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THE EFFECT OF ETHANOL UPON GLUCOSE HOMEOSTASIS

by

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Thesis submitted to the
School of Graduate Studies
of the
University of Ottawa
in partial fulfillment of the
requirements for the degree of
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October 24, 1977

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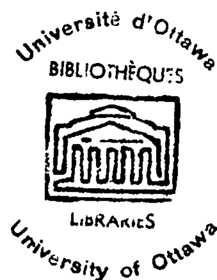

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CHAPTER I

INTRODUCTION

According to Hawkins and Kalant (1972) the metabolic effects of ethanol are of three different types: a) those arising from alterations of cellular processes caused directly by ethanol; b) those arising from interference with neuro-endocrinological regulatory mechanisms, and finally c) pharmacological actions of ethanol on specific cells or processes.

As early as 1941, Brown and Harvey (reported by Madison, 1968) reported six cases of alcohol hypoglycaemia following the ingestion of alcoholic beverages by malnourished, chronic alcoholics. Consistently, low dose ethanol administration has been found to decrease blood glucose concentrations of fasted dogs or humans (Wolfe et al, 1976;Freinkel et al, 1967; Madison, 1968; Field et al, 1963). The syndrome known as "alcohol hypoglycaemia" is by no means restricted to the malnourished nor the chronic alcoholic. Occasionally, well nourished periodic or weekend spree drinkers exhibit this syndrome (Madison, 1968).

This hypoglycaemic effect of ethanol has been attributed to an interference with gluconeogenesis due to decreased concentrations of gluconeogenic substrates (Zakim, 1968) which is associated with the decrease of the $NAD^+/NADH$ ratio

within the cytosol of the hepatocyte (Hawkins and Kalant, 1972; Madison, 1968; Lieber, 1967)

Both ethanol oxidation (specifically the pathway involving the enzyme alcohol dehydrogenase) and gluconeogenesis require the cofactor NAD^+. In the fasted animal whose liver glycogen stores are small, all glucose released originates from gluconeogenesis. In this respect the situation resembles uncontrolled diabetes. The difference between fasting and insulin-deprived diabetes is in the rate at which glucose is being released by the liver. During fasting the rate of hepatic glucose release is less than normal (Cowan et al, 1969) while in diabetic animals it is about double the normal rate (Hetenyi et al, 1961). If the hypoglycaemic effect of ethanol is due to an interference with gluconeogenesis specifically because the demand for NAD^+ exceeds the supply, ethanol is expected to decrease the hepatic glucose production and consequently lower blood glucose levels in the insulin-deprived diabetic.

It is of interest to ascertain whether ethanol interferes with the increase in hepatic glucose production in insulin-induced hypoglycaemia. A massive sympathetic discharge which is triggered by an inadequate supply of combustible substrate to the brain is ultimately responsible for an increase in hepatic glucose production " the hepatic

response to hypoglycaemia " (Hetenyi 1972). The brain of rats is known to contain small amounts of alcohol dehydrogenase (Raskin and Sokoloff 1968) and ethanol is capable of crossing the blood brain barrier. If ethanol or its metabolic products can be utilized by the brain to any significant extent, the hepatic response to hypoglycaemia is expected to be diminished in the presence of ethanol. The hepatic response to hypoglycaemia is not expected to be altered if the brain is unable to utilize ethanol or its metabolic prdoucts unless a significant portion of the excess glucose released by the liver originates from gluconeogenesis.

Therefore, the answer to two questions was sought . Is ethanol capable of interfering with gluconeogenesis to such an extent as to decrease the rate of hepatic glucose production in fasted and diabetic dogs? Can ethanol disrupt the hepatic response to hypoglycaemia either by providing an alternative substrate to glucose or by its possible interference with gluconeogenesis?

CHAPTER II

REVIEW OF THE LITERATURE

A. METABOLIC PATHWAYS OF GLUCOSE METABOLISM

Introduction

The liver, being a versatile organ, is able to respond to various quantities and qualities of nutrients that it receives via the portal vein and hepatic artery. In order to maintain the relatively constant composition of the blood leaving the liver by means of the hepatic veins, the liver must be capable of performing most of the reactions involved in the synthesis, degradation and interconversions of carbohydrates, fats and amino acids (Denton and Pogson, 1976).

It is through the handling of carbohydrates by the liver, specifically by alteration of the rates of both glucose production and utilization, that the concentration of glucose in the plasma is determined (Hetenyi, 1972).

Glycogen Synthesis and Breakdown

The glycogen content of the liver (normal) varies between 0.1% and 10.0%. The actual stores of glycogen in the liver depend upon the nutritional state of the animal. The pathways involving the conversion of glucose to glycogen and glycogenolysis each involve two irreversible steps, specifically

those catalyzed by the enzymes glucose-6-phosphatase and glycogen synthetase for the synthesis of glycogen and glycogen phosphorylase and glucokinase for the degradation of glycogen. Glycogen synthesis and degradation are controlled by the enzymes glycogen synthetase and glycogen phosphorylase respectively. These two enzymes exist in both the active, a, and inactive, b, form. No significant amount of glycogen synthesis can occur during glycogen degradation stimulated by glucagon or epinephrine, due to the fact that increased concentrations of cyclic AMP cause the inactivation of glycogen synthetase as well as the activation of glycogen phosphorylase (Hers et al, 1974).

Regulation of Glycogen Metabolism

Insulin plays an important role in the regulation of glycogen metabolism although its mode of action is still controversial. Insulin inactivates the enzyme glycogen phosphorylase while at the same time activating glycogen synthetase. The amount of glycogen synthetase in the active form is increased; thereby, promoting glycogen synthesis and deposition (Hers et al, 1974).

Epinephrine, which is secreted by the adrenal medulla during severe hypoglycaemia, increases glycogen breakdown. Epinephrine mediated by cyclic AMP activates kinase-kinase. Phosphorylase b is converted to its active form, a, and

glycogen conversion to glucose is facilitated. At the same time, the active form of glycogen synthetase is converted to the inactive form and glycogen synthesis is halted (Turner and Bagnara, 1971)

The mode of action of glucagon is identical with that of epinephrine. Therefore, glucagon facilitates glycogenolysis (Turner and Bagnara, 1971)

Glycolysis

For each mole of glucose converted to two moles of lactate, two moles of ATP are produced. The glycolytic pathway and the reactions therein are noncyclic and all the necessary enzymes are found within the cytosol of the hepatocyte. All but three of the reactions are readily reversible. The irreversible steps are the interconversions of glucose and glucose-6-phosphate; fructose-1-phosphate and fructose-1-6-diphosphate and finally phosphoënolpyruvate and pyruvate. The enzymes involved in these reactions are glucokinase, phosphofructokinase and pyruvate kinase respectively (Larner, 1971)

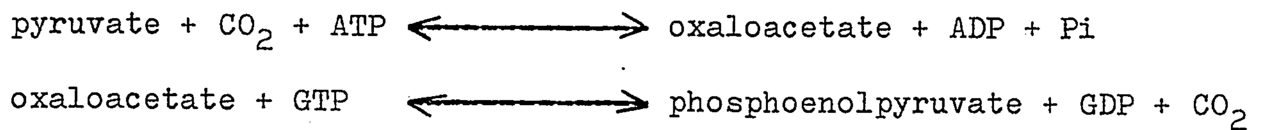
Gluconeogenesis

The major substrates of hepatic gluconeogenesis are lactate, alanine, glutamate and glycerol. Gluconeogenesis is the primary pathway for glucose synthesis when dietary glucose intake is zero or low as it is in fasting or

diabetes (Denton and Pogson, 1976)

In gluconeogenesis, six moles of ATP are required to convert two moles of lactate to one mole of glucose. Gluconeogenesis and glycolysis have all but three reactions in common. These three new reactions involve the enzymes glucose-6-phosphatase, fructose-1-6-diphosphatase and phosphoenolpyruvate carboxykinase (White et al, 1973)

The reactions that ensure the synthesis of phosphoenolpyruvate from pyruvate are catalyzed by pyruvate carboxylase and phosphoenolpyruvate carboxykinase consecutively:



$$\text{pyruvate} + CO_2 + \text{ATP} \longleftrightarrow \text{oxaloacetate} + \text{ADP} + \text{Pi}$$

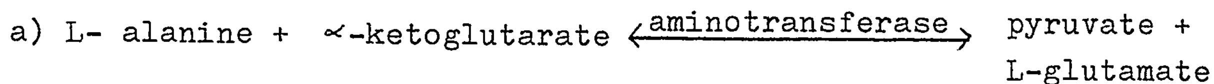
$$\text{oxaloacetate} + \text{GTP} \longleftrightarrow \text{phosphoenolpyruvate} + \text{GDP} + CO_2$$

(Hanson and Garber, 1972)

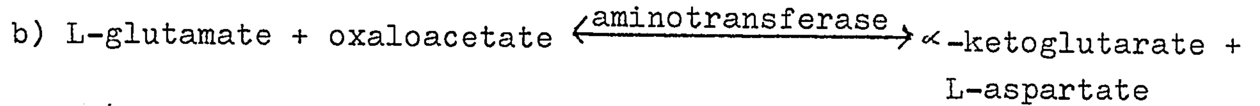
In this way, the liver is able to bypass the thermodynamically unfavourable conversion of pyruvate to phosphoenolpyruvate, the pyruvate kinase step of glycolysis (Hanson and Garber, 1972).

A problem arises in the conversion of pyruvate to phosphoenolpyruvate due to the permeability characteristics of the mitochondrial membrane. Oxaloacetate is formed within the mitochondria and the enzyme necessary for the final conversion of oxaloacetate to phosphoenolpyruvate is located within the cytosol of the hepatocyte. Since oxaloacetate

is unable to freely cross the mitochondrial membrane, malate dehydrogenase within the mitochondria reduces the oxaloacetate to malate. The reaction requires NADH which is oxidized to NAD^+. The malate is able to cross the mitochondrial membrane and enter the cytosol. Within the cytosol, malate is oxidized to oxaloacetate this time by a cytosolic malate dehydrogenase with the reduction of NAD^+ to NADH. The oxaloacetate now within the cytosol is capable of forming phosphoenolpyruvate in the presence of phosphoenolpyruvate carboxykinase. Such would be the situation when lactate or pyruvate are the preferred substrates for gluconeogenesis. If alanine is the preferred substrate for gluconeogenesis the situation is slightly different. Within either the cytosol or mitochondria, alanine is converted to pyruvate and glutamate by means of glutamate-pyruvate transaminase in the presence of α-ketoglutarate. The cytosolic pyruvate enters the mitochondria and is converted to cytosolic phosphoenolpyruvate as described above. The glutamate reacts with oxaloacetate to produce α-ketoglutarate and aspartate. The mitochondrial aspartate is either converted to oxaloacetate and α-ketoglutarate in the presence of glutamate or enters the cytosol and the nitrogen is eliminated via the urea cycle.



a) L- alanine + α-ketoglutarate $\xleftrightarrow{\text{aminotransferase}}$ pyruvate + L-glutamate

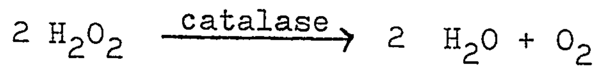


b) L-glutamate + oxaloacetate $\xleftrightarrow{\text{aminotransferase}}$ α-ketoglutarate + L-aspartate

SUM: L-alanine + oxaloacetate $\longleftrightarrow$ pyruvate + L-aspartate

(White et al, 1973)

In the presence of O_2 and H_2O, alanine is deaminated to the ketoacid, pyruvate, and NH_4^+ and H_2O_2. The hydrogen peroxide is converted to H_2O and O_2 via catalase.



$$2\ H_2O_2 \xrightarrow{\text{catalase}} 2\ H_2O + O_2$$

The pyruvate is eventually converted to phosphoenolpyruvate via its entrance into the mitochondria and exit as malate as previously described above (Larner, 1971)

Once phosphoenolpyruvate is produced all the remaining reactions of gluconeogenesis are cytosolic (White et al, 1973).

Regulation of Gluconeogenesis

Starvation, a diet high in fat content and the actions of epinephrine and glucagon are all conducive to gluconeogenesis (Larner, 1971). All of these conditions also release fat reserves. Triglycerides are hydrolyzed to fatty acids and glycerol both of which accumulate in the plasma. The percentage glucose carbon derived from glycerol after a seven day fast is approximately double that reported for the dog in the postabsorptive state (Hall et al, 1976).

At least *in vitro*, free fatty acids play an important role in the regulation of gluconeogenesis. Free fatty acids inhibit several of the irreversible steps in glycolysis such as those involving the enzymes hexokinase and pyruvate kinase. *In vitro*, the key bypass enzymes of gluconeogenesis, pyruvate carboxylase, phosphoenolpyruvate carboxykinase and glucose-6-phosphatase are unaffected by free fatty acid concentrations. All these factors favour gluconeogenesis (Larner, 1971).

Epinephrine, glucagon and the adrenocorticotrophic hormones act by stimulating adenyl-cyclase. The increased concentrations of adenyl-cyclase within the fat cells trigger the process of lipolysis by means of a protein kinase which converts the nonactivated lipase to the active form of the enzyme by phosphorylation with ATP which ultimately increases gluconeogenesis (Denton and Pogson, 1976).

Several steps within the gluconeogenic pathway reduce NAD^+ to NADH. High NADH concentrations inhibit the glycolytic enzyme pyruvate kinase. During glucose production via gluconeogenesis, glucose breakdown is suppressed (Larner 1971).

The concentrations of many enzymes are affected by the various nutritional states. In starvation, the concentration of glucose-6-phosphatase increases several fold while that of glucose kinase is barely detectable (Denton and Pogson, 1976).

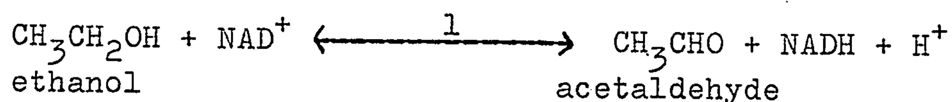
Also the activity of phosphoenolpyruvate carboxykinase within the liver, kidney, adipose and lung tissue increases three to five fold after fasting (Hanson and Garber, 1972). These changes in enzyme activities are alll conducive to gluconeogenesis.

B. THE METABOLISM OF ETHANOL

Metabolic Pathways of Ethanol Oxidation

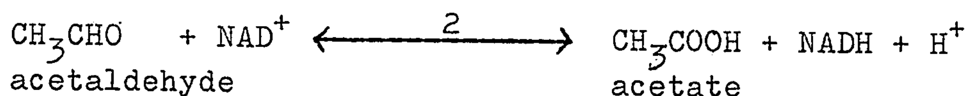
In the intact animal, eighty percent of ethanol oxidation is effected by the liver (Larsen and Krarup,1974; Lieber,1974). There exist at least three different metabolic pathways for ethanol oxidation although it appears that only two, specifically, the alcohol dehydrogenase and the microsomal ethanol oxidizing systems are operative *in vivo* (Lieber et al, 1975; Lieber 1974).

The enzyme alcohol dehydrogenase (ADH) with an *in vivo* Km of 2 mM (Makar and Mannering,1970) is strictly cytosolic and catalyzes the conversion of ethanol to acetaldehyde. Hydrogen is transferred from ethanol to the cofactor NAD^+ effecting its conversion to NADH. Within the mitochondria, acetaldehyde is then converted to acetate, again, with the production of one molecule of NADH. Therefore, the oxidation of one mole of ethanol requires two moles of NAD^+ and produces one mole of acetate and two moles of NADH.



$$CH_3CH_2OH + NAD^+ \xleftrightarrow{1} CH_3CHO + NADH + H^+$$

ethanol   acetaldehyde



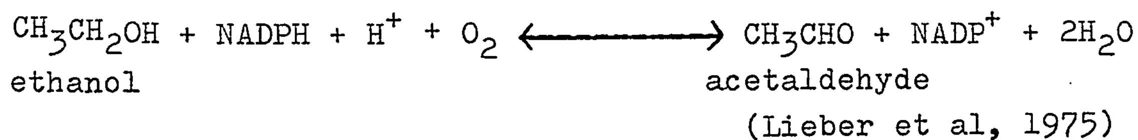
$$CH_3CHO + NAD^+ \xleftrightarrow{2} CH_3COOH + NADH + H^+$$

acetaldehyde   acetate

1- alcohol dehydrogenase
2- acetaldehyde dehydrogenase   (Lieber et al, 1975)

Acetate is released into the blood stream and further metabolized extrahepatically (Forsander, 1970). At ethanol concentrations of less than 10 mM, it is this pathway that is responsible for the majority of ethanol elimination (Thurman et al, 1975).

The use of pyrazole, a potent ADH inhibitor, has led to the proposal of yet another metabolic pathway for ethanol oxidation. Liver tissue extracted from rats administered ethanol for 24 days and then pyrazole were able to oxidize ethanol in vitro (Lieber and DeCarli, 1970). This route of ethanol oxidation is known as the microsomal ethanol oxidizing system, " MEOS ". The in vitro Km of MEOS (8-9 mM) agrees well with the Km of the in vivo pyrazole insensitive pathway (Lieber et al, 1975; Grunnet et al, 1973). This pathway is NADPH dependant and not identical with the pathway that involves catalase-H_2O_2 (Lieber et al, 1975). The overall reaction catalyzed by the microsomal ethanol oxidizing system is:



$$CH_3CH_2OH + NADPH + H^+ + O_2 \longleftrightarrow CH_3CHO + NADP^+ + 2H_2O$$

ethanol   acetaldehyde

(Lieber et al, 1975)

When blood ethanol concentrations are below 20 mM, the alcohol dehydrogenase pathway is the predominant means by which ethanol is eliminated. As ethanol concentrations exceed 20 mM, the contribution of MEOS to ethanol utilization increases.

The third pathway proposed for ethanol oxidation is one involving the enzyme catalase. Catalase is capable of oxidizing ethanol *in vitro* but *in vivo* this pathway would be limited by the rate of H_2O_2 generation within the liver. The rate of H_2O_2 production within the liver is small and even under ideal conditions this pathway would account for at most 2% of the ethanol oxidation rate *in vivo*. It is not possible therefore that catalase could in itself account for the rates of ethanol utilization determined for the pyrazole insensitive pathway and it is uncertain whether this pathway for the elimination of ethanol is at all operative *in vivo* (Lieber et al, 1975).

Rate of Ethanol Utilization

The rate of ethanol utilization equals the amount of ethanol metabolized by the liver and other tissues plus the amount of ethanol eliminated via the breath, sweat and urine. Approximately 2-6% of the administered dose of ethanol is excreted in the urine. If the level of ethanol in the blood

has been elevated for a prolonged time this percentage may be higher (Hawkins and Kalant, 1972).

In the dog, ethanol utilization is a first order reaction and is proportional to the amount of ethanol present in the blood when ethanol concentrations are below 2 mM (Marshall and Fritz, 1953). Between blood ethanol concentrations of 2 and 57 mM, the rate of ethanol utilization follows a zero order reaction and is independent of the concentration present in the blood (Larsen and Krarup, 1974; Marshall and Fritz, 1953; Loomis, 1950). The blood ethanol concentrations that induce respiratory arrest in the dog vary between 86.9 and 147.8 mM (Alcohols and Derivatives, 1970).

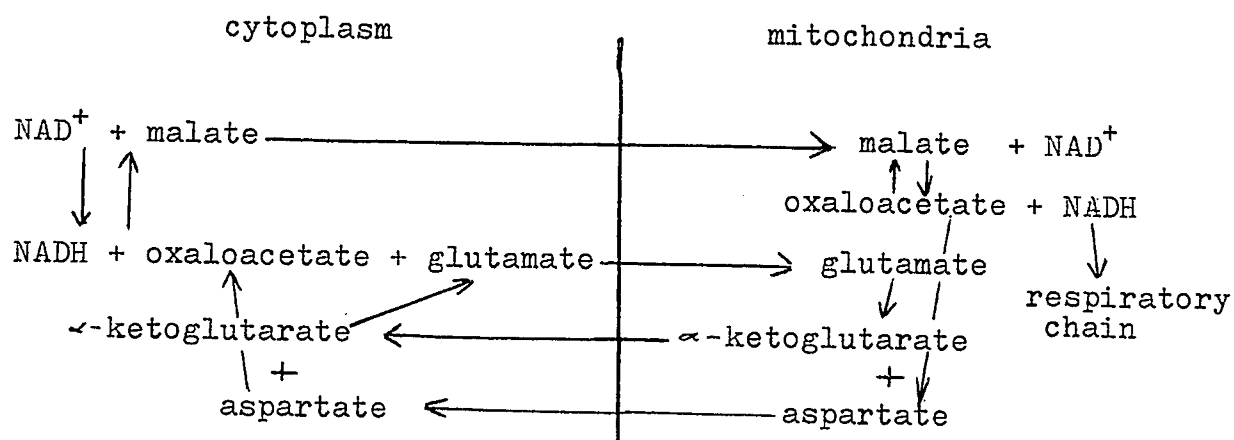
There is no significant difference in the rate of ethanol utilization whether the subject is given the ethanol by slow or fast injection (Nelson and Kinard, 1959). The rate of ethanol utilization in the anaesthetized dog when blood ethanol concentrations vary between 4.4 and 54.0 mM is 0.65 mM/kg·min (Loomis, 1950). The average rate of ethanol utilization in the same dog can vary from day to day and from hour to hour. The mean daily variation in the rate of ethanol metabolism in the same dog may be as high as forty percent (Marshall and Fritz, 1953).

Elimination of Reducing Equivalents

The oxidation of ethanol, specifically via the ADH pathway causes the accumulation of reducing equivalents within the cytosol of the liver cell which is reflected by an increase in the $NADH/NAD^+$ ratio (Guynn and Pielik, 1975; Williamson et al, 1969; Madison et al, 1967; Smith and Newman, 1959).

In the absence of adequate cytosolic hydrogen acceptors (Meijer, et al, 1975), the reoxidation to NAD^+ of at least one of the moles of NADH produced during ethanol oxidation is achieved by "mitochondrial shuttles" since the mitochondrial membrane is impermeable to NADH even in the presence of ethanol (Cederbaum and Rubin, 1975; Grunnet et al, 1973). Several shuttles for the transfer of reducing equivalents from the cytosol to the mitochondria have been proposed. The three most important mitochondrial shuttles are the malate-aspartate, the α-glycerophosphate and the fatty acid shuttle. The shuttling of the reducing equivalents (i.e. H) is carried out by metabolites that pass into the mitochondria, reduce NAD^+ to NADH, then pass into the cytoplasm again in the oxidized form ready to be reduced and then carry another reducing equivalent into the mitochondria. The malate-aspartate shuttle is the one that plays the predominant role in the transfer of reducing equivalents produced during ethanol oxidation.

Malate-Aspartate Shuttle

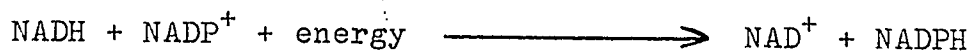


(Cederbaum and Rubin, 1975)

For the proper operation of this shuttle, glutamate and malate must enter the mitochondria while α-ketoglutarate and aspartate exit. Carriers are necessary for these substrate anions to be transferred in and out of the mitochondria (Cederbaum and Rubin, 1975).

The reducing power of the systems that generate NADH can be used to effect the reduction of $NADP^+$ and the concomitant reoxidation of NADH. The transhydrogenation can be effected by mitochondrial respiration in the presence of $NADP^+$.

$$NADH + NADP^+ + energy \longrightarrow NAD^+ + NADPH$$



(White et al, 1973)

Transhydrogenation also occurs within the cytosol. Substrates such as lactate and pyruvate are capable of acting as hydrogen

acceptors to become lactic or pyruvic acid respectively.

Rate-Limiting Step in Ethanol Oxidation

The rate of ethanol utilization is less in the fasted than in the fed animal (Smith and Newman, 1959). In the fasted animal, the oxidation of ethanol is limited by the rate of generation of NAD^+ from NADH generated in the cytosol by alcohol dehydrogenase. The transfer of reducing equivalents from the cytosol to the mitochondria is regulated by the intracellular concentrations of the malate-aspartate and α-glycerophosphate shuttles as well as the flux through the respiratory chain. On the other hand, in the fed animal (postabsorptive state) the mitochondrial reoxidation of NADH is limited due to a decreased availability of ATP for oxidative phosphorylation (Meijer et al, 1975). It is unlikely that the rate limiting step in ethanol oxidation is the enzyme alcohol dehydrogenase itself under any condition (Videla and Israel, 1970).

C. ALCOHOL HYPOGLYCAEMIA

Alcohol hypoglycaemia has been experimentally reproduced in fasted dogs and humans and has been repeatedly ascribed to an inhibition of gluconeogenesis in the presence of low liver glycogen stores (Kreisberg et al, 1971; Freinkel et al, 1967; Lochner et al, 1967).

Unlike the fasted subject whose liver glycogen stores are low, the liver glycogen stores of a subject in the postabsorptive state have not been severely affected by the overnight fast. Since the liver contains sufficient amounts of glycogen and the glycogenolytic action of glucagon is not affected by ethanol, the maintenance of plasma glucose concentrations during ethanol administration could easily be maintained by glycogenolysis in the postabsorptive state (Field et al, 1963). In contrast to the effect of ethanol upon glucose homeostasis in the fasted animal, ethanol is not expected to induce hypoglycaemia after a short, e.g. overnight fast.

Ethanol administration to both humans (Perman, 1958) and dogs (Klingman and Goodall, 1958) stimulates epinephrine release by the adrenal medulla. Epinephrine acts directly upon the beta cells of the pancreas to decrease insulin secretion (Ganong, 1967). Wajchenberg et al (1976) reported that a four hour ethanol infusion (51.2 mM/min) to seventy-two hour fasted humans suppressed endogenous insulin release; yet, alcohol hypoglycaemia was evident. Thus it appears that alcohol hypoglycaemia is not insulin nor epinephrine mediated.

When the livers of forty-eight hour fasted rats were perfused using pyruvate as the substrate, the hepatic glucose production fell 66% in the presence of 10 mM ethanol (Krebs et al, 1969). In forty-eight hour fasted dogs with chronic end to side portal caval shunts, the administration of ethanol

(final blood ethanol concentrations of 4 mM) induced a 58% fall in mean hepatic glucose output (Madison et al, 1967). In three day fasted dogs and humans, the occurance of alcohol hypoglycaemia reportedly caused by an interference with gluconeogenesis has been well documented (Wolfe et al, 1976; Searle et al, 1974; Freinkel et al, 1967; Lochner et al, 1967).

Madison et al (1967) reported that the inhibition of hepatic glucose production induced by ethanol after a prolonged fast was eleviated by the administration of the redox dye methylene blue which oxidizes NADH to NAD^+. Upon the infusion of ethanol, the decrease in the NAD^+/NADH ratio within the cytosol of the hepatocyte is reflected by changes in the reduced and oxidized form of several metabolites, for example, lactate and pyruvate. The increase in the concentration of NADH upon the administration of ethanol alters the relative concentrations of substrates that are both gluconeogenic precursors and components of the mitochondrial shuttle system that aids in the regeneration of cytosolic NAD^+. In addition, substrates such as lactate and glutamate depend upon NAD^+ for their oxidation to pyruvate and their subsequent entrance into gluconeogenesis. Thus it appears that the redox dye methylene blue supplies sufficient NAD^+ for ethanol oxidation and gluconeogenesis. In this way, methylene blue lifts the inhibition of gluconeogenesis imposed by ethanol.

Consequently the hypoglycaemic effect of ethanol has been attributed to an interference with gluconeogenesis in the presence of diminshed liver glycogen stores. The alteration of the NAD^+/NADH ratio within the cytosol of the hepatocyte is the result of an excess production of reducing equivalents from both the operation of gluconeogenesis and ethanol oxidation. The decrease of the NAD^+/NADH ratio ultimately alters the concentrations of substrates that are both gluconeogenic precursors and necessary for the regeneration of NAD^+; thereby, precipitating alcohol hypoglycaemia after a prolonged fast (Zakim, 1968).

Liver glycogen stores are low in insulin-deprived diabetes as in prolonged fasting. The rate of hepatic glucose production in the insulin-deprived diabetic is approximately double that of a fasted animal (Cowan and Hetenyi, 1971; Hetenyi et al, 1961). If alcohol hypoglycaemia is due to an interference of gluconeogenesis in the presence of decreased liver glycogen stores, it is conceivable that ethanol administration to insulin-deprived diabetics would precipitate a decrease in plasma glucose concentrations.

D. HEPATIC RESPONSE TO HYPOGLYCAEMIA

Upon the intravenous infusion of insulin, a well documented pattern of responses occurs. Initially the rate of hepatic

glucose production is suddenly and transiently decreased (Hetenyi et al, 1961). Following this, the induction of hypoglycaemia elicits a massive sympathetic discharge. As a result of this, the rate of hepatic glucose production increases; "the so-called hepatic response to hypoglycaemia". During a continued infusion of insulin, this increase in glucose output is not sufficient to restore plasma glucose concentrations back to normal (Hetenyi et al, 1961;de Bodo et al, 1959). This happens only during the recovery period once the infusion of insulin has stopped (de Bodo et al, 1959).

In 1972 Hetenyi illustrated that mannose, a sugar that is able to replace glucose as a combustible substrate for the brain, abolishes insulin-induced hypoglycaemia. Ethanol too is capable of crossing the blood brain barrier and the brain of at least rats contains small amounts of alcohol dehydrogenase (Raskin and Sokoloff, 1968). If ethanol or its metabolic products are capable of replacing glucose to any significant extent as a combustible substrate for the brain, the hepatic response to hypoglycaemia is expected to be diminished by ethanol administration as it is by mannose.

CHAPTER III

METHODS AND MATERIALS

Animals

All experiments were carried out on 19 mongrel dogs the weights of which ranged between 7.6 and 22.5 kg (average 14.4 kg). The dogs were trained to stand quietly in a Pavlov stand. All dogs were fasted sixteen to twenty hours before the experiments with the exception of one group of dogs fasted for four days. All dogs were maintained on a diet of dry chow containing 23.8% protein, 9.0% fat and 3.3% fibre. This diet was supplemented with meatballs containing 10.6% protein and 14.7% fat. Access to food and water was ad lib. Six of the nineteen dogs were pancreatectomized. Their diet was supplemented by pancreatic enzymes (Festal tablets, Hoechst) and daily injections of protamine-zinc insulin. Insulin was withdrawn from the diabetic animals seventy-two hours before the experiments.

Procedures

Two plastic catheters (Becton-Dickinson PE) were introduced under local (procaine or duracaine) anaesthesia about fifteen to thirty minutes before the experiment. All infusions were given via a catheter inserted into the

cephalic or jugular vein and blood samples were withdrawn via the inferior vena cava by a catheter introduced into the saphenous or femoral vein.

Tracer Methods

Except for six experiments (those coded by 11-13 on Table 12) in which ^{3}H-2-glucose was used, ^{3}H-3-glucose was used as the tracer. The primed tracer infusion technique described by de Bodo et al (1963) was applied in all experiments. Rates of glucose appearance (Ra) essentially identical with the rate of glucose production, and the rates of glucose disappearance (Rd) representing overall glucose utilization and glycosuria were calculated as described by Cherrington and Vranic (1973). The size of the glucose pool was estimated as the product of the apparent distribution space of glucose (V) and the concentration of plasma glucose at the various sampling times. V was taken to be equal to $Q \times CPl / CTRL_g \times SA_o$ where Q = the volume of tracer glucose injected (ml); CPl = the counts per minute of the injected tracer (dpm/ml); $CTRL_g$ = basal glucose concentration in dynamic steady state (mg/ml) and SA_o = specific activity of glucose at t= 0 minutes. (dpm/mg). All calculations including statistical were carried out on a programmable Wang 600 desk calculator.

Experimental Design

Three series of experiments were performed. In all three series of experiments, at t= 0 minutes, two simultaneous infusions were initiated. The priming dose of labelled glucose (25 - 35 µCi) was injected and then followed by an infusion of tracer glucose (0.25 - 0.32 µCi/min in a volume of 0.155 ml/min). The specific activities of the injected tracer for the different series of experiments are as follows: $2.25 \pm 0.11 \times 10^8$ dpm/µg for the four day fasted dogs and $5.74 \pm 0.33 \times 10^7$ dpm/ug for the pancreatectomized dogs and finally $2.12 \pm 0.07 \times 10^8$ dpm/µg (^{3}H-3-glucose) and $2.13 \pm 0.03 \times 10^8$ dpm/µg (^{3}H-2-glucose) in the third series. The other infusion consisted of saline at a rate of 0.45 - 0.62 ml/min. The first period consisted of 100 minutes during which steady state plasma glucose concentrations and the rates of hepatic glucose production and utilization were established. At t= 100 minutes, the second period began.

In the first series of experiments, the effect of an ethanol infusion upon the rates of glucose production and utilization in fasted dogs was tested. Three dogs fasted for four days were employed in this series. At t= 100 minutes, 8 - 16 mM/kg ethanol was injected in a volume of 29 - 41 ml. The injection was immediately followed by an infusion of 0.25 mM/kg min ethanol for another 130 minutes (i.e. until

t= 230 min). In two of the fasted dogs, the experiment was repeated after a fast of the same duration but the priming dose of ethanol was reduced to 2.0 - 2.4 mM/kg in a volume of 29 - 36 ml and 0.04 mM/kg min was infused between t= 100 and t= 230 minutes. The sequence of the paired experiments was at random.

In the second series of experiments, the effect of ethanol upon the rate of hepatic glucose production in diabetic animals was tested. Thirteen experiments were carried out on six pancreatectomized dogs. At least seven days elapsed between the pancreatectomy and the first experiment (Table 1). Each dog being its own control underwent two experiments. At t= 100 minutes in one of the experiments, ethanol diluted in saline was administered as a primed (6.9 - 13.9 mM/kg in a volume of 16 - 31 ml) infusion (0.25 - 0.29 mM/kg min). Ethanol was infused for a period of 100 minutes (i.e. until t= 200 min). In the paired experiment, the infusion of saline was continued until the end of the experiment at t= 200 minutes. The sequence of the two experiments was at random. The sixth dog underwent a third experiment in which neither saline nor ethanol was infused between t= 0 and t= 200 minutes.

In the third series of experiments, the effect of ethanol upon the hepatic response to insulin-induced hypoglycaemia

TABLE 1

ELAPSED TIME (DAYS) BETWEEN PANCREATECOMY AND EXPERIMENTS

Dog	No Infusion (days)	Saline Infusion (days)	Ethanol Infusion (days)
LD-3		7	14
LD-4		15	9
LD-6	23	28	36
LD-7		29	22
LD-8		12	7
LD-9		8	14

was examined in nine normal dogs. Each dog being its own control was subjected to two experiments. At t= 100 minutes, insulin was infused at a rate of 4 - 7 mU/kg min with or without ethanol. The ethanol infusion (0.21 - 0.35 mM/kg min) was primed by the injection of 5.4 - 18.1 mM/kg ethanol in a volume of 30 - 45 ml. At t= 200 minutes, the infusion of insulin or insulin and ethanol was replaced by an infusion of saline which lasted for 30 minutes (i.e. until t= 230 min). The sequence of the paired experiments was at random. Three of the nine dogs underwent a third experiment of identical design in which ethanol alone was infused between t= 100 and t= 200 minutes at a rate of 0.24 - 0.26 mM/kg min. The priming dose of ethanol was 13.9 mM/kg in a volume of 34 ml. All ethanol was diluted in saline.

Blood Sampling

Immediately after having been drawn, the blood was placed in heparinized tubes, mixed and placed in ice. The separate aliquots of blood taken for the determination of radioimmunoreactive insulin were treated in a similar manner. No more than thirty minutes elapsed between the sampling and centrifugation for 15 minutes at 2000 r.p.m. Plasma for the insulin determinations was immediately frozen at -2 to -4 °C while the rest of the plasma was placed in the refrigerator.

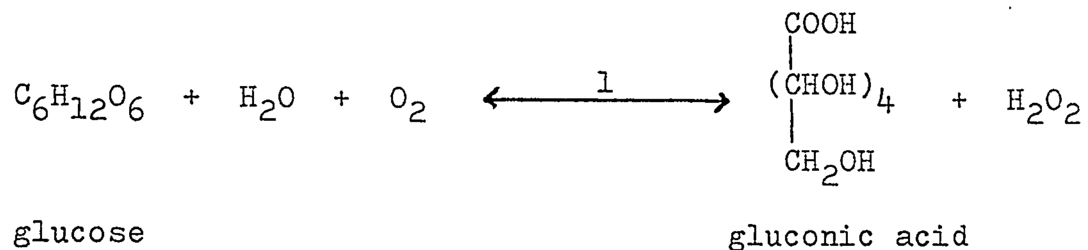
Eight blood samples were drawn during the control period and seven to nine during the second period of the experiment depending upon the individual series. No less than ten and no more than fifteen minutes elapsed between the various sampling times. The total amount of fluid infused during the course of the experiments was approximately 100 ml in excess of that withdrawn during the sampling.

Chemical Determinations

a) Plasma Glucose Determination

The plasma was deproteinized with $Ba(OH)_2$ and $ZnSO_4$ according to the method described by Somogyi (1945).

The enzymatic determination of plasma glucose was carried out using the enzymes glucose oxidase and peroxidase as well as the dye reagent chromagen (Huggett and Nixon, 1957). The determination of glucose is based upon the following equations:



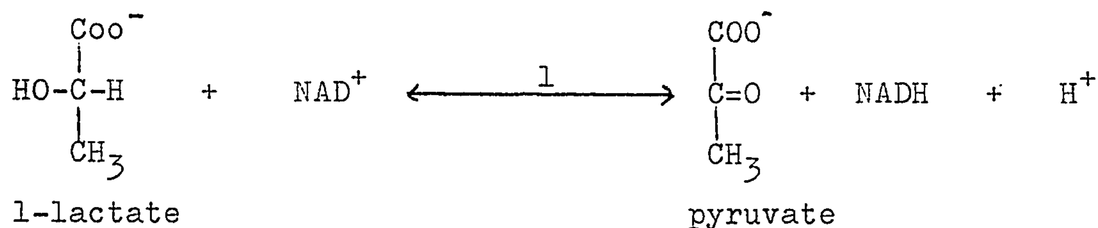
Dye = dianisidine
1 = glucose oxidase
2 = peroxidase

The reaction is complete after one hour incubation at 37°C and the samples are read at 420 Å on a spectrophotometer.

Glucose oxidase is specific for the determination of glucose since for every 100 mg/dl glucose determined only 1% of that is mannose, altrose and galactose (Bergmeyer and Bernt,196

b) Plasma Lactate Determination

A modification of a method described by Hohorst et al (1962) was used to enzymatically determine plasma lactate concentrations. The deproteinization of the plasma was carried out using 6% perchloric acid. The determination of plasma lactate involved the use of a glycine-hydrazine buffer, NAD^+ and the enzyme lactate dehydrogenase. The assay of lactate is based upon the following reaction:



l= lactate dehydrogenase

(White et al, 1973)

After one hour incubation at 25° C the reaction is complete and the samples are read at 340 Å on a spectrophotometer.

All plasma lactate concentrations have been converted to blood lactate concentrations for presentation.

c) Blood Ethanol Determination

A kit designed specifically for the purpose of determining blood ethanol concentrations was purchased from Boehringer-Mannheim. The deproteinization of the blood was carried out using 0.33 N perchloric acid. The enzymatic determination of blood ethanol involved the use of NAD^+, a 75 mM pyrophosphate, pH 8.7; 75 mM semicarbazide; 27 mM glycine buffer and the enzyme alcohol dehydrogenase. Great care was exercised in sealing all tubes to be incubated with rubber stoppers in an attempt to minimize the evaporation of ethanol. The enzymatic reaction that the assay is based upon is as follows:

$$\underset{\text{ethanol}}{C_2H_5OH} + NAD^+ \overset{1}{\longleftrightarrow} \underset{\text{acetaldehyde}}{CH_3CHO} + NADH + H^+$$

1 = alcohol dehydrogenase

(Lieber et al, 1975)

d) Determination of Radioimmunoreactive Insulin (iRi)

A kit manufactured for the purpose of determining plasma iRi concentrations was purchased from Amersham-Searle.

An adaptation of the double antibody method first

described by Hales and Randle (1963) is employed in this kit. In this assay, the second antibody (rabbit anti-(guinea pig γ- globulin) serum) is reacted with the first antibody (guinea pig anti-insulin serum) prior to its use in this procedure. The second antibody retains its ability to bind with the protein insulin and causes the precipitation of the first antibody to yield a microprecipitate which remains in suspension. The cold insulin competes with the ^{125}I-insulin for a limited amount of antibody binding sites and this decreases the amount of labelled antigen bound to the antibody complex. As long as the amount of antibody and labelled insulin is the same in all samples and standards assayed the only variable is the amount of cold insulin. Therefore, unknown amounts of cold insulin can be quantitated by comparing the decrease in antibody binding of the labelled insulin in known standard solutions and in unknown samples. Once the unbound insulin is separated from that bound to the antibody complex, the amount of bound radioactive insulin can be determined by counting the radioactivity of either fraction although the practice was to count the amount of radioactivity bound to the antibody complex (Hales and Randle, 1963).

Initially, the samples or standards are added to the double antibody mixture and incubated for six hours at 2 - 4 ^{o}C. The radioactive insulin is then added and the mixture is

incubated for an additional eighteen hours at the same temperature. The standards and samples are then washed with a 4% bovine serum albumin (fraction V) buffer and filtered using a millipore apparatus. The filter membranes are dried in a vacuum oven. Ten ml of Bray's scintillation fluid is added and the radioactivity is counted on a Liquid Scintillation Spectrometer set for the ^{3}H energy spectrum. It is possible to count the gamma emission of the ^{125}I-insulin in the ^{3}H energy spectrum since the gamma ray is deflected by an atomic electron transferring to the electron some of its energy. This causes the electron to be removed from orbit, becoming a free electron with an energy equal to that of a low energy B particle. This is referred to as the "Compton Effect". (Handbook of Chemistry and Physics, 1971-2).

In order to determine very low plasma iRi concentrations, a modification of the above method was used. The six and eighteen hour incubations as described above were elongated to twenty-four and seventy-two hours respectively.

Calculations

a) Estimation of Ethanol Utilization Rates

The utilization of ethanol was established during the infusion of ethanol from the formula:

$$V \times dC/dt = R(\text{infusion}) - R(\text{utilization})$$

where V= volume of total body water (liters), assumed equal to

0.67 x body weight in kg; C= concentration of ethanol in blood (mMole/l) and R= rates of ethanol utilization or infusion (mM/min). In most experiments, the C versus time curve was found to be linear between t= 125 and t= 200 minutes. This is in accordance with a zero order reaction which describes ethanol kinetics when blood ethanol concentrations exceed 2 mMole/l. The assumptions inherent in the equation are a) the distribution space of ethanol equals the volume of total body water and remains unchanged during the course of the experiment and b) all removal of ethanol is by metabolism only. In light of these assumptions, the calculated rates of ethanol utilization are estimates only.

b) Rates of Glucose Production and Utilization

Polynomial functions were fitted to consecutive triplets of data for both the specific activity of glucose (dpm/µg) versus time and plasma glucose concentrations (mg/ml) versus time curves.

The rates of glucose production (mg/kg·min) and utilization (mg/kg·min) were calculated from the following equations:

$$Ra_t = (F - (p \times C_t \times V \times dSA_t/dt)) \times 1/SA_t$$
$$Rd_t = Ra_t - (p \times V \times dC_t/dt)$$

(Cowan and Hetenyi, 1971)

where Ra is the rate of glucose appearance, F is the infusion

rate of ^{3}H-labelled glucose (1.623 uCi/min), V is the glucose distribution volume calculated from the intercept of the priming injection of tracer, C_t is the plasma glucose concentration (mg/ml) calculated halfway between the first and third points of each triplet and assumed to apply to the central 50% of each triplet, SA_t is the specific activity of glucose (dpm/ug) calculated and applied in the same manner as C_t and finally p is the pool fraction taken to be equal to a portion of the entire glucose pool and has been given the value of 0.65 and 0.87 in the normal and diabetic animals respectively (Cowan and Hetenyi, 1971). The values for dSA_t/dt and dC_t/dt are the calculated first derivatives of the polynomials for SA and C.

Due to the varying time periods between samples and the fact that the SA_t and C_t were assumed to apply to the central 50% of the time period for each triplet, overlaps and gaps occurred in the calculation of SA_t and C_t and consequently Ra_t and Rd_t. These were resolved by calculating the midpoint and the upper and lower limits as previously described. The limits were then adjusted to eliminate the gaps and overlaps by splitting them equally between two time periods and then calculating the new midpoints halfway between the new limits.

c) Extra Release of Glucose

The extra release of glucose was defined as the amount of glucose released during the 100 minute infusion of ethanol or ethanol and insulin minus the basal rate of glucose release during the same period. Extra release was measured as mg/kg·min (Hetenyi, 1972). The extra release of glucose during the infusion of ethanol was calculated and compared with the appropriate infusion in the control experiment, which was saline in the second series and insulin in the third series of experiments.

CHAPTER IV

RESULTS

A. THE EFFECT OF ETHANOL UPON GLUCOSE PRODUCTION IN THE POSTABSORPTIVE STATE

The peak blood ethanol concentration obtained at the termination of the ethanol infusion was 37.9 ± 3.4 mMole/l and the estimated rate of ethanol utilization was 0.13 ± 0.008 mMole/kg·min (Table 2).

When ethanol alone was infused to three normal dogs in the postabsorptive state, no change in plasma glucose concentrations (Table 3), plasma radioimmunoreactive insulin (Table 4), or the rates of glucose appearance or utilization was evident (Figure 1).

B. THE EFFECT OF ETHANOL UPON HEPATIC GLUCOSE PRODUCTION IN FASTED DOGS

The Estimated Rate of Ethanol Utilization

Ethanol was administered as a primed intravenous infusion at rates of either 0.25 or 0.04 mMole/kg·min to dogs fasted for four days. The mean blood ethanol concentrations (calculated between t= 125 and t= 230 minutes) were 0.29 ± 0.02 and 41.8 ± 2.77 mMole/l for the low and high rates of ethanol infusion respectively. The peak ethanol concentrations obtained at the termination of the ethanol infusions (i.e. at t= 230 minutes) are shown on Table 2. Figure 2 illustrates that the

TABLE 2

ESTIMATED RATES OF ETHANOL UTILIZATION
DURING A PRIMED INFUSION OF ETHANOL FOR 100 MINUTES

Metabolic State of Animal	Blood Ethanol Concentration (mMole/l) at the Termination of the Infusion	Estimated Rate of Ethanol Utilization (mMole/kg·min)
Postabsorptive (3)	37.9 ± 3.4	0.130 ± 0.008
Fasted (3)	51.5 ± 3.9	0.067 ± 0.009
Fasted (2)*	4.5 ± 0.4	0.016 ± 0.005
Hypoglycaemic (9)	42.7 ± 3.9	0.107 ± 0.013
Diabetic (6)	38.4 ± 2.6	0.091 ± 0.023

* 0.04 mMole/kg·min was infused. In all other groups the rate of infusion was 0.21 - 0.35 mMole/kg·min.

Number of dogs is in brackets.

Note: In the fasted animals ethanol was infused for 130 minutes. For the sake of comparability the utilization rate of ethanol has been calculated for the first 100 minutes.

Mean values ± s.e.m.-s are shown.

TABLE 3

PLASMA GLUCOSE CONCENTRATIONS (mg/dl) BEFORE, DURING AND AFTER ETHANOL ADMINISTRATION

Metabolic State of Animal	Concentration of Plasma Glucose (mg/dl)			
	Before Infusion	During Ethanol Infusion 0-40 min	During Ethanol Infusion 40-100 min	After Infusion
Postabsorptive (3)	111.17 0.50	114.88 0.93	114.25 1.77	112.13 1.42
Fasted (3)	99.11 2.01	94.81 2.35	102.74** 1.17	
Fasted (2)*	101.33 0.99	88.69 2.56	102.74** 1.63	
Diabetic (postabsorptive)				
Saline (6)	374.19 5.02	356.27 10.53	333.15 10.53	
Ethanol (6)	412.48 16.56	388.51 27.37	387.27 27.65	
Hypoglycaemia (postabsorptive)				
Insulin (9)	104.76 0.85	60.10 4.54	43.34 1.18	54.51 3.73
Insulin and Ethanol (9)	105.86 0.46	64.33 4.76	45.86 2.09	46.61 3.84

Means and s.e.m.-s are shown.
Number of experiments are shown in brackets. * indicates 0.04 mM/kg min ethanol was infused. In all other experiments the rate of infusion was 0.21 - 0.35 mM/kg min.
** indicates ethanol infusion 40 - 120 min.

TABLE 4

PLASMA RADIOIMMUNOREACTIVE INSULIN CONCENTRATIONS (μU/ml) DURING THE INFUSION OF ETHANOL TO DOGS IN THE POSTABSORPTIVE STATE

Plasma Insulin Concentrations at t=

80	100	140	200	230	minutes
14.22 ±2.96	15.00 ±1.53	18.67 ±2.91	16.83 ±3.35	15.30 ±4.11	

Notes: The ethanol infusion took place between t= 100 and t= 200 minutes.

Mean values and s.e.m.-s are shown. n=3.

Figure 1

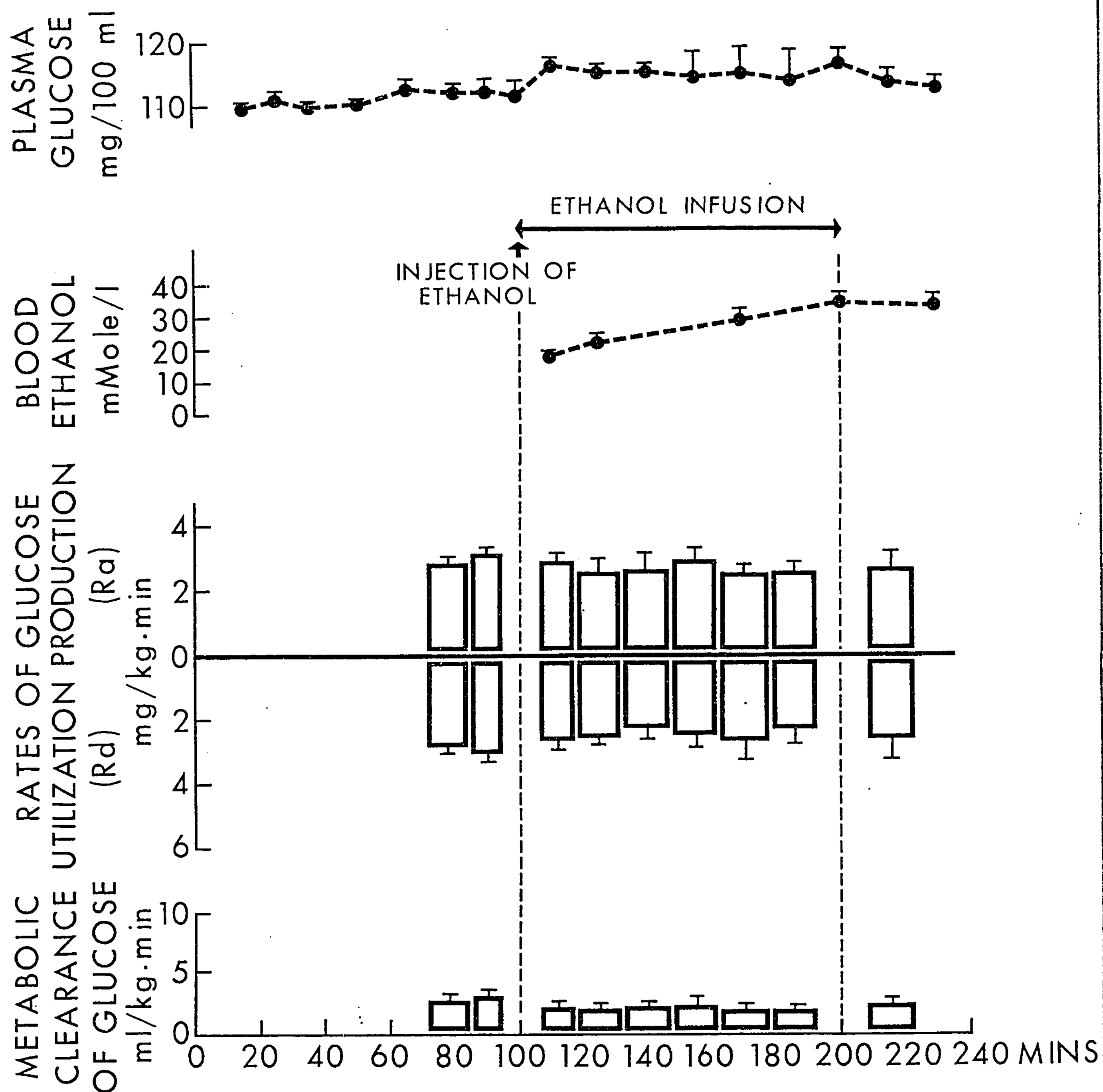


Figure 1: The effect of an intravenous ethanol infusion of 0.24 - 0.26 mMole/ kg·min following a priming dose of 13.9 mMole/kg to normal dogs in the postabsorptive state. Abscissa: time in minutes. Ordinates: as indicated. Mean values ± s.e.m.-s are shown, n=3.

Figure 2

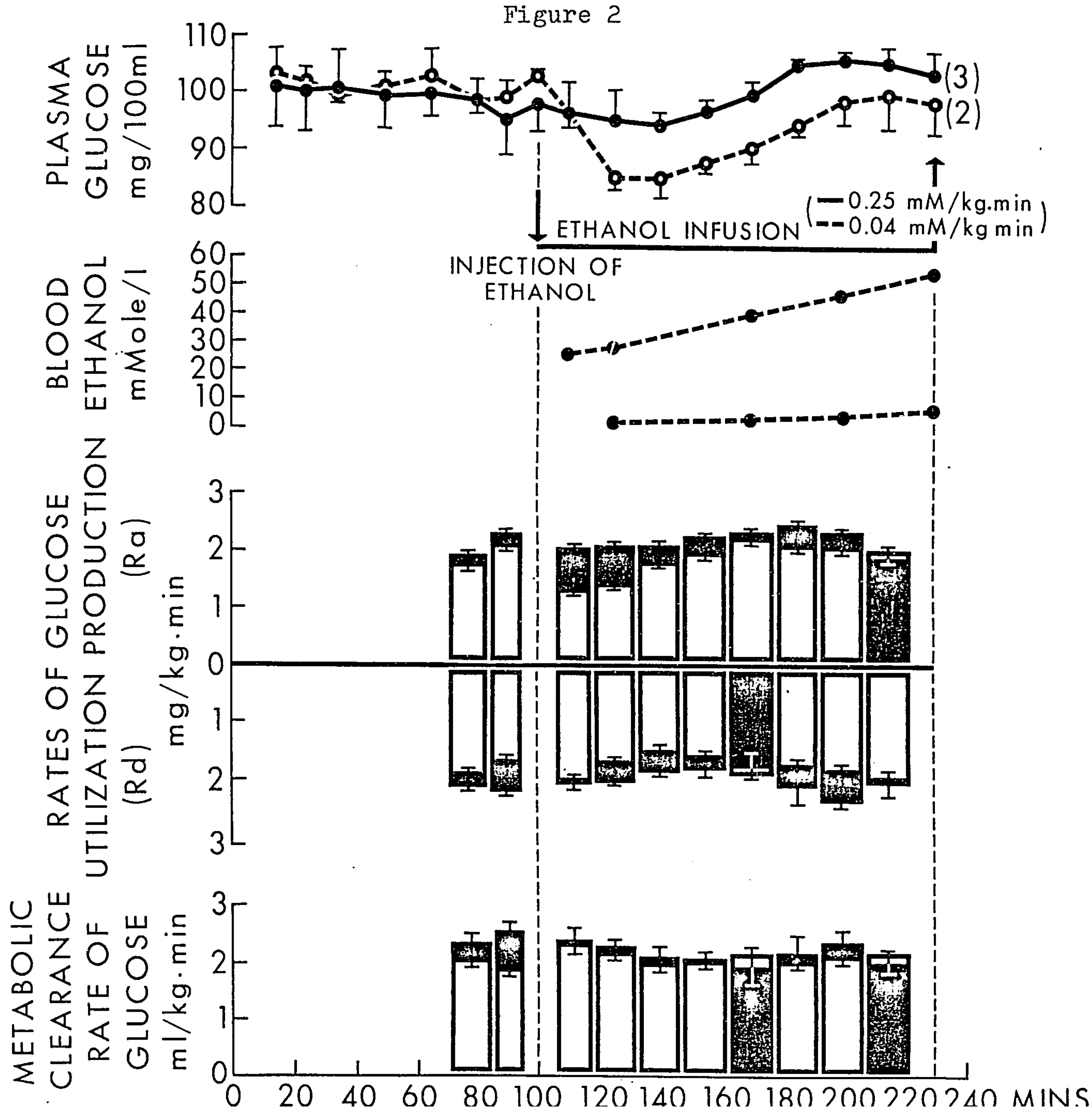


Figure 2: The effect of an intravenous ethanol infusion to dogs fasted for four days. Ethanol was infused either at the rate of 0.25 or 0.04 mMole/kg·min. Abscissa: time in minutes. Four ordinates starting at the top. Ordinate 1: concentration of plasma glucose as mg/100 ml. Ordinate 2: blood ethanol concentrations as mMole/l. Ordinate 3: glucose production (Ra) and utilization (Rd) as mg/kg·min. Ra pointing upward, Rd pointing downward from a common line representing zero. Ordinate 4: metabolic clearance rate of glucose (MCR_g) as ml/ kg·min. Total heights of the columns: Ra, Rd or MCR_g during the infusion of ethanol at 0.25 mMole/kg·min; white columns: Ra, Rd or MCR_g during the infusion of ethanol at 0.04 mMole/kg·min. If the values in the latter type of experiment exceed those in the former, the white columns appear in excess of the black ones. The infusion of ethanol was administered between t= 100 and t= 230 minutes as shown by the broken perpendicular lines. Mean values ± s.e.m.-s are shown.

slope of the ethanol versus time curve is positive for both rates of ethanol infusion; therefore, both rates of ethanol infusion exceeded the rate of ethanol utilization.

Table 2 indicates that the estimated rate of ethanol utilization for the high and low rates of ethanol infusion were 0.067 ± 0.009 and 0.016 ± 0.005 mMole/kg·min respectively. Fasting significantly reduced the estimated rate of ethanol utilization.

Blood Lactate Concentrations

Whether ethanol was infused at the rate of 0.25 or 0.04 mMole/kg·min, the blood lactate concentration rose (Figure 3) The mean lactate concentration was increased from 0.93 ± 0.05 to 2.53 ± 0.55 mMole/l blood-H_2O when ethanol was infused at the higher rate; an increase from 0.64 ± 0.07 to 1.14 ± 0.12 mMole/l blood-H_2O was observed when ethanol was infused at the lower rate (0.04 mMole/kg min) (Table 5). The increase in the blood lactate concentrations was greater at the higher blood ethanol concentrations.

Rates of Glucose Production and Utilization

Figure 2 illustrates the changes in the plasma glucose concentrations during the ethanol infusion at both the rates of 0.25 and 0.04 mMole/kg·min. An analysis of variance of the changes in plasma glucose concentration revealed a significant

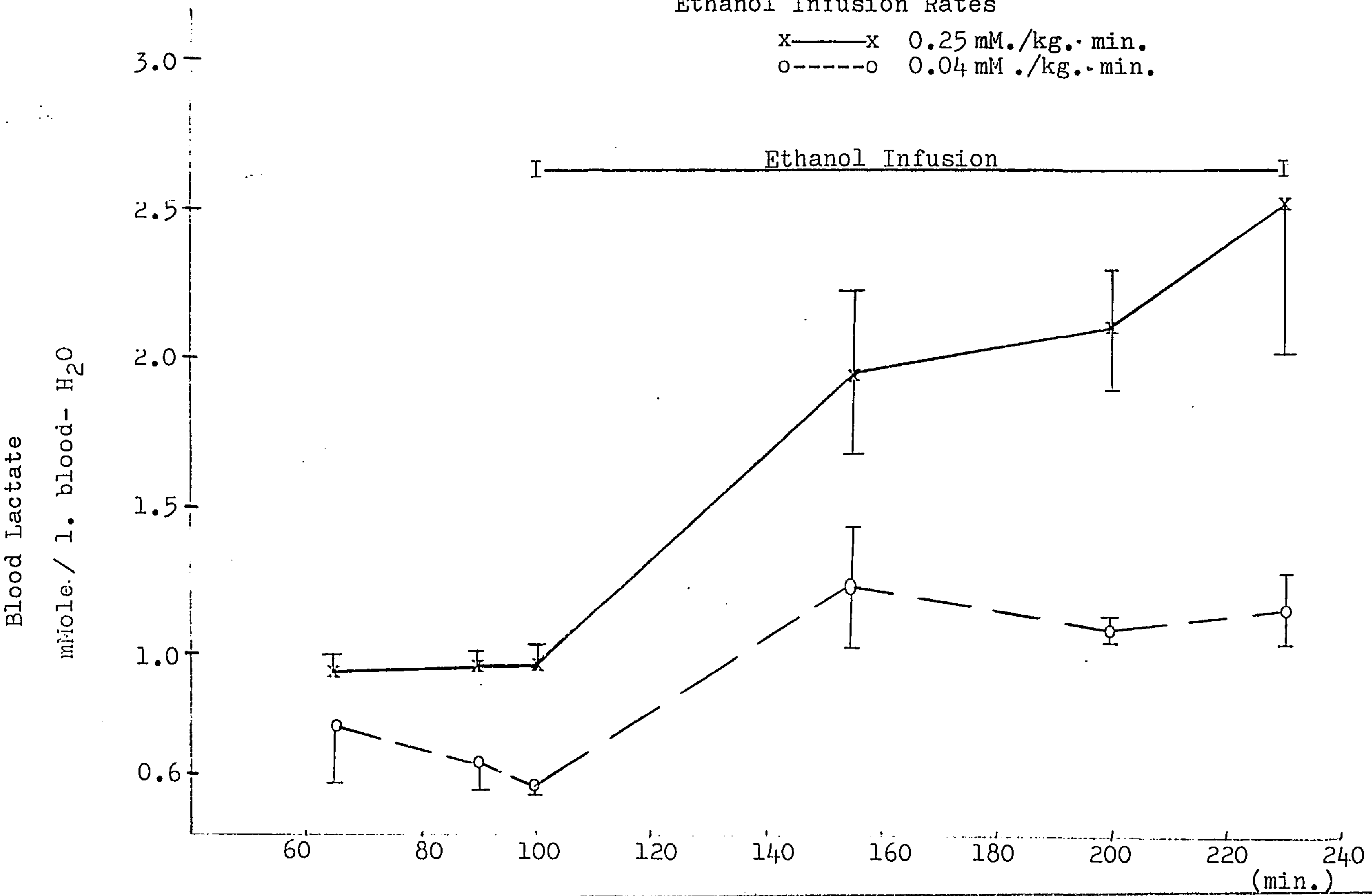

The Effect of Ethanol upon Blood Lactate Concentrations in Fasted Dogs
Figure 3
Ethanol Infusion Rates
x——x 0.25 mM./kg.· min.
o------o 0.04 mM ./kg.· min.
Ethanol Infusion
Blood Lactate
mMole./ l. blood- H_2O
3.0
2.5
2.0
1.5
1.0
0.6
60
80
100
120
140
160
180
200
220
240
(min.)

TABLE 5

CONCENTRATION OF LACTATE IN BLOOD (mMole/l blood-H_2O) BEFORE AND DURING A PRIMED INFUSION OF ETHANOL

State of Dog (rate of infusion)	Concentration of Lactate in Blood: Before Infusion	At Times from the Start of the Infusion: 55 min	100 min	120 min
1. Fasted (3) (0.25 mMole/kg·min)	0.93 ± 0.05	1.90 ± 0.27	2.05 ± 0.23	2.53 ± 0.55
2. Fasted (2) (0.04 mMole/kg·min)	0.64 ± 0.07	1.23 ± 0.24	1.08 ± 0.08	1.14 ± 0.12
3. Diabetic, post-absorptive (0.25 - 0.29 mMole/kg·min)	1.49 ± 0.19	1.92 ± 0.37	2.02 ± 0.37	------

Note: The priming dose of ethanol varied between 8.3 and 16.6 mMole/kg in group one, 2.0 and 2.4 in group two and 6.9 and 13.9 in group three.
Mean values and s.e.m.-s are shown.
Number of dogs used is in brackets.

decrease during the first 55 minutes of the infusion. ($F = 45.27$, $p < 0.01$;Table 6). Between 25 and 55 minutes after the start of the ethanol infusion, the concentration of plasma glucose was significantly lower in the dogs that received ethanol at the lower rate than in dogs to which more ethanol was infused ($t = 4.89$, $p < 0.01$) (Table 7).

The mean basal rate of hepatic glucose production for the three dogs (five experiments) was 2.04 ± 0.07 mg/kg·min. An analysis of variance carried out on the data on Ra before the infusion ($70 \leq t \leq 100$ min) and during the first forty minutes of the infusion in all five experiments (3 dogs) revealed a significant decrease in glucose production. ($F = 6.52$, $p < 0.05$) (Table 8). Compared to the Ra in the dogs that received ethanol at the higher rate, the Ra in the dogs to which less ethanol was infused was significantly less during the first 40 minutes of the infusion ($t = 4.94$, $p < 0.01$) (Table 9). Due to the small number of experiments, the data were not analyzed in more detail. However, after the first initial forty minutes of the ethanol infusion there was no significant difference in the rates of hepatic glucose production whether ethanol was infused at the high or low rate.

Plasma Radioimmunoreactive Insulin Concentrations (iRi)

TABLE 6

PLASMA GLUCOSE CONCENTRATION IN FASTED DOGS

TWO WAY ANALYSIS OF VARIANCE

experiments	1	2	3	4	5
pre-ethanol	96.17 ±3.98 (9)	89.88 ±3.01 (9)	111.29 ±3.93 (9)	100.04 ±2.29 (9)	102.62 ±4.94 (9)
ethanol*	92.79 ±1.85 (3)	88.73 ±2.98 (3)	102.91 ±5.14 (3)	89.99 ±3.75 (3)	87.38 ±8.88 (3)

* indicates that $0 \le t \le 55$ minutes was tested.
For simplicity, mean ± s.e.m.-s of n (number in brackets) is shown.
Note: Dogs 1,2 and 3 received 0.25 mMole/kg·min ethanol while dogs 4 and 5 received 0.04 mMole/kg min ethanol.

Source of Variation	degrees of freedom	sum of squares	mean square	F value
ethanol	1	735.97	735.97	45.27 $p < 0.01$
experiments	4	1577.75	394.44	24.26 $p < 0.01$
interaction	4	428.88	107.22	6.59
error	50	812.94	16.26	

TABLE 7

PLASMA GLUCOSE CONCENTRATIONS IN FASTED DOGS
UNPAIRED STUDENT'S t TEST*

High Ethanol Load	Low Ethanol Load
92.09	86.74
97.43	83.42
105.25	89.15
91.39	81.18
92.14	88.80
97.02	87.45
96.39	
95.24	
98.12	

$t = 4.89$, $p < 0.01$

* indicates that the plasma glucose concentrations in 5 experiments was tested during the first 55 minutes of the ethanol infusion, 0.25 and 0.04 mMole/kg·min.

TABLE 9

RATE OF HEPATIC GLUCOSE APPEARANCE IN FASTED DOGS
UNPAIRED STUDENT'S t TEST *

High Ethanol Load	Low Ethanol Load
1.98	1.40
2.29	1.08
1.80	1.53
2.09	1.17
2.25	
1.70	

$t = 4.94$, $p < 0.01$

* indicates that the rate of hepatic glucose production was tested during the first 55 minutes of the ethanol infusion, 0.25 and 0.04 mMole/kg·min.

TABLE 8

RATE OF HEPATIC GLUCOSE APPEARANCE IN FASTED DOGS
TWO WAY ANALYSIS OF VARIANCE

experiments	1	2	3	4	5
pre-ethanol*	1.97 1.66	1.54 2.54	2.00 2.06	1.92 2.42	2.03 2.49
ethanol**	1.40 1.53	1.08 1.17	2.29 2.09	1.98 2.25	1.80 1.70

* indicates that $70 \leq t \leq 100$ minutes was tested.

** indicates that $0 \leq t \leq 55$ minutes was tested.

Actual values are shown.

Source of variation	degrees of freedom	sum of squares	mean square	F value
ethanol	1	0.56	0.56	6.52 $p < 0.05$
experiments	4	1.13	0.28	3.30 $p < 0.10$
interaction	4	0.69	0.17	2.02
error	10	0.86	0.09	

Note: Dogs 1 and 2 received 0.04 mMole/kg·min ethanol while dogs 3, 4 and 5 received 0.25 mMole/kg min ethanol.

No appreciable change was seen in the plasma iRi concentrations when ethanol was infused at either 0.04 or 0.25 mMole/kg·min (Table 10)

C. THE EFFECT OF ETHANOL UPON HEPATIC GLUCOSE PRODUCTION IN DIABETIC DOGS

Estimated Rate of Ethanol Utilization

Ethanol was administered as a primed intravenous infusion whose rate varied between 0.25 and 0.29 mMole/kg·min. The mean ethanol concentration obtained during the infusion was 20.8 ± 2.5 mMole/l at t= 125 minutes and 38.4 ± 2.6 mMole/l at t= 200 minutes (Table 2). The rate of ethanol infusion exceeded the rate of ethanol utilization as shown by the steady increase of the concentration of ethanol in plasma during the infusion (Figure 4).

Table 2 indicates that the estimated rate of ethanol utilization in diabetic dogs was 0.091 ± 0.023 mMole/kg·min, approximately the same rate as seen in the normal animals in the postabsorptive state. Increased hepatic glucose production had no effect upon the rate of ethanol utilization.

Blood Lactate Concentrations

The lactate concentrations were not affected by the infusion of saline, the mean concentration in blood being 0.65 ± 0.04 mMole/l blood-H_2O. During the infusion of ethanol,

TABLE 10

EFFECT OF ETHANOL ON PLASMA RADIOIMMUNOREACTIVE INSULIN (iRi) CONCENTRATIONS (μU/ml) IN FASTED DOGS

Experiment	iRi Concentrations at t=			
	100	155	200	230 min
LF-1	5	5	5	3
LF-1*	9	4	5	5
LF-2	7	5	9	12
LF-3	6	1	4	3
LF-3*	9	3	1	4

Notes: iRi concentrations at t= 100 min are the mean of three control values, t= 65, t= 90 and t= 100 min.

All rates of ethanol infusion were 0.25 mMole/kg·min except those designated by * which were 0.04 mMole/kg·min.

Figure 4

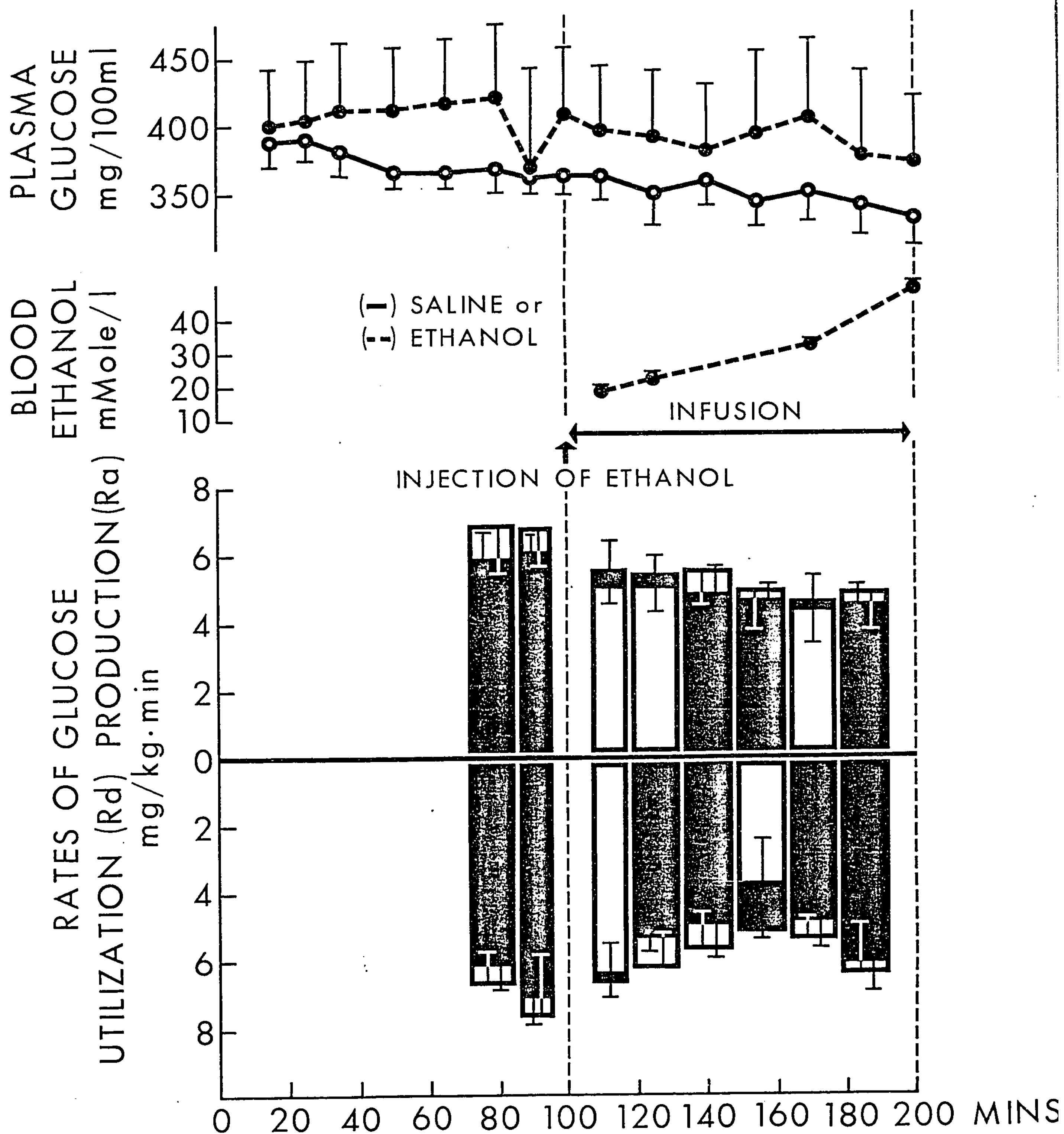


Figure 4: The effect of an intravenous infusion of ethanol or saline to diabetic dogs in the postabsorptive state. Abscissa: time in minutes. See Figure 2 for the description of the ordinates. Solid line: plasma glucose concentrations during the infusion of saline; broken line: same during the infusion of ethanol to the same dogs. Total heights of the columns: Ra or Rd during the infusion of saline only; white columns: Ra or Rd during the infusion of saline plus ethanol (6.9 - 13.9 mMole/kg priming dose followed by 0.25 - 0.29 mMole/kg min). If in the latter type of experiment, Ra or Rd exceeds the respective value found in the saline experiment, the white column appears in excess of the black one and the s.e.m.-s are pointing towards each other. The infusion was given between t= 100 and t= 200 minutes as indicated by the perpendicular broken lines. Average values of 6 pairs of experiments ± s.e.m.-s are shown.

hyperlactacidemia became evident (Figure 5). Upon the administration of ethanol, the blood lactate concentrations rose from the basal 1.49 ± 0.19 mMole/l blood-H_2O to 1.92 ± 0.37 and 2.01 ± 0.31 mMole/l blood-H_2O fifty-five and one hundred minutes respectively after the beginning of the ethanol infusion (Table 5).

Rates of Glucose Production and Utilization

All pancreatectomized dogs had blood sugar levels in excess of 200 mg/dl. The variation of the mean basal plasma glucose concentration between dogs is very high as evidenced by the plasma glucose concentration versus time curves in Figure 4. No significant difference was found in plasma glucose concentrations whether ethanol or saline was infused (Table 3).

The mean basal rate of glucose production for the six pancreatectomized dogs (12 experiments) was 6.40 ± 0.63 mg/kg.min (Table 11). During the infusion of ethanol, the decrease in Ra was not significantly different from that seen during the infusion of saline (Figure 4, Table 3). The overall rate of glucose disappearance (Rd) from the plasma was not affected by the infusion of ethanol.

The extra release of glucose is indicative of the changes in the rate of glucose production which in the pancreatectomized dog reflect changes in hepatic gluconeogenesis Figure 6

Figure 5

Blood Lactate Concentrations in Pancreatectomized Dogs

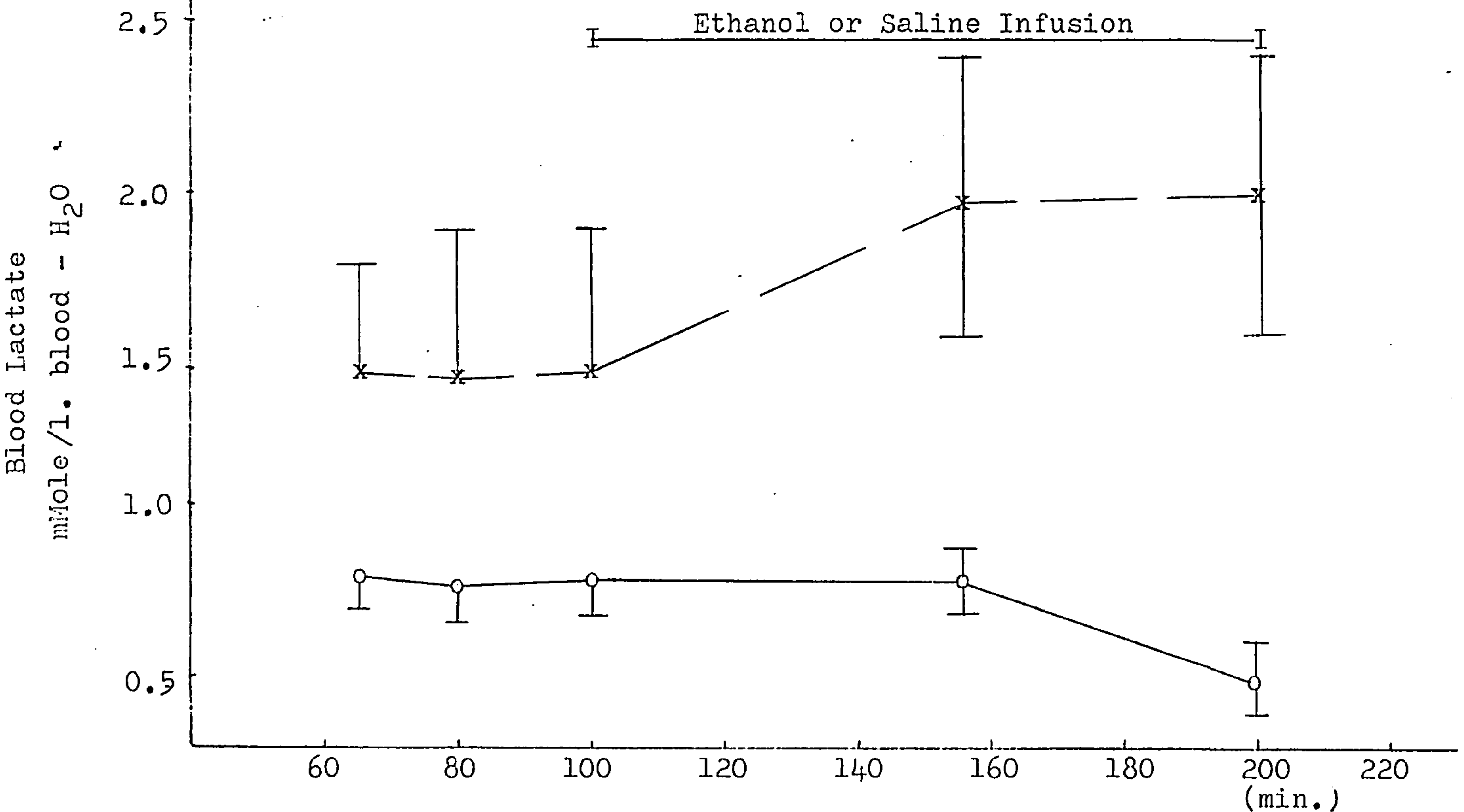


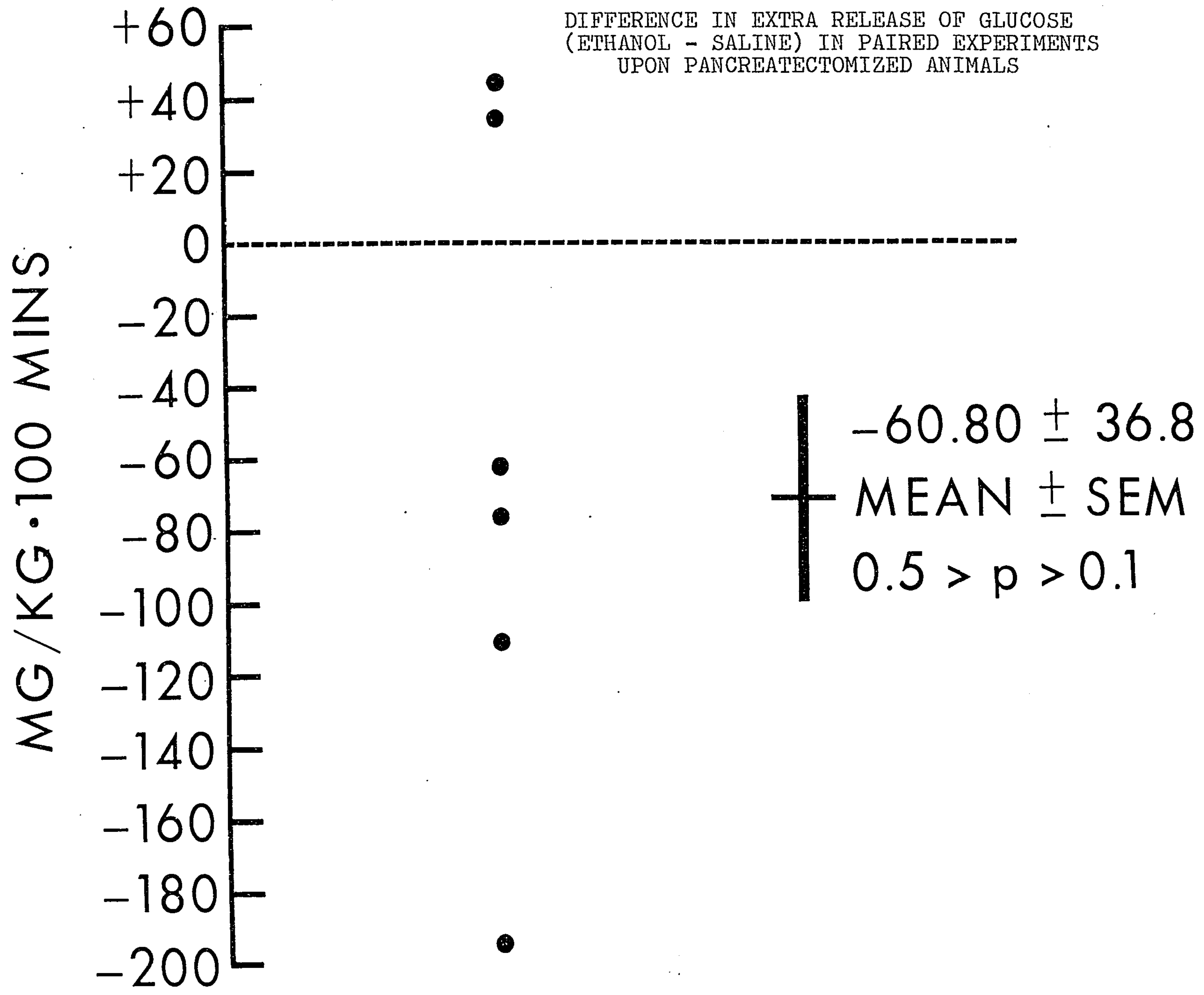
TABLE 11

EFFECT OF ETHANOL ON HEPATIC GLUCOSE PRODUCTION IN DIABETIC DOGS

Experiment	Ethanol Infused	Basal Ra of Glucose (mg/kg·min)	Extra Release of Glucose (mg/kg·100 min)	Difference in "Extra Release" between Paired Experiments (Ethanol -Saline (mg/kg·100 min)
LD-3A	-	5.09	-7.7	-76.9
LD-3B	+	6.05	-84.6	
LD-4B	-	4.11	-185.3	+35.5
LD-4A	+	3.88	-149.8	
LD-6A	-	8.76	-296.0	+43.6
LD-6B	+	11.12	-252.4	
LD-7B	-	5.18	+41.3	-194.1
LD-7A	+	5.24	-152.8	
LD-8B	-	5.78	-147.0	-110.7
LD-8A	+	9.02	-257.7	
LD-9A	-	6.84	-117.3	-62.7
LD-9B	+	5.87	-179.4	
mean		6.40	-	-60.8
± s.e.m.		±0.63	-	±36.8
				(t= 1.65)

Figure 6

DIFFERENCE IN EXTRA RELEASE OF GLUCOSE (ETHANOL - SALINE) IN PAIRED EXPERIMENTS UPON PANCREATECTOMIZED ANIMALS



indicates that four of the six dogs tested showed a negative extra release (ethanol - saline). The mean difference in the extra release was -60.80 $\pm$ 36.8 mg/kg·100 min. Although the overall response of the liver to the infusion of ethanol indicates possible interference with gluconeogenesis, the effect of ethanol is not significant due to the variability of the response ($p > 0.5$) (Table 11). The nonparametric tests of Wilcoxon (Snedecor, 1956) and Gnedenko (Hetenyi, 1955) also failed to indicate a significant effect of ethanol upon glucose production.

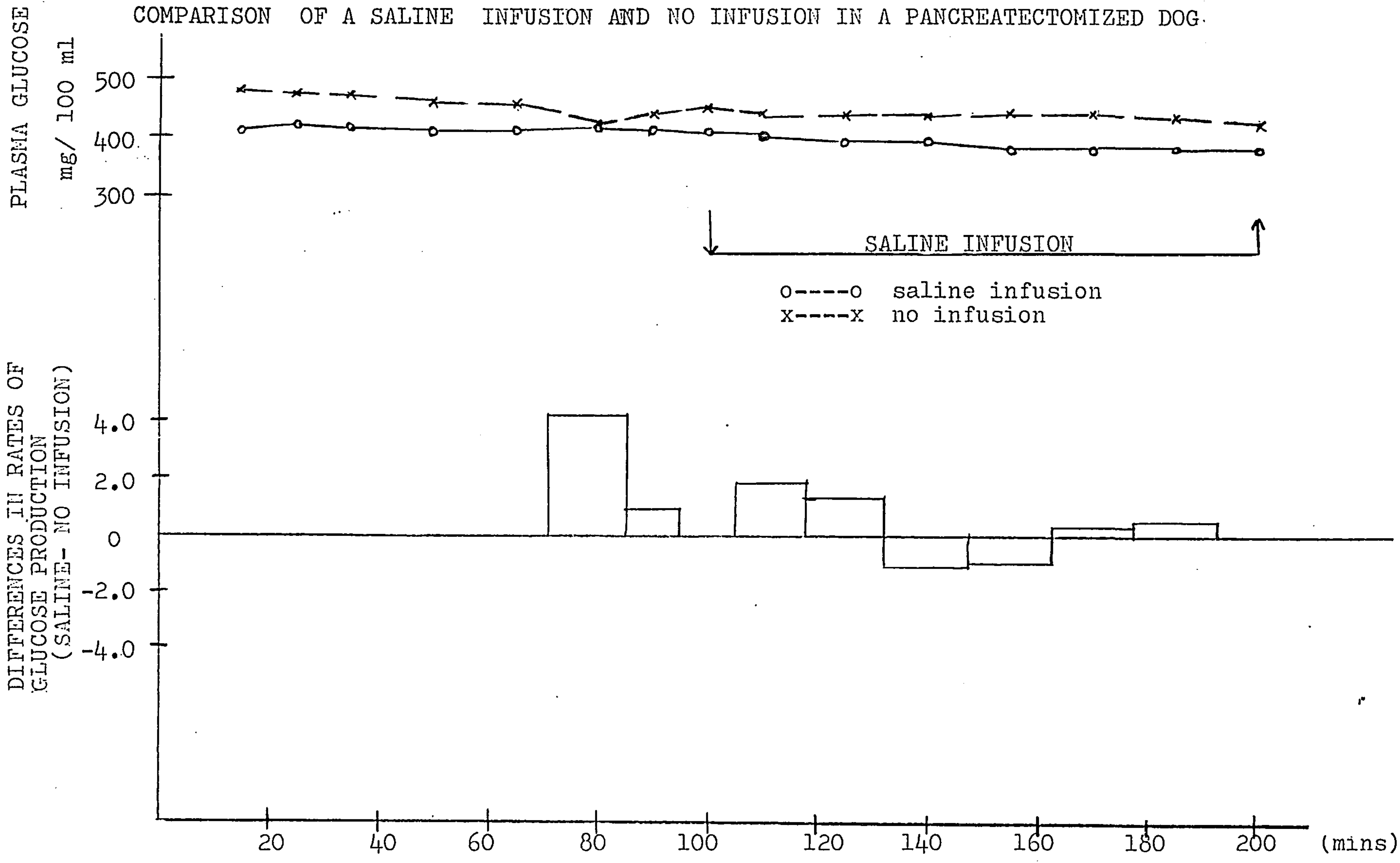
Validity of Saline Control

In an attempt to establish whether the infusion of saline at the rate employed (0.62 ml/min) alters the rate of glucose release, a single experiment was carried out in which neither ethanol nor saline was infused during the course of the experiment.

When the above experiment was compared to one in which saline only was infused to the same dog, no significant difference was found in plasma glucose concentrations nor in the rates of glucose production or utilization (Figure 7). Therefore experiments in which saline was infused represent a valid control for the experiments on pancreatectomized animals.

Figure 7

COMPARISON OF A SALINE INFUSION AND NO INFUSION IN A PANCREATECTOMIZED DOG



D. EFFECT OF ETHANOL UPON THE HEPATIC RESPONSE TO HYPOGLYCAEMIA

Estimated Rate of Ethanol Utilization

The mean ethanol concentration (calculated between t= 125 and t= 200 min) obtained during the infusion of ethanol was 31.8 ± 3.3 mMole/l and the peak ethanol concentration obtained at the termination of the ethanol infusion (at t= 200 min) was 42.7 ± 3.9 mMole/l (Table 2). The slope of the ethanol versus time curve is positive indicating that the rate of ethanol infusion exceeded the rate of ethanol utilization (Figure 8).

The estimated rate of ethanol utilization was 0.107± 0.013 mMole/kg·min which is comparable to the rate observed in the postabsorptive state at normal plasma glucose concentrations (Table 2).

Rates of Glucose Production and Utilization

Upon the infusion of 4 - 7 mU/kg·min insulin, the basal plasma glucose concentrations of 101 to 109 mg/dl dramatically fell and eventually stabilized between 40 and 45 mg/dl. The degree of hypoglycaemia was comparable whether ethanol was infused along with the insulin or not (Figure 8, Table 3).

As shown on Table 12, the mean rate of hepatic glucose production was 2.99 ± 0.20 mg/kg·min when ^{3}H-3-glucose was used as tracer and 3.52 ± 0.21 mg/kg·min when ^{3}H-2-glucose was

Figure 8

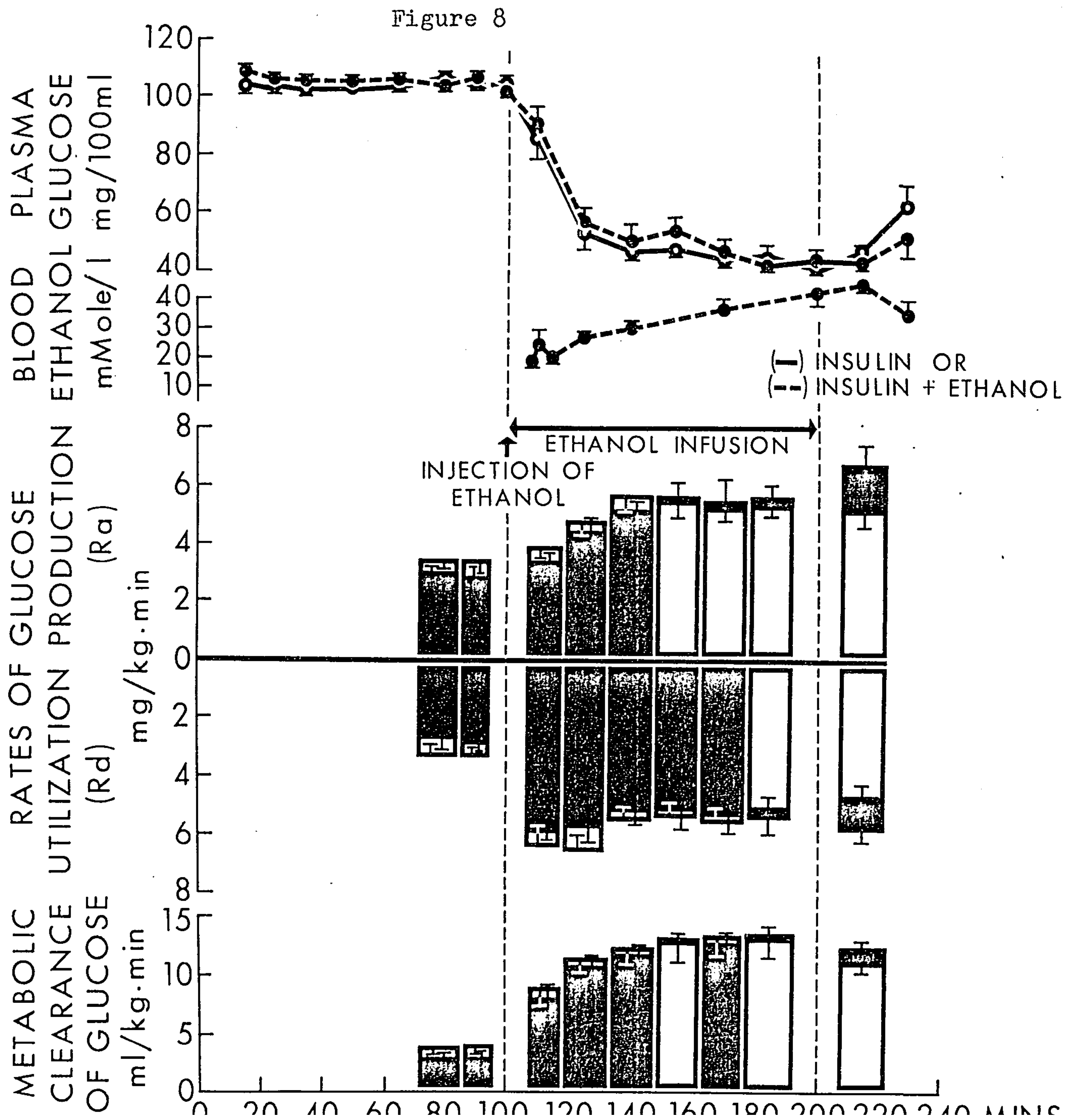


Figure 8: The effect of an intravenous infusion of insulin or insulin and ethanol in the postabsorptive state. Abscissa: time in minutes. See Figure 2 for the description of the ordinates. Solid line: plasma glucose concentrations during the infusion of insulin; broken line; same during the infusion of ethanol plus insulin. Total heights of the columns: Ra, Rd of MCR_g during the infusion of insulin only; white columns: Ra, Rd or MCR_g during the infusion of ethanol and insulin (ethanol administered as a 5.4 - 18.1 mMole/kg priming dose followed by 0.21 - 0.35 mMole/kg·min). If in the latter type of experiment, Ra, Rd or MCR_g exceeds the value found in the insulin experiment, the white column appears in excess of the solid one, and the s.e.m.-s are pointing towards each other. The infusion of ethanol or ethanol plus insulin was given between t= 100 and t= 200 minutes as shown by the perpendicular broken lines. Mean of nine pairs of experiments ± s.e.m.-s are shown.

TABLE 12

EFFECT OF ETHANOL ON THE RATE OF HEPATIC GLUCOSE PRODUCTION DURING INSULIN-INDUCED HYPOGLYCAEMIA

Experiment	Ethanol Infused	Basal Ra of Glucose (mg/kg·min)	Extra Release of glucose (mg/kg·100 min)	Difference of Extra Release between paired experiments (mg/kg·100 min)
1A	-	2.74	395.7	-77.1
1B	+	3.98	318.6	
2A	-	2.45	164.1	-186.6
2B	+	4.58	-22.5	
3A	-	2.47	133.3	-13.2
3B	+	2.38	120.1	
4A	+	3.51	-5.5	-85.3
4B	-	3.19	79.8	
5A	-	2.55	299.2	+73.3
5B	+	2.77	372.5	
6A	-	2.68	164.9	+141.2
6B	+	2.52	306.1	
11A*	-	4.59	214.3	-48.0
11B*	+	4.02	166.3	
12A*	-	3.59	154.6	-26.9
12B*	+	2.83	127.7	
13A*	-	2.68	101.3	+16.3
13B*	+	3.42	117.6	
mean		2.99**	-	-23.0
± s.e.m.		±0.20	-	± 31.6

* In experiments 11, 12 and 13 ^{3}H-2-glucose was used as tracer.
** Mean of experiments 1-6 in which ^{3}H-3-glucose was used as tracer.

used. Upon the induction of hypoglycaemia with insulin, the Ra increased significantly over the basal levels whether ethanol was infused or not (Table 3). Approximately thirty-five minutes after the beginning of the insulin infusion, the elevated Ra stabilized and remained steady until the termination of the infusion. A simultaneous infusion of ethanol did not affect the "hepatic response to hypoglycaemia". During the final thirty minutes of the experiments, when the insulin infusion was terminated (i.e. changed to one of saline), both the rate of glucose production and the plasma level increased (Figure 8). The presence of ethanol in blood appears to have blunted this response (Figure 8).

Ethanol had no significant effect upon the rate of glucose utilization (Figure 8).

The basal metabolic clearance rate of glucose increased approximately five fold upon the infusion of insulin. Once the infusion of insulin was terminated, the metabolic clearance rate of glucose decreased. Ethanol did not affect either of these phenomena (Figure 8).

Extra Release of Glucose

The extra release of glucose is a measure of the response of the liver to hypoglycaemia. Table 12 illustrates the actual extra release for each experiment as well as the absolute differences in extra release (ethanol and insulin - insulin)

for each paired experiment. Six of the nine dogs tested showed a decrease in extra release when ethanol was infused in conjunction with insulin (Figure 9). The mean difference in extra release was -22.94 ± 31.6 mg/kg.100 min (Figure 9). The effect of ethanol upon the hepatic response to hypoglycaemia is not significant ($p > 0.1$) due to the variability of the response. The nonparametric tests of Wilcoxon (Snedecor, 1956). and Gnedenko (Hetenyi, 1955) verify that the effect of ethanol upon the hepatic response to hypoglycaemia is not significant.

Plasma Radioimmunoreactive Insulin Concentrations (iRi)

When ethanol was intravenously infused at the rate of 7 mU/kg min, the iRi concentrations were consistently greater in the presence of ethanol (Figure 10). Mean plasma iRi concentrations were 274.6 ± 72.5 and 248.2 ± 63.7 µU/ml forty and one hundred minutes after the beginning of the insulin infusion. The respective concentrations obtained during the simultaneous infusion of ethanol and insulin were 345.4 ± 63.7 and 335.0 ± 68.7 µU/ml (Figure 10). In spite of the marginally higher plasma iRi concentrations in the presence of ethanol, the metabolic clearance rate of glucose was not greater than those calculated in the experiments where insulin alone was infused (Figure 8).

Figure 9

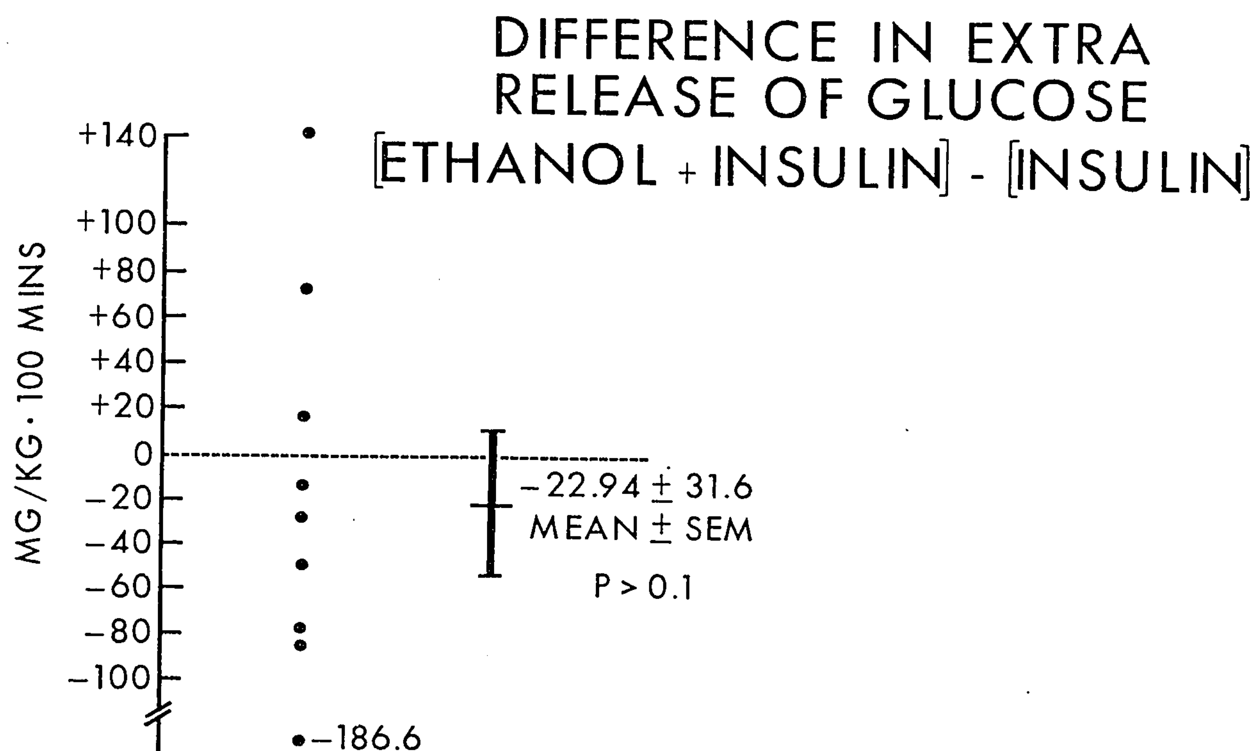


Figure 10

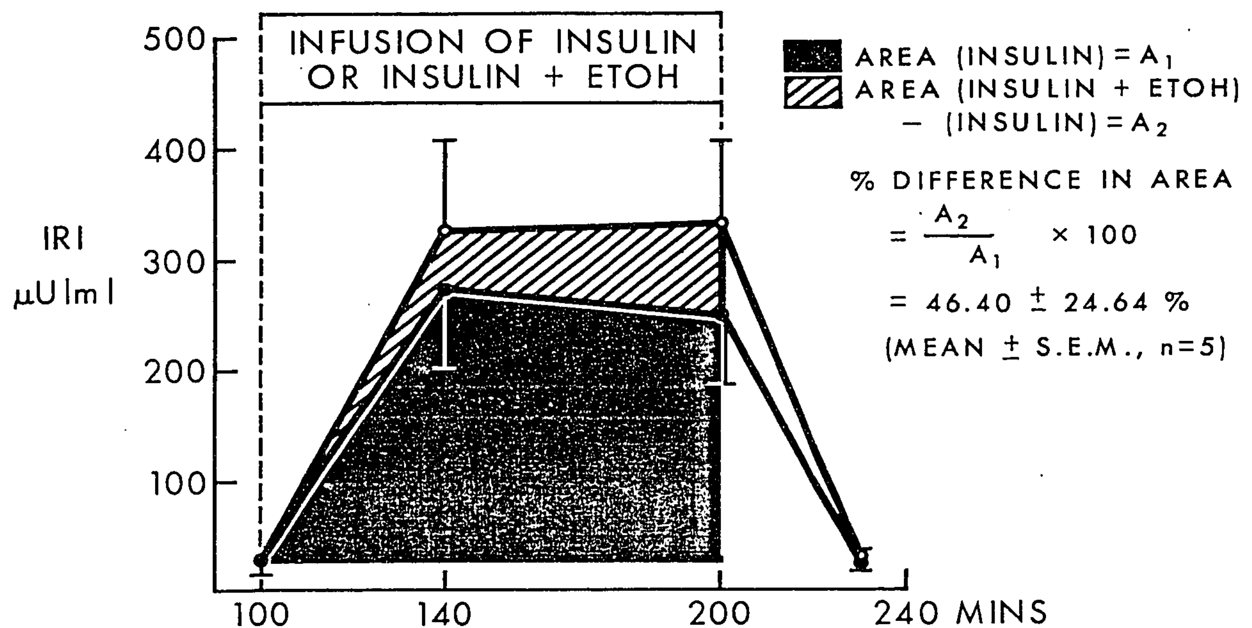


Figure 10: The effect of ethanol upon plasma iRi concentrations during insulin-induced hypoglycaemia in the postabsorptive state. Abscissa: time in minutes. Ordinate: iRi (uU/ml). The solid area indicates the area below the iRi versus time curve during the infusion of insulin. The hatched area equals the difference between the afore mentioned and the area under the iRi versus time curve during the infusion of insulin and ethanol. S.E.M.-s are shown, n= 5. The infusion of insulin or insulin and ethanol took place between t= 100 and t= 200 minutes as indicated by the broken perpendicular lines.

CHAPTER V

DISCUSSION

A. TRACER METHODOLOGY

The rate of hepatic glucose production (predominantly glycogenolysis in the postabsorptive state and hepatic gluconeogenesis in the fasted or pancreatectomized animal) was measured using the isotope dilution principle. The calculation of hepatic glucose production depends upon the observed dilution of circulating labelled glucose with unlabelled glucose released by the liver (de Bodo et àl, 1963).

This method of measuring glucose production and utilization was used since it is noninvasive and easily carried out on trained, conscious dogs.

Both the pathways of glucose synthesis and breakdown share several reactions; thereby, allowing for the cycling of glucose between two metabolites. Such metabolic cycles involve the loss of energy since ATP is reguired in one direction but not in the opposite direction (e.g. glucose conversion to glucose-6-phosphate requires ATP whereas glucose-6-phosphate to glucose does not). Thus they are termed "futile cycles" (Larner 1971). Some isotopes labelled at specific sites can lose their label in these futile cycles thus giving spuriously high values for glucose turnover. For example, ^{3}H-2-glucose loses its label in the reversible interconversion step of glucose-6-phosphate and fructose-6-phosphate. The glucose is phosphorylated,

isomerized and cycled back to glucose-6-phosphate and the label is lost. Glucose labelled in the third position with tritium does not lose its label in this glycolytic step. However, if there was an active futile cycle operating between fructose-6-phosphate and fructose-1,6-diphosphate, ^{3}H-3-glucose would lose its label in this cycle. In the dog, at least in the postabsorptive state, little of the glucose taken up by the liver is re-converted from these glycolytic intermediates; therefore, very little of the ^{3}H-3-glucose can lose its label in this way. ^{3}H-3-glucose gives a very good estimate of glucose turnover. ^{3}H-2-glucose gives estimates of glucose turnover which are approximately 1.5 times that derived using ^{3}H-3-glucose whose values are identical to those calculated using ^{14}C-6-glucose after correction for recycling (Altszuler et al, 1975).

The isolation of glucose labelled with tritium in the second or third position is very straightforward. The content of the filtrate obtained by deproteinization with $Ba(OH)_2$ and $ZnSO_4$ contains all label as ^3H_2O or ^{3}H-labelled glucose. A one ml aliqout of the filtrate is evaporated; thereby, eliminating all the ^3H_2O and then reconstituted with one ml of H_2O prior to the addition of 10 ml of Bray's scintillation fluid (Hetenyi and Mak, 1970).

B. ETHANOL AND ITS METABOLIC EFFECTS

Ethanol is endogenously synthesized only in trace amounts; therefore, ethanol is a compound that is not normally found in any significant quantities within the body. Up to 98% of the ingested ethanol is metabolized within the liver, the organ intimately involved with glucose homeostasis. There is no feedback mechanism present within the body to adjust the rate of ethanol oxidation to the metabolic state of the hepatocyte; consequently, ethanol administration produces striking metabolic imbalances within the liver cell which are reflected by hyperlactacidemia, hyperuricemia, acidosis, enhanced lipogenesis and depressed lipid oxidation (Lieber, 1976).

The effects of ethanol upon the intermediary metabolism of the liver depends upon the amount of ethanol ingested and upon the nutritional state of the subject (Lieber 1976; Wolfe et al, 1976; Guynn and Pielik, 1975).

Liver glycogen stores are not severely affected after an overnight fast; therefore, glycogen can still play a major role in the maintenance of plasma glucose concentrations (Field et al, 1963). The glycogenolytic action of glucagon is not affected by ethanol at least in vitro (Field et al, 1963); furthermore, ethanol increases epinephrine secretion from the adrenal medulla (Klingman and Goodall, 1958; Perman, 1958).

Epinephrine facilitates glycogen breakdown and its conversion to glucose as well as acting directly upon the beta cells of the pancreas to decrease insulin secretion. (Ganong, 1967). In the postabsorptive state, plasma glucose concentrations and the rates of hepatic glucose production and utilization as well as the metabolic clearance rate of glucose were not affected by ethanol administration (Figure 1). The insulin concentration in plasma was not significantly changed by the administration of ethanol (Table 4). These findings are in accordance with those reported by Tennent (1941) and Lieber et al (1962) for ethanol administration after an overnight fast.

Unlike an overnight fast, liver glycogen stores are severely diminished by prolonged fasting (Felig et al, 1969) or in diabetes. The main source of plasma glucose during fasting or in diabetes is hepatic gluconeogenesis although in the two states rates of hepatic glucose production are very different. (Cowan et al, 1969; Hetenyi et al, 1961). The maintenance of glucose homeostasis during starvation requires the availability of an effective enzymatic system and a steady flow of precursor substrate (Felig et al, 1969).

Both the formation of pyruvate as well as its entrance into gluconeogenesis depend upon NAD^+. Gluconeogenic substrates

such as lactate and glutamate depend upon NAD^+ for their eventual oxidation to pyruvate. In order for pyruvate to enter the gluconeogenic pathway, it must be converted to phosphoenolpyruvate. This conversion involves the mitochondrial shuttles which are dependant upon substrates such as malate, aspartate and glycerol-3-phosphate (Meijer et al, 1975). These mitochondrial shuttles are also involved with the elimination of reducing equivalents. Ethanol administration would shift the NAD^+:NADH ratio in favour of NADH, and the activity of the mitochondrial shuttles would be increased in an attempt to eliminate the excess reducing equivalents produced by ethanol oxidation. The rate of ethanol utilization in the animal fasted for forty-eight hours is significantly less than normal (Smith and Newman, 1959) reflecting an inadequate operation of the mitochondrial shuttles due to decreased concentrations of malate and aspartate (Meijer et al, 1975). Ethanol administration to animals whose main source of plasma glucose is gluconeogenesis places an added burden upon the mitochondrial shuttles since pyruvate must not only be converted to phosphoenolpyruvate but excess H produced as a result of ethanol oxidation must be eliminated.

In vitro, ethanol has been shown to inhibit gluconeogenesis (Field et al, 1963). *In vivo*, in humans the concentration of plasma lactate doubled upon the ingestion of ethanol. In spite of this, the turnover rate of lactate remained unchanged and thus its metabolic clearance rate was decreased. This shows a diminished capactity of the liver and the heart to remove lactate from the circulation. This decreased capacity to remove

lactate may reflect a different route of metabolism in the liver as a result of which, less glucose was synthesized from lactate. This would account for Kreisberg's (1971) finding of a lesser conversion of lactate to glucose carbon. Such a decrease in gluconeogenesis indeed may account for the hypoglycaemia observed. Alcohol hypoglycaemia has also been observed in fasted dogs (Madison et al, 1968) although the effect of ethanol upon glucose concentrations is variable (Bleicher et al, 1964).

After a three day fast in humans, plasma glucose concentrations are reduced (Wolfe et al, 1976). Lochner et al (1967) observed in dogs with chronic end to side portal caval shunts decreases in both pre-ethanol venous and arterial plasma glucose concentrations although neither a four nor eight day fast produced comparable results in this study. Two way analysis of variance of the data of plasma glucose concentrations showed a significant effect of ethanol (high and low dose combined) with respect to control values (F=45.27, p 0.01). Low dose ethanol administration lowered the plasma glucose concentration during the first fifty-five minutes of ethanol infusion more than high ethanol loads did (t=4.89, p 0.01). In comparison to high ethanol concentrations, low blood ethanol levels induced a short lived moderate hypoglycaemia due to decreased rates of hepatic glucose production (t-4.94, p 0.01). Lochner et al (1967) reported an extended period of hypoglycaemia upon low dose ethanol administration although the data can be questioned due to the use of anaesthesia and unusual surgical procedures. It has been suggested and

and demonstrated by Searle et al.(1974) that peripheral glucose utilization increases upon ethanol administration to humans fasted for three days. There appeared to be no significant increase in peripheral glucose utilization in this study (Figure 2). As Searle et al (1974) reported, fifty-five minutes after the beginning of the ethanol infusion (0.04 mMole/kg.min) to dogs fasted for four days, the hepatic glucose production escaped the inhibition imposed by ethanol and plasma glucose concentrations returned to pre-ethanol levels (Figure 2). Plasma insulin concentrations remained the same or fell slightly in accordance with that already reported (Searle et al, 1974) and blood lactate concentrations rose upon ethanol administration as found also by Kreisberg et al. (1971). Even though the basal lactate concentration in the four day fasted dogs is variable (Table 5), the levels determined fall within the normal range for the dog. (Cain, 1965).

In the fasted animals to which the small load of ethanol (0.04 mMole/kg.min) was infused, blood lactate concentrations rose although after 120 minutes the lactate level was not markedly different from the basal level obtained for fasted dogs receiving higher ethanol loads (0.25 mMole/kg min) (Table 5). In contrast, the blood lactate concentrations rose throughout the 120 minute high dose ethanol infusion and

the rise was comparable to that detected upon low dose ethanol administration. According to the kinetics of alcohol dehydrogenase, the rise in blood lactate concentrations should have been larger in the dogs administered the lower ethanol load (Krebs, 1968).

In contrast to the four day fasted dogs receiving low doses of ethanol, the fasted dogs when receiving high ethanol loads did not exhibit even the small transient hypoglycaemia. At high blood ethanol concentrations, alcohol dehydrogenase is inhibited by its own substrate (Krebs, 1968). Therefore, as the blood ethanol concentration gradually increases, the blood lactate concentration is expected first to increase then stabilize and finally decrease in reflection of a decreasing production of reducing equivalents that must be eliminated by the mitochondrial shuttles. Even though the blood lactate concentrations did not follow the expected pattern, the mitochondrial shuttles whose operation is already impaired (reflected by below normal rates of ethanol utilization, Table 2) are not severely taxed by high ethanol loads. Also when blood ethanol concentrations are high, a greater proportion of the ethanol is metabolized in the MEOS pathway. This pathway does not require NAD^+; thus, does not generate NADH. Under these conditions, the competition for substrates common to the shuttles and gluconeogenesis would be eleviated and hypoglycaemia would not develop.

An insulin-deprived diabetic dog resembles a dog after prolonged fasting since in both liver glycogen stores are low and all glucose released by the liver has to originate from gluconeogenesis. The major difference between fasted and

diabetic dogs lies in the rate at which glucose is released by the liver. In diabetes, the increased rate of gluconeogenesis increases the demand for NAD^+ and the activity of the mitochondrial shuttles would be elevated to allow for the efficient conversion of pyruvate to phosphoenolpyruvate and its eventual entrance into the gluconeogenic pathway. Upon the administration of ethanol to the diabetic animal, the demand for NAD^+ is further increased and the mitochondrial shuttles must contend not only with an increased rate of conversion of pyruvate to phosphoenolpyruvate but also with the elimination of excess reducing equivalents produced via the oxidation of ethanol to acetate.

Upon the administration of larger ethanol loads (0.25 - 0.39 mMole/kg.min) to insulin-deprived pancreatectomized dogs, the estimated rate of ethanol utilization was comparable to that already determined for animals in the postabsorptive state (Table 2) indicating an abundance of both cytosolic and mitochondrial NAD^+. The lactacidemia produced by the administration of ethanol to diabetic dogs reflects a reduction of NAD^+ to NADH yet the plasma glucose concentration and the rate of hepatic glucose production during the infusion of ethanol paralleled that during the infusion of saline to the same animals. Even though the mitochondrial shuttles were taxed by an elevated rate of hepatic glucose production

and the elimination of excess reducing equivalents produced in ethanol oxidation, sufficient concentrations of malate, aspartate and glycerol-3-phosphate were present to allow for the efficient removal of reducing equivalents and gluconeogenesis was not impaired.

Pancreatectomized animals are volume contracted and the infusion of saline is paralleled by an increase in the size of the glucose space; therefore, the plasma glucose concentration declines (Figure 4) as reported by Cowan (Ph.D thesis, University of Toronto, 1969).

The large dose of insulin which was infused to dogs in the postabsorptive state produced the typical hepatic response to hypoglycaemia as reported by Hetenyi (1972). Ethanol administration (0.21 - 0.35 mMole/kg·min) resulted in estimated rates of ethanol utilization which were comparable to those seen in the postabsorptive state. This finding is in disagreement with the work of Newman and Smith (1959) who reported a 21% increase in the rate of ethanol utilization upon the subcutaneous injection of one I.U. per kg insulin. The plasma glucose concentrations, the rate of hepatic glucose production and the metabolic clearance rate of glucose were not affected by ethanol administration. Since ethanol did not interfere with the hepatic response to hypoglycaemia,

neither glycogen breakdown nor gluconeogenesis (perhaps occurring during the latter phases of insulin administration) were disrupted by ethanol. Unlike mannose, ethanol or its metabolic products can not replace glucose as the combustible substrate for the brain since the increase in the rate of hepatic glucose production during insulin-induced hypoglycaemia was neither diminished or abolished upon ethanol administration.

In each of the paired experiments in which hypoglycaemia was induced by insulin with or without ethanol, the dose of insulin was comparable being given on a weight basis. Even though large dose insulin administration suppresses endogenous insulin release (Rappaport et al, 1972), in five of the six paired experiments the plasma insulin concentrations were greater when ethanol was administered in conjunction with insulin (Figure 10). In spite of the higher insulin concentrations reached during the infusion of insulin and ethanol, the increase in the metabolic clearance rate of glucose was not increased. This points to a marginal decrease in insulin sensitivity in the presence of ethanol.

There are three possible explanations for the higher plasma insulin concentrations in the presence of ethanol: a) the blood ethanol concentrations obtained (42.7 3.9 mMole/l) may decrease hepatic blood flow; b) the enzymes involved with insulin degradation in the dog are NAD^+ dependent, and finally c) ethanol diminishes the rate of utilization of

insulin perhaps by altering membrane structure; thus disrupting the configuration of the insulin receptors making them biologically inactive. Studies involving the effect of ethanol upon mean hepatic blood flow have so far been inconclusive. Stein et al (1963) and Mendeloff (1964) reported that blood ethanol concentrations in the range of 3.04 - 22.36 mMole/l elevated the mean hepatic blood flow in man while a narcotizing dose of ethanol to anaesthetized dogs resulted in no change in mean hepatic blood flow (Symthe et al, 1953). The literature concerning the effect of ethanol upon the mean hepatic blood flow alone is unable to explain the elevated plasma insulin concentrations in the presence of ethanol.

Turning to the second possibility, in the dog insulin is cleared mainly by the kidney and liver (Franckson and Ooms, 1973). The insulin is first split at the disulfide bonds into A and B chains by glutathione-insulin transhydrogenase which is largely present in the microsomes with a small portion located in the plasma membrane fraction. A/B chain proteases which are found in the isolated plasma membrane fraction disrupt the chains into the resultant polypeptides (Varandani and Nafz, 1976). Glutathione-insulin transhydrogenase is $NADPH^+$ dependent (Katzen and Tietze, 1966) while the proteases are not NAD^+ dependent.

Ethanol is a lipid solvent (White et al, 1973) and

thereby would disrupt the lipid layers of the cell membranes. Thus it appears that the most likely cause of the greater insulin concentrations in the plasma in the presence of ethanol would be the direct effect of ethanol upon the mechanism by which insulin is removed from the blood.

SUMMARY

1. The infusion of ethanol (final blood ethanol concentration of 37.9 ± 3.4 mMole/l) did not affect the rate of hepatic glucose production in a dog in the postabsorptive state. Therefore, glycogenolysis was not affected by ethanol.
2. A moderate and transient hypoglycaemia was produced when ethanol was infused at a rate of 0.04 mMole/kg min (final blood ethanol concentration of 4.5 ± 0.4 mMole/l) to dogs fasted for four days. Hypoglycaemia appears to be related to an inhibition of gluconeogenesis specifically at the level of the "mitochondrial shuttles", the mechanism responsible for the elimination of reducing equivalents from the cytosol. The dogs fasted for four and eight days and to which ethanol was infused at a rate of 0.25-0.31 mMole/kg.min did not exhibit hypoglycaemia. This might have been due to an inhibition of alcohol dehydrogenase by high blood ethanol concentrations which in turn reduced the demand upon the mitochondrial shuttles to eliminate excess reducing equivalents.
3. In two dogs fasted for four days, the rate of hepatic glucose production was significantly less when the blood ethanol concentrations were low rather than when they were

elevated.

4. The rate of glucose production, a reflection of gluconeogenesis in the pancreatectomized animal was not affected by ethanol when the final blood ethanol concentration reached 38.4 ± 2.6 mMole/l. Apparently the activity of the mitochondrial shuttles was adequate to regenerate sufficient amounts of NAD^+ for both the operation of gluconeogenesis and ethanol oxidation.
5. Ethanol administration (final blood ethanol concentration of 42.7 ± 3.9 mMole/l) did not affect the "hepatic response to hypoglycaemia". It appears that ethanol could not replace glucose as a combustible substrate for the brain thereby reducing or abolishing the hepatic response to hypoglycaemia.
6. During insulin-induced hypoglycaemia and diabetes, the estimated rate of ethanol utilization was comparable to that in an animal in the postabsorptive state: 0.107 ± 0.013 mMole/kg·min, 0.091 ± 0.023 mMole/kg.min and 0.130 ± 0.008 mMole/kg·min respectively. However, fasting significantly reduced the estimated rate of ethanol utilization whether the dog received a high or low ethanol load: 0.067 ± 0.009 mMole/kg·min and 0.016 ± 0.005 mMole/kg·min respectively.

CHAPTER VI

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CHAPTER VII

APPENDIX I

REAGENTS

A. Plasma Glucose Determination

1. $Ba(OH)_2$

   Add 47.3 g $Ba(OH)_2$ to 1 liter distilled H_2O. When dissolved filter and cap.

2. $ZnSO_4$ $7H_2O$

   Add 50.0 g $ZnSO_4$ $7H_2O$ to 1 liter distilled H_2O.

3. 1 M Phosphate Buffer

   Add 93.7 g of Na_2HPO_4 and 46.9 g NaH_2PO_4 to 1 liter distilled water and adjust pH to 7.0 .

4. Mixed Enzyme Reagent

   Add 12.5 mg glucose oxidase and 5.0 mg horse radish peroxidase to 100 ml 0.2 M phosphate buffer.

5. Chromagen

   Dissolve 100 mg 3-3-dimethoxybenzidine in 10 ml of 95% ethanol.

6. Mixed Enzyme Dye Reagent

   Add 0.5 ml reagent 5 to 100 ml of solution 4. Prepare just before use.

7. Glucose Standard (200 /ml)

   Add 1.0 ml of a 1 g% glucose solution to 49.0 ml of distilled H_2O.

B. Lactate Determination

1. 0.5 M glycine, 0.4 M hydrazine; pH=9.0

   Add 3.75 g glycine, 0.2 g EDTA (Na form) and 5.2 g hydrazine

sulfate to 100 ml distilled H_2O and adjust pH to 9.0

2. 6% Perchloric Acid

   Add 5.14 ml of 70% perchloric acid to 94.86 ml distilled H_2O.

3. NAD^+ (27 mM)

   Add 89.5 mg of NAD^+ to 5 ml of distilled H_2O.

4. Lactate Dehydrogenase (LDH)

   To 0.05 ml of 5 mg/ml protein LDH add 0.95 ml of distilled H_2O.

C. Blôod Ethanol Determination

   (reagents are prepackaged)

1. 75 mM Pyrophosphate Buffer, pH 8.7; 75 mM Semicarbazide; 21 mM Glycine

2. 24 mM NAD^+

3. 30 mg Alcohol dehydrogenase/ml

Reconstitute as per kit instructions.

D. Radioimmunoreactive Insulin (iRi) Determination

   (reagents are prepackaged)

1. Insulin Binding Reagent (anti-insulin serum (guinea pig)) precipitated by anti-guinea pig serum (rabbit).

2. ^{125}I-Insulin

3. Human Insulin Standard

4. Buffer Component

Reconstitute above as per kit instructions.

5. Buffer Component for Washing

   To each 1000 ml of reagent 4 add 39.0 g bovine albumin (fraction V)

APPENDIX II

PLASMA GLUCOSE, BLOOD LACTATE AND BLOOD ETHANOL CONCENTRATIONS AFTER THE TERMINATION OF AN ETHANOL INFUSION TO FOUR DAY FASTED DOGS

After the termination of the 130 minute ethanol infusion to four day fasted dogs (the first series of experiments under "Experimental Design" on page 24) a blood sample was taken hourly for three hours to determine plasma glucose, blood lactate and blood ethanol concentrations.

Whether ethanol was infused at the rate of 0.25 or 0.04 mMole/kg.min, lactacidemia persisted for at least one hour after the termination of the ethanol infusion. When the dose of ethanol was low, the ethanol had disappeared from the blood within two hours and the lactate levels returned to normal. On the other hand, when the load of ethanol was large, the ethanol had not been completely removed from the blood three hours after the termination of the ethanol infusion yet the blood lactate levels had returned to normal (Table 13).

After the termination of the ethanol infusion, the plasma glucose concentrations were normal during the three hour period. Alcohol hypoglycaemia did not develop after the termination of the ethanol infusion (either at the rate of 0.25 or 0.04 mMole/kg·min). Neame and Joubert (1961) reported that alcohol hypoglycaemia did not necessarily occur during the infusion

of ethanol but often appeared six to twenty hours after the ingestion of ethanol. Although the dogs in this series were monitored for three hours after the last ingestion of ethanol, hypoglycaemia did not develop.

TABLE 13

PLASMA GLUCOSE, BLOOD LACTATE AND BLOOD ETHANOL CONCENTRATIONS PRIOR TO AND AFTER THE TERMINATION OF AN ETHANOL INFUSION TO FOUR DAY FASTED DOGS

Time (min)	Plasma Glucose (mg/ 100 ml)		Blood Lactate (mMole/l blood-H_2O)		Blood Ethanol (mMole/l)	
		*		*		*
200	104.94 ±0.27	98.04 ±3.86	2.18 ±0.32	1.08 ±0.08	46.39 ±5.57	3.37 ±0.32
230	99.11 ±1.22	98.19 ±6.20	2.83 ±0.81	1.14 ±0.12	53.46 ±6.79	4.56 ±0.43
290	103.84 ±11.40	98.83 ±3.65	4.62 ±0.21	1.32 ±0.03	47.76 ±4.78	2.49 ±0.95
350	104.62 ±17.16	115.59 ±9.06	3.59 ±0.35	0.66 ±0.32	48.27 --	0.28 --
410	108.38 ±3.98	120.32 ±2.37	0.83 ±0.29	0.61 ±0.03	41.34 ±0.52	--

Notes: The rate of ethanol infusion was 0.25 mMole/kg·min except those values designated by * in which the rate of ethanol infusion was 0.04 mMole/kg·min.

The ethanol infusion took place between t= 100 and t= 230 minutes.

Mean values ± s.e.m.-s are shown.

APPENDIX III

THE EFFECT OF A HIGH ETHANOL LOAD UPON THE RATE OF HEPATIC GLUCOSE PRODUCTION IN AN EIGHT DAY FASTED DOG

The design of the experiment was identical to that already described in the first series of experiments under "Experimental Design" on page 24 except that ethanol was administered for 100 minutes to a dog fasted for eight days. Ethanol was given as a primed (14.5 mMole/kg in a volume of 8.4 ml saline) infusion at the rate of 0.31 mMole/kg.min.

At the termination of the 100 minutes ethanol infusion, the peak blood ethanol concentration attained was 42.98 mMole/l. Figure 11 illustrates that the blood ethanol concentration versus time curve is positive; therefore, the rate of ethanol infusion exceeded the rate of ethanol utilization. The estimated rate of ethanol utilization is 0.095 mMole/kg·min which is similar to that already calculated for normal dogs in the postabsorptive state (Table 2)

The infusion of ethanol did not affect the plasma glucose concentration nor the rates of hepatic glucose production or utilization (Figure 11).

Thus it appears on the basis of a single experiment that the effect of a high ethanol load upon the rates of hepatic glucose production and utilization in a dog fasted for eight days

Figure 11

THE EFFECT OF A HIGH ETHANOL LOAD UPON THE RATE OF HEPATIC GLUCOSE PRODUCTION IN AN EIGHT DAY FASTED DOG

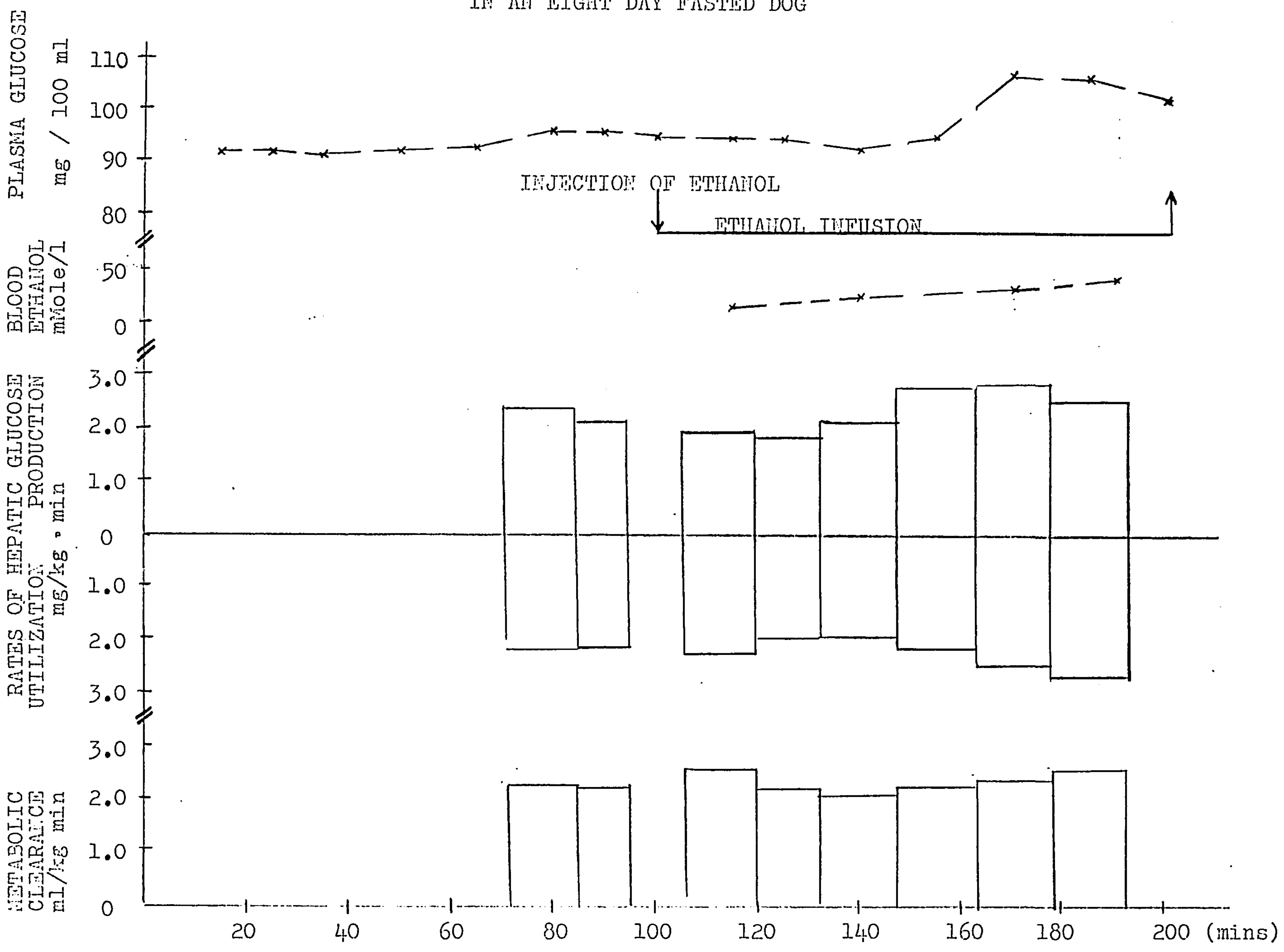

Figure 11: The effect of a high ethanol load upon the rate of hepatic glucose production in an eight day fasted dog. Abscissa: time in minutes. Ordinates starting at the top. Ordinate 1: plasma glucose concentrations as mg/ 100 ml. Ordinate 2: blood ethanol concentrations (mMole/l). Ordinate 3: glucose production (Ra) and utilization (Rd) as mg/kg·min. Ra pointing upwards, Rd pointing downwards from a common line representing zero. Ordinate 4: metabolic clearance rate of glucose as ml/kg·min. Ethanol was administered between t= 100 and t= 200 minutes at a rate of 0.31 mMole/kg·min.

is similar to that already described for high ethanol loads administered to animals fasted for four days. Apparently the eight day fast did not deplete the substrates of the mitochondrial shuttles to any greater extent than a four day fast would do; therefore, hypoglycaemia induced by ethanol did not develop.

APPENDIX IV

THE EFFECT OF A LOW ETHANOL LOAD UPON HEPATIC GLUCOSE PRODUCTION IN A DIABETIC DOG

A paired experiment was carried out on a single pancreatectomized dog. The design of the experiment was identical to that described in the second series under "Experimental Design" on page 25 except that the ethanol was administered as a primed (1.97 mMole/kg in a volume of 29.0 ml of saline) infusion at a rate of 0.04 mMole/kg·min.

At the termination of the 130 minute ethanol infusion, the peak blood ethanol concentration was 2.21 mMole/l. In this case, the estimated rate of ethanol utilization was 0.037 mMole/kg·min which is comparable to the rate of ethanol infusion.

The infusion of a low dose of ethanol to a single pancreatectomized dog had no effect upon the plasma glucose concentration nor upon the rate of hepatic glucose production of utilization (Figure 12).

Thus on the basis of a paired experiment upon a single pancreatectomized dog, the effect of a low dose of ethanol upon hepatic glucose production (mainly derived from hepatic gluconeogenesis in the pancreatectomized animal) is similar to that already described for high ethanol doses in diabetic animals.

Figure 12

THE EFFECT OF A LOW ETHANOL LOAD UPON HEPATIC GLUCOSE PRODUCTION IN A DIABETIC DOG

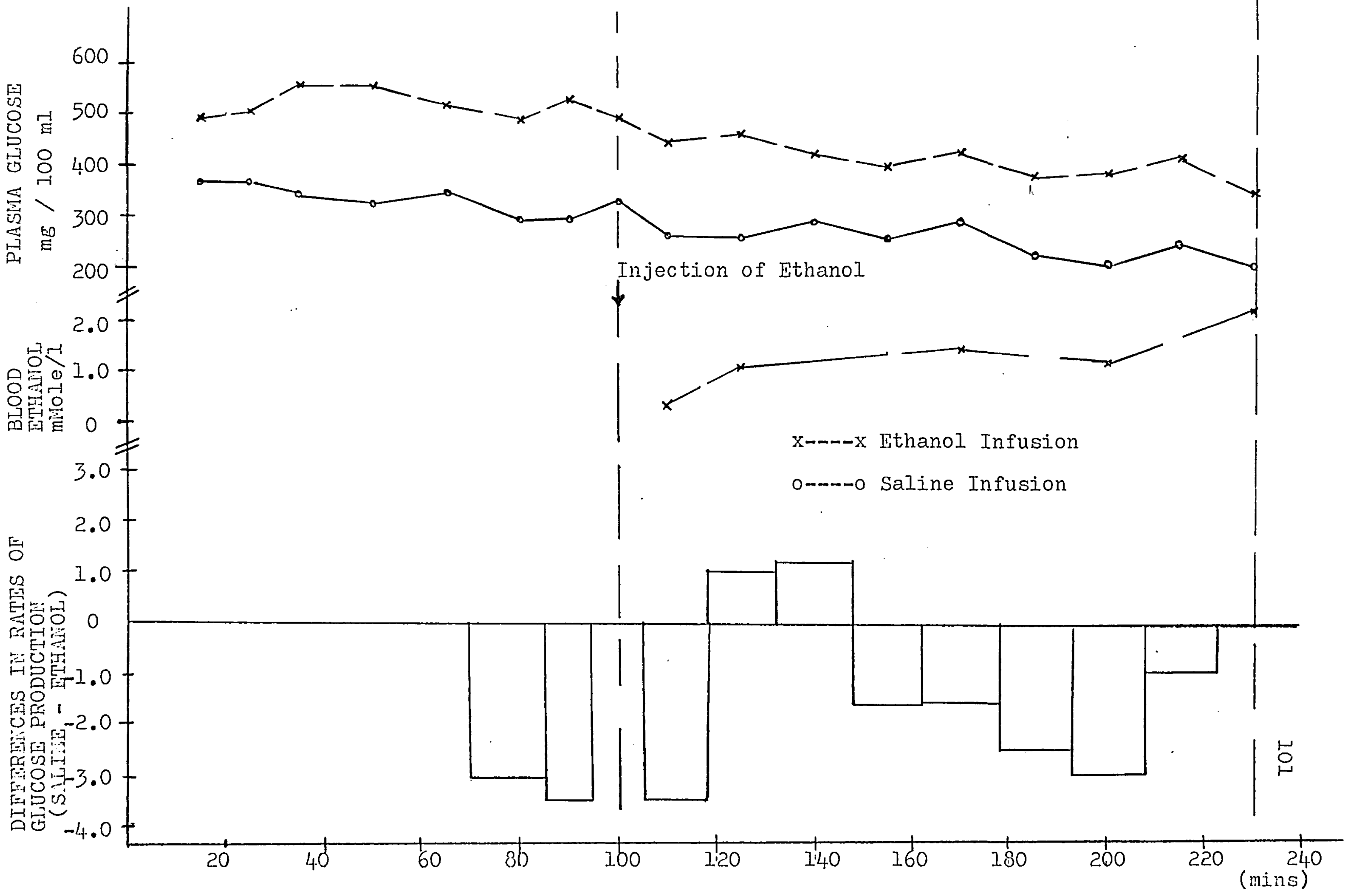

Figure 12: The effect of a low ethanol load upon hepatic glucose production in a diabetic dog. Abscissa: time in minutes. Ordinates starting at the top. Ordinate 1: plasma glucose concentrations as mg / 100 ml. Broken line: plasma glucose concentrations during the infusion of ethanol. Solid line: same during the infusion of saline. Ordinate 2: blood ethanol concentrations (mMole/l). Ordinate 3: Differences in the rates of glucose production (Ra) (saline - ethanol) as mg/kg min. The infusion of ethanol took place between t= 100 and t= 230 minutes as indicated by the perpendicular broken lines. Ethanol was infused at a rate of 0.04 mMole/kg.min.

APPENDIX V

BLOOD PYRUVATE DETERMINATION

A modification of a method first described by Rosenberg and Rush (1966) for the determination of blood pyruvate was used to measure blood pyruvate concentrations in the experiments upon fasted and pancreatectomized dogs.

Within twenty seconds of the blood sampling, the blood was deproteinized using an equal amount of 6% perchloric acid. After centrifugation at 2000 r.p.m. for fifteen minutes, the supernatant was removed. To every three parts of protein free filtrate, one part of a 1.1 M potassium phosphate-potassium hydroxide solution was added. The pH of the solution was then adjusted to pH 7.0-7.5. The tubes were then placed in ice for fifteen minutes to facilitate the precipitation of the potassium perchlorate.

A time course study designed specifically to ascertain the length of time necessary for complete precipitation of potassium perchlorate was carried out. After almost six hours of precipitation, sufficient amounts of potassium perchlorate remained so that only 78.3% of the pyruvate was recovered from the sample (Table 14). In view of this finding, the determination of pyruvate was considered unreliable and the results have not been presented.

TABLE 14

PERCENTAGE RECOVERY OF PYRUVATE

Sample #	Time* (min)	% Recovery of Pyruvate
1	15	73.6
2		74.3
3	30	72.2
4		74.8
5	60	76.9
6		81.3
7	120	72.9
8		79.9
9	290	76.2
10		83.2
11	350	78.3
12		83.6

Note: * indicates time from beginning of potassium perchlorate precipitation.

APPENDIX VI

COMPARISON OF A TWENTY-FOUR AND SEVENTY-TWO HOUR RADIOIMMUNOREACTIVE INSULIN (iRi) ASSAY

A single blood sample was drawn from a normal dog, placed in a heparinized tube and centrifuged. The plasma was then used to determine the iRi concentration by both a 24 and 72 hour insulin assay. Six separate determinations of the same sample were carried out in each assay. The plasma iRi concentrations were 9.78 ± 0.63 and 9.07 ± 0.29 uU/ml for the 24 and 72 hour assay respectively. Thus is appears that the plasma iRi concentrations determined by means of the 72 and 24 hour insulin assay are comparable.

ABSTRACT

The effect fo an intravenous infusion of ethanol upon the rates of glucose production (Ra) and overall glucose utilization (Rd) was examined in conscious dogs. The animals were studied in the postabsorptive state under basal conditions; during insulin-induced hypoglycaemia; in diabetes induced by pancreatectomy or after a four day fast. The rates of glucose production and utilization were calculated using ^{3}H-labelled glucose as tracer. Changes in blood lactate, plasma glucose and radioimmunoreactive insulin concentrations were followed during the administration of ethanol. The estimated rate of ethanol utilization was determined in the four different states studied. The primed infusion of 0.24 - 0.29 mM/kg·min ethanol did not change the plasma glucose concentrations nor the rates of glucose production and utilization in normal or diabetic dogs in the postabsorptive state. A significant decrease in Ra prevailed for fifty-five minutes after the beginning of an ethanol infusion at the rate of 0.04 mM/kg·min to dogs fasted for four days. When the same dogs were again fasted for the same duration and administered ethanol at the rate of 0.25 mM/kg min, there was no significant changes in Ra. When insulin was infused at the rate of 4-7 mU/kg min in the presence of ethanol, ethanol had no effect upon the increase in Ra during insulin-induced hypoglycaemia. During ethanol

infusions to fasted of diabetic dogs, blood lactate concentrations increased and insulin levels did not change. In five of the six paired experiments in which hypoglycaemia was induced with insulin with or without ethanol, the plasma insulin concentrations were consistently higher in the presence of ethanol although the metabolic clearance rates of glucose remained unchanged. The estimated rates of ethanol utilization were reduced by fasting but not in diabetes or during insulin-induced hypoglycaemia. In conclusion, ethanol did not interfere with the elevated rate of gluconeogenesis in diabetes nor did it interfere with the hepatic response to hypoglycaemia.