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ABSTRACT

Title of Dissertation: Control of Hepatic Glucose Metabolism by Oral Hypoglycemic Sulfonylureas

Thomas J. Pillsworth, Jr., Doctor of Philosophy, 1984

Dissertation directed by: Steven Goldstein, Assistant Professor, Department of Pharmacology

The oral hypoglycemic sulfonylureas are widely used in the treatment of noninsulin-dependent diabetes mellitus. Although these drugs acutely stimulate insulin secretion from the pancreas, upon chronic administration blood insulin concentrations return to pre-treatment levels, while hypoglycemia persists. This suggests that these drugs might have extrapancreatic hypoglycemic actions. The important role of the liver in glucoregulation implies that this organ might be a target tissue for the sulfonylureas. In fact, several studies have suggested that these drugs alter hepatic carbohydrate metabolism, although a direct inhibition of hepatic glucose output has not been demonstated. The present study was conducted to investigate the direct actions of the sulfonylureas on: 1) glucose output; 2) glycogen accumulation; 3) levels of gluconeogenic intermediates; and 4) activities of glycogen metabolizing enzymes in isolated rat hepatocytes. Hepatocytes were isolated by collegenase digestion from male, Sprague-Dawley rats and incubated in Krebs-Henseleit-HEPES buffer in the presence of either 30 mM glucose; or 10 mM glucose, 5 mM lactate, 5 mM glutamine, and Minimal Essential Medium amino acid mixture. Hepatocyte glycogen content was measured after precipitation of glycogen with 66% ethanol and conversion to glucose. Glucose output was assessed

by measuring incorporation of [^{14}C]-lactate into [^{14}C]-glucose. Labelled compounds were separated by ion-exchange chromatography in deproteinized samples. Activities of glycogen metabolizing enzymes were assayed in homogenates of hepatocytes by measuring incorporation of [^{14}C]-uridine diphosphoglucose (for synthase) and incorporation of [^{14}C]-glucose 1-phosphate (for phosphorylase) into glycogen. Radiolabelled substrates were separated from radiolabelled glycogen by ion exhange chromatography. Concentrations of gluconeogenic intermediates were measured in deproteinized samples of hepatocytes using standard NAD^+, $NADP^+$, and NADH-coupled enzymatic assays. Chlorpropamide inhibited the production of [^{14}C]-glucose, the incorporation of [^{14}C]-amino acids into [^{14}C]-glycogen, and the accumulation of glycogen in hepatocytes isolated from rats fasted 24 hours. The minimal effective concentrations for these effects were within the therapeutic range for this drug. The effects observed were specific for the hypoglycemic sulfonylureas and could not be extended to include other para-substituted sulfonamides. The inhibition of glucose output and glycogen accumulation appeared to be due to an inhibition of gluconeogenesis, since glycogenolysis was not stimulated by chlorpropamide. Chlorpropamide had no effect on the percentages of glycogen synthase or glycogen phosphorylase in their more active forms and did not alter the total activities of these enzymes. Measuring the concentrations of gluconeogenic intermediates revealed that the sulfonylureas inhibited gluconeogenesis through an inhibition of conversion of malate to phospho(enol)pyruvate. Malate concentrations were elevated while the concentrations of phospho(enol)pyruvate, 2-phosphoglycerate, and 3-phosphoglycerate were depressed in hepatocytes incubated with chlorpropamide.

ATP concentrations were also depressed by chlorpropamide treatment, but only at concentrations twice those needed to inhibit glucose output and glycogen accumulation. The results of this study demonstrate that the oral hypoglycemic sulfonylureas can directly inhibit gluconeogenesis in hepatocytes isolated from fasted animals, which may in part, explain their ability to lower blood glucose in vivo upon chronic administration.

CONTROL OF HEPATIC GLUCOSE METABOLISM

BY ORAL HYPOGLYCEMIC SULFONYLUREAS

by

Thomas J. Pillsworth, Jr.

Dissertation submitted to the Faculty of the Department of Pharmacology
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DEDICATION

To my wife, Michelle Therese Pillsworth for her patience and understanding when I needed her most.

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INTRODUCTION

Background

It has become increasingly clear that most forms of noninsulin-dependent diabetes mellitus (NIDDM) are associated not only with abnormal insulin secretion, but also with some degree of insulin resistance (De Fronzo, et al., 1983). Insulin resistance is defined as either a relative or absolute elevation of insulin levels concomitant with hyperglycemia and can be related to some defect in the target cell sensitivity to the hypoglycemic actions of insulin. This feature of NIDDM was first described by Himsworth, et al. (1942) who noted that in certain patients with NIDDM exogenous insulin was not as effective in decreasing of an oral glucose load compared to the same dosage of insulin administered to normal subjects.

The sulfonylureas are widely used in the treatment of NIDDM and are especially effective in the less severe forms of the disease described above. Although their clinical effectiveness immediately after initiating therapy can be ascribed to their stimulation of insulin secretion from the pancreatic β-cells, a report has indicated that both fasting plasma insulin levels and the insulin response to oral glucose, while initially elevated, return to pre-treatment levels or responses often within a month of treatment (Owens, et al., 1979). Despite their apparent lack of effect on insuln secretion, the metabolic aberrations of NIDDM will remained controlled by the sulfonylureas, as reflected by decreased fasting blood glucose and improved glucose tolerance (Barnes, et al., 1974; Podolsky and Burney, 1979; Ratzmann, et al., 1983).

To explain these findings, a role for the sulfonylureas in ameliorating the insulin resistance in target tissues for this hormone

has been suggested. If the sulfonylureas could decrease glucose production (notably in the liver) or increase glucose disposal (either by storage in the liver or by utilization in peripheral tissues, such as skeletal muscle or adipose tissue) then tissue responsiveness to insulin would be lessened, blood glucose levels would fall, and circulating insulin levels would decrease in response to the decreased stimulus for secretion.

The liver plays an essential role in the control of blood glucose, a role which is compromised in NIDDM since hepatic glucose extraction after feeding is diminished, while elevated glucose output (notably via gluconeogenesis) is found. Both of these abnormalities along with increased substrate inputs to the liver from muscle (amino acids and lactate) and from adipose tissues (glycerol and free fatty acids) combine to make the liver a potential site of control of glucose homeostasis by the hypoglycemic agents. The present study was designed to reevaluate the effects of the sulfonylureas in the liver and to focus on whether these drugs had direct, hormone-independent actions on hepatic glucose output, glycogen metabolism, and gluconeogenic pathways.

Evidence for Extrapancreatic Effects of the Sulfonylureas

Development of the hypoglycemic sulfonylureas was initiated in the early 1940's when Janbon, et al. (1942) observed symptoms of hypoglycemia in patients administered a sufonylurea derivative (RP 2254) for the treatment of typhoid fever. Subsequently, decreased blood glucose concentration was demonstrated in patients treated with this compound. RP 2254 was later shown to stimulate insulin release from the canine pancreas by Loubartieres (1944). Such results strongly influenced development of initial concepts of the mechanism of the blood glucose concen-

tration-lowering action of the hypoglycemic sulfonylureas. The hypothesis that their action during chronic administration was due solely to their effect on insulin secretion was supported by observations that the sulfonylureas were ineffective in decreasing blood glucose in depancreatized animals (Ricketts, et al., 1957).

However, it was later demonstrated that glucose tolerance was increased and fasting blood glucose levels were decreased in patients receiving sulfonylurea therapy for as little as two weeks (Owens, et al., 1979) despite circulating plasma insulin concentrations near or even slightly below pre-treatment levels (Reaven and Dray, 1967; Feldman and Lebovitz, 1971; Duckworth, et al., 1971; Beck-Neilson, et al., 1971). Such findings led to the hypothesis that sulfonylureas might act either directly or in concert with insulin at extrapancreatic sites to lower blood glucose concentration during chronic sulfonylurea administration. Although this hypothesis is not consistant with results of depancreatized animal studies, it is consistant with a sulfonylurea effect on an insulin-sensitive pathway such as gluconeogenesis, glycogen synthesis, anti-proteolysis, or lipogenesis.

Effects of the Sulfonylureas on Insulin Receptor Activity

As noted in the introductory paragraphs, failure to control postprandial and fasting blood glucose levels in mild NIDDM results from poor tissue responsiveness to normal or elevated levels of insulin. A possible explanation for the ability of sulfonylureas to relieve insulin resistance is that they potentiate insulin action by interacting directly at loci of hormone action. A well described phenomenon associated with hyperinsulinemia is down-regulation of insulin receptor number in target tissues (Archer, et al., 1973; Archer, et al., 1975;

Olefsky, 1976; Olefsky and Reaven, 1976). Because of decreased receptor number, concentration-response curves for biological effects of the hormone are shifted rightward so that receptor occupancy and hence, biological responses are decreased at submaximal concentrations of hormone. If sulfonylureas could somehow reverse or inhibit down-regulation of insulin receptor number, insulin resistance based on decreased receptor activity could be reversed or prevented. The effect of sulfonylureas on insulin receptor activity was first studied *in vivo*.

Insulin binding to circulating monocytes taken from noninsulin-dependent diabetics was increased by treatment for six months with chlorpropamide (Olefsky and Reaven, 1976), and tolbutamide (Beck-Nielson, *et al.*, 1979). In each case, the change in insulin binding was associated with insulin levels only slightly above pre-treatment levels and was due to an increase in insulin receptor number approaching non-diabetic levels. While these studies in humans dealt with a tissue not normally considered to be a target of insulin action, analysis of *in vivo* sulfonylurea activity in animals extended these findings to liver and adipose tissue. Increased insulin binding was found in rat liver plasma membranes isolated from rats treated with ADRF-26 (Greenstein, 1979) gliquidone (Bachmann, *et al.*, 1979) or glipizide (Feinglos and Lebovitz, 1978). Each of these drugs is an experimental hypoglycemic sulfonylurea. Joost, *et al.* (1982) demonstrated that insulin binding to adipocytes isolated from rats treated for 7 days with 300-600 mg/kg tolbutamide (20-40 times therapeutic dosage) was increased compared to adipocytes isolated from untreated animals.

In vitro application of sulfonylureas to a number of cultures of established cell lines has not yielded complimentary results.

Although insulin binding to cultured human fibroblasts increased after 16 hours treatment with glipizide (Prince and Olefsky, 1977) others found no such response. Neither Joost, et al. (1982), nor Maloff and Lockwood (1982) could find a direct effect on adipocyte insulin binding with tolbutamide or tolazamide treatment. No effect of glyburide on insulin binding or prevention of down-regulation of insulin receptor number caused by 10^{-8} M insulin was observed in cultured rat hepatocytes (Fleig, et al., 1984). In another in vitro study, Vigneri, et al. (1982) were unable to detect any effect on insulin binding to MCF-7 human breast cancer cells, IM-9 human cultured lymphocytes, H35 Reuber hepatoma cells, or human skin fibroblasts when treated with concentrations of tolbutamide, glibenclamide, glicazide, or glisolamide found to be hypoglycemic in humans.

In combination, the results of these studies indicate that the sulfonylureas do not directly increase insulin binding activity. The in vivo changes in receptor activity that occur with sulfonylurea administration result from decreased insulin secretory demand in response to a lower steady-state plasma glucose concentration. The phenomenon of inverse regulation of insulin receptor activity by circulating insulin concentrations was clearly demonstrated by Olefsky and Reaven (1977). It was known that fasting hyperglycemia and glucose intolerance of noninsulin-dependent diabetics and non-diabetic obese patients are associated with fasting hyperinsulinemia (Olefsky, et al., 1973) and decreased insulin binding to circulating monocytes (Archer, et al., 1973; Archer, et al., 1975) and adipocytes (Olefsky, 1976). Olefsky and Reaven (1977) studied insulin binding to monocytes as a function of fasting insulin levels in normal subjects and in pa-

tients with fasting hyperglycemia and chemical diabetes (impaired glucose tolerance). They observed a statistically significant inverse correlation ($r = -0.90$) between insulin binding and fasting plasma insulin concentration.

At this time it is unclear whether effects of sulfonylureas on insulin binding are important for their hypoglycemic activity *in vivo*. It seems more likely that the sulfonylureas potentiate insulin action indirectly through other blood glucose lowering effects, which eventually result in decreased circulating insulin concentrations and up-regulation of insulin receptor activity.

The possibility that these drugs have hypoglycemic actions of their own has been investigated in three major glucoregulatory tissues: skeletal muscle, adipose tissue, and liver. Each is a potential target tissue for the sulfonylureas and these drugs could have effects in one or more of these tissues that are important for their hypoglycemic activity.

Effects of Sulfonlylureas in Peripheral Tissues

Skeletal Muscle

Although skeletal muscle relies on free fatty acids for the majority of its energy supply, this tissue extracts more glucose from the circulation than any other organ system. Zierler, *et al.* (1963) using the forearm perfusion model were the first to demonstrate that both basal and insulin-stimulated glucose uptake by skeletal muscle is impaired in patients with NIDDM. Subsequently these findings were confirmed by other investigators employing alternate techniques (DeFronzo, *et al.*, 1979; DeFronzo, *et al.*, 1982; Kolterman, *et al.*, 1981; Rizza, *et al.*, 1981). Because impairment of glucose uptake by skeletal

muscle is a contributing factor in the hyperglycemia observed in patients with NIDDM, an action, direct or in concert with insulin that sulfonylureas might have on glucose uptake in skeletal muscle could lower blood glucose levels. Fry and Wright (1957) investigated the direct effect of tolbutamide and carbutamide on glucose uptake in the isolated rat diaphragm. They found no significant effect of either drug on glucose uptake compared to untreated diaphragms. These results were subsequently verified for tolbutamide (Recant and Fisher, 1957; Vallence-Owen, et al., 1959) and chlorpropamide (Cahill, et al., 1957; Seltzer, 1962) in isolated rat diaphragm. Second generation sulfonylureas (gliclazide and glibenclamide) were ineffective in altering glucose uptake by isolated rat diaphragm (Musbah and Furman, 1980). An interesting finding was derived from a study conducted by Feldman and Lebovitz (1969). Tolbutamide alone was ineffective in stimulating glucose uptake in isolated mouse diaphragm, but it potentiated the stimulation of glucose uptake by a physiological concentration of insulin (26 μU/ml). Therefore, the drug did not act directly but potentiated the action of insulin.

Another model system used to examine effects of sulfonylureas on peripheral carbohydrate metabolism is limb perfusion. This model has the advantage over isolated hemidiaphragm in that the skeletal muscle under study is intact and the circulation (hence oxygenation) is maintained. However the limb contains other metabolically active tissues (adipose, connective tissue, capillary endothelium) and is clearly not a homogeneous preparation. The reports concerning the effects of the sulfonylureas on glucose uptake in skeletal muscle using the limb perfusion are conflicting. Butterfield, et al. (1962)

and Zinman and Ogilvie (1972) concluded that there was no direct effect of either chlorpropamide or tolbutamide, respectively in perfused human forearm. Furthermore, neither of these drugs potentiated the effect of insulin on glucose uptake. On the other hand, Daniels and Lewis (1982) reported that tolbutamide marginally increased glucose uptake in rat perfused hindlimb and that tolbutamide substantially potentiated insulin-stimulated glucose uptake.

In summary, sulfonylureas probably do not directly stimulate glucose uptake in skeletal muscle, but rather may potentiate insulin's action in this tissue.

Adipose Tissue

Although adipose tissue (white and brown fat) extracts less than 2 per cent of glucose administered orally or intravenously (Bjorntrop, et al., 1970; Bjorntrop, et al., 1971); adipose tissue can indirectly regulate blood glucose. Hormone sensitive lipase in these tissues catalyzes degradation of stored triglycerides to free fatty acids and glycerol. Free fatty acids are the preferential source of energy for skeletal and cardiac muscle, the use of which spares circulating glucose for use in tissues that use glucose obligatorily (renal medulla, lymphocytes, and erythrocytes) and preferentially (brain). Also, glycerol released from adipose tissue is extracted by the liver where it is converted to glucose through the gluconeogenic pathway. Free fatty acids may be extracted by the liver and then converted to acetyl coenzyme A (acetyl CoA) which enters the tricarboxylic acid cycle or are converted to acetoacetate and its reduced equivalent β-hydroxybutyrate by β oxidation; these are exported from the liver and used as energy sources in other tissues. In obese diabetics

(approximately 80% of Type II diabetics) participation of adipose tissue in the regulation of blood glucose may be increased since the percentage of body fat in these patients is increased. Therefore, it is evident that any perturbation of adipocyte metabolism could ultimately affect blood glucose concentrations.

Administration of sulfonylureas, such as tolbutamide and glibenclamide caused a rapid fall in plasma free fatty acid and glycerol concentrations in Type II diabetics and in normal subjects (Botterman, et al., 1965; Pfeiffer, 1967). This suggested that sulfonylureas might inhibit the release of these metabolites from fat tissue. However, acute administration of sulfonylureas stimulated insulin secretion, and insulin was known to be a potent inhibitor of hormone-sensitive triglyceride lipase (Fain, et al., 1966). Therefore, it was difficult to determine if sulfonylureas directly affected triglyceride metabolism in these subjects or whether this effect was mediated by insulin. Still, several investigators demonstrated that basal and hormone stimulated lipolysis was inhibited by tolbutamide and other sulfonylureas in isolated white and brown fat cells (Stone, et al., 1966; Brown, et al., 1969; Faulhaler, et al.,1969; Ostman, et al., 1970; Rosak, et al., 1972).

The mechanism of inhibition of lipolysis by sulfonylureas is unclear. Agents such as theophylline (Butcher, et al., 1968) and catecholamines (Fain, et al., 1970) stimulate lipolysis in adipose tissue most likely by increasing intracellular concentrations of cyclic adenosine monophosphate (cyclic AMP). Insulin and adenosine decrease lipolysis and intracellular concentrations of cyclic AMP (Butcher, et al., 1968; Schwabe, et al., 1973). Sulfonylureas probably do not

inhibit adipocyte lipase activity through regulation of cyclic AMP since Brown, et al. (1972) demonstrated that tolbutamide decreased epinephrine and theophylline-stimulated lipolysis by 60%, but increased cyclic AMP concentration by 300%. An alternate hypothesis is that inhibition of adipocyte lipolysis by the sulfonylureas is a calcium mediated process. Ebert, et al. (1974) found that calcium-depleted adipocytes became more sensitive to glibenclamide and tolbutamide inhibition of lipolysis when calcium was added back to the medium, than adipocytes that were isolated under normal calcium concentration conditions. They also confirmed the results of Brown, et al. (1969) since intracellular cyclic AMP concentrations were elevated in the presence of the sulfonylureas, while lipolysis was inhibited. These results are interesting since it was shown that increased intracellular cyclic AMP was probably not important in the effect of sulfonylureas on insulin secretion from the pancreas (Shatz, et al., 1978). There is strong evidence for increased intracellular calcium being the mediator of the effects of sulfonylureas in both β-cells and adipocytes. The evidence for a direct inhibition of fat cell lipolysis is convincing and the possibility exists that inhibition of fat cell lipolysis contributes to the drugs' hypoglycemic activity.

In summary, studies of the actions of the sulfonylureas on regulation of intermediary metabolism in peripheral tissues have demonstrated that these drugs may directly inhibit release of free fatty acids from adipose tissue, which can indirectly decrease blood glucose by limiting the supply of glycerol to the liver. However, these drugs have no direct effect on glucose uptake by skeletal muscle. Furthermore, only 15% of an oral glucose load is available for peripheral ulilization,

while the liver extracts 60% in normal (non-diabetic) subjects (Felig, et al., 1975; Bratusch-Marrain, 1980). Thus, it is unlikely that peripheral actions of sulfonylureas would have a significant impact on glycemia, and a regulatory role for these drugs on hepatic carbohydrate metabolism could be suggested.

Sulfonylureas and Hepatic Glucose Management

Glucose is the only source of energy for renal medulla and red blood cells and is the main source of energy for the brain (although this tissue can also obtain a small portion of its energy requirements through metabolism of acetoacetate and β-hydroxybutyrate which can be increased during prolonged starvation). Because short periods of hypoglycemia can irreversibly damage the brain, one of the most important functions of the liver is to maintain normoglycemia. During periods of alternating feeding and fasting this function is accomplished by the liver's ability to store and release glycogen in response to the nutritional status of the animal. However, glycogen stores are depleted after a few hours and to maintain normoglycemia, the liver increases gluconeogenesis in response to increasing blood concentrations of glucagon and decreasing concentrations of blood insulin, while hepatic glycolytic rates are diminished.

Hepatic glucose production is normally regulated by plasma insulin and glucose concentrations. As the concentrations of glucose and insulin increase (e.g., postprandially) hepatic glucose output decreases. In NIDDM, production of glucose by liver does not respond normally to these regulators (Felig and Wahren, 1971; Feinglos, et al., 1974). In these patients, normal or elevated hepatic glucose production occurs despite hyperinsulinemia and plasma glucose concentrations of

200-300 mg/dl.

The liver is the major site of uptake of injested glucose (Bratusch-Marrain, et al., 1975; Felig, et al., 1980) and represents another facet of the liver's glucoregulatory capacity, in addition to its ability to manufacture glucose. Felig, et al.(1978) demonstrated that in NIDDM patients, liver is only approximately 50% as effective in extracting glucose from the circulation as livers of normal subjects.

It is apparent that both hepatic glucose disposal (storage as glycogen and conversion to triglycerides) and production are abnormal and poorly regulated in NIDDM, and are responsible for most of the hyperglycemia both during fasting and postprandially in this disease. Therefore, any interaction of sulfonylureas with hepatic glucoregulatory pathways could result in a normalization of hepatic glucose metabolism.

The first studies to demonstrate hepatic effects of sulfonylureas used rabbit liver slices as a model of hepatic carbohydrate metabolism. Tyberghein, et al. (1956) demonstrated that the rate of spontaneous glucose output from liver slices taken from fed rabbits treated for 4 days with 500 mg/kg tolbutamide was decreased when compared to the rate of glucose output from slices derived from untreated animals. Direct application of 5 mM chlorpropamide to liver slices obtained from fed rats in vitro also resulted in decreased glucose output (Vaughan, 1957) as did perfusion of rat liver with tolbutamide in glucose-free medium (Kaldor and Pogasta, 1960). In vivo measurements in dogs (Shambye and Tarding, 1957) and man (Recant and Fischer, 1957) of hepatic glucose output after injection of tolbutamide by direct measurement from the hepatic vein have produced similar findings, with no measurable effects on peripheral glucose utilization in

either study.

Other direct effects of the sulfonylureas on hepatic metabolism have been identified. Hepatic lipolysis and gluconeogenesis are inhibited by tolbutamide. Both effects may be interrelated as gluconeogenesis from pyruvate depends on an adequate supply of acetyl CoA from stored esterified fatty acids derived from intrahepatic lipolysis (Hems, et al., 1966; Exton, et al., 1969; Williamson, et al., 1969). To separate these effects, Schonborn, et al. (1974) investigated actions of tolbutamide and chlorpropamide on the conversion of fructose to glucose in perfused livers of 18-22 hour fasted rats. Tolbutamide (0.5 mM) and chlorpropamide (0.5 mM) inhibited conversion of fructose to glucose, while conversion of fructose to pyruvate and lactate was increased, indicating that sulfonylurea treatment was either stimulating glycolysis or inhibiting gluconeogenesis, or both. However, these authors did not supply gluconeogenic intermediates more distal to glucose (e.g., lactate, amino acids), which are more commonly extracted from blood by liver. This study was not properly designed since fructose enters the gluconeogenic pathway at the level of triose phosphates and therefore, steps more distal in the gluconeogenic pathway were not investigated. Also this study would have been more complete if the investigators had measured glycogen accumulation since glycogenic precursors can be derived from gluconeogenesis. In the same study, the sulfonylureas decreased hepatic adenosine triphosphate (ATP) concentration as well, but it is difficult to ascertain if the decrease in ATP was biochemically significant and related to the inhibition of glucose production. These livers had

low ATP contents before addition of sulfonylureas to the perfusate. These authors also found a reduction in the β-hydroxybutyrate/acetoacetate ratio in livers perfused with chlorpropamide or tolbutamide. This ratio is an indicator of the mitochondrial ratio of reduced to oxidized nicotinamide adenine dinuecleotide ($NADH/NAD^+$). If this latter ratio were decreased, gluconeogenesis would be inhibited since entry and transport of anionic substrates across mitochondrial membranes is dependent upon NADH. Mitochondria in a more oxidized state would carry out oxidative phosphorylation at slower rates resulting in less ATP production. Decreased ATP levels could explain increased flux of fructose to pyruvate and lactate because ATP is an allosteric inhibitor of pyruvate kinase. Decreased ATP would result in increased pyruvate kinase activity, decreased levels of triose phosphates, and increased production of pyruvate and lactate from fructose.

Murphy and Anderson (1974) found that administration of large doses of tolbutamide (50 mg/kg) to rats with mild diabetes mellitus (plasma glucose of 200-250 mg/dl) induced by administration of streptozotocin (50 mg/kg) could reverse decreased activity of hexokinase caused by the diabetic state. However, no effects of tolbutamide were observed on the activities of pyruvate kinase, glucose 6phosphate dehydrogenase, or phospho(enol)pyruvate carboxykinase activities which were also altered by the diabetic state. No effect of tolbutamide were observed on any enzymes in liver homogenates prepared from severely diabetic animals. These findings led the authors to conclude that sulfonylureas have no direct effect on hepatic carbohydrate metabolism, but this conclusion

may have been based on faulty experimental design. The drugs are more effective in patients with insulin resistance, not decreased plasma insulin levels, as were found in the animals in this study after streptozotocin administration. Alterations in enzyme activities caused by tolbutamide administration may have been obliterated by the methodology used which was to homogenize tissue and to measure enzyme activities directly from samples after ultracentrifugation. A more appropriate method may have been to measure changes in substrate levels which would indirectly indicate alterations in enzyme activities.

Blumenthal and Whitmer (1979) perfused livers derived from rats fasted for 24 hours and demonstrated that chlorpropamide (0.8 mM) inhibited both glucagon-stimulated gluconeogenesis from lactate and glucagon-stimulated increases of cyclic AMP. Glucagon at $5x10^{-10}$ M caused a 250% increase of glucose production from lactate and a similar increase of cyclic AMP concentration. Both of these effects were significantly inhibited by chlorpropamide treatment. Based on the measurement of hepatic content of gluconeogenic intermediates, they determined that chlorpropamide blocked the conversion of pyruvate to oxaloacetate. It was not determined if the effect of chlopropamide on cyclic AMP concentration was causally related to inhibition of glucagon-stimulated gluconeogenesis resulting from chlorpropamide treatment, although it would seem likely since cyclic AMP mediates glucagon stimulation of gluconeogenesis. Chlorpropamide alone caused a slight, but insignificant inhibition on the basal cyclic AMP levels and had no significant effect on basal gluconeogenic rate or ATP levels either in the basal or glucagon-stimulated state. Again, it is difficult to assess if changes of ATP concentrations would occur with chlorpropamide

treatment. ATP levels were already decreased in this perfused liver preparation after a 60 minute pre-perfusion period, which was before chlorpropamide was added to the perfusate (2.05 μmoles/g in these perfusions compared to 2.53 μmoles/g in fresh liver or isolated hepatocytes in suspension) (Krebs et al., 1974).

The hypothesis that sulfonylureas can affect gluconeogenesis was also tested in vivo. Best, et al. (1982) used an isotope dilution method to determine glucose production in a group of noninsulin-dependent diabetics. Glucose production was assessed by injecting [^{3}H]-glucose into the peripheral circulation and subsequently measuring specific activity of [^{3}H]-glucose. Higher rates of glucose production resulted in lower [^{3}H]-glucose specific activity as labelled glucose was taken up into tissue and diluted by unlabelled glucose produced by liver (and kidney). Glucose production was significantly decreased in patients treated with chlorpropamide for three months compared to diabetics not treated with chlorpropamide. Also, fasting blood glucose correlated well with glucose production rates in these patients supporting the hypothesis that elevated hepatic glucose production is a contributing factor in the hyperglycemia observed in NIDDM. In this study, mean pre-treatment plasma insulin concentration in chlorpropamide-treated patients was 18$\pm$ 2 μU/ml and at the end of treatment it was 21$\pm$ 2 μU/ml. Therefore, the results of this experiment support the possibility that the sulfonylureas may act directly to decrease hepatic glucose output in vivo. Whether decreased glucose output caused by treatment with glyburide was the result of an inhibition of gluconeogenesis or glycogenolysis could not be determined. An action on gluconeogenesis is favored since these patients were

subjected to an overnight fast in an attempt to deplete most hepatic glycogen.

As stated above, an effect of chlorpropamide on triglyceride degradation in liver could have profound effects on rates of gluconeogenesis. Acetate groups derived from β-oxidation of fatty acids increase gluconeogenesis by two mechanisms: 1) acetate groups derived from fatty acids condense with citrate and enter the tricarboxylic acid cycle, sparing acetate groups derived from pyruvate and making more pyruvate available for conversion to oxaloacetate by pyruvate carboxylase and eventually to glucose; and 2) increasing the activity of pyruvate carboxylase by combining with coenzyme A to form acetyl CoA, an activator of this enzyme. In fact, it was demonstrated that pharmacologic concentrations of chlorpropamide decreased hepatic lipase activity and increased the content and secretion of triglycerides from both perfused livers (DeBeer, et al., 1976a; DeBeer, et al., 1976b) and isolated hepatocytes (DeBeer, et al., 1977). Therefore, the result of an effect of sulfonylureas on triglyceride metabolism may be to indirectly decrease hepatic glucose production.

Hepatic glycogen metabolism is another glucoregulatory system that could be affected by sulfonylureas resulting in decreased hepatic glucose output. Very early in the development of the sulfonylureas it was found that both fed (Tyberghein, et al., 1956) and fasted (Miller and Dulin, 1956; Baender and Sholz, 1956) rats receiving tolbutamide had higher hepatic glycogen content than untreated animals, but Colwell, et al. (1956) were unable to demonstrate this effect in alloxan-induced diabetic rats. The first study which investigated the role of the sulfonylureas on glycogen metabolism in vitro demonstrated that

incubation of rabbit liver slices in the presence of high concentrations (5 mM) of tolbutamide could block glucagon or epinephrine-stimulated release of glucose from glycogen (Vaughan, 1957). The effect of tolbutamide on glucose release in the absence of epinephrine or glucagon was not investigated, nor were activities of glycogen metabolizing enzymes assayed. Fleig, et al. (1984) found that glyburide had no direct effect on glycogenesis or glycogen content in primary cultures of hepatocytes (cultured without insulin), but glyburide did potentiate the action of insulin to stimulate glycogenesis in hepatocytes preincubated for 24 hours in the presence of 10^{-8} M insulin. Remesar, et al. (1978) demonstrated that total liver phosphorylase activity was decreased with no change in the proportion of phosphorylase in the active (a) form in livers of rats treated for 29 days with either glibenclamide or glipentide (both potent, hypoglycemic sulfonylureas). The results of these studies suggest that the sulfonylureas might act either directly, or as a potentiator of insulin action to decrease blood glucose by increasing the storage of glucose as glycogen in the liver.

On the other hand, chronic administration (2 months; daily administration) of tolbutamide (Prasannan and Augusti, 1973) or glyburide (George and Augusti, 1976) to normal rats resulted in both decreased blood glucose and hepatic glycogen content. Alemay, et al. (1978) treated rats for 29 days twice daily with either tolbutamide or glibentide and on day 27 a fast was begun. On day 29 [^{14}C]-alanine was injected and blood glucose, liver glycogen and incorporation of ^{14}C into glycogen and glucose was measured. The authors found that in animals treated with sulfonylureas both mass of glycogen accumulated

and incorporation of radioactivity into glycogen was decreased. Radioactivity incorporated into blood glucose was also decreased in glipentide treated animals demonstrating that inhibiting hepatic gluconeogenesis results in decreased glucose production and glycogen accumulation in fasted animals.

Mitochondrial Effects of the Sulfonylureas

It has been reported that the sulfonylureas can modify mitochondrial respiration (Katasumata and Hagihara, 1973). In a study of the effects of a series of the sulfonylureas on the respiratory control index (RCI) (the ratio of oxygen consumption in the presence of ADP to oxygen consumption in the absence of ADP) and on the P/O ratio (the ratio of high energy phosphate bonds produced per molecule of oxygen consumed) it was determined that sulfonylureas were efficient uncouplers of oxidative phosphorylation in isolated rat hepatic mitochondria. Chlorpropamide and tolbutamide (3-4 mM) reduced the RCI from 5.3 in untreated mitochondria to 1.0. At similar concentrations both drugs reduced the P/O ratio from 2.0 in control mitochondria to 0.0 in chlorpropamide-treated mitochondria. A P/O ratio of 2.0 was obtained in control mitochondria because succinate was used as substrate which has its electrons funnelled into ubiquinone, thus by-passing site I of the respiratory chain which is the transfer of electrons from NADH to ubiquinone. Supporting these findings are the experiments of Mannaerts, et al. (1974) who showed that over a range of therapeutic drug concentrations sulfonylureas markedly increased hepatic mitochondrial ATPase activity. The same group also found that chlorpropamide caused a shift in mitochondrial pyridine nucleotides to a more oxidized state and ascribed this

effect to the drugs' uncoupling actions (DeBeer, et al., 1974).

Uncoupling of oxidative phosphorylation by the sulfonylureas in vivo could explain decreased hepatic glucose output observed with chronic sulfonylurea treatment. Decreased reduced pyridine nucleotides and cellular ATP could affect carbohydrate metabolism as follows: 1) increased phosphofructokinase (PFK) activity would result since ATP is a negative allosteric inhibitor and ADP and AMP are positive allosteric activators of PFK; 2) increased NAD^+ would stimulate conversion of glyceraldehyde-3-phosphate to 3-phosphoglyceroyl phosphate since NAD^+ is a substrate for glyceraldehyde 3-phosphate dehydrogenase; and, 3) pyruvate kinase and pyruvate dehydrogenase show similar allosteric control as PFK. These effects would all tend to favor glycolysis and inhibit gluconeogenesis. Within the gluconeogenic pathway the two major anaplerotic steps would also be inhibited by sulfonylureas as follows: 1) the shuttle system through the mitochondria for the conversion of pyruvate to phospho(enol)pyruvate is highly NADH dependent; and, 2) fructose diphosphatase activity is markedly reduced by the allosteric inhibitor, AMP. The overall result would be to decrease hepatic glucose output through an inhibition of gluconeogenesis.

Specific Aims

It is evident from the preceding summary concerning the effects of the oral hypoglycemic sulfonylureas on various aspects of intermediary metabolism in insulin-sensitive tissue that the precise role of this class of drugs in the regulation of blood glucose remains undefined. The liver is an extremely important glucoregulatory organ, and several effects of the sulfonylureas on hepatic carbohydrate metabolism have been identified. However, confusion has arisen over their precise mechanism of action partly due to the fact that unsuitable model systems have been used for the study of hepatic carbohydrate metabolism (liver slices and perfusions and *in vivo* studies), because results of studies investigating the effects of acute and chronic sulfonylurea administration to animals and humans are not in agreement, and it is unclear if these drugs affect hepatic carbohydrate metabolism directly, or potentiate the action of insulin.

For this reason a comprehensive, coordinated study has been accomplished in which the direct effects of these drugs on the two major hepatic glucoregulatory pathways, gluconeogenesis and glycogen metabolism were examined.

The specific aims of this study were:

1) The development in our laboratory of the isolated hepatocyte in suspension system as a model for the study of the direct actions of the sulfonylureas on hepatic glucose output and glycogen accumulation. This system has become accepted as the most appropriate model for the study of hepatic carbohydrate metabolism.

2) The evaluation of *in vitro* potencies of the sulfonylureas in this system and their correlation with their *in vivo* therapeutic concentrations. It is important to demonstrate that the bioactive concentrations in isolated hepatocytes was within or near therapeutic concentrations.

3) The determination of the effects of the sulfonylureas on glucose output and glycogen accumulation as a function of incubation time to gain insight into their mechanism of action on these pathways.

4) The evaluation of the effects of both first and second generation sulfonylureas on gluconeogenesis and glycogenesis as a means of investigating whether the relative hypoglycemic potencies of these two subclasses are paralleled in the isolated hepatocyte system. The evaluation para-substituted sulfonamides as a means of determining whether the observed effects of the sulfonylureas are specific to those drugs with hypoglycemic action in vivo.

5) The determination of the effects of the sulfonylureas on glycogen metabolism as a function of initial glycogen content. Liver cells will synthesize glycogen at different rates depending on the initial hepatic glycogen content and this phenomenon is taken advantage of to investigate whether the sulfonylureas affect glycogen synthesis or glycogen degradation.

6) The evaluation of the acute effects of the sulfonylureas on hepatic glycogen metabolizing enzymes. It is possible that the effects of the sulfonylureas on glycogen accumulation is caused by covalent modifications of glycogen synthase and phosphorylase, which can be determined by measuring the percentage of these enzymes in their more active forms.

7) To investigate the effects of chlorpropamide on levels of gluconeogenic intermediates in hepatocytes. This is a valid method for determining which step in gluconeogenesis is affected by a test substance.

8) The determination of the effects of the sulfonylureas on these gluconeogenic intermediates as a function of time and drug concentrations. By correlating these data with the results of other sections of this study (glycogen accumulation and glucose output) further information on the mechanism of action of these drugs could be obtained.

MATERIALS AND METHODS

Materials

Chemicals

Pyruvic acid; glutamic acid; fumaric acid; lactic acid; glucose; glucose standard solution, 1.0 mg/ml; bovine serum albumin, (fraction V, lot numbers: 12F-648, 12F-650); nicotinamide adenine dinucleotide (NAD^+); nicotinamide adenine dinucleotide, reduced form (NADH); nicotinamide adenine dinucleotide phosphate (NADP); trypan blue; potassium hydroxide; rabbit liver glycogen; phospho(enol)pyruvate; D-2-phosphoglyceric acid; D-3-phosphoglyceric acid; dihydroxyacetone phosphate; L-glyceraldehyde 3-phosphate diethylacetal; glucose 6-phosphate; fructose 6-phosphate; frucotose 1,6-diphosphate; o-dianisidine; adenosine monophosphate, sodium salt (AMP); adenosine triphosphate, disodium salt (ATP); caffeine; glucose 1-phosphate; uridine diphosphoglucose (UDPG) were purchased from Sigma Chemical Company, St. Louis, MO.

N-2-Hydroxyethylpiperazine-N'-2-ethanosulfonic acid (HEPES) was purchased from Sigma and Research Organics, Inc., Cleveland, OH. Eagle's (Modified) Minimal Essential Medium amino acid mixture (AAM) was purchased from Flow Laboratories, McLean, VA. Calf thymus deoxyribonucleic acid (DNA) was purchased from Worthington Biochemical Company, Freehold, NJ.

Perchloric acid (70-72%) was purchased from Mallinckrodt, Inc., Paris, KY. Surfasil™ silicone was purchased from Pierce Chemical Co., Rockford, IL. 1,2-Dimethoxyethane (1,2-DME) was purchased from Aldrich Chemical Co., Milwaukee, WI. EP liquid scintillation cocktail was purchased from Beckman Instrument Co., Fullerton, CA. All other chemi-

cals used were of reagent grade and a list of the enzymes used in this study is presented in Table 1.

Radiolabelled Compounds

[^{14}C(U)]-L-amino acid mixture (lot numbers 1080-156, 1431-283; 0.1 mCi/ml); [^{14}C(U)]-D-glucose (lot number 940-017; 13.9 mCi/mmole); [^{3}H(5)]-D-glucose (lot number 1360-260; 16.0 mCi/mmole); [^{14}C(U)]-L-lactic acid (lot numbers 1240-816, 1301-298; 110 mCi/mmole); [glucose-^{14}C(U)]uridine diphosphoglucose (lot number 1104-269; 255 mCi/mmole); α-D-glucose 1-phosphate (lot number 1063-109; 294 mCi/mmole) were purchased from New England Nuclear Co., Boston, MA.

Ion Exchange Resins

AG 1-X8 anion exchange resin (acetate form, 100-200 mesh), AG 3-X4A anion exchange resin (chloride form, 50-100 mesh) and AG 50W-X8 cation exchange resin (hydrogen form, 100-200 mesh) were purchased from Bio-Rad Laboratories, Richmond, CA.

Instrumentation

Spectrophotometers

The spectrophotometers used were either a Gilford 250 (Gilford Instrument Co., Oberlin, OH or an Aminco DW2-a (American Instrument Co., Silver Spring, MD.

Liquid Scintillation Counters

The liquid scintillation counters used were either a Tricarb 2450 (Packard Instument Co., Downers Grove, IL) or an LS7800 (Beckman Instrument Co., Fullerton, CA).

All other instruments used are described in the Methods.

TABLE 1

LIST OF ENZYMES USED

Enzyme	Lot #(s)	Specific Activity (U/mg)	Supplier
Collagenase, Type II	41K221 42C287	164 130	Worthington*
Amyloglucosidase	1341507	14	Boehringer**
α-Amylase	1354667	1000	Boehringer
Glucose Oxidase	62F-0161	1100	Sigma***
Peroxidase	121F-9600	200	Sigma
Lactate Dehydrogenase	96F-9555	730	Sigma
Pyruvate Kinase	82F-9525	390	Sigma
Enolase	78C-9001	40	Sigma
Phosphoglucomutase	61F-9620	545	Sigma
Malic Dehydrogenase	91F-95751	1180	Sigma
Glyceraldehyde 3-Phosphate Dehydrogenase	1193227	100	Boehringer
α-Glycerophosphate Dehydrogenase	1233417	170	Boehringer
Glucose 6-Phosphate Dehydrogenase	22F-80301	320	Sigma
Phoshoglucose Isomerase	42F-96611	590	Sigma
Fructose Diphosphatase	27C-0212	6.2	Sigma
Hexokinase	70F-8035	440	Sigma

* Worthington Biochemical Company, Freehold, NJ
** Boehringer-Mannheim Biochemical Company, Indianapolis, IN
*** Sigma Chemical Company, St. Louis, MO

Methods

Animals and Feeding Schedule

Male, Sprague-Dawley rats (Taconic Farms, Germantown, N.Y.) weighing 250-350 g were used in all studies. Animals had free access to tap water and were maintained 4 rats per cage at constant temperature on a 12 hour light/dark schedule. Hepatocytes were isolated from rats conditioned in one of three nutritional states: 1. Fed animals had free access to standard rat chow (M/R/H 2000, Agway, Inc., Ithica, N.Y.) and were killed between 0800 hours and 1000 hours; 2. Seven to nine hour fasted animals were killed between 1400 and 1600 hours after food was withdrawn at 0700 hours; 3. Twenty-four hour fasted animals were fed between 0700 and 1000 hours for at least 4 days; then the hepatocyte isolation procedure began between 1000 and 1200 hours following a 24 hour fast. Body weights of animals in the latter group (no. 3) increased normally (3-4 g/day) after the first day on this altered feeding schedule.

Hepatocyte Isolation Procedure

Hepatocytes were isolated by collagenase digestion of the liver *in situ* by a modification of the method of Berry and Friend (1969) employing the surgical procedure described by Crisp and Pogson (1972). After animals were anesthetized with pentobarbital (60 mg/kg), a mid-line abdominal incision was made and the viscera laid aside. The right and left renal veins and the inferior vena cava (superior to the junction of the ileac veins) were then ligated. The vena cava was punctured inferior to the junction of the hepatic vein and and the vena cava, and a 14-gauge cannula attached to the perfusion apparatus

(Masterflex no. 7015 peristaltic pump, Cole-Parmer Co., Chicago, IL) was inserted and secured in place with two ligatures. The hepatic portal vein was severed, and the perfusion immediately started at a flow rate of 15-20 ml/min. The thoracic cavity was then opened, the superior vena cava ligated superior to the diaphragm, and the heart punctured to permit outflow of the perfusate. The direction of perfusion was therefore retrograde; the perfusion was non-recirculating. The perfusion was established within 5-10 minutes after opening the abdomen. The time between termination of blood flow and initiation of perfusion was never more than 30 seconds.

Initially, 225 ml of a buffer composed of NaCl, 126.8 mM; KCl, 6.7 mM; HEPES, 10 mM, pH 7.4; pyruvic acid, 4.9 mM; glutamic acid, 4.9 mM; fumaric acid, 4.9 mM; and glucose, 11.5 mM; was passed through the liver. The addition of energy-yielding substrates to the buffer was shown by Zahlten and Stratman (1974) to allow for better hormonal responsiveness of the hepatocytes after isolation compared to cells isolated without such substrates in the perfusate. The buffer was gassed with 100% oxygen to increase oxygen delivery to the tissue and was maintained at 40° which resulted in a temperature of approximately 37° as it entered the liver. The isotonicity of all buffers used in this study was always verified by freezing point depression osmometry before each use. The calcium-free (no calcium added) perfusion had two purposes: 1) blood was removed from the hepatic capillary bed allowing for complete perfusion of the liver; and 2) the calcium-dependent adhesion factor (central plaque region of desmesomes) was disrupted (Amsterdam and Jamieson, 1974). Then, the liver was perfused with another 225 ml of buffer (10-15 ml/min) containing 2.5 mM $CaCl_2$

and 0.05% (w/v) collagenase (Type II) (isolated from Clostridium histolyticum). $CaCl_2$ was present during the second phase of the perfusion because the enzymatic activity of collagenase is calcium-dependent. All collagense lots were tested for activity before use in the preparation of hepatocytes used in experiments. In the course of this study the optimal specific activity of the collagenase preparations was found to be 125-150 U/mg. Preparations with lower specific activities failed to digest the liver, while those with higher specific activities yielded suspensions of hepatocytes which had decreased viability and did not respond to sulfonylureas or hormones, which was most likely due to higher contamination with proteases. During enzymatic digestion, the liver swelled, indicating a breakdown of intercellular adhesions and expansion of the intercellular space. Finally, the liver was excised from the carcass and placed in a 100 mm tissue culture dish containing 40 ml of Krebs-Henseleit-HEPES buffer (KHH) which was composed of: NaCl, 92 mM; KCl, 4.7 mM; $NaHCO_3$, 25 mM; $CaCl_2$, 2.5 mM; KH_2PO_4, 1.2 mM; $MgSO_4$, 1.2 mM, HEPES, 25 mM; and de-fatted (see below) bovine serum albumin (Fraction V) (BSA), 2% (w/v); pH 7.4. This buffer was also gassed with 100% O_2. BSA was included in the incubation buffer for the following reasons: 1) because the suspensions were shaken rapidly to maintain oxygenation, the viscosity of the buffer had to be increased to prevent physical damage to the cells and so prolong cellular viability during the incubations; 2) to bind toxic cellular metabolic products (such as bilirubin); and 3) to mimic the drug binding properties of plasma proteins.

Hepatocytes were released from the liver matrix by gentle mechanical pertubation with a spatula and the cell suspension was

filtered through 253 μm^2 nylon mesh (Tetco, Inc., Elmsford, NY) into a 50 ml polypropylene conical bottom centrifuge tube. The cells were washed three times by centrifugation at 50 x g for 2 min and resuspension in KHH buffer. Viable parenchymal cells sediment more rapidly due to the fact that their density [(1.10-1.14 g/cm^3 (Drochmans, et al.,1977)] is greater than that of Kupffer cells or damaged parenchymal cells which become more bouyant due to fluid uptake. Berg, et al. (1972) found that this method of isolation using low speed centrifugation resulted in a final pellet that consisted almost exclusively of viable parenchymal cells. Less than 2% of the cells in the final cell pellet were Kupffer cells compared to 18% in the total cell suspension immediately after disruption of the liver. Cell number was determined with a Neubauer hemocytometer and cellular viability was assessed either by trypan blue exclusion (Hoskins, et al., 1956) or by leakage of lactate dehydrogenase activity (LDH) (Berg, et al., 1972) in comparison to enzymatic activity retained by the cells. LDH activity was measured according to the method of Bergmeyer, et al. (1963). To measure LDH activity released from hepatocytes 0.05 ml of the supernatant obtained after centrifugation of an hepatocyte suspension at 50 X g for 3 min was added to a cuvette containing (final concentrations): K_2HPO_4/KH_2PO_4 buffer, 47.5 mM, pH 7.5; pyruvic acid, 0.29 mM and; NADH, 0.13 mM; total volume of 1.01 ml. Oxidation of NADH was monitored spectrophotometrically at 340 nm over a five minute period using a Gilford 250 spectrophotometer. Intracellular LDH activity was measured in 0.05 ml samples obtained by disrupting cells collected by centrifugation of suspensions. Cells were disrupted by two 10 second cycles of sonication at maximum power with a Micro-Ultrasonic Cell Disrupter

(Kontes, Inc., Vineland, NJ). The reaction rate of all samples was linear for five minutes after the reaction was started, and LDH activity was calculated from the decrease in optical density during this interval. Cell viability as assessed by the two techniques which were comparable, and the trypan blue exclusion test was used routinely. Cell suspensions were routinely found to contain greater than 90% viable cells, and preparations with cell viabilities of less than 90% at the start of incubation were never used.

Critical factors in the preparation of highly viable hepatocyte suspensions were: 1) the use of animals with a maximum weight of not greater than approximately 350 g, since heavier (older) animals were more likely to develop blood clots in the liver; 2) rapid initiation of perfusion after the surgical procedure was started (to prevent blood clots); 3) adequate oxygenation and adequate flow rate of the perfusate; and 4) suitable preparations of collagenase. Injection of heparin prior to the inititiation of the surgical procedure is often used as an anti-coagulant technique by other investigators, but was not found to be necessary in this study.

Removal of Fatty Acids From Bovine Serum Albumin

Commercial preparations of BSA contain varying amounts of fatty acids which could act as substrates and/or regulators of hepatic intermediary metabolism. For example, it has been shown that the gluconeogenic and ketogenic capability of perfused liver was greatly increased when fatty acids were added to the perfusate (Hems, et al., 1966; Exton, et al., 1969; Williamson, et al., 1969). Therefore, BSA was de-fatted according to the method of Chen (1967). BSA (200 g) was dissolved in 1 l of glass distilled water and the solution adjusted to

pH 3.0 with 1 N HCl. De-fined activated charcoal (100 g) was added to adsorb fatty acids, and the suspension was stirred at 0° for 60 min followed by centrifugation at 20,000 x g for 20 min. The clarified solution was repeatedly filtered through 0.22 μm^2 filters (Millipore Corp., Bedford, MA) until no charcoal was visible on the filter. The final concentration of BSA was determined spectrophometrically at 279 nm (extinction coefficient of 1% solution is 6.67) and the solution was stored at -20° for up to 6 weeks.

Incubation of Hepatocyte Suspensions

Suspensions of hepatocytes, containing 9 x 10^6 cells in 2.5 ml of KHH were added to 25 ml siliconized glass Erlenmeyer flasks. The flasks were flushed with 100% oxygen for 20 sec, stoppered, and shaken at 80 cycles/min in a reciprocating water bath at 37° for 20 min to equilibrate the cells. Flasks were siliconized by rinsing with a 10% (v/v) solution of SurfaSil™ in hexanes, heating for 24 hours at 200°, and rinsing several times with glass distilled water. Siliconization of the flasks was necessary to reduce damage to hepatocyte plasma membranes caused by frictional interaction between the hepatocytes and the glass of the flasks. This treatment of the flasks resulted in extended cellular viability (Zahlten and Stratman, 1974). Substrates, drugs, and hormones were added in 0.5 ml of KHH to achieve a final concentration of 3 x 10^6 cells/ml. When glycogen accumulation and incorporation of radioactivity into glycogen were measured 1.0 ml of cell suspension was removed for initial glycogen content and/or radioactivity determination. The flasks were re-gassed with 100% oxygen before continuing the incubations. At the end of the incubation period, samples of cell suspension were removed for final glycogen determinations

or other assays. Hepatocytes were incubated at a concentration of 3 X 10^6 cells/ml except in experiments designed to measure concentrations of gluconeogenic intermediates; in these a concentration of 1 X 10^7 cells/ml was used. Nyfeler, et al. (1982) demonstrated that hepatocytes incubated under similar conditions at a concentration of 12.5 X 10^6 cells/ml accumulated glycogen at approximately the same rate as cells incubated at a concentration of 3 X 10^6 cells/ml in the present study. Therefore, the increased cell density used in the measurement of gluconeogenic intermediates experiments probably did not alter the metabolism of cell suspensions and data from the different sections in this study are comparable.

Isolation of Hepatic Glycogen and Conversion to Glucose

Glycogen was determined in aliquots of hepatocyte suspensions removed immediately after addition of substrates and again at the end of incubations. Glycogen was isolated from hepatocytes by a modification of the method of Good, et al. (1933). One ml of cell suspension was added to 1 ml of 54% (w/v) KOH, the mixture heated at 99° for 30 min in stoppered tubes, and the samples cooled to room temperature. Because glycogen is soluble in KCO_3 (Van Handel, 1964), preparations of KOH having low KCO_3 were used to improve recovery of glycogen.

In preliminary studies glycogen was converted to glucose by acid hydrolysis. Na_2SO_4 (2% w/v, 0.10 ml) and 4.2 ml of 95% ethanol were added to yield a final concentration of 66% ethanol. Glycogen, which is completely insoluble in 66% ethanol, was precipitated during incubation (in stoppered tubes) for 14-16 hours at room temperature. Glycogen was collected by centrifugation at 2500 X g for 30 min, the pellets washed once with 66% ethanol, recentrifuged, drained, and briefly heated

at 55° to remove trace ethanol. H_2SO_4 (1 N, 0.40 ml) was added, and the samples incubated in stoppered tubes at 99° for 3 hours. Then, 0.60 ml of 0.66 N NaOH containing 100 mM KH_2PO_4/K_2HPO_4 was added to neutralize the hydrolysate. A sample of this hydrolysate was removed for determination of glucose as described below, or added to 10 ml of EP liquid scintillation cocktail for determination of incorporation of radioactivity into glycogen by liquid scintillation spectrophotometry in either a Tricarb B2450 or an LS7800 liquid scintillation counter.

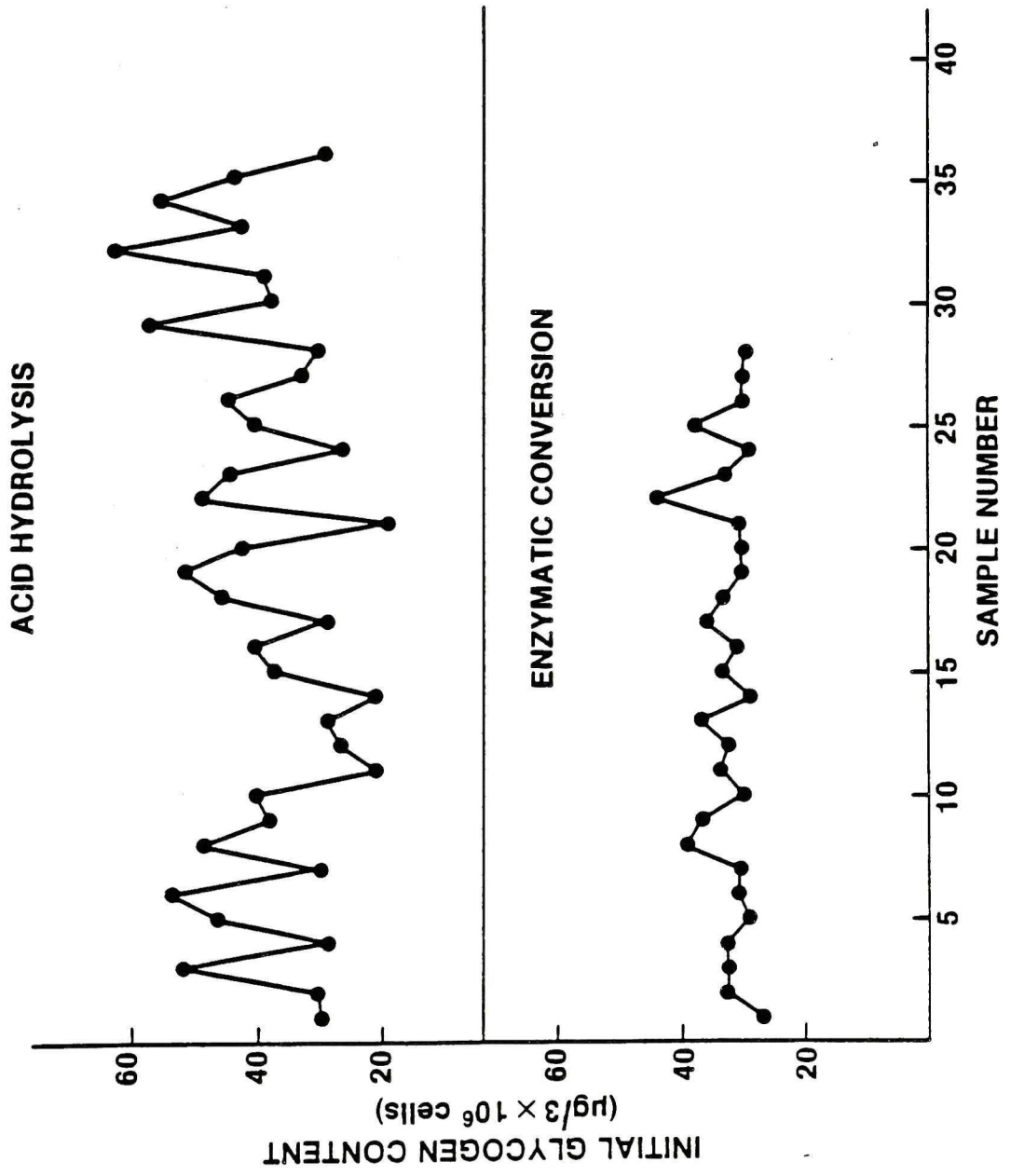
However, it was determined that excessive variability in the determination of small amounts ($\leq$ 100 µg) of glycogen occurred when samples were hydrolysed with acid. To overcome the apparent poor recovery and incomplete hydrolysis of the glycogen derived from hepatocytes, two modifications in the original technique were made. The amount of Na_2SO_4 added per sample was increased from 2 to 10 µg, and 100 µg of rabbit liver glycogen were added to improve precipitation of glycogen by ethanol. Also, glycogen was converted completely to glucose by enzymatic, not acid hydrolysis. The modified procedure follows.

Hepatocytes were treated with KOH as described above. After cooling to room temperature, 0.1 ml of 10% (w/v) Na_2SO_4, 0.1 ml of 0.1% (w/v) rabbit liver glycogen and 4.4 ml of 95% ethanol were added to each tube. Glycogen was precipitated and collected as described above. Glycogen was converted to glucose according to the method of Huijing (1970). Aspirgillus niger amyloglucosidase (1,4 α-glucan glucohydrolase; E.C. 3.2.1.3), (0.5 U) and 0.38 U of porcine pancreatic α-amylase (1,6 α-glucan glucohydrolase; E.C. 3.2.1.1), in 1.0 ml of 100 mM sodium acetate buffer (pH 4.8) were added to the glycogen

pellet. The enzymatic conversion of glycogen to glucose was complete after two hours at 30°. A portion of this digest was removed and used for glucose determination or determination of radioactivity incorporated into glycogen. The sensitivity of the glucose assay used in this study was sufficient to discriminate the hepatocyte glycogen content from the added glycogen.

A comparison of these two methods used in the determination of glycogen in hepatocytes isolated from two separate animals which were fasted for the same length is presented in Figure 1. One ml samples of cell suspension were removed immediately after the addition of substrates in two similar experiments, the only difference being the method of glycogen isolation and its conversion to glucose. Since the same number of cells was added to each flask, the glycogen content should have been the same in each sample. The initial glycogen contents of 3 X 10^6 cells in the two experiments were similar (46.5 µg in the acid hydrolysis experiment vs 33.4 µg in the enzymatic conversion experiment). Although animals from which these hepatocytes were isolated were fasted for the same length of time, it was found that there were slight inter-experimental variation in initial hepatocyte content. The range and SEM found were much different (43.5 µg, 1.8 µg for the acid hydrolysis experiment and 16.1 µg, 0.7 µg for the enzymatic conversion experiment, respectively). This result points out that addition of approximately 100 µg rabbit liver glycogen and increased Na_2SO_4 along with converting glycogen to glucose enzymatically results in less variability, and therefore more accurate experimental results. It was also determined that less than 1 µg of glucose originally present in the incubation buffer (30 mM) was present in the samples after

Fig 1. Comparison of methods of hydrolysis of glycogen to glucose. Each point represents the initial glycogen content of 3 x 10^6 cells in separate aliquots of two hepatocyte suspensions. Glycogen content in the cells represented in the upper panel was determined after hydrolysis of glycogen with 1N H_2SO_4. The bottom panel represents the glycogen content of hepatocytes as determined after conversion of glycogen to glucose with α-amylase and amyloglucosidase as described in the Methods.


ACID HYDROLYSIS
ENZYMATIC CONVERSION
INITIAL GLYCOGEN CONTENT
(μg/3 × 10⁶ cells)
60
40
20
60
40
20
5
10
15
20
25
30
35
40
SAMPLE NUMBER

glycogen isolation and conversion to glucose; therefore, the contribution of glucose present in the incubation buffer to the glucose assay is essentially nil.

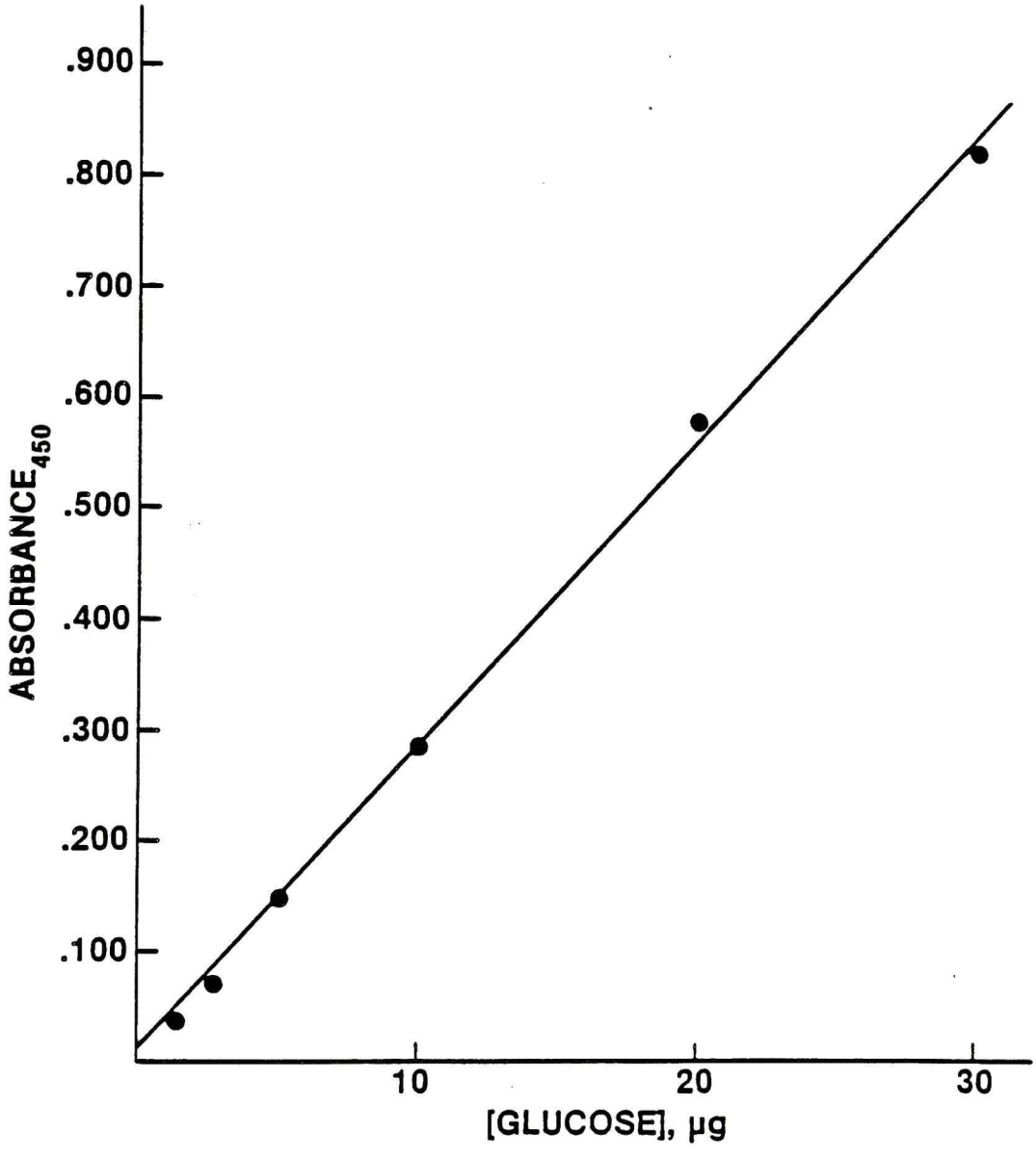
Glucose Determination

Glucose was measured using a modification of the method of Raabo and Terkildsen (1960) as published in Sigma Chemical Co. Technical Bulletin 510. The minimum amount of glucose determined with this assay carried out as described in Bulletin 510 is 5 µg. By concentrating the reactants it was possible to obtain a 4-fold increase in sensitivity so that the typical standard curve (Figure 2) was linear over the range of 1.25 µg to 30 µg ($r^2 \geq 0.99$). This modification allowed for the determination of glycogen content in 3 X 10^6 hepatocytes isolated from fasted rats. A reagent solution containing 12.5 U of Aspirgillus niger glucose oxidase (β-D-Glucose: oxygen 1-oxidoreductase; E.C. 1.1.3.4), 2.5 U horseradish peroxidase (Gluconate: hydrogen peroxide oxidoreductase; E.C. 1.11.1.7), and 0.1 mg o-dianisidine in 1.0 ml of 100 mM potassium phosphate buffer, pH 6.5 was added to a sample of hydrolysate diluted to 1.0 ml with 100 mM potassium phosphate buffer. Glucose standards and reaction mixture blanks were prepared in the same dilution medium to maintain equivalent salt concentrations. The reaction was carried to completion by incubating samples at 37° for 30 min. After cooling to room temperature, the absorbance of oxidized o-dianisidine was monitored at 450 nm.

Measurement of Gluconeogenic Intermediates and Adenosine Triphosphate

All gluconeogenic intermediates and adenosine triphosphate were measured with enzymatic β-nicotinamide adenine nucleotide (NADH,

Fig. 2. Glucose standard curve. The linearity of the glucose determination method was tested utilizing the modifications of the technique of Raabo and Terkildsen (1960) described in the Methods. Each point is the average of duplicate determinations.


.900
.800
.700
.600
.500
.400
.300
.200
.100
ABSORBANCE450
10
20
30
[GLUCOSE], µg

NAD^+, $NADP^+$)-coupled reactions which are according to methods described in Bergmeyer (1963). Hepatocytes (1.2×10^7 cells/ml) were pre-incubated in the absence of substrates, then incubated for 60 min with glucose, 10 mM; lactate, 5 mM; glutamine, 5 mM; and Eagle's (Modified Minimal Essential Medium amino acid mixture (AAM). Estabrook and Maitero (1962) demonstrated that liver contains acid stable aldolase and adenylate kinase that retain significant activity at 4° in the presence of perchloric or trichloroactic acid. Other glycolytic or gluconeogenic enzymes might also remain active under these conditions and could alter the concentrations of gluconeogenic intermediates. Therefore, modifications of standard deproteinization techniques were used in this study to limit the exposure of gluconeogenic intermediates to unprecipitated enzymes. Samples of cell suspension (2.8 ml) was added to 0.154 ml of perchloric acid (PCA), 70-72%, (w/v),(final PCA concentration was 3.6%) contained in glass test tubes that were pre-cooled to -10^0 in ethanol in an insulated flask. The samples were mixed, incubated at -10° for 15 min, and centrifuged at 2,500 x g for 20 min at -10°. The supernatants were removed and neutralized with aproximately 0.2 ml of 5 N KOH, which precipitated perchlorate as $KClO_4$. This mixture was maintained at -10° for 15 min and centrifuged at 2,500 x g for 20 min at -10°. The supernatants were removed and maintained at -10° until used in the assay of gluconeogenic intermediates, or ATP (usually within 1 hour).

Prior to measurement of gluconeogenic intermediates in samples of hepatocytes, standard curves for each intermediate were constructed using authentic compounds. All assays were linear in the range of 1.0 to 30.0 nmoles ($r^2 \geq 0.99$) when measured in the Gilford 250 spectro-

photometer. Because it was anticipated that the cellular content of several of the of gluconeogenic intermediates would be below the detectable sensitivity of the assays using the Gilford 250, several of the standard curves were repeated with amounts of gluconeogenic intermediates varying from 0.1-3.0 nmoles using the Aminco DW2-a spectrophotometer. Over this range of substrate concentrations, the assays were linear ($r^2 \geq 0.99$). The Aminco DW2-a spectrophotometer has two major advantages over the Gilford 250 which result in approximately 100 fold increse in sensitivity. They are: 1) a photomultiplier tube that can amplify signals to a greater extent than that of the Gilford; and 2) this instrument is used in the split-beam mode that allows the electronic components to constantly monitor the difference in signal strength from the sample and reference cuvettes; the Gilford is a single beam instrument and does not have this feature.

Meaurement of Pyruvate, Phospho(enol)pyruvate, D-2-Phosphoglycerate and D-3-Phosphoglycerate

These intermediates were measured according to the method of Czok and Eckert (1963) which is a sequential NADH-coupled assay that measures in order: pyruvate, phospho(enol)pyruvate, 2-phosphoglycerate and 3-phosphoglycerate. The reaction mixture contained, in a final volume of 1.69 ml: triethanolamine-HCl, 45 mM, pH 7.6; KCl, 68 mM; $MgSO_4$, 7.2 mM; adenosine diphosphate, 0.2 mM; 2,3 diphosphoglycerate, 0.1 mM; NADH, 0.2 mM; and 0.60 ml of deproteinized sample. Pyruvate was measured by following the oxidation of NADH after addition of 2.4 U of rabbit skeletal muscle lactate dehydrogenase (LDH) (L-lactate: NAD oxidoreductase; E.C. 1.1.1.27) at 340 nm in a Gilford 250 spectrophotometer. The second intermediate determined within this series of

reactions was phospho(enol)pyruvate (PEP). Rabbit skeletal muscle pyruvate kinase (PK) (ATP: pyruvate-2-O-phosphotransferase; E.C. 2.7.1.40), (1.1 U) was added to catalyze the conversion of PEP to pyruvate. The pyruvate formed by this reaction served as substrate for LDH and the oxidation of NADH was again monitored. The third intermediate measured was 2-phosphoglycerate (2-PG). Rabbit skeletal muscle enolase (E) (2-phosphoglycerate hydrolase; E.C. 4.2.1.11), (0.7 U) was added to catalyze the conversion of 2-PG to PEP. The PEP formed in this reaction served as substrate for PK and LDH and the oxidation of NADH was again monitored. The final intermediate determined in this series of reactions was 3-phosphoglycerate (3-PG). Phosphoglucomutase (2-phospho-D-glycerate phosphotransferase; E.C. 2.7.5.3), (1.8 U) was added to catalyze the conversion of 3-PG to 2-PG. The 2-PG thus formed acted as substrate for E, PK and LDH and the oxidation of NADH was again monitored.

Determination of L-Malate

Malate was measured according to the method of Hohorst (1963). The reaction mixture contained, in a final volume of 1.0 ml: hydrazine, 178 mM; glycine, 446 mM, pH 9.5; NAD^+, 2.5 mM; and 0.2 ml of deproteinized sample. Hydrazine was added in this assay, and arsenate added to the L-glyceraldehyde 3-phosphate assay (see below) to trap the products of the reactions, since the equilibrium for these reactions favors the formation of the substrates. The reaction was started by addition of 2.0 U of porcine heart malic dehydrogenase (L-malate: NAD oxidoreductase; E.C. 1.1.1.37), which catalyzed the reduction of NAD^+ by malate. The reduction of NAD^+ was monitored spectrophotometrically at 340 nm with a Gilford 250.

Determination of L-Glyceraldehyde 3-Phosphate

Glyceraldehyde 3-phosphate was measured according to the method of Racker (1963). The reaction mixture contained, in a final volume of 1.1 ml: glycylglycine, 25 mM, pH 7.4; sodium arsenate, 5 mM; NAD^+, 0.7 mM and 1.0 ml of deproteinized sample. The reaction was started by the addition of 0.2 U of rabbit skeletal muscle glyceraldehyde 3-phosphate dehydrogenase (D-glyceraldehyde 3-phosphate: NAD oxidoreductase; E.C. 1.2.1.12) which catalyzed the reduction of NAD^+ by glyceraldehyde 3-phosphate. The increase in absorbance was monitored at 340 nm with an Aminco DW-2a spectrophotometer.

Measurement of Dihydroxyacetone Phosphate

Dihydroxyacetone phosphate was measured according to the method of Weiland (1963). The reaction mixture contained, in a final volume of 1.1 ml: triethanolamine-HCl, 34 mM; NAD^+, 0.2 mM and 1.0 ml of deproteinized sample. The reaction was started by the addition of 10 U of α glycerophosphate dehydrogenase (glycerol 3-phosphate: NAD 2-oxidoreductase; E.C. 1.1.1.8). The reduction of NAD^+ was monitored spectrophotometrically with an Aminco DW2-a spectrophotometer.

Determination of Glucose 6-Phosphate, Fructose 6-Phosphate and Fructose 6-Phosphate

These intermediates were measured according to the method of Racker (1963). This is a sequential NAD^+-coupled assay that measured in order: glucose 6-phosphate (G6P), fructose 6-phosphate (F6P), and fructose 1,6-phosphate (Fl,6P). The reaction mixture contained, in a final volume of 1.17 ml: Tris-HCl, 100 mM, pH 8.8; $MgCl_2$, 5 mM; ethylenediaminetetraacetic acid (EDTA), 0.2 mM; NAD^+, 0.25 mM and 1.0 ml of deproteinized sample. The reaction was started by the addition of

0.3 U of glucose 6-phosphate dehydrogenase (GPDH) (E.C. 1.1.1.49), prepared from Bakers yeast which catalyzes the reduction of NAD^+ by G6P which was monitored spectrophotometrically at 340 nm with either the Gilford 250 or the Aminco DW2-a spectrophotometers. The second intermediate measured was fructose 6-phosphate. Phosphoglucose isomerase (PGI) (D-glucose 6-phosphate: ketol isomerase; E.C. 5.3.1.9), (0.2 U) prepared from Bakers yeast was added to catalyze the conversion of F6P to G6P. The G6P formed served as substrate for GPDH and the reduction of NAD^+ was monitored. The final intermediate measured was Fl,6P. Fructose diphosphatase (D-fructose 1,6 bisphosphate 1-phosphohydrolase; E.C. 3.1.3.11), (1.4 U) was added to catalyze the conversion of Fl,6P to F6P. The F6P produced served as substrate for PGI and GPDH and the reduction of NAD^+ was again monitored with an Aminco DW-2a spectrophotometer.

Adenosine Triphosphate

Adenosine triphosphate (ATP) was measured according to the method of Lamprecht and Trautchold (1963). The reaction mixture contained, in a final volume of 1.0 ml: triethanolamine-HCl, 40 mM, pH 7.5; $NADP^+$, 0.1 mM; $MgCl_2$, 6.9 mM; glucose, 40 mM; glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate: NADP 1-oxidoreductase; E.C. 1.1.1.49), (10 U) prepared from Bakers yeast; and 0.2 ml of deproteinized sample. The reaction was started by the addition of 1.1 U hexokinase (ATP: D-hexose 6-phosphotransferase; E.C. 2.7.1.1), prepared from Bakers yeast which catalyzes the conversion of glucose to glucose 6-phosphate. The conversion of glucose 6-phosphate to 6-phosphoglucone was catalyzed by glucose 6-phosphate dehydrogenase while $NADP^+$ was reduced to NADPH. The increase in absorbance was monitored at 340 nM

in the Gilford 250.

Determination of Glycogen Synthase and Glycogen Phosphorylase Activities

Glycogen synthase and glycogen phosphorylase activities were measured using a modification of the method of Golden, et al. (1977). Hepatocytes were pre-incubated for 20 min in the absence of substrates, and then incubated for either 2 or 60 min in the presence of glucose, 10 mM; glutamine, 5 mM, lactate, 5 mM; and AAM. At the end of the incubation 1.0 ml of cell suspension was removed and centrifuged at 10,000 x g for 5 sec, the supernatant aspirated and 9 volumes of Tris-HCl, 50 mM, pH 6.8; NaF, 100 mM; EDTA, 10 mM; rabbit liver glycogen, 0.5% (w/v); and dithiothreitol, 5 mM were added to the pellet. To prevent interconversion of the more and less active forms of glycogen synthase and glycogen phosphorylase and to decrease loss of activity due to proteolysis: 1) NaF was added to inhibit phosphatases; 2) EDTA was added to chelate metal ions, which certain proteases are dependent on for activity; 3) glycogen was added to maintain the structure of synthase and phosphorylase, since these enzymes are normally associated with the hepatocyte glycogen granules; and 4) dithiothreitol was added to prevent any oxidation of the synthase and phosphorylase molecules.

Cells were disrupted by two 10 sec cycles of sonication at maximum power with a Micro-Ultrasonic Cell Disrupter. The following conditions were used to measure the total enzyme activity and the activity of the more active forms of the enzymes from which relative percentages of enzymes in their active forms were calculated.

Glycogen Synthase (E.C. 2.4.1.11) Activity Determination

To determine the activity of the more active (a) form of glycogen synthase, 0.025 ml of the homogenate were added to 0.05 ml of reaction mixture containing Tris-HCl, 50 mM, pH 7.5; EDTA, 5 mM; rabbit liver glycogen, 1.0% (to provide primer sites for glycogen synthase); Na_2SO_4, 15 mM; uridine 5'-diphosphoglucose (UDPG), 1.5 mM; and [glucose-^{14}C(U)]UDPG (10,000-15,000 dpm/assay). The incubation time was 12-24 min at 37°. The less active (b) form of glycogen synthase is almost completely inactive in the presence of Na_2SO_4 and in the absence of glucose 6-phosphate. For determination of total synthase activity 0.025 ml of the homogenate was added to 0.050 ml of the above reaction mixture containing 3 mM glucose 6-phosphate, but without Na_2SO_4. The incubation time was 6-12 min at 37°. For either determination, the reaction was stopped by separating [^{14}C]-glycogen from [^{14}C]-UDPG by passing 0.050 ml of the reaction mixture over anion-exchange columns (0.9 x 1.2 cm of AG-1-X8, acetate form, which was poured into siliconized glass disposable pastuer pipettes with 2 cm cut off the top. It was determined that that siliconization of the columns resulted in less resin clinging to the glass which allowed for more consistant bed volumes among the columns. Removing the tops of the columns permitted direct application of the sample on to the resin (no sample ran down the inside of the column). The columns were washed twice with 0.5 ml of glass distilled water and the eluate collected in scintillation vials for determination of [^{14}C]-glucose incorporation into glycogen by liquid scintillation spectrophotometry in either the Tricarb 2450 or LS7800 liquid scintillation spectrophotometer.

Glycogen Phosphorylase (E.C. 2.4.1.1) Activity Determination

Active phosphorylase (a) was determined by adding 0.025 ml of the cell homogenate to 0.050 ml of reaction mixture composed of Tris-HCl, 50 mM, pH 6.8; NaF, 150 mM; rabbit liver glycogen, 1.5% (w/v); glucose 1-phosphate, 15 mM, α-D-[^{14}C(U)]-glucose 1-phosphate, (10,000-15,000 dpm/assay); and caffeine, 0.75 mM. Glycogen phosphorylase b (less active form) has a high affinity for caffeine, an allosteric inhibitor of this enzyme. Therefore, only phosphorylase in the a form is measured in the presence of caffeine. The incubation time was 4-10 min at 37°. For the determination of total phosphorylase activity, 0.025 ml of homogenate was added to 0.050 ml of reaction mixture with the deletion of caffeine and the addition of 7.5 mM adenosine monophosphate (AMP) and 10% (v/v) 1,2-dimethoxyethane (1,2-DME). Uhing, et al. (1979) demonstrated that this organic solvent altered the AMP binding characteristics of glycogen phosphorylase b resulting in activities of this form of the enzyme that were comparable to the a form. 1,2-DME has no effect on the a form. By using 1,2-DME more accurate determinations of total phosphorylase activity were obtained than if the organic solvent was absent. This, in turn resulted in more accurate estimations of the fraction of the enzyme activity present in the active form. The incubation time was 3-5 min at 37°. The activity of glycogen phosphorylase in the presence and absence of 1,2-DME was determined on several occasions. Addition of 10% (v/v) 1,2-DME resulted in approximately 70% stimulation of measurable total phosphorylase activity, with no effect on phosphorylase a activity. As in the glycogen synthase assay, glycogen was isolated with anion exchange columns as described above.

Prior to measuring glycogen metabolizing enzyme activities on samples of hepatocytes the parameters of the columns (recovery of glycogen and non-adsorbable [^{14}C]-glucose 1-phosphate and [^{14}C]-UDPG in the eluate were measured).

Table 2 demonstrates the results of an experiment designed to determine the recovery of glycogen from the columns. Homogenization buffer (0.05 ml) and reaction buffer (0.10 ml) were mixed, (both buffers contain glycogen), 0.05 ml of the mixture was added to 1.0 ml of glass distilled water, while another 0.05 ml was eluted through the column, with 1.0 ml of glass distilled water. The samples were then assayed for glycogen content. Glycogen recovery was calculated as the glycogen content after elution divided by the original glycogen content of the sample. It can be seen that for all reaction buffers, the recovery of glycogen was greater than 98%. Less than 1% of the total amount of [^{14}C]-UDPG and less than 2% of the [^{14}C]-glucose 1-phosphate were not adsorbed to the columns and appeared in the eluate (Table 3). This resulted in a sample:blank ratio of greater than 10:1 in most experiments.

Golden, et al. (1977) suggested that the assays were linear until approximately 15% of the original substrate was converted to glycogen. The linearity of the assays was determined by investigating hepatocyte enzyme activity as a function of time, which revealed that the results of the study of Golden, et al. were confirmed in the present study (Figure 3,4). On the basis of these observations, an estimate for the optimal length of incubation of each assay was made. To control for inter-experimental variation of hepatocyte glycogen metabolizing enzymes, one assay of each type (synthase a, total syntase; phosphory-

TABLE 2

RECOVERY OF GLYCOGEN FROM COLUMNS OF AG 1 X8 ANION EXCHANGE RESIN

Reaction Mixture	Glycogen Placed on Column (μg)	Glycogen Recovered from Column (μg)	Per cent Recovery
Synthase a	382.2 ± 2.4	382.6 ± 4.7	100.1 ± 1.2
Synthase a+b	419.1 ± 2.0	414.1 ± 2.0	98.9 ± 1.9
Phosphorylase a	684.7 ± 8.6	650.7 ± 6.4	96.2 ± 0.8
Phosphorylase a+b	730.4 ± 9.1	708.7 ± 8.5	96.8 ± 2.0

To mimic glycogen synthase and glycogen phosphorylase assay mixtures, the homogenization buffer and the reaction buffer appropriate to each assay were mixed 1:2 (the proportion of each in the usual assay mixture). Of each of these mixtures, 50 μl were eluted through anion-exchange column with 1.0 ml water. Another 50 μl was diluted with 1.0 ml of water directly. Glycogen content in both types of samples was measured after enzymatic hydrolysis to glucose. The values are the mean glycogen content ± S.E.M. in three replicate samples of each assay buffer. The percent recovery was calculated as the glycogen content after elution through the resin divided by the the glycogen present in the samples not eluted through ion-exchange resin.

TABLE 3

APPEARANCE OF [GLUCOSE-^{14}C(U)]-URIDINE 5' DIPHOSPHOGLUCOSE AND α-D-[^{14}C(U)]-GLUCOSE 1-PHOSPHATE IN ELUATE OF AG 1-X8 ANION EXCHANGE RESIN COLUMNS

Reaction Mixture	[Glucose-^{14}C(U)]-Uridine 5' Diphodphoglucose in Eluate (per cent of amount placed on column)
Synthase a	0.32 ± 0.14
Synthase a+b (Total)	0.20 ± 0.07

Reaction Mixture	α-D-[^{14}C(U)]-Glucose 1-Phosphate in Eluate (per cent of amount placed on column)
Phosphorylase a	1.51 ± 0.10
Phosphorylase a+b (Total)	1.42 ± 0.11

Homogenization buffer and reaction buffer containing 10-15,000 dpm ^{14}C-UDPG (glycogen synthase assay) or ^{14}C-G-1-P (glycogen phosphorylase assay) were combined as described in Table 1. After elution of 50 µl of each reaction mixture through separate anion-exchange resin columns with 1.0 ml of water, radioactivity present in the sample was measured. The fraction of the radiolabelled compound not bound to the resin was calculated by comparison with the radioactivity of the compound present in the sample before chromatography. Each value is the mean ± S.E.M. of three determinations.

Fig. 3. Time course of glycogen synthase assay. A homogenate of cells isolated from a 24 hour fasted rat was prepared by sonicating a suspension of cells (3 x 10^6/ml) in 9 volumes of a buffer containing: Tris-HCl, 50 mM, pH 6.8; NaF, 100 mM; EDTA, 10 mM; rabbit liver glycogen, 0.5% (w/v); and dithiothreitol, 5 mM. The activities of glycogen synthase a (●) or total glycogen synthase (O) were determined at 37° as described in the Methods. The points represent the average of two determinations at each time point.

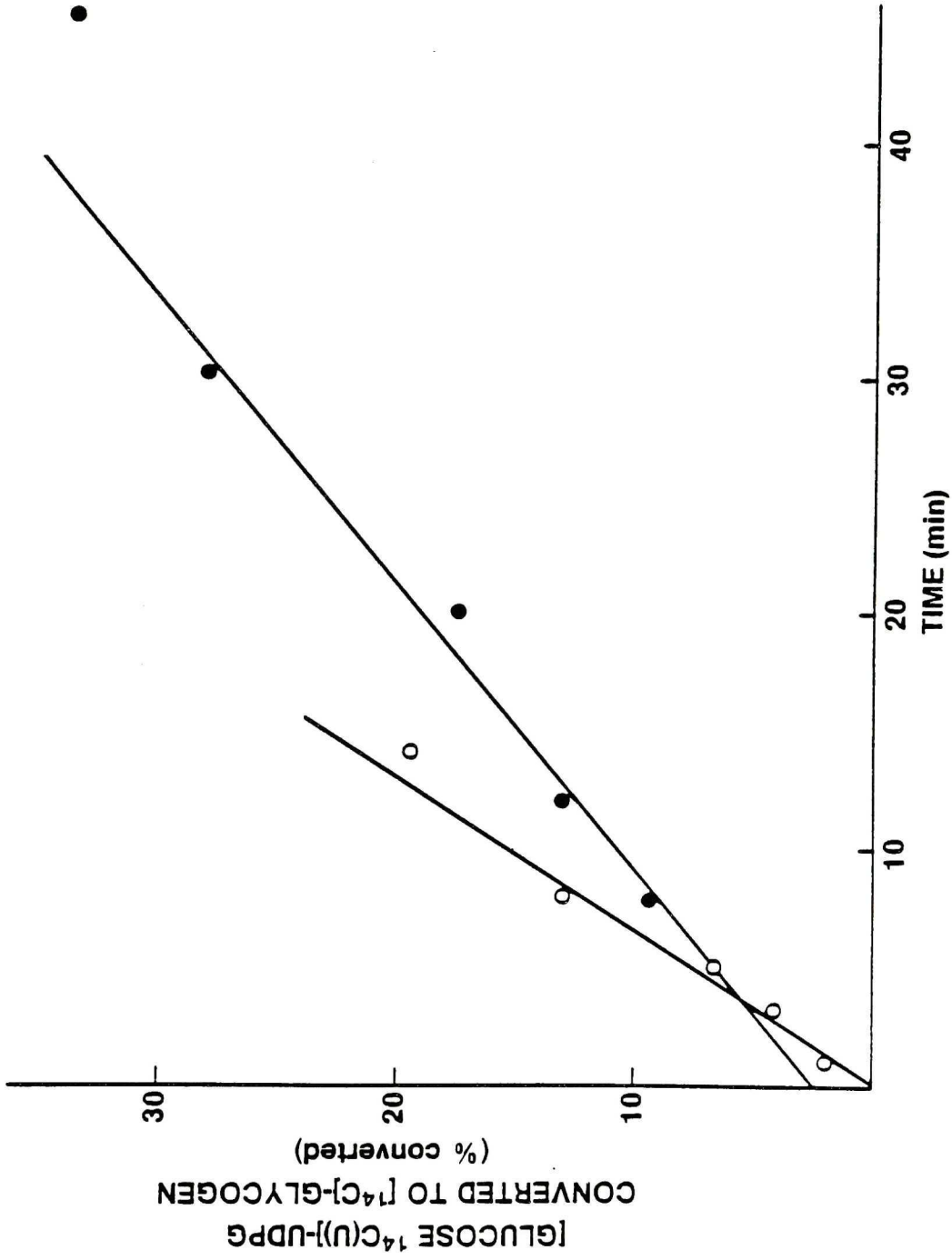
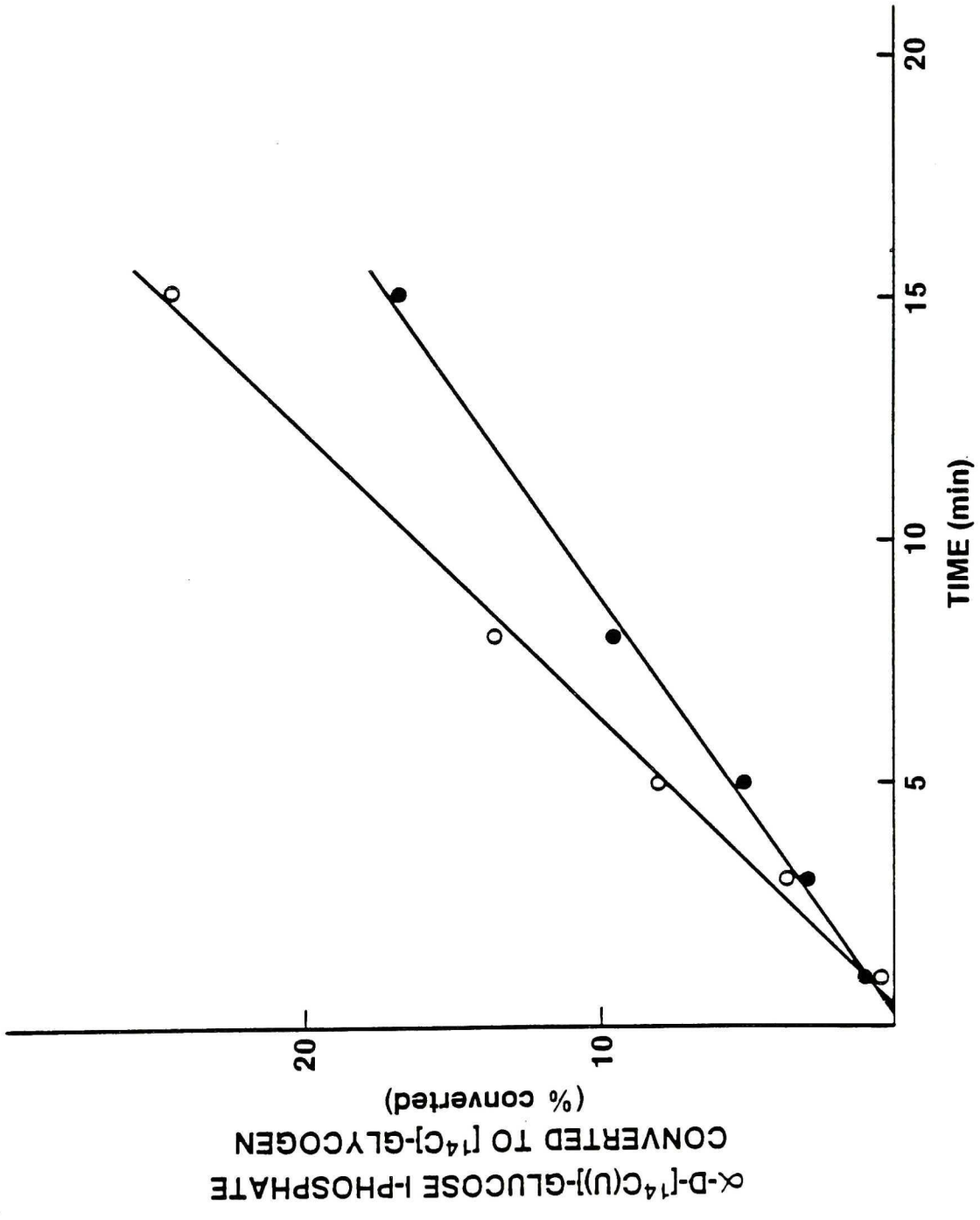

[GLUCOSE 14C(U)]-UDPG
CONVERTED TO [14C]-GLYCOGEN
(% converted)
10
20
30
TIME (min)
10
20
30
40

Fig. 4. Time course of glycogen phosphorylase assay. Hepatocyte homogenates were prepared as described in Fig. 3. The activities of glycogen phosphorylase a (●) or total glycogen phosphorylase (O) were determined at 37° as described in the Methods. The points represent the average of two determinations at each time point.


α-D-[14C(U)]-GLUCOSE 1-PHOSPHATE
CONVERTED TO [14C]-GLYCOGEN
(% converted)
20
10
5
10
15
20
TIME (min)

lase a, total phosphorylase) was carried out on several homogenates from each experiment.

To further demonstrate that inter-experiment variation in cell number would not affect results of these assays, the activities of the glycogen metabolizing enzymes were determined in samples of 1.5 X 10^6, 3.0 X 10^6 and 6.0 X 10^6 hepatocytes. It was anticipated that this concentration range of cells would adequately cover the range of variations in cell number in experiments (inter-experiment cell number variation was usually less than 0.3 X 10^6 cells/ml, or about 10%). The activities of hepatocyte glycogen metabolizing enzymes correlated well with increasing cell number, and as a result it would not be expected that inter-experiment variation in cell number would significantly affect the results of the assays (Table 4). The results of these experiments as well as the results investigating the effect of chlorpropamide on the glycogen metabolizing enzymes were normalized to hepatocyte DNA content.

Determination of Glucose Production from Lactate

Glucose production from lactate was determined according to methods described by Exton and Park (1967) and Claus, et al. (1975). Hepatocytes were pre-incubated for 20 min in the absence of substrates and incubated with glucose and gluconeogenic precursors as in the measurement of gluconeogenic intermediates experiments but with the addition of 0.05 μCi/ml [^{14}C(U)]-lactic acid. At the end of the incubation period (60 min), 1.0 ml of cell suspension was added to 0.5 ml of 0.15 M BaOH to deproteinize the samples. $ZnSO_4$ (5% w/v, 0.5 ml) was added to neutralize the samples which were incubated at 4° for 15 min and centrifuged at 2,500 x g for 20 min at 4°. One ml of the

TABLE 4

CORRELATION OF GLYCOGEN SYNTHASE AND GLYCOGEN PHOSPHORYLASE ACTIVITIES WITH HEPATOCYTE CONCENTRATION

Hepatocyte Concentration (cells/ml)	Synthase a Activity (U/g wet wt. cells)	Synthase a+b Activity (U/g wet wt. cells)	% Synthase a/a+b
1.5×10^6	0.160	0.360	0.444
3.0×10^6	0.147	0.373	0.394
4.5×10^6	0.204	0.420	0.486

Hepatocyte Concentration (cells/ml)	Phosphorylase a Activity (U/g wet wt. cells)	Phosphorylase a+b Activity (U/g wet wt. cells)	% Phosphorylase a/a+b
1.5×10^6	1.120	6.417	0.175
3.0×10^6	1.343	5.765	0.232
4.5×10^6	1.257	7.000	0.190

Hepatocyte suspensions (1.5, 3.0, or 6.0 x 10^6 cells/ml) were homogenized as described in Fig. 3. The activities of glycogen synthase a, toatal glycogen synthase, glycogen phosphorylase a, and total glycogen phosphorylase were determined at 37° after 12, 6, 6, and 4 min of incubation, respectively. 1 unit=1 μmole of either [glucose-^{14}C (U)]-UDPG or α-D-[^{14}C (U)]-glucose 1-phosphate converted to [^{14}C]-glycogen.

supernatant was added to 0.6 ml of AG 50W-X8 anion exchange resin, and 0.3 ml of AG 3-X4A cation exchange resin, contained in plastic liquid scintillation vials. The suspensions were shaken at 40 cycles/min for 30 min at room temperature, transferred to plastic centrifuge tubes and briefly centrifuged at 50 x g to yield a resin-free supernatant. One ml of supernatant was removed and the resin treatment repeated. One ml of the final supernatant was added to 10 ml of Beckman EP liquid scintillatlion cocktail for analysis of ^{14}C incorporation into glucose by liquid scintillation spectrometry.

A series of experiments were conducted to investigate the recovery of glucose and the percentage of non-adsorbable lactate. One ml of KHH containing 10 mM glucose, 0.1 μCi/ml [^{3}H]-glucose, 5 mM lactate and 0.05 μCi/ml [^{14}C]-lactate was deproteinized and treated twice with the resins. The recovery of [^{3}H]-glucose was 100.4 $\pm$ 0.8% and 0.36 $\pm$ 0.04% of the total [^{14}C]-lactate was not adsorbed to the resin. The sample:blank ratio for most experiments was 60-100:1.

Treatment of Ion Exchange Resins

All ion exchange resins used in this study were used in the ionic form supplied by the manufacturer, except AG 1-X3A (glucose output experiments). This resin was only obtainable in the chloride form, but it was necessary to treat the de-proteinized samples with the hydroxide form since chloride has a much higher affinity for the resin than lactate (Bio-Rad Chromatography, Electrophoresis, Immunochemistry, and HPLC catalogue, Jan., 1984, pp. 14). The interconversion of the ionic forms was accomplished by washing the resin (retained by filter paper in a Buchner funnel) with 5 bed volumes of 1 N NaOH. The completeness of conversion was tested by collecting 5 ml of effluent,

acidifying with 0.5 ml of HNO_3, and adding 0.5 ml of 1% (w/v) $AgNO_3$, which precipitated the chloride as $AgCl_2$. The absence of a precipitate indicated complete conversion (no chloride in the effluent). The converted resin was washed with 5 volumes of glass distilled water, or until the pH of the effluent was $\leq$ 7.0.

Deoxyribonucleic Acid Determination

Hepatocyte deoxyribonucleic acid (DNA) content was determined according to the methods described by Burton (1956, 1968). A concentrated hepatocyte suspension (0.5 ml containing 1.8 x 10^7 cells/ml) was diluted to 3.0 ml with glass distilled water. PCA (70-72%, 0.154 ml) was added to the suspensions, the samples were mixed and incubated at 4° for 15 min. The samples were centrifuged at 2,500 x g for 20 min at 4° and the supernatants discarded. The pellets were re-suspended in 2.0 ml of PCA, incubated at 4° for 15 min and recentrifuged. This treatment was repeated, the pellets were resuspended in 4.0 ml of 0.5 N PCA and the samples were heated at 90° for 20 min in stoppered tubes. The samples were cooled to 4°, centrifuged at 2,500 x g for 20 min at 4°. A standard DNA solution was prepared by dissolving calf thymus DNA (1 mg/ml) in 5 mM NaOH, and further diluting this solution 1:1 with 1 N $HClO_4$. Immediately prior to use, this solution was heated at 70° for 15 min. One ml samples of the supernatants were added to 2.0 ml of a reagent that contained (final concentrations): acetaldehyde, 0.008 % (w/v); diphenylamine, 89 mM (re-crystalized from hot hexane); acetic acid, 17.4 N; and sulfuric acid, 0.27 N. The samples were mixed and incubated at 25-30° in the dark for 16-20 hours. The absorbance was monitored, spectrophotometrically, at 600 nm in the Gilford 250 spectrophotometer.

RESULTS

Establishment of hepatocyte model system

Preliminary studies were conducted to establish that the hepatocytes prepared by the collagenase digestion technique were viable, metabollically active, and responsive to hormonal stimuli.

An experiment was designed to monitor both leakage of a soluble enzyme activity, lactate dehydrogenase (LDH), from hepatocytes and the abililty of cells to exclude trypan blue during the preparative steps. *In vivo*, the measurement of LDH activity in the plasma is not a specific indicator of hepatic damage since other tissues (erythrocytes, skeletal muscle, etc.) also contain this enzyme. However, *in vitro* leakage of LDH activity is a commonly accepted indicator of viability of isolated hepatocyte. Exclusion of trypan blue, although more accurately described as an indicator of cell membrane integrity, is often utilized as a measure of cell viability.

Hepatocytes were isolated from an 8 hour fasted rat and the cells were suspended in substrate-free (KHH) at 37°. Samples of the supernatants and pellets of cell suspensions after successive stages in cell preparation were removed for determination of leakage of LDH activity. The results of this experiment are shown in Table 5. The activity of LDH in the supernatants was 21.4%, 6.1%, and 4.0% of that contained in the hepatocyte pellet after the first, second, and third washes, respectively. Approximately 95% of the hepatocytes excluded trypan blue after the third centrifugation (the stage of isolation prior to beginning incubations in later experiments). Hepatocytes incubated at a concentration of 3×10^6 cells/ml in KHH containing

TABLE 5

ASSESSMENT OF HEPATOCYTE VIABILITY BY RELEASE OF LACTATE DEHYDROGENASE ACTIVITY

Stage in Isolation Procedure	Lactate Dehydrogenase Activity (U/ml)
Supernatant After First Centrifugation	2.24
Supernatant After Second Centrifugation	0.64
Supernatant After Third Centrifugation	0.42
Cell Pellet After Third Centrifugation	10.40

Lactate dehydrogenase activity was measured in supernatants obtained during isolation of hepatocytes from the liver of an 8 hr fasted rat and in a homogenate of the cells after the final preparative step. A 50 µl aliquot of each supernatant or of the homogenate was incubated with potassium phosphate buffer, 47.5 mM, pH 7.5; pyruvic acid, 0.29 mM; and NADH, 0.13 mM. The decrease in optical density of the solution associated with the oxidation of NADH was monitored at 340 nm. Lactate dehydrogenase activities of these preparations are listed as units/ml (1 unit=1 µmole of pyruvic acid converted to lactic acid per min).

10 mM glucose retained approximately 97%, 95%, and 90% of LDH activity after 10, 30, and 60 minutes of incubation, respectively. Approximately 90% of hepatocytes excluded trypan blue after 60 minutes of incubation. Therefore, the results of this experiment demonstrate that the exclusion of trypan blue and leakage of LDH activity correlate well in this hepatocyte system, which is in agreement with the results of Edmondson and Bang (1981). It was concluded that the preparative techniques yielded suspensions of hepatocytes that were acceptable for use in further studies.

Other investigators have argued that a more accurate indicator of hepatocyte viability is the ability of cells to conduct metabolic processes that involve transport of substrates through various intracellular compartments, such as gluconeogenesis, drug metabolizing activity, albumin synthesis, etc. (Drochmans, et al.,1978; Elliot, et al., 1975). Demonstration of the metabolic integrity of hepatocytes utilized in this study was accomplished by isolating cells from an 8 hour fasted rat, pre-incubating the cells for 20 minutes at a concentration of 3×10^6 cells/ml in the absence of substrates, incubating the cells in various concentrations of glucose, and finally measuring accumulation of glycogen. The rates of glycogen accumulation by hepatocytes were 4.08 ± 0.98 µmoles/g/hr, 15.22 ± 1.78 µmoles/g/hr, and 22.31µmoles/g/hr when the cells were incubated with 10, 20, or 30 mM glucose, respectively. These values are in agreement with the results of Seglen (1973) who reported glycogen accumulation of approximately 3, 9, and 22 µmoles/g/hr when hepatocytes were incubated with 10, 20, or 35 mM glucose, respectively.

Hepatic glycogenolysis is markedly stimulated by hypoxia in

hepatocytes (Seglen, 1974; Bollen, et al., 1983) and perfused liver (Sharma, et al., 1980; Walli, et al., 1973; Lutaya, et al., 1983). Since hepatocytes in this study accumulated significant quantities of glycogen during the 60 minute incubation period, adequate oxygenation of the cells was evidently maintained.

Hepatic glycogen metabolism in vivo is hormonally responsive and isolated hepatocytes should respond to the appropriate hormones as would be predicted by those hormones' in vivo action on glycogen metabolism. Figure 5 demonstrates that glycogen accumulation in hepatocytes isolated from 8 hour fasted rats, pre-incubated for 20 minutes in the absence of substrates and incubated for 60 minutes with 10 mM glucose, 5 mM lactate, 5 mM glutamine, Eagle's (Modified) Minimal Essential Medium amino acid mixture (AAM) and 0.8 mg/ml bacitracin (to prevent proteolysis of added hormones) decreased in response to glucagon treatment at both physiological (1×10^{-10} M) and supraphysiological concentration (1×10^{-8} M) by $65.4 \pm 5.0\%$ and $94.4 \pm 2.7\%$, respectively. Insulin, at a supraphysiological concentration (1×10^{-8} M) did not significantly increase glycogen accumulation ($110.0 \pm 14.1\%$ of control), nor could it counteract the effect of 1×10^{-10} M glucagon. Figure 6 demonstrates that glucagon and insulin had similar effects on ^{14}C incorporation from [^{14}C]-amino acids into glycogen. Glucagon decreased the incorporation of ^{14}C into glycogen by $59.0 \pm 4.4\%$ at 10^{-10} M and $92.3 \pm 4.1\%$ at 1×10^{-8} M. Insulin treatment of hepatocytes did not result in a stimulation of incorporation of ^{14}C into glycogen ($107.1 \pm 14.0\%$ of control), nor could it counteract the effect of glucagon on ^{14}C incorporation into glycogen.

The results of these experiments demonstrate that: 1) the

Fig. 5. The effects of glucagon and insulin on glycogen accumulation in isolated hepatocytes. Hepatocytes were isolated from an 8 hr fasted rat. The cells were pre-incubated for 20 min in the absence of substrates and then incubated for 60 min with glucose, 10 mM. lactate, 5 mM; glutamine, 5 mM; and amino acid mixture with glucagon and/or insulin as indicated. Glycogen accumulation is expressed as the per cent of glycogen accumulated in the absence of hormones. The height of the bars represent the mean per cent glycogen accumulation $\pm$ S.E.M. determined in hepatocytes isolated from 3 rats (mean control glycogen accumulation = 36.2 $\pm$ 6.4 µg/3 x 10^6 cells).

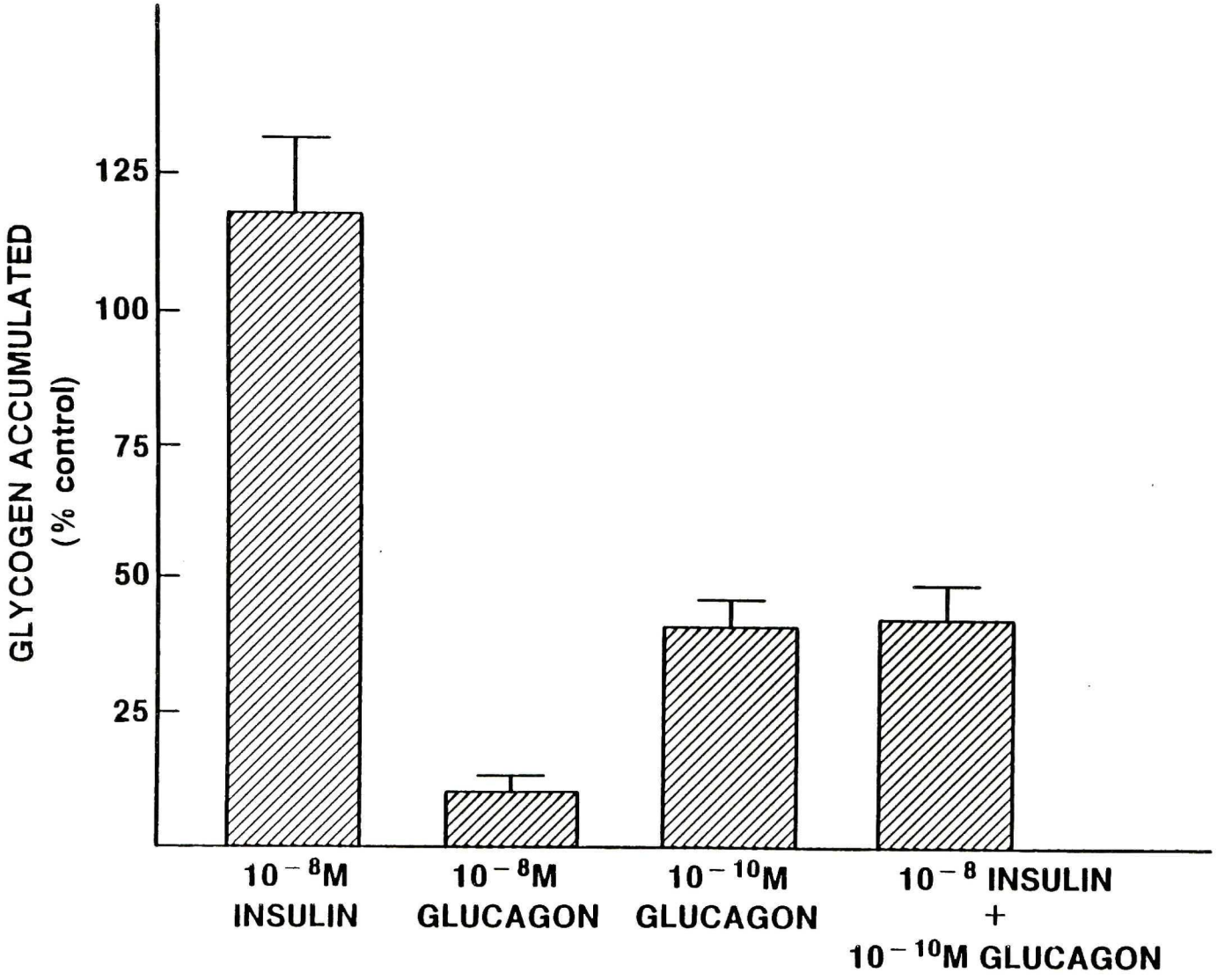
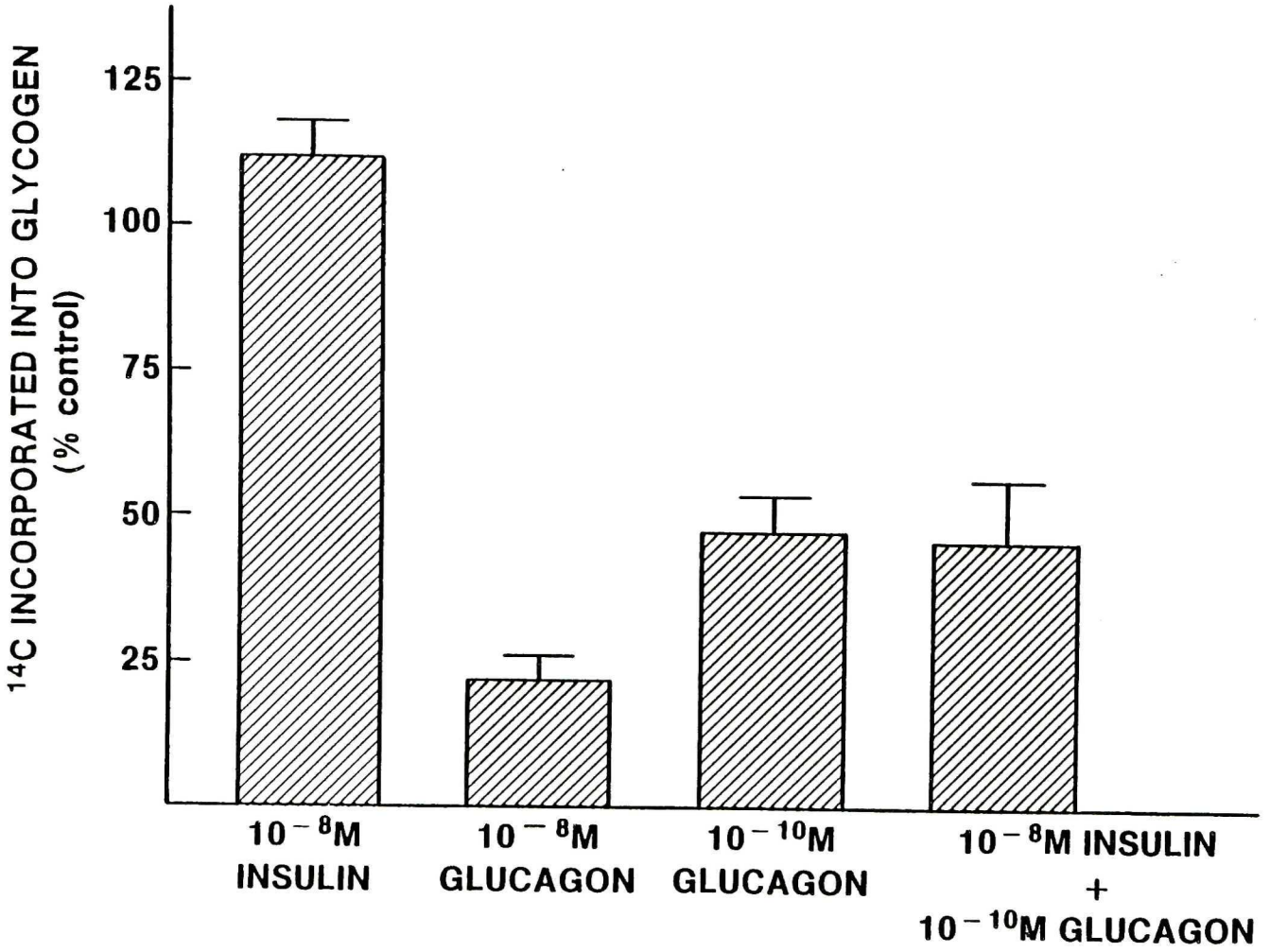

GLYCOGEN ACCUMULATED
(% control)
125
100
75
50
25
10 − 8M
INSULIN
10 − 8M
GLUCAGON
10 − 10M
GLUCAGON
10 − 8 INSULIN
+
10 − 10M GLUCAGON

Fig. 6. The effect of glucagon and insulin on ^{14}C incorporation from [^{14}C]-amino acids into glycogen in isolated hepatocytes. Hepatocytes were isolated and incubated as in Figure 5. ^{14}C incorporated into glycogen is expressed as the percent ^{14}C incorporated into glycogen in the absence of hormones. The height of the bars represent the mean per cent incorporation of ^{14}C into glycogen $\pm$ S.E.M. determined in hepatocytes isolated from 3 rats (mean contol incorporation of ^{14}C into glycogen = 1730 $\pm$ 241 dpm/3 x 10^6 cells).


^{14}C INCORPORATED INTO GLYCOGEN
(% control)
125
100
75
50
25
10^{-8}M INSULIN
10^{-8}M GLUCAGON
10^{-10}M GLUCAGON
10^{-8}M INSULIN + 10^{-10}M GLUCAGON

procedure for the isolation of hepatocytes used in this study results in a population of cells that was routinely $\geq$ 95% viable immediately before the start of incubation as assessed by two commonly used indicators of cell viabillity; 2) 90% of hepatocytes incubated under the conditions described remained viable for at least 60 minutes; 3) not only was the structural integrity of hepatocytes maintained (evidenced by exclusion of trypan blue and retention of LDH activity), but they were also metabolically intact, since they responded to increasing glucose concentrations by increased glycogen accumulation; and 4) hepatocytes responded to glucagon treatment by decreasing glycogen accumulation and incorporation of ^{14}C into glycogen, but did not respond to insulin treatment by increasing glycogen deposition or incorporation of ^{14}C into glycogen. These results may be explained as follows: Glucagon will both stimulate gluconeogenesis and glycogenolysis, therefore it would be expected that glycogen accumulation in hepatocytes would be decreased and glucose output from cells would be increased. On the other hand, insulin stimulates glycogenesis, but inhibits gluconeogenesis and since these cells are relying on gluconeogenic precursors for glycogen accumulation, the effects of this hormone on the two pathways counteract each other resulting in no net gain in glycogen accumulation or incorporation of ^{14}C from [^{14}C]-amino acids into glycogen. Also it is apparent that the gluconeogenic pathway in cells isolated from 8 hour fasted rats is more sensitive to glucagon than insulin since the latter hormone could not counteract the effect of glucagon.

The results of the previously described experiments led to the conclusion that suspensions of viable hepatocytes could be isolated that could be used for the study of the effects of the sulfonylureas

on hepatic carbohydrate metabolism.

Preliminary results on the effect of chlorpropamide on glucose output and glycogen accumulation in hepatocytes

Previously reported *in vitro* experiments demonstrated that sulfonylureas decreased release of glucose from liver slices (Vaughan, 1957). *In vivo*, it was demonstrated that glyburide inhibits hepatic glucose output in NIDDM patients (Best, *et al.*, 1981). However, the validity of the liver slice model for the study of hepatic carbohydrate metabolism has been questioned, and the results of *in vivo* experiments are complicated because of the drugs' ability to stimulate insulin secretion. In order to clarify the effects of sulfonylureas on hepatic glucose output and storage, a series of experiments was designed to investigate the effect of 2.0 mM chlorpropamide on glucose formation and glycogen accumulation in hepatocytes.

The basis for selecting the feeding state of the animals used as a source of liver cells, for using the particular substrate mixture, and for utilizing a particular sulfonylurea are as follows. The cells used in this study were isolated from 24 hour fasted rats, preincubated for 20 minutes in the absence of substrates, and incubated in the presence of 10 mM glucose, 5 mM lactate, 5 mM glutamine, AAM, and 0.05 μCi/ml [^{14}C(U)]-lactic acid. Hepatocytes were isolated from 24 hour fasted rats, since Seglen (1974) demonstrated that hepatocytes isolated from rats in this nutritional state would form glucose from gluconeogenic precursors and store glucose as glycogen at maximal rates. Hepatocytes isolated from fed animals accumulated glycogen at 50% of the rate found in hepatocytes isolated from 24 hour fasted rats while hepatocytes isolated from 40 hour fasted animals would not accumulate

glycogen at significant rates. Glucose and gluconeogenic precursors were chosen as glycogenic substrates because Katz, et al. (1976, 1979) demonstrated that maximum rates of glycogen synthesis were obtained when hepatocytes were supplied glucose, lactate and amino acids. Chlorpropamide was chosen as a model sulfonylurea because: 1) chlorpropamide is equally potent as tolbutamide and both these drugs are more potent that other first generation sulfonylureas, acetohexamide or tolazamide; 2) chlorpropamide is the most soluble of the first generation sulfonylureas and 3) it has the slowest rate of hepatic metabolism in vivo, and these drugs have metabolites with varying hypoglycemic potencies which could complicate the interpretation of results of experiments using liver preparations, such as isolated hepatocytes. In these preliminary experiments, incubation of hepatocytes with 2.0 mM chlorpropamide inhibited the conversion of [^{14}C]-latate to [^{14}C]-glucose by 44.1 $\pm$ 0.6% and glycogen accumulation by 52.8 $\pm$ 0.5%. These results demonstrated that chlorpropamide directly affected hepatic carbohydrate metabolism independently of hormonal mediation but the site(s) of action remained unidentified. The effect on glucose output was expected, since no previous studies either in vitro or in vivo have demonstrated that the sulfonylureas increase glucose output, but the effect on glycogen metabolism was not neecessarily expected in light of previous studies. Since these results were reproducible, but the concentration of chlorpropamide used was 2-4 fold higher than therapeutic concentrations for this drug (Izzo, 1957; Sheldon, et al., 1965; Melander, et al., 1978), further studies were carried out to characterize the effect of chlorpopamide and other sulfonylureas on glucose output from hepatocytes. Results of studies involving further

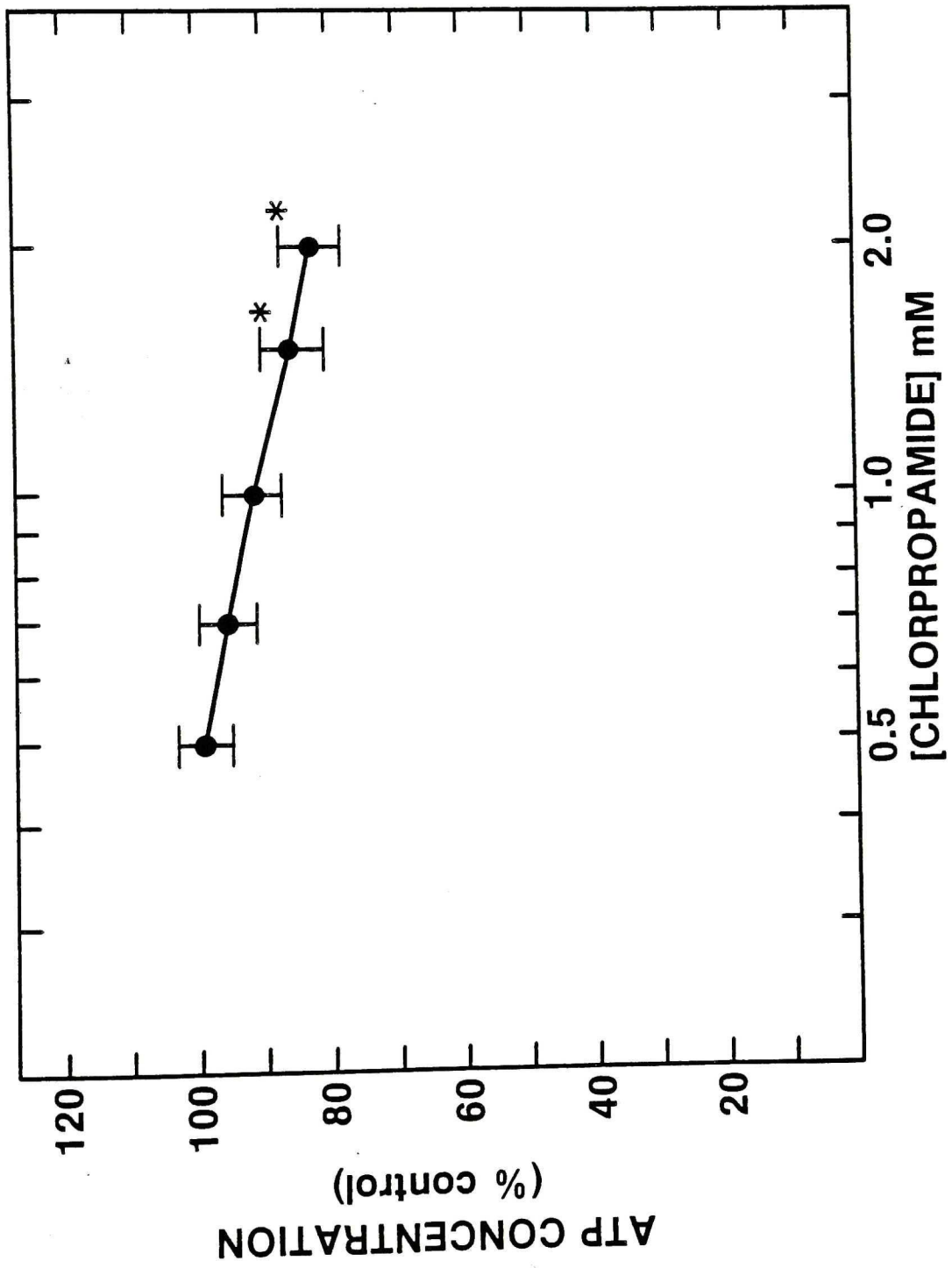
characterization of the effects of the sulfonylureas on glycogen metabolism will be presented later.

Glucose output studies

In order to demonstrate that the inhibition of hepatic glucose output by chlorpropamide could have biological significance, a series of experiments was carried out to investigate the concentration-response relationship, the time course, and the chemical specificity of the effect of chlorpropamide and other sulfonylureas on the conversion of [^{14}C]-lactate to [^{14}C]-glucose. To maintain incubation conditions consistant with those of the glycogen accumulation experiments to be presented later, 10 mM glucose was present in the medium. Because the assay used to measure mass of glucose could not discriminate between the amount of glucose produced by 3 X 10^6 cells/ml/hr and the 10 µmoles/ml glucose already in the buffer, incorporation of [^{14}C]-lactate into [^{14}C]-glucose was used as a measure of glucose production instead of measuring the mass of glucose produced from gluconeogenic precursors. Furthermore, this assay is a measure of glucose output, since the intracellular concentration of non-phosphorylated glucose is below detectable levels.

Hepatocytes were isolated from 24 hour fasted rats, pre-incubated for 20 minutes in the absence of substrates, and incubated for 60 minutes with glucose, gluconeogenic precursors, 0.05 µCi/ml [^{14}C(U)]-lactate, and increasing concentrations of chlorpropamide. The results of these experiments are presented in Figure 7. Chlorpropamide inhibited the conversion of [^{14}C]-lactate into [^{14}C]glucose at all concentrations tested (0.5, 0.7, 1.0, and 2.0 mM). Hepatocytes incubated with 0.7 mM chlorpropamide produced significantly ($p<.05$) less glucose (19.1% inhib-

Fig. 7. Relationship of conversion of [^{14}C]-lactate into [^{14}C]-glucose to chlorpropamide concentration in isolated hepatocytes. Hepatocytes were isolated from 24 hr fasted rats and pre-incubated for 20 min in the absence of substrates. The cells were incubated with glucose and gluconeogenic precursors including 0.05 µCi/ml [^{14}C]-lactate for 60 min in the presence of chlorpropamide at the concentrations indicated in the figure. Each point represents the mean per cent of control conversion of [^{14}C]-lactate into [^{14}C]-glucose $\pm$ S.E.M. determined with hepatocytes isolated form 5 rats (mean control conversion of [^{14}C]-lactate into [^{14}C]-glucose = 42.6 $\pm$ 0.9 µg/3 x 10^6 cells). *, $p < 0.05$; **, $p < 0.01$, cells incubated with chlorpropamide vs control incubations.

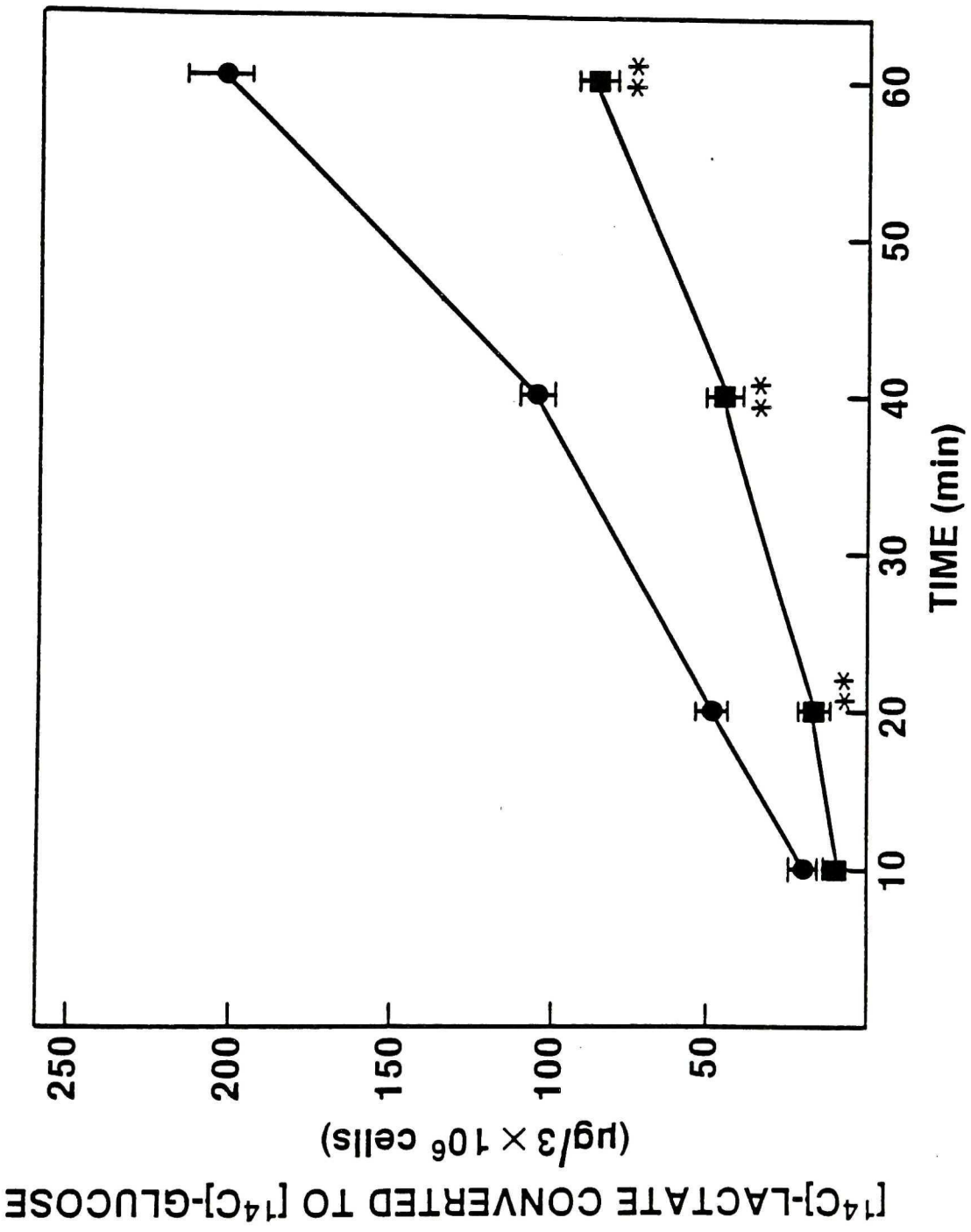

ATP CONCENTRATION
(% control)
120
100
80
60
40
20
0.5
1.0
2.0
[CHLORPROPAMIDE] mM
*
*

ition) compared to hepatocytes incubated in the absence of chlorpropamide. The difference between chlorpropamide treated and untreated cells became significant at the $p<.01$ level when hepatocytes were incubated with 1.0, 1.5 and 2.0 mM chlorpropamide (the inhibition was 31.9%, 41.6% and 48.4% for these drug concentrations, respectively). Important facts that emerged from this study were: 1) the inhibition of glucose output by chlorpropamide was a concentration-dependent phenomenon; 2) this inhibition did not require the presence of an anabolic hormone such as insulin; and 3) the concentration of chlorpropamide necessary to significantly inhibit glucose output from hepatocytes (0.7 mM) was within the therapuetic concentration range of this drug.

To relate temporally the effect of chlorpropamide on glucose output with the effects of other metabolic processes (presented later) a study was performed to investigate the effect of 2.0 mM chlorpropamide on glucose output from hepatocytes as a function of time (Figure 8). Chlorpropamide did not inhibit glucose output at 10 min, but did significantly ($p<.01$) inhibit glucose output from hepatocytes at 20, 40 and 60 min by 63.7%, 56.3% and 57.2%, respectively. That the inhibition of glucose production was relatively constant as a function of time after the inhibition became evident indicates that the effect of chlorpropamide on glucose output was a steady-state inhibition that developed quite in the incubation period, and was not an accumulating effect. This could be explained by a blockade of a reaction in the gluconeogenic pathway. Studies relavent to this possibility wil be presented later.

To demonstrate that chlorpropamide did not have a general cytotoxic effect on isolated hepatocytes, which could be evident by

Fig. 8. Time-dependent inhibition of glucose output by chlorpropamide. Hepatocytes were isolated and incubated as described in Fig. 7, except the hepatocytes were incubated for 10, 20, 40, or 60 min in the presence (■) or absence (●) of 2.0 mM chlorpropamide. Values shown are the mean μ g [^{14}C]-lactate converted to [^{14}C]-glucose ± S.E.M. as determined in 3 separate experiments. **, P 0.01; chlorpropamide-treated compared to control incubations at each time point.


[14C]-LACTATE CONVERTED TO [14C]-GLUCOSE
(μg/3 × 10^6 cells)
250
200
150
100
50
10
20
30
40
50
60
TIME (min)
**
**
**

decreased glucose output as the viability of cell suspensions decreased during the incubations, an experiment was conducted to investigate the effect of 5.0 mM chlorpropamide on the release of lactic dehydrogenase (LDH) activity from hepatocytes isolatated from an 8 hour fasted rat and incubated in the presence of 10 mM glucose. Treatment of hepatocytes with this concentration of chlorpropamide (2.5 fold higher than that used in any experiment in this study) did not result in significant increases of LDH leakage activity from hepatocytes (Table 6). It can be concluded that the effect of this drug on glucose output was not due to an ability of the drug to decrease the viability of suspensions of hepatocytes.

All clinically useful sulfonylureas are hypoglycemic *in vivo*. The only difference in hypoglycemic action among these drugs is their potency, with no known differences in mechanism of action in any system studied including the chronic effects on insulin release, inhibition of triglyceride lipase, apparent regulation of insulin receptor activity, etc. If the inhibition of glucose output described in hepatocytes was an action relevant to their *in vivo* hypoglycemic action, a series of sulfonylureas would be expected to have *in vitro* activity that reflected *in vivo* potencies. Therefore, a series of experiments was conducted to investigate the the effect of a second sulfonylurea, tolbutamide and a non-hypoglycemic sulfonylurea, HOE 17,710 on the conversion of [^{14}C]-lactate to [^{14}C]-glucose in hepatocytes isolated from 24 hour fasted rats. Chlorpropamide and tolbutamide are approximately equipotent in hypoglycemic potency *in vivo*. The difference in dosage (usually 3 fold more tolbutamide is administered/unit time for equivalent hypoglycemic response) is mostly attributable to the more rapid metabolism of

TABLE 6

LACK OF A CYTOTOXIC ACTION OF CHLORPROPAMIDE ON ISOLATED HEPATOCYTES

Incubation Time	Lactate Dehydrogenase Activity (U/ml)
10 min (Control)	0.36
10 min (Chlorpropamide)	0.58
30 min (Control)	0.55
30 min (Chlorpropamide)	0.61
60 min (Control)	0.86
60 min (Chlorpropamide)	1.14

Hepatocytes were isolated from an 8 hr fasted rat. After pre-incubation without substrates for 20 min, 10 mM glucose was added with or without 5.0 mM chloropropamide. At 10, 30, and 60 min, hepatocytes were removed from each suspension by centrifugation and lactate dehydrogenase activity was assayed in the supernatants. The units of lactate dehydrogenase activity are μmoles of pyruvate converted to lactate per min.

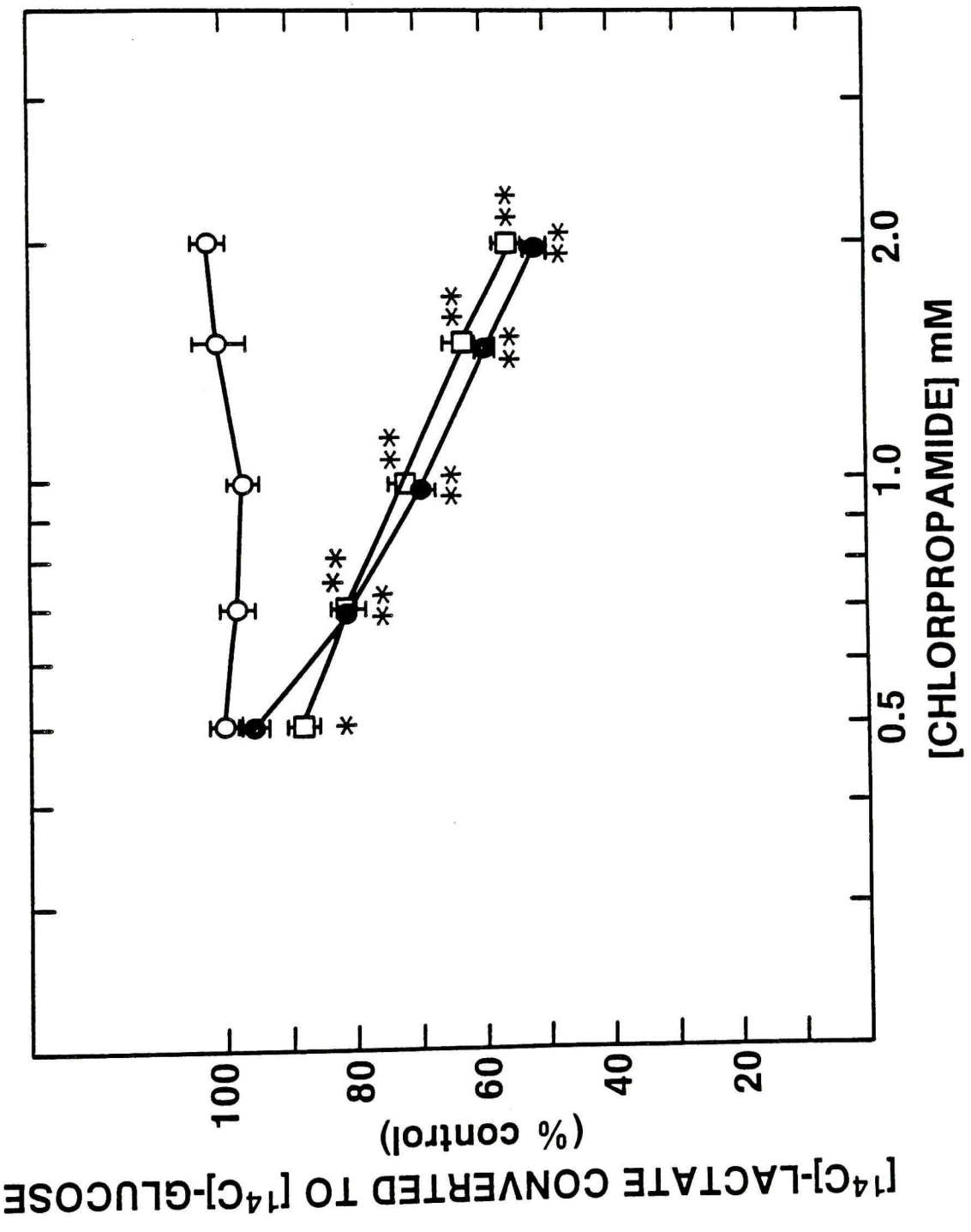
tolbutamide to non-hypoglycemic metabolites than chlorpropamide (Melander, et al;, 1978). In the present studies, these drugs were approximately equipotent in inhibiting the conversion of [^{14}C]-lactate to [^{14}C]-glucose (Figure 9). There were no significant differences in activity at any concentrations tested except 0.5 mM, at which tolbutamide significantly ($p<.05$) inhibited glucose output, but chlorpropamide did not. On the other hand, the non-hypoglycemic sulfonylurea, HOE-17,710 did not decrease the conversion of [^{14}C]-lactate to [^{14}C]-glucose at any concentration tested.

These results demonstrate that glucose output from hepatocytes isolated from fasted rats and incubated with glucose and gluconeogenic precursors is inhibited only by hypoglycemic sulfonylureas and this effect on glucose output from hepatocytes may relate to the in vivo hypoglycemic activity of the drugs.

Glycogen Accumulation

To this point, chlorpropamide was shown to inhibit the conversion of [^{14}C]-lactate to [^{14}C]-glucose, and that this effect was specific for hypoglycemic sulfonylureas. Another important hepatic glucoregulatory pathway in vivo is the metabolism of glycogen. Approximately 60% of the glucose absorbed after an oral glucose load is extracted by the liver, which stores a large portion of the glucose as glycogen. An action of the sulfonylureas to augment hepatic glycogen storage or decrease its catabolism might be important for their hypoglycemic action. In fact, the sulfonylureas had previously been shown to decrease glycogenolysis in liver slices (Vaughan, 1957) and perfused liver (Kaldor and Pogasta, 1960). On the other hand, animals administered sulfonylureas for 60 days had lower hepatic glycogen content

Fig. 9. Comparison of the effects of hypoglycemic and non-hypoglycemic sulfonylureas on glucose output from isolated hepatocytes. Hepatocytes were isolated and incubated as in Fig. 6, except that in addition to chlorpropamide (●), the effects of tolbutamide (□) or HOE-17,710 (O) on glucose output were also examined. Each point is the mean per cent of control conversion of [^{14}C]-lactate into [^{14}C]-glucose $\pm$ S.E.M. as determined using hepatocytes isolated from 3 rats (mean control glucose output = 47.3 $\pm$ 1.0 µg/3 x 10^6 cells). *, $P<0.05$; **, $P<0.01$ for treated vs control cells.


[14C]-LACTATE CONVERTED TO [14C]-GLUCOSE
(% control)
100
80
60
40
20
0.5
1.0
2.0
[CHLORPROPAMIDE] mM

(Prasannan and George, 1973; George and Augusti, 1976) than untreated animals. A major goal of this study was thus a clarification of the direct action of the sulfonylureas on hepatic glycogen metabolism. Since in the present study, treatment of hepatocytes isolated from 24 hour fasted rats with 2.0 mM chlorpropamide decreased glycogen accumulation by approximately 50% compared to cells incubated without the drug, studies were conducted to further characterize the effect of the sulfonylureas and other para-substituted sulfonamides on hepatocyte glycogen metabolism. Hepatic gluconeogenesis and glycogen metabolism are closely related, especially in the nutritional state of fasting-re-feeding. Hepatocytes isolated from 24 hour fasted rats and incubated in the presence of glucose and gluconeogenic precursors are in such a state, since a large portion of the glycogenic pool is derived from gluconeogenesis. The possibility existed, therefore, that further information on the effect of chlorpropamide on hepatic carbohydrate metabolism could be obtained by investigating the effects of these drugs on glycogen metabolism in hepatocytes.

As in the glucose output studies, the initial examinations of the effects of chlorpropamide on glycogen accumulation in this section were designed to investigate the concentration-response relationship of the effect of chlorpropamide on glycogen accumulation and incorporation of radioactive carbon from [^{14}C]-amino acids into glycogen. Figure 10 demonstrates that the mass of glycogen accumulated was significantly ($p<.05$) inhibited by 22.6% and 44.2% at concentrations of 1.0 and 2.0 mM chlorpropamide, respectively. The incorporation of ^{14}C from [^{14}C]-amino acids were significantly ($p<.05$) inhibited by 12.9%, 39.7% and 61.8% at 0.1, 1.0 and 2.0 mM, respectively (Figure 11). Although both processes were inhibited by chlorpropamide, the inhibition

Fig. 10. Dependence of glycogen accumulation in isolated hepatocytes on chlorpropamide concentration. Hepatocytes were isolated and incubated as described in Fig. 7. Samples for the determination of glycogen were removed at the start of each incubation and again after 60 min. Glycogen accumulation is the difference between hepatocyte glycogen content measured at the beginning and the end of incubation. Each point represents the mean per cent of control glycogen accumulation $\pm$ S.E.M. as determined in 3 different isolated hepatocyte preparations (mean control glycogen accumulation = 80.1 $\pm$ 3.0 µg/3 x 10^6 cells). **, $P<0.01$ compared to glycogen accumulation in the absence of chlorpropamide.

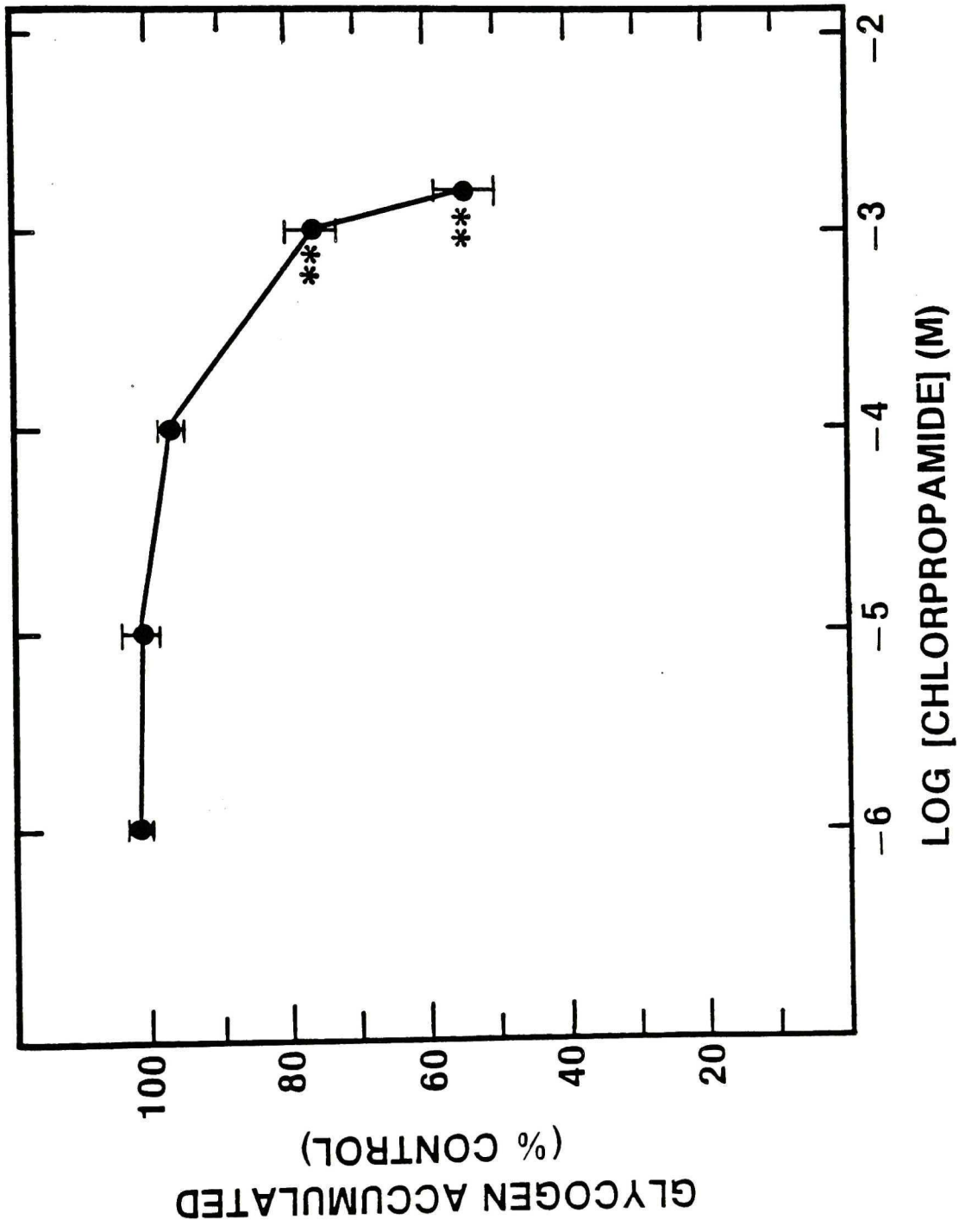
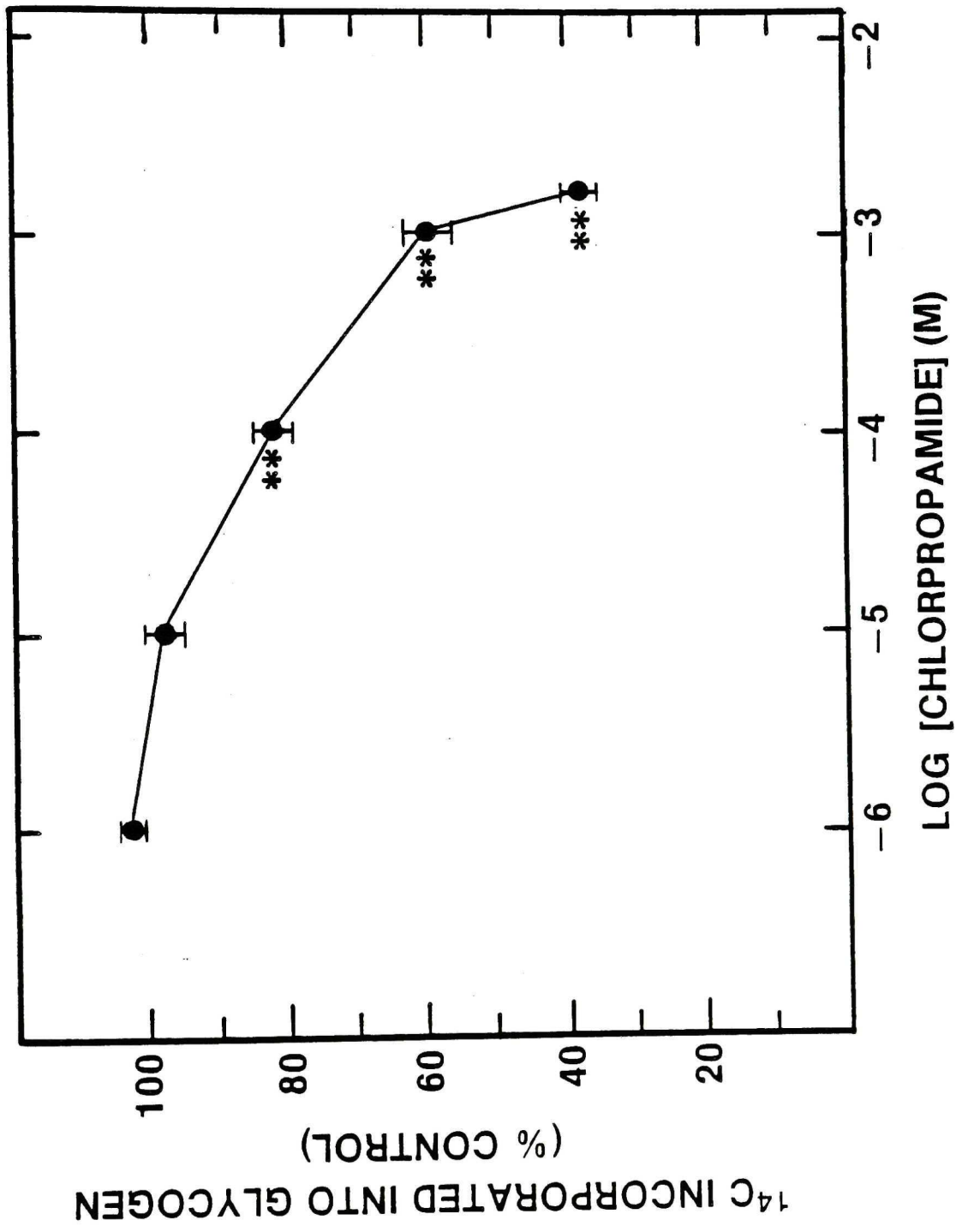

GLYCOGEN ACCUMULATED
(% CONTROL)
100
80
60
40
20
**
**
-6
-5
-4
-3
-2
LOG [CHLORPROPAMIDE] (M)

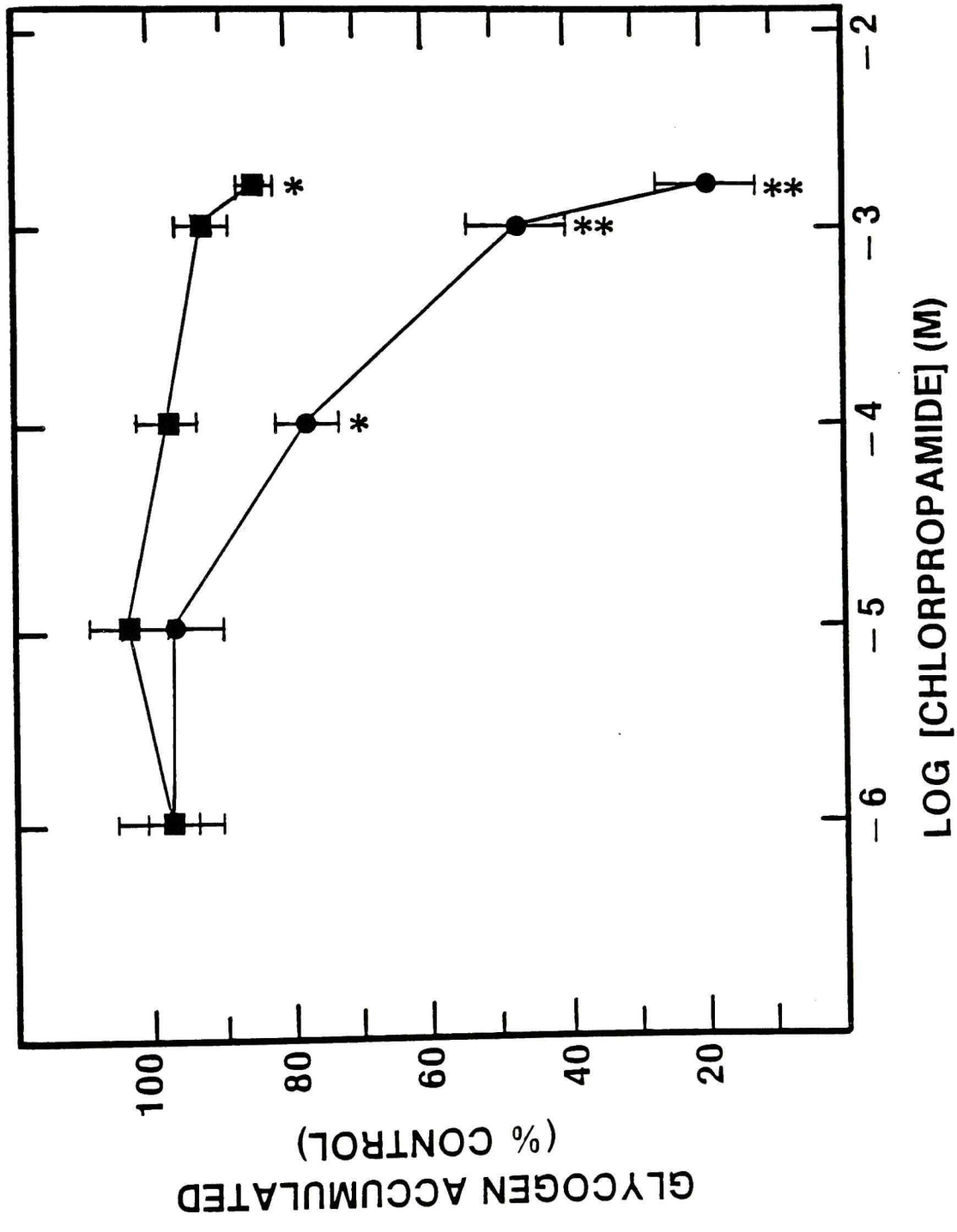
Fig. 11. Dependence of incorporation of ^{14}C from [^{14}C]-amino acids into glycogen in isolated hepatocytes on chlorpropamide concentration. Hepatocytes were isolated and incubated as in Fig. 7. In addition, [^{14}C]-amino acid mixture (0.1 μCi/ml) was added. An aliquot of the glycogen hydrolysate was removed for liquid scintillation counting; counts incorporated into glycogen is the difference between cpm in 60 min and 0 min aliquots. Values shown are mean per cent of control cpm incorporated into glycogen ± S.E.M. as determined in 3 experiments (mean control ^{14}C incorporation into glycogen = 1305 ± 28 dpm/ 3 x 10^6 cells). **, $P<0.01$, compared to ^{14}C incorporated into glycogen in the absence of chlorpropamide.


^{14}C INCORPORATED INTO GLYCOGEN
(% CONTROL)
100
80
60
40
20
**
**
**
−6
−5
−4
−3
−2
LOG [CHLORPROPAMIDE] (M)

of incorporation of radioactive glycogenic precursors into glycogen was significantly ($p<.05$) greater than the inhibition of glycogen accumulation at 1.0 and 2.0 mM. This difference in apparent activity may be a result of the ability of the sulfonylureas to inhibit gluconeogenesis, but not the incorporation of glucose into glycogen. Also it may demonstrate the ability of these drugs to inhibit hepatic transaminases. Bornstein (1957) found that tolbutamide inhibited a cell-free preparation of alanine transaminase isolated from rat liver cytosol, although the concentrations needed to observe this effect ($\geq$ 1.0 mM) may not be attained intracellularly. The results of these experiments demonstrate that chlorpropamide inhibits glycogen accumulation and incorporation of glycogenic precursors into glycogen in hepatocytes within or near therapuetic concentrations, but it was impossible to determine from these data if the effect of chlorpropamide on glycogen metabolism was due to increased glycogenolysis or decreased glycogen synthesis.

To assess which of these processes was affected, two experimental approaches were employed to control initial glycogen content, and hence relative rates of glycogen synthesis and degradation. A comparison of the action of chlorpropamide on glycogen accumulation in hepatocytes isolated from either 24 hour fasted or fed animals is presented in (Figure 12). Chlorpropamide significantly decreased glycogen accumulation in hepatocytes islolated from fasted animals at concentrations of 0.1, 1.0 and 2.0 mM. The drug was ineffective in decreasing glycogen accumulation in cells isolated from a fed animals except for a small (13.8%), but statistically significant ($p<.05$) decrease at 2.0 mM, the highest concentration of chlorpropamide tested. Hepatocytes isolated

Fig. 12. The relationship of chlorpropamide inhibition of glycogen accumulation to initial glycogen content. Hepatocytes were isolated from a 24 hr fasted rat (●) or a fed rat (■) and incubated as in Fig. 10. Values shown are the mean per cent of control glycogen accumulation ± S.E.M. as determined in 3 separate experiments (mean control glycogen accumulation = 20.3 ± 2.1 and 86.6 ± 3.6 μg/3 x 10^6 cells for hepatocytes isolated from fed and fasted rats, respectively). *, $P<0.05$, **$P<0.01$ compared to glycogen accumulation in the absence of chlorpropamide.


GLYCOGEN ACCUMULATED
(% CONTROL)
100
80
60
40
20
-6
-5
-4
-3
-2
LOG [CHLORPROPAMIDE] (M)
*
**
*
**
*

from fed animals initially contained approximately 50 times more glycogen than those isolated from 24 hour fasted rats (15.1 ± 0.2 mg/g wet weight vs 0.3 ± 0.02 mg/g wet weight). The rate of hepatic glycogenesis is dependent upon glycogen content (Seglen, 1977). Glycogen can control rates of glycogen accumulation since it is an inhibitor of phosphorylase a phosphatase (Stalmans, et al., 1971). This inhibition results in a higher percentage of glycogen phosphorylase in the more active a form, higher rates of glycogen degradation, and lower overall rates of glycogen accumulation. Cells with high initial glycogen content (cells from fed animals) synthesize glycogen at much lower rates than cells isolated from 24 hour fasted rats. Therefore, it was concluded from these experiments that chlorpropamide decreased glycogenesis and did not accelerate glycogenolysis. If chlorpropamide stimulated glycogenolysis it would have had equal or greater effectiveness in decreasing glycogen accumulation in cells isolated from fed animals.

It was possible, however, that some hormonal influences persisted throughout the isolation and incubations of the previously described experiments. The higher circulating insulin levels in fed rats could interfere with the observed effect of of chlorpropamide on glycogen accumulation, so an alternate strategy was employed to modify initial hepatocyte glycogen content. Hepatocytes were isolated from 8 hour fasted rats and cells isolated from each animal were divided into two aliquots allowing for subsequent modifications of initial glycogen content. One group of cells were isolated and pre-incubated as usual (at 37° in the absence of substrates) while the other group of cells were isolated at 2-4° in the presence of 8 mM glucose with no pre-incubation in order to prevent glycogenolysis. The cells isolated at 37°

had an initial glycogen content of 1.3 ± 0.09 mg/g wet weight, while the cells isolated at 2-4° had an initial glycogen content of 8.2 ± 0.2 mg/g wet weight. Figure 13 demonstrates that at 1.0 mM chlorpropamide, glycogen accumulation was 55% of control in the cells with low initial glycogen but was 80.5% of control in the cells with high initial glycogen. These experiments also demonstrated that the effect of chlorpropamide on glycogen accumulation is most likely due to inhibition of glycogen synthesis and not stimulated glycogenolysis.

Further evidence for inhibition of glycogen synthesis by chlorpropamide was obtained by quantitating hepatocyte glycogen accumulation as a function of time after addition of substrate. Compared to untreated cells, the extent of glycogen accumulation in cells treated with 2.0 mM chlorpropamide was significantly ($p<.05$) inhibited by 52.2%, 55.7%, and 56.3% at 20, 40, and 60 minutes, respectively (Figure 14). Incorporation of ^{14}C from [^{14}C]-amino acids into glycogen was also significantly ($p<.05$) inhibited by 54.3%, 54.4% and 62.1% at these times by treatment with chlorpropamide (Figure 15).

As in the glucose output experiments, it was necessary to demonstrate that other sulfonylureas as well as chlorproamide were active in inhibiting glycogen accumulation in isolated hepatocytes. Concentration-response studies indicated that chlorpropamide and tolbutamide were approximately equipotent in inhibiting glycogen accumulation in hepatocytes isolated from 24 hour fasted rats (Figure 16). On the other hand, glyburide, a second generation hypoglycemic sulfonylurea was more effective than either of the first generation sulfonylureas in inhibiting glycogen accumulation; this was expected based on their relative potencies *in vivo*. Whereas chlorpropamide and tolbutamide

Fig. 13. The relationship of chlorpropamide inhibition of glycogen accumulation to initial glycogen content as altered by post-isolation incubation conditions. Hepatocytes were isolated from an 8 hr fasted rat, washed and pre-incubated at 37° in the absence of substrates for 20 min (●) or washed 2-4° in the presence of 8.0 mM glucose and not pre-incubated (▲). Both sets of cells were then incubated for 60 min with 10.0 mM glucose (final concentration) and gluconeogenic precursors. T_{60}/T_0 is the ratio of hepatocyte glycogen content after 60 min of incubation to the initial glycogen content. T_{60}/T_0 greater than 1.0 indicates net glycogen accumulation. The values shown are the mean $T_{60}/T_0 \pm$ S.E.M. as determined in 4 separate experiments. *, $P<0.05$, **, $<P$ 0.01, compared to T_{60}/T_0 in the absence of chlorpropamide.

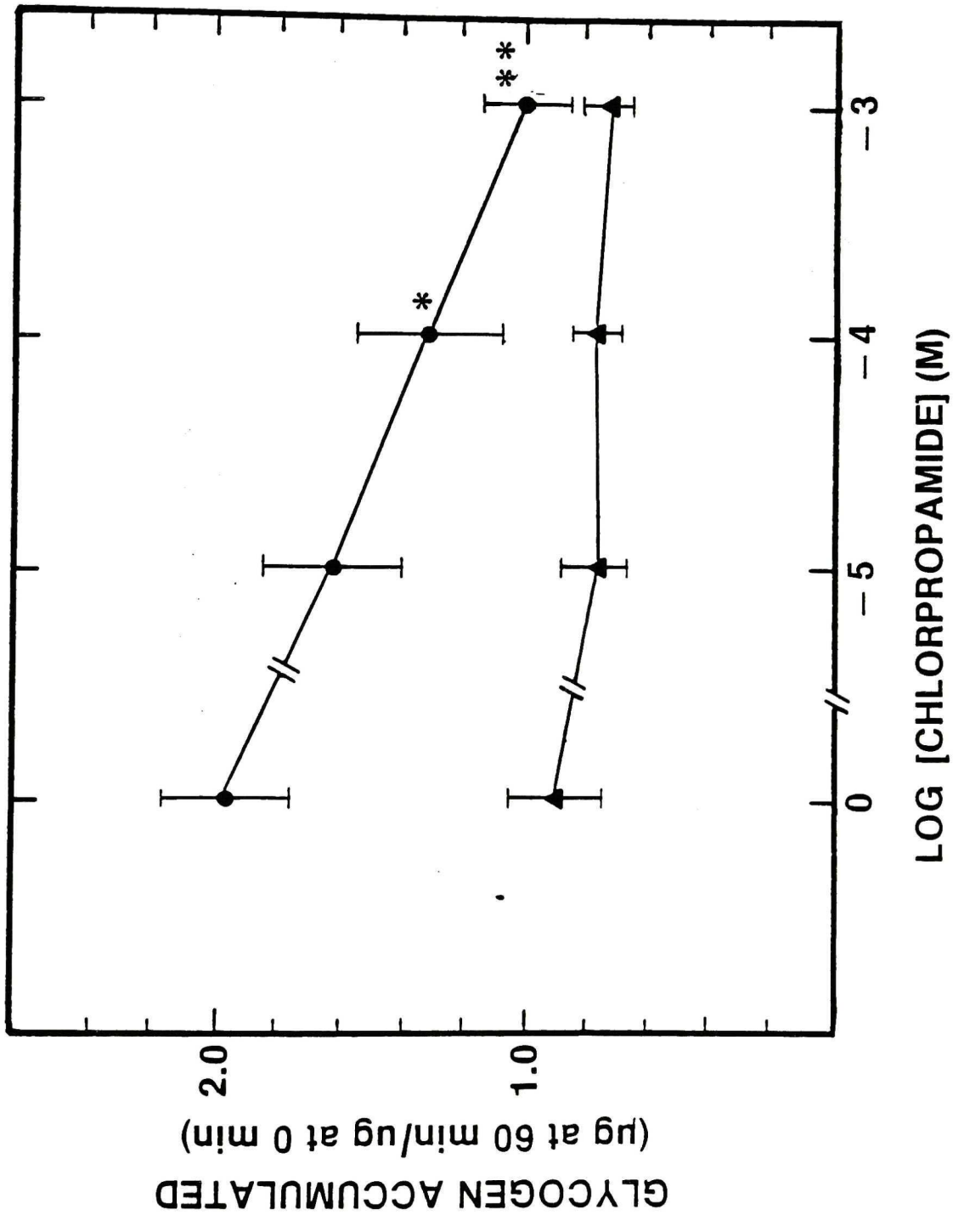

GLYCOGEN ACCUMULATED
(μg at 60 min/μg at 0 min)
2.0
1.0
0
−5
−4
−3
LOG [CHLORPROPAMIDE] (M)
*
**

Fig. 14. Time-dependent inhibition of glycogen accumulation by chlorpropamide in isolated rat hepatocytes. Hepatocytes were isolated and incubated as in Fig. 8. Hepatocytes were incubated in the presence (■), or absence (●) of 2.0 mM chlorpropamide. Values shown are the mean glycogen accumulated ± S.E.M. as determined in 3 experiments. *, $P<0.05$, **, $P<0.01$ compared to glycogen accumulated in the absence of chlorpropamide in suspensions incubated for the same length of time.

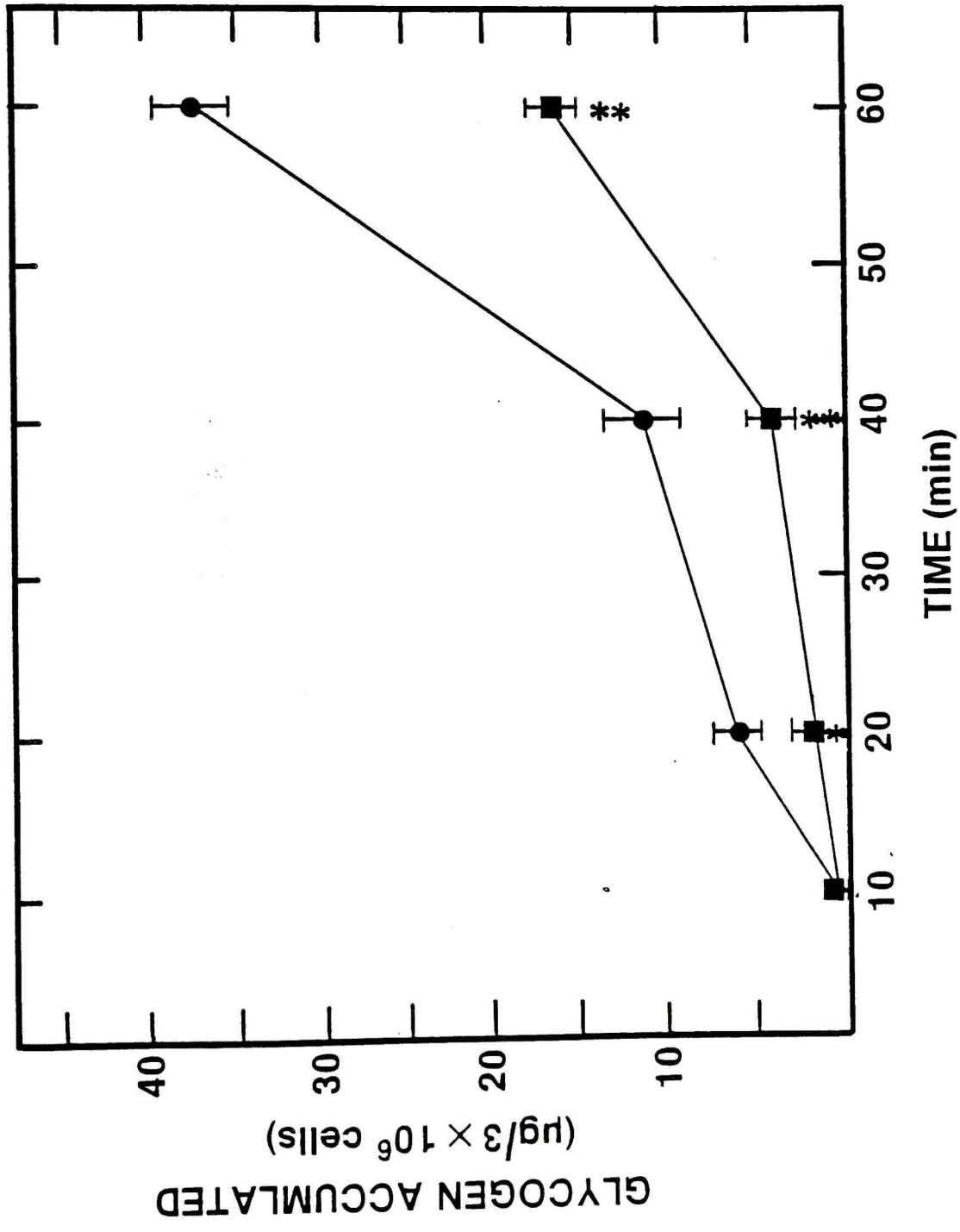

GLYCOGEN ACCUMLATED
(μg/3 × 10⁶ cells)
10
20
30
40
TIME (min)
10
20
30
40
50
60
**
***
*

Fig. 15. Time dependent inhibition by chlorpropamide of ^{14}C incorporation from [^{14}C]-amino acids into glycogen. Hepatocytes were isolated and incubated as in Fig. 14, except that hepatocytes were incubated in the presence of 0.1 μCi/ml [^{14}C]-amino acid mixture. Values shown are the mean ^{14}C incorporation into glycogen ± S.E.M. as determined in 3 experiments. *, $P<0.05$; **, $P<0.01$ compared to ^{14}C incorporated into glycogen in the absence of chlorpropamide for the same length of time.

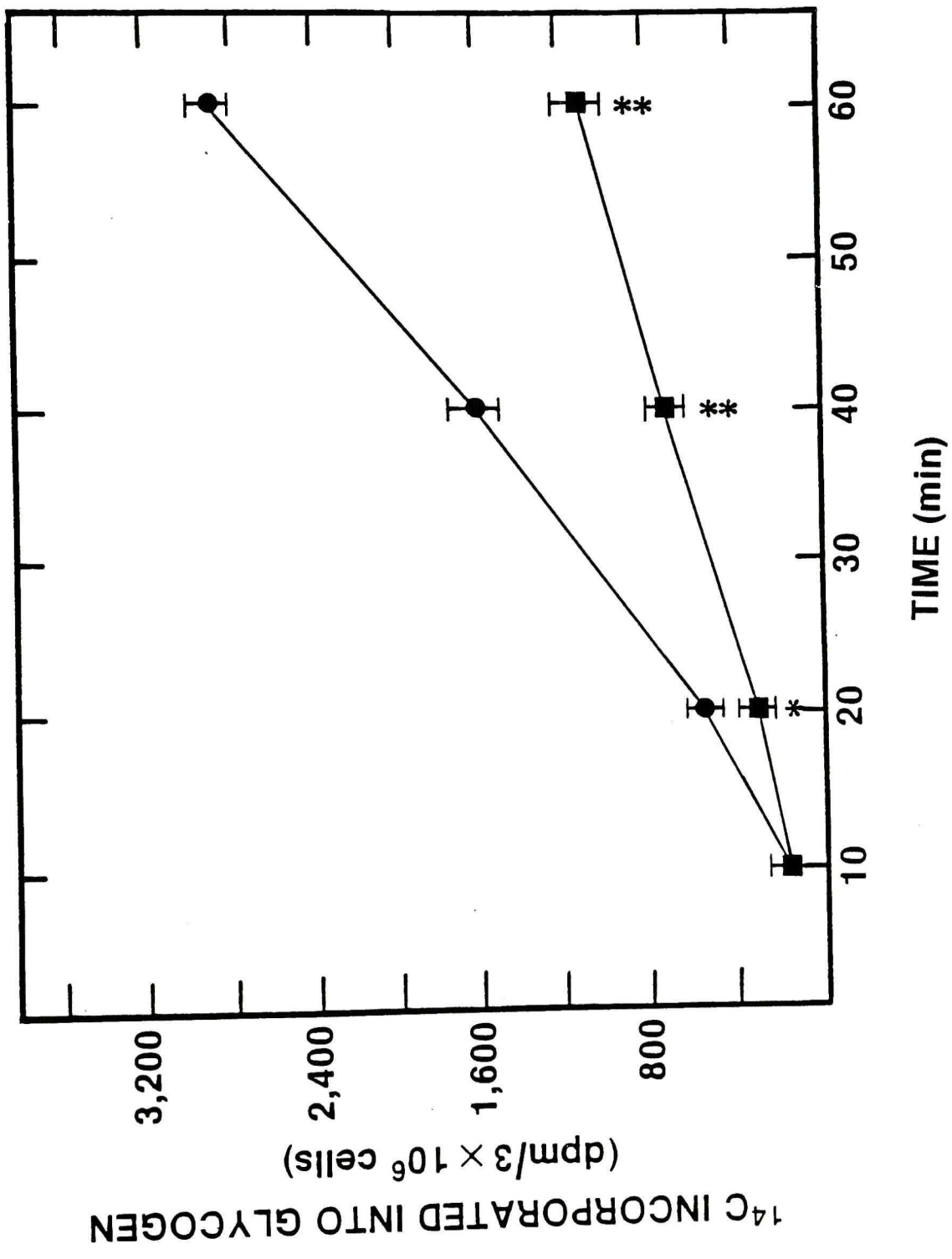
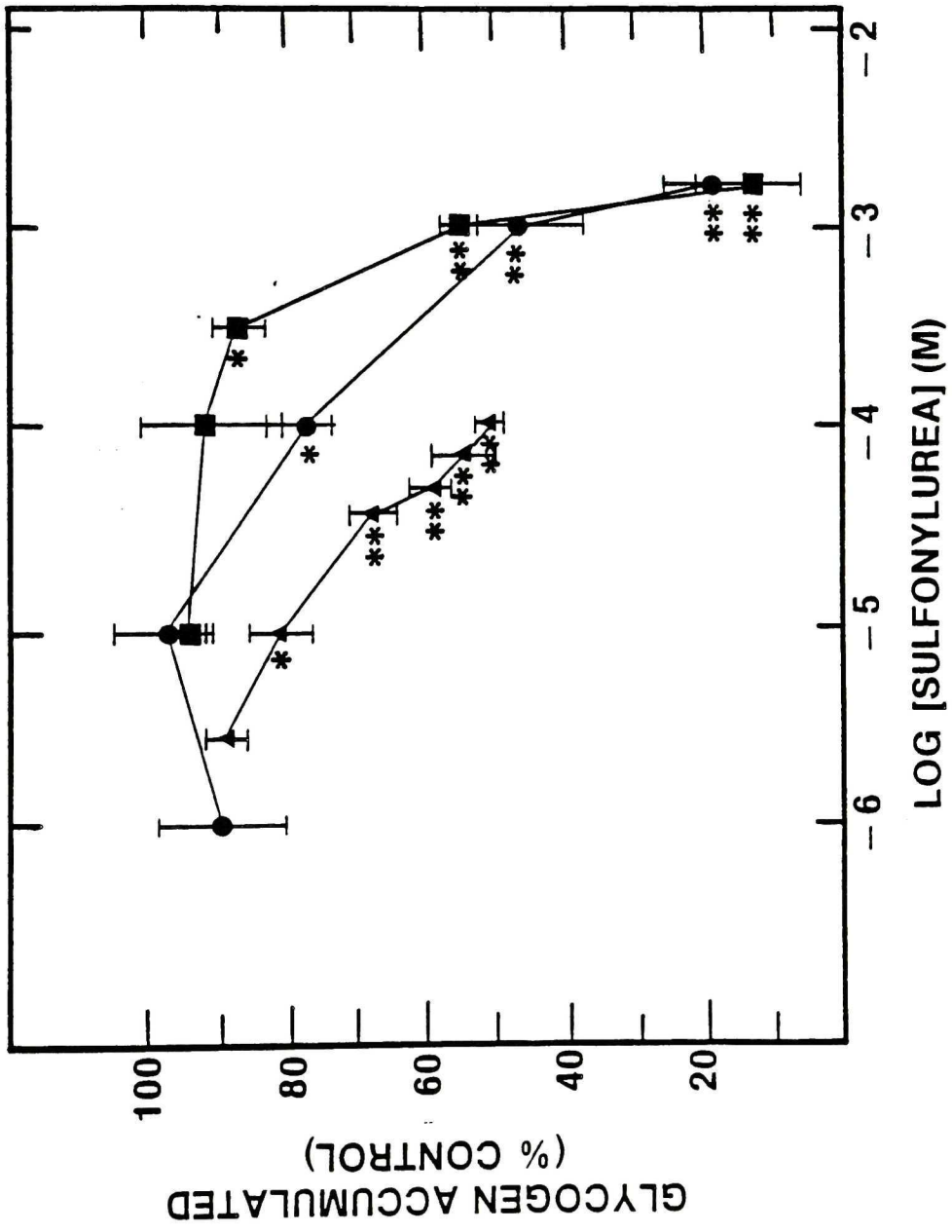

14C INCORPORATED INTO GLYCOGEN
(dpm/3 × 10^6 cells)
3,200
2,400
1,600
800
10
20
30
40
50
60
TIME (min)
*
**
**

Fig. 16. The effects of chlorpropamide, tolbutamide, and glyburide on glycogen accumulation. Hepatocytes were isolated and incubated as in Fig. 10 except that in addition to chlorpropamide (●), the effects of tolbutamide (■) or glyburide (▲) on glycogen accumulation were also examined. Values shown are the mean per cent of control glycogen accumulation ± S.E.M. as determined in three separate experiments (mean control glycogen accumulation = 97.5 ± 3.5 μg/3 x 10^6 cells).
*, $P<0.05$; **, $P<0.01$ compared to glycogen accumulation in the absence of sulfonylureas.


GLYCOGEN ACCUMULATED
(% CONTROL)
100
80
60
40
20
-6
-5
-4
-3
-2
LOG [SULFONYLUREA] (M)

inhibited glycogen accumulation by approximately 50% at 1.0 mM, glyburide at 0.1 mM was equally inhibitory. At 0.1 mM, a concentration normally found in the serum of patients treated chronically with chlorpropamide (Izzo, 1959; Sheldon, et al., 1965; Melander, et al., 1978) or tolbutamide (Baird and Duncan, 1957; Izzo, 1959; Sheldon, et al., 1965) the drugs inhibited glycogen accumulation by 22.6% and 7.5%, respectively. Glyburide significantly decreased glycogen accumulation at 0.01 mM and above, which is a concentration 10-50 fold higher than that found clinically (0.2-0.9 μM) (Sartor, et al., 1980; Sartor, et al., 1982; Matsuda, et al., 1983).

To further assess the specificity of the action of the sulfonylureas on glycogen accumulation, a series of para-substituted sulfonamides was tested. The effects of the two highest concentration of each test drug on glycogen accumulation are reported in Table 7. Only the hypoglycemic sulfonylureas tested (chlorpropamide, tolbutamide and glyburide) inhibited glycogen accumulation within or near their steady-state therapeutic concentrations. Neither the poorly hypoglycemic tolbutamide metabolite, carboxytolbutamide, nor the non-hypoglycemic sulfonylurea, HOE 17,710, inhibited glycogen accumulation. Of the clinically useful para-substituted sulfonamides tested, only the diuretics hydrochlorothiazide (at 1.0 and 2.0 mM) and furosamide (at 1.0 mM) were effective at decreasing hepatocyte glycogen accumulation. The inhibitory concentrations of the diuretics that decreased glycogen accumulation were approximately 100 and 500 times the therapuetic concentrations for furosamide and hydrochlorothiazide, respectively (Andreasen and Mikkelsen, 1977).

These experiments demonstate that the relative potencies of the

TABLE 7

SPECIFICITY OF INHIBITION OF GLYCOGEN ACCUMULATION CAUSED BY PARA-SUBSTITUED SULFONAMIDE COMPOUNDS

Drug	Concentration (mM)	Glycogen Accumulation (% control)
Chlorpropamide	0.1	78.2 ± 3.9*
	1.0	47.2 ± 7.9**
Tolbutamide	1.0	58.8 ± 3.4**
	2.0	7.0 ± 0.5**
Glyburide	0.01	81.0 ± 3.2*
	0.03	68.0 ± 2.4**
Carboxytolbutamide	0.1	106.1 ± 2.5
	1.0	101.4 ± 5.0
HOE 17,710	1.0	100.0 ± 3.5
	2.0	103.3 ± 5.6
Hydrochlorothiazide	1.0	85.1 ± 5.7
	2.0	77.0 ± 10.2*
Furosamide	0.1	104.2 ± 1.8
	1.0	77.5 ± 3.2
Diazoxide	0.1	107.2 ± 2.6
	1.0	99.0 ± 3.8
Sulfadiazine	0.7	92.7 ± 1.7
	1.0	93.1 ± 3.6

Hepatocytes were isolated from 24 hr fasted rats and pre-incubated for 20 min in the absence of substrates. The cells were then incubated with glucose and gluconeogenic precursors in the presence of the indicated drugs for 60 min. Glycogen accumulation was measured as in Fig. 10. Values shown are the mean per cent of control glycogen accumulation in the absence of added drugs ± S.E.M. as measured in three or more experiments. *, $P<0.05$; **, $P<0.01$ compared to control glycogen accumulation.

sulfonylureas in vivo are reflected in this system and that the effect of the sulfonylureas on glycogen accumulation could not be extended to include other para-substituted sulfonamides.

In the present study hepatocytes isolated from 24 hour fasted rats would not accumulate glycogen when incubated with 30 mM glucose alone, nor would they incorporate [^{14}C]-glucose into glycogen in the absence of gluconeogenic precursors (data not shown). Hepatocytes isolated from 8 hour fasted animals did accumulate glycogen when incubated in the presence of either a high concentration of glucose only (30 mM) or a lower concentration of glucose (10 mM) along with gluconeogenic precursors. To gain further information on the locus (loci) of action of the sulfonylureas on hepatic carbohydrate metabolism a series of experiments were carried out in which hepatocytes isolated from an 8 hour fasted rat were incubated with either 30 mM glucose alone, or 10 mM glucose, 5 mM lactate, 5 mM glutamine, and AAM (Table 8). Cells incubated in the presence of 30 mM glucose accumulated 61 µg glycogen/3 x 10^6 cells/60 minutes, whereas glycogen was accumulated at 103 µg/3 x 10^6 cells/60 minutes when gluconeogenic precursors were added with 10 mM glucose. Chlorpropamide at concentrations of 0.1 mM and 1.0 mM decreased glycogen accumulation by 33.2% and 49.5%, respectively, in cells supplied both glucose and gluconeogenic precursors. Although there was a similar trend in hepatocytes incubated with 30 mM glucose only, chlorpropamide was less effective in decreasing glycogen accumulation under these substrate conditions; only at 1.0 mM was there a significant decrease in glycogen accumulation in these cells (28.4%).

To this point it was demonstrated that: 1) chorpropamide de-

TABLE 8

THE EFFECT OF CHLORPROPAMIDE ON GLYCOGEN ACCUMULATION IN HEPATOCYTES INCUBATED WITH GLUCOSE ONLY OR GLUCOSE AND GLUCONEOGENIC PRECURSORS

Chlorpropamide Concentration (M)	Glycogen Accumulated in Hepatocytes Incubated with Glucose and Gluconeogenic Precursors (T_{60}/T_0)	Glycogen Accumulated in Hepatocytes Incubated with Glucose Only (T_{60}/T_0)
0	1.96 ± 0.20	1.48 ± 0.12
1×10^{-5}	1.62 ± 0.21	1.42 ± 0.13
1×10^{-4}	1.31 ± 0.24*	1.20 ± 0.22
1×10^{-3}	0.99 ± 0.14**	1.06 ± 0.12*

Hepatocytes were isolated from 8 hr fasted rats and pre-incubated in the absence of substrates for 20 min. The cells were then incubated for 60 min with glucose, 30 mM (glucose only) or in the presence of glucose , 10 mM; lactate 5 mM; glutamine, 5 mM; and AAM (glucose and gluconeogenic precursors). Glycogen accumulation (T_{60}/T_0) was calculated as in Fig. 13. The values listed are the mean T_{60}/T_0 ± S.E.M. as determined in 3 separate experiments. *, $P<0.05$; **, $P<0.01$ compared to T_{60}/T_0 in the absence of chlorpropamide for each substrate condition.

creased both glycogen accumulation and the conversion of gluconeogenic precursors to glucose; 2) the inhibition of glycogen accumulation was most likely due to decreased glycogenesis and not accelerated glycogenolysis; 3) the relative potencies and specificity relationships found in vivo were reproduced in this system; and 4) the common pathway affected in both glucose output and glycogenesis was most likely gluconeogenesis.

Effect of Chlorpropamide on Glycogen Metabolizing Enzymes

Although the effect of the sulfonylureas on glycogen accumulation and glucose output seemed to be caused by inhibition of gluconeogenesis, the results of the study of Remesar (1978) suggested that these drugs might also directly affect the glycogen metabolizing enzymes. To test this possibility, the activities of glycogen synthase and glycogen phosphorylase were determined in hepatocytes which were isolated from 24 hour fasted rats, pre-incubated for 20 minutes in the absence of substrates, and incubated with glucose and gluconeogenic precursors for 60 minutes in the presence of various concentrations of chlorpropamide. The results of these experiments are presented in Table 9. Chlorpropamide had no effect on the the total assayable activity of either of these enzymes tested in this system, nor did this drug alter the percentage of these enzymes in the active form. The incubation time in these experiments was 60 minutes. It has been demonstrated by other investigators, however, that agents that alter the percentages of hepatic glycogen metabolizing enzymes such as glucagon, epinephrine, phenylephrine, etc. act in as little a 2-3 minutes (Hutson, et al., 1976; Studer and Borle, 1982). To assess the responsiveness of the hepatocytes isolated and incubated under the present conditions to

TABLE 9

EFFECT OF CHLORPROPAMIDE ON HEPATOCYTE GLYCOGEN SYNTHASE AND GLYCOGEN PHOSPHORYLASE ACTIVITIES

Chlorpropamide Concentration (mM)	Synthase a Activity (U/mg DNA)	Synthase a+b Activity (U/mg DNA)	Synthase a/a+b
0.0	0.66 ± 0.03	1.21 ± 0.04	0.55 ± 0.03
1.0	0.68 ± 0.03	1.14 ± 0.04	0.60 ± 0.03
1.5	0.67 ± 0.01	1.21 ± 0.03	0.56 ± 0.03
2.0	0.65 ± 0.03	1.16 ± 0.06	0.57 ± 0.03
Chlorpropamide Concentration (mM)	**Phosphorylase a Activity (U/mg DNA)**	**Phosphorylase a+b Activity (U/mg DNA)**	**Phosphorylase a/a+b**
0.0	7.9 ± 0.9	18.7 ± 0.6	0.41 ± 0.04
1.0	7.8 ± 0.9	20.1 ± 0.3	0.39 ± 0.05
1.5	7.8 ± 0.3	19.9 ± 0.5	0.40 ± 0.01
2.0	7.5 ± 0.5	17.7 ± 0.3	0.43 ± 0.03

Hepatocytes were isolated from 24 hr fasted rats and pre-incubated for 20 min in the absence of substrates. The cells were then incubated with glucose and gluconeogenic precursors in the presence of 0, 1.0, 1.5, or 2.0 mM chlorpropamide. After 60 min, samples were removed for determination of glycogen metabolizing enzymes activities as described in Methods. Values shown are the mean enzyme activity ± S.E.M. as determined in 4 experiments. No significant differences in any of the chlorpropamide-treated incubations were observed. 1 unit=1 μmole of either [glucose-^{14}C(U)]-UDPG or α-D-[^{14}C(U)]-glucose 1-phosphate converted to [^{14}C]-glycogen.

known modifiers of synthase and phosphorylase activities, and to test the effect of chlorpropamide over a shorter time interval, hepatocytes were preincubated for 20 min in the absence of substrates and incubated for 2 minutes with glucose and gluconeogenic precursors and either 2.0 mM chlorpropamide or 1 X 10^{-5} M phenylepherine. Phenylepherine treatment of hepatocytes resulted in a increase of 12% of phosphorylase in the active form and a decrease of 16% of synthase in the active form, results which agree with Hutson, et al. (1976) and Studer and Borle (1982). On the other hand, chlorpropamide was ineffective in altering total phosphorylase or synthase activities, or altering the percentage of these enzymes in their more active forms. Thus, these results demonstrate that the effects of the sulfonylureas on glycogen accumulation cannot be explained by covalent modifications of these enzymes, which could alter their activities. Rather, when considered with results presented on the effect of the sulfonylyureas on glucose output and glycogen accumulation experiments these results further point to a decrease in substrate supply for glycogen synthesis and infer an inhibition of gluconeogenesis in hepatocytes by the sulfonylureas.

Effects of Chlorpropamide on the Gluconeogenic Pathway

By measuring the changes in the levels of hepatic gluconeogenic intermediates the step in gluconeogenesis that is affected by a test substance could be identified. Thus, if a catalytic conversion is inhibited, then it would be expected that the concentration of the substrate for the enzyme in question would increase in treated vs untreated tissue, while the concentration of the product of the enzymatic reaction would decrease.

To directly examine the role of the sulfonylureas in hepatic gluconeogenesis, hepatocytes were isolated from 24 hour fasted rats, pre-incubated for 20 min in the absence of substrates, incubated for 60 min with glucose and gluconeogenic precursors in the presence or absence of 2.0 mM gluconeogenesis. At the end of the incubation period, samples of hepatocyte suspensions were removed and the concentrations of gluconeogenic intermediates measured. Figure 17 demonstrates that in cells treated with 2.0 mM chlorpropamide the concentration of malate was increased by 42.1 $\pm$ 10.0 % while the concentrations of phospho(enol)-pyruvate, 2-phosphoglycerate, and 3-phosphoglycerate were decreased by 36.6 $\pm$ 12.0 % , 58.1 $\pm$ 14.4 %, and 23.4 $\pm$ 7.2 %, respectively. Also, treatment of hepatocytes with 2.0 mM chlorpropamide resulted in a statistically significant decrease in ATP concentration (3.21 μmoles/g in untreated cells and 2.81 μmoles/g in treated cells). The concentrations of the other intermediates measured in this study were not significantly altered by chlorpropamide treatment. Although the decrease in the concentration of 2-phosphoglycerate is greater than that of phospho(enol)pyruvate, the difference between these two intermediates is not statistically significant, and the results of these experiments seem to indicate that chlorpropamide is inhibiting the conversion of malate to phospho(enol)pyruvate. The precise mechanism of this inhibition cannot be determined from these results, but the more likely possibilities will be discussed in the Discussion.

To correlate the changes of the concentration of intermediates with drug levels found *in vivo*, concentration-response relationships were carried out. It can be seen from Figure 18 that there were concentration-dependent changes in the levels of malate, phospho(enol)pyruvate,

Fig. 17. The effect of chlorpropamide on the concentrations of gluconeogenic intermediates in isolated hepatocytes. Hepatocytes were isolated from 24 hour fasted rats and pre-incubated in the absence of substrates for 20 min. Cells were then incubated with glucose and gluconeogenic precursors in the presence (●) or absence of 2.0 mM chlorpropamide. After 60 min, samples were removed for measurement of gluconeogenic intermediates. The abbreviations for the intermediates and the mean control concentrations of each in nmoles/g wet weight cells are as follows: PYR=pyruvate (1860 ± 114), MAL=malate (849 ± 79), PEP= phospho(enol)pyruvate (251 ± 26), DHAP=dihydroxyacetone phosphate (6.4 ± 0.8), G3P=glyceraldehyde 3-phosphate (15.8 ± 1.7), F16P=fructose 1,6-diphosphate (33.1 ± 3.6), F6P=fructose 6-phosphate (60.8 ± 7.3), and G6P=glucose 6-phosphate (186.4 ± 20.8). Values shown are the mean per cent of control gluconeogenic intermediate concentration ± S.E.M. as determined in three or more experiments. *, $P<0.05$; **, $P<0.01$ compared to cells incubated in the absence of chlorpropamide.

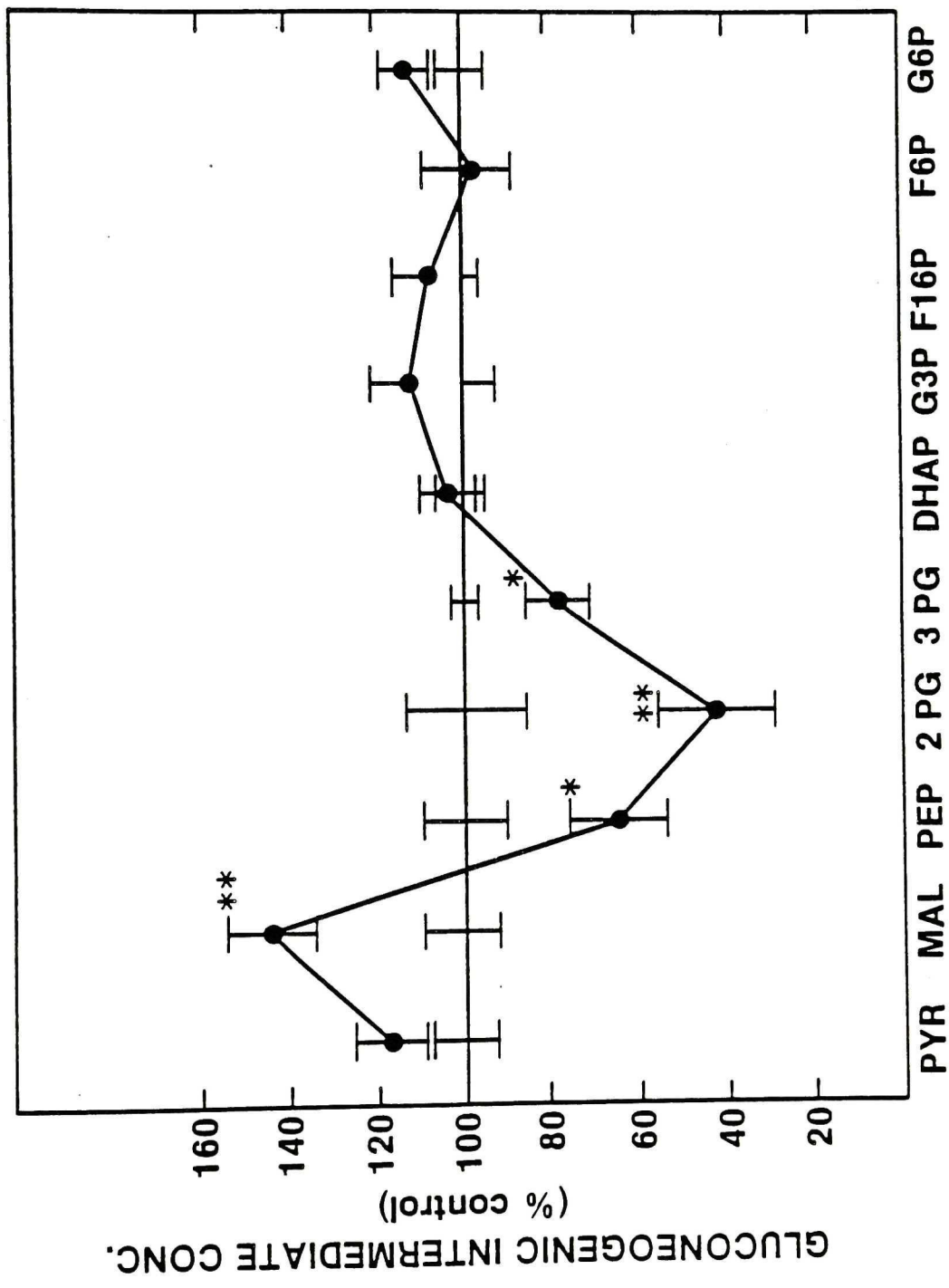
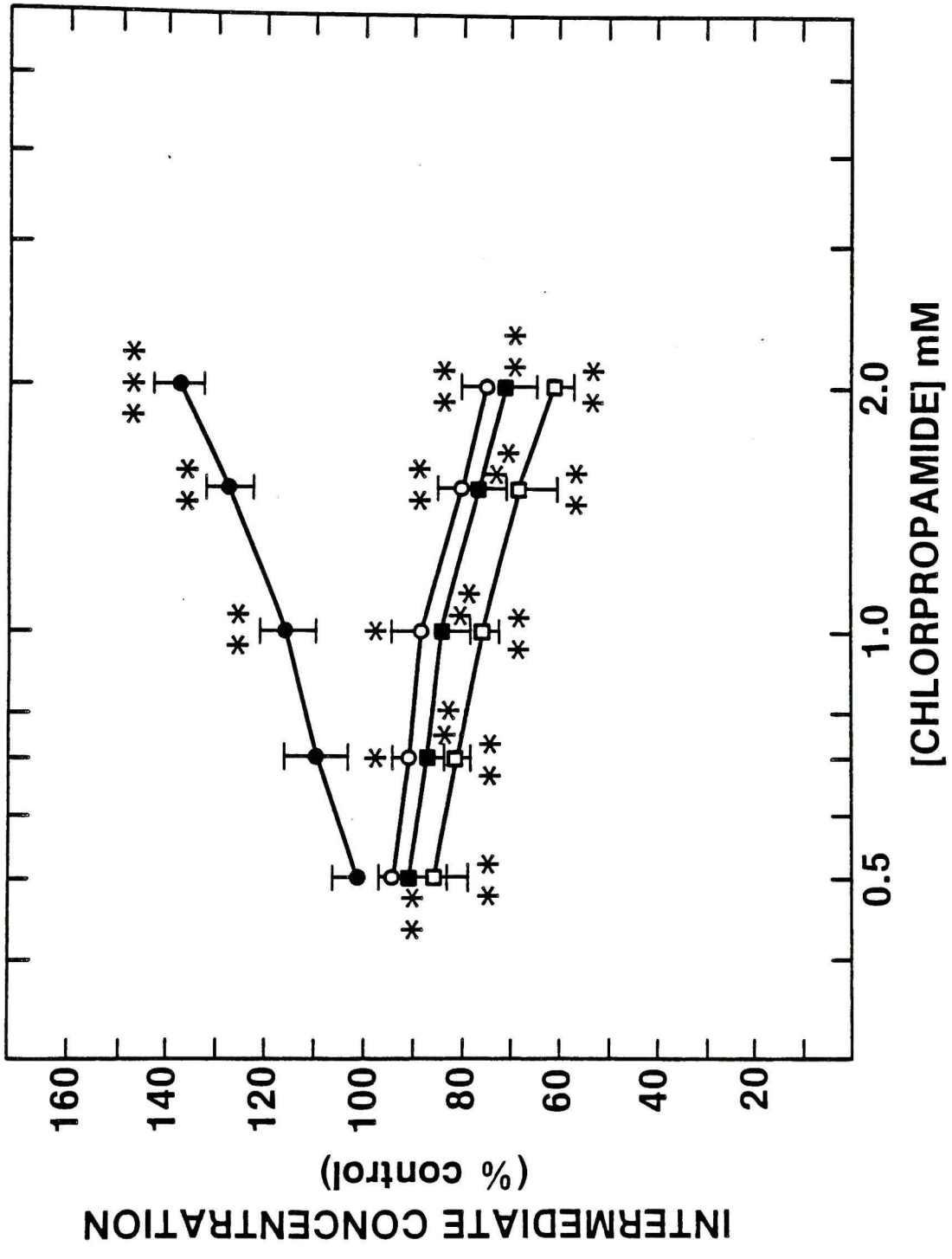

GLUCONEOGENIC INTERMEDIATE CONC.
(% control)
160
140
120
100
80
60
40
20
PYR MAL PEP 2 PG 3 PG DHAP G3P F16P F6P G6P
**
*
**
*
*

Fig. 18. Relationship of hepatocyte content of malate, phospho(enol)pyruvate, 2-phosphoglycerate, and 3-phosphoglycerate to chlorpropamide concentration. Hepatocytes were isolated and incubated as in Fig. 17. except that the effect of 0.5, 0.7, 1.0 and 1.5 mM chlorpropamide on MAL (●), PEP (■), 2-PG (□), and 3-PG (○) concentrations were assessed. Values shown are the mean per cent of control gluconeogenic intermediate concentration ± S.E.M. as determined in three experiments (mean control concentrations of intermediates were 862 ± 69, 28.3 ± 2.6, 85.3 ± 8.9, and 468.8 ± 59.1 nmoles/g wet weight cells for MAL, PEP, 2-PG, and 3-PG, respectively.) *, $P<0.05$; **, $P<0.01$ compared to control incubations.


INTERMEDIATE CONCENTRATION
(% control)
160
140
120
100
80
60
40
20
0.5
1.0
2.0
[CHLORPROPAMIDE] mM

2-phosphoglycerate, and 3-phosphoglycerate. The concentration of malate became significantly greater than contol at 1.0 mM chlorpropamide, while the level of 3-phosphoglycerate became significantly decreased at a similar concentration. The concentrations of phospho(enol)pyruvate and 2-phosphoglycerate decreased significantly below control levels at 0.7 mM, while the concentration of 3-phosphoglycerate became significantly less than control at 1.5 mM. The concentration of ATP in these hepatocytes became significantly depressed from control hepatocyte levels at 1.5 and 2.0 mM chlorpropamide (Figure 19). The changes in the concentrations of these intermediates and ATP at 2.0 mM chlorpropamide in the present experiments were not significantly different from those found in the previously described experiments (Figure 17).

To temporally relate the apparent inhibition of gluconeogenesis by chlorpropamide with its actions on glycogen accumulation and glucose output, the effect of chlorpropamide on the levels of malate, phospho-(enol)pyruvate, 2-phoshoglycerate, and 3-phosphoglycerate as a function of time was assessed. It can be seen from Figures 20 and 21 that the maximal changes in the concentrations of these gluconeogenic intermediates and ATP occurred within 10 min of addition of the drug, a shorter time than that needed for apparent blockade of glucose output or glycogen accumulation. Therefore, it is evident that the alterations in gluconeogenic intermediates, an indicator of the blockage of gluconeogenesis results in the decreased production of glucose and accumulation of glycogen caused by chlorpropamide observed in this study. The concentration needed to cause significant alterations in gluconeogenic intermediates is also consistant with the concentrations that caused decreases in the glycogen accumulation and glucose output.

Fig. 19. Relationship of content of ATP in hepatocytes to chlorpropamide concentration. Hepatocytes were isolated and incubated as in Fig. 17. After 60 min of incubation, samples were removed for the determination of hepatocyte ATP concentration. Values shown are the mean per cent of control ATP concentration $\pm$ S.E.M. as determined in three experiments (mean control ATP concentration was 3.21 $\pm$ 0.14 µmoles/g wet weight cells). *, $p<0.05$; **, $P<0.01$ compared to control incubations.

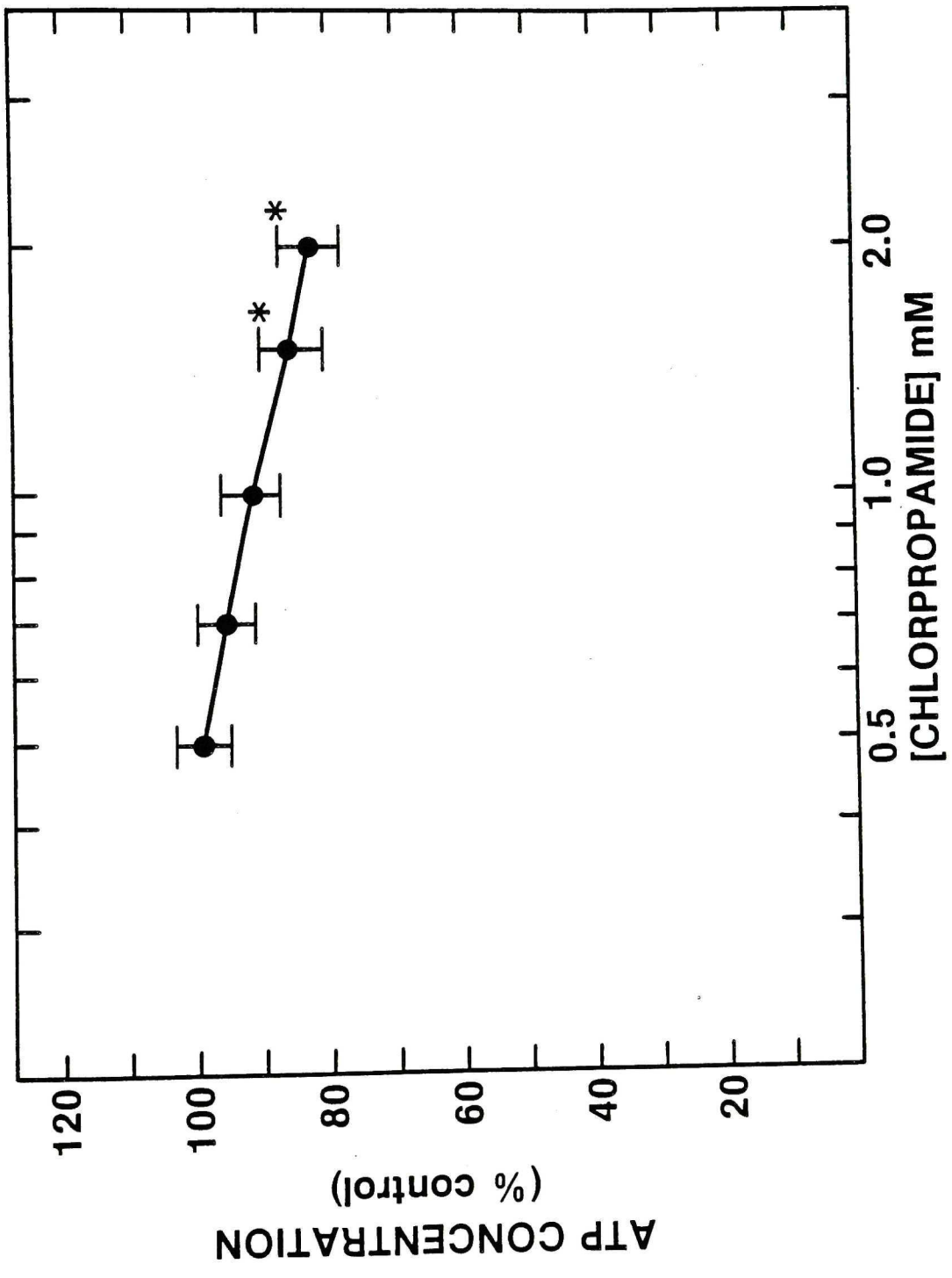

ATP CONCENTRATION
(% control)
120
100
80
60
40
20
0.5
1.0
2.0
[CHLORPROPAMIDE] mM
*
*

Fig. 20. Time-dependent changes in the concentrations of malate, phospho(enol)pyruvate, 2-phosphoglycerate and 3-phosphoglycerate with chlorpropamide. Hepatocytes were isolated from 24 hr fasted rats and pre-incubated for 20 min in the absence of substrates. The cells were then incubated for 10. 20, 40, or 60 min with glucose and gluconeogenic precursors in the presence or absence of 2.0 mM chlorpropamide. Samples were then removed for determination of malate (●), phospho(enol)pyruvate (■), 2-phosphoglycerate (□), or 3-phosphoglycerate (○) content. Each point represents the mean per cent of control gluconeogenic intermediate concentration ± S.E.M. as determined in three experiments (mean control concentrations of intermediates were 828.9 ± 73, 29.5 ± 4.1, 88.9 ± 5.7, and 490.0 ± 50.3 nmoles/g wet weight cells for MAL, PEP, 2-PG, and 3-PG, respectively. *, $P<0.05$; **, $P<0.01$ compared to control incubations at the same time point.

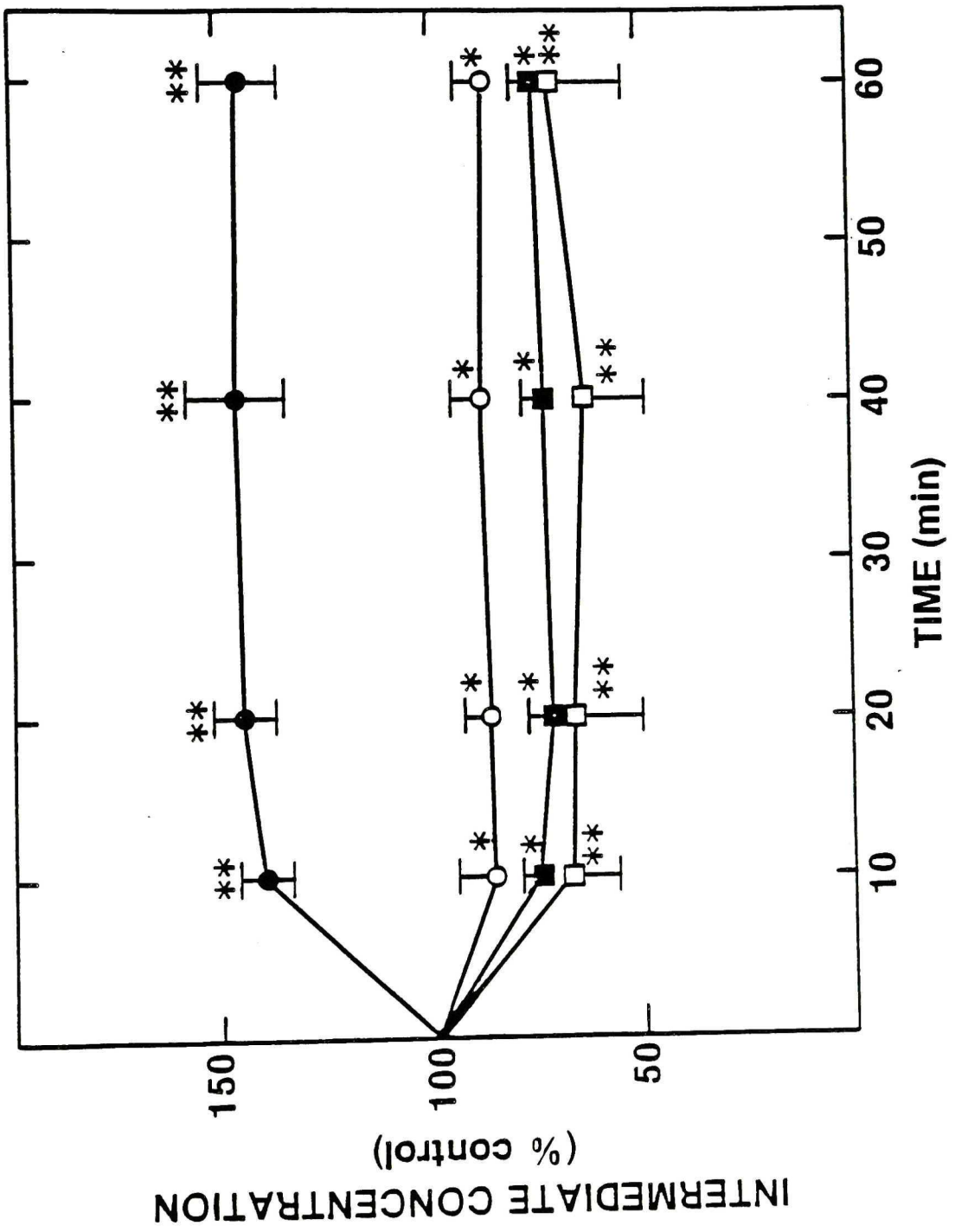
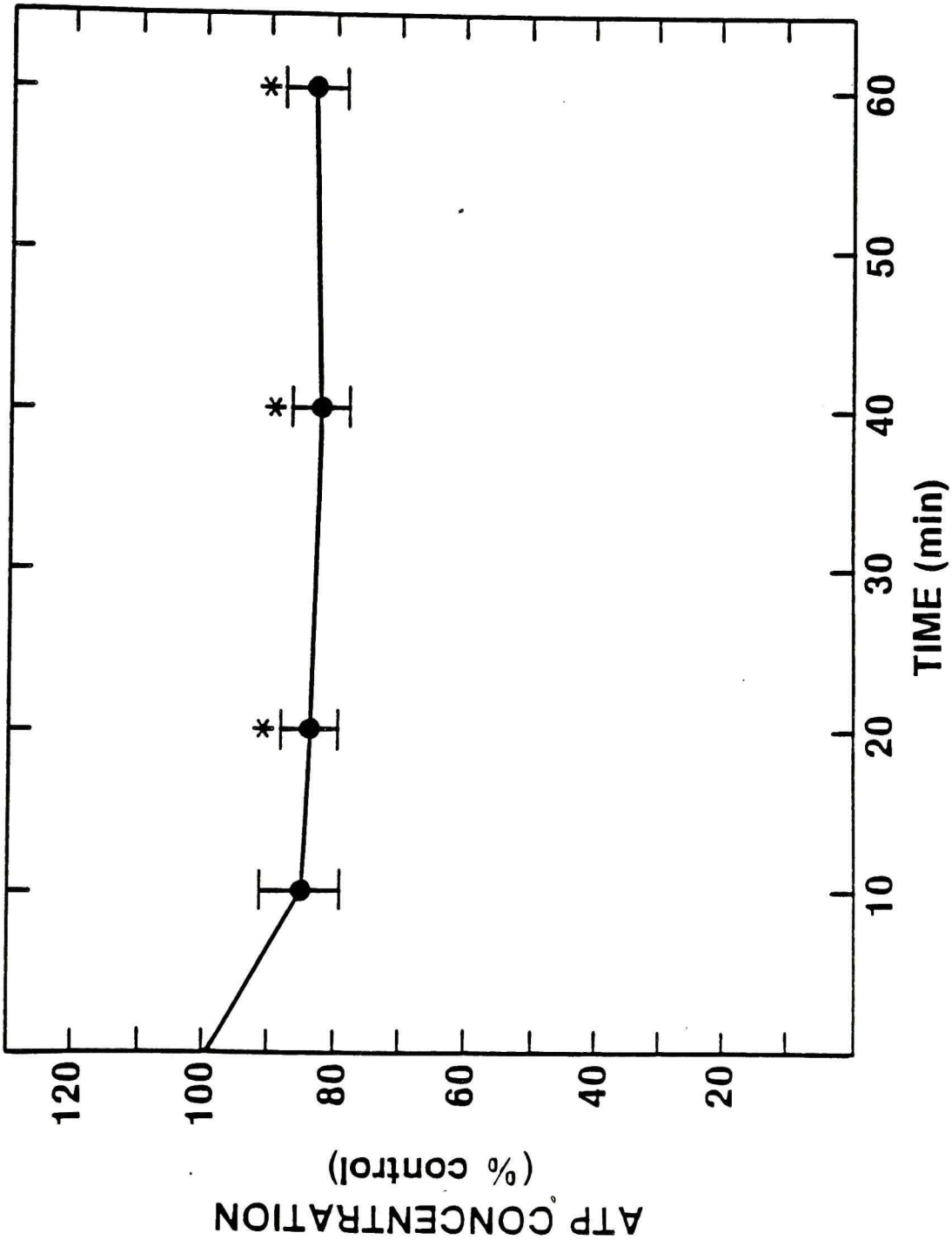

INTERMEDIATE CONCENTRATION
(% control)
150
100
50
10
20
30
40
50
60
TIME (min)

Fig. 21 Time-dependent changes in hepatocyte ATP content in the presence of chlorpropamide (2.0 mM). Hepatocytes were isolated and incubated as in Fig. 20 except that samples were removed for measurement of ATP at 10, 20, 40, or 60 min. Each point is the mean per cent of control ATP concentration $\pm$ S.E.M. as determined in three animals (mean control ATP concentration = 3.26 $\pm$ 0.17 µmoles/g wet weight cells. *, $P<0.05$; compared to control incubations at the same time point.


ATP CONCENTRATION
(% control)
120
100
80
60
40
20
10
20
30
40
50
60
TIME (min)
*
*
*

Correlation of the effect of chlorpropamide on glycogen accumulation and glucose output

If glycogen accumulation in hepatocytes isolated from 24 hour fasted rats is dependent upon gluconeogenesis as suggested by Katz, et al., (1976, 1979) and others, then cells from animals in this nutritional state incubated with gluconeogenic precursors should both accumulate glyocgen and release glucose. Furthermore, rates of these two processes should not be independent of each other since they rely on a common pathway (gluconeogenesis) for substrate input. If an agent inhibits gluconegenesis, it should decrease both glucose output and glycogen accumulation. Figures 22 and 23 are transformations of data obtained from studies of the effect of chlorpropamide on glycogen accumulation and glucose output previously presented (time courses). It is evident that in cells isolated from 24 hour fasted rats and incubated with gluconeogenic precursors that glycogen accumulation and glucose output are not independent variables. The mass of glucose and glycogen (r^2=0.88, P<.001) and incorporation of labelled substrates into glycogen and glucose (r^2= 0.98, P<.001) are positively correlated demonstrating that they both rely on a common substrate supply. Also it can be seen from these figures that chlorpropamide decreased both processes, further supporting the hypothesis that the sulfonylureas inhibit gluconeogenesis in isolated hepatocytes.

Dependencece of the inhibition of glycogen accumulation by chlorpropamide on the gluconeogenic precursors available

At 2.0 mM, chlorpropamide inhibited the incorporation of ^{14}C from both [^{14}C]-amino acids and [^{14}C]-lactate to the same extent. Glycogen accumulation as a function of time in hepatocytes isolated from 8 hour fasted rats and incubated with 10 mM fructose and 10 mM

Fig. 22. Correlation of glycogen accumulation with glucose output in isolated hepatocytes incubated in the presence or absence of chlorpropamide. This figure is a transformation of the data found in Figs. 8 and 14. Hepatocytes were isolated and incubated in the presence (○) or absence (●) of 2.0 mM chlorpropamide as described in those figures. Each point represents the mean glycogen accumulated plotted against the mean glucose produced at the same time point within each experiment. The correlation between glycogen accumulation and glucose output was statistically significant (r^2=0.88, P<.001).

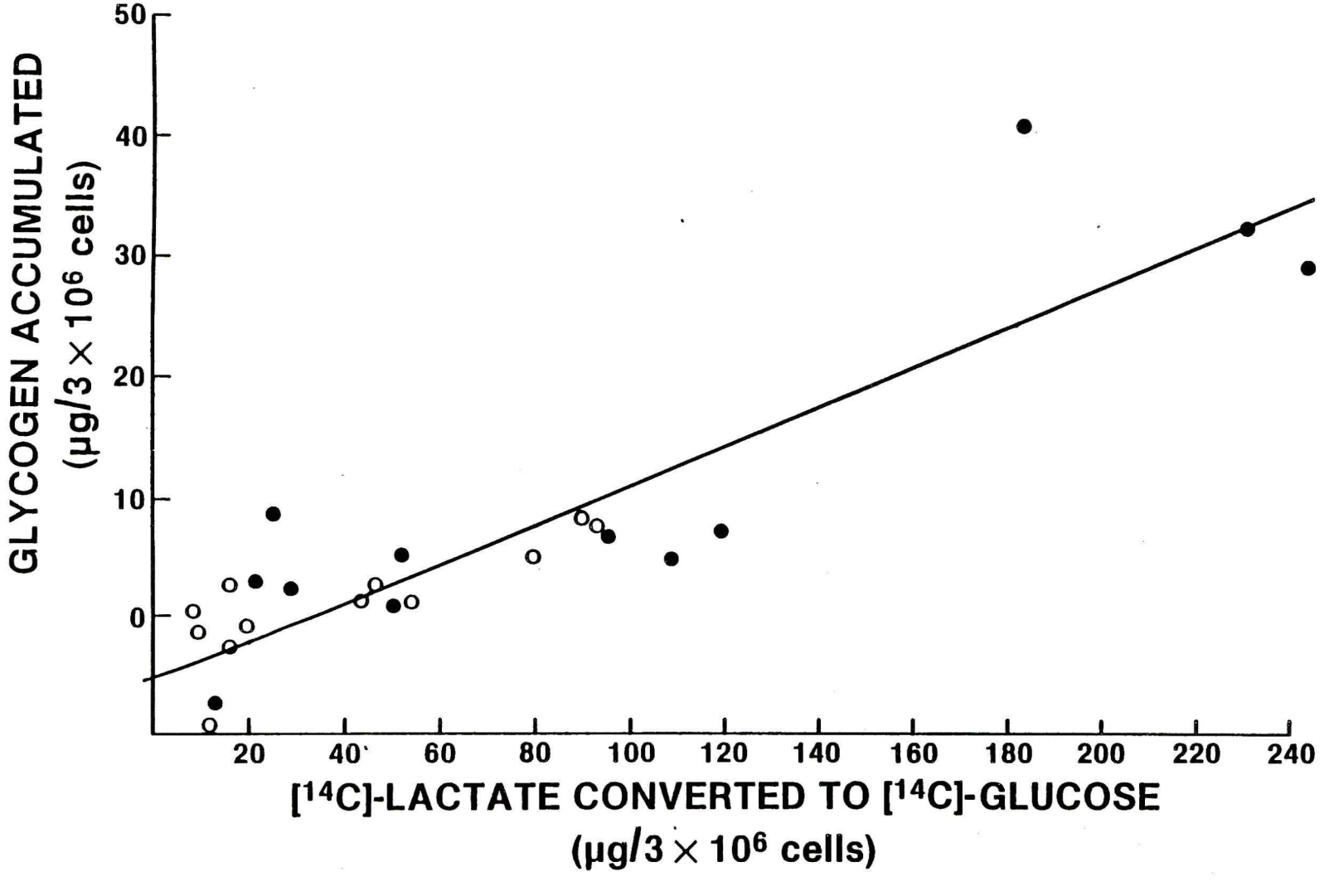

GLYCOGEN ACCUMULATED
(µg/3 × 10⁶ cells)
50
40
30
20
10
0
20
40
60
80
100
120
140
160
180
200
220
240
[¹⁴C]-LACTATE CONVERTED TO [¹⁴C]-GLUCOSE
(µg/3 × 10⁶ cells)

Fig. 23. Correlation of ^{14}C incorporated into glycogen from [^{14}C]-amino acids with [^{14}C]-lactate conversion to [^{14}C]-glucose in hepatocytes in the presence or absence of chlorpropamide. This figure represents a transformation of the data taken from Figs. 8 and 15. Hepatocytes were isolated and incubated in the presence (○) or absence (●) of 2.0 mM chlorpropamide as described in those figures. Each point represents the mean ^{14}C incorporated into glycogen from [^{14}C]-amino acids plotted against the mean [^{14}C]-lactate converted to [^{14}C]-glucose at the same time point within each experiment. The correlation between labelling of glycogen and glucose was statistically significant ($r^2=0.98, P<.001$).

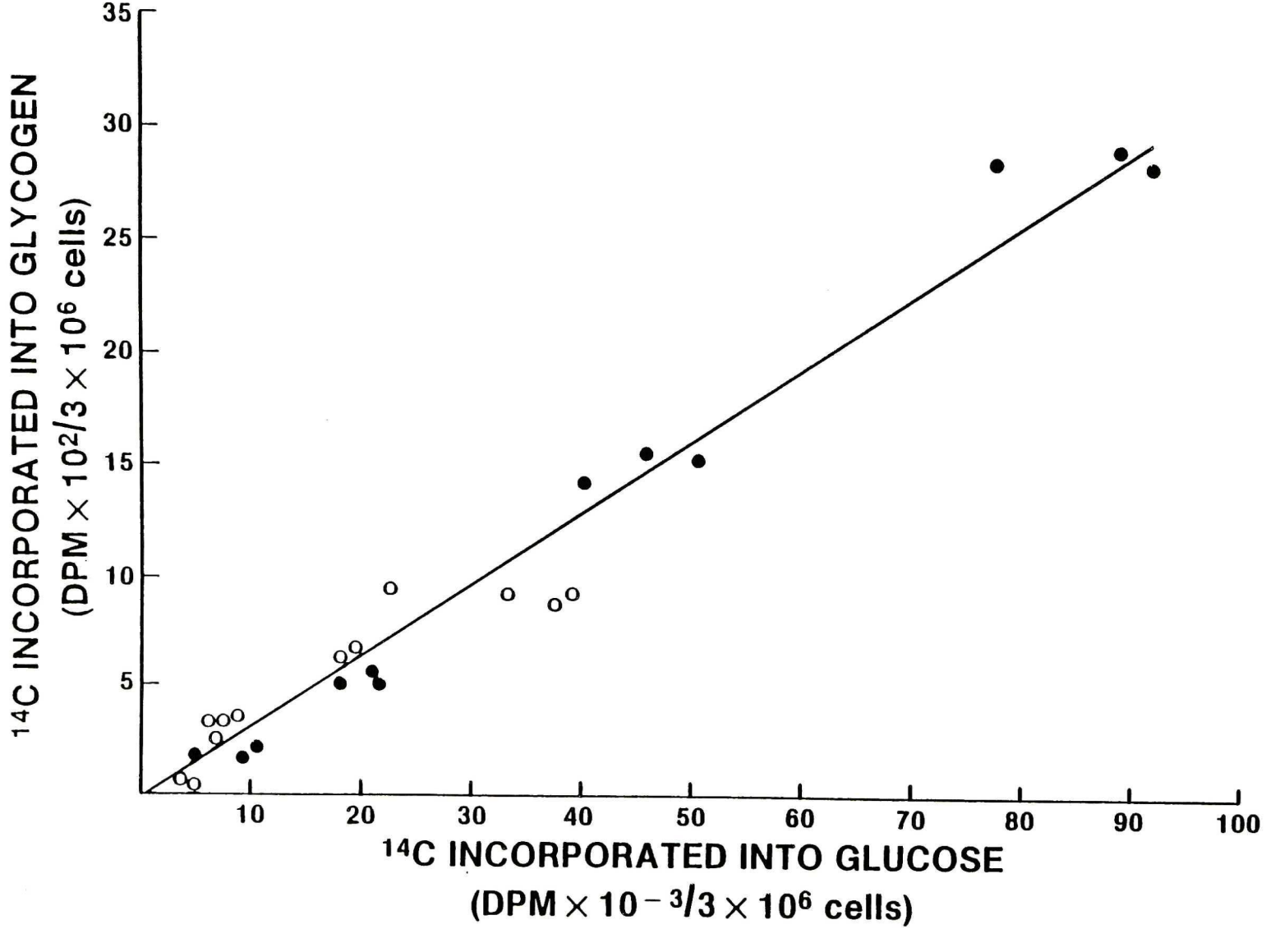
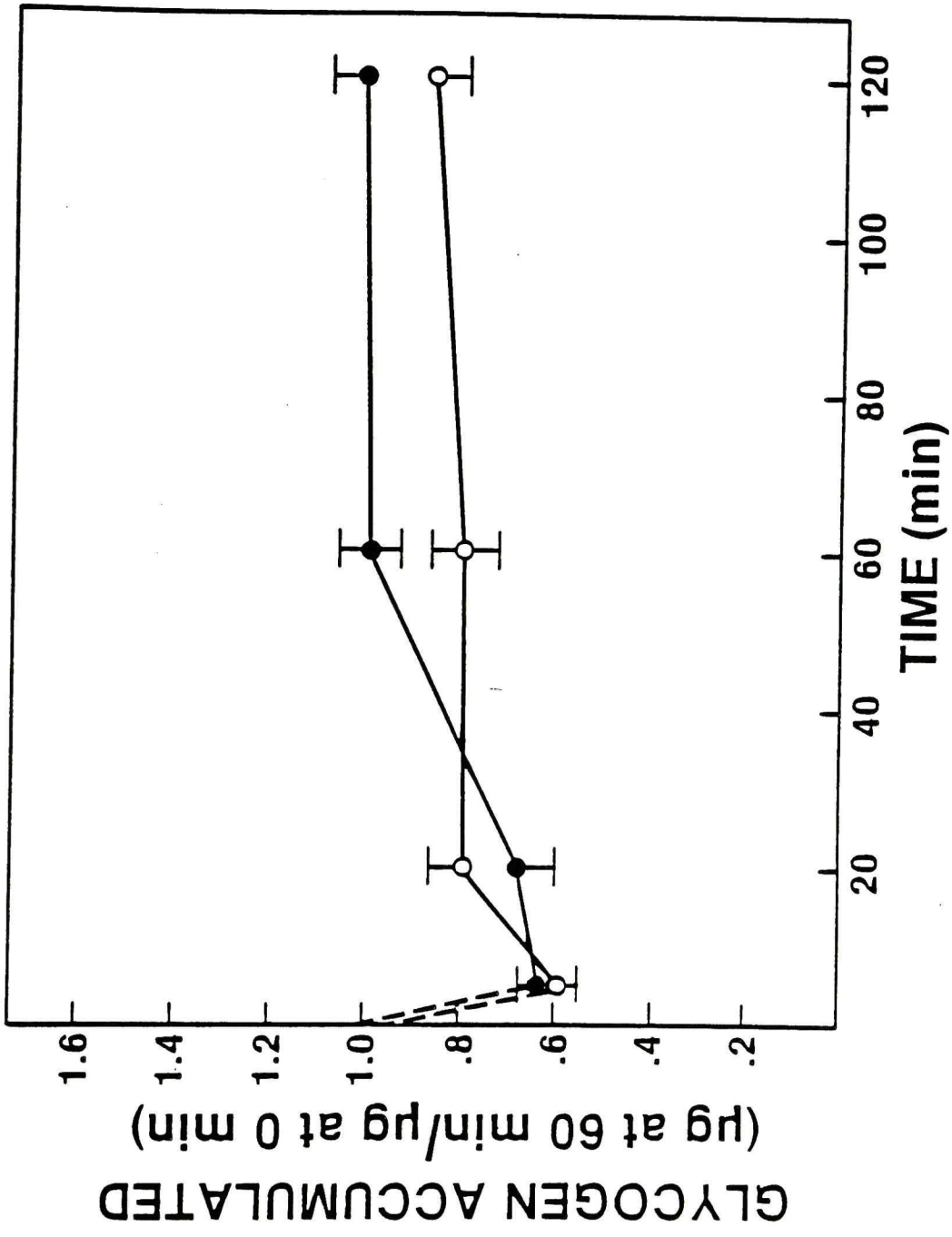

^{14}C INCORPORATED INTO GLYCOGEN
(DPM × 10^{2}/3 × 10^{6} cells)
35
30
25
20
15
10
5
10
20
30
40
50
60
70
80
90
100
^{14}C INCORPORATED INTO GLUCOSE
(DPM × 10^{-3}/3 × 10^{6} cells)

Fig. 24. Effect of chlorpropamide on glycogen accumulation in hepatocytes incubated with glucose and fructose as a function of time. Hepatocytes were isolated from 8 hour fasted rats and pre-incubated in the absence of substrates for 20 min. The cells were then incubated with glucose, 10 mM; and fructose, 10 mM for 5, 10, 20 or 60 min in the presence (○) or absence (●) of 2.0 mM chlorpropamide. Glycogen accumulation was calculated as in Fig. 13. The values shown are the mean $T_{60}/T_0 \pm$ S.E.M. as determined in three experiments. No statistically significant differences between chlorpropamide-treated and control incubations were found at any time point.


GLYCOGEN ACCUMULATED
(µg at 60 min/µg at 0 min)
1.6
1.4
1.2
1.0
.8
.6
.4
.2
20
40
60
80
100
120
TIME (min)

glucose was investigated. Figure 24 demonstrates that there was an initial drop in glycogen content followed by a net glycogen synthetic phase; neither of these processes was affected by chlorpropamide. Fructose enters gluconeogenesis at the level of the triose phosphates. Since there was no effect of chlorpropamide on glycogen accumulation supported from this sugar, further evidence is put forth for the inhibition of glucose output and glycogen accumulation by the sulfonylureas being a result of an inhibition of the conversion of malate to phospho-(enol)pyruvate.

DISCUSSION

One of the more attractive hypotheses for the mechanism of the persistant *in vivo* hypoglycemic effect of the sulfonylureas is that they either diminish hepatic glucose production or decrease the amount of glucose deposited as glycogen in the liver. While previous reports suggested that the sulfonylureas might decrease hepatic glucose output, none of these studies have shown this conclusively because the model system used (liver slices, perfused livers, and acute *in vivo* administration) may have been inappropriate for the study of the direct actions of the drugs on hepatic carbohydrate metabolism. The results reported here demonstrate that the sulfonylureas directly inhibit the production of glucose by the liver under conditions where abnormal cellular metabolism was avoided. The site of this inhibition has been identified, as well.

Choice of the Model System

Several reasons may be suggested for the use of hepatocytes in the study of hepatic carbohydrate metabolism. Primary suspension cultures of hepatocytes retain the characteristics of subcellular localization of substates, enzymes, etc., and maintain energy and substrate gradients. Homogenates are useful for the study of isolated reactions, but the complex regulation of pathways may be lost when compartmetalization is destroyed upon homogenization. Hepatocytes allow for easy manipulation of substrates and test substances, and several experimental conditions can be compared simultaneously in cells isolated from a single animal. Liver slices also have this advantage, however, in

perfused livers one can never be certain that the effect of a treatment will not carry over to subsequent experimental conditions.

Evidence for the superiority of the isolated hepatocyte system in the study of hepatic carbohydrate metabolism over the perfused liver and liver slices is derived from the work of Krebs, et al. (1974). These authors found that hepatocytes in suspension were more active in O_2 consumption, urea synthesis, ketone body formation, and gluconeogenesis than perfused livers. In comparison studies, Hems (1966) and Krebs (1970) found that ATP and total adenine nucleotide concentrations were 2.53 μmoles/g and 3.68 μmoles/g, respectively in fresh liver. The values for ATP and total adenine nucleotide concentrations in hepatocytes incubated for 60 minutes in the absence of substrates were identical to those found in fresh liver. Presumably, these cells relied on glycogen and stored triglycerides for maintanance of energy charge. In contrast, livers perfused for 60 minutes in the absence of substrates contained 2.05 μmoles/g ATP and 3.15 μmoles/g total adenine nucleotides. Liver slices incubated under conditions which maintain the integrity of the hepatocyte adenine nucleotide pathway contained 0.5 μmoles ATP/g and 0.91 μmoles/g total adenine nucleotides. Krebs and Hems both concluded that an important factor in the maintanance of cellular energy charge in vitro is delivery of oxygen to the tissue. When tissue oxygen content is suboptimal, and there is a deficiency in the quantity of oxygen that can act as the final electron acceptor in oxidative phosphorylation, less ATP is produced per glucose molecule traversing the glycolytic pathway. That oxygen content in hepatocytes incubated under the conditions of the present study was maintained is evident from the results presented in Fig. 21. ATP con-

tent in hepatocytes was not less than 3.08 μmoles/g in cells pre-incubated for 20 minutes in the absence of substrates. The concentration of ATP rose to approximately 3.24 μmoles/g after incubation for 10 minutes with glucose and gluconeogenic precursors. No change in ATP concentration was measured in cells incubated for a total of 20, 40, or 60 minutes under these conditions.

The absolute and relative concentrations of adenine nucleotides are critical in the regulation of hepatic carbohydrate metabolism. Experimental model-induced changes in these effectors may have altered the results of the previous *in vitro* studies investigating the effects of the sulfonylureas on hepatic carbohydrate metabosism. ATP is a substrate for the kinases involved in the regulation of glycogen metabolizing enzymes, and is a positive allosteric effector of pyruvate carboxylase (Barritt, *et al.*, 1971). It is also an allosteric inhibitor of pyruvate kinase, phosphofuctokinase (Lehninger, 1975), the active (*a*) (Maddaiah and Madsen, 1966) and inactive (*b*) (Tan and Nuttal, 1975) forms of glycogen phosphorylase, and phosphorylase *a* phosphatase (Khandelwal, 1977). Also decreased ATP concentrations may be an indication of poor viabililty of the preparation. It is evident, therefore, that the interpretation of results obtained employing perfused livers and, especially, liver slices may not yield an accurate indication of the true effect of the sulfonylureas on hepatic carbohydrate metabolism.

The substrate conditions and fast length of the animals were chosen to obtain maximum rates of hepatocyte glycogen accumulation. The source of the glucosyl residue generated as substrate for glycogenesis is variable and depends on the nutritional status (a function of the fast length of the animal). When hepatocytes were isolated from

16-24 hour fasted animals, the cells obtained accumulated no glycogen when incubated with 10 mM glucose, a concentration normally found in the portal vein postprandally (Nuttall, et al., 1983). Boyd, et al. (1981) were unable to demonstrate net glycogen accumulation in hepatocytes isolated from 18 hour fasted rats and incubated with 20 mM glucose. Those results are in agreement with the results of the present study, since hepatocytes isolated from 24 hour fasted animals and incubated with 30 mM glucose and 0.1 μCi/ml [^{14}C-(U)]-glucose for 60 minutes did not accumulate glycogen, nor did these cells incorporate significant amounts of [^{14}C]glucose into [^{14}C]-glycogen. Katz, et al., (1976, 1979) found that hepatocytes isolated from 18-24 hour fasted rats accumulated glycogen at maximal rates when incubated with glucose (10 mM) and gluconeogenic precursors. In their system, three substrate components were necessary for maximum rates of glycogen synthesis in cells isolated from fasted rats: 1) physiological concentrations of glucose; 2) lactate; and 3) gluconeogenic amino acids, all of which contributed approximately equally as glycogenic precursors. Glutamine was the preferential glycogenic amino acid, but alanine and asparagine also supported glycogen accumulation. Glutamine and alanine were approximately equally effective in supporting glycogen accumulation in the present study (data not shown), but glutamine was used in most experiments based on the work of Katz, et al. This mixture presents a more physiological situation than stimulating glycogen accumulation with extreme concentrations of glucose (30-50 mM) because such concentrations are not normally attained in the portal circulation (never in normal rats and only rarely in uncontrolled insulin-dependent diabetic animals). Also lactate and amino acids are always found in the circu-

lation, with the concentration of lactate and amino acids rising during exercise and fasting, respectively. A fast length of 24 hours was chosen since Seglen (1974) and others have demonstrated that hepatocytes isolated from animals in this nutritional state synthesize glycogen at maximal rates compared to the release of free glucose.

As previously mentioned, hepatic gluconeogenesis is elevated in in the fasting state, and also postprandially in patients with NIDDM (Best, et al., 1982). Such high rates of glucose production are only observed in hepatocytes isolated from normal rats if fasted for 24-36 hours. This nutritional state results in gluconeogenic rates similar to those observed in patients with NIDDM. Therefore, hepatocytes isolated from 24 hour fasted rats are an appropriate model for the study of the effects of the sulfonylureas on hepatic glucose production.

Glucose Output Studies

The present study was conducted to determmine the direct effect of the oral hypoglycemic sulfonylureas on gluconeogenesis. The total glucose produced from non-carbohydrate moities consists of the glucose released from the liver and that which is deposited as glycogen. Preliminary studies were carried out to investigate the effect of the sulfonylureas on glycogen accumulation and glucose output and when an inhibition of both pathways was found, the effects of these drugs on the individual pathways were further characterized.

Chlorpropamide inhibited the production of glucose in hepatocytes in a concentration-dependent manner. The minimal effective concentration needed to significantly decrease glucose output (0.7 mM) was at the higher limit of the therapeutic range of this drug (Izzo, 1957; Sheldon, et al, 1965; Melander, 1978). However, all of the studies

which investigated the pharmacokinetic properties of the sulfonylureas relied on sampling of peripheral blood. It is probable that peak concentrations of these drugs in hepatic portal blood were higher than in the periphery, since these drugs are administered orally.

The results of the experiments of this section also demonstrated that the two first-generation sulfonylureas tested, chlorpropamide and tolbutamide were approximately equipotent in inhibiting glucose output. (Fig. 9). This observation supports the finding of Melander, et al. (1978) who found that the intrinsic potencies of these two drugs in vivo were similar in spite of the need to administer 2-5 fold more tolbutamide per unit time to achieve the same degree of hypoglycemia. The clinical differences in potencies of these drugs can be ascribed to chlorpropamide's incomplete metabolism and slower elimination rate. On the other hand, HOE-17,710 a non-hypoglycemic sulfonylurea was ineffective in decreasing the production of glucose supporting the hypothesis that the inhibition of glucose output by the hypoglycemic sulfonylureas in isolated hepatocytes may relate to their hypoglycemic activity in vivo.

Although, the inhibition of glucose output (gluconeogenesis) from hepatocytes observed in the present study is in agreement with the observation that the sulfonylureas directly inhibited glucose release from liver slices (Vaughan, 1957; Kaldor and Pogasta, 1960), it is doubtful that the two experimental models are comparable. The slices were obtained from fed animals in both of these earlier studies, in contrast to hepatocytes which were isolated from fasted rats in the present study. Also, the slices were incubated in the absence of substrates or 10 mM glucose only; in the present study hepatocytes

were incubated with glucose and gluconeogenic precursors. The effect of the sulfonylureas in the studies using liver slices was primarily on glycogenolysis, while an inhibition of gluconeogenesis in hepatocytes by chlorpropamide is implicated in the present study.

A direct inhibition of hepatocyte glucose output by the sulfonylureas is also in agreement with the observed inhibition of glucose output by the sulfonylureas in noninsulin-dependent diabetics by Best, et al. (1981). Hepatic glucose production after an overnight fast in patients chronically treated with glyburide was decreased compared with untreated diabetics.

Most studies investigating the in vivo effects of the sulfonylureas on hepatic carbohydrate metabolism were conducted after acute administration. The resulting added circulating insulin probably interfered with the assessment of any extrapancreatic actions of the drugs. The study of Best, et al. was carried out long enough after the initiation of sulfonylurea therapy to allow plasma insulin concentrations to return to pre-treatment levels (Owens, et al., 1979). The results of the study of Best, et al. indicated that the sulfonylureas might either directly inhibit hepatic glucose production or potentiate the action of insulin in the liver. When the normalization of insulin concentarations after prolonged sulfonylurea administration is considered, the results of the present study are in close agreement with those obtained in the in vivo system.

Two studies were carried out in perfused livers to investigate the effect of chlorpropamide on hepatic glucose metabolism. In the first study Schonborn, et al. (1974) supplied the liver with fructose and observed that both chlorpropamide and tolbutamide treatment (0.5 mM)

resulted in a decrease in the conversion of this substrate into glucose, while the conversion of fructose to pyruvate and lactate was stimulated. Blumenthal and Whitmer (1981) perfused livers with lactate and found that chlorpropamide (0.8 mM) significantly inhibited glucagon-stimulated gluconeogenesis but did not significantly decrease basal (non-glucagon-stimulated) gluconeogenesis. In both of these studies, ATP concentrations were less than in fresh liver (or in hepatocytes as prepared in the present study) and the decreased ATP concentrations in these preparations may be an indication that regulation of carbohydrate metabolism was abnormal. It is interesting that in the study of Blumenthal and Whitmer, chlorpropamide decreased glucagon-stimulated gluconeogenesis. Glucagon has been shown to increase intramitochondrial ATP concentration (Halestrap, 1978; Tithradge, et al., 1978) and if the sulfonylureas act by affecting mitochondrial function, that effect may be more evident in perfused livers in which mitochondrial ATP concentrations are elevated. Also, these investigators perfused the livers with a different substrate supply than the hepatocytes were supplied with in the present study, which may also partly explain the discrepency in results.

Glycogen Accumulation Studies

Preliminary experiments in this study indicated that chlorpropamide inhibited glycogen accumulation. Since the precursors for glycogen accumulation are provided through gluconeogenesis in fasted animals, further studies were conducted to characterize the effects of the drugs on glycogen accumulation as a second measure of gluconeogenesis.

As in the glucose output experiments, chlorpropamide inhibited glycogen accumulation in a concentration-dependent manner. The minimum effective concentration in most series of experiments was found to be

0.1 mM (Figs. 11, 12, 13, 16; Table 7, 8). In one particular set of experiments, (Fig. 10) the minimal effective concentration was found to be 1.0 mM. However, at that concentration, glycogen accumulation was inhibited by 22.6 %, and it is possible that a significant inhibition may have occurred at a concentration of between 0.1 mM and 1.0 mM, which were not measured. The discrepencies in minimum effective concentrations may be explained as follows. The sulfonylureas are all highly plasma protein bound, with chlorpropamide being approximately 95% bound to plasma albumin. BSA was used in these experiments and Brown and Crooks (1979) demonstrated that different lots of BSA had varying sulfonylurea binding capacities. Different lots of BSA were used in the present study and it can be calculated that if the sulfonylurea binding capacity was increased by 2%, it would result on a decrease of approximately 40% in available (unbound) chlorpropamide. Further evidence for this hypothesis is put forth by the fact that there were no significant differences in viability or rate of glycogen accumulation in the experiments where a difference in sensitivity to chlorpropamide was observed (data not shown).

Tolbutamide and chlorpropamide were approximately equipotent in inhibiting glycogen accumulation (Fig 16). Glyburide, a sulfonylurea with hypoglycemic potency of 100-500 times that of chlorpropamide or tolbutamide (Sartor, et al., 1980; Sartor, et al., 1982; Matsuda, et al., 1983) was also tested, but had only 10 times the potency of the other sulfonylureas in inhibiting glycogen accumulation. It was necessary to use glyburide as a fine suspension in polyethylene glycol 400/phosphate buffer (100 mM) in a ratio of 3:2 (v/v) due to its poor solubility in aqueous solution. Possibly less drug was available than

calculated.

The data presented in Table 7 demonstrates that the inhibition of glycogen accumulation was specific for the sulfonylureas and could not be extended to include the other para-substituted sulfonamides tested. An analysis of the structure-activity relationships of the compounds tested revealed that the sulfonylurea group of the oral hypoglycemic agents was most likely necessary for the inhibition of glycogen accumulation in hepatocytes. A common chemical feature of carboxytolbutamide and HOE-17,710 is that they both have polar aliphatic side chains. Carboxytolbutamide contains a carboxyl group at either C3 or C4 of the side chain (Thomas and Ikeda, 1966), while HOE-17,710 is identical to tolbutamide except that the aliphatic side chain is shortened from four to two carbons and the terminal carbon has a hydroxyl group attached to it (Hellman, 1981). These modifications of the tolbutamide molecule may result in increased polarity such that these compounds cannot effectively enter hepatocytes. Glyburide and the other second generation sulfonylureas are more hydrophobic than chlorpropamide or tolbutamide due to the replacement of aliphatic side chains with aromatic groups. Their increased potency may be due to their ability to enter cells more readily resulting in higher intracellular concentrations. Sulfadiazine, the diuretics, and diazoxide do not contain the sulfonylurea moiety, which may explain their lack of hypoglycemic activity and ineffectiveness in inhibiting glycogen accumulation. The inhibition of hepatocyte glycogen accumulation resulting from treatment with hydrochlorothiaze and furosemide may be due to a cytotoxic effect of these drugs, since a toxic effect caused by overdose of furosemide in humans is hepatic necrosis (Mitchell, et

al., 1974). Concentrations of furosemide and hydrochlorothiaze that are commonly achieved in patients treated with these drugs (1-10 um) (Andreasen and Mikkelsen, 1977) had no effect on hepatocyte glycogen accumulation in the present study (data not shown).

Results of the studies designed to control rates of glycogen synthesis by altering initial glycogen content of hepatocytes (Figs. 12, 13) demonstrated that the sulfonylureas inhibited glycogen synthesis and did not stimulate glycogenolysis. The effect of chlorpropamide on hepatocyte glycogen accumulation was also more pronounced when cells were incubated with glucose and gluconeogenic precursors. Taken together, these experiments supply further evidence that the effect of the sulfonylureas on hepatocyte glycogen accumulation was a result of an inhibition of gluconeogenesis.

The results of the glycogen accumulation experiments are in agreement with the studies of George and Augusti (1973) and Prasannan and Augusti (1976) who found that normal rats treated for 60 days with either chlorpropamide or glyburide had lower hepatic glycogen content than untreated animals. This treatment period was probably long enough to allow plasma insulin levels to return to pre-treatment levels. Therefore, the insulin concentration that the livers of treated animals were exposed to probably did not affect the results of these experiments. Furthermore, increased plasma insulin levels would result in increased hepatic glycogen content. The effect of acute sulfonylurea administration would also explain the results of Baender and Sholz (1956) and Miller and Dulin (1956) who found that the hepatic glycogen content of rats treated with a single dose of chlorpropamide was elevated compared to untreated animals. The difference between the acute in vivo actions

of the sulfonylureas on hepatic glycogen content and the drugs' effects on glycogen accumulation in isolated hepatocytes was investigated in the the present study. The results of Baender and Sholz and Miller and Dulin were verified in the present study. As a bioassay to assess the bioactivity of different lots of chlorpropamide used in this study, rats were given two daily doses of chlorpropamide and the content of hepatic glycogen was measured at the end of the second day. Animals treated with 100 mg/kg chlorpropamide had lower blood glucose and higher hepatic glycogen content after the second dose of chlorpropamide (data not shown).

The results of experiments investigating the effects of chlorpropamide on the incorporation of [^{14}C]-lactate and [^{14}C]-amino acids into glycogen in the present study are in agreement with the results of the study of Alemay, et al. (1978). These authors found that in animal fasted for the last two days of a 29 day regime of daily tolbutamide administration, less ^{14}C from an injection of [^{14}C]-alanine (a suitable gluconeogenic precursor) was incorporated into blood glucose and liver glycogen than in untreated animals fasted for the same length of time. Again, based on the study of Owens, et al. (1979) it may be predicted that plasma insulin levels in treated animals had returned to pre-treatment levels and that the effect of tolbutamide on gluconeogenesis was extrapancreatic. It could not be determined in the study of Alemay, et al. however, if the drug was acting directly or potentiating the hepatic effects of insulin.

Early studies which demonstrated that the sulfonylureas could inhibit the breakdown of glycogen in liver slices cannot be considered valid for reasons previously described. The present study may be the

first to demonstrate the ability of this class of drugs to directly alter hepatic carbohydrate metabolism independent of concommitant insulin action.

Effect of Chlorpropamide on Glycogen Synthase and Glycogen Phosphorylase

Although an inhibition of hepatocyte gluconeogenesis was suggested by the results of the glucose output and glycogen accumulation experiments, it was also possible that the sulfonylureas were altering hepatic glycogen accumulation by regulating the activity of the glycogen metabolizing enzymes. This possibility was considered based on the results of the study of Remesar, et al. (1978) who found that animals treated for 30 days with tolbutamide had less glycogen phosphorylase in the more active (a) form, with no change in total phosphorylase activity. When hepatocytes were incubated for either 3 or 60 min with 2.0 mM chlorpropamide in the present study, no significant effect on the total activities or the percentages of glycogen synthase or glycogen phosphorylase in their more active (a) forms was measured. On the other hand, hepatocyte glycogen synthase and glycogen phosphorylase did respond to treatment with phenylepherine for 3 minutes by increasing the percentage of glycogen phosphorylase in the a form and decreasing the percentage of glycogen synthase in the a form. Hutson, et al. (1976) and Studer and Borle (1983) found similar changes in synthase and phosphorylase activity upon 3 minute treatment with phenylephrine. It must be pointed out, however, that these enzyme assays as performed in this study do not completely rule out a regulatory effect of the sulfonylureas on these enzymes. These assays detect only covalent modifications (changes in the phosphorylation state) of the enzymes which would be apparent by a change in the proportion of the more active

forms of the enzymes relative to total activity; the assays could not detect changes in activities resulting from non-covalent modifications (increase or decrease in allosteric effectors, such as AMP, ATP or glucose 6-phosphate, or limitation of substrate supply, etc.). The same experimental limitations were faced by Remesar, et al., and the reason for the discrepency in the results of that study and the present one is unclear. It may be that the effect on glycogen phosphorylase results from chronic treatment that could not be detected in isolated hepatocytes during a short incubation period. Also, the effect of the sulfonylureas on the glycogen metabolizing enzymes may not be direct and might require the presence of insulin.

Measurement of Gluconeogenic Intermediates and ATP

Crossover studies (described earlier) have been used sucessfully for the investigation of the interactions of various substances with a variety of metabolic pathways. This technique was applied to the present study to identify the step in gluconeogenesis affected by the sulfonylureas. The levels of gluconeogenic intermediates, and more importantly the relative concentrations of the intermediates, in untreated cells agree with the results of the study of Zelski, et al. (1978). Measurement of the concentration of gluconeogenic intermediates in hepatocytes incubated with 2.0 mM chlorpropamide revealed that the drug caused an elevation of malate concentration and a decrease in the concentration of phospho(enol)pyruvate (PEP), 2-phosphoglycerate (2-PG), and 3-phosphoglycerate (3-PG) (Fig. 17). Subsequent to these initial observations, it was found that treatment with chlorpropamide resulted in changes in intermediate concentrations in a concentration-dependent manner (Fig. 18).

The study of the effect of 2.0 mM chlorpropamide on gluconeogenic intermediates yielded an unexpected result. Although the concentrations of phosph(enol)pyruvate, 2-phosphoglycerate and 3-phosphoglycerate were significantly decreased by incubation of hepatocytes with 2.0 mM chlorpropamide, there were no significant changes in the concentrations of other intermediates more proximal to glucose. It cannot be concluded from the results of this study how this phenomenon occurs, but several hypotheses can be suggested. It is possible that the K_m's of the enzymes for their substrates is much greater than the available concentrations of intermediates which would result in small (possibly unmeasurable in the present study) decreases in the concentrations of intermediates causing significant decrease in enzymatic activity. This would then result in decreased flux of gluconeogenic precursors to glucose and glycogen. Although the concentration of glucose 6-phosphate was unchanged by chlorpropamide, the drug may have altered the sequestration of this intermediate in the endoplasmic reticulum resulting in less substrate available for glucose production and glycogen synthesis.

Only two studies (Shonborn, et al., 1974 and Blumenthal and Whitmer, 1981) have investigated the effects of the sulfonylureas on levels of gluconeogenic intermediates. Both of these studies utilized perfused liver models. The results of those studies are not in total agreement with the present results. Shonborn, et al. perfused livers with 10 mM fructose in the presence or absence of glyburide. Glyburide treatment resulted in a reduction in the concentrations of fructose 6-phosphate, glucose 6-phosphate, 2-PG, 3-PG, ATP, ADP, and AMP. Blumenthal and Whitmer perfused livers with 10 mM lactate in the presence and absence of chlorpropamide. Chlorpropamide treatment resulted in no

significant changes in intermediate concentrations, but did block the glucagon-stimulated increases in concentrations of malate, PEP, 2-PG, and 3-PG. It is interesting however, that in this study chlorpropamide treatment of perfused livers (in the absence of glucagon) caused a trend toward changes in malate, PEP, 2-PG, and 3-PG concentrations that are similar to the changes of levels of gluconeogenic intermediates caused by chlorpropamide in the present study. These authors also found that glucagon caused significant alterations of pyruvate and malate concentrations, but not changes in the concentrations of other intermediates more proximal to glucose, while the flux of lactate to glucose was stimulated. As in the present study, control of gluconeogenesis by physiological or pharmacological agents seems to occur at early steps. These two studies also investigated the effect of the sulfonylureas on glucose output and the reasons for the discrepency in results in the measurement of gluconeogenic intermediates experiments in the present study and the results of Shonborn, et al. and Blumenthal and Whitmer have been discussed previously.

Correlation of Sulfonylureas Actions on Various Aspects of Hepatic Carbohydrate Metabolism

There is no doubt that the sulfonylureas inhibited glucose output and glycogen accumulation in isolated hepatocytes in this study. Figures 22 and 23 demonstrate that these two processes are dependent on each other when hepatocytes isolated from 24 hour fasted rats are supplied with gluconeogenic precursors. Strong evidence favoring an inhibition of gluconeogenesis as the locus of action of the sulfonylureas comes from direct measurement of concentrations of gluconeogenic intermediates and the actions of the drugs on glycogen accumulation when gluconeogenic substrates are supplied with glucose. Further evidence

for an inhibition of gluconeogenesis is derived from a comparison of the time courses of the effect of chlorpropamide on glucose output (Fig. 8), glycogen accumulation (Fig. 14), and incorporation of ^{14}C into glycogen (Fig. 15) and changes in the concentrations of gluconeogenic intermediates (Fig 20), and ATP (Fig. 21). The figures depicting the effect of chlorpropamide on glucose ouput, glycogen accumulation and incorporation of ^{14}C from [^{14}C]-amino acids are nearly superimposable, while the effect of the drug on the levels of gluconeogenic intermediates and ATP are quite different. It is evident that the ability of the sulfonylureas to inhibit glucose output and decrese glycogen accumulation in hepatocytes is the result of a primary effect on the rate of gluconeogenesis. The changes of gluconeogenic intermediate levels are nearly maximized within 10 minutes after addition of chlorpropamide, while the effect of the drugs on glucose output and glycogen accumulation is still increasing from 10 to 20 min of incubation. If the concentrations of chlorpropamide that resulted in changes in intermediate concentrations are compared with effective concentrations on glucose output and glycogen accumulation, it can be seen that they are very similar. In combination, these comparisons further support the findings that the sulfonylureas inhibit gluconeogenesis in hepatocytes isolated from 24 hour fasted rats.

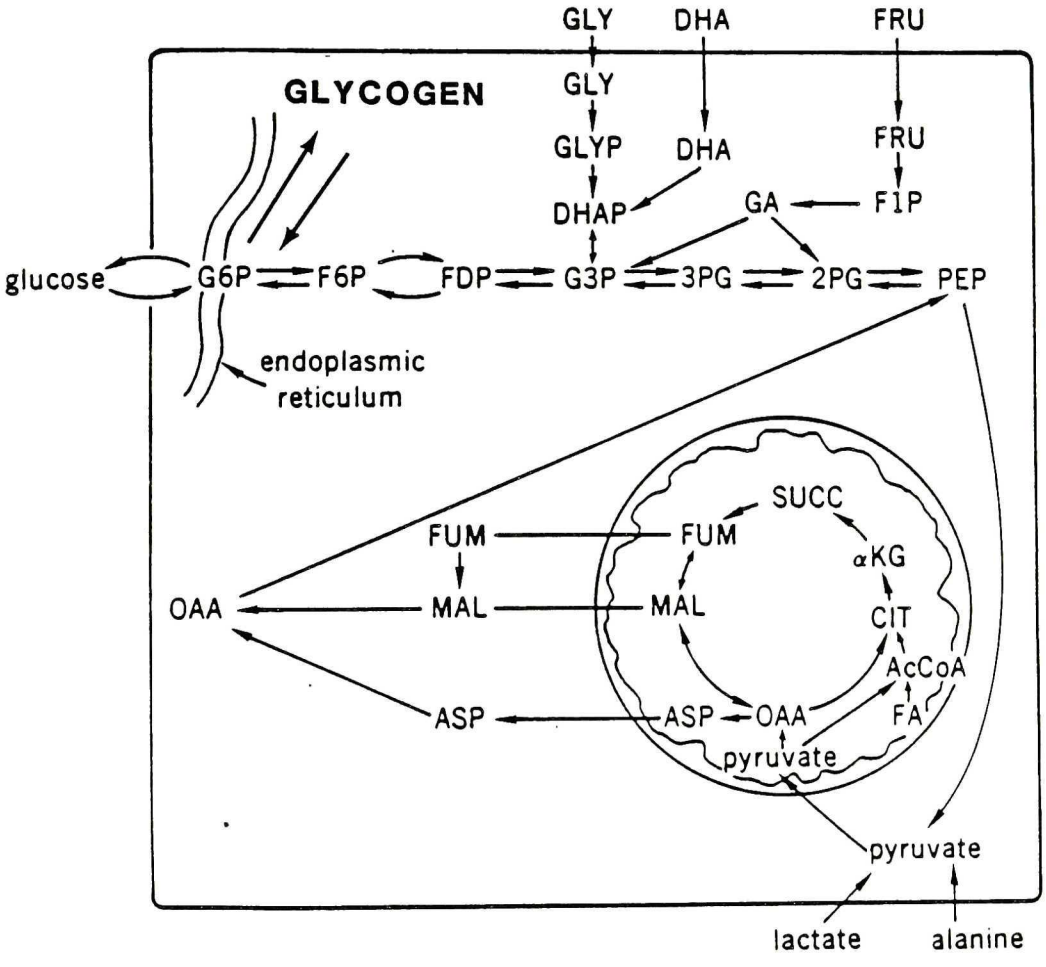
Mechanism of Action of the Sulfonylureas on Hepatic Carbohydrate Metabolism

The results of this study demonstrate that the sulfonylureas inhibit glycogen accumulation and glucose output primarily through an inhibition of gluconeogenesis. Furthermore, the study supports the hypothesis of Katz, et al. (1979) who suggested that hepatic glycogen

synthesis in the 24 hour fasted rat is derived not only from extracellular glucose, but from glucose produced de novo via gluconeogenesis.

An examination of the pathway of gluconeogenesis (presented schematically in Figure 25) and its regulation indicates two control points which sensitive to regulation both by intracellular metabolites and exogenous agents. Most steps in glycolysis are reversible and the gluconeogenic pathway utilizes these enzymes in the reverse direction to form glucose. However, there are two steps, the conversion of pyruvate to phospho(enol)pyruvate and the conversion of fructose 1,6-bisphosphate to fructose 6-phosphate which are irreversible due to their large positive standard free energy changes (Lehninger, 1975). The first step involves the participation of the mitochondria, while the latter step does not. Gluconeogenesis begins with the conversion of lactate to pyruvate, and the conversion of gluconeogenic amino acids to pyruvate or oxaloacetate via tricarboxylic acid cycle intermediates. Glycerol is converted to glyceradehyde 3-phosphate via dihydroxyacetone phosphate. In the initial gluconeogenic reaction, pyruvate is converted to oxaloacetate by pyruvate carboxylase. This mitochondrial enzyme is stimulated by acetyl CoA and high ATP/ADP ratios (Barritt, et al., 1975). Oxaloacetate is converted to malate by the mitochondrial form of malate dehydrogenase at the expense of the oxidation of NADH. The malate formed is transported out of the mitochondria by the tricarboxylic acid shuttle that exchanges malate for citrate. The malate in the cytosol is reconverted to oxaloacetate by the cytosolic form of malate dehydrogenase (which reduces NAD^+). The second regulatory step at the first control point of gluconeogenesis is the conversion of oxaloacetate to phospho(enol) pyruvate by phospho-

Figure 25. Pathways of glycolysis and gluconeogenesis. AcCoA, acetyl coenzyme A; ASP, aspartate; CIT, citrate; DHA, dihydroxyacetone phosphate; FA, fatty acid; FDP, fructose diphosphate; F1P, fructose 1-phosphate; F6P, fructose 6-phosphate; FRU, fructose; FUM, fumarate; GA, glyceraldehyde; GLY, glycerol; GLYP, gycerol phosphate; G3P, glyceraldehyde phosphate; G6P, glucose 6-phosphate; αKG, α-ketoglutarate; MAL, malate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; 2PG, 2-phosphoglycerate; 3PG, 3-phosphoglycerate; SUCC, succinate. (Reproduced from Krauss-Friedmann, 1984).


GLY
DHA
FRU
GLYCOGEN
GLY
GLYP
DHA
FRU
DHAP
GA
F1P
glucose
G6P
F6P
FDP
G3P
3PG
2PG
PEP
endoplasmic
reticulum
SUCC
FUM
FUM
αKG
OAA
MAL
MAL
CIT
AcCoA
ASP
ASP
OAA
FA
pyruvate
pyruvate
lactate
alanine

(enol)pyruvate carboxykinase (PEPCK) which is not regulated by ATP, but uses GTP as a source of high energy phosphate for the phosphorylation of oxaloacetate. This enzyme is not regulated by any effectors in rat liver where 90% of PEPCK is cytosolic (Hanson and Garber, 1972), but exerts its control on gluconeogenesis by its presence in relatively low amounts in the cell, and having a K_m for oxaloacetate that is slightly higher than the concentration of this substrate normally found in the cytosol (Ballard, 1970; Walsh and Chen, 1971). The long-term regulation of PEPCK is a result of the production or destruction of enzyme molecules. Induction of diabetes in rabbits with alloxan resulted in a marked increase in the levels of hepatic PEPCK (Huibregtse, 1976), as did culturing isolated rat hepatocytes with glucagon (Shudt, 1980). Culturing Rueber H35 hepatoma cells in the presence of insulin results in a decrease of PEPCK concentration within 24 hours (Barritt and Wicks, 1971).

The second point of control in gluconeogenesis is at the site of conversion of fructose 1,6-bisphosphate to fructose 6-phosphate which is catalyzed by fructose 1,6-bisphosphatase. The activity of this enzyme is stimulated by ATP and citrate and is inhibited by ADP, AMP (Lehninger, 1975) and fructose 2,6-bisphosphate (Van Shaftingen and Hers, 1981; Pilkis, et al., 1981). These effectors act in an opposite manner on phosphofructokinase, the enzyme that catalyzes the reverse reaction.

It is reasonable to assume that the sulfonylureas would inhibit gluconeogenesis at one of these control points. Possible mechanisms for these effects are: 1) the drugs act through a second messenger (cAMP, calcium, or both); 2) they alter the concentration of an allosteric regulator for one of the control points; 3) they reduce the

concentration of substrates for one of the reactions; and/or 4) they directly inhibit one of the enzymes that are unique to the gluconeogenic pathway. Each of these possibilities will be discussed integrating reports from the literature, the results of the present study, and suggestions for experiments that could be conducted to further investigate each possibility.

Several second messenger systems have been identified through which hormones and pharmacological agents cause their ultimate effect. Studies in pancreatic and adipose tissue have demonstrated that the sulfonylureas can interact with two of these systems. The sulfonylureas increase the concentration of cAMP in pancreatic β-cell *in vitro* by stimulation of adenylate cyclase (Howell and Montague, 1973; Kuo, *et al.*, 1973) and inhibition of phosphodiesterase (Goldfine, 1971; Ashcroft, *et al.*, 1972). However, increased cAMP concentrations apparently do not mediate the effects of these drugs in this tissue since the sulfonylureas decrease proinsulin production upon long-term treatment (Shatz, *et al.*, 1978) while agents such as theophylline which increase intracellular cAMP concentrations in islet tissue increase the production of proinsulin (Shatz, *et al.*, 1973). It is unlikely that the sulfonylureas could inhibit glucose output and glycogen accumulation through changes in intracellular cAMP concentrations. Agents such as glucagon which act through increasing cAMP levels stimulate the rates of gluconeogenesis and glycogenolysis, which results in increased glucose output. This effect is contrary to the results of the present study. One study has investigated the effects of the sulfonylureas on hepatic intracellular cAMP levels in perfused livers. Blumenthal and Whitmer (1981) found that chlorpropamide inhibited the glucagon-stimulated

increase in intracellular cAMP, but had no effect on basal levels of this effector. The results of studies investigating the effects of the sulfonylureas on intracellular cAMP levels in various tissues are conflicting, and it is unlikely that this transduction system is important for the effect of the sulfonylureas on hepatic carbohydrate metabolism.

Another second messenger system capable of mediating the actions of agents which might not enter cells is the regulation of intracelluar calcium concentration. Increased cytosolic calcium may be derived from the extracellular compartment or released from intracellular sequestration sites such as mitochondria or rough endoplasmic reticulum. The sulfonylureas increase the intracellular concentration of calcium in pancreatic tissue, and this action seems to be important for the drugs' ability to stimulate insulin release (Hellman, et al., 1975; Henquin, 1980; Kalkoff, et al., 1983). In adipose tissue, the sensitivity of the isolated adipocyte to the antilipolytic action of the sulfonylureas is dependent on the concentration of calcium in the medium (Ebert, et al., 1974). Therefore, it may be proposed that the sulfonylureas could alter hepatic glucose metabolism by regulating the intracellular concentration of calcium. However, agents which increase the intracellular concentration of calcium in liver in vivo, and hepatic tissue in vitro, such as phenylepherine or vasopressin, have similar ultimate effects as agents that increase intracellular cAMP levels. Also, Ochs and Lardy (1981) demonstrated that cytosolic calcium regulates the transfer of reducing equivalents to the mitochondria in a concentration-dependent manner. This process is an integral step in gluconeogenesis. To definitely rule out the possibility that

these drugs effect hepatic carbohydrate metabolism through alterations in intracellular calcium concentrations (either an increase or decrease) the intracellular concentration of calcium in hepatocytes treated with sulfonylureas could be monitored using a new, non-invasive technique. (2-(-bis (carboyxmethyl)-amino-5 methylphenoxy-methyl-6-methoxy-8-bis (carboxymethyl)-aminoquinoline tetrabis-(acetoxymethyl) ester (Quin 2/AM) is a flourescent indicator that enters hepatocytes where the tetra-acetoxymethyl ester group is cleaved by intracellular esterases, trapping Quin 2 within the cytosol. This compound has the ability to indicate ionized calcium concentration since its flourescence is inversely proportional to calcium concentration. These experiments have not been done, but are an obvious extension of this study.

However, it is possible to monitor changes of cytoplasmic calcium concentrations by following the activities of enzyme systems whose activity is altered by calcium. Closer examination of the results of the experiments investigating the effect of the sulfonylureas on glycogen metabolizing enzymes in the present study. All agents that alter intracellular concentrations of cAMP or calcium (or both) cause rapid, reversible changes in the proportion of glycogen synthase and glycogen phosphorylase in the more active (a) form. Instead, chlorpropamide (2.0 mM) had no effect on the proportion of either synthase or phosphorylase in the more active form (Table 9).

When hepatocytes are incubated with varying concentrations of a depletor of intracellular ATP pools (D,L,-ethionine) (Wilkening, et al., 1975) the rate of gluconeogenesis from lactate correlates with the intracellular ATP concentration. As discussed above, the two rate limiting steps of gluconeogenesis that are regulated by ATP are the

conversion of pyruvate to phospho(enol)pyruvate and the conversion of fructose 1,6-bisphosphate to fructose 6-phosphate. In the present study, treatment of hepatocytes with chlorpropamide resulted in a concentration-dependent decrease in ATP concentration (Fig 19). These results are in agreement with Katsumata and Hagihara (1973) who found that the sulfonylureas could decrease the respiratory control index and the ratio of high energy phosphate molecules formed per oxygen molecule consumed in isolated rat liver mitochondria. These two measurements indicated that the sulfonylures uncoupled oxidative phosphorylation. It is unlikely however, that this effect can explain the drugs' hypoglycemic action since the concentrations of drugs needed to cause this effect ($\geq$ 1.0 mM) are probably not achieved intracellularly when the drugs are administered in vivo. Also, the 15% decrease in hepatocyte intracellular ATP concentration resulting from exposure to 2.0 mM chlorpropamide observed in the present study may not be biochemically important for the following reasons. According to the crossover theorum, if an enzymatically catalyzed reaction is inhibited, the concentration of substrate for the reaction should increase, while the concentration of product decreases. An examination of Fig. 17 demonstrates that neither pyruvate nor fructose 1,6-bisphosphate were elevated by exposure of hepatocytes to 2.0 mM chlorpropamide for 60 min. Also, the concentrations of the products of these reactions should be decreased, and although oxaloacetate was not measured, the concentration of fructose 6-phosphate was not significantly decreased. Treatment of hepatocytes with this drug concentration caused a 55% inhibition of glucose output at the same time point, demonstrating that the drugs inhibited gluconeogenesis without apparent effect on

reactions in gluconeogenesis that are regulated directly by ATP.

Further evidence for the lack of direct effects of intracellular ATP concentration is derived from a closer examination of the results of experiments investigating the dependence of the inhibition of glycogen accumulation by chlorpropamide on the initial hepatocyte glycogen content. ATP is an inhibitor of glycogen phosphorylase a and b and glycogen phosphorylase phosphatase. A decrease in hepatocyte ATP concentration resulting from chlorpropamide treatment would relieve the inhibition of these enzymes and result in stimulated glycogenolysis. Since there was a much attenuated effect of chlorpropamide in cells with relatively high initial glycogen content (Figs. 12, 13) it can be surmised that decreased hepatocyte ATP concentration was not biologically significant for the drug's ability to inhibit glycogen accumulation and glucose output. There is the possiblility, however, that there are important, as yet undefined, regulatory processes in gluconeogenesis that are more sensitive to adenine nucleotide concentrations than decarboxylation of pyruvate or the dephosphorylation of fructose 1,6-bisphosphatase.

The results presented in Fig. 19 suggest that chlorpropamide might inhibit PEPCK activity, since the hepatocyte concentration of malate is increased, while the concentrations of PEP, 2-PG, and 3-PG are depressed. It may also be inferred from these data that the activity of of malate dehydrogenase is inhibited by treatment with chlorpropamide since this enzyme converts malate to oxaloacetate before oxaloacetate is converted to PEP. This action on malate dehyrogenase is unlikely, since this enzyme has the lowest contol strength in the gluconeogenic pathway. Control strength is the dependence of flux of substrates

through a pathway on the activities of the individual enzymes. If an enzyme has no control strength, increasing its activity will not result in increased flux through the pathway (Heinrich, et al., 1977). Measurement of hepatocyte content of oxaloacetate would not clarify this possibility, since mitochondrial malate dehydrogenase converts oxaloacetate to malate, while the cytosolic form catalyzes the opposite reaction. To determine if malate dehydrogenase was inhibited, mitochondria could be separated from cytosol after treatment with chlorpropamide and oxaloacetate could be measured in each fraction. Elevated mitochondrial and decreased cytosolic oxaloacetate concentrations would be an indication of decreased malate dehydrogenase activity.

The most likely target enzyme for the sulfonylureas is PEPCK. This assumption is based on the data of the present study and previous reports of the ability of PEPCK to control overall rates of gluconeogenesis (Rognstad, 1979). This enzyme is also important for the long-term regulation of gluconeogenesis since its concentration is hormonally regulated. It is unlikely that the decreased ATP concentration resulting from the treatment with the sulfonylureas decreased PEPCK activity because this enzyme is not regulated by ATP in mammalian tissue. However, the high energy phosphate needed for the phosphorylation of oxaloacetate is derived from GTP. Most cellular GTP is formed in the tricarboxcylic acid cycle (decarboxylation of succinyl CoA to succinate) in the mitochondria, or is a product of the conversion of ATP to GTP by adenine transkinase in the cytosol. It is possible that the drugs could interfer with the tricarboxcylic acid cycle or the activity of adenine transkinase, resulting in decreased cellular GTP concentration. This possibility could be determined by first measuring the cellular

concentration of GTP and then the concentrations of tricarboxcylic acid cycle intermediates and cytosolic adenine transkinase activity.

The sulfonylureas might also directly inhibit PEPCK activity. In fact, a specific inhibitor of PEPCK has been identified (3-mercapto-picolinic acid) (Jomain-Baum, et al., 1974). This compound decreases gluconeogenesis in isolated hepatocytes in a concentration-dependent manner and has a K_i of approximately 70 μM. Treatment of hepatocytes with this compound also results in a decrease in the concentration of PEP and an increase in the concentration of malate, thus resembling the results of the present study. The direct in vitro effect of the sulfonylureas on PEPCK activity could be assessed, since the enzyme in rat cytosol and in purified form has similar inhibitor sensitivity and K_m for substrates as in the intact cell (Mac Donald, 1978).

The final possibility is that the sulfonylureas can interact with the carrier systems of the mitochondrial matrix. After malic dehydrogenase (mitochondrial form) converts oxaloacetate to malate, malate is transported out of the mitochondria to the cytosol by a tricarboxylate acid (malate exchanged for citrate) transporter. This translocation is not energy-dependent. The sulfonylureas might specifically interfer with the transport of malate out of the mitochondria. Other pharmacologic agents that can inhibit translocators have been identified. Atractyloside and bongkrekic acid inhibit the adenine nucleotide carrier, which translocates ATP and ADP across the mitochondrial membrane (Arinze and Hanson, 1980). Since the sulfonylureas are lipophilic, it might also be suggested that they non-specifically alter the mitochondrial membrane (fluidity, permiability, etc.) resulting in decresed efficiency of the translocators. If the activity of the translocators were inhibited, the fact that cellular malate concen-

trations are increased and PEP, 2-PG, and 3-PG concentrations are decreased after chlorpropamide treatment could be explained. Intramitochondrial malate would increase and less oxalaoacetate would be available for PEPCK activity. The most reliable method of testing this hypothesis would be to isolate cells and separate mitochondria from cytosol. If the translator activities were depressed by chlorpropamide treatment there should be an elevation of mitochondrial malate concentration and and a decrease in cytosolic malate concentration.

From the preceding discussion of the possible mechanisms of action of the sulfonylureas, it is evident that these drugs are acting at a site that is under complex regulation, and only after careful experimental examination is conducted, will the precise mechanism of action be identified.

Relationship of In Vitro Effects of the Sulfonylureas with Their In Vivo Hypoglycemic Activity

A hypothesis for the relationship of the *in vitro* effects of the sulfonylureas observed in the present study with their *in vivo* hypoglycemic activity will be presented here.

The acute hypoglycemic activity of the sulfonylureas is a result of their ability to increase insulin secretion. However, many *in vitro* studies suggest that the sulfonylureas also have direct effects that can partially explain their hypoglycemic activity during chronic administration. These extrapancraetic effects (most notably decreased hepatic glucose output) may occur immediately upon administration of these drugs and the decreased hepatic glucose production provides less stimulus for insulin secretion from the pancreas. This effect gradually decreases blood insulin concentration over the course of weeks and as the

insulin levels return to pre-treatment concentrations, the tissue resistance to insulin present in NIDDM also declines through the mechanism of autoregulation of insulin receptor number. The end result would be a new set-point of blood glucose and insulin concentrations, which is maintained as long as the drugs are administered. Support for this hypothesis comes from: 1) the present study which clearly demonstrated the ability of the sulfonylureas to directly inhibit glucose output from hepatocytes isolated from normal rats; 2) the study of Owens, et al. (1979) which demonstrated that after 2-3 weeks of sulfonylurea treatment that blood glucose concentrations were decreased and insulin concentrations returned to pre-treatment levels in patients with NIDDM; 3) chronic sulfonylurea therapy results in increased insulin receptor binding activity in NIDDM patients; and 4) hepatic glucose output is decreased in patients treated with sulfonylureas for three months.

The question may be asked however, "Why are the sulfonylureas ineffective in the treatment of insulin-dependent diabetes mellitus (IDDM), if they have direct hepatic actions which should decrease blood glucose concentrations?" I propose that the sulfonylureas are only effective in insulin-sensitive pathways that have been exposed to minimal concentrations of insulin. It may be necessary for the pathway to be "insulin-primed" in order to be a suitable target for sulfonylurea action. Evidence supporting this hypothesis is provided by two clinical studies conducted in patients with IDDM. It was found that in a group of juvenile diabetics sulfonylurea therapy was effective in lowering blood glucose for approximately one week after the onset of the disease (De Belle, et al., 1967). During this period, no increase in insulin secretion could be measured in the patients' blood

by radioimmunoassay. Subsequently, the sulfonylureas become less effective, until after six months, the drugs had lost all hypoglycemic activity. Also in a group of patients with IDDM who had very low levels of circulating insulin, administration of insulin caused a significant decrease in hepatic glucose production, demonstrating that the gluconeogenic pathway was still insulin-sensitive. When the study was repeated with the administration of a single dose of 2 mg of glyburide, there was a 100% decrease in the hepatic production of glucose (Martin, 1971). These data may be related to the results of the present study in the following manner. PEPCK is known to be hormonally regulated and low levels of insulin are needed to prevent an increase in the concentration of PEPCK. In the present study an inhibition of PEPCK by the sulfonylureas was indicated as their mechanism of action to inhibit hepatic gluconeogenesis. If in patients with prolonged IDDM PEPCK concentrations become elevated, control of this enzyme by the sulfonylureas might be attenuated. This could explain their failure to lower blood glucose after six months treatment in patients with IDDM in the study of De Belle, et al.

Interesting experiments that would lend further support for this hypothesis would be to divide a group of rats into three groups: 1) control; 2) streptozotocin-induced diabetic; and 3) diabetics animals maintained on low levels of insulin. After a suitable period of time (animals made diabetic by streptozotocin injection can generally be kept alive for 3-4 weeks), the experiments conducted in IDDM patients (De Belle, et al.; Martin) could be repeated in these animals. It would be expected that the hypoglycemia and decreased hepatic glucose output in response to the sulfonylureas administration would be greater

in the animals maintained on insulin than those which were untreated. Another valuable experiment would investigate the *in vitro* effects of the sulfonylureas in hepatocytes isolated from animals in groups described above.

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