic carn., oleic acid plus carnitine.

5 hour cultures, by 98% for 24 hour cultures, and by over 300% for 42 hour cultures (both the 98% and the 300% values are significant at $p \leq .05$). Details of the action of dcAMP are given in Table IX. Two interesting observations arise from these data. Firstly, two fresh liver samples had very high TTA levels, viz. 1.54 and 1.30 µg pHPP/mg protein/minute. During 24 hours of culture the enzyme level decreased by 3% in one sample and increased by only 37% in the other. dcAMP elevated the TTA level by a relatively small amount in each sample. Secondly, there appears to be a gestational age (weight) range in which dcAMP has its greatest relative influence, viz. 1.37 to 3.14 gm.

As indicated in Figure 30, PEPck was increased by 178% by dcAMP (Significant at $p < .01$). This enzyme level is significantly reduced by both oleic acid and oleic acid plus carnitine by 51% and 44% respectively (both are significant at $p < .01$). PK and G6PD were variable, there being no significant change in either with any agent (see Figures 30 and 31). Oleic acid plus carnitine inhibited G6Pase by 28%; this is significant at $p < .01$ (see Figure 31). Neither acetyl CoA nor acetyl carnitine had any significant influence on any of the enzymes assayed (see Table X).

Two inhibitors of FFA oxidation, viz. deoxycarnitine and citral (in the form of citral sodium bisulfite), were tested in the cultures. Citral caused a consistent increase, by an average of 57%, in TTA and an average decrease of 13% in the PEPck level (both are significant at $p < .05$). The influence on PK was variable. When citral was combined with dcAMP, the results were

variable for PEPck; however, the action of dcAMP on TTA was at least partially inhibited (see Figure 32). Citral caused an average increase of 57% in the level of TTA and dcAMP caused a 150% increase. Citral plus dcAMP resulted in only a 96% increase in the level of TTA.

The results with deoxycarnitine were extremely variable. If the only samples of PEPck considered are those where dcAMP dramatically increases the level of this enzyme, i.e. by 300% or more, then deoxycarnitine does partially inhibit the action of dcAMP. Deoxycarnitine causes an 18% decrease in the level of PEPck, dcAMP causes a 406% increase, and dcAMP plus deoxycarnitine causes a 218% increase. Deoxycarnitine was also partially effective in inhibiting the action of dcAMP on TTA. Deoxycarnitine caused a 64% increase in the TTA level and dcAMP a 114% increase. Deoxycarnitine plus dcAMP caused only a 106% increase in TTA.

Petri Dish Incubation:

These short term incubations were undertaken to determine how rapidly the level of cytosol enzymes change when fetal liver is placed in culture. Sereni and Sereni (34) reported that the level of TTA in fetal rat liver may increase up to 5 fold during 72 hours of culture; however, their first measurement was not made until 12 hours after the start of the incubation and they did not indicate if any change occurred in the protein level of the tissue.

The increase in the TTA level appears to depend on the stage of development of the fetus (see Figure 33). This confirms

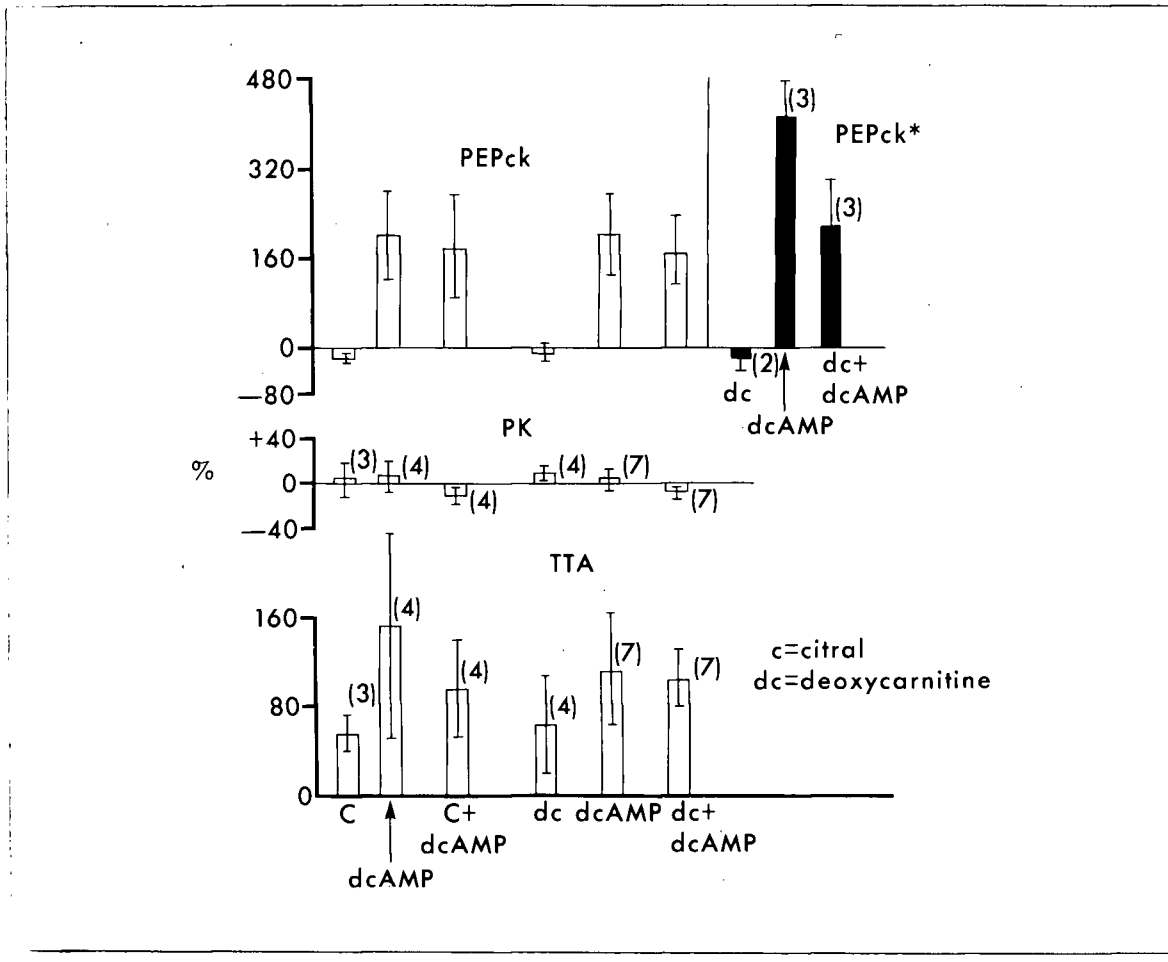


FIGURE 32: Influence of deoxycarnitine (3×10^{-4}M), citral (3×10^{-5}M), and dcAMP (9×10^{-4}M) on the percentage change in cytosol TTA, PEPck, and PK in fetal rat liver (1 to 3 days prior to term). The tissues were incubated for 24 hours with the agents added for the last 5 hours. The columns show Means ± SEM (vertical lines) with the number of fetuses in parentheses. c, citral; dc, deoxycarnitine.

* Samples where dcAMP increased PEPck by at least 300%.

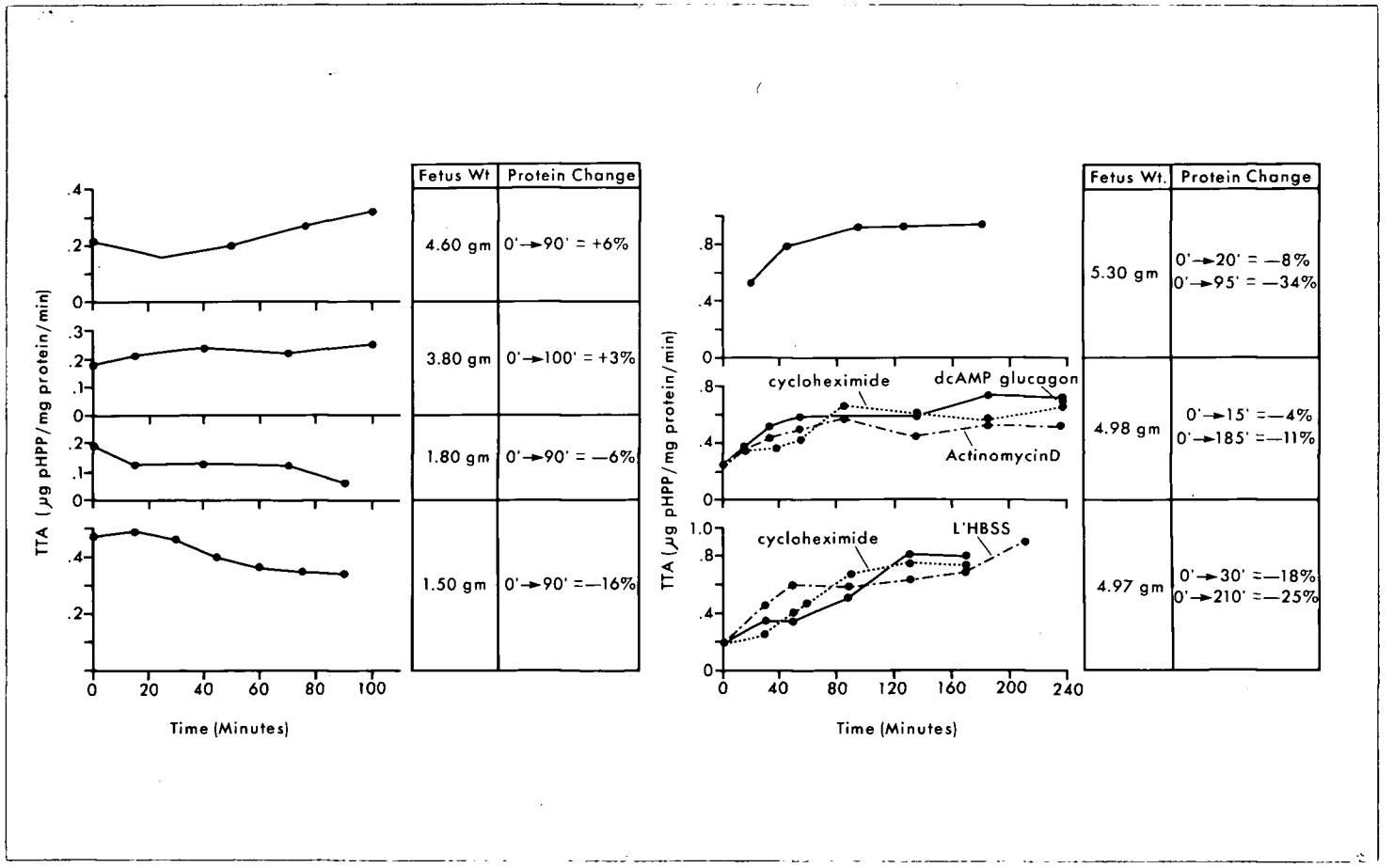


FIGURE 33: Influence of time of incubation on changes in the level of fetal rat liver cytosol TTA. Note the examples of the use of actinomycin D (8×10^{-5}M) and cycloheximide (3×10^{-5}M). Also note the example of the use of LHBSS in place of the standard Eagle's MEM+HBSS as medium. dcAMP (9×10^{-4}M) and glucagon (10^{-4}) were added to two separate samples of the 4.98 gm fetus liver tissue at the start of the experiment. After 240 minutes the TTA levels were essentially no different than the controls.

the same finding by Sereni and Sereni (34). In young fetuses, i.e. the 1.5 and 1.8 gm animals, there was a decrease in TTA during 90 minutes of incubation; whereas, in fetuses 3.80 to 5.30 gm (term) there was an increase in level ranging from 110% to 300%. The maximum protein decrease was 34%; the decrease was 25% for the sample in which the TTA increased by 300%. The actions of cycloheximide and actinomycin D were variable. Both agents initially caused lower levels of TTA. With cycloheximide there was then an increase of enzyme relative to the control with a later decrease. Actinomycin D caused consistently lower enzyme levels; however, this difference was only barely detectable until after two hours of incubation. There were higher enzyme levels between time 0 and 80 minutes when LHBSS was substituted for the medium usually used, viz. Eagle's MEM + HBSS. After this period of time the reverse occurred. Lastly, neither dcAMP nor glucagon had any significant influence on the level of TTA during 240 minutes of incubation.

The enzymes PEPck, PK, G6PD, and G6Pase were assayed in three different experiments (see Figure 34). The protein levels remained relatively constant during the 100 minutes of incubation. PEPck decreased by a factor of 10 in the youngest fetus and by a factor of 5 in the two older fetuses. The G6Pase level remained constant in the youngest fetus and decreased by a factor of approximately 2 in the other fetuses. The PK level approximately doubled during the 100 minutes of incubation. The G6PD increase ranged from 21% for the 3.80 gm fetus to 40% and 65% respectively for the 4.60 and 1.80 gm animals.

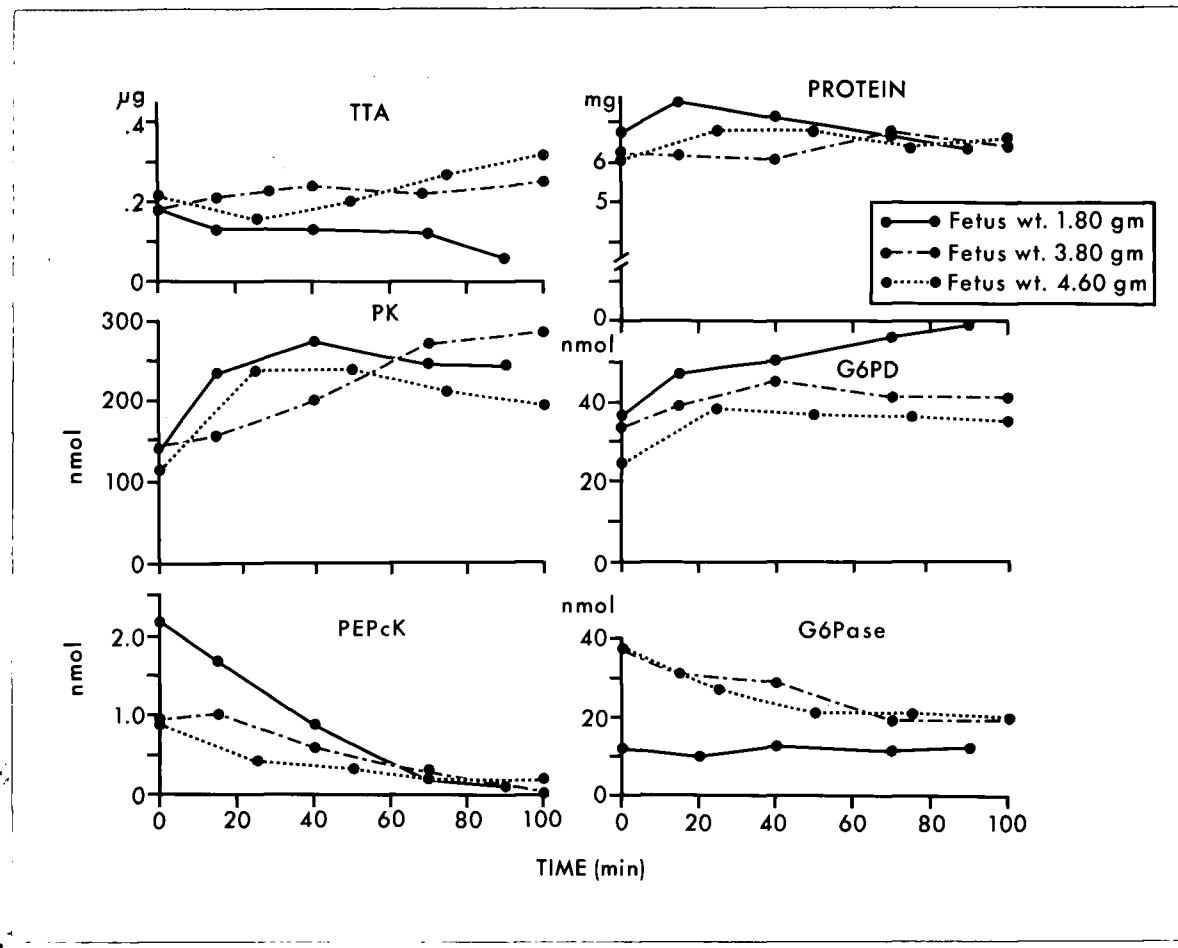


FIGURE 34: Influence of time of incubation on changes in the level of fetal rat liver protein, TTA, PK, PEPck, G6PD, and G6Pase. The ordinate for PK, PEPck, G6PD, and G6Pase is n moles/mg protein/min; for TTA is μg pHPP/mg protein/min; and for protein is mg/ml.

Microsomal Incubation:

The rapid rate by which the assayable level of TTA can be increased is outlined in these experiments. Figure 35 illustrates the influence of the agents dcAMP, oleic acid, and acetyl CoA on the level of TTA in microsomal suspensions incubated for 45 minutes. These compounds resulted in enzyme increases of 101%,48%, and 39% respectively. (dcAMP is significant at $p < .01$; oleic acid and acetyl CoA are significant at $p < .05$). For three of the fetuses the ratios of cytosol enzyme, i.e. the 105,000 x g supernatant TTA, to that released from the microsomes by the addition of dcAMP were calculated. The ratio for the 4.00 gm fetus was 3.5:1, for the 2.88 gm fetus was 1.5:1, and for the 4.00 gm fetus was 6:1. A greater quantity of TTA was, therefore, always present in the cytosol than could be released by the addition of dcAMP to the microsomal suspension.

Comparison of Human and Rat Liver Enzyme Changes In Vitro

To facilitate a comparison of human and rat liver enzyme levels, the mean values, which were previously noted, have been assembled in Tables XIII and XIV. The spontaneous change for all enzymes is opposite in the human and rat with the exception of TTA in fetal material older than 16.7 weeks of gestation.

dcAMP causes a substantially greater increase in the level of TTA and PEPck in rat liver than in human liver. Oleic acid plus carnitine stimulated a 19% increase in PEPck in the human; whereas, it caused a 44% decrease in this enzyme level in the rat (Significant at $p < .05$ and $p < .01$ respectively).

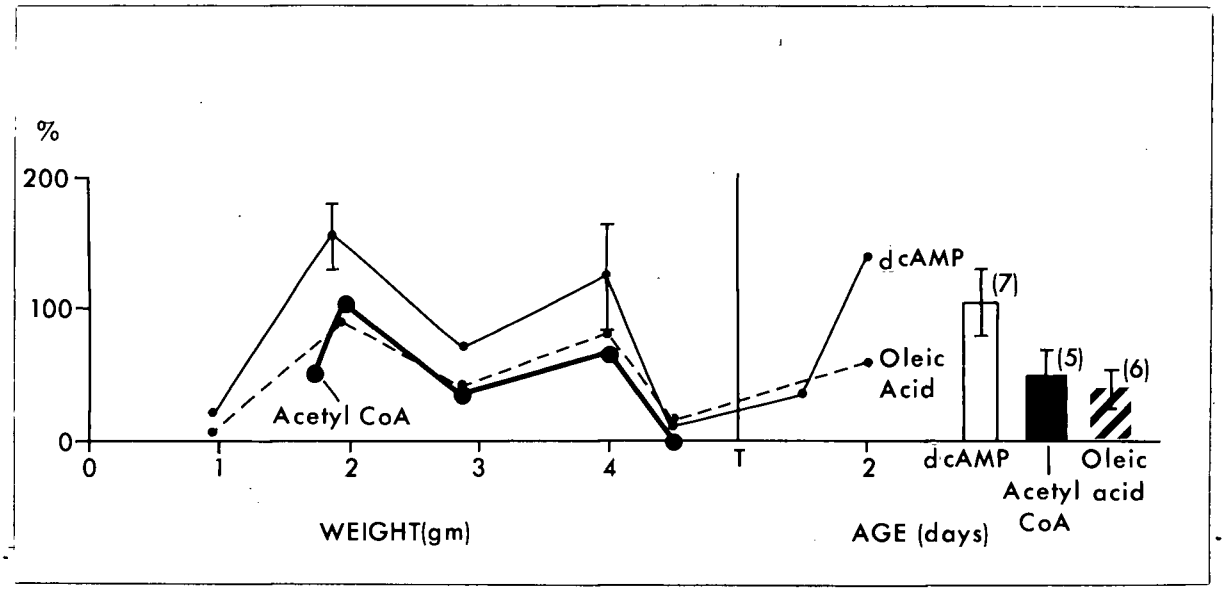


FIGURE 35: Percentage increase in the release of TTA from the rat fetal liver microsomal cell fraction after 45 minutes incubation with dcAMP (9×10^{-4}M), oleic acid (10^{-4}M), or acetyl CoA (8×10^{-4}M). The columns show the Mean ∓ SEM values for all animals used. Abscissa: Weight in gm (fetal) or age in days (postnatal). T, term.

TABLE XIII

Comparison of Human and Rat Fetal Liver Cytosol Enzyme Changes in 24 Hour Cultures

Enzyme	Test Agent	% Change Test Agent/Control Human	Rat
Protein	spontaneous	-42.9 ∓ 2.9(22)**	-59.2 ∓ 2.3(27)**
TTA	spontaneous	Δ -13.3 ∓ 9.5(20)	439.4 ∓ 78.3(21)**
PEPck	spontaneous	+172.4 ∓ 55.1(7)**	-52.7 ∓ 9.1(10)**
PK	spontaneous	-13.1 ∓ 4.4(7)*	31.8 ∓ 11.4(11)*
G6PD	spontaneous	-39.1 ∓ 4.6(16)**	26.9 ∓ 15.5(7)
TTA	dcAMP	30.2 ∓ 8.0(20)**	98.2 ∓ 37.8(9)*
	0 + c	1.7 ∓ 4.2(18)	-9.2 ∓ 7.3
	acetyl CoA	4.8 ∓ 10.0(5)	15.0 ∓ 36.6(4)
PEPck	dcAMP	66.1 ∓ 18.1(7)**	178.4 ∓ 58.2(9)**
	0 + c	19.3 ∓ 8.5(7)*	-44.1 ∓ 8.6(7)**
	acetyl CoA	37.5 ∓ 12.8(5)*	-4.5 ∓ 8.8(2)
PK	dcAMP	-6.1 ∓ 3.5(7)	3.0 ∓ 5.3(10)
	0 + c	1.0 ∓ 3.6(7)	7.3 ∓ 2.2(3)*
	acetyl CoA	2.4 ∓ 4.1(5)	3.8 ∓ 4.0(4)
G6PD	dcAMP	1.88 ∓ 4.1(16)	0.33 ∓ 7.6(6)
	0 + c	1.00 ∓ 3.8(15)	-4.6 ∓ 6.3(5)
	acetyl CoA	-6.4 ∓ 5.4(5)	--

The test agents were added for the last 5 hours of the incubation period. dcAMP (9×10^{-4}M), oleic acid (10^{-4}M), carnitine (5×10^{-5}M), and acetyl CoA (8×10^{-4}M). The values are Means ∓ S.E.M. with the figures in parentheses showing the number of fetuses used. The human fetuses were 8 to 21 weeks gestational age and the rat fetuses 1 to 3 days prior to term. 0 + c = oleic acid + carnitine.

Δ (6.8 to 16.5 weeks gestation -30.0 ∓ 6.3(16)
(16.7 to 26.2 weeks gestation +53.5 ∓ 13.6(4)

* Significant at $p < .05$ relative to the control

** Significant at $p < .01$ relative to the control

TABLE XIV

Comparison of Human and Rat Liver TTA Changes in Microsomal Incubations

Enzyme	Test Agent	% Change Test Agent/Control	
		Human	Rat
TTA	dcAMP (9×10^{-4}M)	245.0 ∓ 59.3(3)**	101.4 ∓ 24.5(8)**
	oleic acid (10^{-4}M)	93.3 ∓ 34.9(3)*	39.0 ∓ 16.1(6)*
	acetyl CoA (8×10^{-4}M)	276.0 ∓ 0.0(1)	47.6 ∓ 14.8(5)*

The incubation period was 45 minutes with the test agents added for the full period. The values are Means ∓ S.E.M. with the figures in parentheses showing the number of fetuses used.
* Significant at $p < .05$ relative to the control.
** Significant at $p < .01$ relative to the control.

Acetyl CoA caused a significant increase (38%) in the level of PEPck in the human; however, in the two rat samples assayed there was a decrease in the level of PEPck when acetyl CoA was added to the culture medium.

All agents, viz. dcAMP, oleic acid, and acetyl CoA caused an increase in the level of TTA in incubated microsomal suspensions. The percentage increase in the assayable TTA was at least twice as great in the human in all samples (see Table XIV).

DISCUSSION

Tissue Culture System

We were unable to establish a tissue culture system as is conventionally used, i.e. placing pieces of tissue on rafts or grids so that only the lower surface is in contact with the medium and then placing the dishes with the tissue in an incubator with a circulating gas phase (37,72,76), since the incubator facilities were simply not available. Therefore, a closed system was established. This system, using 250 ml disposable flasks with 2.5 ml of medium, proved to be successful for the short term 24 hour cultures required. The tissue under histological analysis remained satisfactory in appearance during this period and the spontaneous change, e.g. up to a 5 fold increase in rat liver cytosol TTA, as well as the induced change in enzyme levels indicated active cell metabolism.

It was not necessary to add agents such as fetal calf serum, which again is conventional at least for long term cultures (76), and we found it unnecessary to increase the glucose level of the medium as has been done by Wicks (37) and Räihä (72). To maintain the pH, however, the bicarbonate level was doubled. The consistent decrease in pH with Eagle's MEM plus HBSS attests to the rapid metabolism of the liver tissue.

Electron micrographs would be required for a detailed study of the structural changes that occur in the tissue after different periods in culture. This type of study has been undertaken by Trump et al. (92), and Goldblatt et al. (93) with 10 to 14 week old mouse tissue. Their system consisted of maintaining 1 to 3 mm

liver slices in a closed sterile bottle at 37°C. The tissue rested on glass rods which in turn were placed on filter papers moistened with 0.85% saline. The authors found that plasma membrane changes could be noted as early as 15 minutes after the start of their experiments; there was also a loss of mitochondrial matrix granules within this period. They concluded that the hepatic cells die sometime between the onset of incubation and 24 hours. Räihä et al. (72) reported that they were unable to successfully maintain postnatal rat liver in culture. No other successes are known by the author for the maintenance of postnatal liver tissue.

Wicks (37) has reported, for his grid and circulating gas system with fetal rat liver, that during 2 days of culture there is a) a change in tissue color from red to tan, b) a dramatic loss of erythrocytes (these have been washed into the medium), c) a loss of cytoplasmic vacuolisation (perhaps due to decreased glycogen), and d) a loss of hematopoietic elements. Wicks states, however, that the hepatocytes remain healthy even after 52 hours in culture. The TTA level approximately doubled during 48 hours of culture; the author states that this also suggests that the hepatocytes are viable since blood cells are devoid of this enzyme. Räihä et al. (72), using a system similar to that of Wicks, reported that during 72 hours of culture the hepatocytes in both human and rat fetal liver did not change in appearance; however, there was a loss of erythrocytes and hematopoietic cells. Neither Wicks nor Räihä and his coworkers outline any data to indicate the degree of protein decrease between fresh and cultured material; however, Wicks states that a decrease does occur.

It appears that the protein loss (or apparent loss) may be due to a) dilution because of the diffusion of culture medium into the tissue, b) red cell and thus hemoglobin removal, c) serum washout, and/or d) liver parenchymatous and blood-forming tissue autolysis. The specific percentage decrease due to each of these is not known.

With respect to the culture system, one is able to conclude that it is relatively simple, inexpensive, and successful.

Human Fetal Liver and Kidney Basic Enzyme Data - A Comparison with Rat and Pig Liver

As was noted in the Introduction, very little is known about the change in enzyme levels with gestational age in the human. In an attempt to at least partially solve this problem we have collected data on cytosol protein, PEPck, TTA, PK and G6PD for the liver and kidney from 7 to 21 weeks gestational age. We also have data on the mitochondrial PEPck levels during this period. Important factors in choosing the liver and kidney were, that they are the main gluconeogenic organs in the human, they provide sufficient material to enable a number of enzyme determinations to be undertaken, and the liver has been intensively studied in the rat thus enabling us to compare the human and rat. Also, before we could undertake a study of enzyme induction in human fetal liver the basic enzyme changes with age had to be known.

There is a very marked decrease in the cytosol level of TTA in both the human fetal liver and kidney between 9 and 21 weeks of gestation. The decrease in the liver, for example, is by a factor of 13. The specific reason for this change is not known.

As with rat liver, this enzyme increases in level quite noticeably after birth (15); again, the reason is not known.

In contrast to the results of Diesterhaft et al. (16) for adult human liver and by Ballard and Hanson (58) for fetal rat liver, data from this thesis study indicate that the cytosol fraction of human fetal liver has a much greater quantity of PEPck than does the mitochondrial fraction. This ratio, however, decreases with gestational age so that by term, or at least in the postnatal period, the almost equal cytosol-mitochondrial distribution found by Diesterhaft for adults may be achieved. Söling et al. (50) suggest that both cytosol and mitochondrial PEPck are involved in gluconeogenesis in the adult guinea pig; this is in contrast to the adult rat which does not have the mitochondrial enzyme. The human thus appears to be similar to the guinea pig in this respect.

The changes in enzyme levels in rat liver during the perinatal period have been well documented (9,12,14,35,90). The results from this thesis work, for the enzymes studied, are comparable to those in the literature.

The fetal levels of liver cytosol protein, TTA, PK, and G6PD are quite similar in human, pig, and rat. The levels of PEPck are also similar in the human and rat; however, the fetal pig value is about 7 times greater. One could speculate, therefore, that the process of gluconeogenesis is more active in the fetal pig than in the human or rat. Also, the increase in the level of this enzyme at birth in the pig is only 65%, which confirms the finding of Tildon et al. (87); whereas, it increases

in the rat by a factor of 20 which is similar to the finding of Ballard and Hanson (58). In the human liver, PEPck increases by a factor of 4 at birth according to Räihä and Lindros (94).

There was no significant change in the PK level in the human liver or kidney during the period of gestation studied; also, there was no significant change in the kidney G6PD. The liver G6PD does, however, appear to peak prior to 9 weeks of gestation with a lesser peak at 19 to 20 weeks. According to Mino et al. (89) this enzyme is at its maximum level at approximately 21 weeks of gestation then decreases until term. These authors indicate that the adult level is approximately the same as the level at term.

The Mechanism(s) of Enzyme Induction with Emphasis on Free Fatty Acids and their Oxidation Products

The mechanisms by which the level of enzymes such as PEPck and TTA increase so dramatically at birth in the rat and to a lesser degree in the human are still unknown. The blood hormone levels, e.g. glucagon, corticosterone, and epinephrine, as well as substrate levels, e.g. glucose and FFA are also known to change at birth (5,6,9,17,43).

Most enzyme induction or at least activation is thought to involve cAMP as well as hormones and substrates. The mechanisms by which these agents interact is a key problem. Ballard (90) and Greengard (35), for example, have suggested that the termination of the external glucose supply at birth may result in increased glucagon release which in turn could cause an increase in cAMP resulting in increased glycogenolytic and gluconeogenic enzyme levels or activation. Williamson et al. (49) have suggested, from work with adult rat liver, that FFA are involved

in the activation of at least some of the gluconeogenic enzymes. Exton et al. (48), however, believe that FFA are not significant in the regulation of hepatic gluconeogenesis.

This thesis study was undertaken to determine if FFA or their oxidation products are involved in the change in the level or activation of some enzymes involved directly or indirectly in glucose metabolism and to determine to what degree the results of enzyme induction studies in fetal rat liver are applicable to human fetal liver. Rat liver has been the main mammalian organ used in enzyme induction studies. We used both human and rat fetal liver. Our experimental approach was primarily through the use of tissue cultures; the advantages and disadvantages of the various in vivo and in vitro approaches were previously outlined in the Introduction.

Spontaneous Enzyme Changes:

There were a number of changes in enzyme levels simply due to culturing human fetal liver. The possible reasons for the protein decrease were previously discussed. It is difficult to state to what degree the protein change influenced the TTA, PEPck, PK, and G6PD levels. Erythrocyte PK and G6PD activities are relatively high; however, there is no detectable TTA or PEPck in these cells. PK, G6PD, and TTA (in fetuses 9 to 16 weeks gestational age) activities decreased in culture; whereas, PEPck increased as did TTA (but only in fetuses greater than 16.5 weeks). Perhaps the decrease in PK and G6PD was partly due to the loss of red blood cells. The only other study, to our knowledge, which reports a change in human liver enzyme levels due to culturing is by Räihä et al. (72). They found that TTA

in fetuses 14 to 24 weeks increased by approximately 40% during 40 hours in culture. We observed an inverse relationship between the fresh tissue level of TTA and the change in culture. This enzyme is at a low level in the fresh tissue of older fetuses; at this time the potential exists for an increase to occur due to culturing. This potential is not present in younger tissue due perhaps to inactive receptors and/or adenyl cyclase.

We found that the level of cytosol PEPck almost trebled in 24 hour human fetal liver cultures. The significance of this will be discussed later.

As with human liver, the protein levels consistently decreased in fetal rat liver in culture. In rat cultures, unlike human cultures, the level of TTA dramatically increased during 24 hours of incubation. This more than 5 fold increase is initiated almost immediately, at least for fetuses near term, after the tissue is placed in culture medium (see Figure 33). Sereni and Sereni (34) have reported on the spontaneous increase in this enzyme due to culturing. These authors hypothesise that a repressor is in some way released; this is perhaps logical since none of the inducing agents are effective until the tissue has been in culture for a number of hours. Wicks (37) has reported that the TTA level approximately doubled in fetal rat liver during 48 hours of culture. He did not discuss this in detail except to state that it was an indication of hepatocyte viability. Again, unlike the human enzyme, cytosol PEPck in fetal rat liver consistently decreased when placed in culture; the average decrease was 53% after 24 hours. PK and G6PD increased in level by approximately 30% in 24 hour rat cultures.

From a comparison of human and rat fetal liver enzyme levels in culture it appears that in human tissue, gluconeogenesis, i.e. glucose formation, was stimulated. Two main enzymes concerned with glucose utilization, viz. PK and G6PD, increased in level in rat liver; whereas, PEPck which is associated with the formation of glucose decreased. The opposite occurred in cultures of human fetal liver in that G6PD decreased; whereas, PEPck increased.

Induced Enzyme Changes:

PEPck: The induction of PEPck in rat fetal liver cultures by catecholamines, glucagon, and cAMP has been well established by Wicks (33,39,40); we have confirmed these results using dcAMP. The action of the hormones is thought to be mediated by cAMP; however, there is no direct evidence for this. Many workers have suggested that the increased level of PEPck at birth may initiate the capacity for gluconeogenesis in the rat; the same has been suggested for the human (1,33). Räihä and Lindros (94) reported that the PEPck level in human liver 2 days after birth is about 4 times greater than that between 3 to 7 months of gestational age. The adult level is approximately 10 times greater. The importance of PEPck is also underlined by the fact that F1,6DPase is approximately 50% of the adult level by 16 weeks of gestation and PC is two-thirds of the adult level by this age (94). These enzymes, therefore, should not be rate limiting. Gennser et al. (95) indicate that the G6Pase level in liver from fetuses 13 to 21 weeks gestational age is approximately 20% of that in the adult; thus, this is also perhaps a key enzyme in the regulation

of glucose formation. In their studies of clamping the umbilical cord for a period of 10 minutes in women undergoing therapeutic abortions, Gennser and his coworkers found that the fetal liver glucose level almost doubled in this period; glycogen, phosphorylase, and G6Pase levels did not significantly change. Villee (96,97), from incubation studies with liver slices involving C^{14} pyruvate has suggested that the gluconeogenic pathway may exist in human fetal liver at least after 12 to 15 weeks of gestation.

No other workers, to our knowledge, have studied human fetal liver PEPck induction in vitro and we know of no reports of chemical agents influencing this enzyme in the fetus while in utero. As well as the 3 fold increase in PEPck in human liver due to culturing, as previously noted, we were able to increase the level by an additional 66% with dcAMP. Thus, relative to the fresh tissue approximately a 4.5 fold increase in this enzyme was achieved.

Our data indicate that the ability of the human fetus to initiate gluconeogenesis may exist long before term. This could be very important in that a decrease in the maternal supply of glucose to the fetus could be compensated for by the fetus producing its own supply at least for a short period. Further support for the proposal that the level of PEPck can be greatly increased in the human fetus was obtained from a study of the liver from a fetus the mother of whom had received prednisolone for 8 days prior to abortion. The PEPck level was 33 times higher than normal. This finding also underlines the dramatic side effects that agents given to a mother can have on the fetus.

Cytosol PEPck was increased in human liver cultures by adding oleic acid plus carnitine or acetyl CoA; the former increased the level by 19% and the latter by 38%. The role of acetyl CoA as an activator in the PC reaction in gluconeogenesis is well known (32). It is possible that acetyl CoA acted directly at this point and FFA indirectly through the production of acetyl CoA in β-oxidation. There are reports that acetyl CoA will not readily cross cell membranes (8); perhaps the concentration used in this thesis study was great enough to partially overcome this problem.

In contrast to human tissue, oleic acid plus carnitine resulted in a decrease in the level of PEPck by an average of 44% in rat fetal liver cultures. Acetyl CoA had no significant influence. The reason for the positive action of oleic acid and acetyl CoA in human tissue and the lack of this in rat tissue may reside in the degree of activity of the carnitine transferases. Hahn and Skala (98) and Augenfeld and Fritz (56) have reported that the activities of acetylcarnitine and acylcarnitine transferase are very low in rat fetal liver and increase rapidly after birth. Hahn (75) has determined that the activity of these enzymes in 10 to 20 week human fetal liver is relatively much higher than that found in the fetal rat. These enzymes are involved with FFA oxidation.

Another approach to the study of the action of FFA on enzyme induction is through the use of inhibitors. We found that citral, an inhibitor of β-oxidation of FFA, had no significant influence on PEPck induction in fetal rat liver cultures. Deoxycarnitine, an inhibitor of carnitine transferases, inhibited the effect of dcAMP in rat liver by almost 50%; however, this only occurred with

samples in which dcAMP caused at least a 300% increase in the enzyme, thus the significance of the result is questionable.

TTA: This has perhaps been the most intensively studied enzyme in induction work with fetal rat liver. It increases in level dramatically at birth and has been induced in culture by glucocorticoids, catecholamines, glucagon, and cAMP (33,34,37, 40,72). We have also confirmed these resulted with glucagon and dcAMP. As with PEPck, the mechanism of induction of TTA is not known; however, cAMP is thought to be intimately involved (33).

Kretchmer et al. (15) reported a 4 fold increase in TTA in human liver at birth and an additional 4 fold increase in the adult. The only report of induction experiments in tissue culture is by Räihä et al. (72). They had success with only one agent, viz. triamcinolone; this was with a 28 week fetus where TTA increased 4 fold.

We were able to increase the level of TTA by an average of 30% when dcAMP was added to liver cultures of fetuses 9 to 21 weeks gestation. The reason for this partial success with dcAMP in contrast to the lack of success reported from Räihä's laboratory may have been due to the difference in the culturing systems. Also, Räihä and his coworkers cultured their tissue for at least 60 hours and added the inducers for the last 18 hours. It will be recalled that our tissues were incubated for 24 hours with the inducers added for the last 5 hours.

The increased level of fetal rat liver TTA was approximately 100% when dcAMP was added to the cultures during the last 5 hours of a 24 hour incubation period. No increase occurs in 1 to 4 hour incubations; this confirms Wicks' (37) report that the

tissue must be in culture for a number of hours before induction by agents such as cAMP will occur. Both Wicks (40) and Sereni and Sereni (34) have suggested that a number of hours in culture may be required to remove or inactivate an inhibitor.

The reason for the more pronounced influence of dcAMP on rat tissue than on human material may reside in the state of development of the liver. Rat tissue had completed at least 80% of its gestational period; whereas, the human tissue had completed only 25 to 50% of its gestation. The fact that Räihä et al. (72) had a 4 fold TTA increase in a 28 week fetus further supports this speculation. The TTA level in the liver from the fetus exposed to prednisolone, which as previously noted had a 33 fold increase in PEPck, was 47% higher than the average normal fetus for the same age. Perhaps the rather modest increase was a result of the very early stage of development of the fetus, viz. 12.5 weeks.

There was no significant change in the TTA level when either FFA or acetyl CoA was added to tissue cultures of human or rat liver; however, two inhibitators of FFA oxidation, viz. citral and deoxycarnitine, partially inhibited the effect of dcAMP on TTA induction in rat tissue. Thus, FFA and/or acetyl CoA may have some influence on the level of active TTA in liver. Further support for this conclusion was obtained from in vitro experiments with the microsome-polysome fraction of both human and rat fetal liver.

Chuah and Oliver (29) have reported that it is possible to stimulate the release of TTA from preloaded polysomes in 1 and 2 day old rat livers by incubating the microsome-polysome cell

fraction at 37°C for 45 minutes with cAMP. We have confirmed these results using dcAMP and have also observed, for fetal rat liver, that there is a 2 fold increase in active TTA with dcAMP and approximately a 50% increase with acetyl CoA or oleic acid. The ratio of normal cytosol TTA to that released by dcAMP is approximately 5 to 1. TTA can also be released from the human fetal liver microsomal fraction by the action of dcAMP, acetyl CoA, or oleic acid. Assayable TTA is increased in this fraction by about 3.5 fold for both dcAMP and acetyl CoA and by almost a factor of 2 for oleic acid. The quantity of enzyme is low compared with that normally found in the cytosol, viz. approximately one-tenth using dcAMP; however, this could be significant as a reserve of enzyme which is very readily made available with the proper stimulus. In human and rat fetal liver cAMP and FFA (probably mainly in the form of acetyl CoA) appear to function in the release of TTA from the microsomal cell fraction.

From studies undertaken in connection with this thesis, involving both tissue and microsomal incubations, there is a definite indication that initially release of TTA from the microsomal cell fraction occurs followed by de novo enzyme synthesis. In support of this concept, Wicks (33) has demonstrated fairly conclusively, for rat fetal liver, that there is new enzyme synthesis mediated by cAMP and Chuah and Oliver (29) have presented evidence for the release of TTA from the microsome-polysome fraction in postnatal animals. The process of rapid enzyme release followed by de novo synthesis may be very important at birth when a dramatic increase in TTA occurs.

PK: This enzyme begins to decrease in level in rat liver at or slightly before term. It increases after weaning to the adult level (13,14). Weber et al. (54,55) using an adult rat liver cell-free system, found that FFA and acetyl CoA inhibit PK. We found no significant influence of FFA, acetyl CoA, or dcAMP on fetal human or rat liver PK in culture. It is logical that the activity of this enzyme must be reduced if gluconeogenesis is to occur since under normal conditions the activity ratio of PEPck to PK was approximately .010 for our human fetal livers and .015 for the rat livers. An explanation for the apparent lack of inhibition in our system could be that the inhibition is lost when the tissue is homogenised and enough time had not elapsed during the 5 hour induction period for a significant decrease in the PK level to occur.

The PK level in the 12.5 week human fetus which had been exposed to prednisolone for 8 days in utero was 30% lower than the level in normal fetuses of the same approximate age. This decrease is rather insignificant when compared with the 33 fold PEPck increase; however, it is the greatest change we have observed that has been caused by a PEPck inducing agent.

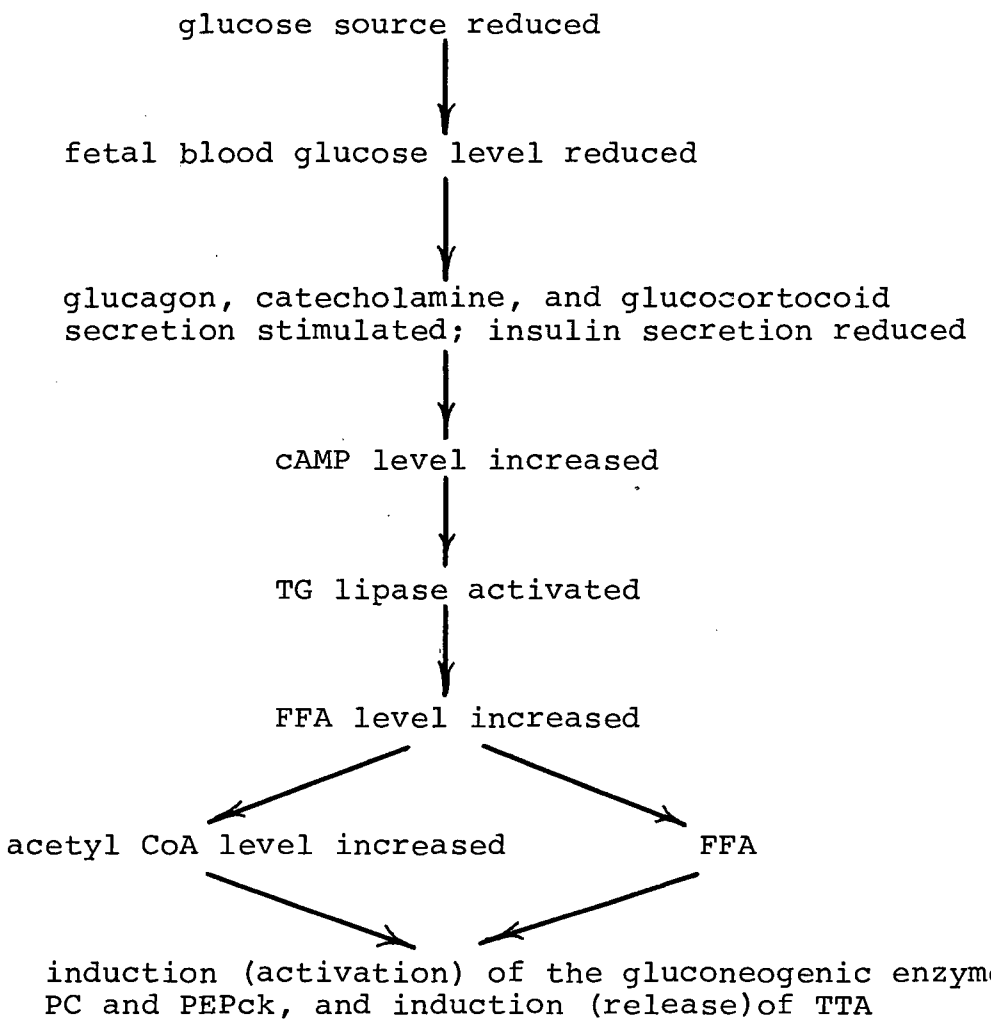
G6PD: This enzyme begins to decrease in level in rat (14) and human (89) liver before term. G6PD is at its lowest level in adult animals. We found no significant influence of dcAMP, acetyl CoA, or FFA on this enzyme in either human or rat cultures; however, the human fetus exposed to prednisolone, which has been previously described, had a 36% lower G6PD level than normal.

Rudak et al. (65) recently reported that either dcAMP or glucagon injected every 6 hours over a 2 day period inhibits the in vivo 4 fold increase in liver G6PD which occurs when adult rats are fed a high carbohydrate diet. Thus, it appears that the enzyme can be inhibited at least in vivo. It is difficult to speculate on the reason why the fetal liver enzyme in vitro was not inhibited; perhaps the tissues were not exposed to dcAMP for a sufficient period of time.

FFA and Acetyl CoA: These agents appear to be involved in the induction of the gluconeogenic pathway at least in human fetal liver. Fetal rat liver enzymes did not respond positively to FFA or acetyl CoA in our experiments; however, as has been reported by Williamson et al. (49) there may be a response in adult tissue. In contrast to human fetal liver, rat fetal liver has very low levels of carnitine transferases; the levels, however, increase rapidly at birth (56,98). This would be ample reason for the lack of response in the fetal rat.

Both FFA and acetyl CoA caused TTA to be released from the microsomal fraction of human and rat fetal liver. It is difficult to speculate on the need for increased levels of this enzyme; however, perhaps gluconeogenesis is indirectly involved since transamination is one of the reactions in providing carbon skeletons for the production of glucose from amino acids.

One mechanism of induction of the gluconeogenic process, at least in the human fetus, appears to be as follows:



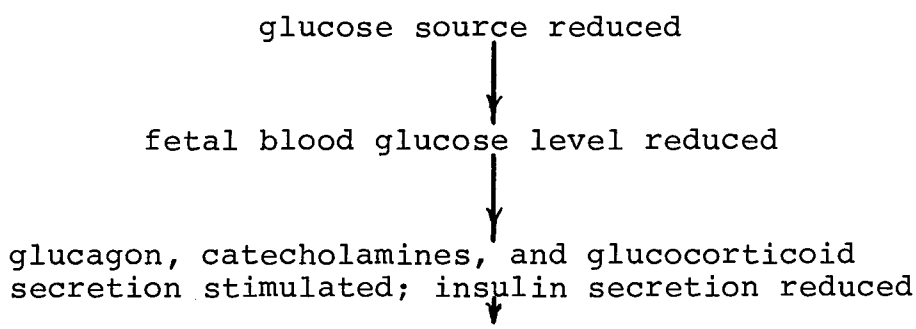
FFA and/or acetyl CoA may, therefore, be involved with the increase in the level of TTA, PEPck, and PC at birth when the external glucose source for the fetus is terminated.

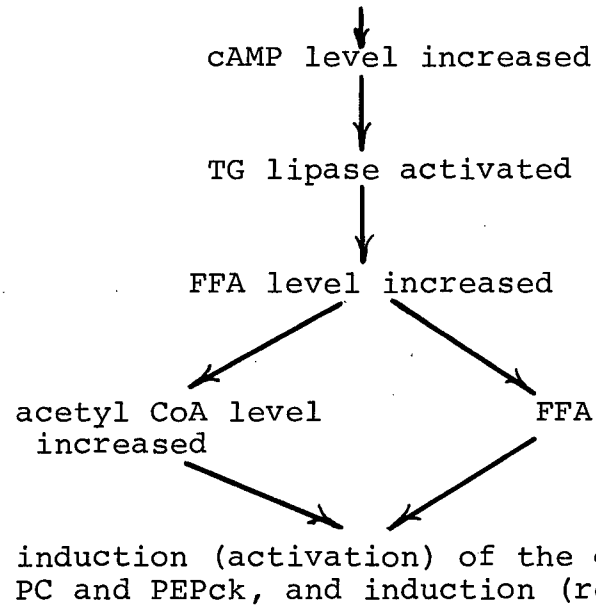
SUMMARY

As was outlined in the Introduction, our objectives were (a) to determine if FFA or their oxidation products are involved in the induction of enzymes, mainly TTA and PEPck, in human and rat fetal liver, (b) to determine if it is possible to induce enzymes in fetal human liver as it is in rat liver, and (c) to collect as much basic enzyme data as possible on the changes in enzyme levels in human fetal liver and kidney during gestation.

Our experimental approach was mainly through the use of tissue cultures. We established a culture system whereby it is possible to study the spontaneous or induced change in enzyme levels in human and rat fetal liver segments. The system is short term, i.e. of approximately 24 hours duration, and can be established with a minimum of facilities.

FFA or acetyl CoA caused increased levels of PEPck in human fetal liver. Also, from work with inhibitors of FFA oxidation, viz. deoxycarnitine and citral, there was an indication that the inducing effect of dcAMP on fetal rat liver cytosol PEPck and TTA can be partially blocked with these agents. One mechanism of induction of the gluconeogenic process, at least in the human fetus, appears to be as follows:





FFA and/or acetyl CoA may, therefore, be involved with an increase in the level of TTA and PEPck at birth when the external glucose source for the fetus is terminated.

We also found that it was possible to release TTA from the microsomal cell fraction of human and rat fetal liver with dcAMP, acetyl CoA, and oleic acid. This released enzyme was approximately 10% of the quantity normally present in human liver cytosol and approximately 20% of that present in rat liver cytosol. The suggestion is made that a reserve of TTA is available for rapid release; this is later complemented by de novo enzyme synthesis. This process may be very important at birth when a rapid dramatic increase in the level of TTA occurs.

It was possible to increase the level of the cytosol enzymes PEPck and to a lesser extent TTA in human fetal liver as it was in fetal rat liver. PEPck was increased in vitro approximately 4.5 fold and TTA by about 30%. The importance of the induction of these enzymes to fetal gluconeogenesis is stressed.

We observed that there was a 33 fold increase in the PEPck level in a fetus which had been exposed to the glucocorticoid prednisolone *in utero*. The importance of this induction to the gluconeogenic process is noted; also the importance of the possible side effects of therapeutic agents given to a mother on enzymes in the fetus is stressed.

As gestational age increased from 10 to 21 weeks, there was approximately a 14 fold decrease in the cytosol level of TTA in the fetal human liver and kidney. There was a 3 fold decrease in liver cytosol PEPck between 10 and 13 weeks gestational age; this enzyme level then remained relatively constant. The ratio of liver or kidney cytosol to mitochondrial PEPck was 25 to 1 in the tenth week of gestation and decreased to 6 to 1 by the eighteenth week. Thus, with increasing gestational age the mitochondrial enzyme appeared to increase in significance. The levels of kidney and liver cytosol PK and G6PD did not significantly change from 10 to 21 weeks of gestation.

Lastly, comparisons between the fetal liver enzymes TTA, PEPck, PK, and G6PD in the human, pig, and rat indicated that the levels were similar for all except PEPck; this enzyme was approximately 7 times greater in pig than in the human or rat.

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APPENDIX I

Source of Chemical Agents

S = Sigma Company SM = Schwarz-Mann EK = Eastman-Kodak

C = Calbiochem O = Otuska (Osaka) NEN = NEN Chemicals

albumin (C)

acetyl CoA (S)

α-ketoglutaric acid (C)

ADP (C)

Citral (EK)

cycloheximide (C)

dcAMP (S)

deoxycarnitine (S)

d-carnitine (O)

dl-carnitine (O)

Fiske-Subbarow reagent (S)

glucagon (C)

GSH (C)

G6P (C)

IDP (S)

imidazole (EK)

l-carnitine (O)

LDH (C)

linolenate (C)

MDH (C)

acid molybdate solution (S)

NADP (C)

NADH (C)

$NaHCO_3$ (C^{14}) (C-Atomic)

PEP (S)

PK (C)

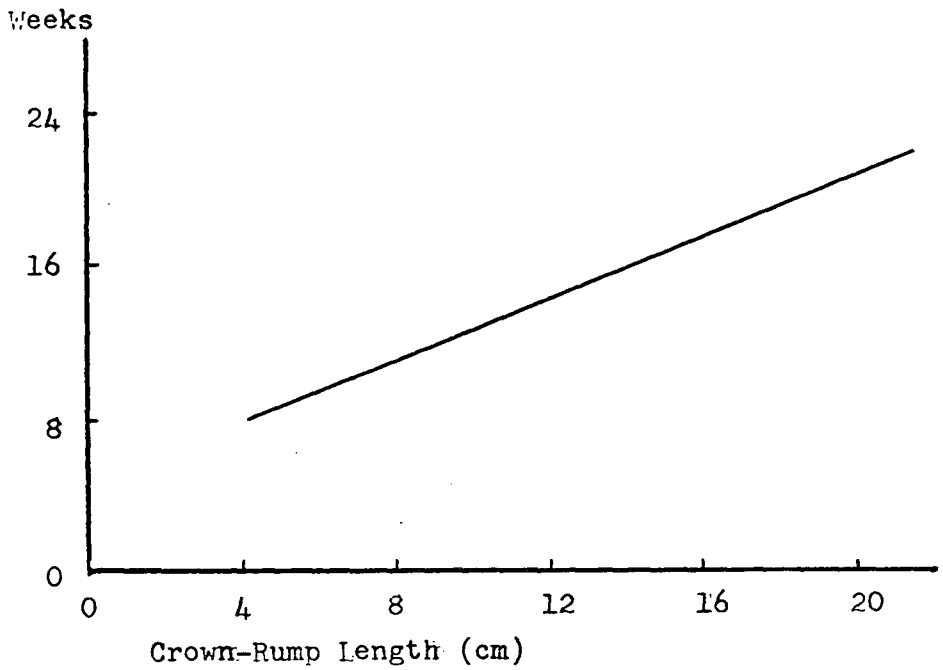
PLP (SM)

POPOP (NEN)

PPO (S)

TEA (C)

APPENDIX II



Age crown-rump-length relationship for the human fetus. Ordinate: Weeks after ovulation (menstrual age minus 14 days). Modified from Shepard et al. (99).

APPENDIX III

Fetal Rat Age-Weight Relationship

Fetus Weight (gm)

0	1	2	3	4	5	6
	-4	-3	-2	-1	Term (22 days)	

Fetus Age (days prior to term)

APPENDIX IV

Compenents of the Culture Media

Eagle's Minimum Essential Medium

	Concentration	
	Milligrams per 1000 ml.	Approx. equiv. in millimoles
L-Arginine	105	0.6
L-Cystine	24	0.2
L-Histidine	31	0.2
L-Isoleucine	52	0.4
L-Leucine	52	0.4
L-Lysine	58	0.4
L-Methionine	15	0.1
L-Phenylalanine	32	0.2
L-Threonine	48	0.4
L-Tryptophan	10	0.05
L-Tyrosine	36	0.2
L-Valine	46	0.4
L-Glutamine	292	2.0
Choline	1	
Nicotinic acid	1	
Pantothenic acid	1	
Pyridoxal	1	
Riboflavine	0.1	
Thiamine	1	
i-Inositol	2	
Folic acid	1	
Glucose	2000	
NaCl	8000	
KCl	400	
$CaCl_2$	140	
$MgSo_4 7H_2 0$	100	
$MgCl_2 6H_2 0$	100	
$Na_2HPO_4 2H_2 0$	60	
KH_2PO_4	60	
$NaHCO_2$	350	
Phenol red	20	

(This version is based on HBSS)

APPENDIX IV (CONT'D)

Substance	EBSS g/l	HBSS LHBSS g/l
NaCl	6.80	8.00
KCl	0.40	0.40
$CaCl_2$	0.20	0.14
$MgSO_4 7H_2O$	0.10	0.10
$MgCl_2 6H_2O$		0.10
$NaH_2PO_4H_2O$	0.125	
$Na_2HPO_4 2H_2O$		0.06
KH_2PO_4		0.06
Glucose	1.00	1.00
Pehnol Red	0.05	0.02
$NaHCO_4$	2.20	0.35
Gas phase	5% CO_2 in air	Air

Eagle's MEM, HBSS, and EBSS were obtained from Grand Island Biological Co. LHBSS was prepared in this laboratory.

APPENDIX V

Action of Inhibitors

Deoxycarnitine: This is a structural analogue of carnitine. It functions as a competitive inhibitor in the oxidation of FFA by competing with acylcarnitine for binding on the acylcarnitine transferase (100).

Citral: This compound is thought to inhibit β-oxidation of FFA because of its branched chain nature, i.e. it enters the oxidation cycle; however, the oxidation stops at the branch point thus giving rise to the accumulation of the resulting shorter chain FFA (101).

Cycloheximide: This compound acts at the site of peptide elongation. It prevents the transfer of the peptide chain to the adjacent tRNA-amino acid unit (102).

Actinomycin D: This acts at the site of RNA polymerase. It prevents the polymerisation of ribonucleotides into mRNA (103).