Effect of Exogenous Insulin and its Relationship to Carbohydrate Intake in the Metabolic Response of Starved-Refed Rats

by

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ABSTRACT

Studies were conducted with adult male rats to assess effects of exogenous insulin on metabolic response to refeeding following starvation. In experiments 1, 2 and 3, the response of hepatic and epididymal adipose tissue glucose-6-phosphate dehydrogenase (G-6-PDH) and malic enzyme (ME) activities during refeeding following starvation in rats either injected intraperitoneally with insulin (2-4 units/kg body weight/day) or a placebo of bovine serum albumin, used to dilute insulin, was compared with that of non-injected rats. Activities of G-6-PDH and ME failed to respond consistently to insulin when measured at 24 to 72 hours of refeeding on a high carbohydrate diet. Placebo injections tended to decrease hepatic G-6-PDH and ME activities. In experiment 4, hepatic and epididymal adipose tissue G-6-PDH and ME activities and _in vitro_ acetate-1-^{14}C incorporation into lipid of non-injected rats were compared with those of rats injected subcutaneously with 4 units undiluted insulin/100 g body weight/day. All rats were fasted for 72 hours and then refed either a high protein or high carbohydrate diet for 48 hours. Total food intake during refeeding was recorded for each rat. Insulin increased activities of G-6-PDH and ME in adipose and hepatic tissues. Hepatic ME activity in rats refed the high protein diet exhibited the greatest increase in activity to insulin;

$2\frac{1}{2}$ fold that of non-injected controls. Increases in adipose tissue but not liver appeared to be due to increased food consumption of rats receiving insulin. Substitution of protein for carbohydrate in the diet resulted in lower hepatic ME activities in both injected and non-injected rats. Acetate-1-^{14}C incorporation was not markedly affected by insulin or variation in level of dietary carbohydrate. These results indicate that insulin plays a role in G-6-PDH and ME induction in starved-refed rats which does not appear to be related to rate of lipogenesis.

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LIST OF ABBREVIATIONS

DNA	–	deoxyribonucleic acid
G-6-PDH	–	glucose-6-phosphate dehydrogenase
HMP	–	hexosemonophosphate
HMPD	–	hexosemonophosphate shunt dehydrogenase
ME	–	malic enzyme
NADP	–	nicotinamide-adenine dinucleotide phosphate
NADPH	–	reduced form of NADP
PEP	–	phosphoenolpyruvate
RNA	–	ribonucleic acid

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I. GENERAL INTRODUCTION

Many dietary and hormonal conditions have been shown to affect enzyme concentrations and activities. Glucose-6-phosphate dehydrogenase and malic enzyme, both of which have been implicated with hyperlipogenesis in adipose and hepatic tissue during refeeding of fasted rats, appear to be regulated by levels of dietary protein, fat and carbohydrate. Hormonal involvement in the regulation of these enzymes is indicated by the fact that thyroxine, cortisone, and insulin have been shown to alter their activity levels. It has been suggested that insulin, whether it is secreted in response to dietary conditions, or administered to the animal, can produce a pronounced rise in the biosynthesis of hepatic G-6-PDH. However, there is some question as to whether biosynthesis occurs in response to insulin *per se*. It has been reported that the increase in G-6-PDH in the livers of fasted-refed rats is directly proportional to the calories consumed as carbohydrate and the effect of insulin is due to increased consumption of carbohydrate in response to a stimulation of appetite by insulin.

The present study was undertaken to further clarify the role of insulin in the regulation of G-6-PDH and malic enzyme in the liver when varying levels of dietary carbohydrate are given to fasted rats. Adipose tissue was

similarly examined since this tissue also has been demonstrated to respond to fluctuations in diet, and only one study has been reported concerning the effect of exogenous insulin on the activities of malic enzyme and glucose-6-phosphate dehydrogenase in the adipocyte. In addition, the study investigated the correlation of food intake and enzyme activity level.

II. STATUS OF THE PROBLEM

Recent research has attempted to determine whether a relationship exists among nutritional and hormonal factors in the induction of G-6-PDH and malic enzyme. Insulin, for example, has been suggested to be involved in the induction of G-6-PDH and malic enzyme since the activities of these enzymes decrease in metabolic states of hypoinsulinism such as diabetes and starvation, and are restored by insulin and refeeding respectively. It is also possible that a relationship exists between diet and insulin. Freedland et al. (1966) found that exogenous insulin was more effective in increasing hepative activity of G-6-PDH and malic enzyme when high protein or high fructose diets were given than when a high glucose diet was fed. The response to insulin with the fructose and protein diets might be due to the fact that the diets evoke less endogenous insulin secretion than glucose. Weber (1966), for example, observed that the activity of G-6-PDH increased in a dose-dependent fashion when starved rats were injected with insulin during refeeding on a lab chow diet.

There is controversy, however, concerning what acts as the inducer of enzyme activity in starved-refed animals. The increase in activity of malic enzyme with exogenous insulin could be the result of an increased demand

for NADPH for hyperlipogenesis (Rosmos and Leveille, 1971). Holten *et al.* (1971) have proposed that the increase in hepatic G-6-PDH can be accounted for by the increased consumption of carbohydrate due to the stimulation of the appetite by insulin.

Increases in the activity of G-6-PDH upon refeeding and/or insulin treatment and increases in malic enzyme activity upon refeeding of fasted rats involves *de novo* enzyme synthesis (Potter and Ono, 1961; Weber, 1966; Lyons *et al.*, 1970). The increase in protein production could be due to the apparent direct action of insulin in protein synthesis, such as renewal of cellular RNA (Steiner and King, 1966) or aggregation of polysomes (Wittman *et al.*, 1969).

It is important to increase our knowledge concerning the regulation of G-6-PDH and malic enzyme, since these enzymes appear to supply NADPH necessary for fatty acid synthesis. The mechanisms regulating hyperlipogenesis must be understood in order to control diseases such as cardiovascular disorders and arteriosclerosis which have been shown to accompany the physiological stress of realimentation following starvation in experimental animals.

III. REVIEW OF LITERATURE

A. Relationship of Enzyme Activity and Lipogenesis

The rate of lipogenesis in rat liver and adipose tissue has been correlated with concomitant changes in the activities of various enzymes related to fatty acid synthesis, such as citrate cleavage enzyme, acetyl CoA carboxylase, fatty acid synthetase and certain NADP-linked dehydrogenases. This present discussion will be limited to the association of two enzymes, glucose-6-phosphate dehydrogenase and malic enzyme, with lipogenesis.

An association between generation of NADPH via the HMP shunt and lipogenesis, has been noted under a variety of metabolic conditions. Tepperman and Tepperman (1958) postulated that the availability of NADPH limited fatty acid synthesis. They suggested that hyperlipogenesis in starved-refed rats depended upon increased activity of the HMP shunt to produce the necessary reduced coenzymes. However, more recent evidence disclosed that only 50-60% of the reduced NADP used for fatty acid synthesis was generated in the conversion of hexose monophosphate to pentose phosphate (Flatt and Ball, 1964).

Malic enzyme which generates NADPH in the conversion of malate to pyruvate, could play an important role in

lipogenesis since the activity of this enzyme is altered by conditions which influence lipogenesis. ME activity and lipogenesis fall upon fasting and rebound above normal in the liver (Pande et al., 1964; Shrago et al., 1963; Tepperman et al., 1964) and adipose tissue (Wise and Ball, 1964) upon refeeding. Wise and Ball (1964) observed that in diabetes, as in starvation there was a lowered rate of lipogenesis in liver and adipose tissue with a concomitant decrease in malic enzyme activity. Young et al. (1964) and Pande et al. (1964) have suggested that malic enzyme activity is more closely related to lipogenesis than HMP shunt, since ME increased more than G-6-PDH during refeeding of starved rats, although the pattern of change was similar for the two enzymes.

Although NADPH is required for lipogenesis, many studies suggest that the supply of reduced coenzymes is not a primary control mechanism for lipogenesis. Abraham et al. (1962) noted that 24 hours after pancreatecomy of rats, lipogenesis from acetate was severely depressed, but G-6-PDH activity did not decrease. Wakil and Bressler (1962) observed that addition of NADPH or NADPH-generating systems to liver preparations from diabetic or starved animals did not restore the activity of fatty acid synthesizing enzymes. However, this failure to restore lipogenesis in diabetic rats, could be due to enzymatic defects of lipid metabolism.

Gellhorn and Benjamin (1964) demonstrated that the microsomal enzymatic conversion of stearate to oleate which requires molecular oxygen and NADPH stops in diabetes, but is corrected by insulin therapy. Deficiencies in *de novo* fatty acid biosynthesis from acetate and microsomal monoenoic fatty acid synthesis in adipose tissue and liver of alloxan-diabetic rats also have been observed (Gellhorn and Benjamin, 1966). Insulin *in vitro* slightly increased the rate of acetate incorporation but did not affect olefin synthesis. In agreement with Wakil and Bressler (1962), the provision of additional ATP or NADPH did not change the desaturating enzyme activity. However, when insulin was administered to the intact animal, this enzyme increased to normal levels, which Gellhorn and Benjamin have suggested to be the result of renewal of cellular RNA.

A lack of relationship between NADPH-generating dehydrogenase activity and fatty acid synthesis has been proposed by studies that demonstrate conflicting rates of lipogenesis and NADPH activities. McDonald and Johnson (1965) found that refeeding a high carbohydrate, 4% protein diet greatly depressed the response of G-6-PDH but had no effect on the apparent rate of hepatic lipogenesis. Tepperman *et al.* (1968) observed that lipogenesis was the same when a protein-free diet was substituted for a diet containing 33% protein, in the refeeding of starved rats

although there was almost complete suppression of the G-6-PDH rise in activity and a partial suppression of malic enzyme. Recent evidence has verified further this lack of relationship. Yeh and Leveille (1970) found that fatty acid synthesis measured *in vivo* or *in vitro* in chicken liver, decreased within 30 minutes after food withdrawal, and was depressed by about 90% after 2 hours of food deprivation. Following a fast of 2 hours, fatty acid synthesis returned to normal after 30 to 60 minutes of refeeding, but the specific activity of ME did not change during the short period of fasting and refeeding. Sullivan *et al.* (1971) found that after a 48-hour fast the *in vivo* rate of lipogenesis in rat liver was maximal at 5 hours after meal initiation and a minimum at 24 hours, when the rate had declined to 3% of the five hour level. However, malic enzyme and G-6-PDH activities have been shown to continue to climb to a peak at 72 hours in the hepatic tissue of rats refed a high glucose diet (Tepperman and Tepperman, 1958 and 1965; Wise and Ball, 1964). Sullivan *et al.* (1971) suggested that the maximum lipogenic enzyme activities may not be completely expressed since 24 hours after feeding, only 7 to 8% of the lipogenic enzyme pool was used to produce the *in vivo* rate; the *in vitro* rate of lipogenesis was 13 to 15 times the observed *in vivo* rate. The control of *in vivo* lipogenesis indicates that enzyme activities are not the

only control of lipogenesis but other regulators or repressors such as concentration of substrates, activators and inhibitors which may be masked in _in vitro_ studies, are at work.

Although HMP dehydrogenase and malic enzyme activities may not be necessary for the initiation of fatty acid synthesis, subsequent increases in activities are probably the result of the increased demand for NADPH to support accelerated fatty acid synthesis. The lack of association between lipogenesis and these enzymes, based on the time at which changes occur when dietary and hormonal conditions are altered, supports the "pull" hypothesis (Tepperman and Tepperman, 1963) which states that hyper-lipogenesis is the primary event and the increase in shunt enzymes secondary.

III. B. Effect of Starvation and Refeeding on Enzyme Activity

Starvation and refeeding following inanition have repeatedly been shown to be accompanied by marked changes in the activities of a number of enzymes. Most reports indicate that enzymes in the hexose-monophosphate shunt system, for example, decrease in activity during starvation to levels approximately 30% of those seen in non-starved controls (Tepperman et al., 1958; Weber et al., 1962; Pande et al., 1964; McDonald et al., 1965). However, Garza et al. (1970) found fasting for 48 hours did not significantly change HMPD activity. During realimentation on a high carbohydrate diet, the activity of G-6-PDH rises sharply within 48 to 72 hours to levels 3 to 4 times those of non-starved controls. There is an initial lag period of approximately 12 hours before activity starts to increase which has been postulated to be due to the necessity for de novo enzyme synthesis (Tepperman et al., 1958; Potter and Ono, 1961; McDonald et al., 1965). Holten et al. (1971), for example, found considerable variation in the lag phase between the start of feeding a new diet in non-starved rats and the induction of G-6-PDH synthesis. They have postulated that the duration of the lag phase prior to the increased rate of enzyme synthesis depends partially upon how soon animals start to eat the new diet. They noted,

for example, that fasting decreased the lag phase and caused a greater increase in the rate of G-6-PDH synthesis. The fasted rats began to eat sooner and consumed more food than non-fasted rats. Insulin-treated rats also had short lag phases. Similarly, these rats began to eat sooner probably because of the stress of low blood glucose.

The level of activity of G-6-PDH is dependent upon the dietary treatment of the rat prior to feeding the specified diet. Johnson and Sassoon (1967) observed a greater increase in G-6-PDH activity upon feeding a high carbohydrate diet following starvation than following transfer from a high fat diet which suggested that starvation may change the formation of the enzyme synthesizing mechanism, making it more sensitive to induction. However, Watt (1970) found that the method of producing weight loss (starvation or diet restriction) made no difference on the metabolic response to refeeding in either liver or adipose tissue. She postulated that the weight loss rather than starvation *per se* may be the condition which triggers the marked metabolic response observed during refeeding of a high carbohydrate diet.

Malic enzyme which is involved in the conversion of malate to pyruvate responds in a similar manner to G-6-PDH during starvation and refeeding. The activity of hepatic and adipose malic enzyme decrease to about one-half

the normal activity after a 48-hour fast (Young *et al.*, 1964). Young *et al.* (1964) also noted that malic enzyme activity (expressed in μmoles of NADPH produced/minute/mg protein) was higher in the adipose tissue than in the hepatic tissue of fasted-refed rats. Similarly, Pande *et al.* (1964) found that G-6-PDH activity (umoles of NADPH produced/minute/mg protein) in adipose tissue was three times that of hepatic tissue of rats fasted and refed a carbohydrate-rich diet for 48 hours.

The higher activities in adipose tissue could be due to the greater need for NADPH for lipid production. These observations suggest that adipose tissue could play a more important role than the liver in lipogenesis in the rat, especially during refeeding.

The influence of high carbohydrate diets on the activity of G-6-PDH and malic enzyme of meal fed or fasted-refed rats does not appear to be connected with its function as an energy source. Tepperman *et al.* (1965) observed that refeeding a high fat, carbohydrate-free diet following fasting, did not elevate activity of HMP shunt dehydrogenases. Likewise, both G-6-PDH and malic enzyme activities were depressed when meal fed rats were given a high fat, carbohydrate-free diet (Leveille and Hanson, 1966).

Sassoon *et al.* (1968) noted that when amount of diet was restricted during refeeding of starved rats, or

when the animals were refed diets varying in carbohydrate content, their hepatic G-6-PDH increased directly with their carbohydrate intake. The lower the carbohydrate intake, the lower the enzyme levels. Holten et al. (1971) also observed that a straight line relationship existed for the rate of G-6-PDH synthesis and caloric consumption at each of the steady state enzyme levels. The steady state level of the enzyme was determined by the ratio of the rate constants for enzyme synthesis and degradation on a variety of dietary regimens.

From these studies, it appears that carbohydrate is the prime dietary inducer of the overshoot in the level of G-6-PDH and malic enzyme. However, other nutritional and hormonal factors which are discussed in the following sections also appear important.

III. C. *De Novo* Enzyme Synthesis

When discussing enzyme induction, it is important to consider whether biosynthesis of new protein occurs. The marked increase in the activities of G-6-PDH and malic enzyme during refeeding of a high carbohydrate diet to previously fasted rats could be the result of *de novo* enzyme synthesis.

Potter and Ono (1961) observed that administration of puromycin, an agent known to block protein synthesis at the level of translation, prevented the increase in G-6-PDH normally observed during refeeding following starvation. Johnson and Sassoon (1967) noted that actinomycin D, an agent which blocks the transcription of DNA to RNA, thereby controlling messenger RNA synthesis, also prevented the increase in G-6-PDH described above. It appears that the effector, that is, the compound which at the molecular level causes the enzyme induction, could be working at the transcription level from DNA to RNA.

Carbohydrate appears to be the dietary inducer of the increase in enzyme activity, when a dietary inducer is defined as the dietary component which leads to an increase in enzyme level. The lag in induction of G-6-PDH following refeeding suggests the probability of a sequence of events between the refeeding of the high carbohydrate diet and the induction of enzyme synthesis.

Holten *et al.* (1971) have assumed that after a lag phase, an immediate increase in the rate of G-6-PDH synthesis occurs which is maintained for several hours. Based on this assumption, they calculated a theoretical curve using a kinetic method. This curve fit the experimental data fairly well, indicating that during the early phases of G-6-PDH induction, the increase in the rate of synthesis is achieved quite rapidly. The validity of this kinetic method awaits further testing. However, using this method, Holten *et al.* studied whether the changes in hepatic G-6-PDH levels produced by carbohydrate diets were the result of a change in either the rates of synthesis or degradation of the enzyme. They first found that the rates of enzyme synthesis and degradation at the low steady state level (pellet-fed rats) were both very low. They found that the rate of G-6-PDH synthesis observed in pellet-fed rats was increased 30-fold by feeding a high carbohydrate diet, and if the rat had been fasted for 2 days there were increases of 73- to 103-fold. Feeding the carbohydrate diet also increased the rate of degradation. The half-life for G-6-PDH degradation *in vivo* for pellet-fed rats was 69 hours, but the half-life decreased to 15 hours when the rats were fed the high carbohydrate diet. From this data, it appears probable that G-6-PDH is regulated by changes in both the rates of synthesis and degradation.

Less work has been conducted concerning _de novo_ synthesis of malic enzyme in rat liver during refeeding a high carbohydrate diet. Lyons _et al._ (1970) observed that the equilibrium constant for enzyme degradation (K) for malic enzyme in a normal rat was 0.29 $days^{-1}$ and the rate of enzyme synthesis (S) was 0.69 enzyme units/gm liver/day. During adaptive hyperlipogenesis both K and S rose to 0.84 and 20.9, respectively; resulting in apparent net enzyme synthesis.

Biosynthesis of new enzyme protein also has been suggested by studies with various levels of dietary protein. The increased activity of G-6-PDH in the hepatic and adipose tissue upon refeeding fasted rats does not occur when low-protein diets (Potter and Ono, 1961; McDonald and Johnson, 1965; Oliver, 1967; Johnson and Sassoon, 1967) or protein-free diets are fed (Tepperman _et al._, 1968; Jomain and Hanson, 1969; Garza _et al._, 1970). Since Wittman _et al._ (1969) have postulated that protein is not necessary for polysomal aggregation, the protein could be necessary for the formation or release of the peptide chain. Lack of protein could also alter the synthesis of insulin which appears necessary for protein synthesis (see section III. E.).

A discrepancy arises between the activities of malic enzyme and G-6-PDH during the refeeding of protein-deficient diets to fasted rats. Oliver (1967) observed that

malic enzyme activity in the liver was not appreciably affected by a high carbohydrate, protein-deficient diet. However, Tepperman et al. (1968) observed a partial suppression of the activity of malic enzyme in the liver on a high carbohydrate, protein-free diet. Furthermore, there is a difference between responses of malic enzyme in hepatic and adipose tissue. Dietary protein appears to be a necessity for malic enzyme activity in the adipose tissue since activity remained low during refeeding of a protein-free diet (Jomain and Hanson, 1969).

Vaughan and Winters (1964) found that there was an increase in hepatic G-6-PDH activity with a higher protein content in the diet suggesting that this enzyme increases linearly with protein content. However, Potter and Ono (1961) noted that increasing the level of dietary protein above 18% did not enhance the overshoot in G-6-PDH activity seen in fasted-refed rats. Tepperman et al. (1968) found that the increase in G-6-PDH activity during refeeding on a high protein, carbohydrate-free diet was only one-half that seen on a 33% casein, 67% glucose diet. However, the rise in activity with a high protein, carbohydrate-free diet was to a level $3\frac{1}{2}$ times that of non-starved controls. This rise in activity could be related to the increase in hepatic RNA levels which diminish during fasting. In mammals, the principal source for pentose sugars to be used in the

formation of nucleic acids, appears to be glucose metabolism via the HMP shunt. By using a steroid inhibitor which prevented synthesis of G-6-PDH, Tepperman *et al.* (1968) have concluded that the rise in G-6-PDH activity on a high protein, carbohydrate-free diet was the result of *de novo* enzyme synthesis. The fact that there could be new enzyme synthesis without carbohydrate conflicts with Wittman's theory (see section III. E. 2.) that both glucose and insulin are necessary for polysomal aggregation.

Malic enzyme activity also is suppressed in liver and adipose tissue of rats when dietary protein is increased (Cohen *et al.*, 1966; Leveille, 1967). Yeh *et al.* (1969) noted that increasing dietary protein from 15 to 35% depressed malic enzyme activity in growing chicks. Allee *et al.* (1970) demonstrated that increasing dietary protein from 12 to 24% resulted in a depression of malic enzyme but not G-6-PDH activity in the adipose tissue of pigs. The suppressing effect of high-protein diets on enzyme activity could be due to changes in the level of dietary carbohydrate, since protein was increased by diminishing the glucose. These observations support the concept that carbohydrate is the primary dietary inducer of *de novo* synthesis of G-6-PDH and malic enzyme.

III. D. Effect of Insulin on Enzyme Activity

1. Gluconeogenic and Glycolytic Enzymes

Fatty acid synthesis has been demonstrated to have a direct relationship with the glycolytic rate in fed animals (Ballard et al., 1967; Zakim et al., 1968). Fasting and alloxan-diabetes can "turn-off" fatty acid synthesis while insulin repletion of the alloxan-diabetic rat or refeeding of the fasted rat can restore fatty acid synthesis.

Glucokinase, a key glycolytic enzyme, is directly inducible by insulin (Salas et al., 1963). Glucokinase in rat liver disappears in diabetes or after fasting and reappears within a few hours after insulin administration or refeeding, respectively. Since actinomycin inhibits this induction, the formation of messenger RNA appears to be involved.

Salas and associates reported that hexokinase in the liver, in contrast to glucokinase, showed no decrease in alloxan-diabetes. However, Machiya et al. (1969) found that two of the four types of hexokinase decreased in alloxan-diabetes and were restored by insulin. Furthermore, the hexokinases exhibited different patterns of response in different tissues. No response to insulin was seen in liver 1 hour after administration but activity

returned to normal levels within 24 hours. On the other hand, response of the enzyme was seen in the epididymal fat pad 1 hour after insulin administration but activity had not returned to normal by 24 hours. The difference in pattern of response to insulin could be associated with the mode of action of insulin in the two tissues.

Insulin has also been suggested as being a suppressor of enzyme induction (Young *et al.*, 1964). Activity of PEP carboxykinase, a gluconeogenic enzyme increased when fasted rats were refed with carbohydrate-free or protein-free diets but decreased in liver and adipose tissue upon refeeding a balanced diet. In fact, Jomain and Hanson (1969) postulated that the absence of protein in the diet alters the synthesis of insulin, and a lack in insulin synthesis would result in an increase in the activity of PEP carboxykinase. Young *et al.* (1964) stated that insulin appeared to be essential for the repression of this enzyme, although carbohydrate was also necessary since PEP carboxykinase was not decreased in fasted rats when insulin was administered alone.

Young *et al.* (1964) noted that a competition existed among malic dehydrogenase, malic enzyme and PEP carboxykinase for the substrate, oxaloacetate. Competition of this type could exert a concerted effect in lipogenesis, since elevated PEP carboxykinase levels caused by lack of

carbohydrate and insulin would divert oxaloacetate to carbohydrate formation while elevated malic enzyme would divert this 4 carbon acid to pyruvate, a reaction that results in the production of NADPH. The concept that carbohydrate and insulin could result in an increase in malic enzyme is discussed in section III. D. 2.

Weber (1966b) found that pyruvate kinase, another glycolytic enzyme, decreased in diabetes. Insulin restored the enzyme to normal levels. Since the insulin-induced rise was blocked by actinomycin, Weber concluded that *de novo* enzyme synthesis was involved. He formulated the hypothesis that insulin acts as an inducer in the biosynthesis of the hepatic glycolytic enzymes and a suppressor of the gluconeogenic enzymes.

Other studies have contradicted this hypothesis and demonstrated that insulin is not necessary for the suppression of PEP carboxykinase or the induction of pyruvate kinase (Freedland, 1966; Sillero *et al.*, 1969). When diabetic rats were fed diets rich in fructose and/or glycerol, both of which are triosephosphate precursors that by-pass glucokinase and phosphofructokinase, PEP carboxykinase and pyruvate kinase levels were normal, indicating that insulin *per se* was not necessary for the suppression or induction of these enzymes. The parallel induction of glucokinase and pyruvate kinase by administering

insulin to diabetic animals is probably a sequential process involving hormonal induction of glucokinase by insulin and secondary metabolite induction of pyruvate kinase by some glycolytic intermediate. PEP carboxykinase could perhaps be repressed by an intermediate from fructose-diphosphate to phosphoenolpyruvate.

These studies indicate that the mechanism of action of insulin in enzyme induction is certainly more complex than previously believed and considerable caution must be exercised in ascribing a direct role for insulin in this induction process.

2. Malic enzyme and Glucose-6-phosphate dehydrogenase

The presence of insulin may be an important factor in producing changes in the enzymatic pattern of various tissues during dietary and other physiological changes. Glucokinase has been shown to be directly inducible by insulin (Salas *et al*., 1963). Glycolytic enzymes such as pyruvate kinase and phosphofructokinase also have been shown to increase in a dose dependent fashion when diabetic rats are injected with insulin (Weber *et al*., 1966b).

Recently, research has focused on G-6-PDH induction in an attempt to establish whether a relationship is involved between nutritional and hormonal factors. Glock and McLean (1955) were the first to suggest that G-6-PDH

was responsive to insulin since activity of the enzyme decreased in alloxan-diabetic rats and returned to normal following insulin administration.

The HMP shunt activity in adipose tissue also appears to be influenced by insulin. Milstein (1955) observed that adipose tissue from alloxan-diabetic rats had a reduced capacity to oxidize glucose, with the HMP shunt pathway being depressed more than the Embden-Meyerhof pathway. Winegrad and Renold (1958) noted that insulin caused adipose tissue to oxidize more glucose by both the Embden-Meyerhof and the HMP shunt pathways.

Weber (1966) suggested that insulin was a specific inducer of G-6-PDH because the activity of this enzyme decreases in metabolic states of hypoinsulinism such as diabetes and starvation but is restored by insulin and refeeding respectively. Weber (1966) noted that injection of starved rats with 1 unit of insulin/day/100 gm body weight during refeeding on a lab chow diet, resulted in almost twice the G-6-PDH activity of comparable non-injected, starved-refed rats in 48 hours. Treatment of animals with a higher dose of insulin caused an even more marked increase in G-6-PDH activity. 6-P-gluconate dehydrogenase activity showed a similar but less marked increase.

The insulin-induced rise in G-6-PDH appears to involve <u>de novo</u> synthesis of new RNA because the response to

insulin in alloxan-diabetic rats could be blocked by actinomycin (Weber, 1966). However, there is controversy concerning what acts as the inducer in starvation and refeeding. Leveille (1967) has suggested that changes in the activity of G-6-PDH are secondary to alterations which increase the demand for NADPH. The increase in G-6-PDH activity during refeeding following fasting, for example, occurs after the increase in lipogenesis. Therefore the increase in G-6-PDH activity seen in fasted animals refed a high carbohydrate diet or in diabetic rats treated with insulin could be the result of an increased demand for NADPH and not due to a direct response to insulin *per se*.

Tepperman *et al.* (1965) demonstrated that HMP shunt and malic enzyme activity increased in the livers of rats fed diets containing hydrogenated fat but did not increase for those fed corn oil, when both diets contained identical amounts of glucose. They proposed that a component of the signal for eliciting increased NADP-linked enzyme activity was an increased rate of oxidation of NADPH attendant on monoene formation and chain lengthening. Essential fatty acid deficiency in the mouse also has been found to increase hepatic G-6-PDH activity (Allmann *et al.*, 1965). However, these changes in activity reported by Tepperman *et al.* and Allmann *et al.* might be just activation of enzyme already present, rather than new enzyme synthesis *per se*.

Holten *et al.* (1971) observed that at a low steady state level of G-6-PDH (pellet-fed rats) 4 units of insulin per day per 100 g of body weight or less did not result in a significant increase in the activity of G-6-PDH whereas 8 units caused a maximal response. When insulin (10 I.U./day/ 100 g of body weight) was administered to rats previously adapted to an intermediate steady state level of G-6-PDH (fed sucrose diet for 8 days) and to rats with a high steady state level of this enzyme (fasted for 48 hours and fed sucrose diet for 6 days), the combination of insulin and diet resulted in approximately the same final enzyme specific activity regardless of the initial enzyme level. The greatest change produced by insulin was in those animals who started with an intermediate enzyme level (non-fasted).

Holten observed that the half-life of G-6-PDH degradation was 15 hours when rats were fed a high carbohydrate diet. *In vivo*, this half-life was unchanged by insulin. Since the increase in G-6-PDH activity in the preceding experiment was not caused by a change in the rate of enzyme degradation (the half-life was 15 hours before and after insulin treatment), it was concluded that insulin must have increased the rate of synthesis of G-6-PDH. Holten and associates also suggest that insulin may not directly regulate the level of G-6-PDH *in vivo* since the amount of insulin required to produce a significant increase

in the enzyme is greater than that which one could call a physiological dose. They found a highly significant correlation between the steady-state level of G-6-PDH and the caloric intake of carbohydrate, and suggest that the effect of insulin is due to the increased stimulation of the appetite by insulin and the resulting greater consumption of carbohydrate. Therefore, the effect of insulin could be an indirect effect upon appetite rather than a direct stimulation of G-6-PDH synthesis.

Sassoon *et al.* (1968) also have questioned the action of insulin in the rise in activity of G-6-PDH. They refed starved rats a high carbohydrate diet, with one group receiving alloxan, a second group alloxan and insulin replacement, and another receiving a supplement of insulin (4 USP units ip. 24 and 16 hours before killing). When the enzyme levels were plotted against caloric intakes of whole diet, the influences of the insulin treatment appeared to be indistinguishable from effects on appetite. They suggested that the rise in the level of G-6-PDH in the liver cells of rats starved and refed protein-adequate diets is chiefly dependent on the amount of carbohydrate ingested and not on the amount of insulin present.

However, Freedland *et al.* (1966) have suggested that there is a relationship between diet and amount of insulin present in states that result in an increase in the

activity of G-6-PDH (micromoles per min per 100 g of body weight). The activity of G-6-PDH increased in the hepatic tissue of rats fed glucose, high protein, or high fructose diets when they were injected with insulin for 5 days. The activity of the G-6-PDH of the rats on the high protein diet increased from 27.9 to 65.3, and on the high fructose, the increase was from 51.8 to 106.0 while the changes for those fed the glucose diet was highly variable with the average increase being only 22.0 to 38.0. There is the possibility that insulin was more effective with the high protein and high fructose diets, because these diets would be expected to evoke less insulin secretion. Malic enzyme activity did not increase in response to insulin in the group fed the glucose diet but showed increases from 14.4 to 18.6 and 52.6 to 69.0 in the high protein and high fructose groups, respectively. It appears that insulin administration had a more marked effect on G-6-PDH activity than on malic enzyme activity.

Nevertheless these observations by Freedland and others, suggest that malic enzyme also could be influenced by insulin. Conditions such as fasting and diabetes (hypoinsulinism), for example, lead to excess glucogenesis with enhancement of phosphopyruvic carboxykinase activity and depression of malic enzyme activity in the liver of rats (Fitch and Chaikoff, 1962; Mehlman _et al._, 1971;

Shrago *et al.*, 1963; Young *et al.*, 1964). Young *et al.* (1964) noted that after three days of insulin treatment, hepatic malic enzyme activity of alloxan-diabetic rats increased to above normal levels. Mehlman *et al.* (1971) observed that malic enzyme activity in the adipose tissue was greatly decreased in diabetic rats and increased over normal level in insulin-treated diabetic rats. Rosmos and Leveille (1971) found that malic enzyme activity was at an extremely low level in the alloxan-diabetic pig and was increased by insulin administration. However, restoration of lipogenesis occurred prior to the restoration of malic enzyme activity. Conversely, insulin withdrawal resulted in a significant depression in lipogenesis prior to a decrease in malic enzyme. These observations suggest that insulin may be necessary for the biosynthesis of malic enzyme but the inducer is some other factor such as the demand for NADPH required for fatty acid synthesis.

III. E. Insulin and Protein Synthesis

In all cells of the body, proteins are in a continual state of turn-over, being formed from amino acids on the one hand and on the other being split back into amino acids. The total amount of protein stored in body tissues is a function of the balance between these two processes. Protein is stored in the presence of insulin and depleted in its absence. Increased protein storage is not a secondary response relative to the well known effect of insulin on carbohydrate metabolism because studies in which carbohydrate metabolism was strictly controlled have demonstrated that exogenous insulin still has a moderate effect in increasing protein synthesis, especially in the liver. Part of this effect is probably due to the fact that insulin increases the transport of amino acids through cell membranes in a similar manner to its effect on the transport of glucose. However, insulin appears to play other roles in protein synthesis, which are discussed in the following sections.

1. Effect on DNA and RNA

The present discussion will be confined to starvation, refeeding, and insulin and how these factors may relate to tissue enzyme activities. It is conceivable that changes in enzyme levels accompanying fasting and refeeding

are related to the availability of energy and/or cellular levels of RNA. Changes in nucleic acid content in response to fasting and refeeding have been noted in various tissues such as adipose, liver, and kidney (Cooper _et al._, 1953; Summers and Fisher, 1962; Kazdová _et al._, 1968; Garza _et al._, 1970).

Braun _et al._ (1966) found that fasting resulted in a decrease in total RNA content with a concomitant reduction in the ratio of RNA/DNA in adipose tissue. The absolute amount of DNA remained constant but the synthesis and turnover of DNA was diminished. In addition, fasting for 48-72 hours resulted in a decrease in the rate of synthesis of heavy or ribosomal RNA in adipose tissue (Benjamin _et al._, 1966).

Refeeding a standard laboratory diet, which is known to restore protein synthesis also resulted in an increase in RNA content of adipose tissue to levels above those of non-fasted controls (Kazdová _et al._, 1968). DNA synthesis also occurs in adipose tissue during refeeding following fasting. Kazdová _et al._ (1967) observed DNA levels 72-340% above those of non-fasted controls after 72 hours of refeeding. The enhanced synthesis of DNA is associated with smaller, more numerous fat cells which have increased synthetic activity and increased sensitivity to hormones such as insulin and adrenalin. Similarly, Benjamin

et al. (1966) showed that the synthesis of ribosomal RNA was restored to control levels within 7 hours of refeeding rats a high glucose diet. By contrast, when rats were refed a high fat, carbohydrate-free diet, the RNA and DNA levels did not increase (Kazdová *et al.*, 1968). However when insulin was injected during the period of refeeding of the high fat, carbohydrate-free diet, the DNA content of the adipose tissue increased appreciably whereas RNA content was not affected. These findings suggest that the basic mechanism whereby insulin and glucose bring about an induction of enzyme synthesis is through the stimulation of cellular RNA.

In the liver, as with adipose tissue, total liver DNA concentration was found to remain constant during starvation while RNA content decreased substantially (Summers and Fisher, 1962). On repletion with a standard diet RNA content increased. However DNA synthesis did not rise until after a lag period of several days (Mendes and Waterlow, 1958). Garza and associates (1970) found that RNA also increased to slightly above control levels when a high-protein, carbohydrate-free diet was refed to starved rats. Stimulation of RNA synthesis under these circumstances could be brought about by insulin because dietary protein stimulates insulin secretion (Floyd *et al.*, 1966). Glucose, *per se*, on the other hand does not appear necessary

for the renewal of RNA synthesis. When a high-carbohydrate, protein-free diet was given, liver RNA levels did not change from those of the fasted rats (Garza _et al._, 1970). These findings are difficult to rationalize because Benjamin _et al._ (1966) showed that feeding a high glucose diet restored RNA levels in the adipose tissue. Perhaps the apparent contradiction among these findings, especially as they relate to the different tissues being examined (viz. liver vs. adipose), could be clarified if the RNA content in adipose tissue also was measured during refeeding with a high-protein, carbohydrate-free diet.

Studies concerning the effect of exogenous insulin on RNA levels in the liver during starvation and refeeding have not been conducted. However, Freedland and associates (1966) observed that non-starved rats fed on standard laboratory or high protein diets and injected with insulin over a 5-day period had higher total liver protein levels than non-injected control rats. The rats on the high protein diet with exogenous insulin had the highest total liver protein content which suggests that dietary protein and insulin might be more important factors than glucose in protein synthesis.

Many studies have been conducted concerning the effect of insulin treatment on the diabetic liver. Steiner _et al._ (1959) noted that during the first three days of

insulin treatment, the liver increased at least 2.5 times in weight and 1.7 times in total DNA content. Steiner and King (1966) found that within 30-60 minutes after insulin injections, the specific activity of liver RNA began to rise with an increase in total content of RNA. The synthesis of DNA did not begin until 24-31 hours after insulin administration. However, an increase in DNA specific activity could be measured by 24 hours, and by 72 hours, total DNA had increased approximately 70% (Younger *et al.*, 1966). There was no significant increase in average DNA concentration per nucleus while the synthesis of new DNA was proceeding. Increased amounts of total liver protein were detected as early as 12 hours after insulin injections but a larger increment occurred after 36 hours, during the period of rapid cell proliferation, just as with total RNA content. The diabetic liver closely resembles the starved liver in that it does not synthesize normal amounts of proteins.

As discussed in section III. C. *de novo* synthesis of G-6-PDH and malic enzyme could be occurring during refeeding following starvation. The increases in RNA and protein synthesis during refeeding and/or insulin administration could partly reflect the new synthesis of these enzymes.

2. Effect on Rat Liver Polysome Profiles

Protein synthesis in *in vitro* cell-free amino acid

incorporating systems has been shown to be directly related to the distribution of ribosomal aggregates by size, or as they are commonly called polysome profiles (Sox *et al.*, 1966). It has been proposed that the rate of protein synthesis *in vivo* also depends on the degree of aggregation of the polysomes. Because the physical properties of polysomes shift under conditions known to affect protein synthesis, the physical form of the polysomes could be a primary regulator in the rate of protein synthesis.

Rat liver polysomes disaggregate on fasting or undernutrition with a gradual decrease in the heavy polysomes (aggregate of 5 or more ribosomes) and an increase in the lighter species. The amount of polysomal disaggregation that occurs during the first 48 hours of fasting varies considerably among animals but the polysome patterns of 60-80 hour fasted rats are very similar (Wittman *et al.*, 1969). Liver ribosomes from starved rats are less proficient in protein synthesis. Ribosomes from the livers of rats starved for one week have been found to incorporate proportionately less amino acids *in vitro* than ribosomes from the livers of rats that had been starved for the same period and then refed a complete diet for 10 to 15 hours (Sox *et al.*, 1966).

There is considerable controversy pertaining to which dietary constituents participate in the regulation of

polysome aggregation. Baliga *et al.* (1968) reported that amino acid concentration, especially tryptophan concentration, was a necessary factor in regulating attachment of free ribosomes to messenger RNA in a cell-free system. Wunner *et al.* (1966) have suggested that the supply of amino acids also regulates polysomal aggregation *in vivo*. The polysome patterns in the livers of rats fed a tryptophan-deficient mixture showed a distribution shift with a decrease in large aggregates and an increase in the smaller aggregates, especially disomes. Thomson *et al.* (1953) noted that when starved rats were refed, there was a five-fold decrease in tryptophan concentration which could be due to its utilization in polysome aggregation. The mechanism of tryptophan in protein synthesis is not understood but it could be acting other than as a protein constituent.

However, the depletion of the availability of amino acids may not be the cause of polysome disaggregation. Webb *et al.* (1966) showed that refeeding a protein-free diet to fasted rats restored the disaggregation of the polysomes. These authors concluded that the energy rather than the nitrogen supply determines the state of polysome aggregation. However, energy *per se* does not appear to be the determining factor since refeeding diets high in fat content did not restore the liver polysomes of fasted rats.

The presence of insulin seems to be necessary,

since after its administration to the diabetic rat, the polysome distribution rapidly shifted towards the heavier species (Wittman _et al._, 1969). Wool _et al._ (1967) observed that when alloxan-diabetic rats were treated with insulin, _in vitro_ protein synthesis returned to normal levels.

Insulin could be a factor in polysome aggregation when a high protein, carbohydrate-free diet is given to fasted rats (as in Webb's study, 1966), since Floyd _et al._ (1966) have found that some amino acids as well as glucose stimulate insulin release.

Insulin, _per se_, however does not appear to be the only factor required for polysomal aggregation since administration of insulin to fasted rats did not restore the polysome patterns (Wittman _et al._, 1969). Wittman _et al._ (1969) noted that insulin and glucose together, but not separately caused an increase in ribosomal aggregation. When laboratory chow was fed to rats fasted for 60 to 65 hours, the polysomes were restored in 8 to 10 hours, but when glucose was refed, the polysomes were restored sooner. The delayed effect of refeeding suggests that glucose must first be absorbed and then in turn it stimulates the release of insulin. The necessity of glucose and insulin together for polysome aggregation to occur may explain why the activities of G-6-PDH and malic enzyme in the adipose tissue did not increase when Fábry _et al._ (1969) administered insulin

(1 unit to 12.5 units/kg body weight/day) to rats fasted and refed a high fat, carbohydrate-free diet for 72 hours.

However, what factor or factors are important in polysomal aggregation is still controversial. Glucose, in the presence of insulin may make tryptophan available for polysome aggregation. Therefore, the critical factor may still be the availability of tryptophan (anonymous, 1970).

There also is controversy regarding the correlation between *in vivo* amino acid incorporation and polysome aggregation. As previously mentioned amino acid incorporation *in vitro* is parallel to polysome aggregation (Baliga *et al.*, 1968) and various reports appear to confirm the theory of polysomal regulation of amino acid incorporation *in vivo* (Oler *et al.*, 1969; Webb *et al.*, 1966). However, Wittman *et al.* (1971) observed a lack of correlation between *in vivo* amino acid incorporation and polysome aggregation in rat liver. Fasted and fed rats which have differing degrees of hepatic polysome aggregation, incorporated amino acids *in vivo* at similar rates. They have suggested that the liver can control quantitatively and perhaps qualitatively protein synthesis which is not reflected in polysome aggregation. Because of the controversy of these reports, it appears that our knowledge must be expanded before the importance of insulin and polysome aggregation in protein synthesis is

clearly understood.

IV. OBJECTIVES OF RESEARCH

The present study was undertaken to further clarify the role of insulin in the regulation of glucose-6-phosphate dehydrogenase and malic enzyme in the liver of fasted-refed rats. Adipose tissue also was examined since very few studies have investigated the activities of G-6-PDH and malic enzyme in this tissue. Two dietary levels of carbohydrate were used (68% glucose, 18% protein and 21% glucose, 65% protein) to investigate the possible interaction of diet and insulin in the regulation of these enzymes. Fatty acid synthesis also was examined by measuring *in vitro* sodium acetate incorporation into fat.

In addition, daily food intakes were recorded in order to correlate food intake and enzyme activity since Holten *et al.* (1971) have suggested that the increase in hepatic G-6-PDH is directly proportional to the calorie consumption of carbohydrate and the response to exogenous insulin is the result of a stimulation of the appetite.

It was hoped through these studies to further establish the function of insulin and its relationship to composition of the diet fed, in the metabolic responses accompanying refeeding following starvation. In other words, the purpose of the project was to determine if exogenous insulin would affect lipogenesis and the

activities of G-6-PDH and malic enzyme and, if so, would the increase be due only to higher carbohydrate consumption.

V. EXPERIMENTAL PROCEDURE

A. Design of Experiment

Adult male rats (180-210 g) purchased from Woodlyn Farms Ltd., Guelph, Ontario were used in all experiments. In the initial preliminary study, 30 rats were paired on the basis of similarity of weight. The rats were starved for 72 hours and then three pairs were refed the fat-free diet for 12 hours, three pairs for 36 hours, and six pairs for 48 hours. During realimentation, one rat in each pair was injected with insulin intraperitoneally (2 units/kg body weight) one-half hour before feeding. The maximum number of injections administered was three; one at the commencement of refeeding and one each at 12 and 24 hours of refeeding.

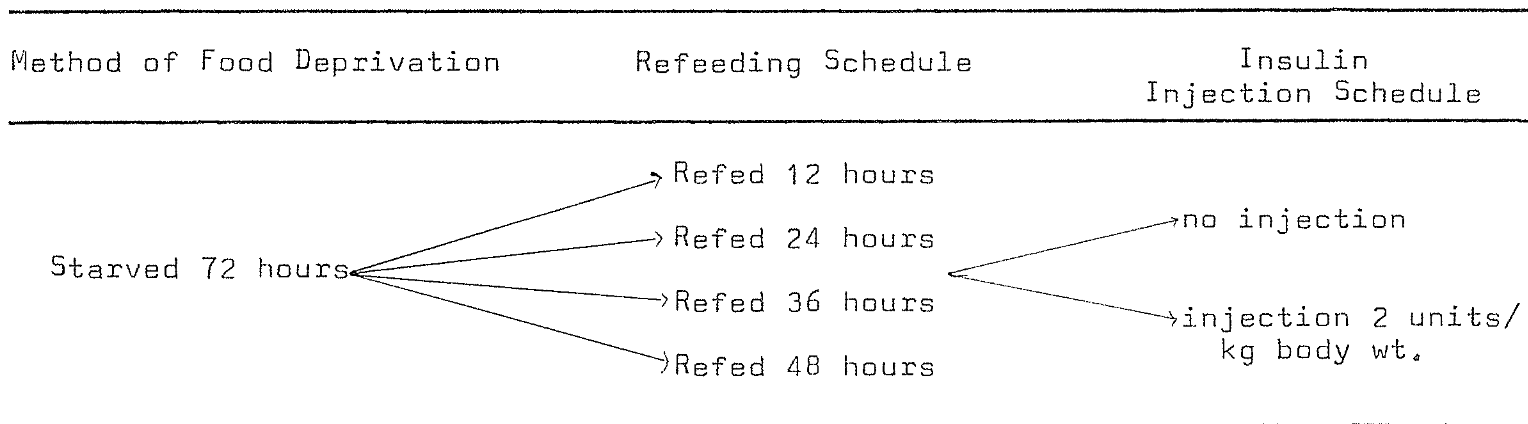
The format of the second preliminary study was similar to Experiment 1, except one rat in each pair received a placebo injection of 3% albumin solution, and the other an injection of insulin (2 units/kg body weight). In addition, the refeeding periods were changed to 24, 48 and 72 hours.

In the third experiment, 80 rats were purchased in three shipments. The arrangement of rats was changed from pairs to groups of four. In each group, one rat received no injection, one a placebo injection, one an insulin injection of 2 units/kg body weight, and one an

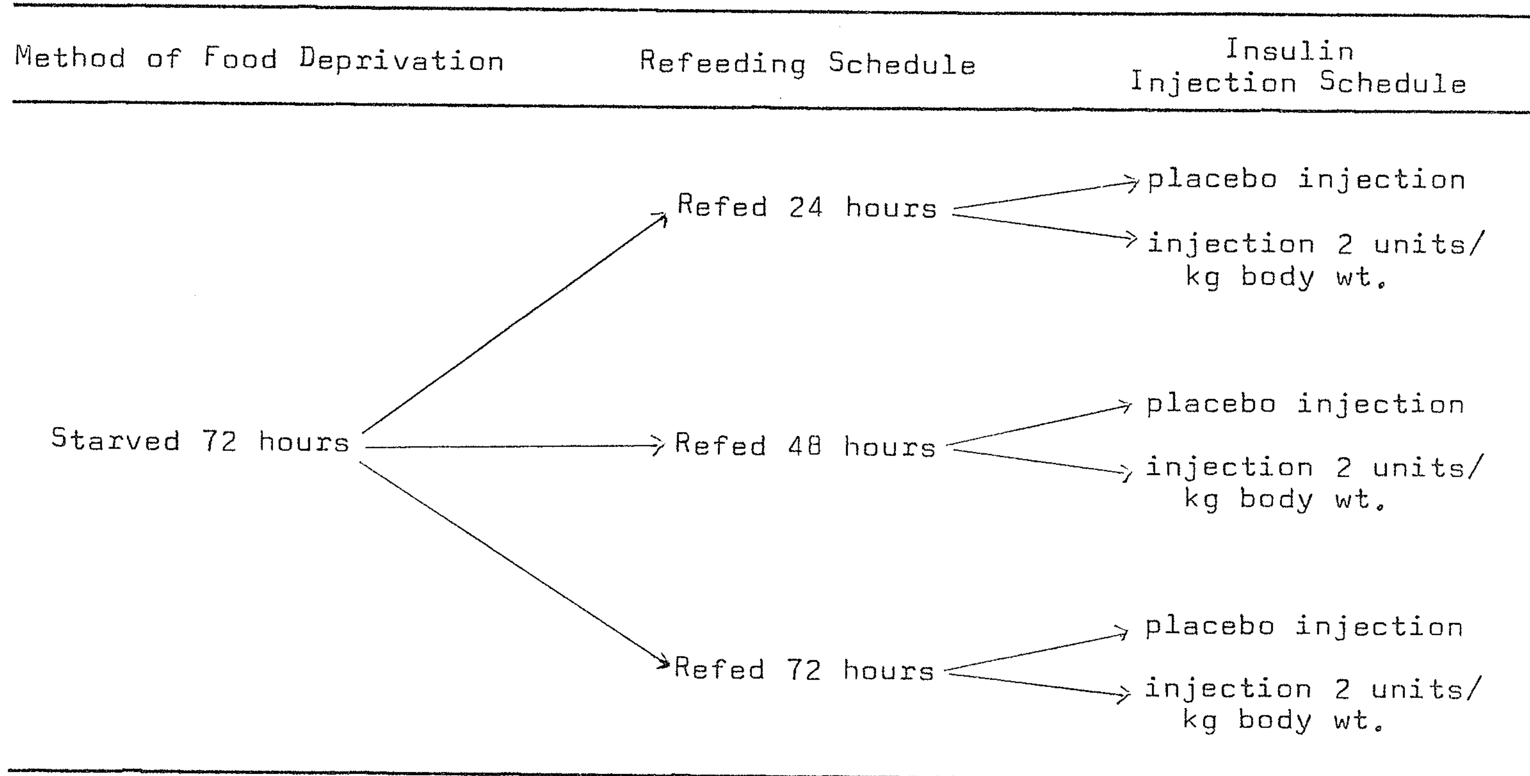
insulin injection of 4 units/kg body weight. Two groups were refed for 24 hours, and two groups for 48 hours. Four rats were assigned as starved controls and four as controls fed standard laboratory chow.

Thirty-six rats were purchased in one shipment for the fourth experiment. The rats were arranged in groups of six rats per treatment. After starvation for 72 hours, two groups were refed the high-protein diet for 48 hours with one group receiving additional insulin injected subcutaneously (4 units/100 g body weight/day). Another two groups were treated similarly except they were refed a high carbohydrate diet. Six rats were used as starved controls, and six as fed controls. The rats were slaughtered at the end of the respective protocols.

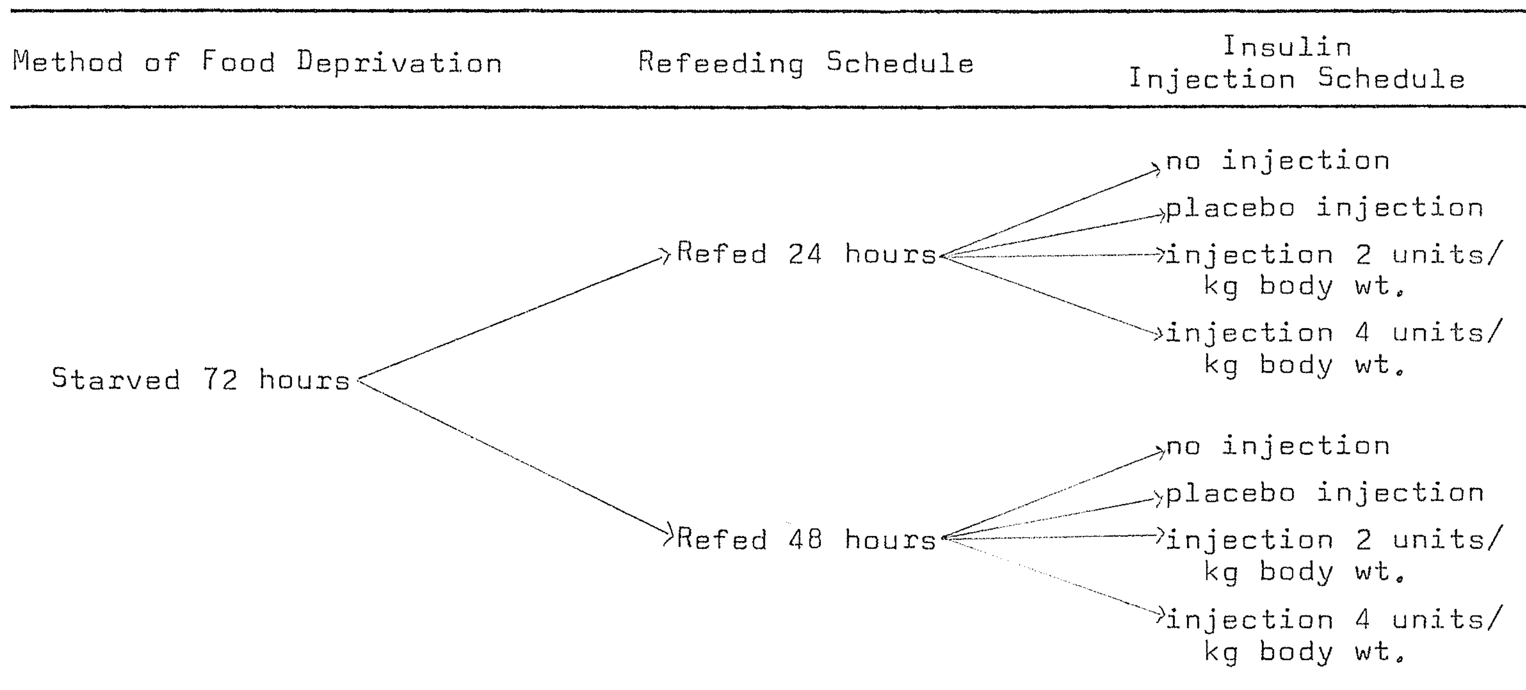
DESIGN OF EXPERIMENT 1



DESIGN OF EXPERIMENT 2



DESIGN OF EXPERIMENT 3

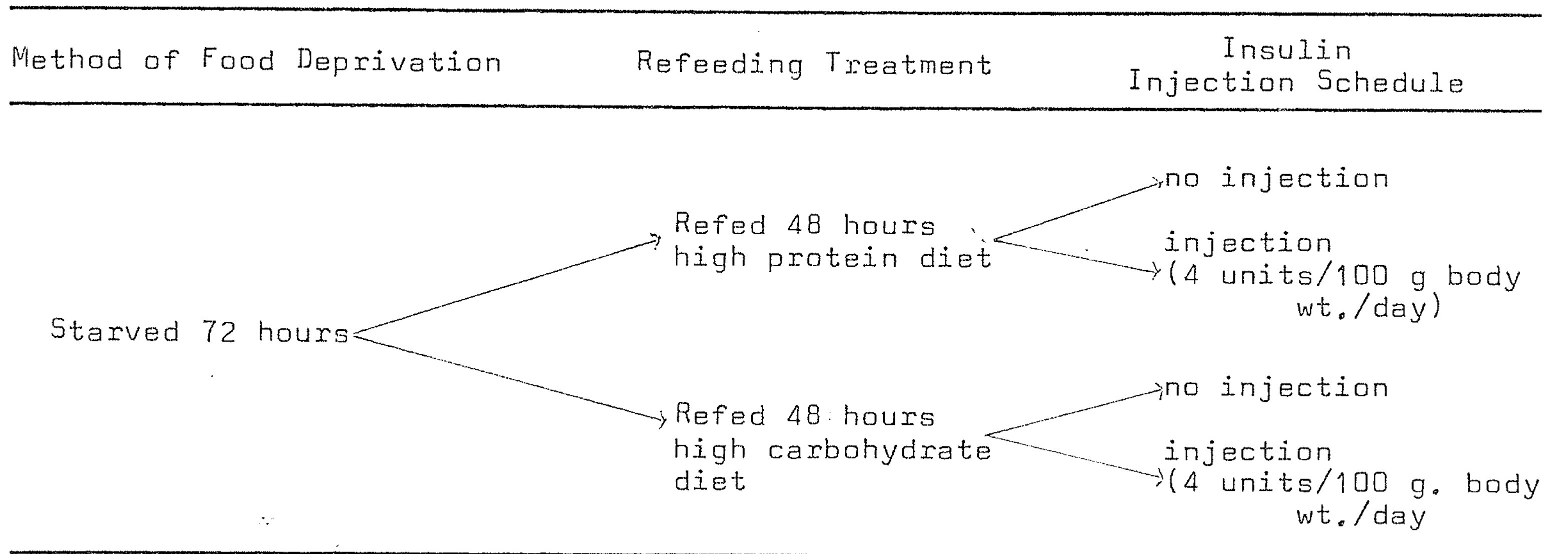


<u>Controls</u>

Starved - 3 days

Fed *ad lib.* - laboratory chow

DESIGN OF EXPERIMENT 4



<u>Controls</u>

Starved - 3 days

Fed *ad lib.* - laboratory chow

V. B. 1. Diets

The diets (tables 1 and 2) used in the present study were similar to those previously used in the Department of Foods and Nutrition, University of Manitoba (Watt, 1970). Substitution was made on the basis of 4 cal/g for glucose, 4 cal/g for protein, 9 cal/g for coconut oil and 0 cal/g for cellulose. The diets were mixed in a Hobart mixer[1] and stored in a refrigerator at 5°C.

V. B. 2. Insulin Preparation

In the first three experiments, two concentrations of insulin[2] were prepared; 2 units/cc of solution and 4 units/cc of solution. A 3% bovine serum albumin[3] solution was used to dilute insulin. The amount of insulin administered was dependent upon body weight of the rat and the treatment protocol; 2 units/kg body weight/day or 4 units/kg body weight/day. The 3% albumin solution was used for the placebo injections and was injected at a level of 1 cc/kg body weight.

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[1]The Hobart Manufacturing Co., Ltd., Ontario, Canada.

[2]Protamine Zinc Insulin, 40 units/cc. Connaught Medical Research Laboratories, University of Toronto, Ontario, Canada.

[3]Albumin Bovine Serum, Sigma Chemical Co., St. Louis, U.S.A.

The insulin, for the fourth experiment, was not diluted, and was injected at a level of 4 units/100 g body weight/day.

TABLE I

Composition of diet

Ingredients	Fat-Free Diet		
	Amount of Ingredient (g/100g)	Calculated Calorie Values (Cals/100g)	(% Total Cals)
Soybean protein	18.0	72.0	19.66
DL-methionine	0.6	2.4	0.66
Vitamins[1]	0.6	--	--
Minerals[2]	4.0	--	--
Glucose	73.0	292.0	79.69
Alphacel	3.8	--	--
	100.0	366.4	100.01

[1]Vitamin mix provided per 100g of diet: vitamin A, 540 I.U.; vitamin D, 28.32 I.U.; DL-alpha-tocopherol succinate, 3 I.U.; menadione, 1.35 mg; inositol, 3 mg; choline chloride, 45 mg; niacin, 2.7 mg; riboflavin, 0.6 mg; pyridoxine HCL, 0.6 mg; thiamine HCL, 0.6 mg; calcium panthothenate, 1.8 mg; D-biotin, 12 µg; folic acid, 5.4 µg; vitamin B_{12}, 0.8 µg; dextrose, 0.54 g.

[2]Salt mix provided per 100g of diet: sodium chloride, .43 g; potassium citrate ($K_3C_6H_5O_7.H_2O$), .95 g; dipotassium phosphate (K_2HPO_4), .31 g; dicalcium phosphate ($CaHPO_4.2H_2O$), 1.42 g; calcium carbonate ($CaCO_3$), .67 g; magnesium carbonate ($MgCO_3$), .16 g; iron sulphate ($FeSO_4.7H_2O$), 59.5 mg; copper sulphate ($CuSO_4.5H_2O$), 0.71 mg; manganous sulphate ($MnSO_4.H_2O$), 5.5 mg; potassium iodide, 0.18 mg; zinc sulphate ($ZnSO_4.7H_2O$), 7.28 mg.

TABLE 2

Composition of diets for Experiment 4

Ingredients	High Glucose Diet			High Protein Diet		
	Amount of Ingredient (g/100g)	Calculated Calorie Values (Cals/100g)	(% Total Cals)	Amount of Ingredient (g/100g)	Calculated Calorie Values (Cals/100g)	(% Total Cals)
Soybean protein	18.0	72.0	19.67	18.0	72.0	19.67
Casein	--	--	--	47.0	188.0	51.36
DL-methionine	0.6	2.4	.66	0.6	2.4	.66
Vitamins	0.6	--	--	0.6	--	--
Minerals	4.0	--	--	4.0	--	--
Coconut oil[1]	2.0	18.0	4.91	2.0	18.0	4.91
Glucose	68.4	273.6	74.75	21.4	85.6	23.38
Alphacel	6.4	--	--	6.4	--	--
	100.0	366.0	99.99	100.0	366.0	99.98

[1]Coconut oil - Canada Packers Ltd., Winnipeg, Manitoba.

V. C. Feeding and Management of Experimental Animals

Rats were allowed to adjust to their new environment for a pre-experimental period during which they were housed 4 to 6 per cage. The cages were wire-floored, measuring 24 x 12 inches. The animals were fed <u>ad libitum</u> a commercial laboratory diet[1] and tap water was available at all times from water bottles located at the front of each cage. The temperature in the air-conditioned room fluctuated between 68-70°F. The lighting was controlled so that darkness occurred between 7:00 p.m. and 7:00 a.m.

During the experimental trials, rats were housed in individual wire-floor cages measuring 8 x 12 inches. All rats were starved for 72 hours and realimination was achieved by feeding 20 g of diet daily; 10 g each at 9:00 a.m. and 9:00 p.m. from feed containers (6 oz glass jars) wired to the front of each cage. The specified injections (see section A) were made one-half hour prior to feeding. Rats were weighed at the commencement of starvation, again before refeeding and at the time of slaughter. The rats were slaughtered by decapitation using a guillotine.[2]

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[1] Rat Lab Chow: Ralston Purina Co., St. Louis, Missouri, U.S.A.

[2] Harvard Apparatus Inc., Dover, Massachusetts, U.S.A.

V. D. Preparation of Tissue for Chemical Analyses

The livers and epididymal fat pads were quickly excised following decapitation of the rats. The livers were rinsed with cold tap water, blotted with filter paper and weighed.

Liver slices were prepared with a Stadie-Riggs tissue slicer,[1] blotted on filter paper, weighed (150 to 250 mg) and used in the *in vitro* acetate-1-^{14}C incorporation into fatty acids. Segments of the epididymal fat pad (100 to 150 mg) were dissected with scissors and fatty acid synthesis was assayed in the same manner as hepatic tissue.

Approximately 2.0 g of remaining liver (a composite of a piece from each lobe) was minced with scissors and homogenized in a buffer (1:9 w/v) (McDonald and Johnson, 1965) using a Potter-Elvejhem tissue homogenizer.[2] Similarly, the epididymal fat pads were weighed and homogenized in 0.15 molar KCL solution (1:9 w/v) using a Virtis 23 tissue homogenizer.[3]

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[1]Arthur H. Thomas Company.

[2]Arthur H. Thomas Company.

[3]Virtis Research Co., Model 23, Gardiner, New York, U.S.A.

Both homogenates were centrifuged at 0°C in a refrigerated centrifuge.[1] The speeds were 10 minutes at 1,475 x g, 10 minutes at 10,800 x g and 20 minutes at 45,900 x g. The overlaying lipid was removed from the supernatant fractions which were kept for later enzyme and protein analyses.

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[1]Model B-20 International Equipment Co., Needham Hts., Mass., U.S.A.

V. E. Chemical Analyses

1. Protein Assay

Protein content of the liver and adipose tissue supernatants was measured by the method of Lowry *et al.* (1951) using commercial Folin Ciocalteau Reagent.[1] Bovine serum albumin diluted with physiological saline (80 ug protein per ml) served as the protein standard. The liver and adipose tissue supernatants (1:9 w/v) had been frozen for storage. After thawing, 0.1 ml of liver was diluted with 1.9 ml of 0.25 N NaOH to dissolve the protein and 2.0 ml of 0.25 N HCl to neutralize the solution. Final dilution of the supernatants was 1:360 w/v. Similarly, 1.0 ml of fat pad supernatant was diluted with 2 ml of 0.25 N NaOH and 2 ml of 0.25 N HCl to give a 1:5 w/v dilution. Duplicate determinations were made on each supernatant. Colorimetric measurements were read in a Unicam SP600 Series 2 spectrophotometer[2] at a wavelength of 750 mu.

2. Enzyme Assays

The G-6-PDH activity was assayed by the method of Lohr and Waller (1963) and the malic enzyme activity by the method of Ochoa (1955). The procedures outlined by Watt

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[1]Fisher Scientific Co., Toronto, Ontario.

[2]Unicam Instruments Ltd., Cambridge, England.

(1970) were used, except the temperature in the cuvette chamber was raised to 37°C.

3. <u>Lipid Analyses</u>

a. Determination of *in vitro* acetate-1-^{14}C incorporation into lipid by liver slices and epididymal adipose tissue segments.

Duplicate samples (ca. 100 mg of wet tissue) of epididymal adipose tissue and liver slices were incubated in 25 ml Erlenmeyer flasks containing 3.30 ml of Krebs-Ringer phosphate-bicarbonate buffer (pH 7.4). The buffer contained, per 3.3 ml, 30 µmoles sodium acetate, 15.0 µmoles glucose, 0.3 units insulin and 0.89 uC sodium acetate-1-^{14}C. The flasks were stoppered with ground glass stoppers and incubated at 37°C for three hours in a shaking water bath. The reaction was stopped by adding 1.0 ml of six N HCl to each flask. The flasks were cooled and the tissues were recovered by filtering the contents of each flask through Whatman No. 1 filter paper. Filtration was carried out under gentle suction with a water aspirator. The flasks were rinsed with 2 portions (2-4 ml each) of 0.1 N HCl and the washings were filtered by pouring them over the tissues. The filter papers with tissues were transferred to 25 x 200 cm test tubes and extracted with 15.0 ml of chloroform and methanol mixture (2:1 v/v). The tubes were stoppered and left to stand for 48 hours, during which time they were vigorously shaken three times daily. Then 14.0 ml of

methanol and water mixture (5:9 v/v) was added to each test tube. The solution in the tubes was mixed and the filter paper was removed. The two phases in the test tube were allowed to separate for at least 2 hours. The upper layer consisting of methanol and water was removed by aspiration. The chloroform layer was washed once with 14.0 ml distilled water. An aliquot of the chloroform layer in each test tube was transferred to a counting vial and evaporated to dryness. Ten ml of Scintillation liquid containing 5.0 g of PPO[1] and 0.3 g POPOP[2] per litre of toluene, was added to each dry vial. The samples were counted in a Liquid Scintillation spectrometer[3] and the total radioactivity in each sample was expressed as dpm/g tissue on the basis of counting efficiency determined by the channel ratios.

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[1]PPO : 2-5 diphenylozazole - Packard Instrument Inc., Dovers Grove, Illinois, U.S.A.

[2]POPOP : 2,2-p-phenylene biz (5-phenylozazole) - Packard Instrument Inc., Dovers Grove, Illinois, U.S.A.

[3]Model 8260 - Nuclear Chicago Instruments, Des Plaines, Illinois, U.S.A.

V. F. Statistical Analyses

Statistical analyses for the first three experiments were performed according to the method of Student's Paired T-Test.

In the fourth experiment, analyses of variance, covariance and Student's T-Test were used (Steel and Torrie, 1960).

VI. RESULTS AND DISCUSSION

A. Results (Experiments 1, 2 and 3)

Experiment 1 was undertaken to assess the effect of exogenous insulin on G-6-PDH and malic enzyme activities in hepatic and adipose tissues of starved rats refed a high carbohydrate diet. The activities of these enzymes showed the customary increase during refeeding, with the hepatic levels at 48 hours 4- to 7-fold those at 12 hours of refeeding. Hepatic activities of G-6-PDH and malic enzyme were significantly higher ($P < .10$, $P < .05$ respectively) after 48 hours of refeeding in animals receiving exogenous insulin (Table 3). Although the increase was not significant, mean activities for G-6-PDH in liver and in adipose tissue at 36 and 48 hours were higher for the insulin-injected rats.

Experiment 2 was conducted to determine whether the response in the liver at 48 hours was due to insulin *per se* or to some other factor(s), such as the albumin solution or excitability produced by handling the animals during intraperitoneal injections. The control group received equivalent placebo injections of albumin. The results summarized in Table 4 indicate that there were no significant differences in hepatic or adipose dehydrogenase activities among the groups receiving exogenous insulin and the groups given the placebo injections, which suggested that the response to

Table 3

Experiment 1

Effect of exogenous insulin on enzyme activities during refeeding following starvation

Hours Refed	Enzyme Activity (η moles NADPH produced/mg protein/min.)			
	Liver		Epididymal Adipose Tissue	
	No Insulin	Insulin (2 units/kg rat/day)	No Insulin	Insulin (2 units/kg rat/day)
	G-6-PDH Activity			
12	26.0± 7.9[1]	28.2±21.8	87.0±25.2	101.2±120.4
24	74.1±49.7	48.6±17.8	59.2±22.5	46.5± 28.2
36	83.9±49.2	103.4±20.2	86.8±33.2	104.3± 52.1
48	112.8±60.7[2]	181.6±84.3[2]	99.8±56.2[2]	114.8± 52.6[2]
	Malic Enzyme Activity			
12	21.9± 6.1	23.7± 7.1	152.9±39.2	107.9± 18.0
24	34.7±10.1	60.6±31.3	153.0±78.0	96.4± 40.2
36	81.4±11.8	82.3±23.8	286.7±97.1	266.9±167.4
48	84.3±30.7[2]	132.9±35.3[2]	240.5±127.3[2]	298.3± 87.1[2]

[1]Mean ± S.D. of 3 rats except where indicated.

[2]Mean ± S.D. of 6 rats.

Table 4

Experiment 2

The effect of placebo vs. insulin injections on enzyme activities during refeeding following starvation

Hours Refed	Enzyme Activity (η moles NADPH produced/mg protein/min.)			
	Liver		Epididymal Adipose Tissue	
	Placebo	Insulin (2 units/kg rat/day)	Placebo	Insulin (2 units/kg rat/day)
	G-6-PDH Activity			
24	53.1±21.3[1]	50.9±17.4	51.3±22.6	84.7± 26.6
48	110.8±25.2	133.9±38.5	92.5±13.7	92.4± 31.2
72	126.6±26.6	102.6±43.7	84.6±58.9	80.9± 32.2
	Malic Enzyme Activity			
24	31.0± 6.0	45.9±22.6	113.2±75.7	204.0±113.4
48	91.5±19.5	66.8±25.1	150.3±35.9	190.6± 89.7
72	117.1±17.5	104.5±20.6	180.6±45.0	248.2± 81.7

[1]Mean ± S.D. of 4 rats.

insulin in experiment 1 was the consequence of factor(s) other than insulin.

The basic object of experiment 3 was to compare the response of dehydrogenase enzymes in insulin-injected, starved-refed rats with that of non-injected or placebo injected starved-refed animals. G-6-PDH and malic enzyme activities tended to be considerably higher in both liver (Table 5) and adipose tissue (Table 6) in the present experiment than that observed in experiments 1 and 2. This was especially true for animals refed for 48 hours. There were no significant differences among insulin-injected and non-injected rats for G-6-PDH or malic enzyme in liver or fat pads although activities tended to be higher for animals given 4 units of insulin per day. However, activities of hepatic G-6-PDH were signifcantly ($P < .05$) higher at 24 hours for the rats injected with 4 units of insulin than for rats receiving placebo injections. The slightly higher activities in rats that received 4 units of insulin together with lower activities for those given the placebo, account for these observations. These results represent a further variation from the responses observed in the first 2 experiments. In addition, the data was further complicated by the fact that the activities of G-6-PDH and malic enzyme in the livers of placebo-injected rats were significantly ($P < .05$) lower at 24 hours of refeeding than those of the non-injected group (Table 5).

Table 5

Experiment 3

Effect of placebo and insulin injections on hepatic enzyme activities during refeeding following starvation

Hours Refed	Enzyme Activity (η moles NADPH produced/mg protein/min.)			
	No injection	Placebo	Insulin (2 units/kg/rat/day)	Insulin (4 units/kg rat/day)
			G-6-PDH Activity	
24	122.9±34.9[1]	74.5±27.7	98.7±22.8	126.3±49.8
48	268.9±54.1	279.4±39.1	257.2±56.1	311.6±91.6
			Malic Enzyme Activity	
24	85.6±30.8	60.9±26.8	73.1±28.9	98.4±32.4
48	201.4±39.1	172.2±61.3	178.3±44.0	199.3±34.9

Controls	G-6-PDH Activity	Malic Enzyme Activity
Starved 3 days	25.7±3.0[2]	18.3±3.6[2]
Ad libitum-fed lab chow	30.5±2.5[2]	28.1±3.9[2]

[1]Mean ± S.D. of 8 rats except where indicated.

[2]Mean ± S.D. of 4 rats.

Table 6

Experiment 3

Effect of placebo and insulin injections on enzyme activities in epididymal adipose tissue during refeeding following starvation

Hours Refed	Enzyme Activity (η moles NADPH produced/mg protein/min.)			
	No injection	Placebo	Insulin (2 units/kg/rat/day)	Insulin (4 units/kg rat/day)
			<u>G-6-PDH Activity</u>	
24	88.1±10.9[1]	96.2± 26.8	93.2± 23.2	111.2± 24.4
48	134.8±18.3	142.6± 24.4	150.1± 22.9	160.5± 25.9
			<u>Malic Enzyme Activity</u>	
24	178.3±51.2	147.0± 44.0	158.5± 68.2	174.9± 54.9
48	338.2±77.5	280.6±124.2	369.0±119.1	322.7±106.2

Controls	G-6-PDH Activity	Malic Enzyme Activity
Starved 3 days	78.5± 3.9[2]	87.6±20.9[2]
Ad libitum-fed lab chow	112.3±19.2[2]	180.4±17.2[2]

[1]Mean ± S.D. of 8 rats except where indicated.

[2]Mean ± S.D. of 4 rats.

VI. B. Discussion (Experiments 1, 2 and 3)

There are several possibilities why the activities of malic enzyme and G-6-PDH failed to respond consistently to the presence of exogenous insulin. The increase in hepatic activities of these enzymes in experiment 1 could have been a genuine response. However, the effect of the insulin could not be repeated even when the dosage was doubled in experiment 3.

Nevertheless, an appropriate level of exogenous insulin may not have been used in these experiments. The blood glucose level of rats (150 - 250 g) fed a standard laboratory chow has been reported to be 82.0 mg/100 ml and is reduced to one-fifth that level by fasting (Braun _et al._, 1967). Controversy exists concerning appropriate dosage levels of exogenous insulin for a response to occur in G-6-PDH activity. Although insulin at a level of 1 unit/kg body weight/day produced a considerable drop in blood glucose level of unfasted rats, this level failed to cause an increase in the activities of G-6-PDH and malic enzyme in the adipose tissue of starved-refed rats (Fabry _et al._, 1969). Holten _et al._ (1971) have suggested that the dosage level must be higher than the endogenous insulin secretion, which is 2-3 units/day (Wagle _et al._, 1965) for a response in dehydrogenase activity, since in their experiments 4 units of insulin/100 g body weight/day failed to increase the hepatic G-6-PDH activity

of chow-fed rats. However, the appropriate dosage level may differ depending on dietary treatment. Weber (1966) observed a two-fold increase in hepatic activity of G-6-PDH when starved rats were given insulin at a level of 1 unit/100 g body weight during refeeding.

The inconsistencies observed in the present study could be due to other factors such as the dilution of insulin. Unequal dosages could have been administered since mixing the insulin with albumin resulted in a suspension rather than a solution. The presence of the albumin also could have affected the enzymes since malic enzyme and G-6-PDH activities were significantly lower in the placebo group as compared to the non-injected group in experiment 3. Although researchers (Freedland _et al._, 1966; Fábry _et al._, 1969) have reported injecting insulin intraperitoneally, this method also could have altered the results since it created a high degree of excitability in some animals.

Modifications were made in experiment 4, because of the inconsistencies arising in the first 3 experiments. The insulin was not diluted and was injected subcutaneously at a higher dosage level of 4 units/100 g body weight/day.

VI. C. Results (Experiment 4)

During the course of the present study, a paper was published by Holten et al. (1971) which suggested that the action of insulin in increasing the hepatic activity of G-6-PDH was due to a stimulation of appetite thus resulting in increased consumption of carbohydrate rather than a direct effect of insulin on G-6-PDH enzyme. Therefore, the present study on the effect of exogenous insulin on dehydrogenase activity during refeeding following starvation was further modified to include two dietary levels of carbohydrate during the refeeding period. The low carbohydrate diet, hereafter designated as a high protein diet, was formulated by substituting 47% casein for an equivalent amount of glucose in the high carbohydrate diet. In addition, daily feed intake was recorded for each rat.

The number of rats per treatment was reduced to five from the original six, since the dosage level used in this study (4 units/100 g body weight/day) produced insulin coma in one rat on each diet. One non-injected rat on each diet was eliminated randomly so analyses of variance could be performed. The level of insulin that a rat can adjust to seems highly variable since Holten et al. (1971) had reported using levels as high as 16 units/100 g body weight/day with no reports of ill effects.

Exogenous insulin (4 units/100 g body weight/day,

subcutaneous) resulted in significantly higher G-6-PDH ($P < .01$, $P < .01$) and malic enzyme ($P < .01$, $P < .05$) activities in both hepatic and adipose tissue when rats were refed either the high protein or high carbohydrate diet for 48 hours following starvation (Tables 7 and 8, appendix tables I to IV). Weber (1966) also found that hepatic G-6-PDH activity increased when insulin was administered during refeeding of fasted rats. Although no reports were found concerning the effect of exogenous insulin on hepatic malic enzyme activity during refeeding following fast-induced hypoinsulinism, the present results agree with the observations of Young _et al_. (1964) and Mehlman _et al_. (1971) who found insulin treatment increased malic enzyme activity in alloxan-diabetic rats. The significant increases in activities of G-6-PDH and malic enzyme in the adipose tissue are not in accordance with the observations by Fábry _et al_. (1969) who found that the activities of these enzymes did not increase when fasted rats were given crystalline insulin during refeeding.

Malic enzyme activity in the livers of rats receiving insulin during refeeding on a high protein diet was $2\frac{1}{2}$ times the activity of non-injected animals fed this diet (Fig. 1). Insulin did not cause as pronounced an increase in malic activity in rats refed the high glucose diet. Part of the difference may be due to the lower activity of the enzyme in rats refed the high protein diet. By contrast, response of

Table 7

Effect of diet composition and exogenous insulin on liver enzyme activities following starvation and refeeding for 48 hours[1]

Diet Refed	Enzyme Activity (η moles NADPH produced/mg protein/min)	
	No Insulin	Insulin (4 units/100 g body wt./day)
		G-6-PDH Activity
High protein	190.2±59.5[2]	306.5±26.7
High glucose	204.7±54.2	317.0±14.2
		Malic Enzyme Activity
High protein	59.2±26.8	142.4±33.7
High glucose	125.0±46.5	197.9±35.7

Controls	G-6-PDH Activity	Malic Enzyme Activity
Starved 3 days	18.1± 7.5[3]	13.9± 4.2[3]
Ad libitum-fed lab chow	29.5±11.7[3]	18.0± 4.7[3]

[1]Analysis of variance for this data is shown in Appendix Tables I and II.

[2]Mean ± S.D. of 5 rats.

[3]Mean ± S.D. of 6 rats.

Table 8

Effect of diet composition and exogenous insulin on enzyme activities in epididymal adipose tissue following starvation and refeeding for 48 hours[1]

Diet Refed	Enzyme Activity (η moles NADPH produced/mg protein/min)	
	No Insulin	Insulin (4 units/100 g body wt./day)
		G-6-PDH Activity
High protein	116.2± 31.9[2]	147.8±78.2
High glucose	105.1± 30.2	172.6±20.5
		Malic Enzyme Activity
High protein	217.0±129.7	267.6±52.1
High glucose	197.2±108.9	346.3±58.1

Controls	G-6-PDH Activity	Malic Enzyme Activity
Starved 3 days	79.8± 27.8[3]	101.2±70.6
Ad libitum-fed lab chow	97.6± 20.6	120.2±34.4

[1] Analysis of variance for this data is shown in Appendix Tables III and IV.

[2] Mean ± S.D. of 5 rats.

[3] Mean ± S.D. of 6 rats.

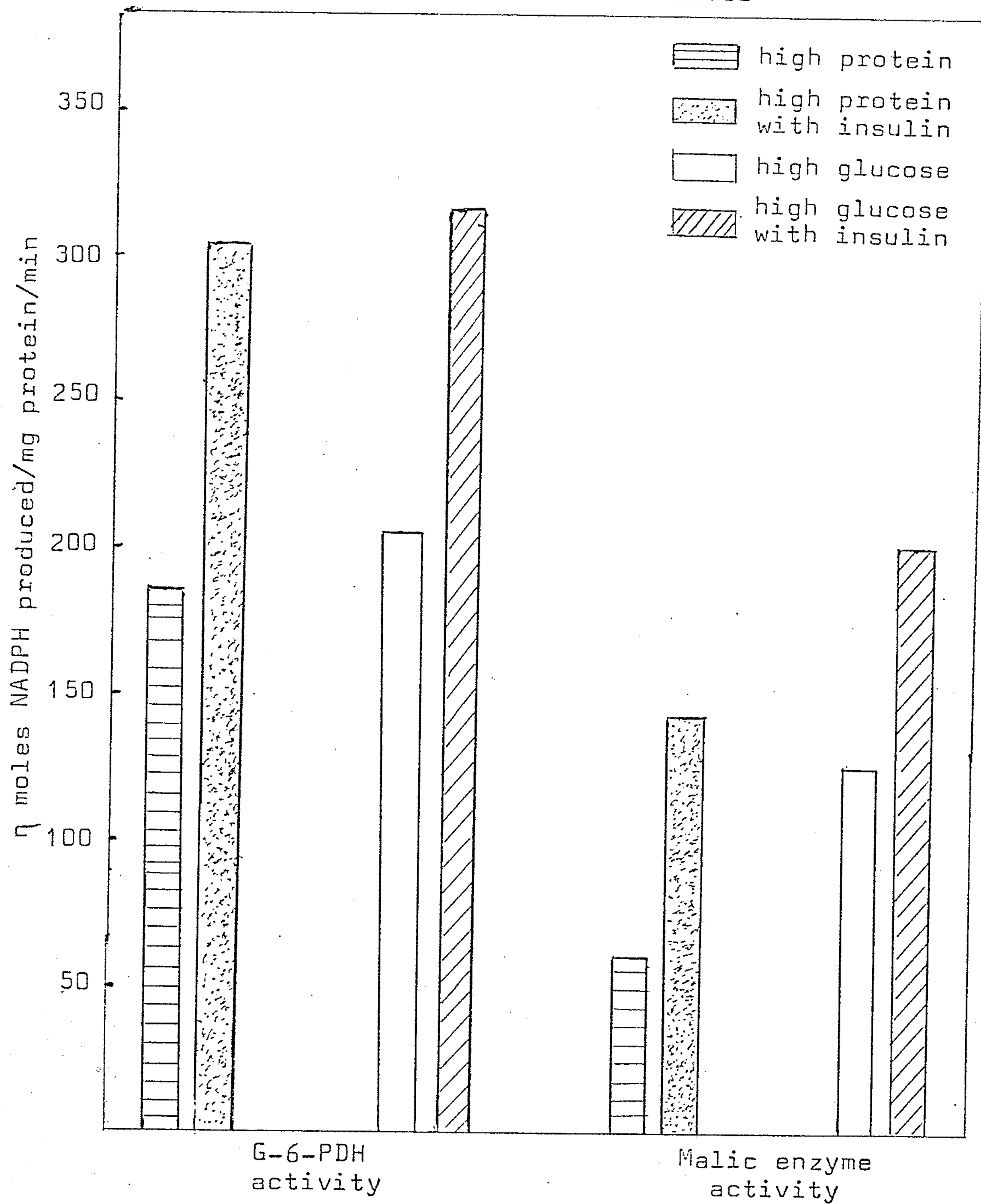

Figure 1. Effect of diet composition and exogenous insulin on liver enzyme activities during refeeding for 48 hours following starvation. Each bar is the mean activity of 5 rats.

G-6-PDH to insulin was similar whether the animals were refed the high glucose or high protein diet. Freedland et al. (1966) also observed increases in activities of G-6-PDH and malic enzyme in livers of unfasted rats given exogenous insulin during feeding on a high protein diet. However, they found that only G-6-PDH activity increased in response to insulin when the rats were fed a high glucose diet. Method of treating the animals may be important because malic enzyme activity in the liver also increased for rats fed the high glucose diet in the present study; where the rats were fasted prior to diet manipulation.

Malic enzyme activity in the adipose tissue (Fig. 2) followed a pattern almost opposite to that of the liver. Both enzymes increased more when insulin-treated rats were fed high glucose instead of high protein diets.

Rats injected with insulin consumed significantly ($P < .05$) more food during refeeding irrespective of diet, than the non-injected rats (Table 9, appendix table V). Holten et al. (1971) have suggested that the increased food consumption is due to lower blood glucose levels of insulin-treated rats, thereby causing a stimulation in appetite. Although they have proposed that increased hepatic G-6-PDH activity was the result of increased carbohydrate consumption, similar responses to insulin on both the high and low carbohydrate diets in our study suggested that the response might

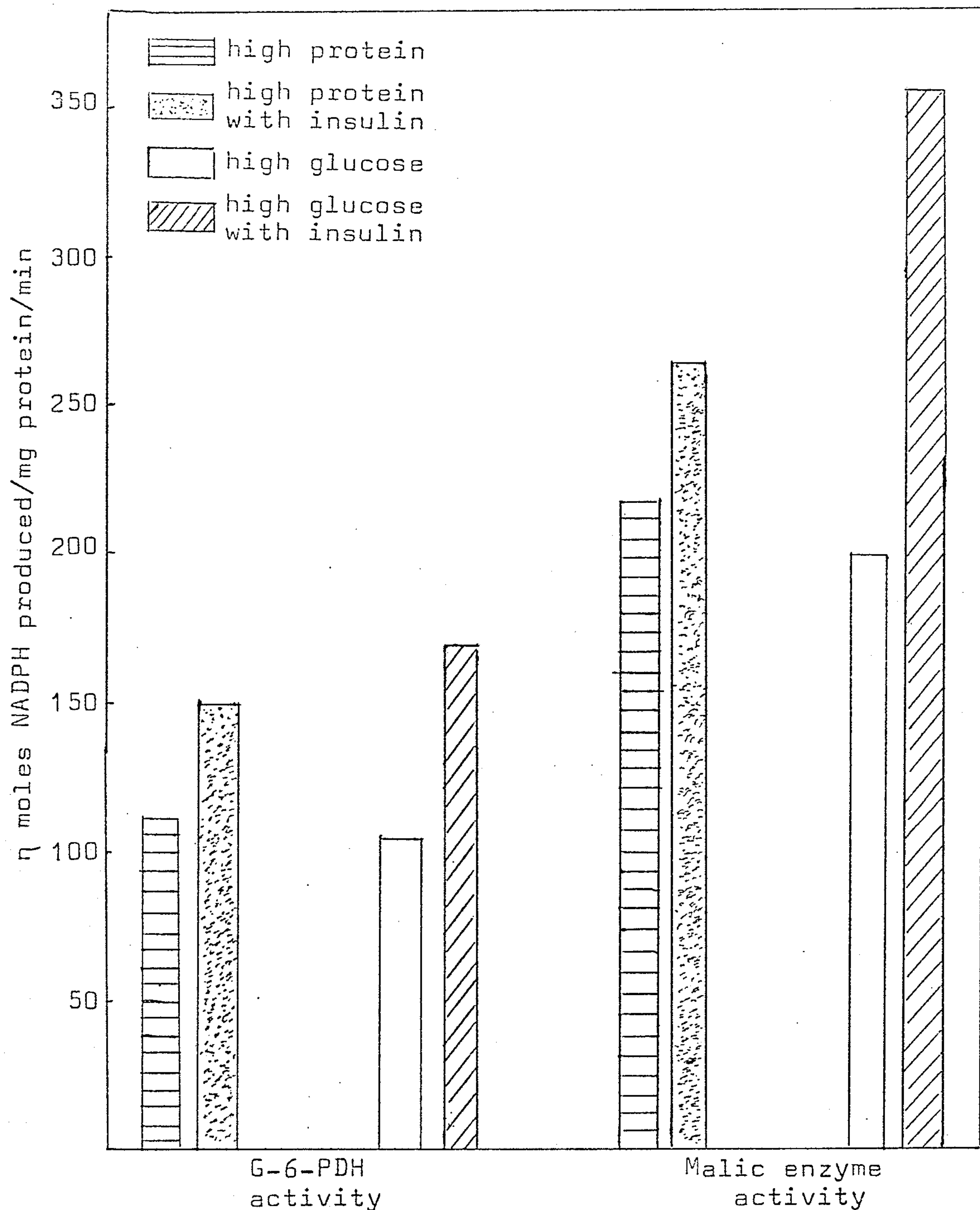

Figure 2. Effect of diet composition and exogenous insulin on enzyme activities in epididymal adipose tissue during refeeding for 48 hours following starvation. Each bar is the mean activity of 5 rats.

Table 9

Food consumption during 48-hour refeeding period following starvation[1]

Diet Refed	Food Intake (g)[2]	
	No Insulin	Insulin (4 units/100 g body wt./day)
High protein	31.9±5.1[3]	33.7±1.7
High glucose	33.7±3.3	39.2±1.1

[1]Analysis of variance for this data is shown in Appendix Table V.

[2]Total food consumed out of 40 g allotted during 48-hour refeeding period.

[3]Mean ± S.D. of 5 rats.

be due to increased caloric consumption from either protein or carbohydrate. Thus, the data from the present study was subjected to analyses of covariance (Appendix tables 6 to 9) to determine whether the response to insulin was due to increased caloric consumption. Hepatic G-6-PDH and malic enzyme activities in the insulin-treated rats were still significantly ($P < .01$) higher than those of non-injected rats. However, the activities of these enzymes in adipose tissue of rats receiving additional insulin were no longer significant.

Hepatic malic enzyme activities were significantly ($P < .01$) lower for rats refed a high protein than for those fed the high glucose diet (Table 7, appendix table II). Since rats on the high protein diet consumed significantly ($P < .05$) less food than those on a high glucose diet (Table 9, appendix table V), the data was assessed by analysis of covariance. The lower hepatic malic enzyme activity was still significant ($P < .05$) which is in agreement with Cohen _et al_. (1966) and Leveille _et al_. (1967).

G-6-PDH activities were similar in liver or adipose tissue of refed rats regardless of carbohydrate levels of the diet. These results are at variance with those of Potter and Ono (1961) who noted that increasing protein content above 18% lowered activity and Sassoon _et al_. (1968) who found that activity increased directly with carbohydrate intake in starved-refed rats.

The synthesis of lipid as shown by acetate-1-^{14}C incorporation was not significantly increased in adipose or hepatic tissue of rats given additional insulin (Table 10). Lipid synthesis tended to be lower in the livers of rats fed high protein than in those fed the high glucose diet but the differences were not significant. Acetate-1-^{14}C incorporation was higher in adipose tissue than in liver which agrees with previous observations in our laboratory by Watt (1970).

Table 10

The effect of starvation and refeeding for 48 hours on *in vitro* acetate-1-^{14}C incorporation into lipid

Diet Refed	Acetate-1-^{14}C Incorporation (dpm x 10^3/g tissue)	
	No Insulin	Insulin (4 units/100 g body wt./day)
		Liver
High protein	21± 7[1]	17± 4[2]
High glucose	30±11	42±19
		Epididymal Adipose Tissue
High protein	380±217	232±116[2]
High glucose	222±130	108± 31
Controls	Liver	Adipose Tissue
Starved 3 days	20.9± 4.1	59.5±65.0
Ad libitum-fed lab chow	27.8±19.3	103.7±70.3

[1]Mean ± S.D. of 4 rats except where designated.

[2]Mean ± S.D. of 3 rats.

VI. D. Discussion (Experiment 4)

The relationship between insulin and the induction of G-6-PDH and malic enzyme appears to be an excellent example of the complex interaction among hormonal and nutritional factors in physiological control. Holten et al. (1971) have proposed that the increase in hepatic G-6-PDH activity in response to exogenous insulin is simply the result of a stimulation in appetite thus causing increased consumption of carbohydrate. The present study suggests that this hypothesis is too simple for hepatic tissue because hepatic G-6-PDH and malic enzyme activities still were significant ($P < .01$) when the activities of the insulin treated rats were adjusted for greater food consumption by analysis of covariance.

The possibility that insulin acts as a specific inducer of G-6-PDH and malic enzyme is debatable. Weber (1966) suggested that hepatic G-6-PDH activity is extremely sensitive to the amount of insulin present, since there was an increase in hepatic G-6-PDH activity when alloxan-diabetic rats were starved and refed, which he assumed to be the result of the release of a small amount of insulin from the remaining B cells. However, in the present study, a physiological dose of insulin (2 to 4 units/kg body weight/day) was ineffective in increasing activities of G-6-PDH and malic enzyme. In fact, a pharmacutical dose of 40 units/kg

body weight/day was necessary to increase the activities of these enzymes. However, an exogenous dose of insulin is not directly comparable to an endogenous secretion because initial rate of breakdown, rate of release, etc. of exogenous insulin would not come under normal physiological control.

Hepatic malic enzyme activity appeared to be slightly more sensitive than G-6-PDH to insulin levels. Response of malic enzyme activity was less when rats were refed the high protein diet, which would be expected to elicit less endogenous insulin secretion than the high glucose diet. Malic enzyme also showed a greater response than G-6-PDH in the liver of rats receiving insulin during refeeding ona high protein as compared to a high glucose diet. These two results suggest that a decrease in insulin not carbohydrate may be responsible for the lower malic enzyme levels in non-injected animals.

Although carbohydrate has been suggested as the prime dietary inducer of G-6-PDH activity during hyperlipogenesis, the role of protein in the induction of this enzyme has not been extensively researched. The fact that G-6-PDH activity in hepatic and adipose tissue of fasted-refed rats did not decrease when dietary protein was substituted for carbohydrate, disagrees with the proposal of Sassoon and Johnson (1968) that G-6-PDH activity varied directly with carbohydrate intake, and that protein was not a dietary inducer of this

enzyme. However, their hypothesis is questionable since unlike the present study they confounded their diets by substituting fat as well as protein for carbohydrate. Nevertheless, Garza and associates (1970) have reported a suppression in the "overshoot" of G-6-PDH activity in the liver when fasted rats were refed a high protein, carbohydrate-free diet. The possible role of insulin in the induction of G-6-PDH and malic enzyme and the relationship of carbohydrate to this response might be elucidated if insulin were administered to fasted rats during refeeding with diets containing graded levels of dietary carbohydrate including a high protein, carbohydrate-free diet.

The observations of the present study suggest that adipose tissue responds differently than liver when exogenous insulin is administered to fasted-refed rats. Although injection of insulin markedly increased the activities of malic enzyme and G-6-PDH in adipose tissue, subjection of the results to analysis of covariance showed this response was primarily the result of increased food consumption by the insulin-injected rats. However, the adipose tissue appears to respond more slowly than liver to dietary and hormonal influences during refeeding of fasted rats. Furthermore, Watt (1970) found the activities of G-6-PDH and malic enzyme was still increasing in the adipose tissue of rats at 120 hours, whereas the activities in the liver decreased after

72 hours of refeeding fasted rats a high-carbohydrate diet. Validation of the results in the present study which showed the response to insulin in adipose tissue was the result of increased food intake awaits further studies where the refeeding period is considerably extended beyond 48 hours.

The present experiment indicates a lack of association between lipogenesis and NADPH-generating enzyme activity. Although there was a pronounced increase in activities of G-6-PDH and malic enzyme in adipose and hepatic tissue when insulin was administered to fasted-refed rats, the rate of *in vitro* acetate-1-^{14}C incorporation into lipid was not affected. Halperin *et al.* (1971) noted that inclusion of insulin in the assay medium caused a 30-fold increase in the *in vitro* rate of glucose conversion to fatty acid in white adipose tissue. They also found that insulin increased the supply of substrate (pyruvate) and directly increased pyruvate incorporation into fatty acids by a mechanism distinct from the known stimulation of glucose transport. Failure to find a response to insulin injection in the present study suggests that the insulin *in vivo* has no effect on lipogenesis, although the action of insulin may have been marked by physiological regulators such as concentration of substrate, activators and inhibitors of enzyme, etc. which would not be factors in *in vitro* studies.

The observations in this experiment also disagree

with the hypothesis of Leveille (1967) that changes in activities of malic enzyme and G-6-PDH with insulin administration are secondary to alterations in the rate of lipogenesis. Rosmos and Leveille (1971) further supported this proposal by demonstrating that malic enzyme activity increased with insulin administration in the alloxan-diabetic rat, but only after the rate of fatty acid synthesis had increased. Nevertheless, the observations of the present study suggest that insulin-induced increases in G-6-PDH and malic enzyme activities in adipose and hepatic tissue are not due to the increased demand for NADPH, although comparison of diabetic and normal animals is difficult. However, confirmation of these results require additional studies where the assays are also carried out at 12 and 24 hours after refeeding to see if insulin induces an early response in lipogenesis which is no longer evident at 48 hours.

VII. SUMMARY

Four experiments were conducted to determine the effect of exogenous insulin on the activities of G-6-PDH and malic enzyme in the hepatic and adipose tissues of starved-refed rats.

In the first three experiments, insulin at a dosage level of 2 to 4 units/kg body weight/day injected intraperitoneally failed to consistently increase the activities of G-6-PDH and malic enzyme. These results were further complicated by the fact that a placebo injection of bovine serum albumin used to dilute the insulin tended to depress the hepatic G-6-PDH and malic enzyme activities during refeeding.

However, when the dosage level of insulin was increased to 4 units/100 g body weight/day, and the insulin was injected subcutaneously, G-6-PDH and malic enzyme activities were profoundly increased in both hepatic and adipose tissues of starved rats refed either a high protein or high glucose diet for 48 hours. Rats injected with insulin had marked increases in food consumption irrespective of diet. When the results were assessed by analyses of covariance, the increases in hepatic G-6-PDH and malic enzyme were still significant. On the other hand, the effect of insulin on G-6-PDH and malic enzyme activities in the adipose tissue

was primarily the result of increased food intake suggesting a different mode of action of insulin in liver and adipose tissue.

The rate of *in vitro* acetate-1-^{14}C incorporation into lipid did not increase when rats were administered insulin, thus displaying a lack of association between lipogenesis and NADPH-generating enzyme activities. These results suggest that the increases in hepatic G-6-PDH and malic enzyme activities with insulin are not secondary to the increased rate of lipogenesis.

A relationship between diet and insulin was suggested in the present study since hepatic malic enzyme showed a greater response to exogenous insulin during refeeding on the high protein diet which would be expected to elicit less endogenous insulin secretion than a high glucose diet. Furthermore, the response of malic enzyme in liver was appreciably lower when dietary protein was substituted for carbohydrate in the diet refed to fasted rats.

The results of this study suggest that insulin could play an important role in the induction of G-6-PDH and malic enzyme. However, further research must be conducted before the mechanism of action of insulin in enzyme synthesis in fasted-refed rats is clarified.

VIII. APPENDIX TABLES

APPENDIX TABLE I

Analysis of variance for G-6-PDH activity in liver

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Significance $P < 0.01$
Total	19	95603.23			
Within treatment	16	29550.87	1846.93		
Between treatment					
1. Diet glucose vs. protein	1	782.13	782.13	.42	ns[2]
2. Insulin insulin treated vs. non-insulin treated	1	65250.46	65250.46	35.33	s[1]
3. Interaction diet x insulin	1	19.78	19.78	.01	ns

Variance ratio $n_1 = 1$, $n_2 = 16$
- 1 Percent Points for Distribution of $F = 8.53$
- 5 Percent Points for Distribution of $F = 4.49$

[1]significant [2]not significant

APPENDIX TABLE II

Analysis of variance for malic enzyme activity in liver

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Significance $P < 0.01$
Total	19	70128.11			
Within treatment	16	21164.51	1322.78		
Between treatment					
1. Diet glucose vs. protein	1	18356.18	18356.18	13.88	s[1]
2. Insulin insulin treated vs. non-insulin treated	1	30454.33	30454.33	23.02	s
3. Interaction diet x insulin	1	153.08	153.08	.12	ns[2]

Variance ratio $n_1 = 1$, $n_2 = 16$

- 1 Percent Points for Distribution of $F = 8.53$
- 5 Percent Points for Distribution of $F = 4.49$

[1] significant [2] not significant

APPENDIX TABLE III

Analysis of variance for G-6-PDH activity in epididymal adipose tissue

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Significance $P < 0.01$
Total	19	23850.96			
Within treatment	16	9711.60	606.97		
Between treatment					
1. Diet glucose vs. protein	1	235.57	235.57	.39	ns[2]
2. Insulin insulin treated vs. non-insulin treated	1	12288.90	12288.90	20.25	s[1]
3. Interaction diet x insulin	1	1614.89	1614.89	2.66	ns

Variance ratio $n_1 = 1$, $n_2 = 16$
- 1 Percent Points for Distribution of F = 8.53
- 5 Percent Points for Distribution of F = 4.49

[1]significant [2]not significant

APPENDIX TABLE IV

Analysis of variance for malic enzyme activity in epididymal adipose tissue

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Significance $P < 0.05$
Total	19	205383.37			
Within treatment	16	139087.88	8692.99		
Between treatment					
1. Diet glucose vs. protein	1	4353.90	4353.90	.50	ns[2]
2. Insulin insulin treated vs. non-insulin treated	1	49821.16	49821.16	5.73	s[1]
3. Interaction diet x insulin	1	12120.43	12120.43	1.39	ns

Variance ratio $n_1 = 1$, $n_2 = 16$
- 1 Percent Points for Distribution of $F = 8.53$
- 5 Percent Points for Distribution of $F = 4.49$

[1] significant

[2] not significant

APPENDIX TABLE V

Analysis of variance for food consumption

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Significance $P < 0.05$
Total	19	312,75			
Within treatment	16	161,52	10.09		
Between treatment					
1. Diet					
glucose vs. protein	1	66,87	66.87	6.62	s[1]
2. Insulin					
insulin treated vs. non-insulin treated	1	67,45	67.45	6.68	s
3. Interaction					
diet x insulin	1	16.91	16.91	1.67	ns[2]

Variance ratio $n_1 = 1$, $n_2 = 16$

- 1 Percent Points for Distribution of F = 8,53
- 5 Percent Points for Distribution of F = 4,49

[1] significant

[2] not significant

APPENDIX TABLE 6

Analyses of covariance for G-6-PDH activity in liver

Source of Variation	d.f.	SS for x (food consumption)	SP (xy)	SS for y (enzyme activity)	d.f.	Deviations SS	MSE
Total	19		3349.21				
Error	16	161.52	1040.84	29550.87	15	19314.59	1287.64
1. Diet glucose vs. protein	1	66.87	228.69	782.13			
2. Insulin insulin treated vs. non-insulin treated	1	67.45	2097.96	65250.46			
3. Interaction diet x insulin	1	16.91	-18.29	19.78			
Diet + error	17	228.38	1269.53	30332.99	16	23275.95	
Insulin + error	17	228.97	3138.80	94801.33	16	51773.27	

	d.f.	SS	MS	F value	Significance ($P < 0.01$)
Adjusted diet	1	3961.36	3961.36	3.08	ns[2]
Adjusted insulin	1	32458.68	32458.68	25.21	s[1]

Variance ratio $n_1 = 1$, $n_2 = 15$
- 1 Percent Points for Distribution of F = 8.68
- 5 Percent Points for Distribution of F = 4.54

[1] significant

[2] not significant

APPENDIX TABLE 7

Analyses of covariance for malic enzyme activitiy in liver

Source of Variation	d.f.	SS for x (food consumption)	SP (xy)	SS for y (enzyme activity)	d.f.	Deviations SS	MSE
Total	19		3257.24				
Error	16	161.52	762.86	21164.51	15	17561.39	1170.76
1. Diet glucose vs. protein	1	66.87	1108.66	18356.18			
2. Insulin insulin treated vs. non-insulin treated	1	67.45	1433.28	30454.33			
3. Interaction diet x insulin	1	16.91	-47.56	153.08			
Diet + error	17	228.38	1871.52	39520.69	16	24184.27	
Insulin + error	17	228.97	2196.14	51618.84	16	30554.72	

	d.f.	SS	MS	F value	Significance
Adjusted diet	1	6622.88	6622.88	5.66	s[1] ($P < 0.05$)
Adjusted insulin	1	12993.33	12993.33	11.10	s[1] ($P < 0.01$)

Variance ratio $n_1 = 1$, $n_2 = 15$
-1 Percent Points for Distribution of F = 8.68
-5 Percent Points for Distribution of F - 4.54

[1]significant

APPENDIX TABLE 8

Analyses of covariance for G-6-PDH activity in epididymal adipose tissue

Source of Variation	d.f.	SS for x (food consumption)	SP (xy)	SS for y (enzyme activity)	d.f.	Deviations SS	MSE
Total	19		1997.69				
Error	16	161.52	796.11	9711.60	15	5787.54	385.84
1. Diet glucose vs. protein	1	66.87	125.51	235.57			
2. Insulin insulin treated vs. non-insulin treated	1	67.45	1129.26	12288.90			
3. Interaction diet x insulin	1	16.91	-53.19	1614.89			
Diet + error	17	228.38	921.62	9947.17	16	6228.05	
Insulin + error	17	228.97	1925.37	22000.49	16	5810.29	

	d.f.	SS	MS	F value	Significance ($P < 0.05$)
Adjusted diet	1	440.51	440.51	1.14	ns[1]
Adjusted insulin	1	22.76	22.76	.05	ns

Variance ratio $n_1 = 1$, $n_2 = 15$
- 5 Percent Points for Distribution of $F = 4.54$

[1]not significant

APPENDIX TABLE 9

Analyses of covariance for malic enzyme activity in epididymal adipose tissue

Source of Variation	d.f.	SS for x (food consumption)	SP (xy)	SS for y (enzyme activity)	d.f.	Deviations SS	MSE
Total	19		6231.90				
Error	16	161.52	3406.39	139087.88	15	67246.13	4483.08
1. Diet glucose vs. protein	1	66.87	539.57	4353.90			
2. Insulin insulin treated vs. non-insulin treated	1	67.45	1833.21	49821.16			
3. Interaction diet x insulin	1	16.91	452.72	12120.43			
Diet + error	17	228.38	3945.97	143441.78	16	75263.99	
Insulin + error	17	228.97	5239.61	188369.04	16	68468.61	

	d.f.	SS	MS	F value	Significance ($P < 0.05$)
Adjusted diet	1	8017.86	8017.86	1.79	ns[1]
Adjusted insulin	1	1222.48	1222.48	0.27	ns

Variance ratio $n_1 = 1$, $n_2 = 15$
– 5 Percent Points for Distribution of $F = 4.54$

[1]not significant

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