REGULATION OF THE SYNTHESIS OF KEY GLUCONEOGENIC AND GLYCOLYTIC ENZYMES BY BIOTIN

A Thesis Presented to the
Faculty of Graduate Studies
University of Manitoba

In Partial Fulfilment
of the Requirements
for the Degree of
MASTER OF SCIENCE

by
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THIS THESIS IS DEDICATED TO MY LATE TEACHER, ENDOCRINOLOGIST,

PROFESSOR OF INTERNAL MEDICINE, ZHOU XIAN-TENG

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ABSTRACT

Previous studies from our laboratory have shown that biotin, similar to insulin, can increase enzyme activities of hepatic glucokinase (GK), phosphofructokinase and pyruvate kinase (PK) in diabetic and glucocorticoid-treated rats. These effects could be blocked by actinomycin D. Recent studies have indicated that biotin can increase transcription of GK in starved rats. In this study, the possible role of biotin in the regulation of GK, PK and phosphoenolpyruvate carboxykinase (PEPCK) was investigated in streptozotocin-induced diabetic rats. Time course studies showed that the hepatic PEPCK mRNA was decreased by 85% 3 hour after biotin administration. No significant change was found in kidney PEPCK mRNA concentration. Parallel studies with insulin indicated that biotin had a regulatory effect similar to that of insulin on hepatic PEPCK mRNA, although the magnitude of supression by biotin was less. In accordance with changes in hepatic PEPCK mRNA, the activity of liver PEPCK was also decreased in a time dependent fashion. The transcriptional activity of hepatic PEPCK gene, as measured by nuclear run-on assay, was decreased by 57% within 30 minutes of biotin administration. In addition, preliminary results indicated that GK mRNA could be induced at 1 and 3 hour intervals following biotin injection. However, no changes have been found in the concentration of hepatic PK mRNA with the treatment of biotin.Determination of serum insulin level of diabetic rats after biotin treatment suggests that the effects of biotin on hepatic GK and PEPCK is not the result of induced release of endogenous

insulin in diabetic rats.

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ABBREVIATIONS

ATP	Adenosine-5′-Triphosphate
cAMP	Cyclic adenine-3,5-monophosphate
cGMP	Cyclic guauine-3,5-monophosphate
CTP	Cytidine-5′-triphosphate
DNA	Deoxyribonucleic acid
d-GDP	deoxy-Guanine-5′-diphosphate
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(ß-aminoethylether)N, N,N′,N′-tetraacetic acid
GK	Glucokinase
GTP	Guanine-5′-triphosphate
hnRNA	Heterogeneous nuclear ribonucleic acid
IDP	Inosine 5′-diphosphate
mRNA	messenger RNA
NADH	Nicotinamide adenine dinucleotide, reduced form
NAD	Nicotinamide adenine dinucleotide
PEPCK	Phosphoenolpyruvate carboxykinase
PK	Pyruvate kinase
PMSF	Phenyl methyl sulfonyl fluoride
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
TRIS	Tris (hydroxymethyl) aminomethane
tRNA	Transfer RNA
UV	Ultra Violet
UTP	Uridine-5′-triphosphate

Part I. Literature review: Properties and regulations of hepatic and renal glucose metabolism

1. General discussion of substrate cycles of gluconeogenic and glycolytic pathways.

Hepatic and renal glucose production and utilization involve the movement of substrates through three major substrate cycles (Figure 1). The process, glycolytic and gluconeogenic pathways, are closely coupled due to the common intermediate substrates they use in the pathways. In some of the intermediate steps, the same enzyme catalyzes reversible reactions. In other steps (as indicated in the Figure 1) the bi-directional reactions are catalyzed by different enzymes. These steps form the major substrate cycle and are the key pionts for regulation of these two opposite pathways. While the first substrate cycle, formed by the interconversion steps of glucose and glucose-6-phosphate, is relatively simple in terms of one reversible reaction catalyzed by two enzymes, the fructose-6-phosphate and fructose-1,6-bisphosphate substrate cycle is complicated by a branched substrate cycle which facilitates the interconversion between fructose-6- phosphate and frucose-2,6-bisphosphate (Pilkis and El-Maghrabi, 1988). Though several allosteric effectors may influence fructose-1,6-bisphosphatase and 6-phosphofructo-1-kinase activity, allosteric regulation at this cycle is now thought to be mediated primarily by changes in the level of fructose-2,6-bisphosphate (Claus et al., 1984; Hue, 1987;

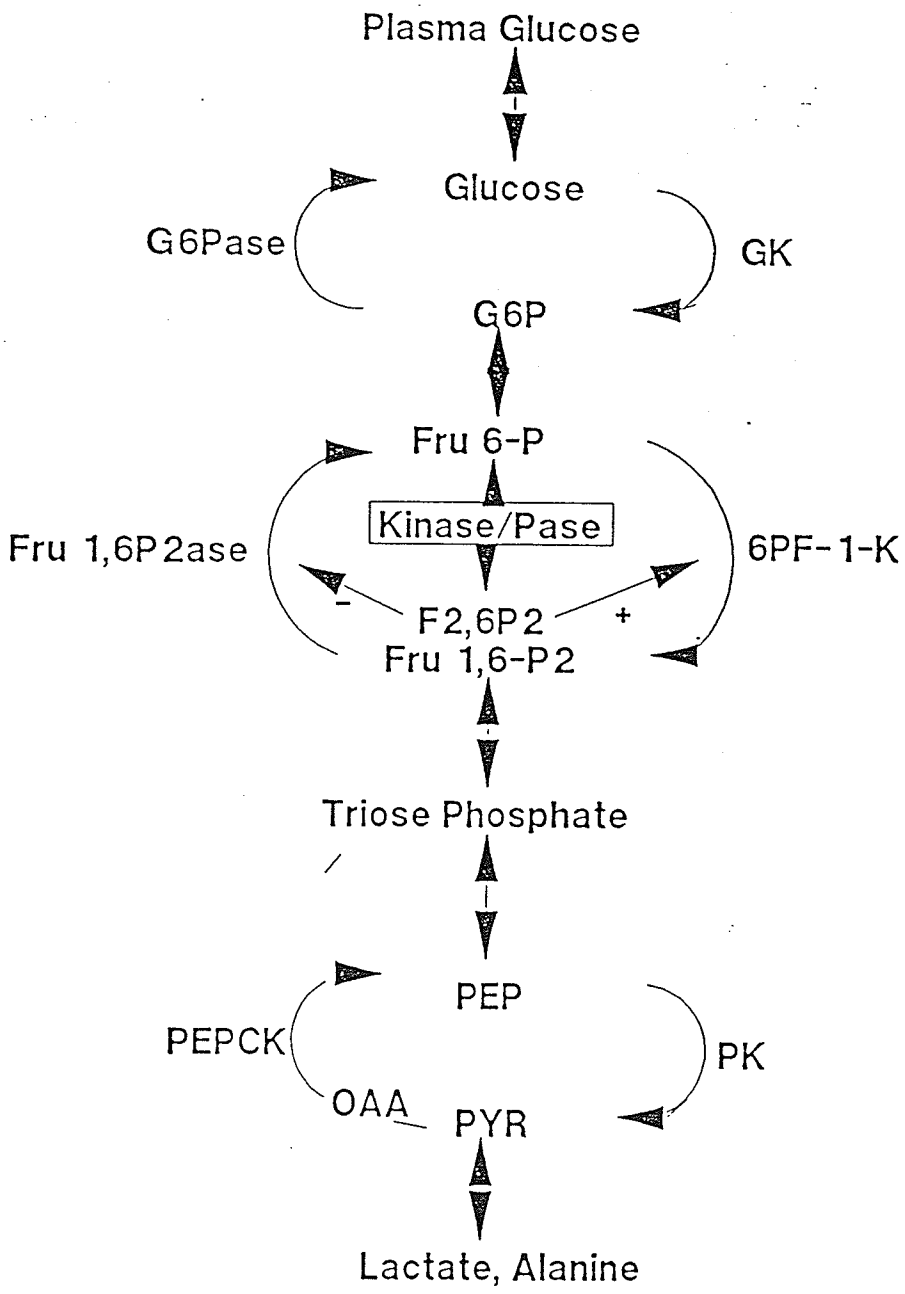

Figure 1. The substrate cycles and enzymes involved in glucose metabolism. G6P, glucose 6-phosphate; Fru 6-P, Fructose 6-phosphate; F2,6P2, Fructose-2,6-bisphosphate; Fru 1,6-P2, fructose-1,6-bisphosphate; PEP, phosphoenolpyruvate; PYR, pyruvate; OAA, oxaloacetate; G6Pase, glucose-6-phosphatase; Fru 1,6P2ase, fructose-1,6-bisphosphatase; 6PF-1-K, 6-phosphofructo-1-kinase; Kinase/Pase,6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; GK, glucokinase; PK, pyruvate kinase; PEPCK, phosphoenolpyruvate carboxykinase.

Pilkis et al., 1987). Fructose-2,6-bisphosphate is a potent allosteric activator of 6-phosphofructo-1-kinase (Pilkis et al., 1981a; Van Schaftingen et al., 1981; Uyeda et al., 1981) and acts synergistically with AMP to oppose the inhibitory action of ATP and citrate (Pilkis et al., 1982; Uyeda et al., 1982; Claus et al., 1983, 1984; Van Schaftingen et al., 1981; Uyeda et al., 1981). The sugar bisphosphate is thought to bind to the same allosteric site that binds fructose-1,6-bisphosphate (Kitajima and Uyeda, 1983). Fructose-2,6-bisphosphate is also a potent competitive inhibitor of fructose-1,6-bisphosphatase and acts synergistically with AMP to inhibit that enzyme (Pilkis et al., 1982; Uyeda et al., 1982; Claus et al., 1983, 1984). The mechanism of fructose-2,6-bisphosphate inhibition is controversial. There is evidence that fructose-2,6-bisphosphate either acts as a substrate analog and binds to the active site (Pilkis et al., 1981b) or binds to a discrete allosteric site (Van Schaftingen and Hers, 1981) or binds to both sites (Meek and Nimmo, 1983). The synthesis and degradation of this key regulatory sugar bisphosphate in liver is catalyzed by a unique bifuctional enzyme (Pilkis et al., 1983a; El-Maghrabi and Pilkis, 1984). The two activites of this enzyme determine the steady-state level of fructose-2,6-bisphosphate and glycolytic and gluconeogenic flux in liver (Claus et al., 1984).

In the third substrate cycle, conversion of pyruvate into phosphoenolpyruvate represents the initial steps for gluconeogenesis. Some precursor substances of gluconeogenesis

(amino acid, lactate) enter the cell by carrier-mediated membrane transport systems, and are converted to pyruvate in the cytoplasm. Pyruvate enters the mitochondria by a transport system and, along with that generated in the mitochondria, is converted to oxaloacetate by pyruvate carboxylase. The fate of the mitochondrial oxaloacetate varies in different species, depending in large part on the distribution of the enzyme PEPCK between cytosol and mitochondria (Tilghman et al., 1976). In the rat and mouse liver, it is converted to malate and/or aspartate, and those metabolites are transported to the cytosol where they are reconverted to oxaloacetate. The oxaloacetate is directly converted to phosphoenolpyruvate by PEPCK, which is predominantly a cytosolic enzyme in rat and mouse liver. In pigeon, chicken and rabbit liver, PEPCK is located predominantly in the mitochondria, and oxaloacetate is directly converted to phosphoenolpyruvate and then transported to the cytosol. In the human, guinea pig, sheep, and cow liver, the enzyme is distributed about equally between the mitochondria and the cytosol.

2. General aspects of the regulation of glycolysis and gluconeogenesis

Both liver and kidney are responsible for blood glucose homeostasis which they accomplish by fine-tuning of metabolic pathways of glycogen and glucose synthesis and degradation in response to dietary and hormonal stimuli (Hers et al., 1977; Claus and Pilkis,

1981; Hers, 1976). Several remarkable features in the regulation of glycolysis and gluconeogenesis make it possible for these two processes to satisfy the high demand of maintaining blood homeostasis under different situations. First, the major regulatory steps in glycolytic and gluconeogenic pathways are these key enzymes located at the three major substrate cycles of the two processes (Pilkis and El-Maghrabi, 1988). These enzymes catalyze uni-directional reactions and constitute only a small number of the total enzyme group in the glucose metabolic pathways, thus making the regulation of gluconeogenesis and glycolysis in liver and kidney more efficient. Second, the metabolic pathways of glycolysis and gluconeogenesis are subject to the regulation of many dietary and hormonal stimuli (Pilkis and El-Maghrabi, 1988). By this feature, the production and utilization of glucose in liver and kidney are functionally connected to demand of other organs and ensure the satisfactory supply of glucose to the whole body. Third, hormones and other stimuli could influence some key enzyme activity at several levels. For example, insulin and glucagon have opposite effects on the phosphorylation status of hepatic PK which has positive or negative regulatory effects on PK activity. In addition, insulin and glucagon also modulate the production of allosteric effectors of PK such as fructose-1,6-bisphosphate and indirectly regulate PK activity. Moreover, both hormones regulate gene expression of PK gene and influence the stability of PK mRNA (Pilkis and El-Maghrabi, 1988). Through these different mechanisms, these hormones modulate PK activity in a effective, precise and

well-balanced way. Fourth, glucose metabolism is a coordinated process, and hormones provide the integration and coordination. Increased insulin level would increase the activities of key gluconeogenic enzymes such as PEPCK, fructose-1,6-bisphosphatase and glucose-6-phosphatase, and suppress activities of GK, phosphofructokinase and PK. Increased glucagon opposes the effects of insulin on these enzymes. In conditions favoring gluconeogenesis (the combination of high plasma glucagon, catecholamine, and glucocorticoid levels with a low plasma insulin level, as occurs when animals are starved or fed a low carbohydrate diet), there is an increase in the activities of PEPCK, Fructose-1,6-phosphatase and glucose-6-phosphatase and a coordinate decrease in the activities of PK, phosphofructokinase and GK. Reciprocal changes in the activity of these enzymes occur when animals are fed a carbohydrate diet, particularly after a prolonged fast. In this situation, the plasma insulin concentration increases, levels of the counterregulatory hormones decrease, and glycolysis predominates.

3. Characteristics of hepatic gluconeogenesis and glycolysis and their regulation

Liver is the most important organ in maintaining blood glucose homeostasis and possesses all the typical properties of gluconeogenic and glycolytic pathways mentioned above. The regulation of gluconeogenesis / glycolysis in liver can be divided

into three categories. The first involves regulation of supply of substrate. All gluconeogenic substrates as well as glucose reach the liver in subsaturating concentrations. In perfused rat livers or in isolated hepatocytes, saturation of the system for gluconeogenesis with lactate or pyruvate occurs at concentrations well above the physiological range (half maximal rate at 2 mM lactate or 1 mM pyruvate versus physiological concentrations of 1 and 0.1 mM), rendering gluconeogenesis greatly dependent upon substrate concentration (Exton and Park, 1967; Ross et al., 1967). On the other hand, liver does not glycolyze at high rates except when in anoxia or given a large excess of glucose (Woods and Krebs, 1971; Brunergraber et al., 1973). The net glycolytic rate in the presence of physiological concentrations of glucose is close to 0.5 µmol of lactate / minute / g and can be increased four fold when glucose concentration is between 20 to 40 mM (Woods and Krebs, 1971; Hue, 1982). Glucose by itself is therefore a regulator of liver glycolysis. It has been proposed that the major function of liver glycolysis is not to provide ATP but to allow the transformation of carbohydrate into fat (Krebs, 1972; Woods and Krebs, 1971).

The second category of regulation is connected with the minute to minute regulation of pathway flux due to changes in the phosphorylation state and / or allosteric effectors of key enzymes through the action of hormones such as insulin, glucagon and catecholamines. Several general mechanisms are involved in this

category of regulations. The first involves those hormones (glucagon, ß-adrenergic agonists) that interact with plasma membrane receptors that are specific for each hormone and are coupled to adenylate cyclase. The activation of this membrane bound enzyme results in elevation of intracellular cAMP. This, in turn, leads to activation of cAMP-dependent protein kinases that catalyze the phosphorylation of a number of protein substrates. The end result of this cascade of events is stimulation of gluconeogenesis and inhibition of glycolysis (Claus and Pilkis, 1981; Hue, 1981; Claus et al., 1984). The second mechanism involves those hormones that act via changes in intracellular Ca^{++} levels. These hormorms (α-adrenergic agonist, vasopressin and angiotension) interact with their own specific plasma membrane receptor to generate two intracellular messengers, myoinositol-1,4,5-triphosphate and 1,2-diacylglycerol (Exton, 1985). The elevation in intracellular Ca^{2+}, in combination with calmodulin and / or other effectors, leads to activation of a number of Ca^{2+}-linked protein kinases including Ca^{2+} / calmodulin-dependent protein kinases, phosphorylase kinase and protein kinase C. These protein kinases also catalyze phosphorylation of a number of protein substrates that leads to gluconeogenic and glycolytic flux that are similar to those seen with hormones that act through cAMP. Insulin, on the other hands, opposes the action of the above hormones to phosphorylate various protein substrates and to stimulate gluconeogenesis. One mechanism probably involves its ability to activate cAMP phosphodiesterase, which results in lower cAMP levels (Loten et al ., 1978). Precisely

how this effect on phosphodiesterase is brought about or how insulin oppose the action of Ca^{2+}-linked hormones is not known. Another mechanism relates to the regulation of allosteric effector level. For example, the level of hepatic fructose-2,6-bisphosphate has been shown to be under acute hormonal control. Addition of glucose or cAMP to hepatocytes results in a dramatic fall in the level of the compound (Van Schaftingen et al., 1980; Richards and Uyeda, 1980; Hue et al., 1981; El-Maghrabi et al., 1982). Insulin , which acts by counteracting glucagon's effect to elevate cAMP , opposes the action of glucagon to lower fructose-2,6-bisphosphate levels (Pilkis et al., 1983; Richards and Uyeda, 1982). Fructose-2,6-bisphosphate can be thought of as a master switching signal between gluconeogenesis and glycolysis. In states where fructose-2,6-bisphosphate levels are high, the activity of and flux through 6-phospho-fruco-1-kinase is high, frucose-1,6-bisphosphatase is inhibited and glycolytic flux predominates. When fructose-2,6-bisphosphate levels are low, fructose-1,6-bisphosphatase activity and flux are enhanced, 6-phosphofructo-1-kinase is inhibited, and gluconeogenic flux predominates. The ensuing changes in the level of frucose-1,6-bisphosphate, because of its effects on pyruvate kinase, serves to coordinate the control of both substrate cycles.

The third category involves the very important but relatively slow (hours to days) adaptive changes in enzyme activities due to regulation of gene expression, protein synthesis, and/or degradation. Again hormones play important roles in this type of

regulation and they exert their effects in a highly integrated pattern. Under conditions of starvation or low carbohydrate diet, a combination of high plasma glucagon, catecholamine and glucocorticoid levels with a low plasma insulin level results in increased activity of PEPCK, fructose-1,6-bisphosphatase and glucose-6-phosphatase and a coordinate decrease in the activity of PK, 6-phosphofructo-1-kinase and GK. Reciprocal changes in the activities of these enzymes occur when animals are fed a carbohydrate diet, particularly after a prolonged fast. In this situation, the plasma insulin concentration increases and the levels of the counter regulatory hormones decrease, and glycolysis predominates.

The effects of hormones on regulation of gene expression are consonant with their established physiological actions. Insulin induces the mRNAs that encode glycolytic enzymes and repress the mRNAs that encode gluconeogenic enzymes. cAMP has opposite effects. Both can increase or decrease transcription (Granner and Pilkis, 1990). Whereas insulin and cAMP affect all of these mRNAs, other hormones may have a more restricted action. For example, glucocorticoid hormones may play an important role in increasing PEPCK and bifuctional enzyme mRNA and have a permmissive action in the regulation of PK mRNA (Granner and Pilkis, 1990).

Contrary to the better understanding of hormone effects on regulation of gene expression, relatively little is known about

how mRNA stability is regulated. It has been reported PEPCK and PK mRNAs can be stabilized by agents that increase the rate of transcription of these genes (Decaux et al., 1989; Hod and Hanson, 1988; Petersen et al., 1989). Under appropriate metabolic signals, this dual control provides a long-term increase in PEPCK mRNA and protein.

It has been found that glucose is an important regulatory molecule. Glucose is required for the effects of insulin on PK (Decaux et al., 1989) and bifuctional enzyme genes and for the effect of glucocorticoids on the latter. Since these RNAs encode enzymes that catalyze intermediate or distal reactions in the glycolytic pathway and the regulation GK gene transcription by insulin is glucose independent (Iynedjian et al., 1989a), it is possible that a glucose metabolite is the active agent and that this is generated as a consequence of insulin's stimulation of GK gene transcription. The increased catabolism of glucose could account for the fact that insulin is necessary but not sufficient for the induction of PK (Decaux et al., 1989) and the bifuctional enzymes. Regulation of the gluconeogenic enzymes by insulin appears to be glucose-independent (Granner and Pilkis, 1990).

4. Characteristics of renal gluconeogenesis and its regulation

Although glycolysis occurs in all living cells, gluconeogenesis operates only in liver and kidney. Since the discovery of renal

gluconeogenesis, it has become apparent that several significant differences exist with regard to this process in the two organs (Wirthensohn and Guder, 1986). The liver has been regarded as a more important source of glucose under most physiological circumstances, however, the kidney may contribute as much as 50% of blood glucose produced under certain situations such as starvation, metabolic acidosis and diabetic acidosis in the rat and human (Kida et al., 1978; Owen et al., 1969) but not in dog (Steiner et al., 1968; Costello et al., 1973; Roxe, 1972). One of the characteristics of renal glucose metabolic pathway is the differential distribution of this activity in kidney. It is found that renal gluconeogenesis is confined to the cortex wheareas glycolysis occurs primarily in the medullary structures (Cohen and Kamm, 1981). Compare to liver, renal gluconeogenesis has different substrate requirements. For example, although lactate, pyruvate, glutamate, glutamine, proline, propionate and glycerol are substrates in both organs, alanine and serine are gluconeogenic in liver only (Wirthensohn and Guder, 1986). Primary substrates for renal gluconeogenesis appear to be glutamine, glutamate, α-ketoglutarate and other di- and tricarboxlic acids which do not readily cross liver membranes (Klahr et al., 1972; Krebs et al., 1983; Nishiitsutsuji-Uwo et al., 1967). In both liver and kidney, glucocorticoids and catecholamine regulate the process of gluconeogenesis. Parathyroid hormone seems to be a unique stimulator for renal gluconeogenesis (Schoolwerth et al., 1988). Somatostatin, which inhibits glucagon-stimulated gluconeogenesis

in liver (Hers and Hue, 1983), has been shown to stimulate glucose production in rat renal cortical slices (Lupianez et al., 1979). Additional stimuli for glucose synthesis in kidney include starvation and metabolic acidosis. Similar to hepatic gluconeogenesis, the rate-limiting enzymes in the pathway including PEPCK, fructose-1,6-bisphosphatase and glucose-6-phosphatase are the regulatory steps by most effecors. But in kidney, no mechanism other than substrate concentration has been shown to be important in the control of glucose-6-phosphatae. Another important feature of renal gluconeogenesis is that this process is functionally related to ammoneagenesis, sodium and phosphate transport (Schoolwerth et al., 1988).

5. The regulation of GK

Upon entering the heptocyte, glucose is converted to glucose-6-phosphate by the enzyme GK. GK, a member of the hexokinase family, is unusual in that its Km for glucose is 5mM whereas that of HK I-III (GK is Hexokinase IV) varies between 0.1 to 0.001 mM (Middleton, 1990). In the hepatocyte, glucose is efficiently converted to glucose-6-phosphate because GK, unlike hexokinase I-III, is not subject to feedback inhibition by the reaction products. It has been shown (Weinhouse, 1976) that in rats glucokinase is localized in both the soluble portion of a liver extract as well as in particular fraction which contains the plasma membrane components. Most of the activity is localized in the

soluble fraction and it is this component that is induced by increased serum insulin after meal. However, it is found that in the domestic fowl, it is the GK, which associated with plasma membrane, that is regulated by hormones or dietary factors (Klandorf et al., 1986).

GK activity is not altered by post-transcriptional modification but has been reported to be modulated by a Fructose-6-phosphate sensitive and fructose-1-phosphate sensitive protein (Van Schaftigen, 1989). Upon purification of this 60,000 Da protein, it is found that the regulatory protein inhibits GK by forming a complex with this enzyme in the presence of fructose-6-phosphate, and that fructose-1-phosphate antagonises this inhibition by preventing the formation of the complex (Vander Cammen and Van Schaftingen, 1990). However it is generally agreed that the physiological significant changes in activity of GK are entirely due to changes in the amount of protein. In diabetic animals, where gluconeogenesis is unrestrained and glycolysis is incooperative, GK mRNA is very low and the GK gene is inactive. Within 30-60 minutes after the injection of insulin into a diabetic rat or after addition to primary cultures of hepatocytes, a 20-30-fold increase in GK gene transcription occurs and GK mRNA increase accordingly (Sibroski and Seitz, 1984; Iynedjian et al., 1987, 1988; Andreone et al., 1989; Magnuson et al., 1989). Glucagon (or its intracellular messenger: cAMP) inhibits GK gene transcription and overides the stimulatory effect of insulin (Magnuson et al., 1989;

Iynedjian et al., 1988, 1989a). These effects are not dependent on the ambient glucose concentration (Iynedjian et al., 1989a). Glucocorticoid has no effect on hepatic GK activity in normal fed rat but increases the activity in fed adrenalectomized rats to normal level (Weinhouse, 1976). In primary cultures of neonatal rat hepatocytes, dexamethasone alone does not induce GK mRNA but enhances the response to insulin and decreases the response to T_3. Similar to insulin, the addition of tri-iodothyronine to the medium results in induction of GK mRNA. The effects of tri-iodothyronine is dose dependent and additive to the effect of insulin (Narkewicz et al., 1990).

Isolation of a specific GK cDNA, combined with extensive amino acid sequence information, leads to the elucidation of the primary struture of the protein (Andreone et al., 1989). A comparison of this with the sequences of yeast hexokinases and a partial sequence of mammalian hexokinase I show that the ATP-binding and glucose-binding domains are highly conserved between members of the hexokinase family (Andreone et al., 1989). A further analysis of mammalian hexokinase I confirmes a long standing hypothesis about the origin of the hexokinases. A primordial enzyme, similar to the yeast hexokinase (and GK) gave rise , by gene dupliction with tandem ligation, to mammalian hexokinase I, and presumably, to hexokinase II and III (Nishi et al., 1988; Schwab and Wilson, 1989). A comparison of the structure of the hexokinase II and hexokinase III genes with that of GK gene should provide an

additional test of this hypothesis.

Although hexokinases have been found in nearly all tissues (Middleton, 1990), GK is only expressed in liver and pancreatic ß-cells. The regulation of glucokinase activity in pancreatic ß-cells is remarkably different from that of hepatic GK, and is to "serve" the circulating glucose level and allow flux through glycolysis which control the synthesis and secretion of insulin (Meglasson and Matschinsky, 1984; Ashcroft, 1980). Despite marked tissue-specific differences in regulation, it has been confirmed that there is only one copy of the GK gene in rat (Iynedjian et al., 1989a; Magnuson et al., 1989). The GK gene in hepatic and pancreatic ß-cells uses different first exons (1^H and $1^ß$ are separated by 12 kb) which means that different transcriptional initiation sites, promoters and regulatory elements are functional in these cells and the alternative splicing results in different primary transcripts (Magnuson, 1990).

Attempts to identify hormone response elements in the hepatic Gk promoter have, to date, been unsuccessful. However, 5' DNA sequence of rat GK gene has been cloned and several putative promotor elements including a probable "TATA" box, a Sp1 binding site, a sequence similar to the insulin responsive element in the promoter of PEPCK, and several elements related to liver specific gene expression are speculated (Magnuson, 1989) but the function of these elements has not been verified.

6. The regulation of PK

PK plays a central role in glycolysis by regulating the flux through the phosphoenolpyruvate /pyruvate cycle. Phosphorylation and allosteric effectors are important in the acute regulation of PK (Pilkis and El-Maghrabi, 1988). PK is an allosteric enzyme that exhibits homotropic cooperativity with regard to its substrate phosphoenolpyruvate, is allosterically activated by fructose-1,6-bisphosphate and is inhibited by ATP and alanine (Eugstorm, 1978). Both the cAMP-dependent protein kinase (Eugstorm, 1978; Riou et al., 1978; Pilkis et al., 1976; Ishibashi and Cottam, 1978; Garrison and Wagner, 1982) and Ca^{2+} /calmodulin-dependent protein kinase (Schworer et al., 1985; Soderling et al., 1986) catalyze the phosphorylation of rat liver L-PK and phosphorylation results in inhibition of L-PK activity that is characterized by an increase in the Km for phosphoenolpyruvate. Inhibition is seen when the enzyme is assayed with low phosphoenolpyruvate concentrations but is overcome by high substrate concentrations or by the allosteric activator fructose-1,6-bisphosphate. In addition to its effects on enzyme activity, fructose-1,6-bisphosphate also inhibits the rate of cAMP-dependent protein phosphorylation of pyruvate kinase (El-Maghrabi and Pilkis, 1985; Claus et al., 1979).

The Ca^{2+} / calmodulin-dependent protein kinase catalyzes phosphorylation of pyruvate kinase at two sites: one on the same

serine residue as that phosphorylated by the cAMP-dependent protein kinase and the second on a threonine residue (Schworer et al., 1985). Both sites are located near the amino terminus, and the amino acid sequence has been shown to be LRRASVAQLIQE (Soderling et al., 1986). Since the two phosphorylation sites are only five residues apart, it is not surprising that phosphorylation by either kinase results in the same change in kinetic properties. However, there is no evidence that the threonyl residue phosphorylated in vitro by the Ca^{2+} / calmodulin protein kinase is also phosphorylated in vivo (Connelly et al., 1987).

A number of hormones have been shown to alter pathway flux by modulating the phosphorylation state of PK. For example, glucagon acts to raise cAMP levels, which leads to phosphorylation and inhibition of PK and to decreased recycling of phosphoenolpyruvate to pyruvate and thus to enhanced flux toward glucose. In addition to glucagon, the phosphorylation state of PK in intact cells has been shown to be regulated by insulin, catecholamines, antigiotension and vasopressin (Claus and Pilkis, 1981; Claus et al., 1983, 1984; Garrison and Wagner, 1982). Phosphorylation of the enzyme yields an inactive form that is less sensitive to activation by fructose-1,6-bisphosphate and more sensitive to inhibition by alanine and ATP (Van Den Berg et al., 1980; Garrison and Vagner, 1982). These effects favour gluconeogenesis because of decreased recycling of phosphoenolpyruvate to pyruvate. Addition of glucagon to liver cells is known to lower fructose-1,6-bisphosphate, as

well, thus providing an amplication mechanism for further decreasing PK flux (Pilkis et al., 1976). In the dephosphorylated form, all these effectors are reversed and glycolysis predominates. Since the rate-limiting portion of the gluconeogenic pathway is located between pyruvate and phosphoenolpyruvate (Exton, 1972; Exton and Park, 1969; Williamson et al., 1969) and since pyruvate kinase is the only enzyme in that part of the pathway regulated by phosphorylation, PK is now thought to be a major site of acute hormone action for the gluconeogenic pathway.

The chronic regulation of the liver form of PK (L-PK) mRNA by hormones and dietary factors is extremely complex. Hepatic PK activity and mRNA, decreased in starvation and diabetes, are restored to normal by refeeding a high carbohydate diet and insulin administration (Miyonaga et al., 1982; Pool et al., 1982). Glucagon, acting via cAMP, inhibits transcription of the L-PK gene, and it also accelerates the degradation of L-PK mRNA (Noguchi et al., 1985; Vaulont et al., 1986). Carbohydrates stimulate mRNA accumulation (Noguchi et al., 1985; Vaulont et al., 1986). This appears to involve transcription and mRNA stabilization (Noguchi et al., 1985; Vaulont et al., 1986) and the permissive effect of thryroid hormones and glucocorticoids may be required (Noguchi et al., 1985). The stimulation effect of insulin is slow in onset, and it requires ongoing protein synthesis, so induction of another gene product may be a prerequisite (Noguchi et al., 1985). L-PK gene expression is stimulated by the combination of glucose and insulin

in primary cultures of adult rat hepatocytes provided that thyroid hormone and glucocorticoids are present (Decaux et al., 1989). Neither glucose nor insulin work by themselves. Glucagon inhibits the synthesis of L-PK mRNA in cultured hepatocytes (Decaux et al 1989), as it does in vivo (Vaulont et al., 1986). Glucagon also increases the rate of degradation of L-PK mRNA whereas glucose / insulin increase the stability of this mRNA in hepatocytes (Decaux et al., 1989).

The two L-PK isozymes (L' and L) are encoded by a single gene (Noguchi et al., 1987). The L-PK gene utilizes alternative first exons to generate the L' and L primary transcripts, whereas the other 10 exons are common to both. Consensus cAMP responsive element sequences are present in the L-PK gene but are far upstream (-2.3kb) and Downstream (+5.8 kb) from the capsite (Cognet et al., 1987). A sequence resembling glucocorticoid responsive element is also identified. No studies of the function of these putative regulatory elements have been reported.

7. The regulation of PEPCK

PEPCK catalyzes the first step of gluconeogesis which is common to pyruvate and a number of amino acids. Its intracellular distribution varies between species (Tilghman et al., 1976) and both the mitochondrial and the cytosolic enzymes are 74,000 Da monomers (Chang and Lane,1966; Ballard and Hanson, 1969). However,

they differ in their kinetic and immunochemical properties (Nordlie and Lardy, 1963; Holten and Nordlie, 1965; Ballard, 1970). The apparent Km of the rat cytosolic enzyme for oxaloacetate is in the micromolar range (Ballard, 1970; Walsh and Chen, 1971) and about the same as the cytosolic concentration of oxaloacetate for which values ranging from 5 to 50 μM have been reported (Siess et al., 1977, 1982; Tischler et al., 1977). Similar to that of hepatic PEPCK, renal PEPCK intracellular distribution is different among species. For example, in rat kidney, the enzyme is located almost exclusively to the cytosolic compartment whereas in man, dog and rabbit, a greater percentage of the total enzyme is located in the mitochondria. The subcellular distribution of the enzyme is similar both in liver and kidney for any given species (Hers and Hue, 1983; Flores and Alleyne, 1971). However, in mammal, only the cytosolic form of the enzyme adapts to dietary or hormonal stimuli whereas the mitochondrial enzyme appears to be constitutive (Tilghman et al., 1976). It has been confirmed that the cytosolic PEPCK from rat liver, adipose tissue and kidney cortex are immunologically identical (Ballard and Hanson, 1969; Longshaw and Pogson, 1977).

Short term regulation of PEPCK, affected by ferrous and mangneous (Bentle and Lardy, 1976; Brinkforth et al., 1981) ions, has been proposed. Lardy and Merrifield (1981) and Merrifield and Lardy (1982) have suggested an important role for a so-called ferroactivator protein. According to this proposal, activity is stimulated by release of ferrous ions from the mitochondria. This

release may, in turn, be mediated by pH or hormonal stimulated changes in cytosolic free Ca^{2+} content and may require mediation via the putative ferroactivator. However, as discussed by Hers and Hue (1983) several difficulties are apparent in accepting this mechanism as being important physiologically.

The hepatic gene is subject to positive and negative regulation by dietary factors and hormones.In contrast to the glycolytic enzymes, hepatic PEPCK activity is markedly reduced in the carbohydrate-fed animal and is increased in fasted animals (Tilghman et al., 1976). These changes are largely, if not entirely, due to coincident changes of plasma glucagon and insulin. The increased plasma insulin following a carbohydrate meal results in a decreased rate of PEPCK synthesis (Andreone et al., 1982; Granner et al., 1983). This is directly due to the fact that insulin rapidly inhibits trancription of the PEPCK gene (Granner et al., 1983; Sasaki et al., 1984). This effect, studied most extensively in H4II E hepatoma cells, occurs in minutes, is mediated through the insulin receptor, is promptly reversed upon removal of insulin from the culture medium, and does not require ongoing protein synthesis (O'Brien and Granner, 1990). The elevated plasma glucagon (or intracellular cAMP) characteristic of the fasting condition induces PEPCK synthesis (Wicks et al., 1972; Iynedjian and Hanson, 1977). This too is related to enhanced transcription from PEPCK gene (Granner et al., 1983; Sasaki et al., 1984; Lamers et al., 1982), although cAMP also stabilizes PEPCK mRNA against degradation (Hod

and Hanson, 1988). Glucocorticoid hormones, named for their ability to promote gluconeogenesis, also increase PEPCK by stimulating transcription of the gene (Sasaki et al., 1984) and they also stabilize PEPCK mRNA (Petersen et al., 1989). The effects of glucocorticoids and cAMP on transcription are additive (Sasaki et al., 1984). In rat liver and H4II E cells, insulin inhibits basal and cAMP, glucocorticoid stimulated transcription, and the effect of insulin is dominant (Sasaki et al., 1984; Granner et al., 1983). However, in primary cultures of hepatocytes (Christ et al., 1988; Iynedjian et al., 1989a) and regenerating liver (Mohn et al., 1990), the effect of insulin is no longer dominant. Recently, retinoic acid, a derivative of vitamin A, has been shown to stimulate PEPCK gene transcription (Pan et al., 1990; Lucas et al., 1991).

PEPCK gene of kidney is also subject to hormonal regulation but the response to hormonal stimuli may be different as compared to the liver. For example, PEPCK is stimulated by glucocorticoids, cAMP and catecholamines in both kidney and liver, while parathyroid hormone and growth hormone stimulate enzyme synthesis in kidney only. Glucagon, vasopressin and insulin regulate the enzyme in liver but not in kidney (Schoolwerth et al., 1988; Pollock, 1989). In addition, rat renal PEPCK mRNA can be induced by respiratory and metabolic acidosis, and this induction correlate with increased transcription of the gene coding for the enzyme (Cimbala et al., 1982).

The basal hepatic PEPCK gene promoter, whose major fuctional region is from -460 to +73 of the PEPCK gene, consists of three major elements, CAAT box, combined basal enhancer element and cAMP responsive element, and a TATA box (Quinn et al., 1988). The PEPCK gene cAMP regulatory element (-98 to -80) can confer cAMP responsiveness when linked to a chimeric gene containing an enhancerless SV40 promoter and the CAT structural gene (Roesler et al., 1989). Footprinting analysis showed that cAMP regulatory element 1 in the PEPCK promoter (-90 to -84 bp) was protected from DNase I digestion by nuclear proteins from rat liver (McGrane et al., 1990). The cAMP regulatory element 1 from the PEPCK promotor can interact with several different binding proteins, including cAMP regulatory element binding protein (CREB), Jun / Fos and CCAAT / enhancer binding protein (C / EBP) (Park et al., 1990; Yamamoto et al., 1988). All these proteins have transcriptional regulatory capacity. On the other hand, C / EBP not only binds to cAMP regulatory element 1, but also to another two regions within the promotor sequence, namely, P_3 and P_4 (Park et al., 1990). It was found that , a block mutation, which prevents the binding of transcriptional factors to the sequence in cAMP regulatory element 1, did not completely inhibit cAMP responsiveness of a chimeric CAT gene in hepatoma cells. This suggests that other elements in the PEPCK promotor are also involved in cAMP regulation of transcription (Liu and Hanson, 1991).

Insulin is the major negative factor in the control of PEPCK gene expression and is a dominant controlling signal, since it can inhibit PEPCK gene transcription even in the presence of cAMP (Magnuson et al., 1987). The cis sequence necessary to confer negative regulation of PEPCK gene transcription by insulin appears to be contained in a region of promoter from -455 to the start site of transcription (Magnuson et al., 1987). Recently, O'Brien et al.(1990) reported that the sequences between -415 and -400 bp contain an insulin regulatory element which can confer negative regulation by insulin to a heterologous promotor. This region of the promotor has been shown to be involved in the stimulatory effects of glucocorticoids on PEPCK gene transcription (O'Brien et al., 1990). Since the negative effect of insulin is only apparent when glucocorticoid were used to stimulate transcription of PEPCK gene (O'Brien et al., 1990), it is possible that insulin interacts with the positive stimulation of transcription caused by glucocorticoids. However, the region of the promotor between -415 to -400 bp is not sufficient to account for the total negative effect of insulin on PEPCK gene transcription, since sequences 3' to this region of the PEPCK gene are also involved in the response of the gene to insulin(O'Brien et al., 1990).

Glucocorticoid hormones stimulate PEPCK gene transcription through a uniquely complex glucocorticoid response unit (GRU). This GRU spans about 110 base pair (-465 to -350), and it consists of two accessory factor binding sites (AF1 and AF2) and two glucocorticoid

receptor binding sites. All are required for a maximal response to glucocorticoids (Granner and Pilkis, 1990).

8. Regulation of glycolytic enzymes by biotin

A defect in the utilization of glucose by the biotin-deficient rat was reported by Dakshinamurti et al. (1962). In a further study, it is found that there is a 40-45% reduction in liver glucokinase activity in biotin-deficient rats in comparison with the control group. Biotin administration returns the GK activity to the control levels regardless whether the rats are fed high-carbohydrate diet or low carbohydrate diet. The same situation stands when the experiments were done in starvation and refeeding cycle (Dakshinamurti and Cheah-Tan, 1968a). In earlier work (Mistry et al., 1962), it was shown that insulin treatment increased glucose utilization in the biotin deficient-rat. However, when insulin and biotin were given together, there was no further increae in liver GK activity (Dakshinamurti and Cheah-Tan, 1968a). Late on, more studies confirmed that biotin treatment markedly increased hepatic GK activity in starved, diabetic and glucocorticoid-treated rats in non-biotin-deficient state (Dakshinamurti et al., 1970; Dakshinamurti and Cheah-Tan, 1968b; Dakshinamurti and Hong, 1969). Studies on other glycolytic enzymes in diabetic and glucocorticoid-treated rats indicated that biotin administration also increased phosphofrucokinase and PK activities but had no effects on a bifuctional enzyme like phosphohexokinase isomerase. However the

magnitude of response of phosphofrucokinase and PK was less when compared with that of GK (Dakshinamurti et al., 1970; Dakshinamurti and Hong, 1969).

It was found that the increase in hepatic GK activity following biotin administration is prevented by puromycin or actinomycin D which suggests that the effect of biotin on GK is mediated through increase of enzyme protein and that it probably involves the synthesis of new RNA (Dakshinamurti et al., 1970). Using primary culture of hepatocytes from adult rat, Spence and Koudelka (1984) have found that the addition of biotin at a concentration of 10^{-6} M results in a 4-fold increase in the enzyme activity of GK. The maximum response was observed 6 hours following the addition of biotin to culture medium and the induction of GK activity was preceded by an increase in the intracellular level of cGMP. The addition of 8-bromo-cGMP to the culture medium also increased the activity of GK and its effect was not additive with respect to the effect of biotin. The effect of biotin upon the activity of GK could be mimicked by including glucose in the culture medium. When hexose utilization by the hepatocytes was blocked by the addition to the culture medium of N-acetylglucosamine, the induction of GK by biotin was unaffected whereas the induction brought about by glucose was not observed. Previous study with the use of protein synthesis inhibitor by Spence et al. (1981) indicated that the induction of GK activity by cGMP was the result of an increase in the synthesis of enzyme protein and this appeared to arise as the

result of a change in the translation efficiency of existing message. However, the effects of biotin upon the level of mRNA coding for GK, measured using a cell free translation assay, indicated that there was an increase in the amount of translatable message. The authors speculate that the increased amount of translatable message may be due to a shift from a non-translatable message to a translating pool of mRNA for GK (Spence and Koudelka, 1984).

In an attempt to clarify the mechanism by which biotin induces hepatic GK activity, Chauhan and Dakshinamurti (1991) reported that GK mRNA increased remarkably 1 hour after biotin administration to starved rats and the maximum GK activity was detected 2 hours after biotin injection. Although the mRNA level of GK decreased more than 3-folds by 2 hours after biotin injection, the drop of GK activity was much slower and 2/3 of the maximum activity was retained even 12 hours later. This result is consistent with report (Watford, 1990) that hepatic GK has a long half-life (more than 30 hours). Nuclear run-on assay confirmed that the rate of transcription of GK gene increased rapidly after injection of biotin and a 6-fold increase is observed by 45 minutes after biotin administration. Compatible with the rapid decrease of GK mRNA, the transcription rate decreased rapidly after initial induction by biotin and reached undetectable level by 2 hours after biotin treatment. This study indicates clearly that biotin induce hepatic GK at the transcriptional level. Also, in the same study, it was found that

the transcription rate of hepatic PEPCK gene decreased by 60% 30 minutes after biotin injection to these starved rats (Chauhan and Dakshinamurti, 1991).

9. Objectives of the present investigation

It is well established that both starvation and diabetes lead to the similar adaptive response in the metabolism of glucose, that is, the activities of key enzymes of glycolytic pathway are depressed and the activities of key gluconeogenic enzymes are abnormally increased. Refeeding to starved rats or injection of insulin to diabetic rats would reverse the abnormality in these enzyme activities. In both cases, insulin level is the key factor resulting in changes of these enzyme activities (Pilkis and El-Maghrabi, 1988; Granner and Pilkis, 1990). It has been confirmed that GK is defective in biotin-deficient rat (Dakshinamurti et al., 1962) and insulin treatment increases glucose utilization in these rats (Mistry et al., 1962). On the other hand, it has also been found that biotin injection can reverse the abnormally low level of hepatic GK activity in starved and diabetic rats and increase hepatic PK and phosphofrucokinase in diabetic rats (Dakshinamurti et al., 1970; Dakshinamurti and Cheah-Tan, 1968a). Furthermore, biotin was found to reciprocally regulate transcription of GK gene and PEPCK gene in the liver of starved rats (Chauhan and Dakshinamurti, 1991). Based on this evidence, it seems obvious that (1) biotin seems to be having an effect on these hepatic glycolytic

enzymes and PEPCK gene transcription similar to that of insulin. (2) biotin, again similar to insulin, coordinately regulates these enzymes in diabetic rats. Thus it is of interest to know: (1) whether biotin influences the activities of key gluconeogenic enzymes in diabetic rats, and (2) whether biotin regulates these gluconeogenic and glycolytic enzymes, similar in starved rats, at transcriptional level.

Part II. Materials and Methods

1. Chemicals

Radioimmunoassay kit for rat insulin was obtained from NOVO Biolab. Random primer DNA labelling systems and oligo (dT) - cellulose type 7 were purchased from Bethesda Research Laboratories. (α-^{32}P) dGTP, (α-^{32}P) UTP and $NaHC^{14}O_3$ were obtained from New England Nuclear Corp. Nitroplus 2000 (Nitrocellulose Hybridization Transfer Membrane MSI, 0.45 micron) was from Fisher. Casein, dextrose and MOPS (Morpholinopropane Sulfonic Acid) were from United States Biochemical Corp. Formamide was purchased from Terochem. Lab. Ltd. Phenol and isopropanol were purchased from Fisher. Ethyl Ether Anhydrous ACS was from Canlab. Industrex Mannal Developer and replenisher, and Rapid Fixer with hardener were from Kodak. Yeast tRNA was from Bethesda Research Laboratory. Chemstrips, RNase A,

RNase Guard and Restriction enzymes were purchased from Boeheringer Mannheim Biochemicals. Biotin, insulin (from bovine pancreas) and most other chemicals were purchased by Sigma.

2. Animals

Male Sprague-Dawley rats (150-200 g body weight) were used in these experiments. When the rats arrived, they were kept in cages with free access to drinking water and lab chow for 2-3 days in order to make the rats adpat to new environment. On experiment days, the rats were first anesthetized with ethyl ether and injected with strepzotocin (dissolved in 150 mM sodium citrate solution. pH 4.5) at a dose of 80 mg / Kg body weight intravenously. Whole blood glucose levels of the rats were measured 48 hours after streptozotocin treatment with Chemstrips. Only these rats with blood glucose level more than 350 mg% were used in the following experiments. Diabetic rats were fed high-glucose diet (90% dextrose and 10% casein) for 2 days with free access to drinking water. Biotin (1 mg / Kg body weight) or insulin (20 u /Kg body weight) was administrated intraperitoneally at this time. Following this, at specified time pionts, rats were killed by a blow to the head and decapitated for isolation of the liver and kidney.

3. Assay of PEPCK

3. 1. Preparation of tissue samples - Livers were homogenized in

4 volumes of buffer containing (in final concentrations) 10 mM Tris/HCl, pH. 7.0, 50 mM KCl, 1 mM EDTA, 0.25 M sucrose. The homogenate was centrifuged at 10,000 g for 30 minutes, and the supernatant was assayed for PEPCK activity immediately or stored at - 20°C for next enzyme assay.

3. 2. Radiochemical method - This method is based on procedures given by Lane et al., (1969) for ^{14}C-bicarbonate fixation assay with modifications. The IDP and Mn^{2+} dependent carboxylation of phosphoenolpyruvate results in the formation of ITP and oxaloacetate. The reaction velocity is followed, in the presence of NADH and malate dehydrogenase, by determining the rate of $H^{14}CO_3$ into malate (acid stable 14C activity). The assay medium (total volume in 1 ml) containing (in final concentration) 100 mM imidazol (CL^-) buffer, pH. 6.6, 50 mM $NaH^{14}CO_3$ (approximately 10^5 CPM per micromole), IDP 1.25 mM, M_nCL_2 1.0 mM, GSH 2.0 mM, NADH 2.5 mM. Malate dehydrogenase 5 units and liver supernatant containing 0.5 to 1 mg protein. Phosphoenolpyruvate with a final concentration 1.25 mM was added to initiate the reactions. The assays were carried out at 30°C for 15 minutes, then vials moved to a fume-hood and 1 ml of this reaction solution was transfered to a scintillation counting vial, and baked at 85°C for at least 60 minutes (until the solution was dried up), then 0.5 ml d.d.H_2O and 5 ml of Sincinti verse II was added to the vial and counted by a Beckman LS 3081 Liquid Scintillation System. Reaction mixture without enzyme or IDP were used as blanks.

3. 3. Spectrophotometric method - To determine the PEPCK activity in crude enzyme preparation, a protocol described previously (Petrescu et al., 1979) was used with modifications. The enzyme assay mixture in a volume of 1 ml contains the following components in final concentration: 50 mM Tris/HCl buffer, pH. 7.0, 2 mM $MnCL_2$, 2.5 mM phosphoenolpyruvate, 100 mM $NaHCO_3$ (freshly prepared and saturated with CO_2 before use), 5 units malate dehydrogenase, 0.15 mM NADH, and liver cytosol containing 0.3 to 0.6 mg protein. dGDP (0.4 mM) was added to start the reaction. The decrease in absorbance was monitored at 340 nm and 25°C for 3 minutes. Enzyme activity increased linearly during this time period. For each sample, there was a separate control without addition of dGDP. The decrease in absorbance after subtraction of that due to endogenous oxidization of NADH was used to calculate enzyme activity. 1 unit enzyme activity converts 1 nmole NADH to NAD/minute.

4. Protein Determination

Protein was determined by dye binding method using the reagent supplied by Bio-Rad (Bradford, 1976; Biorad Technical Bulletin, 1977). Bovine serum albumin was used to construct the standard curve.

5. Determination of rat serum insulin concentration

A radioimmunoassay kit specific for rat insulin was used for this purpose. 100 µl NaFAM (0.04 mM sodium phosphate, 0.6% NaCl, 6% human albumin, pH. 7.3) containing insulin standard ranging from 0 to 4 ng/ml or rat serum were mixed with 100 µl anti-porcine insulin Guinea pig serum (1:18000) and incubated for 24 hours at 4°C. Then 100 µl of ^{125}I-(tyr A 19)-human insulin was added to each of tubes and incubated for additional 4 hours at 4°C. A volume of 1.6 ml of 95% (V/V) ethanol was added to all tubes (still at 4°C). Vortex mixed and centrifuged for 10 minutes at 2000 g. One ml of the supernatant from each tube was transfered to plastic vials stoppered and count on a LKB 1271 Riagamma Automatic Gamma counter. The insulin concentration of the unknown sample was determined by referring to the standard curve.

6. Isolation of total RNA and poly $(A)^+$ RNA from rat liver and kidney

6. 1. Isolation of total RNA - Total RNA was isolated from tissues using guanidinium thiocyanate and phenol extraction, as decribed by Chomczynski and Sacchi (1987). In general, immediately after isolation of liver or kidney, 10 to 15 g liver or 5 to 10 g kidney tissue was homogenized in 15 ml of D solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH. 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) by a polytron. 1/10 volume of 2 M sodium acetate, pH 4.0, 2/10 volume of chloroform, and equal volume of water saturated phenol were added respectively and throughly mixed.

This tissue suspension was shaked vigorously for 20 seconds and cooled on ice for at least 20 minutes. After centrifuged at 10,000 g for 20 minutes at 4°C, the aqueous phase containing RNA was transfered to fresh tube, mixed with equal volume of isopropanol and placed at -20°C for at least two hours. The sample was subjected to centrifugation again at 10,000 g at 4°C for 20 minutes. RNA pellet was dissolved in 2 ml of solution D, mixed with equal volume of isopropanol and kept at -20°C for 60 minutes. RNA was pelleted through centrifugation at 8,000 g for 15 minutes at 4°C and was resuspended in 75% ethanol. Aliquots of the sample were transfered to eppendorf tubes, centrifuged at 4°C. The sample was washed once with 75% ethanol, vacuume dried and dissolved in certain volume of double autoclaved dd.H_2O for future analysis.

6. 2. Isolation of poly $(A)^+$ RNA - Poly $(A)^+$ RNA isolation from total RNA was by chromatography on oligo (dT)-cellulose (type 7, Pharmacia LKB Biotechnology Inc.) (Edmonds et al., 1971; Aviv and Leder, 1972).

A: Column chromatography - 0.5 to 1 g of oligo (dT)-cellulose was equilibrated with sterile loading buffer (20 mM Tris / HCl, pH 7.6, 0.5 M NaCl, 1 mM EDTA). A column with 1 to 2 ml of volume was packed and washed sequentially with 3 column-volumes each of sterile water, 0.1 M NaOH and 5 mM of EDTA, and sterile water. After checking pH. of the eluent (less than 8.0), column was washed with 5 volumes of sterile loading buffer. RNA sample (1.5 mg) in

water was added an equal volume of 2 x loading buffer and heated for 15 minutes at 65°C for 15 minutes. Sample was cooled on ice and applied to the column. Eluent was collected, heated at 65°C, cooled ,and reapplied to the column again. The column was washed with 5 column-volumes of loading buffer, poly $(A)^+$ RNA was eluted with 2 to 3 column volumes of sterile TE buffer pH. 7.6. Elution fraction containing poly $(A)^+$ RNA (usually 2 to 4 ml) was added with sodium acetate (3 M, pH. 5.0) to a final concentration of 0.3 M. Poly $(A)^+$ RNA was precipitated by addition of 2.2 volumes of 95% ethanol. Sample was placed at -70°C overnight and centrifuged at 10,000 g at 4°C for 45 minutes. The supernatant was discarded, pellet was dried and dissolved in 0.4 ml of sterile water. Fourty-five µl of 3 M sodium acetate and 1 ml 95% ethanol were mixed with the pellet solution in a eppendorf tube and kept at -70°C for at least 2 hours. After centrifugation by a table eppendorf centrifuge for 30 minutes at 4°C , the supernatant was discarded and the RNA pellet was dissolved in 25 µl of sterile water.

B: Batch absorption method for isolation poly $(A)^+$ RNA - This procedure is a modification of chromatography method. After dissolving total RNA in loading buffer (equal amount of RNA and equal volume of buffer for every sample), 1 ml of oligo (dT)-cellulose solution (previously equilibrated with 1 x loading buffer and containing about 100 µl of oligo (dT)-cellulose) was added to each sample and centrifuged at 1500 g for 4 minute at room temperature. The pellet was washed 4 times with 5 ml of loading

buffer and poly $(A)^+$ RNA was eluted with four 1 ml of washes of sterile TE buffer. The following steps were the same as that described above in the column method.

6. 3. Determination of total or poly $(A)^+$ RNA concentration - The exact concentration of RNA in a sample was determined by measuring the absorbance of a diluted RNA sample at 260 nm using the formula: 1 OD_{260} = 40 µg / ml RNA (Sambrook,et al., 1989). It was found that it was often necessary to heat RNA sample at 65°C for 10 to 15 minutes in order to dissolve RNA completely and get an accurate determination.

7. Electrophoresis of RNA and RNA blotting

Total RNA electrophoresis was done on 1% (w/v) agarose gel containing 6.3% formamide, 1 x GRB (1 X GRB contains 40 mM morpholinopropanesulfonic acid, pH. 7.0, 10 mM sodium acetate, pH. 5.5 and 1 mM EDTA) and 0.5 µg / ml ethidium bromide. Preparation of RNA for gel electrophoresis is as follows: to 9 µl RNA sample containing 30 µg of total RNA was added 20 µl deionized formamide, 7 µl of formaldehyde and 4 µl 5 X GRB. The sample was then incubated at 65°C for 30 minutes to denature any secondary structure, after which it was cooled rapidly to prevent reannealing. Four µl 10 x gel loading buffer containing 50% glycerol, 0.1% bromophenol blue and 0.1% xylene cyanol was added and RNA samples were applied to gel for electrophoresis at 30 volts

overnight.

Poly $(A)^+$ RNA was fractionated on a minigel which had the same composition as that of total RNA gel except smaller size (10 X 7 x 1 cm). Usually 7 µg of poly $(A)^+$ RNA was used for RNA preparation. Sample treatment was the same as that of total RNA except the volume of any components was half of that for total RNA preparation. After loading , RNA sample was run into the gel dry (the gel was not submerged in 1 x GRB) for 15 minutes at 150 volts. Following this, 1 x GRB was added to totally cover the gel, then electrophoresed for 6 hours at 30 volts. Fluorescence photography was performed of the gel with the help of UV-light box after the mini gel was soaked in water overnight (for total RNA gel this soaking was not necessary).

The RNA in the gel was blotted by capillary transfer onto NitroPlus hybridization transfer membrane using 20 x SSC (1 x SSC: 0.15 M NaCl, and 0.015M sodium citrate, pH. 7.0) buffer. The membrane was first placed next to the gel followed in order by 2 pieces of Whatman filter paper, a stack of paper towels, a glass plate and a weight. Two days later, the blotted membrane was peeled off from the gel, air dried, and baked at 80°C for 2 hours under vaccum.

8. Labelling of cDNA probe with (^{32}P)-dCTP

All labelling reactions were performed using a random primer DNA

labelling system (Bethesda Reseach Laborotories). Reactions were carried out according to the kit instructions with a few modifications. Briefly, 100 to 500 ng DNA insert in 23 µl dd.H_2O was heated at 100°C for 5 to 10 minutes and cooled rapidly on ice. Two µl of dATP, dGTP, and dTTP solution (0.5 mM in 3 mM Tris/HCl pH. 7.0, and 0.2 mM EDTA) was added separately and followed by addition of 15 µl random primer buffer mixture (0.67 M HEPES, 0.17 M Tris/HCl , 17 mM $MgCl_2$, 33 mM 2-mercaptoethanol, 1.33 mg/ml bovine serum albumin, 18 OD unit / ml oligodeoxyribonucleotide primers (hexamer fraction, pH. 6.8), 5 µl (α-^{32}P) dCTP (3000 Ci / mmol, 10 µCi / µl) and 1 µl of Klenow fragment (large fragment of DNA polymerase I, 3 units/µl). The reaction was performed in a volume of 50 µl for 1 to 2 hours at room temperature and terminated by 5 µl of stop buffer (0.2 M EDTA, pH. 7.5). In the case of PEPCK or ß-actin probes, the labelled DNA was separated from the unincorporated free nucleotide by centrifugation of the reaction mixture though 1 ml of sephadex G-50 saturated with TE buffer (pH. 7.6) in a 1 ml tuberculin syringe at 2000 rpm for 4 minutes. As for GK probe, the reaction solution passed though a 5 ml Sephsdex G-50 column equilibrated with TE buffer (pH. 7.6). The labelled DNA was collected by a eppendorf tube. The average specific activity of each labelled probe was about 10^8 cpm/µg. Before hybridization, the probe was boiled for 10 minutes to separate the DNA strands followed by rapid cooling to prevent renaturation.

9. Hybridization of RNA membrane with radioactive probe

Prehybridization was performed for at least 4 hours at 42°C in 50% formamide, 5 x Denhardt's solution (0.1% each of Ficoll, polyvinylpyrrolidone,and bovine serum albumin), 5 x SSPE (0.75 NaCl, 50 mM NaH_2PO_4, 5 mM EDTA), 0.1% SDS, 100 µg/ml salmon sperm DNA. Hybridization was performed in the same solution for 24 hours with ^{32}P-labelled cDNA probe at 42°C. After hybridization , filter was washed twice for 10 minutes each in 400 ml of 2 x SSC, 0.1% SDS at room temperature and three times for 60 minutes in 250 ml of 0.2 SSC, 0.1% SDS at 65°C. Filters were dried and exposed to Kodak X-OMAT X-ray films with an intensifing screen for 3 hours to 3 days at -80°C. Quantification of PEPCK, PK, GK, albumin, ß-actin mRNA was accomplished by densitometric scanning of the autoradiograms.

10. Nuclear run on assay

10. 1. Isolation of transcriptionally active nuclei from rat liver - Livers were homogenized in 5 volumes of buffer A (60 mM KCl, 15 mM NaCl, 0.5 mM EGTA, 2 mM EDTA, 15 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer, pH. 7.5, 0.3 M sucrose, 0.5 mM spermidine, 0.15 mM spermine, and 14 mM 2-mercaptoethanol. Homogenate was filtered through four layers of cheesecloth. To this filtered homogenate, about 20 ml of buffer B (components were same as buffer A except that the concentration of sucrose, EGTA, and EDTA was 2 M, 0.1 M and 0.1 mM respectively) was added to a total volume of 30 ml and shaked by reversing the tube

several times. The resulting solution was then carefully layered on top of 10 ml buffer B which served as a cushion (bottom layer). The samples were centrifuged in a SW-28 rotor at 22,000 rpm for 1 hour at 4°C to pellet the nuclei. The supernatant containing membrane fragments and other organelles was aspirated with a pipette and discarded. The nuclei were washed with 1 ml glycerol storage buffer (20 mM Tris/HCl buffer, pH. 7.9, 75 mM EDTA, 0.85 mM DTT, 0.125 mM phenylmethylsulfonylfluoride (PMSF), 50% (V/V) glycerol, adjusted to pH. 7.4) and centrifuged in a eppendorf vial. The pellet was then suspended in 0.2 ml of storage buffer and frozen immediately at -70°C until the in vitro elogation step started.

10. 2. In vitro elongation and isolation of hnRNA - The run-on transcription reaction in isolated nuclei was carried out at 25°C for 30 minutes in a reaction mixture containing 50 mM Tris / HCl, pH. 8.0, 5 mM $MgCl_2$, 100 mM KCl, 0.05 mM EDTA, 20% glycerol, 0.04 mg/ml creatine phosphokinase, 8.8 mM creatine phosphate, 4 mM dithiothreitol, 0.5 mM CTP, 0.5 mM GTP, 1.0 mM ATP, 0.5 unit/ml RNA guard and 1 mCi of (α-^{32}P)-UTP (800 Ci / mmol). Labelled nuclear RNA was isolated based on the method of Chomczyski and Sacchi (1987). To 200 µl of in vitro elongation assay solution, 800 µl solution D (same solution as mentioned in RNA isolation section). The solution was then sheared by several passes through a 22-gauge needle, and transfered to two vials by equal volume. Fifty µl sodium acetate (pH. 4.0), 100 µl chloroform and 0.5 ml of water

saturated phenol were added sequentially to each tube, and shaked vigorously. After cooling for 20 minutes on ice, the samples were centrifuged in a eppendorf centrifuge at 4°C for 15 minutes and the aqueous phase was transfered to a new eppendorf tube, 30 µg tRNA then added to each tube, followed by addition of 0.5 ml isopropanol. The samples were kept at -70°C overnight and centrifuged to get the RNA pellet. RNA pellets coming from the same tissue sample were pooled together and subjected to extraction once more using the same procedure described above. After second extraction, the radiolabelled nuclear RNA was dissolved in 20 µl 10 M ammonium acetate, 80 µl sterile water and 300 µl 75% ethanol were added to the RNA solution and placed at -70°C for 30 minutes. The RNA precipitated was collected by centrifugation and the RNA pellet was dried under vaccum. RNA pellet was dissolved by addition of 110 µl 50% deionized formamide and heated at 65°C for 15 minutes. The radioactivity in 1 µl of RNA sample was determined by scintillation counting.

10. 3. Hybridization of run-on transcripts to filter-bound plasmid DNA. - 5 µg of plasmids containing cDNA inserts for ß-actin, albumin, PEPCK and Bluescript were linearized using the appropriate restriction enzymes and denatured by treating with 0.3 M NaOH at 37°C for 30 minutes. The mixture was then neutralized with an equal volume of 2 M ammonium acetate. Plasmid DNA was spotted onto NitroPlus 2000 using a slotblot apparatus (Bio-Dot^{R}, Bio-Rad) and rinsed with 500 µl of 1 M ammonium acetate. The filter was air-

dried and then baked at 80°C for 2 hours under vaccum. Filter strips were prehybridized for 8 hours at 42°C in a solution containg 50% formamide, 0.8 M NaCl, 34 mM sodium phosphate buffer, pH. 6.5, 0.12% Ficoll 400, 0.12% bovine serum albumin. 0.12% polyvinylpyrrolidone, 0.1% SDS, 100 µg / ml denatured salmon testes DNA and 100 µg / ml poly $(A)^+$ oligonucleotide. Prior to hybridization, ^{32}P labelled RNA was heated for 3 minutes in a boiling water bath, cooled quickly then added to fresh hybridization solution. Hybridization was carried out with $3x10^7$ cpm in hybridization solution similar to the prehybridization solution in a final volume of 1.5 ml at 45°C for 72 hours. Filters were washed twice in 2xSSC, 0.1% SDS at 25°C and 4 times for 60 minutes in 0.2xSSC at 65°C. Autoradiography and densitometric scanning were carried out as with Northern blot hybridization.

11. Isolation and purification of DNA fragments and plasmids

11. 1. cDNA probes and vectors - PEPCK cDNA insert was cloned in PBR322, albumin cDNA was in PUC119, ß-actin probe was cloned in PUC119, GK and PK cDNA were carried in PBR322 and Bluescript, respectively. All these plasmids are provided by Dr. Chauhan.

11. 2. Large scale plasmid preparation - large scale preparations of plasmid DNA were performed according to the procedures of Maniatis et al. (1989). Ten µl DH5α E. Coli transformed with the appropriate plasmid was inoculated into 20 ml LB medium (Luria -

Bertani; medium containing 1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract and 1% (w/v) NaCl, pH. 7.0) with appropriate kind and amount of antibiotics, and incubated at 37°C overnight. The entire bacterial culture was then inoculated into 750 ml of LB medium and incubated at 37°C until an OD_{600} value of 0.6 was reached. This usually required about 4 hours. At this point, 4 ml of 4 mg / ml chloramphenicol in ethanol was added, and the cells were incubated overnight in a rotary shaker / incubator at 3000 rpm. The cell suspension was transfered to a 500 ml Beckman centrifuge bottle and spun in a GS-A rotor at 4000 x g for 15 minutes at 4°C. The supernatant was discarded while the pellet was drained and resuspended in 75 ml ice-cold of solution: 25 mM Tris/HCl (pH. 8.0), 50 mM glucose, 10 mM EDTA. To this solution, 15 ml of 0.2 M NaOH and 1% SDS was added, mixed by gently inverting the tube several times and kept on ice for 10 minutes. Following this, 10 ml of ice-cold solution of 5 M potassium acetate (pH. 4.8) was added and mixed the contents by sharply inverting the tube several times and incubated on ice for 10 minutes. The sample was then centrifuged for 30 minutes at 10,000 x g at 4°C in a SS34 rotor to spin down bacterial debris and precipitated high molecular weight DNA. The supernatant was saved and 0.6 volume of isopropanol was added followed by mixing and incubation for 15 minutes. Plasmid DNA was recovered by centrifugation at 12,000 x g for 30 minutes at room temperature in a SS34 rotor. The supernatant was discarded and the pellet was dissolved in 8 ml TE buffer (pH. 8.0). To each ml of DNA solution, exactly 1 g of solid cesium chloride was added

and mixed gently until all the salt is dissolved. 1 ml of ethidium bromide (10 mg / ml in water) was added to every 10 ml of cesium chloride solution containing DNA except one and mixed well. The mixture was then transfered to a Quick-Seal™ centrifuge tube (16 cm x 76 cm). The tube then was filled up completely with cesium chloride - ethidium bromide solution without plasmid DNA, balanced and sealed with a Beckman heat sealer. The tube was placed in a Beckman Ti 70.1 rotor and centrifuged at 55,000 rpm for 18 hours at 22°C, following which, the speed was reduced to 45,000 rpm for a further 1 hour. Two bands were usually visible under UV-light . The bottom band (closed circular plasmid DNA) was collected to a 15 ml Falcon tube by pucturing the side of the centrifuge tube with a 21 guage needle at a position just below the band. The ethidium bromide in the plamid solution was removed by extraction 5 to 7 times with water saturated equal volume of 1-butanol. The aqueous bottom layer containing the plasmid was transfered to a fresh tube and the volume was made up to 5 ml with dd.H_2O. To precipitate the plasmid, 500 µl 3 M sodium acetate (pH. 5.0) and 10 ml 95% ethanol was added and mixed. After keeping at -20°C overnight, the plasmid was recovered by centrifugation at 11,000 rpm for 30 minutes at 4°C in a SS34 rotor. The pellet was dried under vaccum and dissolved in 100-200 µl TE buffer (pH. 8.0). The plasmid samples were stored at -20°C, the concentration of the plasmid DNA solution was determined by measuring the absorbance of a diluted sample at 260 nm using the formula: 1 OD_{260} = 5µg / ml DNA.

11. 3. Linearization of plasmid for nuclear run-on assay - The plasmid was first cut with the appropriate restriction endonucleases at 37°C overnight using roughly 1.5 unit / μg DNA. The total reaction volume was usually 0.5 ml. Following digestion, 250 μl phenol (equilibrated with 0.1 M Tris / HCl buffer, pH. 8.0, containing 0.1% hydroxyquinoline and 0.2% ß-mercaptoethanol) and 250 μl chloroform were added, and then mixed. After centrifugation, the top aqueous solution was transfered to fresh vial (0.5 ml), DNA was then precipitated using sodium acetate and ethanol. Pellet was dried, dissolved in sterile water and the concentration was determined (same as in the section of large scale plasmid preparation.

11. 4. Isolation of cDNA fragment from plasmid - Procedures for plasmid DNA digestion were the same as in plasmid linearization section. The mixture containing the fragments was size fractionated on 1% (w/v) agarose gels in 1 x TAE buffer (0.04 M Tris / acetate, 1 mM EDTA, pH. 8.0) containing ethidium bromide (7 μl of 10 mg / ml solution per 150 ml gel). After running the samples overnight at 30 volts, the gel was visualized by UV light and the band of interest cut out from the gel. The gel slice containing the band was then transfered to the inner tube of a self-made filtration centrifuge unit (a hole was made at the bottom of the 0.5 ml volume eppendorf tube, a small piece of glass wool was fit at the bottom of this tube. The 0.5 ml eppendorf tube was then fit into a 1.5 ml volume eppendorf tube and autoclaved) and centrifuged at 6,000 rpm

for 10 minutes. The 0.5 ml volume of eppendorf tube with glass wool and agarose was discarded. The resulting solution containing DNA fragment was measured, DNA was precipitated with salt and ethanol, washed once more with 75% ethanol, dried, dissolved in water for concentration determination and radiolabelling.

Part III. Results

1. Regulatory influence of biotin on PEPCK in diabetic rat

1. 1. Time course studies on suppression of hepatic mRNA after biotin administration - In starved rats, biotin induces increased transcriptional rate of GK gene as early as 15 minutes after injection and the maximum level of mRNA is seen at 60 minutes following biotin treatment (Chauhan and Dakshinamurti, 1991). This suggests that the induction of GK gene expression by biotin does not require new protein synthesis. In order to increase the opportunity of detecting any effect of biotin on the level of PEPCK mRNA, whether the change is caused by a mechanism similar to that of Gk or by mechanism which needs new protein formation, a 1 to 10 hours time course study was carried out. Initially, the rats were kept for 7 days after making them diabetic (rats were with free access to drinking water and rodent chow). Results from the first experiment indicated that mRNA level of PEPCK was significantly decreased at 1 to 2 hour time period following biotin administration (Figure 2). However, in a following experiment, we

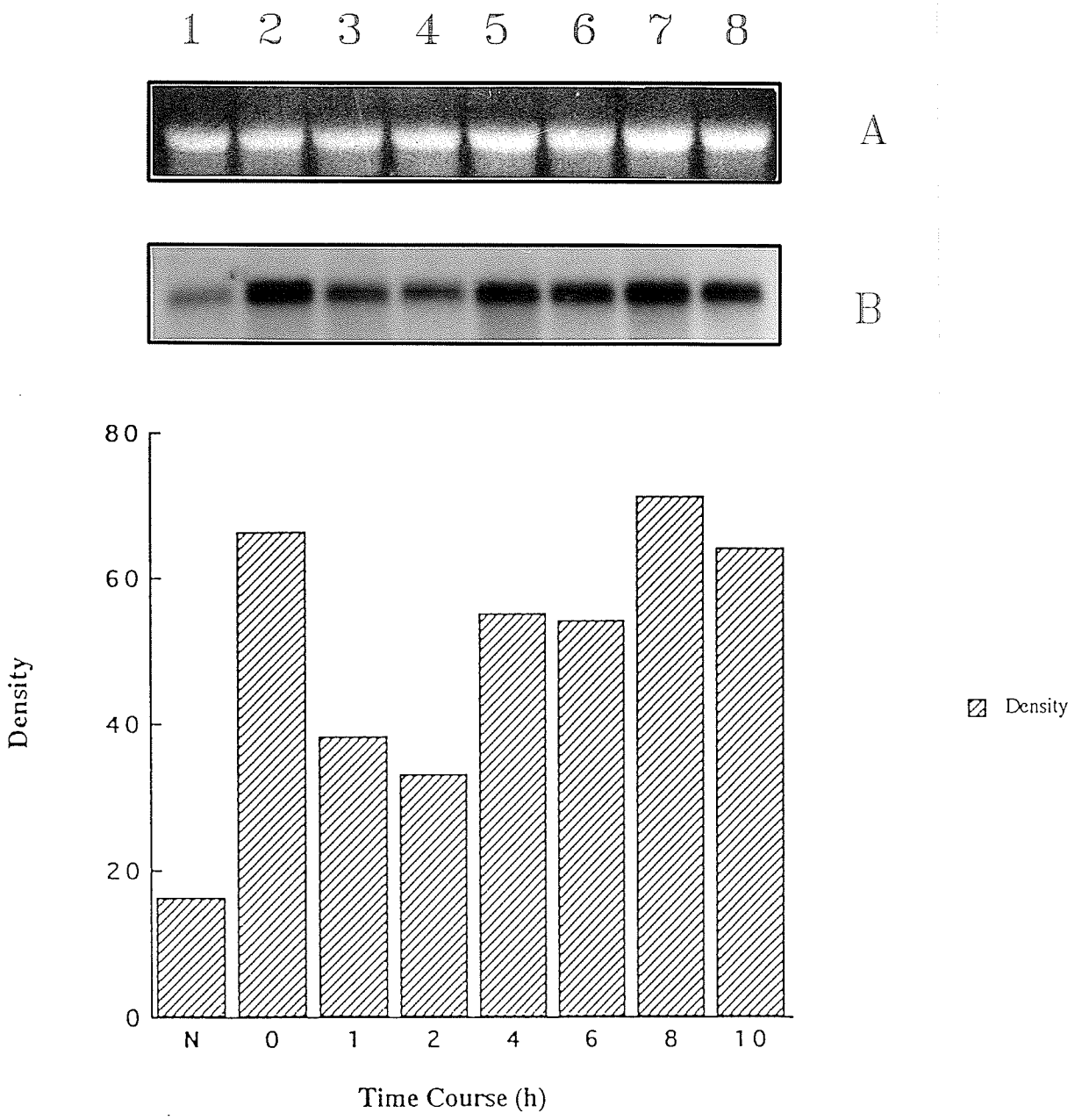

Figure 2. Time course of deinduction of hepatic PEPCK mRNA by biotin in diabetic rats.
In this experiment, diabetic rats were kept for 7 days before the experimental day. Other experimental procedures are described in "Materials and Methods". A, 28S rRNA of rat liver. B, PEPCK mRNA of rat liver. Lane 1, normal rat. Lane 2, diabetic control. Lane 3, diabetic rat 1 h after biotin treatment. Lane 4, diabetic rat 2 h after biotin treatment. Lane 5, diabetic rat 4 h after biotin treatment. Lane 6, diabetic rat 6 h after biotin treatment. Lane 7, diabetic rat 8 h after biotin treatment. Lane 8, diabetic rat 10 h after biotin treatment. Diagram under B indicates the relative absorbance value of each band at corresponding time point in B.

found the responsiveness of some diabetic rats to biotin was less efficient (Figure 3). Previous report from our laboratory (Dakshinamurti et al., 1970) indicated that biotin induced GK activity only with the rats that were diabetic for less than 5 days. Following the same line of consideration, the rats were sacrificed 3 days after becoming diabetic. Northern blots from these samples showed some improvement in terms of deinduction response of hepatic PEPCK mRNA to biotin, but certain individual variation still existed. To minimize individual variation and maximize the response of PEPCK mRNA to biotin, the rats were fed high glucose diet (90% dextrose and 10% casein) for two days within the three days of their diabetic life and liver samples (the same weight) from several rats were pooled together for each time point. Results from these samples showed a consistent decrease of PEPCK mRNA after biotin administration (Figure 4). To define the recovery time of hepatic PEPCK mRMA after injection of biotin, the time course study was extended to 5 hours in the following experiments. As indicated in Figure 5, the relative abundance of PEPCK mRNA in diabetic rats is about 10-fold higher in comparison with that of non-diabetic rats. After biotin administration, hepatic PEPCK mRNA started to decrease at 1 hour and dropped to 15% of the non-biotin-injected control level by 3 hours after the injection. The concentration of hepatic PEPCK mRNA recovered significantly by the end of this 5 hour time course study (Table 1).

1. 2. Influence of biotin on kidney PEPCK mRNA in diabetic rats -

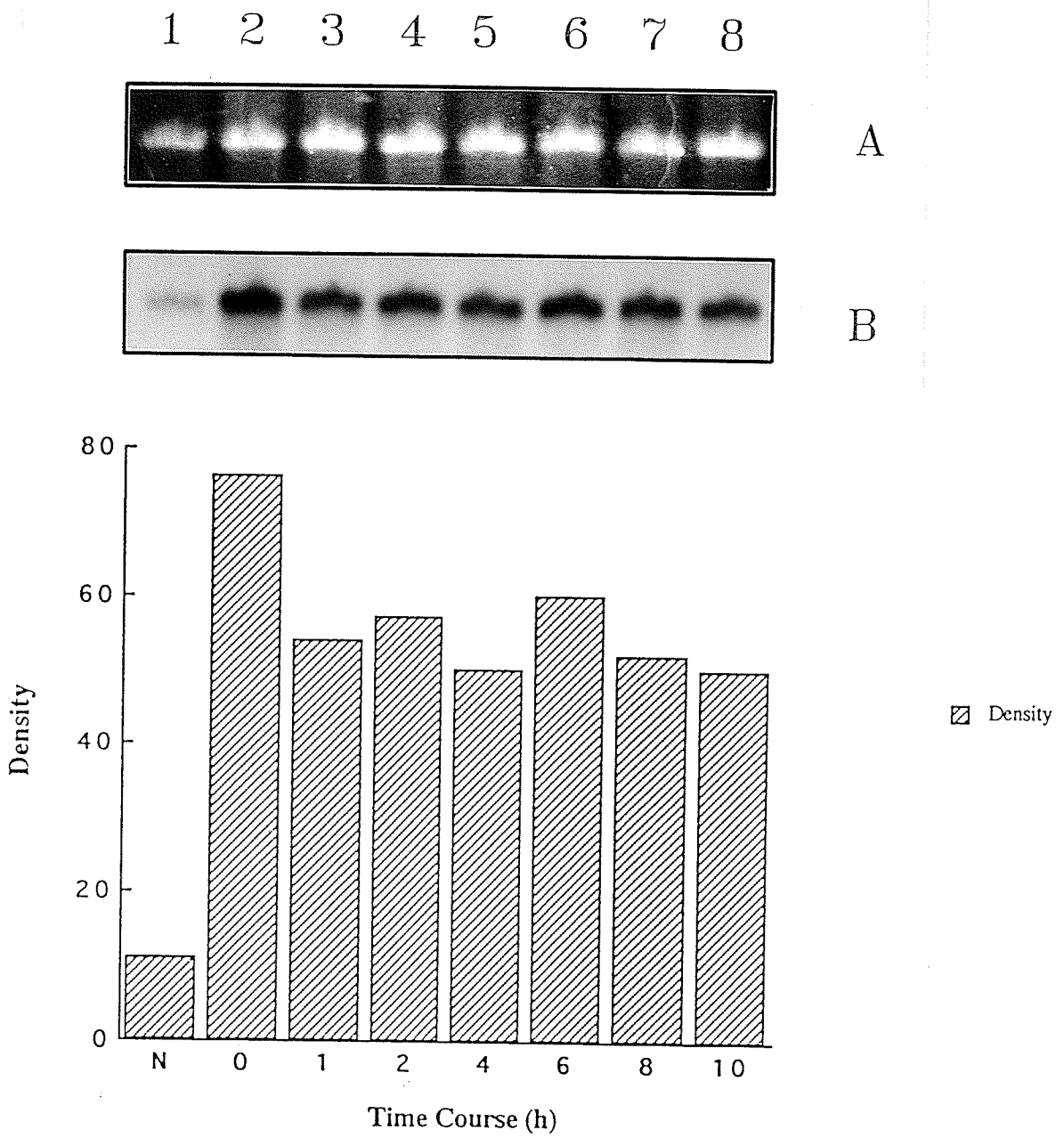

Figure 3. Time course of deinduction of hepatic PEPCK mRNA by biotin in diabetic rats.
Experimental conditions and procedures are same as Figure 2. A, 28S rRNA of rat liver. B, PEPCK mRNA of rat liver. Lane 1, normal rat. Lane 2, diabetic control. Lane 3, diabetic rat 1 h after biotin treatment. Lane 4, diabetic rat 2 h after biotin treatment. Lane 5, diabetic rat 4 h after biotin treatment. Lane 6, diabetic rat 6 h after biotin treatment. Lane 7, diabetic rat 8 h after biotin treatment. Lane 8, diabetic rat 10 h after biotin treatment. Diagram under B indicates the relative absorbance value of each band at corresponding time piont in B.

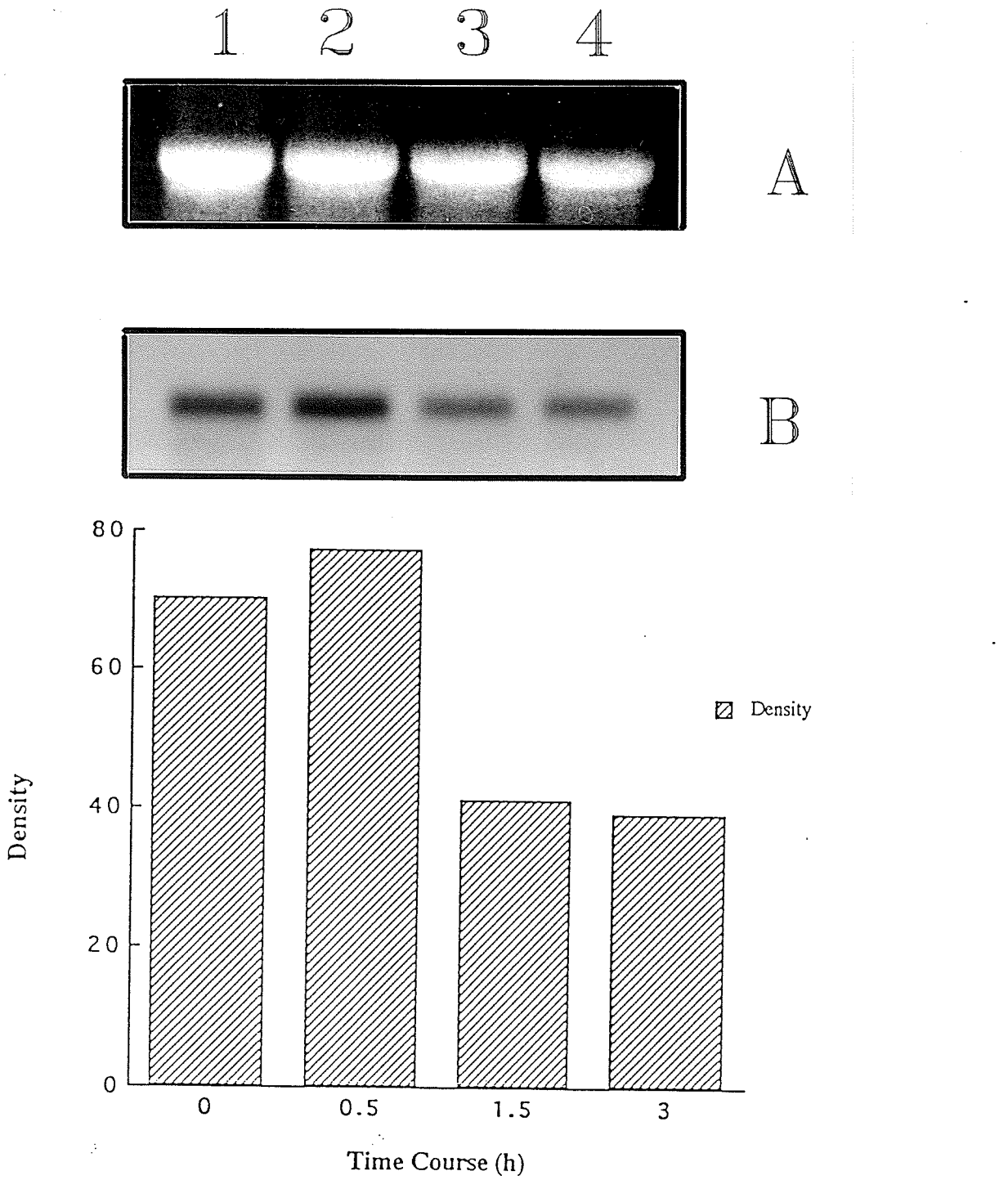

Figure 4. Time course of deinduction of hepatic PEPCK mRNA by biotin in diabetic rats.
Rats were used for biotin treatment and RNA isolation 3 days after they became diabetic. Before experimental day, 2 days of high-glucose diet was given to the diabetic rats. Other experimental procedures are same as Figure 2. A, 28S rRNA. B, PEPCK mRNA of rat liver. Lane 1, diabetic control. Lane 2, diabetic rat 30 min after biotin treatment. Lane 3, diabetic rat 1.5 h after biotin treatment. Lane 4, diabetic rat 3 h after biotin treatment. Lane 6, diabetic rat 6 h after biotin treatment. Lane 7, diabetic rat 8 h after biotin treatment. Diagram under B indicates the relative absorbance value of each band at corresponding time piont in B.

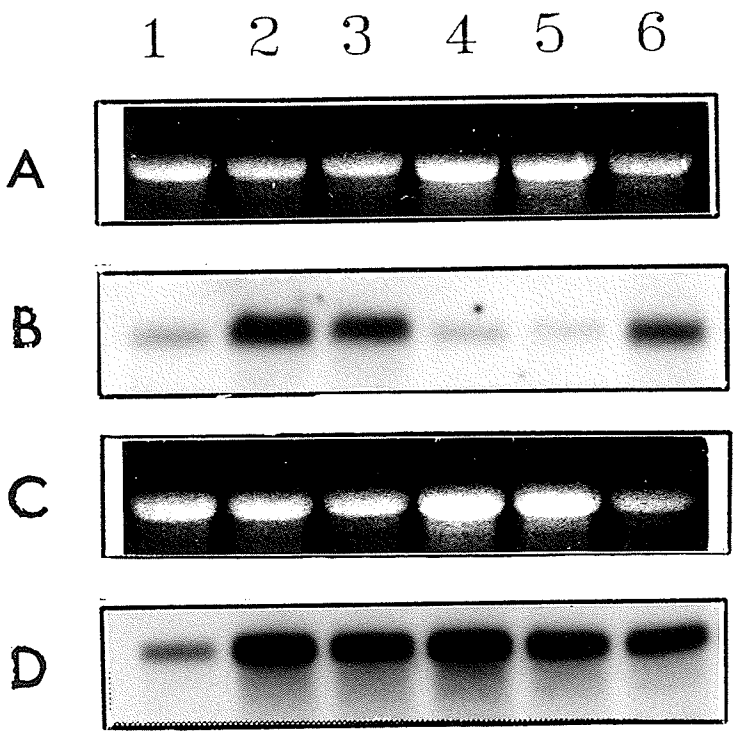

Figure 5. Effects of biotin on PEPCK mRNA concentrations in liver and kidney of diabetic rats.
The experimental procedures are described in "Materials and Methods". A, 28S rRNA of rat liver. B, PEPCK mRNA of rat liver. C, 28S rRNA of rat kidney. D, PEPCK mRNA of rat kidney. Lane 1, normal rat. Lane 2, diabetic control. Lane 3, diabetic rat 1 h after biotin treatment. Lane 4, diabetic rat 2 h after biotin treatment. Lane 5, diabetic rat 3 h after biotin treatment. Lane 6, diabetic rat 5 h after biotin treatment.

Table 1. Time course of the effects of insulin or biotin on liver and kidney PEPCK mRNA concentration in diabetic rats. Experimental details are described under "Materials and Methods". The height of the absorbance peaks corresponding to the mRNA bands was calculated and expressed in arbitrary units. Values represent the mean and standard deviation of three or four completely separate experiments. Student's T test was used for statistical calculation.

Time after treatment (h)	Insulin Treatment				Biotin Treatment			
	Liver		Kidney		Liver		Kidney	
	PEPCK mRNA	%	PEPCK mRNA	%	PEPCK mRNA	%	PEPCK mRNA	%
0	82± 7	100	66±12	100	72±14	100	49± 7	100
1	22± 9*	27	57± 6	100	61±22	85	49± 6	100
2	18±12*	22	58± 3	88	26± 7*	36	52±10	106
3	9± 2*	11	55±11	83	11± 2*	15	59± 1	120
5	29±20*	35	53±10	80	51± 4#	71	50± 8	102
7	72±11	88	55±11	83	N.D.		N.D.	

*: In comparison with the values of 0 h, $P < 0.01$
#: In comparison with the values of 0 h, $P < 0.05$
N.D. Not determined

It is well known that kidney tissue is rich in PEPCK activity (Schoolwerth et al., 1988). Renal PEPCK mRNA level was investigated after biotin injection. As indicated in Figure 5 and Table 1, the PEPCK mRNA abundance of kidney in diabetic rat increased significantly but no suppression of PEPCK mRNA was found after biotin injection.

1. 3. Hepatic PEPCK activity after administration of biotin in diabetic rats - Hepatic PEPCK activity following suppression of mRNA was determined in a 24 hour time period. The initial and maximum decrease of PEPCK activity was shown at 3 hour and 5 hour respectively after biotin injection which indicated a 2 hour time lag from the decrease of mRNA level in both cases. The activity returned to the pre-injection level by 10 hours after biotin treatment (Table 2).

1. 4. Results of nuclear run-on assay - The run-on transcription experiments were used to estimate the relative rates of liver PEPCK gene transcription at various time intervals following biotin administration to diabetic rats. The results are shown in Table 3. Hybridization with (α-^{32}P)-UTP labelled RNA transcripts to vector PBR322 DNA was negligible. The transcription of the actin gene, included as internal control, was not influenced by biotin administration. Biotin suppressed the transcription rates of hepatic PEPCK gene by 55% at 30 minutes (Table 3) and gradually, the rate of transcription increased back to the original level

Table 2. Time course of the effects of insulin or biotin on hepatic PEPCK activity of diabetic rats.
Experimental details are described under "Materials and Methods". Data given are the mean and standard deviation of four complete separate experiments. Student's T test was used for statistical calculation.

Time after treatment (h)	Hepatic PEPCK activity after administration of insulin (U/mg Protein)	Hepatic PEPCK activity after administration of biotin (U/mg Protein)
Non-diabetic control	6.6±1.2	6.6±1.2
0	19.4±1.5	19.4±1.5
1	20.2±1.7	20.4±1.2
3	10.0±3.5*	11.4±1.0*
5	8.9±4.6*	9.2±3.3*
7	8.2±3.1**	13.1±3.8#
10	8.3±2.1*	18.2±1.7
14	8.9±3.6*	25.5±1.5
24	18.3±0.5	21.3±3.1

\# In comparison with the values of 0 h, $P < 0.05$
* In comparison with the values of 0 h, $P < 0.01$
** In comparison with the values of 0 h, $P < 0.001$

Table 3. Biotin induced suppression of hepatic PEPCK gene transcription.
Nuclei were isolated at specified times after biotin administration. The procedures for isolation of nuclei and nuclear run-on assay are described under "Materials and Methods". The radiolabeled transcripts were hybridized to filter-bound plasmids containing cDNA for PEPCK and ß-actin respectively. Specific transcripts from the two genes were detected by autoradiography of the filters. Values in this table are in arbitrary units obtained by densitometric scanning of the autoradiograms. Data are given as the mean and standard deviation of four completely separate experiments. Student's T test was used for statistical calculation.

Time after biotin injection (min)	Rate of gene transcription			
	PEPCK	%	ß-actin	%
0	70±14	100	12.3±9.0	100
15	44±6.6#	63	13.0±7.8	106
30	32±5.6*	45	14.0±5.6	114
45	51±11	72	16.0±3.7	130
60	63±11	90	15.0±2.5	122

In comparison with the values of 0 h, $P < 0.02$
* IN comparison with the values of 0 h, $P < 0.01$

(Figure 6; Table 3).

2. Regulatory effect of insulin on hepatic and kidney PEPCK mRNA

2. 1. Suppression of PEPCK mRNA of liver and kidney by insulin in diabetic rats - parallel studies with insulin have been done to compare to the results with biotin treatment. The concentration of of hepatic PEPCK mRNA decreased significantly during the 1 to 5 hour time period and the timing of initial and maximum deinduction closely matched that induced by biotin. However, insulin suppression hepatic PEPCK mRNA was stronger and and longer lasting (Figue 7; Table 1). Kidney PEPCK mRNA was not regulated by insulin.

2. 2. Effect of insulin on the suppression of hepatic PEPCK activity in diabetic rats - Compatible with the deinduction pattern of mRNA, PEPCK activity decreased markedly after insulin treatment (Table 2). After reaching maximum inhibition, the enzyme activity kept at this low level for a relatively long period and returned to pre-injection level by 24 hours, an inhibitory duration much longer than that seen after biotin treatment.

3. The effect of biotin on hepatic GK mRNA in diabetic rats

Because GK mRNA in liver is less abundant, poly(A)$^+$-RNA was isolated and used for Northern blot analysis. After determination of abundance of GK mRNA at each time point, the same blot was

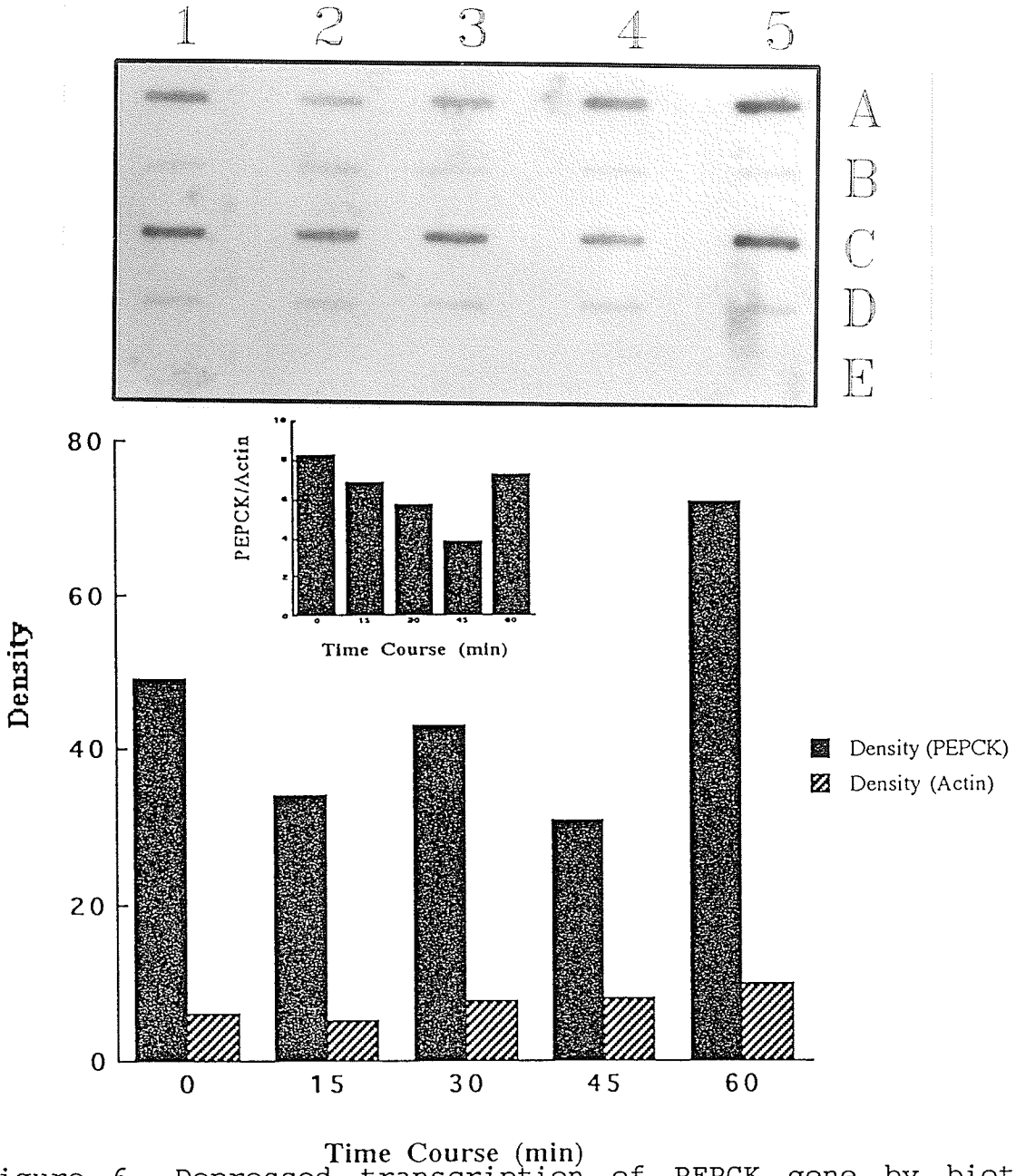

Figure 6. Depressed transcription of PEPCK gene by biotin in diabetic liver.
Nuclear run-on assays were used for estimating the relative trancription rate of PEPCK gene after biotin treatment. The principle and experimental procedures are described in "Materials and Methods". A, in vitro labelled nuclear albumin mRNA. B, in vitro labelled nuclear PK mRNA. C, in vitro labelled nuclear PEPCK mRNA. D, in vitro labelled nuclear actin mRNA. E, pBR322 plasmid. Actin mRNA was used as internal control, pBR322 was used for background control. Lane 1, diabetic control. 2, diabetic rat 15 min after biotin treatment. Lane 3, diabetic rat 30 min after biotin treatment. Lane 4, diabetic rats 45 min after biotin treatment. Lane 5, diabetic rat 60 min after biotin treatment. Diagram under the autoradiograph indicates the relative values of density absorbance for PEPCK and actin at specified time pionts. The ratio value of PEPCK/actin is also included in the diagram for easier comparison.

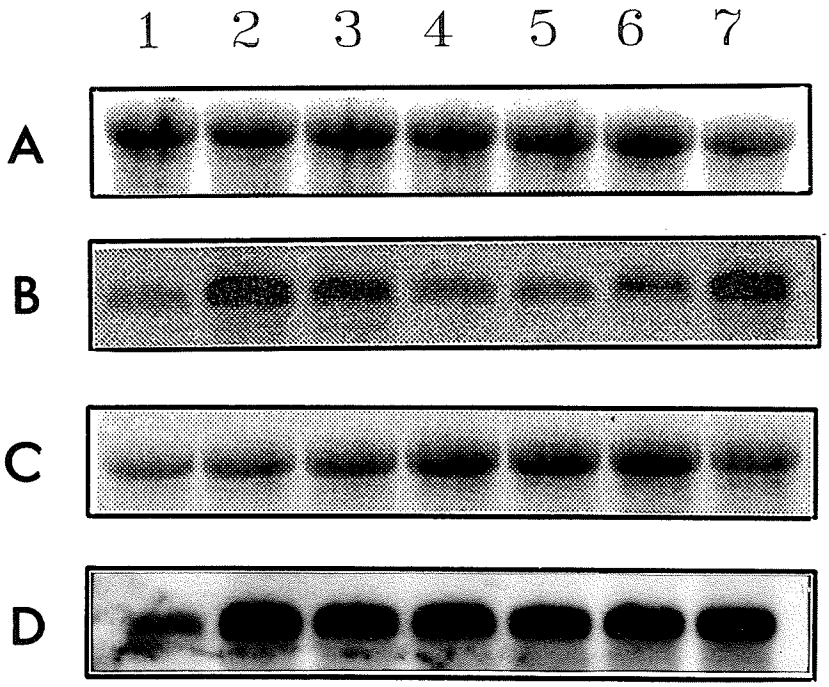

Figure 7. Effects of insulin on PEPCK mRNA concentrations in liver and kidney of diabetic rats.
The experimental procedures are described in "Materials and Methods". A, 28S rRNA of rat liver. B, PEPCK mRNA of rat liver. C, 28S rRNA of rat kidney. D, PEPCK mRNA of rat kidney. Lane 1, normal rat. Lane 2, diabetic control. Lane 3, diabetic rat 1 h after insulin treatment. Lane 4, diabetic rat 2 h after insulin treatment. Lane 5, diabetic rat 3 h after insulin treatment. Lane 6, diabetic rat 5 h after insulin treatment. Lane 7, diabetic rat 7 h after insulin treatment.

deprobed and reprobed with ß-actin cDNA to serve as a internal control. Preliminary results indicated that hepatic GK mRNA from diabetic control rats was undetectable. There were two main peaks of GK mRNA induction by biotin (1 hour and 3 hour after biotin injection) and certain variations in terms of response time point and magnitude existed (Figure 8; Table 4).

4. Serum insulin levels of diabetic rats after biotin injection

The constant concentration of serum insulin after biotin administration to diabetic rats is shown in Figure 9. Diabetic rats had less than one hundredth the level of serum insulin found in corresponding normal rats. Injection of biotin to the diabetic rat did not influence the serum insulin level in the diabetic rats (Figure 9).

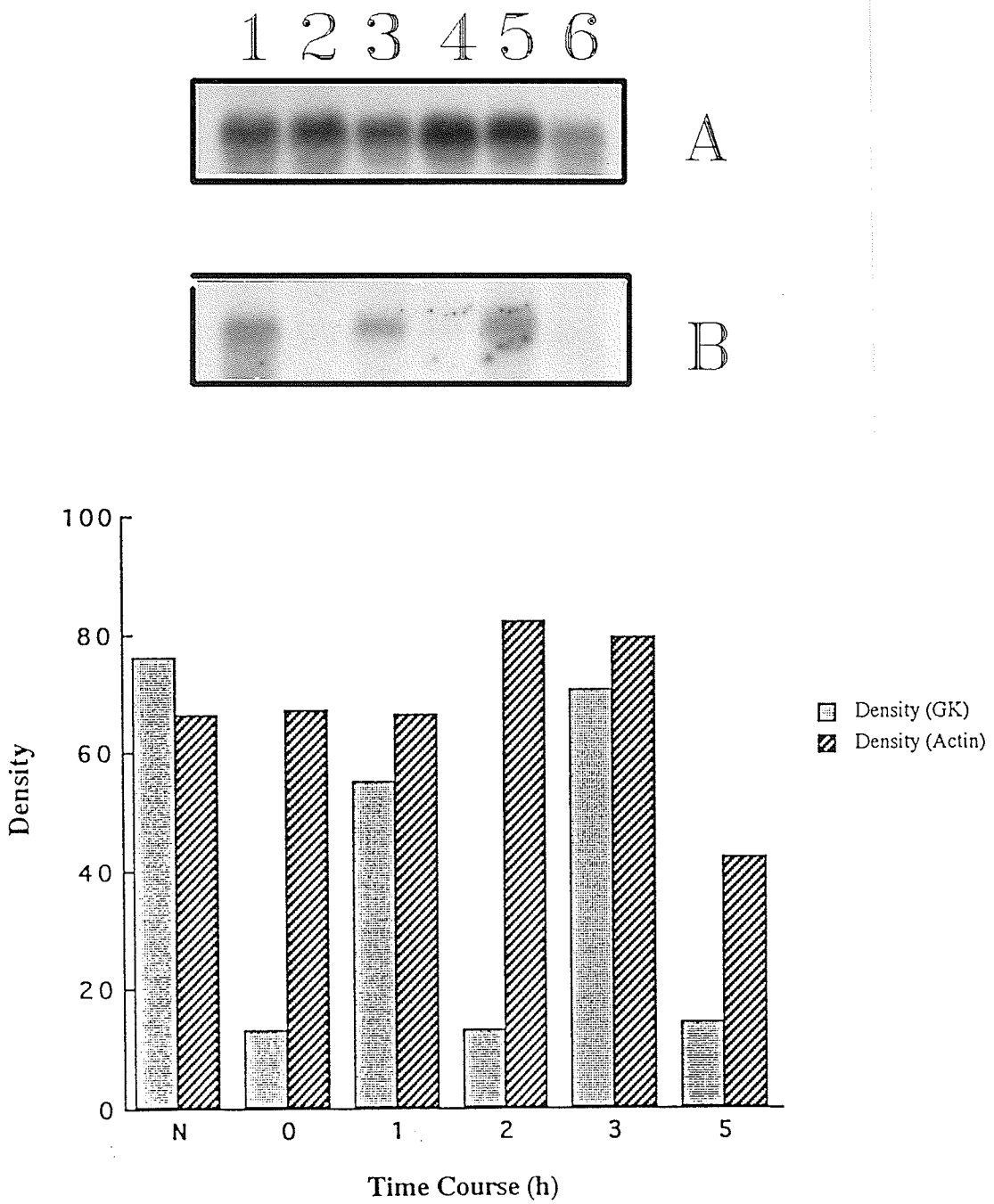

Figure 8. Effects of biotin on hepatic GK mRNA concentrations of diabetic rats.
The experimental procedures are described in "Materials and Methods". A, ß-actin mRNA of rat liver. B, GK mRNA of rat liver. Lane 1, normal rat. Lane 2, diabetic control. Lane 3, diabetic rat 1 h after biotin treatment. Lane 4, diabetic rat 2 h after biotin treatment. Lane 5, diabetic rat 3 h after biotin treatment. Lane 6, diabetic rat 5 h after biotin treatment.

Table 4. Time course of the effects of biotin on induction of GK mRNA in diabetic rats.
Poly $(A)^+$ RNA was used for this study (experimental details are described under "Materials and Methods". ß-actin was used as internal control. The height of the absorbence peaks corresponding to the mRNA bands was calculated and expressed in arbitary units. Values represent the mean and standard deviation of three completely separate experiments. Student's T test was used for statistical calculation.

	GK(units)		ß-actin(units)	
Time Course (h)	mRNA	%	mRNA	%
Normal	81±6.1	100	43±19	100
0	14±5.1	17.6	53±17	123
1	45±20	55.3	45±24	104
2	10±3.0	12.3	57±30	132
3	52±21*	62.7	54±23	125
5	11±5.0	13.9	41±11	95

* In comparison with the value of 0 h, $P < 0.05$

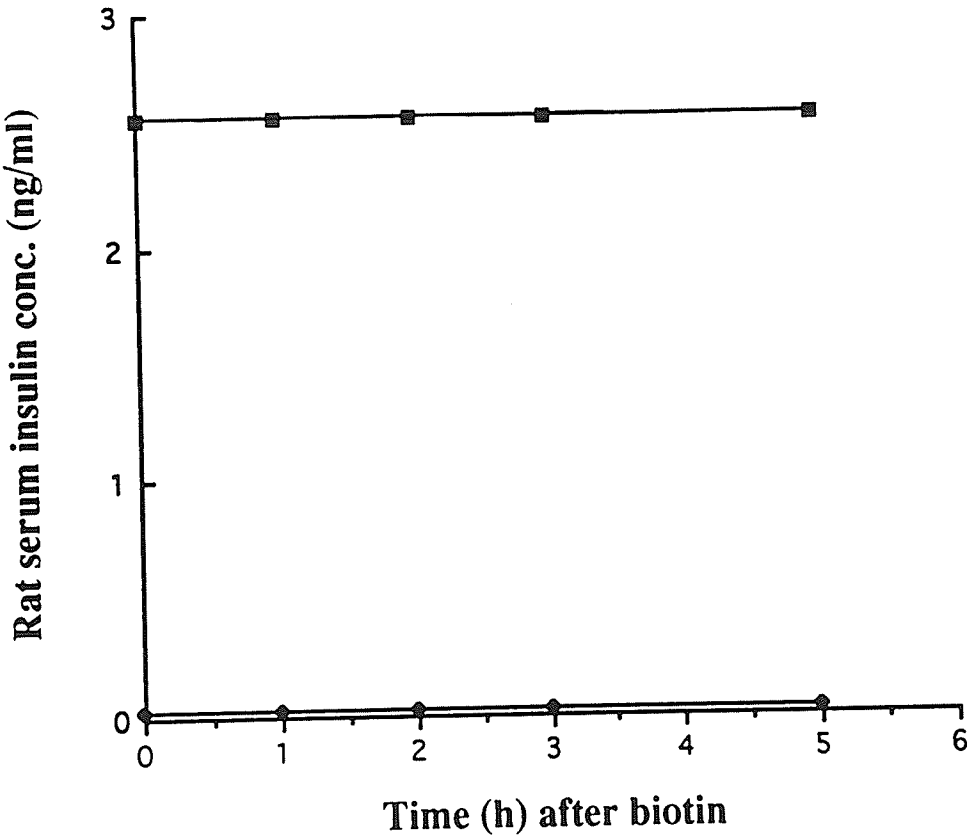

Figure 9. Rat serum insulin concentration after biotin administration.
Serum from 4 to 5 rats was pooled for each time point before and after biotin administration and used for insulin concentration measurement. The experimental procedures are described in "Materials and Methods". ■ represents serum insulin concentration of normal rats. • represents serum insulin concentration of diabetic rats.

Part IV. Discussion

1. The validity of using diabetic rats induced by streptozotocin as a diabetic research model

Streptozotocin is a glucose-containing anologue of the N-nitroso compound, methylnitrosourea and a well-documented diabetogenic agent in laboratory animals (Byrne and Schein, 1981). Because of the strong diabetogenic effect of this drug to rat (Mossman, et al., 1985), streptozotocin was used to make rats diabetic in this study. Streptozotocin exhibits selective toxicity to B cells of pancreas in some species of mammals animal (Byrne and Schein, 1981). It is unclear why streptozotocin shows a specificity for B cell, although there is evidence to suggest that the presence of the glucose moiety is important (Mossman et al., 1985). It has been found that streptozotocin-induced diabetogenicity is ameliorated when 3-O-methylglucose or 2-deoxyglucose, the non-metabolized analogues of glucose, are administered to rodents (Rossini et al., 1977; 1978). Large amount of glucose also prevent hyperglycemia and necrosis of B cells after injection into rats of either alpha or beta anomers of streptozotocin (Rossini et al., 1977). When injected into rodents at equivalent concentrations, streptozotocin is found in insular tissue at 3.8 fold higher amounts than methylnitrosourea (Anderson et al., 1974). These data suggest that the structural glucose renders N-nitroso compounds with a unique specificity for B cell.

Streptozotocin is metabolized in liver and eleminated through

kidney. Injection of streptozotocin leads to rapid acumulation of this drug in liver and kidney, and hepatotoxicity and proximal renal tubular injury, when a large dose is given, have been reported (Weiss 1982; Evan et al., 1984). However, it is found that with a dose of 60 mg/kg body weight, streptozotocin induces severe diabetes in rat without any detectable kidney injury (Evan et al., 1984).

In the present study, the amount of streptozotocin used to induce diabetes in rats was the same as that of the majority of scientific reports using diabetic rats. Thus, one can assume that at this dose range of streptozotocin, no obvious toxicity of this drug to tissues other than B cell of pancreas would happen. In addition, in the case of starved rats where the physiological changes of hormone (mainly insulin and glucagon) status are similar to that in streptozotocin induced diabetic rats, the enzyme activities and mRNA levels of hepatic GK, PK and PEPCK and renal PEPCK have the same pattern of change as that of the streptozotocin induced diabetic rats (Pilkis and El-Maghrabi 1988; Granner and Pilkis 1990), and this , from another aspect, provides evidence that the change of these gluconeogenic and glycolytic enzyme activities are due to diabetic status rather than drug toxicity.

2. The regulatory effect of biotin on hepatic PEPCK mRNA in diabetic rats

Previous studies from our laboratory have shown that biotin,

similar to insulin, can increase enzyme activities of hepatic GK, phosphofructokinase and PK in diabetic rats and the effect of biotin on GK could be blocked by actinomycin D (Dakshinamurti et al., 1970). Recent studies has indicated that biotin can increase transcription of GK gene in starved rats (Chauhan and Dakshinamurti, 1991). In both diabetic and starved rats, insulin regulates these key enzymes of glycolytic and gluconeogenic pathway in a highly integrated way (Pilkis and El-Maghrabi, 1988). Gene expressions of these enzymes are one of the major steps regulatd by insulin and other hormones, and in the case of GK and PEPCK, modulation of gene expression is almost the only regulation step (Granner and Pilkis, 1990). Since biotin coordinately increases the three key glycolytic enzyme activities in diabetic rats, it is of interest to investigate the possible effects of biotin on the key gluconeogenic enzmyes.

In the present study, PEPCK, due to its important role in the regulation of gluconeogenesis, was investigated for the effect of biotin. Time course studies indicated that there was a significant decrease of hepatic PEPCK mRNA concentration 1 hour after biotin administration and by 3 hours of this time course study, the abundance of hepatic PEPCK mRNA dropped by 6-fold when compared with diabetic control. Injection of insulin to diabetic rats led to a similar supression of hepatic PEPCK mRNA, but the effect of insulin was stronger and longer lasting in comparison with that of biotin. The in vivo time course of repression of PEPCK mRNA by

insulin in this study is consistent with other reports (Cimbala et al., 1982; Beale et al., 1984).

It has long been recognized that the depression of hepatic PEPCK synthesis by insulin in diabetic rat needs the coadministration of glucose (Tilghman et al., 1974; Cimbala et al., 1981). More recent studies confirm that the repression of hepatic PEPCK mRNA by insulin also requires feeding glucose to diabetic rats (Cimbala et al., 1982). Glucose feeding could be replaced by feeding the diabetic rats with rodent chow (Beale et al., 1984). Cimbala et al. (1982) have investigated the so-called "glucose-effect". They found that insulin or glucose given separately had no effect on PEPCK mRNA 90 minutes after administration. Glucose plus insulin caused a marked reduction in the levels of translatable mRNA for the enzyme, although the blood glucose concentration was essentially in the same range as that noted in diabetic rats not given glucose by gavage. It was also found that the role of glucose could be substituted by fructose feeding or injection of somatostatin. Because of the relatively low level of blood glucose in insulin-alone treated diabetic rat and the fact that somatostatin may inhibit the secretion of glucagon, they hypothesized that insulin administration alone would lead to acute hypoglycemia and in turn hypoglycemia would stimulate the secretion of glucagon (Unger and Dobbs, 1978) and thus antagonize insulin's ability to inhibit PEPCK synthesis. Somatostatin can inhibit the secretion of glucagon (Unger and Dobbs, 1978) and thus can facilitate the insulin effects

on PEPCK. However, several lines of evidence potentially disagree with this hypothesis. (1) In their studies, the blood glucose level of rats treated with insulin alone was in the same range as that in rats treated with insulin plus glucose, and within the normal limits of blood glucose concentration. It has been reported that hypoglycemia would stimulate secretion of glucagon only when blood glucose falls below 50 mg% (Dobbs, 1981). Thus, it seems that the relatively low (but still above the normal low limit) blood concentration in insulin-alone treated diabetic rats can not constitute an effective stimulation for glucagon secretion. (2) Many in vitro studies have confirmed that insulin exerts a dominant negative effect on hepatic transcription (Sasaki et al., 1984; Granner et al., 1983) over the effects of many other hormone including glucagon. Thus, the hypothesis that the increased secretion of glucagon diminishes the effect of insulin on hepatic PEPCK seems contradictory to this observation. (3) Somatostatin has many other fuctions besides inhibition of glucagon secretion (Dobbs, 1981), and thus the mechanism by which somatostatin injection facilitates the effect of insulin on suppression of PEPCK synthesis is difficult to define under this circumstances. In the present study, we have found that the two-days feeding of high-glucose diet seems essential to maximize the effect of biotin on hepatic PEPCK mRNA repression. It is possible that glucose would change the metabolic status of the diabetic rats, which in turn influences the response of PEPCK to biotin in these rats.

Hepatic and renal PEPCK are immunologically similar (Longshaw and Pogson, 1972; Iydejian et al., 1975), but each enzyme has a unique pattern of hormonal regulation (Schoolwerth et al., 1988). For example, translatable mRNA levels of hepatic but not renal PEPCK are markedly increased by administration of N^6, $O^{2'}$-dibutyryladenosine 3':5'- monophosphoric acid (Cimbala et al., 1982; Iynedjian et al., 1975). Acidosis induces the enzyme activity in kidney but not in liver (Cimbala et al., 1982; Iynedjian et al., 1975). PEPCK translatable mRNA and enzyme activity in both tissues can be induced by glucocorticoids (Sasaki et al., 1984; Iynedjian and Hanson, 1977; Shargo et al., 1963). On the other hand, insulin rapidly deinduces hepatic PEPCK mRNA (Cimbala et al., 1981) and has no effect on the kidney. We have found that both insulin and biotin shared the same tissue specificity of regulating hepatic but not renal PEPCK. The selective effect of biotin on hepatic rather than renal PEPCK provides additional information on the diverse regulatory mechanism of hepatic and renal PEPCK regulation.

The reciprocal changes between the levels of serum insulin and hepatic PEPCK mRNA in normal and diabetic rats indicated the dominant role of insulin, which is consistent with the results obtained in in vitro studies (Sasaki et al., 1984). The constant low endogenous serum insulin levels both before and after biotin injection excluded the possibility that the in vivo effect of biotin on hepatic PEPCK mRNA was secondary to the effect of biotin

on the release of endogenous insulin.

In studies on the 24 hour follow-up of PEPCK activity in both insulin and biotin treated rats, we found that the enzyme activity of the untreated diabetic rat was three times higher than that of the normal control. Though both insulin and biotin decreased hepatic PEPCK mRNA during the first hour after biotin injection, a decrease of enzyme activity was not seen till three hours after the treatment. Injection of biotin led to a maximum suppression of mRNA at three hour and enzyme activity at 5 hour post-injection. Compatible with the changes in the concentration of mRNA, enzyme activity reached the pre-injection level by 10 hour of the time course study. However, administration of insulin resulted in inhibition of the enzyme activity even 14 hours after the injection.

It is well known that insulin decreases PEPCK mRNA by reducing the transcription rates of PEPCK gene (Sasaki et al., 1984). To investigate the role of biotin in the regulation of PEPCK gene expression, nuclear run-on assay was carried out during 0 to 60 minutes after injection of biotin. Suppression of PEPCK gene transcription was found within 15 minutes of injection of biotin and maximum inhibition was seen at 30 minutes. The transcription rates of hepatic PEPCK gene return to the pretreated level by 1 hour after biotin administration. In H4IIE hepatoma cells, addition of insulin to the culture medium led to a significant decrease of

PEPCK mRNA within 1 hour and the maximum inhibition was seen between 2 to 4 hour (Granner et al., 1983). In vitro nuclear run-on assay using nuclei from H4IIE cell treated with inducer indicated that 15 minutes after addition of insulin, the transcription rate of PEPCK gene was decreased by 50% (Sasaki et al., 1984). The time course of insulin effect on PEPCK in both cultured cells and rat liver, as well as the biotin effect on diabetic rat liver are parallel. Furthermore, the time course of transcriptional inhibition is also comparable between the insulin effect on the hepatoma cells and the biotin effect on the diabetic rat liver. This suggests that both biotin and insulin exert their effect on PEPCK through a mechanism which does not require new protein synthesis.

3. The induction of hepatic GK mRNA in diabetic rats

Preliminary results from the present study indicated that biotin administration induced a marked increase of GK mRNA at 1 hour and 3 hour time points in the time course studies. Because of the big variations between indiviual rats, only at 3 h point the increase of GK mRNA has statistic significance when comparing with the mRNA level of diabetic control. It is otfen found that the GK mRNA level is too low to be detected quanitatively by the present method, thus a more sensitive method (such as the nuclease protection assay or quantitative RT-PCR) should be used in the future study. In starved rats, the appearance of GK mRNA is as early as 45 minutes

after injection of biotin and by 2 hour, the induced hepatic GK mRNA basically disappeared (Chauhan and Dakshinamurti, 1991). Induction of hepatic GK mRNA by insulin in diabetic rats is also rapid and the amount of GK mRNA increases dramatically by 1 hour after insulin injection and the abundance of specific mRNA continues to increase until 8 hours of the initial dose of insulin (Iynedjian et al., 1988). The time course of induction GK mRNA in the present study roughly fit the time ranges of these in starved rat with biotin and diabetic rats treated with biotin or insulin and imply relavent regulatory pattern among them.

4. The regulation of hepatic PK by biotin

Unlike GK and PEPCK, phosphorylation and allosteric effectors are important in the acute regulation of hepatic PK (Pilkis and El-Maghrabi, 1988). Insulin increases transcription of PK gene and prolongs the half life of PK mRNA. The stimulatory effect of insulin is slow in onset. In diabetic rats, no remarkable PK mRNA can been seen till 10 hours after insulin administration and the accumulation of PK mRNA continues even 16 hours after insulin treatment (Noguechi et al., 1985). However, no changes in the level of hepatic PK mRNA were found after biotin administration to diabetic rats even the time course studies went to 24 hours. It has been found that biotin can increase PK activity in diabetic rat liver (Dakshinamurti et al., 1970), but the magnitude of the increase is relatively smaller than that of GK activity. Another

possible reason for lack of the observation of PK mRNA changes after biotin treatment is that under the present diabetic and dietary situation, the PK gene is insensitive to biotin.

5. General discussion

The reciprocal regulation of glycolytic and gluconeogenic key enzyme activities suggests an important function of biotin in the metabolism of glucose in liver. Upon administration of biotin, the activities of glucokinase, phosphfrucokinase and pyruvate kinase incrase and hepatic PEPCK activity decreases, which leads to increased glucose utilization and decreased glucose production in diabetic status and corrects the high level of blood glucose concentration.

Current evidence suggests that biotin, a water-soluble vitamin, has very complicated and diverse biological fuctions rather than just serving as a CO_2 carrier in certain carboxylation reactions (Dakshinamurti and Chauhan, 1989). A number of cell lines maintained in continuous culture in defined serum free media were shown to require biotin for cell growth (Takaoka and Katsuta, 1971; Higuchi and Robinson, 1973). Dakshinamurti and Chalifour (1981) and Chalifour (1982) using serum that was rendered biotin deficient by avidin-sepharose chromatography showed a biotin requirement for Hela cells, human fibroblasts, and Rous sarcoma virus-transformed baby hamster kidney cells based on the viability, biotin content

and activities of biotin-dependent and independent enzymes (Chalifour and Dakshinamurti, 1982a, 1982b). Further study by Bhullar and Dakshinamurti (1985) confirmed that there was a significant decrease in the incorporation of leucine into protein of the homogenate or cytosol of biotin-deficient Hela cells as compared to cells grown in a biotin supplemented medium. With the addition of biotin to the biotin-deficient medium, the rate of incorporation of radiolabelled leucine into proteins increased by two-fold. The important cellular effects of biotin are also suggested in that biotin can promote cell differentiation in cell culture. The 3T3-L1 subline derived from 3T3 mouse fibroblast cell line has the capacity to differentiate into a cell type having the characteristics of adipotes (Green and Kehinde, 1974) when reaching a resting stage. The process of differentiation can be accelerated by biotin (Rosen et al., 1979). It was found that the differentiation of this cell line paralleled a corresponding rise in the activity of key enzymes of the fatty acid biosynthetic pathway. The increase of many enzyme activities have been shown to result from specific translatable mRNAs (Spiegelman and Farmer, 1982; Wise et al., 1984). This increase correlated with a marked rise in nuclear run-on transcription rates for these mRNAs during differentiation (Bernlohr et al., 1985). In addition, biotin can specifically induce the production of some proteins. For example, dietary biotin is reported to induce the production of biotin binding protein BBP1 and BBP2 in egg yolk, and the induction of biotin binding proteins may involve in gene expression (White and

Whitehead, 1987). Moreover, biotin also works on non-biotin related proteins such as guanylate cyclase and RNA polymerase II (Dakshinamurti and Chauhan, 1989). Although it has suspected that the induction of many proteins by biotin involves in the synthesis of new RNAs, recent work by Chauhan and Dakshinamurti (1991) was the first one which supplied direct evidence that biotin can regulate gene transcription. The results from the present studies add additional evidence that biotin selectively affects expression of some genes. At present, the detailed mechanism by which biotin regulates gene expression is not clear, but results from previous research imply some mechanisms of how biotin regulates gene expression.

A nuclear biotin-binding protein has been isolated in our laboratory (Bhullar, 1985). This protein specifically binds to biotin and is negative for carboxylase activity. So far the fuction of this nuclear biotin-binding protein is not clear, but it is conceivable that biotin may use the analog mechanism of steroid hormones to interact with gene regulatory elements. In this model, the biotin binding protein serves as a transcriptional factor, and upon binding of biotin to this factor, it is activated and interacts with regulatory DNA elements of some particular genes and modulate the transcription of these genes. Thus it is of interest to isolate the 5'-flanking sequence from GK or PEPCK genes and by using gel retardation assay to determine whether this nuclear binding protein binds to particular sequences in this region. The

next step may involve in making a construct, introducing it into cells expressing the nuclear biotin binding protein and examining the function of this nuclear protein in regard to the gene expression from a particular promoter. The facts that biotin influences the transcription status of both hepatic GK and PEPCK genes within 60 minutes suggests that the regulatory process does not require new protein synthesis and this is a support for using gel retardation assay to investigate the property of the nuclear biotin-binding protein.

On the other hand, another model is also possible for the action of biotin on gene regulation. Vesely (1982; 1984) reported that biotin enhanced guanylate cyclase activity in various rat tissues. Spence and Koudelka (1984) found that the induction of GK activity by biotin in primary culture of rat hepatocyte was preceded by an increase in the intracellular level of cGMP. cGMP is one of the important secondary messagers and changes in cGMP concentrations cause diverse biological effects in cells (Walter, 1984). For example, cGMP has been shown to involve in cell growth, DNA and RNA synthesis (Zeilberg and Goldberg, 1977). Furthermore, it has been found that cGMP profoundly influences carbohydrate metabolism in rat liver (Spence, 1984). Thus, it is difficult to exclude that biotin may affect GK and PEPCK gene expression through the cGMP signal transduction pathway.

Among vitamins, the role of retinoic acid and vitamin D_3 in the

regulation of gene expression of a number of genes is well established (McDonnell et al., 1987; Petkovich et al., 1987; Giguere et al., 1987). The amino acid sequences of retinoic acid and vitamin D_3 receptors are homologous to the receptors for steroid hormones and thyroid hormones, and they use the same mechanism as that of steroid and thyroid hormones to regulate genes (McDonnell et al., 1987; Umesono et al., 1988). It has been reported that retinoic acid and thyroid hormone can use the same responsive element to induce gene expression (Umesono et al., 1988). Functions of regulating gene expression by water soluble vitamines are less well studied. However, current evidence suggests that water-soluble vitamines are also important in this aspect. Besides biotin, it has been reported that the transcription of carnitine palmitoyltransferase in riboflavin deficient rat can be regulated by riboflavin (Brady et al., 1988). Thiamine, another water-soluble vitamin, can repress the level of Pho4-mRNA which codes for a minor expressed cell surface acid phosphatase in yeast (Schweingruber et al., 1986). Though these vitamin effects, similar to that of biotin, needs more study, the current evidence suggests that participation in gene regulation may be a common phenomenon.

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