Effect of Dietary Fat and Diet Restriction versus Inanition on the Metabolic Response of Rats during Refeeding

by

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ABSTRACT

Studies with adult male rats compared the effect of 4-day starvation or diet restriction to 5 g daily for 8 days and the effect of a fat-free or 12% fat diet, fed during the restriction and refeeding periods, on the metabolic response during refeeding. The metabolic response was also examined in controls that were either starved or restricted on either diet but not refed and in those fed either diet ad libitum for ten days. Hepatic and epididymal adipose tissue glucose-6-phosphate dehydrogenase and malic enzyme activities and in vitro acetate-1-^{14}C incorporation into fatty acids were assayed in control rats and in starved or restricted rats refed either the fat-free or 12% fat diet for 48, 72, 96 and 120 hours. Total hepatic lipid was also extracted from all rats. Neither method of inducing weight loss nor diet fed during restriction influenced the refeeding response. Inclusion of fat in the diet refed had a marked effect only on hepatic NADP-linked enzyme activity. Refeeding the fat-free diet 120 hours increased liver G-6-P dehydrogenase and malic enzyme activities 18 to 25 times that of starved or restricted controls. The 12% fat diet suppressed activity to a 5-fold increase. Fat pad G-6-P dehydrogenase activity increased 3- to 6-fold irrespective of diet fed, while malic enzyme activity increased 8-fold when the fat-free diet was refed and 5-fold when the 12% fat

diet was refed. Hepatic ^{14}C incorporation into fatty acids showed a rise at 48 and 72 hours refeeding, decreasing to one-half that of starved or restricted rats at 120 hours refeeding. Incorporation by fat pads increased throughout refeeding. Refeeding the fat-free diet increased liver lipid concentrations to a maximum level at 72 hours which were three times those of ad libitum-fed controls. No increase in liver lipid concentrations were observed in rats refed the corn oil diet.

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LIST OF ABBREVIATIONS

Co A - coenzyme A

NADP - nicotinamide-adenine dinucleotide phosphate

NADPH - reduced form of NADP

NAD - nicotinamide-adenine dinucleotide

NADH - reduced form of NAD

EMH - embden-meyerhof

HMP - hexosemonophosphate

PEP - phosphoenolpyruvate

G-6-P - glucose-6-phosphate

G-6-phosphatase - glucose-6-phosphatase

6-P-gluconate - 6-phosphogluconate

F-1, 6-diphosphatase - fructose 1, 6-diphosphatase

ATP - adenosine triphosphate

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I. GENERAL INTRODUCTION

Hyperlipogenesis in adipose tissue and liver is the most striking metabolic response observed during refeeding following starvation. However, there is some question whether hyperlipogenesis is the cause or the effect of hypertension and of elevated enzyme activities which also accompany refeeding following starvation. Composition of the diet refed can modify the nature and extent of these responses. Physiological stress due to unregulated refeeding tends to be most severe in animals refed a high-carbohydrate, low-fat diet and least severe when fat or high-protein diets are fed. The effects of dietary fat have been shown to depend not only on the fatty acid composition of the fat, but also on the level of fat in the diet, the length of time it is fed, the species and age of the animal being studied, whether fat is added to the diet at the expense of carbohydrate and whether the studies are conducted *in vivo* or *in vitro*.

These observations are of some concern to humans since total starvation is prescribed for weight reduction in the chronic obese although the more common practice used by the general public is restriction of food intake. The indication by Johnson and Sassoon (1967) that starvation must precede carbohydrate refeeding to obtain an 'overshoot' in enzyme activities suggested that the consequences of

ad libitum-refeeding might be different following diet restriction than following starvation. Therefore the present study was undertaken to determine whether the metabolic response to unregulated food intake was the same following severe calorie restriction as that observed following starvation. In addition, the study investigated the effect of feeding a high-fat diet during restriction and refeeding on the response accompanying *ad libitum* refeeding.

An understanding of the effect of diet composition on lipogenesis is necessary before appropriate measures can be effectively applied to control the physiological stress (ie. hypertension, cardiovascular disease and arteriosclerosis) resulting from realimentation following starvation. Furthermore, an investigation into the effects of different weight reduction regimens on body metabolism might reveal a regimen that is as effective as starvation in promoting weight loss but is not accompanied by the undesirable effects associated with refeeding following starvation.

II. REVIEW OF LITERATURE

A. Introduction

Meal-fed and fasted-refed animals are well-suited for studying the pathway of fatty acid formation because high rates of lipid synthesis can be easily induced in the tissues of these animals. Although the liver has been classically regarded as the major if not exclusive site of fat synthesis, there is the opinion that the adipose tissue is equal to or more important than the liver in fat synthesis. Liver and adipose tissue recently have been shown to differ not only in their capacity to synthesize fat but also in their ability to synthesize glucose via gluconeogenesis. These differences may arise from the presence or absence of key enzymes in glucose and fat metabolism in the liver and adipose tissue. Therefore the control and extent of lipogenesis and gluconeogenesis in the liver and adipose tissue would be influenced by differences in enzyme activity, substrate and inhibitor concentrations and hormonal response. All of these factors have been shown to be influenced by the composition of the diet which is refed following a period of fasting.

To more clearly elucidate and understand the metabolic pathway for fat synthesis it has been traditional to investigate the activity of enzyme systems responsible for mediating key steps in the fatty acid synthetic pathway

and the dietary factors involved in regulating the activity and synthesis of these enzymes. Recent concepts suggest however that availability and type of substrate may control fatty acid synthesis through its influence on the balance between production of reduced coenzymes and re-oxidation of these coenzymes during fat synthesis. Although these and other suggestions have been proposed for the control of fatty acid synthesis, the exact mechanism is not clear. Further investigation into the mechanism regulating fatty acid synthesis is necessary before appropriate measures can be applied to control the physiological stress resulting from realimentation following starvation.

II. B. The Importance of Adipose Tissue in Fatty Acid Synthesis

To understand fatty acid synthesis it is necessary to identify the chief sites of synthesis, the relative importance of each, and the mechanisms involved in regulating synthesis in each site. This knowledge is necessary before appropriate measures can be applied effectively to control fatty acid synthesis at the sites where it occurs.

Favarger (1965) concluded that the liver and adipose tissue are the principal sites of fatty acid synthesis although other organs and tissues possess this ability. However, a direct comparison of the role of the liver with that of adipose tissue is difficult because the former is a well-defined organ and the latter is a tissue distributed throughout the organism. Moreover, the turnover rate for fatty acids in the two tissues is different.

Nevertheless, the liver has been classically regarded as the major, if not exclusive, site of fat synthesis. This idea may have arisen because the half-life of hepatic fatty acids was reported much shorter than the half-life of fatty acids in other tissues, except for intrascapular brown adipose tissue where the half-life of fatty acids was similar to that in liver. The increase in fatty acids in other tissues during lipogenesis was presumed to be brought about by transport from liver via the blood stream.

However Favarger (1965) cited studies suggesting the

adipose tissue was equal to or more important than the liver in fat synthesis. Brown adipose tissue, which is not a storage type of adipose tissue, was found more specialized and more active than the liver with respect to lipogenesis on the basis of its greater mass. Although brown adipose tissue is a peculiar variety of adipose tissue it was suggested that white adipose tissue was also a metabolically active tissue, not simply an inert reserve for the deposit of fat synthesized elsewhere. This was based on the demonstration that lipogenesis was not impaired in hepatectomized rats and the observation that adipose tissue possessed a high respiratory quotient.

Adipose tissue has also been shown as the major site of the lipogenic change induced by meal-feeding rats and mice. Under normal conditions adipose tissue from rats and mice accounted for approximately 50% of total fatty acid synthesis whereas it accounted for 95% during meal-feeding when lipogenesis was markedly enhanced (Favarger, 1965; Jansen et al., 1966; Leveille, 1967a and 1967b).

The distribution of hexose monophosphate shunt dehydrogenase activity in various rat tissues has been related to the lipogenic capacity of these tissues; (Fritz, 1961) with higher activities being observed in adipose tissue than in liver (Hollifield and Parson, 1962; Young et al., 1964; Pande et al., 1964). Recent experiments also suggest this may be true for other enzymes related to

lipogenesis. The activity of the fatty acid synthesizing enzymes (acetyl Co A carboxylase and fatty acid synthetase), citrate cleavage enzyme, NADPH-generating enzymes, and other enzymes related to carbohydrate-induced lipogenesis (hexokinase, pyruvate kinase, alpha-glycerophosphate dehydrogenase, pyruvate carboxylase) increased in adipose tissue but not liver of meal-fed rats in a manner similar to fatty acid synthesis (Chakrabarty and Leveille, 1968 and 1969; Hollifield and Parson, 1962). Young et al. (1964) and Pande et al. (1964) have suggested that malic enzyme activity is more closely related to lipogenesis than hexose monophosphate shunt dehydrogenase activity since this enzyme increased more than glucose-6-phosphate dehydrogenase during refeeding following fasting although the pattern of change was similar for the two enzymes. The concentration of malic enzyme has also been found to be higher in adipose tissue than liver (Pande et al., 1964; Young et al., 1964; Wise and Ball, 1964). Therefore, the adipose tissue of the rat appears to play a major role in lipid synthesis, especially under conditions of enhanced lipogenesis. This view is supported by its enzymatic capacity to carry out lipogenesis and its much greater overall mass relative to liver.

Adipose tissue also appears to play a central role in fatty acid synthesis in the pig as shown by its adaptive lipogenic and enzymatic response to refeeding following fasting (O'Hea and Leveille, 1969a). Like the rat, adipose

tissue from ad libitum-fed pigs possessed higher glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and malic enzyme activities than the liver.

On the other hand, the situation is reversed in the chicken where in contrast with the rat, mouse and pig, hepatic tissue accounts for over 70% of de novo fatty acid synthesis (Leveille et al., 1968). Like the rat, the chicken responds to fasting and refeeding and meal-feeding with an increased rate of lipid synthesis but unlike the rat this occurs in the liver (Leveille, 1969; Leveille et al., 1968). Meal-feeding in the pig, in contrast with the rat and chicken, produced no change in lipogenesis or the complementary enzymes (O'Hea and Leveille, 1969b).

These data illustrate that in studies on lipogenesis care must be taken in the choice of experimental animal and tissue. Unless fatty acid synthesis in the rat is studied in both liver and adipose tissue, difficulties will arise in interpreting the physiological significance of the data. In the chicken and pig however, lipogenesis is confined to one site. An awareness of these species differences is a prerequisite to the study of the influence of diet on lipogenesis.

II. C. The Pathways of Carbohydrate Metabolism in Liver and Adipose Tissue

Weber (1965) concluded that the carbohydrate-metabolizing enzymes present in adipose tissue were essentially the same as those in the liver, only the specific activities were 50% lower. The HMP shunt dehydrogenase enzymes were an exception to this general condition (Pande et al., 1964).

The fact that the glycolytic pathway was less active in the adipose tissue than in the liver may be due to low phosphohexoisomerase and lactic dehydrogenase activities, the first and last enzymes in the glycolytic path from G-6-P (Weber, 1965). In addition, low aldolase activity may limit the rate of glycolysis in adipose tissue.

The limited ability of adipose tissue to manufacture and store glycogen in comparison with the capacity of the liver, may be due to low phosphoglucomutase activity (Weber, 1965). In addition, the absence of glucose-6-phosphatase and fructose-1, 6-diphosphatase activities in adipose tissue impedes gluconeogenic production of glucose for glycogen synthesis (Weber, 1965; Pande et al., 1964; Leveille, 1966). However, the presence of these enzymes in organs such as liver facilitates glucose for release to blood and for manufacture of glycogen. Gluconeogenic production of glucose then appears to have a more important role in liver than in adipose tissue, because the liver is more involved

in glycogen synthesis and glucose release to the blood than adipose tissue.

On the other hand, lipid production has been suggested as the special function of the adipose tissue (see section II. B.). HMP shunt dehydrogenase and malic enzyme activities, necessary for NADPH generation, were higher in this tissue than the liver from rats (Pande et al., 1964; Young et al., 1964). Weber (1965) noted that G-6-P dehydrogenase activity was five times more active in the adipose tissue than in hepatic tissue. In addition, malic enzyme activity was higher than G-6-P dehydrogenase activity in adipose tissue. G-6-P dehydrogenase activity was also higher than 6-phosphogluconate dehydrogenase in rat adipose tissue while the opposite relationship existed in the liver. The same was true for human liver and adipose tissue. On the other hand, Pande et al. (1964) found both G-6-P and 6-phosphogluconate dehydrogenase activities equal in the adipose tissue. Nevertheless, the pentose phosphate pathway appears to be the main metabolic route that G-6-P follows in adipose tissue.

It is apparent then, that the predominant metabolic pathways in the adipose tissue are the pentose phosphate shunt and glycolytic pathways while in the liver glycogenesis, glycogenolysis, gluconeogenesis and glycolysis predominate. The pathways that predominate in each tissue are in line with the need for glucose production by liver

and lipid synthesis in adipose tissue.

II. D. The Metabolic Response of Liver and Adipose Tissue During Fasting

Triglyceride synthesis occurs in adipose tissue even during fasting. The importance of re-esterification in adipose tissue from the fasting animal is confusing since re-esterification is counter to the primary function of fasting tissue; namely, lipolysis (Reshef et al., 1969; Shapiro et al., 1957; Stein and Stein, 1961).

Nevertheless, maintenance of a steady state equilibrium in the presence of continuing lipolysis requires a constant supply of substrate from which alpha-glycerophosphate can be generated and energy for esterification derived (Steinburg et al., 1961). In fasted liver this requirement is met by gluconeogenic production of glucose, the main precursor of glyceride-glycerol. Since adipose tissue lacks glycerokinase (Shapiro et al., 1957; Stein and Stein, 1961; Steinburg et al., 1961) and gluconeogenic enzymes (see section II. B.), it can neither utilize free glycerol nor produce glucose to meet this requirement. However, it can adapt enzymatically and convert pyruvate, certain gluconeogenic amino acids (alanine, aspartic) and lactate to glyceride-glycerol via the dicarboxylic acid shuttle—a pathway similar to gluconeogenesis in the liver (Reshef et al., 1969; Ballard et al., 1967).

The existence of this pathway in adipose tissue has been shown in vitro. Adipose tissue has sufficient pyruvate

carboxylase activity (mitochondrial, 70%; cytoplasmic, 30%) to generate mitochondrial oxaloacetate for: (1) the citric acid cycle; (2) fatty acid synthesis via the citrate cleavage pathway; and (3) the transhydrogenation sequence of the shuttle (Reshef et al., 1969; Ballard and Hanson, 1967; Steinburg et al., 1961). Although Young et al. (1964) failed to observe any PEP carboxykinase activity in adipose tissue Ballard et al. (1967), using a different assay procedure, found sufficient activity in the cytoplasm to support pyruvate conversion to glycerol via oxaloacetate.

Reduced NADP necessary for lipogenesis from pyruvate during fasting is derived from the coupling of three reactions: (1) pyruvate conversion to oxaloacetate via pyruvate carboxylase intramitochondrially; (2) oxaloacetate conversion to malate; and (3) malate passage to the cytosol and conversion to pyruvate with NADPH production by malic enzyme. The NADH necessary to drive this transhydrogenation reaction is derived from oxidative decarboxylation of some pyruvate via pyruvate dehydrogenase (Kneer and Ball, 1968).

The shuttle pathway is operative during fasting coincident with the need for gluconeogenesis in liver and re-esterification in adipose tissue. It follows that pyruvate carboxylase and PEP carboxykinase activities in both tissues are enhanced during fasting. The shuttle pathway probably operates very slowly during refeeding on a

balanced or high-carbohydrate diet since both enzymes were either depressed or not elevated from the level in untreated animals (Jomain and Hanson, 1969; Young et al., 1964; Reshef et al., 1969; Ballard et al., 1967; Ballard and Hanson, 1967). Leveille (1967) also noted that the shuttle pathway is increased in rats fed a high-fat (56%) diet in accord with the increased need for gluconeogenesis. In periods of glucose deprivation then, the prime function of the shuttle is glucose production in liver and glyceride-glycerol production in adipose tissue.

The operation of the dicarboxylic acid shuttle in hepatic gluconeogenesis is in agreement with observations sited by Young et al. (1964) where the response of PEP carboxykinase to various treatments was similar to the response of gluconeogenic enzymes (eg. G-6-phosphatase, F-1, 6-diphosphatase, glutamic-pyruvate transaminase), but opposite to lipogenesis and enzymes related to lipogenesis (eg. malic enzyme). Furthermore, PEP carboxykinase activity may play a key role in the control of gluconeogenesis since PEP carboxykinase response to dietary alterations was more rapid than other gluconeogenic enzymes.

These observations illustrate that liver and adipose tissue differ in their metabolic response during fasting due to differences in the activity of key carbohydrate-metabolizing enzymes and also physiological need. These differences suggest that the mechanisms controlling the

major metabolic pathways may not be identical in the two tissues.

II. E. Current Concepts in the Control of Fatty Acid Synthesis

The biosynthetic pathway for fatty acid synthesis is similar in liver and adipose tissue (Wise and Ball, 1964; Martin and Vagelos, 1965). However the control and extent of lipogenesis in each site may be different because of differences in enzyme activity (see section II. C.), substrate and inhibitor concentrations, and hormonal response.

1. Relationship of Enzyme Activity and Lipogenesis

A close association between production of NADPH, via the HMP shunt, and lipogenesis has been observed under a variety of conditions. This relationship has been extensively reviewed and discussed by several workers (Fritz, 1961; Jomain and Hanson, 1969; Masoro, 1962). The apparent association between HMP shunt dehydrogenase activity and lipogenesis led Tepperman and Tepperman (1958 and 1963) to propose a mechanism, based on the reduction of NADP, for the control of lipogenesis in the livers of starved-refed rats. They suggested that the availability of NADPH limited fatty acid synthesis and that 'supernormal' lipogenesis depended upon increased pentose cycle activity to generate adequate reduced coenzymes.

On the other hand, Flatt and Ball (1964) suggested that the pentose cycle of adipose tissue furnished only about 63% of the reducing equivalents necessary for fatty

acid synthesis. Subsequently, Kornacker and Ball (1965) suggested that another source of reducing power in adipose tissue might be the coupling of malic dehydrogenase (EC 1.1.1.37) and malic enzyme (EC 1.1.1.40) in what was termed the malate transhydrogenation cycle.

Malic enzyme activity has been suggested to play a key role in lipogenesis. The activity of this enzyme in liver and adipose tissue of the rat was altered by experimental conditions which are known to affect lipogenesis such as fasting, refeeding, diabetes and lactation (Wise and Ball, 1964; Pande et al., 1964). Goodridge (1969 and 1968), working with chicks, also concluded that the changes in malic enzyme activity are adaptive in nature; with activity increasing or decreasing as lipogenesis increases or decreases. Furthermore, malic enzyme has been suggested to be more intimately involved in fat synthesis than the HMP shunt dehydrogenase enzymes. The magnitude of the change in activity of shunt dehydrogenase enzymes in response to dietary manipulation was lower than that for malic enzyme although dietary alterations produced parallel changes in lipogenesis, malic enzyme and HMP shunt dehydrogenase activity (see section II. B.).

The function for malic enzyme in lipogenesis is interrelated to pyruvate carboxylase activity as well as malic dehydrogenase. Ballard and Hanson (1967) have demonstrated that oxaloacetate, used in the formation of

citrate, can be regenerated from pyruvate in the presence of mitochondrial pyruvate carboxylase in both adipose tissue and liver. In fact, replenishment of oxaloacetate by this reaction was essential for the continuous functioning of the citrate cleavage pathway and malate transhydrogenation cycle in liver and adipose tissue. Ballard and Hanson (1967) also proposed that cytoplasmic pyruvate carboxylase activity functioned in a separate 'short circuit' of the citrate cleavage pathway thereby generating NADPH in the cytoplasm without acetyl Co A formation. These results suggest that the malate transhydrogenation cycle and the HMP shunt may together, rather than either alone, provide the NADPH necessary for fat synthesis.

Not all evidence is in agreement with the possibility that the supply of NADPH limits lipogenesis although NADPH is required for lipogenesis (Fritz, 1961; Masoro, 1962; Tepperman and Tepperman, 1963). Bortz et al. (1963) and Abraham et al. (1959 and 1962) observed that the capacity for NADPH production in liver homogenates from alloxan diabetic, pancreatectomized, or fat-fed rats was similar to that of control animals, even though rate of fat synthesis was low. Wakil and Bressler (1962), in agreement with this conclusion, noted studies where the addition of NADPH or NADPH-generating systems to liver preparations from diabetic or starved animals failed to restore the activity of fatty acid synthesizing enzymes.

Further evidence indicating a lack of relationship between NADPH-generating dehydrogenase activity and fatty acid synthesis is based on the occasional association of high rates of lipogenesis with low NADPH dehydrogenase activities. McDonald and Johnson (1965) found that G-6-P dehydrogenase activity was reduced while lipogenesis was elevated in liver from starved rats refed a high-carbohydrate, low-protein diet (4%) when compared to the level produced by refeeding a high-carbohydrate, high-protein (18%) diet. Tepperman et al. (1968) also found that livers from rats refed a protein-free diet possessed a high capacity for lipogenesis in spite of minimal increases in HMP dehydrogenase enzymes, malic enzyme and citrate cleavage enzyme. Similarly, Leveille (1969) demonstrated a lack of association between lipogenesis and HMP shunt dehydrogenase activity in chicks refed a high-carbohydrate, low-fat diet following a three-day fast. One and two days of refeeding following starvation increased hepatic fatty acid synthesis to 275% and 364% of normal. Pentose pathway dehydrogenase activity, which was relatively low in the chick, was not greatly influenced by fasting and refeeding. Malic enzyme activity tended to parallel fatty acid synthesis but the magnitude of the change was much less pronounced. Malic enzyme exhibited a small 'overshoot' upon refeeding with activity returning to control values after two days.

In contrast with these studies Tepperman and Tepperman (1958) observed that HMP shunt dehydrogenase activity not only increased but continued to climb after lipogenesis had levelled off in livers from refed rats. Furthermore, Tepperman and Tepperman (1965) observed high levels of hepatic NADP-linked enzymes when lipogenesis was low. The physiological significance of this 'overshoot' and the signal which initiates it is not well understood. NADP generation could not act as the signal for increased NADP-linked enzyme activity because lipogenesis and consequently NADPH utilization is low. Tepperman and Tepperman (1965) have suggested however that the higher NADPH oxidase activity observed in liver homogenates from coconut oil-fed rats, could account for increased NADP generation and this in turn could stimulate NADP-linked enzyme activity. Moreover, they suggest that the higher rate of desaturation of saturated fatty acids to monoenoic acids, which occurs in liver from coconut oil-fed rats, may lead to increased NADPH and oxygen utilization and in turn increased NADP-linked enzyme activities in the absence of lipogenesis. This theory is complicated however, by the fact that fatty acid elongation and desaturation occur intramitochondrially while NADPH production is extramitochondrial.

Lack of relationship between lipogenesis and HMP shunt dehydrogenase activity also was reported by Leveille (1966) in meal-fed rats and chicks. In this instance, fatty

acid synthesis was elevated for some time before dehydrogenase activities increased. These observations are in line with those by Tepperman and Tepperman (1963) and Allmann et al. (1965) in starved-refed rats. Similarly, the lack of association observed between citrate cleavage enzyme activity and fatty acid synthesis argues against a regulatory function for this enzyme in fat synthesis (Foster and Srere, 1968), although this function has been proposed repeatedly (Leveille and Hanson, 1966; Kornacker and Ball, 1965). Hepatic fatty acid synthesis in rats made alloxan diabetic or fasted for 24 hours decreased to negligible levels prior to any change in citrate cleavage enzyme activity. Refeeding a high-carbohydrate diet after the fast resulted in a marked increase in fatty acid synthesis before any change was observed in citrate cleavage activity. These data agree with observations in pig adipose tissue (O'Hea and Leveille, 1969) and chick liver (Goodridge, 1968).

This apparent lack of association among metabolic pathways, based on the time at which changes occur in response to altered dietary or hormonal environments, tends to support the 'pull hypothesis' (Tepperman and Tepperman, 1963) which states that hyperlipogenesis is the primary event and the increase in shunt enzymes secondary. However one should not be misled to believe that apparent dissociation constitutes proof of a lack of relationship, for two related biochemical processes need not always be in phase with each

other. Although HMP dehydrogenase activity may not be essential for the initiation of fatty acid synthesis, subsequent increases in activity probably are necessary and reflect the increased demand for NADPH to support accelerated fatty acid synthesis. Similarly, citrate cleavage enzyme activity may increase secondary to lipogenesis to insure adequate acetyl Co A generation in the cytoplasm. Data on citrate cleavage enzyme is confounded however, by the fact that citrate cleavage enzyme activity under all conditions is generally several hundred to a thousand times greater than the activity of the fatty acid synthesizing enzymes (Foster and Srere, 1968). If citrate is involved in the control of fatty acid synthesis, the passage of citrate from the mitochondria may be under physiological control (ie. diet, hormones).

These studies suggest that factors other than inhibition of activity or loss in amount of enzymes may limit the rate of fatty acid synthesis *in vivo*. These factors may include the concentration of substrates, activators and inhibitors which may be masked in *in vitro* enzyme assays.

2. Concentration of Substrates

Wakil and Bressler (1962) sited studies where hepatic fatty acid synthesizing activity was restored in the diabetic animal by pretreatment with insulin, in the fasted

animal by provision of glucose and insulin, and in the starved animal by refeeding. Glucose, the main source of carbon atoms for fatty acid synthesis, was probably limiting in these instances since NADPH-generating systems do not appear to limit lipogenesis (see section II. E. 1.).

If glucose and insulin are not limiting for lipogenesis, Del Boca and Flatt (1969) suggest that fatty acid synthesis in adipose tissue is limited by the ability to generate acetyl Co A from glucose. They postulate that conversion of glucose to acetyl Co A may be limited by an insufficient supply of oxidized coenzymes (NAD and NADP) which are necessary for the continuous functioning of the pentose and EMH pathways. Therefore glucose conversion to acetyl Co A may be limited by the reactions involved in oxidizing reduced coenzymes, such as fatty acid synthesis, since the rate of production of reduced coenzymes during glucose conversion to acetyl Co A exceeds the rate at which they are utilized for fatty acid synthesis. Consequently, the total capacity of the tissue for fatty acid synthesis can not be attained with glucose as substrate because the supply of acetyl Co A is insufficient due to a slowdown of the EMH and pentose pathways as a result of a deficiency of oxidized coenzymes.

The proposal that the supply of oxidized coenzymes limits fatty acid synthesis stems from observations by Flatt and Ball (1966) and Del Boca and Flatt (1969). They found

that there was a 60% increase in *in vitro* fatty acid synthesis in adipose tissue from *ad libitum*-fed rats when the medium contained acetate-U-^{14}C (15mM) in addition to glucose-U-^{14}C (10mM) at physiological concentrations of insulin. Acetate in the presence of glucose and insulin also resulted in an 83% increase in glucose flow through the pentose cycle while the EMH pathway remained at the same rate as when glucose and insulin were present alone. Consequently, the pentose cycle supplied 95% of the NADPH necessary for fat synthesis under these conditions but only 65% when glucose was the sole substrate. The additional NADPH required for fat synthesis from glucose would be provided by transhydrogenation of NADH, produced via the EMH pathway, to NADPH via the malate cycle (see section II. E. 1.).

Flatt and Ball (1966) concluded that fatty acid synthesis was not limited by the activity of the enzymes involved in lipogenesis (ATP-citrate lyase, malic enzyme, pyruvate carboxylase, fatty acid synthetase, acetyl Co A carboxylase). Similar conclusions were arrived at by Reshef *et al.* (1969) who noted that glyceride-glycerol synthesis and lipogenesis increased in adipose tissue from fasted rats when the incubation medium contained pyruvate-3-^{14}C and by Jomain and Hanson (1969) who demonstrated that raising the concentration of pyruvate in the incubation medium from 0.25 to 25mM resulted in 400- and 3000-fold increases in fatty

acid synthesis in adipose tissue from fasted rats and fasted rats refed a protein-free diet, respectively.

Pyruvate, in contrast to acetate, can act as a substrate for fat synthesis in the absence of glucose because pyruvate metabolism can furnish the reduced coenzymes necessary for fatty acid synthesis (Wise and Ball, 1964). To meet this and other requirements for fatty acid synthesis, the pyruvate pool would be divided between (1) conversion to acetyl Co A via pyruvate dehydrogenase and (2) carboxylation to oxaloacetate via pyruvate carboxylase. These two pathways provide not only substrate (acetyl Co A) and reduced coenzymes (NADH and NADPH) for fat synthesis, but also replenish oxaloacetate necessary for the continuous functioning of the TCA cycle, citrate cleavage pathway and malate transhydrogenation cycle. The quantity of fat synthesized when pyruvate is the sole substrate would be less than half that which is possible when glucose is the substrate because pyruvate is channelled in this fashion.

Glucose is the common physiological precursor for fat synthesis in non-ruminant animals although fat can be synthesized from glucose, pyruvate or acetate in the presence of glucose or pyruvate. Formation of acetyl Co A from pyruvate or acetate and conversion of acetyl Co A to fat would not be limited in the same way as when glucose is the substrate (Del Boca and Flatt, 1969). Conversion of pyruvate to acetyl Co A produces one reduced coenzyme (NADH)

while each condensation of two molecules of acetyl Co A utilizes two reduced coenzymes (NADPH). However, conversion of acetate to acetyl Co A does not produce reduced coenzymes. Therefore, the action of acetate and pyruvate on glucose conversion to fat may be due to the utilization of excess reduced coenzymes (NADH) formed during acetyl Co A production from glucose. Consequently, when glucose and acetate or pyruvate are present in an incubation medium more oxidized coenzymes would be provided for the continuous functioning of the pentose cycle (NADP), the malate cycle (NAD and NADP) and triose-phosphate conversion to acetyl Co A (NAD) than when glucose is present alone. Therefore these three pathways would be accelerated and fatty acid synthesis increased (Flatt and Ball, 1966).

The observations of Flatt and Ball (1966) and Del Boca and Flatt (1969) explain the accelerating effect of acetate and pyruvate on lipogenesis from glucose in adipose tissue from fasted rats. It appears that the type of substrate (pyruvate, glucose, acetate) limits <u>in vitro</u> lipogenesis and not the enzymatic capacity of the tissue. Thus supply of acetyl Co A may control lipogenesis when glucose is the substrate through its influence on the balance between production of reduced coenzymes during glucose catabolism and re-oxidation of these coenzymes during fat synthesis. This hypothesis suggests that the ability to reoxidize reduced coenzymes may be more important in the

conversion of glucose to fat than the ability to generate NADPH.

In addition to the substrates already mentioned, the availability of L-alpha-glycerophosphate in tissues has been suggested as a factor regulating lipogenesis. The removal of fatty acids by esterification with glyceride-glycerol to form triglycerides decreases the possibility of free fatty acids inhibiting fatty acid-synthesizing enzymes (Howard and Lowenstein, 1965). However, glyceride-glycerol production is ultimately limited by the concentration of precursors (ie. glucose and pyruvate).

Glucose appears to be the main precursor of glycerol (Leveille, 1967). In the presence of glucose, pyruvate is preferentially converted to acetyl Co A and glucose to glyceride-glycerol. In the absence of glucose, pyruvate is converted to glyceride-glycerol. Consequently during fasting, the availability of substrate may limit the rate of glyceride-glycerol formation and as a result triglyceride synthesis (Leveille, 1969; Tepperman and Tepperman, 1964; Gellhorn et al., 1962). Fasting overloads the esterifying system with fatty acids mobilized from the depot stores and accelerates the depletion of glyceride-glycerol synthesized in limited amounts from glucose which was formed via glycogenolysis. Restricted generation of alpha-glycerophosphate under these conditions would limit re-esterification of mobilized fatty acids present in the liver. Thus concentration

of free fatty acids or their acyl Co A derivatives would increase and may limit lipogenesis (see section II. E. 3.). Refeeding carbohydrate could enhance lipogenesis by increasing the concentration of glycerophosphate thereby decreasing the concentration of free fatty acids and their derivatives (Kornacker and Lowenstein, 1965). These observations further support the suggestion that substrate availability is the factor limiting lipogenesis.

3. Concentration of Activators and Inhibitors

The carboxylation of acetyl Co A to malonyl Co A classically has been regarded as the rate-limiting step in fatty acid biosynthesis, because fatty acid synthetase activity has been shown to be much higher than acetyl Co A carboxylase activity (Bortz et al., 1963; Ganguly, 1960). Other observations have also suggested that fatty acid synthesis might be controlled through the carboxylation of acetyl Co A. Formation of the active polymeric form of acetyl Co A carboxylase has been shown to be promoted in vitro by citrate (Zakim and Herman, 1969; Charkrabarty and Leveille, 1969; Vagelos and Martin, 1963) while the catalytically inactive protomeric form was favored in the presence of malonyl Co A (Numa et al., 1966; Gregolin et al., 1968). This safeguard against excessive malonyl Co A production would provide a reasonable one-step control of fatty acid biosynthesis.

However, recent observations indicate that the control of fatty acid synthesis may not be through control of the carboxylation of acetyl Co A. Chang *et al.* (1967) reported that acetyl Co A carboxylase and fatty acid synthetase activities were approximately equal in liver from mice, chickens and rats. On the other hand, Chakrabarty and Leveille (1969) found acetyl Co A carboxylase activity greater than fatty acid synthetase activity in adipose tissue, but not the liver, from meal-fed as compared to nibbling rats. However, activity was similar in liver and adipose tissue from *ad libitum*-fed rats. Furthermore, no condition has been reported in which changes in carboxylase level *per se* account fully for changes in the rate of fatty acid synthesis. For example, Korchak and Masoro (1962) found a 50% reduction in carboxylase activity accompanied a 99% depression in fatty acid synthesis in livers from fasted rats. These observations suggest that changes in the level of enzymes catalyzing fatty acid synthesis from malonyl Co A also merit further study to determine whether they represent possible sites for the control of fatty acid synthesis.

Acetyl Co A and malonyl Co A may exert a specific type of metabolic control over glucose and fat homeostasis although fatty acid synthesis may not be controlled through the carboxylation of acetyl Co A to malonyl Co A. Acetyl Co A or pyruvate may be the branch-point between glucose and fat metabolism. Ready availability of carbohydrate leads to

rapid rates of fatty acid synthesis and esterification, whereas high fatty acid concentrations spare oxidation of glucose and favor gluconeogenesis. Acetyl Co A generated when fatty acid synthesis is low as a result of fat-feeding, diabetes, or fasting has been shown to inhibit pyruvate kinase and glucokinase activity but activate pyruvate carboxylase activity (Weber et al., 1967a). Pyruvate carboxylase together with PEP carboxykinase activity could initiate gluconeogenesis (see section II. D.). This inhibition of glycolysis and activation of gluconeogenesis in vitro, together with malonyl Co A inhibition of fatty acid synthesis, may indicate a specific type of metabolic control over glucose and fat homeostasis in vivo.

In addition to acetyl Co A and malonyl Co A, other fatty acyl Co A derivatives of fatty acids and free fatty acids formed during fasting, fat-feeding and diabetes have been implicated in feedback control of hepatic and adipose tissue fatty acid synthesis through in vitro inhibition of acetyl Co A carboxylase activity (Bortz et al., 1963; Bortz, 1967; Dorsey and Porter, 1968; Tubbs and Garland, 1964; Korchak and Masoro, 1964; Bortz and Lynen, 1963; Korchak and Masoro, 1962). These free fatty acids and their acyl Co A derivatives may function by interfering directly with the citrate-dependent association of inactive protomeric acetyl Co A carboxylase subunits to form the active polymeric enzyme or indirectly through inhibition of citrate

synthetase (Wieland and Weiss, 1963).

Weber et al. (1967b) noted that octanoate inhibited a number of glycolytic enzymes (glucokinase, phosphofructokinase, pyruvate kinase), in addition to lipogenic enzymes (fatty acid synthetase, alpha-glycerol phosphatase, alpha-glycerol phosphate dehydrogenase), but had no effect on gluconeogenic enzymes (G-6-phosphatase, F-1, 6-diphosphatase, pyruvate carboxylase and PEP carboxykinase). Enzymes that participate in both glycolysis and gluconeogenesis (lactate dehydrogenase, aldolase, phosphohexoisomerase) also were unaffected. Weber et al. (1967a) contend that this represents a specific inhibition. Pyruvate kinase inhibition is important in order to prevent recycling which would occur under normal conditions when pyruvate kinase activity is high in comparison with PEP carboxykinase activity. Therefore, like acetyl Co A and malonyl Co A the concentration of other fatty acyl Co A derivatives and free fatty acids in vivo could regulate carbohydrate and fat metabolism during fasting, fat-feeding, and diabetes by specifically decreasing fatty acid synthesis and glycolysis but not gluconeogenesis.

Similarly a degree of selectivity in the action of free fatty acids is indicated by the observation that there was also a selective protection from free fatty acid inhibition of glucose-phosphorylating enzymes (hexokinase, glucokinase) and phosphofructokinase only by their substrates

glucose and fructose-6-phosphate, respectively (Lea and Weber, 1968). Increasing the concentration of substrate increased protection for each enzyme. Lea and Weber conclude that at normal physiological concentrations of glucose, up to 10 mM, protective action would be small but significant and it would be related to the concentration of free fatty acids present. The altered conformation of the active site of the enzyme due to substrate binding, may render the enzyme less susceptible to inhibition (Taketa and Pogell, 1966). This substrate specificity in protection against the inhibitor of an enzyme further supports the suggestion that levels of substrates and inhibitors are important in the regulation of enzymes involved in carbohydrate and fat metabolism in the cell.

However, if these fatty acids and their derivatives function as metabolic regulators of fat metabolism, they must be selective in their action. Taketa and Pogell (1966) and Dorsey and Porter (1968) have argued against ascribing a regulatory role *in vivo* to free fatty acids and their acyl Co A derivatives on the basis of their inhibitory effect on a wide range of enzyme systems. In addition to the enzymes already mentioned, Pande and Mead (1968) noted oleate inhibition of G-6-phosphatase, an important gluconeogenic enzyme. This observation is at variance with that of Weber *et al.* (1968) and argues against the contention that free fatty acids specifically inhibit enzymes regulating

glycolysis, but not those regulating gluconeogenesis. Moreover, the conclusion of Weber *et al.* (1967b) that free fatty acids inhibit NADPH-generating enzymes and therefore lipogenesis selectively is unjustified since malic enzyme was not inhibited and isocitrate dehydrogenase, which has not been shown important in the production of NADPH for lipogenesis, was markedly inhibited by free fatty acids. This observation further supports the suggestion that NADPH-generating enzyme activity is not associated with fat synthesis (see section II. E. 1.). The question arises then whether fatty acids function as metabolic regulators of carbohydrate and fat metabolism since they appear to act as non-specific enzyme inhibitors.

Lea and Weber (1968) have suggested that glucose phosphorylation may be the glycolytic step most sensitive to the action of fatty acids since higher laurate concentrations were required for 50% inhibition of lactate production when G-6-P, rather than glucose, was used as substrate. If this is the site of control *in vivo*, the organism would appear inefficient by inhibiting other enzymes. This also argues against *in vivo* control by free fatty acids.

Nevertheless, elevated levels of long chain free fatty acids and fatty acyl Co A derivatives are associated with low rates of fatty acid synthesis and enzyme activity *in vitro*. There is some question however about the mechanism whereby free fatty acids inhibit fatty acid synthesis

and whether the control is direct. *In vitro* studies suggest that *in vivo* control of fatty acid synthesis may be influenced by the concentration and chain length of free fatty acids, the length of time the enzyme is exposed to the fatty acids at each concentration and the substrate level. Lea and Weber (1968) noted that percent inhibition of glucokinase, hexokinase, phosphofructokinase, glycerophosphate dehydrogenase and citrate synthetase increased with longer periods of incubation of the assay mixture with fatty acids prior to starting the reaction and with increasing concentrations of fatty acids. At concentrations less than 1 mM, long chain fatty acids were more inhibitory than medium chain fatty acids. Bortz and Lynen (1963) and Lea and Weber (1968) noted that long chain fatty acids (myristic, palmitic, stearic) were 5 to 10 times more inhibitory than octanoate and laurate. In fact medium chain fatty acids may not have a regulatory role *in vivo*, in contrast with long chain fatty acids, since high concentrations are necessary to achieve inhibitory effects. Consequently dietary fats, which vary in their fatty acid composition, would have different effects on enzyme regulation.

Dorsey and Porter (1968) reported that inhibition of pigeon liver fatty acid synthetase activity by palmityl Co A *in vitro* depended upon the presence of a critical mixed micellar concentration of palmityl Co A and on the molar ratio of inhibitor (ie. Co A ester) to protein (ie. enzyme).

They concluded that the molar ratio requirement eliminated the possibility of a site-specific inhibition of fatty acid synthetase activity and suggested the inhibition was a function of the detergent properties of palmityl Co A. Inhibition may occur through the formation of one or more mixed micelles of protein and palmityl Co A. This may explain the *in vitro* action of free fatty acids and fatty acyl Co A esters on other enzymes (Srere, 1965). The greater effect of long chain unsaturated fatty acids could result from their greater detergent action. Detergent anions (hydrophilic region) could attack and neutralize cationic regions of the enzyme (positively-charged arginine and lysine groups). Thus the enzyme molecule would swell and dissociate due to disruption of the hydrophobic regions.

It is appealing to suggest that the detergent action of free fatty acids or acyl Co A derivatives of free fatty acids may operate as a control mechanism *in vivo*. However, direct extrapolation from *in vitro* to *in vivo* conditions is difficult. The inhibitory or stimulatory action of a compound *in vitro* may merely indicate non-specific chemical or physical effects rather than specific cellular action. Since sodium lauryl sulfate, another strong detergent, acts in a similar manner to palmityl Co A on fatty acid synthetase it is unlikely that palmityl Co A, and other acyl Co A derivatives, can be considered physiological agents of control (Dorsey and Porter, 1968). This conclusion is

further supported by the observation by Masoro (1965) that myristate inhibited the fatty acid synthesizing enzymes more than palmitate and oleate more than myristate. It appears that free fatty acid inhibition of fatty acid synthesis *in vivo* can not be a specific, feedback-type of inhibition since palmitate, the final product of fatty acid synthesis in mammalian systems, is a better detergent but was shown less inhibitory than shorter chain fatty acids which are minor components of fat synthesis.

These results suggest that the regulatory role of malonyl Co A in fatty acid synthesis (via acetyl Co A carboxylase) and of acetyl Co A in gluconeogenesis is physiologically more important than the inhibitory effect of other fatty acyl Co A derivatives or free fatty acids. In fact, Vijayvargiya and Singhal (1969) suggest that acetyl Co A may be the main fatty acyl Co A inhibitor *in vivo* and that other fatty acids are converted to it before inhibition occurs. They noted that alpha-glycerophosphate dehydrogenase activity from rat adipose tissue was inhibited to a greater extent when acetyl Co A rather than octanoate was added directly to the assay mixture before the addition of the substrate. Since preincubation of the assay mixture with octanoate resulted in a greater inhibition it was suggested that octanoate may have given rise to acetyl Co A which in turn might produce the observed pronounced inhibition of alpha-glycerophosphate dehydrogenase. This may explain the

effects seen by Lea and Weber (1968) wherein preincubation of the assay medium with octanoate, laurate, myristate, palmitate and elaidate was necessary for free fatty acid inhibition of enzyme activity. They suggested that the lack of inhibition which occured with direct addition of free fatty acids to the assay medium was due to substrate present in the assay medium which protected the enzyme from free fatty acid inhibition.

Although several suggestions have been proposed for the control of fatty acid synthesis, the exact mechanism is not clear. Recent concepts however, favor substrate availability.

II. F. Effects of Diet Composition on Enzyme Activity and Lipogenesis

Meal-fed and fasted-refed animals are well-suited for studying the pathway of fatty acid formation because high rates of lipid synthesis can be induced in the liver and adipose tissue of these animals. Composition of the diet fed can have a profound influence on this induction. Induction of hyperlipogenesis has been extensively studied and several excellent reviews are available on the subject (Masoro, 1962; Leveille and Hanson, 1966).

1. Carbohydrate

The presence of carbohydrate seems essential for acceleration of lipogenesis and activation of enzymes associated with glucose metabolism and fat synthesis in adipose tissue and liver of meal-fed and fasted-refed rats. A marked metabolic response as a consequence of meal-feeding a high-carbohydrate diet or refeeding a balanced or high-carbohydrate, low-fat diet following starvation has been reported by many workers (Hollifield and Parson, 1962; Leveille and Hanson, 1966; Leveille, 1967; Young et al., 1964; Pande et al., 1964; Tepperman and Tepperman, 1958, 1963 and 1964). High-carbohydrate diets have been suggested as the dietary inducer of G-6-P dehydrogenase, malic enzyme, glucokinase, citrate cleavage enzyme, and fatty acid synthesizing enzymes (acetyl Co A carboxylase and fatty acid

synthetase) in liver and adipose tissue from meal-fed and starved-refed rats (Leveille and Hanson, 1966; Leveille, 1967; O'Hea and Leveille, 1969a; McDonald and Johnson, 1965; Potter and Ono, 1961; Pande et al., 1964; Tepperman and Tepperman, 1958; Ballard and Hanson, 1967; Bortz and Lynen, 1963; Ganguly, 1960). The effect of carbohydrate on lipogenesis and the activity of associated enzymes does not appear to be related to its role as an energy source because adipose tissue and liver from rats meal-fed or fasted-refed a high-fat, carbohydrate-free diet exhibited neither elevated rates of lipogenesis nor enzyme activity (Leveille and Hanson, 1966; Tepperman and Tepperman, 1965).

Carbohydrate also appears to be involved in the regulation of PEP carboxykinase and pyruvate carboxylase activity—key enzymes in the dicarboxylic acid shuttle. Carbohydrate, together with insulin, appears to be the major factor controlling PEP carboxykinase levels in liver and adipose tissue of fasted-refed rats. Young et al. (1964) noted that hepatic PEP carboxykinase activity increased 2-fold in 24 hours when rats were transferred from a lab chow diet to a 0% sucrose, 10% corn oil, 90% casein diet. Glucose and glycerol administration decreased the activity to levels below normal while DL-alanine, D-serine, DL-serine and D-glycerate and dietary lipids were without effect. Jomain and Hanson (1969) also noted that refeeding rats a balanced diet reduced PEP carboxykinase activity in

adipose tissue to the level found in fed rats. However, PEP carboxykinase activity in adipose tissue from rats refed a protein-free diet (84% dextrin) was five times as great as that in tissue from animals refed a balanced diet following fasting. These two studies demonstrated that PEP carboxykinase activity increased when fasted animals were refed with carbohydrate-free or protein-free diets but decreased upon refeeding balanced diet containing carbohydrate. Jomain and Hanson (1969) suggested that the absence of protein in the diet altered the synthesis of insulin. Insulin appears to be essential for the repression of PEP carboxykinase activity by carbohydrate (Young et al., 1964) and therefore a lack of insulin synthesis would cause an increase in the activity of this enzyme. Moreover, PEP carboxykinase activity was not decreased in fasted rats when insulin was administered alone; indicating that carbohydrate, together with insulin, is necessary for the suppression of this enzyme in liver and adipose tissue.

Pyruvate carboxylase activity in liver and adipose tissue has been shown to respond differently to dietary alterations; suggesting that this enzyme has a different metabolic function in each tissue. Only cytoplasmic pyruvate carboxylase was adaptive in rat liver; being stimulated by fasting and decreased by refeeding a chow diet ad libitum (Reshef et al., 1969). Mitochondrial pyruvate carboxylase activity showed little variation with feeding

schedule.

Reshef et al. (1969) found that dietary conditions had very little effect on either mitochondrial or cytoplasmic pyruvate carboxylase activity in adipose tissue although ad libitum fat-feeding has been reported to decrease cytoplasmic pyruvate carboxylase activity (Ballard and Hanson, 1967). These observations are in line with the suggestion that cytoplasmic pyruvate carboxylase activity is involved in gluconeogenesis in the liver and lipid synthesis in the adipose tissue via the dicarboxylic acid shuttle pathway (see section II, D.).

Acetyl Co A appears to be the factor controlling pyruvate carboxylase activity in liver and adipose tissue since pyruvate carboxylase activity was stimulated by acetyl Co A in both tissues (Ballard and Hanson, 1967). The effect of high-fat diets on pyruvate carboxylase activity may be explained through the action of acetyl Co A. Fat-feeding would decrease pyruvate carboxylase activity in the adipose tissue because acetyl Co A concentration decreases due to low rates of fatty acid synthesis and an elevated rate of lipolysis. On the other hand, hepatic acetyl Co A concentrations would increase and stimulate pyruvate carboxylase activity which would in turn stimulate gluconeogenesis. These observations suggest that control of cytoplasmic pyruvate carboxylase activity of liver and adipose tissue by carbohydrate and fat is via acetyl Co A.

Carbohydrate then, appears to be the dietary inducer of a number of enzymes involved in carbohydrate and fat metabolism. However, the expression of induction is related to the level of protein and fat in the diet, in addition to the level of carbohydrate.

2. Protein

The effects of dietary protein on lipogenesis and the associated enzymes have not been extensively studied. De novo protein synthesis may account for the marked increase in HMP shunt dehydrogenase activity observed in adipose tissue and liver during refeeding of starved rats since the increase was prevented by the administration of puromycin (Potter and Ono, 1961) and feeding low-protein (Potter and Ono, 1961; McDonald and Johnson, 1965; Oliver, 1967; Methfessel et al.; Johnson and Sassoon, 1967) or protein-free diets (Tepperman et al., 1968; Jomain and Hanson, 1969). The decrease in dehydrogenase activity however, was not associated with a decrease in hepatic lipogenesis (McDonald and Johnson, 1965; Oliver, 1967; Tepperman et al., 1968) but was associated with low rates of fatty acid synthesis in adipose tissue (Jomain and Hanson, 1969). These observations support the suggestion that HMP shunt dehydrogenase activity and dietary protein are not necessary for the accumulation of lipid in liver, but may be necessary in adipose tissue. Nevertheless HMP shunt dehydrogenase

activity was increased by refeeding a higher protein diet after fasting, presumably via de novo protein synthesis (Vaughan and Winders, 1964; Thoys et al., 1968).

In contrast with HMP shunt dehydrogenase enzymes, Oliver (1967) noted that hepatic malic enzyme was not depressed by refeeding rats a protein-deficient diet while Tepperman et al. (1968) reported only partial suppression during refeeding on a high-carbohydrate, protein-free diet. These observations indicate that malic enzyme activity, like lipogenesis, may be independent of protein supply. In fact, these observations have served as a basis for the suggestion that malic enzyme is more closely associated with lipogenesis than HMP shunt dehydrogenase enzyme activity. However in contrast with liver, malic enzyme was low in adipose tissue from rats refed a high-carbohydrate, protein-free diet (Jomain and Hanson, 1969). Therefore, dietary protein may have a different effect on malic enzyme activity in the liver and adipose tissue.

Increasing dietary protein to very high levels has been shown to suppress fatty acid synthesis and enzyme activity in liver and adipose tissue of rats (Cohen and Teitelbaum, 1966; Masoro et al., 1950; Leveille, 1967). Yeh and Leveille (1969), working with growing chicks, also demonstrated that increasing dietary protein from 15 to 35% depressed malic enzyme activity and in vitro hepatic lipogenesis by about 75%. Allee et al. (1970) also noted

that lipogenesis and malic enzyme activity, but not G-6-P dehydrogenase or 6-P-gluconate dehydrogenase activity, were depressed in adipose tissue from growing pigs when the protein content of the diet was increased from 12 to 24%.

The suppressing effect of high-protein diets on fatty acid synthesis and enzyme activity could be attributed to changes in the level of dietary carbohydrate since protein was increased at the expense of glucose. Therefore, lipogenesis may be limited by the availability of substrate (carbohydrate) and an imbalance between the production and utilization of reduced coenzymes. If glucose is limiting in the high-protein diets, the production of cytoplasmic NADH and NADPH might be reduced, thereby limiting fatty acid synthesis. Furthermore, if glucose production via gluconeogenesis is enhanced, cytoplasmic NADH would be utilized and further reduce that available for transhydrogenation. Long chain fatty acids do not appear to be involved in the suppression which results when high-protein diets are fed since Yeh and Leveille (1969) showed that high-protein diets depressed both the rate of fatty acid synthesis and the level of circulating free fatty acids. Therefore, the availability of cytoplasmic reduced coenzymes may be the major factor limiting fatty acid synthesis when high levels of dietary protein are fed.

These observations further support the suggestion that carbohydrate, as opposed to protein, is the dietary

inducer of the 'overshoot' in enzyme activity and lipogenesis which occurs during refeeding following starvation. An adequate supply of protein appears essential for the induction since refeeding protein-free or low-protein, high-carbohydrate diets failed to induce hyperlipogenesis or increased activity of associated enzymes.

The effects of dietary protein level on enzyme activity have also been related to its effects on polysome aggregation since the rate of protein synthesis appears to be dependent on the amount of aggregation. The suppressing effect of high-protein diets on lipogenesis and enzyme activity may be due to a deficiency of carbohydrate which appears necessary for polysome aggregation (Wittman et al., 1969). Wittman et al. (1969) showed that polysome aggregation was completely restored when glucose was refed to starved rats. Insulin in conjunction with glucose appears necessary to stimulate polysome aggregation. Insulin administration to alloxan diabetic rats, restored the polysome pattern while administration to fasting or fat-refed rats had no effect (Wittman et al., 1969). However, Wittman and Miller (1970) found that although aggregation qualitatively required both glucose and insulin, quantitatively aggregation was more dependent on the amount of glucose. The importance of glucose and insulin acting together may explain the failure of insulin-supplemented rats fed a 50% fat, carbohydrate-free diet to induce a change in malic enzyme

and the combined G-6-P and 6-P gluconate dehydrogenase activities of adipose tissue from rats refed following a 72-hour fast (Fabry et al., 1969). The effect of glucose was not due to energy supply per se, since isocaloric fat-feeding had no effect (Wittman et al., 1969). The lack of polysome aggregation due to fat-feeding may explain the suppressing effect of fat diets on enzyme activity (see section II. F. 3.).

The failure of low-protein, or protein-free diets to induce enzyme synthesis when refed to starved animals is not due to a lack of polysome aggregation since polysomes were restored in fasted rats refed a protein-free diet (Wittman et al., 1969). Therefore, exogenous protein or amino acids do not appear to be essential for restoration of polysome patterns. In this instance synthesis of protein or its release from the aggregated polysome may be limiting. In fact, the effect of protein does not appear to be mediated through either carbohydrate or insulin because polysome aggregation was evident. These observations also suggest that the enzymatic response seen during refeeding may merely represent increases or decreases in enzyme activity rather than new synthesis.

Insulin appears to be more important in adipose tissue than liver (Weber et al., 1965). In hepatic tissue there is a free permeation of glucose to tissue cells without the need for insulin, whereas insulin is of major

importance for this function in adipose tissue. Weber et al. (1965) suggest that the effect of insulin would be to activate a 'limiting transport mechanism' rather than phosphorylation of sugar. However, O'Hea and Leveille (1969c and 1970) showed that pig adipose tissue fatty acid synthesis was relatively insensitive to added insulin in vitro in contrast to rat tissue. This suggests that in the pig the role of insulin in glucose utilization may not be as critical as in other species.

Insulin levels have been shown to be depressed in the pancreas under the gluconeogenic conditions of fasting, fat-feeding and diabetes when fatty acid levels are elevated. Insulin appears to be required for the induction of key glycolytic enzymes (Lea and Weber, 1968). Therefore the inhibitory action of free fatty acids on these enzymes would reinforce and amplify the effect of depressed insulin levels on enzyme activity. This might provide an enzyme-basis for the decrease in glycolysis during these conditions.

These observations suggest that dietary changes could influence the balance between enzyme synthesis and degradation through its effect on polysome aggregation and protein synthesis. An adequate supply of exogenous protein or amino acids appears necessary for protein synthesis to occur whereas polysome aggregation is more dependent on an adequate supply of insulin and carbohydrate.

3. Lipids

There are conflicting reports in the literature regarding the effect of dietary fat on lipogenesis. Critical evaluation of these reports suggests that any alteration induced by the inclusion of fat in the diet will depend not only on the fatty acid composition of the fat, but also on the level of fat in the diet, the length of time it is fed, the species and age of the animal being studied, whether fat is added to the diet at the expense of carbohydrate and whether the studies are conducted *in vivo* or *in vitro*.

Diets high in fat have been shown to depress not only fatty acid formation *in vivo* and *in vitro* by rat liver slices and adipose tissue but also the activity of several enzymes related to lipogenesis (Young *et al.*, 1964; Leveille and Hanson, 1966; Bortz *et al.*, 1963; Hill *et al.*, 1958; Leveille, 1967; Hill *et al.*, 1960). For example in adipose tissue, Di Giorgio *et al.* (1962) found that high fat diets (48%) depressed fat synthesis *in vitro* regardless of the type of dietary fat (safflower, corn, olive, hydrogenated cottonseed, butter, coconut). Fabry *et al.* (1969) found that refeeding fasted rats a diet containing 50% lard also suppressed enzyme activity in adipose tissue to prefast levels from the high level induced by refeeding a diet containing 60% sucrose. Similarly Leveille and Hanson (1966) found no increase in lipogenesis or enzyme activity in adipose tissue from rats meal-fed a diet containing 57%

hydrogenated fat. Hausberger and Milstein (1955) concluded that there was an inverse relationship between the level of fat in the diet and lipogenesis in epididymal adipose tissue from fed rats. Furthermore, Whitney and Roberts (1955) have reported a similar decrease in the capacity to incorporate acetate-2-^{14}C to fatty acids in liver slices from rats fed high-fat diets (50%) two to three months and Brice et al. (1956) observed that rats fed a high-fat diet (40%) for three weeks had a lower capacity to convert acetate-2-^{14}C into fatty acids. Yeh and Leveille (1969) also noted that hepatic lipogenesis was depressed in growing chicks fed high fat diets (20% Crisco) ad libitum.

The anti-lipogenic effects in response to added dietary fat could be attributed to either a reduction in carbohydrate or an increase in fat because dietary fat is generally substituted with carbohydrate. Nevertheless, dietary fat appears to have a specific inhibitory effect on lipogenesis unrelated to a decrease in carbohydrate intake. At lower levels of dietary fat than those sited above, the ameliorating effect of dietary fat on fatty acid synthesis and enzyme activity has been associated with the degree of unsaturation of the fat. In fact Hill et al. (1958 and 1960) observed that as little as 2.5% dietary corn oil depressed hepatic lipogenesis in ad libitum-fed rats. Di Giorgio (1962) also found the least active lipogenesis in adipose tissue from 40 to 50g rats fed a diet containing 12%

safflower oil and the most active lipogenesis in those fed coconut oil. Fatty acid synthesis was also reduced in non-fasted mice which were fed a diet containing 10% corn oil or safflower oil for three days when compared to those fed a fat-free diet (Sabine et al., 1969). Coconut oil, tricaprylin, tripalmitin, triolein, oleic acid and mineral oil had little effect. A diet containing 15% corn oil also suppressed the usual adaptive hyperlipogenesis accompanying fasting and refeeding. In addition, lipogenesis and NADP-generating enzyme activity (G-6-P dehydrogenase, 6-P-gluconate dehydrogenase, malic enzyme) were depressed in adipose tissue from growing pigs fed a diet containing 13% corn oil (Allee et al., 1970).

The inhibitory effect of unsaturated fats on fatty acid synthesis has been attributed to the linoleic acid content of the fat. Allmann and Gibson (1965) and Allman et al. (1965) reported that elevated fatty acid synthesis, G-6-P dehydrogenase and fatty acid synthetase activities were lowered within two days in livers of essential fatty acid-deficient mice and fasted rats that were refed a fat-free diet supplemented with corn oil or methyl linoleate, but not methyl palmitate or methyl oleate. Arès (1969) also found dietary ethyl linoleate had a suppressing effect on *in vivo* glucose-U-^{14}C incorporation into liver lipids and NADP-generating dehydrogenase activities (G-6-P and malic enzyme) of starved-refed rats while ethyl oleate had a variable

effect and ethyl stearate responded in a similar manner as a low-fat diet. Inkpen *et al.* (1969) also speculated that linoleic acid was a key factor in the cellular control of hepatic lipogenesis (see section II. G.). Therefore, a specific regulatory function for linoleate in hepatic fatty acid synthesis has been postulated.

However the effects of linoleate *per se* have been questioned because other unsaturated fatty acids have been shown as effective as linoleic acid in alleviating the signs of essential fatty acid deficiency. Mohrhauer and Holman (1963) found arachidonate more effective than linoleate in remedying the effects of essential fatty acid deficiency while Uchiyama *et al.* (1967) showed that *in vitro* desaturation of stearic-^{14}C to oleic acid was inhibited to a similar extent by the addition of linoleic, arachidonic or alpha-linolenic acid. Furthermore, Korchak and Masoro (1964) and Steiner and Cahill (1966) found that exogenous oleic acid and linoleic acid inhibited *in vitro* lipogenesis to a similar extent. In addition, Chu *et al.* (1969) noted that arachidonate and alpha-linolenate, as well as linoleate, exerted a regulatory effect on fatty acid synthetase and G-6-P dehydrogenase activity. The effectiveness of alpha-linolenate in modifying induced hyperlipogenesis was suggested by Muto and Gibson (1969) who found alpha-linolenate would permit normal growth in young rats fed a fat-free diet. Muto and Gibson (1969) also reported that

feeding gamma-linolenate, the product of linoleyl Co A desaturation, reduced fatty acid synthetase activity toward normal in young rats previously fed a fat-free diet. Yet Chu et al. (1969) suggested that linoleate was more effective than alpha-linolenate or arachidonate in overcoming the signs of essential fatty acid deficiency. They found that phospholipid linoleate and arachidonate were increased above the level of the seven-day depleted animals when linoleate was refed to essential fatty acid deficient rats, while neither phospholipid linoleate nor arachidonate levels were increased when alpha-linolenate was refed and only phospholipid arachidonate increased when arachidonate was refed.

The effects of fat on lipogenesis and enzyme activity are further confused by studies which show corn oil does not suppress hepatic lipogenesis in chicks (Goodridge, 1969) and others which suggest that saturated fats are as effective as unsaturated fats in suppressing fatty acid synthesis. Hill et al. (1958) found that dietary corn oil, vegetable oil, hydrogenated vegetable oil, and lard were equally effective in suppressing in vitro hepatic fatty acid synthesis in rats. Similarly, Tepperman and Tepperman (1965) found coconut oil as effective as corn oil in reducing fatty acid synthesis by liver slices from rats that had been: fed fat seven days followed by a 48-hour fast; in similar animals refed a high-carbohydrate, fat-free diet for

48 hours; and in non-fasted controls. Bhattathiry (1966) also found that 10% dietary palm oil or olive oil, neither of which contain appreciable amounts of linoleic acid, depressed *in vitro* hepatic fatty acid synthesis in weanling and adult rats when compared to fatty acid incorporation by rats fed a fat-free diet for thirty days. These diets however, did not depress synthesis to the same extent as reported by Sabine *et al.* (1969) for highly unsaturated fat diets. Wiley and Leveille (1970) noted that the activity of the pentose phosphate path and citrate cleavage enzyme in liver and adipose tissue from rats fed a diet containing 2% corn oil was decreased more than 50% by feeding diets containing 12% corn oil, lard or coconut oil for three weeks. Lipogenesis by liver slices was depressed approximately 75%, relative to the control group, by feeding either corn oil or lard but not by coconut oil whereas lipogenesis in adipose tissue was depressed to the same extent by all three fats.

On the other hand, Wiley and Leveille (1970) noted that lipogenesis was not depressed in liver and adipose tissue from weanling rats fed a diet supplemented with 12% medium-chain triglycerides for three weeks relative to controls. The pentose phosphate pathway and citrate cleavage enzyme activities were approximately 20% lower in liver and adipose tissue from the group fed 12% medium-chain triglycerides than in the control group. In contrast, Bottino *et al.* (1965) noted that the inclusion of either 15%

tripalmitin, triolein, or trilinolein in the diet greatly depressed *in vivo* acetate incorporation into carcass lipids. However, rats were fed these diets for one to three months following a fat-free diet which reduced the level of linoleic acid in their depot fat to around 5%. Reiser *et al.* (1963) also noted that saturated triglycerides (trimyristin and tripalmitin) were almost as effective as trilinolein and safflower oil in suppressing hepatic fatty acid synthesis *in vivo*. In fact, triglycerides containing short-, medium- and long-chain fatty acids all depressed synthesis. However, these diets contained 30% fat and were fed for only two weeks.

The effects of dietary fat on lipogenesis and enzyme activity are variable and inconclusive. The precise mechanism whereby dietary fats stimulate or suppress these and other parameters must await further study.

II. G. Changes in Total Fatty Acid Composition During Starvation and Refeeding

The classical signs of an essential fatty acid deficiency syndrome appear in liver in less than ten weeks following linoleate restriction, and in ten or more weeks in tissues other than the liver. However, Allmann and Gibson (1965) and Allmann et al. (1965) observed metabolic changes analogous to those in animals showing outward signs of essential fatty acid deficiency in less than five days with mice that were fed a fat-free or saturated fat diet (2% coconut oil and 1% cholesterol). A similar syndrome was apparent in 48-hour starved rats that were refed a fat-free diet for 24 and 48 hours. They concluded that the liver registers the first and most profound response in these essential fatty acid deficient states. The early linoleic acid depletion in liver may have far-reaching effects on other tissues because the liver plays a central role in the metabolism of the animal.

In both studies Allmann and co-workers found rapid depletion of liver linoleate coincident with elevated rates of lipogenesis and elevated activities of enzymes associated with lipogenesis. Hyperlipogenesis was reflected in a shift in the total fatty acid composition which included an accumulation of saturated (palmitic) and monounsaturated fatty acids (palmitoleic, oleic) and a decrease in stearic, linoleic acid, and arachidonic. Only small amounts of

eicosatrienoic acid were found. These changes occurred principally in the triglyceride fraction of liver lipids, although Chu et al. (1969) noted changes in the phospholipid fraction as a result of feeding linoleate, alpha-linolenate or arachidonate following an essential fatty acid deficiency. Reiser et al. (1963) also found increased rates of monoenoic fatty acid synthesis in rats fed a fat-free diet. Addition of methyl linoleate or fats containing linoleate to the diet caused both hepatic enzyme activity and linoleic acid levels to return toward normal. The altered liver lipid composition however, was not completely restored. Allmann and Gibson (1965) and Gellhorn and Benjamin (1964) noted that the epididymal fat pad displayed the same general adaptation, indicating that the changes were not confined to the liver tissue.

Tepperman and Tepperman (1965) presented evidence to suggest that the rapid desaturation and chain elongation of fatty acids which occur during the apparent essential fatty acid deficiency induced by feeding saturated fat diets probably explain the alterations in lipid composition that they observed. These changes in lipid composition were also similar to those observed by Allmann and co-workers (1965). Liver slices from both coconut and corn oil-fed rats contained similar amounts of palmitic acid. However, in vitro studies showed that the per cent recovery of acetate-1-^{14}C was less in 16:0 than in 18:1 and 16:1 fatty acids in livers

from coconut oil-fed rats while the per cent recovery of radioactivity was greater in the 16-carbon acids (16:0 and 16:1) than in 18-carbon acids (18:0 and 18:1) from rats fed unsaturated fat.

Recently, Inkpen et al. (1969) presented evidence that 9-desaturation of saturated fatty acids and 6-desaturation of unsaturated fatty acids are accomplished by different enzyme complexes and that these enzyme complexes are controlled by different mechanisms. They concluded that linoleate may exert a negative feedback action upon desaturation. Inkpen et al. reported that 9-desaturase but not 6-desaturase activity decreased (as shown by decreased desaturation of stearate) during fasting and in alloxan diabetes. Nine-desaturase activity was stimulated while 6-desaturase was suppressed by refeeding a diet where the only source of calories was glucose (93%) or hydrogenated coconut oil (87%). Similar changes also accompanied treatment of the alloxan-diabetic animal with insulin. By contrast, casein (97%) reversed the responses. Both desaturase systems were stimulated by feeding glucose (79%) together with casein (16%). The response of the 9-desaturase to feeding hydrogenated coconut oil and safflower oil (20% plus 55% glucose and 18% casein) were inversely related to the proportion of safflower oil in the fat that was fed, while the 6-desaturase was not affected. They also found that 9-desaturase activity was relatively high in hepatic

microsomes from essential fatty acid deficient rats. These results suggested that linoleate (approximately 70% of the fatty acids in safflower oil) may exert a negative feedback action upon desaturation.

These results are also in line with the suggestion by Allmann and co-workers that linoleic acid is a key factor in the mechanism regulating lipogenesis especially in essential fatty acid deficient states. The ability of methyl linoleate-supplementation to alleviate the signs of essential fatty acid deficiency may be due to a regulatory effect exerted by linoleic on 9-desaturase activity.

Brenner and Peluffo (1966) have also suggested that the active center of the 9-desaturase enzyme may be capable of reacting with many unsaturated fatty acids, since a 20-fold excess of either oleic or linoleic suppressed desaturation of saturated fatty acids in their system. Brenner and Peluffo also suggested that the 9-desaturase enzyme may have an active center similar to that of the 6-desaturase enzyme. Therefore, oleic, linoleic, and linolenic acids may not only inhibit 9-desaturation of stearic acid but also compete for a common 6-desaturase enzyme and be converted to other unsaturated fatty acids in the linoleic acid series.

However, Brenner and Peluffo did not measure enzyme activity and Inkpen et al. did not work with the individual unsaturated fatty acids. Therefore, their data can only suggest that these unsaturated fatty acids regulate

9-desaturase activity by negative feedback inhibition.

Nevertheless, if the effects seen are due to linoleic acid regulating 9-desaturase activity and if it is true that other unsaturated fatty acids also regulate this enzyme in a similar manner, it is not surprising then that other unsaturated fatty acids have been shown capable of remedying the signs of essential fatty acid deficiency. Brenner and Peluffo (1966) also observed that there was an order of preference of fatty acids for attachment to the 9-desaturase. This may explain the observation by Chu *et al.* (1969) that linoleic acid was more effective in alleviating the signs of essential fatty acid deficiency than alpha-linolenic or arachidonic acids.

Many of the hepatic enzymes that play a role in glucose conversion to triglycerides are depressed by fasting and are elevated beyond normal levels in response to subsequent refeeding. If the desaturase enzymes are essential for triglyceride synthesis perhaps the metabolic response during refeeding is primarily a result of 9-desaturase stimulation or inhibition by dietary components and the increase or decrease in other enzymes a secondary effect.

III. STATUS OF THE PROBLEM

Hyperlipogenesis in adipose tissue and liver, coincident with elevated activity of enzymes frequently associated with fat synthesis, is the most striking metabolic aberration observed during refeeding following starvation (Young et al., 1964; Tepperman and Tepperman, 1958 and 1964; McDonald and Johnson, 1965; Sabine et al., 1969).

Composition of the diet refed can modify the nature and extent of these responses (Potter and Ono, 1961; Young et al., 1964; O'Hea and Leveille, 1969). Physiological stress due to unregulated refeeding tends to be most severe when fat or high-protein diets are refed (Smith et al., 1964).

The ameliorating effect of dietary fat on fatty acid synthesis appears to be associated with the degree of unsaturation of the fat (Di Giorgio, 1962; Sabine et al., 1969) or the amount of linoleic acid in the fat (Allmann et al., 1965; Allmann and Gibson, 1965; Inkpen et al., 1969). The effects of linoleate per se have been questioned however, because other unsaturated fatty acids such as arachidonic and alpha-linolenic have been shown as effective as linoleic acid in alleviating the signs of essential fatty acid deficiency (Chu et al., 1969; Mohrhauer and Holman, 1963; Muto and Gibson, 1969).

The observation by Hill et al. (1958) that as little

as 2.5% dietary corn oil depressed hepatic lipogenesis in ad libitum-fed rats agrees with the above concept. On the other hand, Goodridge (1969) found that dietary corn oil did not suppress hepatic lipogenesis in chicks while other studies, including that of Hill et al. (1958), have shown saturated fats as effective as unsaturated fats in suppressing lipogenesis (Fabry et al., 1969; Tepperman and Tepperman, 1965; Bhattathiry, 1966; Reiser et al., 1963; Bottino et al., 1965).

For many years a relationship between NADP-linked dehydrogenase activities and fatty acid synthesis has been postulated. Recently, Jomain and Hanson (1969) reported parallel behavior between NADP-dehydrogenase activity and fatty acid synthesis in adipose tissue. By contrast, Tepperman and Tepperman (1968) found no evidence of parallel behavior for these pathways in liver. Nevertheless, the distribution of dehydrogenase activity in rat tissues has been related to their lipogenic capacity (Fritz, 1961; Young et al., 1964). Adipose tissue has also been proposed as the major site of fatty acid synthesis, especially under conditions of enhanced lipogenesis such as meal-feeding (Leveille, 1967; Hollifield and Parson, 1962). This coincides with the observation that adipose tissue was richer in dehydrogenase activity than the liver (Hollifield and Parson, 1962; Young et al., 1964; Pande et al., 1964). Malic dehydrogenase (NADP-linked) activity was also higher in

adipose tissue than in liver (Young et al., 1964; Pande et al., 1964). This observation, together with the greater increase in malic dehydrogenase activity than G-6-P dehydrogenase during refeeding following fasting, led Young et al. (1964) to conclude that malic dehydrogenase activity is more intimately related to fat synthesis than hexose monophosphate shunt dehydrogenase activity. These observations do not prove however, that the control of lipogenesis is dependent upon fluctuations in dehydrogenase activity and that the same control exists in adipose tissue and liver.

These observations are of some concern to humans since total starvation is prescribed for weight reduction in the chronic obese (Lawlor and Wells, 1969). Although restriction of food intake is the more common practice used by the general public, it is not known whether the effects due to unregulated food intake following severe calorie restriction are the same as those observed following starvation. Johnson and Sassoon (1967) have suggested that starvation must precede carbohydrate refeeding to obtain an 'overshoot' in enzyme activity. However, their study was confounded by the fact that the non-starved animals were fed a carbohydrate-free, high-fat diet prior to refeeding on a high-glucose diet.

Thus there is considerable confusion surrounding induced hyperlipogenesis. There is not only a question of the effect of severity of food deprivation but also the

effect of diet composition during refeeding following starvation. These effects would be influenced by different periods of fasting and refeeding, different levels and types of dietary fat and carbohydrate, different species and ages of animals, and *in vivo* or *in vitro* conditions of the experiment.

IV. OBJECTIVES OF RESEARCH

The present study was undertaken to determine whether the metabolic response to unrestricted food intake was the same following severe calorie restriction as that observed following starvation. In other words, an attempt was made to determine whether weight loss induced by diet restriction would predispose an animal to the same metabolic response during ad libitum feeding as that observed during refeeding following starvation.

In addition, the study investigated the effect of composition of the refeeding diet on the response observed during refeeding. An attempt was made to clarify the effect of refeeding an unsaturated fat on the response during realimentation following diet restriction and starvation. Allmann et al. (1965) had observed that the changes in early linoleic acid-deficiency in mice appeared to be the same as those accompanying refeeding of a fat-free diet following starvation in rats. These changes included hyperlipogenesis and a fall in linoleate concentration of liver lipids. It was therefore of interest to determine whether the 'refeeding response' of animals restricted on the fat-free diet would be greater and that of animals restricted on the 12% corn oil diet less than the refeeding response of animals which had been starved.

The response to realimentation also was examined

over a longer period of time than reported previously, in an attempt to determine whether the 'overshoot' in lipogenesis and NADP-dehydrogenase activities would return to the levels of control animals fed the same diet ad libitum without prior deprivation.

In addition, the metabolic response to refeeding was examined in liver and adipose tissue in an attempt to determine whether the effect of diet on fatty acid synthesis would be the same in the two major sites of fatty acid synthesis. This knowledge is necessary before appropriate dietary measures can be applied effectively to control the physiological stress resulting from realimentation.

V. EXPERIMENTAL PROCEDURE

A. Design of Experiment

Ninety-two adult male rats (200 to 275g) purchased in four shipments (Woodlyn Farms Ltd., Guelph, Ontario) were randomly assigned to 29 treatments. Following the pre-experimental period (see section V. C.) each rat was weighed. Two groups of four animals were fed ad libitum either a fat-free or 12% corn oil diet for ten days and slaughtered. The remaining rats were assigned to three groups comprised of 28 animals each and either starved four days or restricted to five grams daily of the fat-free or 12% corn oil diet for eight days.

A preliminary study had shown that these periods of starvation and diet restriction resulted in a 20 to 30% loss of body weight in 200 to 275 g rats. In addition, five grams of ration was found to be approximately one-fourth to one-fifth the normal daily food consumption.

At the completion of the starvation period, four of the starved animals were slaughtered, 12 were refed ad libitum the fat-free diet, and 12 refed ad libitum the 12% corn oil diet. Three rats refed the fat-free diet and three refed the corn oil diet were slaughtered following 48, 72, 96 and 120 hours of refeeding. The 28 animals in each of the two restricted groups were similarly subdivided at the end of the restriction period and either slaughtered or fed

DESIGN OF EXPERIMENT

METHOD OF FOOD DEPRIVATION	REFEEDING SCHEDULE	NOTATION
Starved (4 days)	fat-free	S - r0
	12% corn oil	S - r12
Restricted-fat-free (5 g./day for 8 days)	fat-free	R0 - r0
	12% corn oil	R0 - r12
Restricted-12% corn oil (5 g./day for 8 days)	fat-free	R12 - r0
	12% corn oil	R12 - r12

CONTROLS

Starved - 4 days	S
Restricted - 5 g. fat-free - 8 days	R0
Restricted - 5 g. 12% corn oil - 8 days	R12
Fed ad lib. - fat-free - 10 days	A0
Fed ad lib. - 12% corn oil - 10 days	A12

ad libitum either the same diet that was fed during the restriction period or the alternate diet for 48, 72, 96 or 120 hours.

Six animals were slaughtered each day. Rats from the same treatment were slaughtered on different days in order to distribute the variation within treatments over several days. At least one starved, restricted or ad libitum-fed control was included in the six to check the accuracy of assay procedures and equipment.

V. B. Diets

The 12% corn oil diet was similar to the fat-free diet except that 12% corn oil was substituted for an isocaloric amount of glucose (Table I). Substitution was made on the basis of 4 Cal/g for glucose, 9 Cal/g for corn oil and 0 Cal/g for cellulose. Diets were mixed using a Hobart mixer[1] and stored at -10°C. Butylated hydroxytoluene was added to both diets as an extra precaution against oxidative changes.

[1] The Hobart Manufacturing Co., Troy, Ohio, U.S.A.

TABLE I

Composition of diets

Ingredients	Fat-Free Diet			12% Fat 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	19.66	18.0	72.0	19.67
DL-methionine	0.6	2.4	0.66	0.6	2.4	0.66
Vitamins[1]	0.6	--	--	0.6	--	--
Minerals[2]	4.0	--	--	4.0	--	--
Corn oil[3]	--	--	--	12.0	108.0	29.50
Glucose	72.9	291.6	79.67	45.9	183.6	50.16
Alphacel	3.8	--	--	18.8	--	--
BHT[4]	0.1	--	--	0.1	--	--
	100.0	366.0	99.99	100.0	366.0	99.99

[1]Vitamin mix provided per 100g of diet: vitamin A, 540 I.U.; vitamin D, 60 I.U.; DL-alpha-tocopherol, 3 I.U.; menadione, 1.35 mg; inositol, 3 mg; choline chloride, 45 mg; para-amino benzoic acid, 3 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, 0.012 mg; folic acid, 0.054 mg; vitamin B_{12}, 0.81 µg; dextrose, 0.54 g.

[2]Salt mix provided per 100g of diet: sodium chloride, 432 mg; potassium citrate ($K_3C_6H_5O_7.H_2O$), 945 mg; dipotassium phosphate (K_2HPO_4), 309 mg; dicalcium phosphate ($CaHPO_4$, $2H_2O$), 1,420 mg; calcium carbonate ($CaCO_3$), 368 mg; magnesium carbonate ($MgCO_3$), 163 mg; 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 ($ZnSO_4$), 4.08 mg.

[3]Mazola: Canada Starch (Best Foods)

[4]Butylated hydroxytoluene: Sigma Chemical Co., St. Louis, Missouri. U.S.A.

V. C. Feeding and Management of Experimental Animals

Rats were housed in individual, wire-floor ($\frac{1}{2}$-inch screen) cages measuring 8 x 12 inches. Feed containers (six-ounce glass jars) were attached to the front of the cage in an attempt to minimize feed wastage. Water bottles, located at the front of the cage, were filled with fresh water daily. Temperature in the room fluctuated between 70 and 80°F. Feed consumption and weight change were recorded daily for each animal.

The rats were allowed to adjust to their new environment for a five-day pre-experimental period during which they were fed ad libitum a commercial laboratory diet[1]. Starvation, diet restriction and ad libitum refeeding were initiated at 8:30 a.m. Feed containers were removed from the animal cages during starvation whereas diet restriction was accomplished by feeding five grams of ration daily; two feedings of 2.5 g each at 8:30 a.m. and 5:00 p.m. Following the specified treatment protocol (see section V. A.) rats were decapitated using a guillotine[2] and the tissue to be assayed rapidly excised.

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[1]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

Livers were rapidly excised, rinsed with cold tap water, blotted on filter paper and weighed on a top-loading balance[1]. Epididymal fat pads were removed carefully to exclude epididymus and blood vessels and weighed.

Thin slices of liver were prepared with a Harvard tissue slicer[2], blotted on filter paper, weighed (75 to 150 mg) on an analytical balance[3] and <u>in vitro</u> acetate-1-^{14}C incorporation into fatty acids determined according to the method of Leveille (1967) (see section V. E. 3.a.i.). Segments from the periphery of the epididymal fat pad (75 to 150 mg) were dissected with scissors and fatty acid synthesis assayed in a similar manner.

A 2.0 to 2.2 g sample (a composite of a piece from each lobe) was cut from the remaining liver, minced with scissors and homogenized in 18 volumes of cold buffer (McDonald and Johnson, 1965) for one minute using a motor-driven, Teflon-glass homogenizer[4]. Similarly, a 2.0 to 2.2 g sample of epididymal fat pad (a composite of a piece from each pad) was homogenized in cold 0.15 molar KCL buffer (1:9 w/v) for one and one-half minutes using a Lourdes

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[1]Model 2254-Sartorius Werke, Gottingen, West Germany.
[2]Model 140-Harvard Apparatus Inc., Dover, Massachusetts, U.S.A.
[3]Model 2403-Sartorius Werke, Gottingen, West Germany.
[4]Arthur C. Thomas Inc., Philadelphia, Pennsylvania, U.S.A.

multi-mix homogenizer[1].

Both homogenates were centrifuged at 0°C for 10 minutes at 1,475 x g, 10 minutes at 10,800 x g, and 20 minutes at 45,900 x g in a refrigerated centrifuge[2]. The resulting supernatant fractions were carefully separated from any overlaying fat and retained for enzyme and protein analyses. All procedures were performed at 0 to 4°C, except for weighing the tissue.

The remainder of each liver was stored at -10°C in three-ounce, screw-cap glass jars for later total lipid analyses by the method of Bligh and Dyer (see section V. E. 3. b.).

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[1]Model MM-Lourdes Instrument Corp., Brooklyn, New York, U.S.A.

[2]Model RC 2-B-Ivan Sorvall Inc., Norwalk, Connecticut, U.S.A.

V. E. Chemical Analyses

1. Protein Assay

Protein content of the supernatant fractions from liver and epididymal adipose tissue was determined by the method of Lowry et al. (1951) using Folin and Ciocalteau Reagent purchased commercially[1]. Bovine serum albumin stock solution was further diluted with physiological saline 1:1 (v/v) for use as the protein standard. The liver and adipose tissue supernatants (1:9 w/v) were further diluted with physiological saline to give 1:351 (w/v) and 1:81 (w/v) dilutions, respectively. Colorimetric measurements on duplicate samples of diluted supernatant and albumin were read at 750 mμ in a Unicam SP600 Series 2 spectrophotometer[2].

2. Enzyme Assays

The combined glucose-6-phosphate and 6-phosphogluconate dehydrogenase activity referred to as G-6-P dehydrogenase assay, was determined by the method of Lohr and Waller (1963) and malic enzyme activity by the method of Ochoa (1955). These procedures involved a measurement of the rate of formation of NADPH at 340 mμ in a Unicam SP800A UV spectrophotometer[3], equipped with a SP20 Series recorder and a constant temperature (30°C) cuvette chamber. G-6-P

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[1] Fisher Scientific Co., Toronto, Ontario.
[2] Pye Unicam Ltd., Cambridge, England.
[3] Pye Unicam Ltd., Cambridge, England.

dehydrogenase and malic enzyme activities were expressed as nanamoles NADPH produced/minute/mg protein.

The reaction cell (total volume 3.0 ml) for the G-6-P dehydrogenase assay contained 2.75 ml glycylglycine buffer, pH 7.4 (229 μmoles); 0.05 ml NADP (2.72 μmoles); 0.1 ml G-6-P (1.424 μmoles) and 0.1 ml supernatant. The reaction cell for the malic enzyme assay contained 2.25 ml glycylglycine buffer, pH 7.4 (187 μmoles); 0.2 ml $MnCl_2$ (3.33 μmoles); 0.05 ml NADP (2.72 μmoles); 0.40 ml L-malate, pH 7.4 (0.4 μmoles) and 0.1 ml supernatant. Assays were run with a reference cell containing all the reagents added to the reaction cell except the substrate (G-6-P or L-malate) which was replaced by an equivalent volume of buffer.

The enzyme reaction was initiated by adding undiluted supernatant (1:9 w/v) from control rats and diluted liver and adipose supernatants (1:63 w/v and 1:36 w/v, respectively) from refed animals to the reaction cell. Greater supernatant volumes were required to obtain satisfactory activity measurements with some treatments. In these cases, total volume was maintained by a decrease in buffer volume.

3. Lipid Analyses

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

i. Incubation of tissue

Duplicate slices of liver and epididymal adipose tissue were incubated in 25 ml Erlenmeyer flasks containing

three ml Krebs-Ringer, phosphate-bicarbonate buffer (Ca-free, pH 7.4). The buffer contained, per ml: 10 μmoles sodium acetate, 5 μmoles glucose, 0.1 unit insulin and 0.297 μC sodium acetate-1-^{14}C.

The flasks, equipped with ground glass stoppers, were incubated at 37°C in an atmosphere of air in a shaking water bath[1] for three hours. At the termination of incubation, the reaction was stopped by adding one ml of 30% (w/v) methanolic KOH (95 ml methanol: 30 g KOH in five ml water) to each flask. Flask contents were transferred, together with two one-ml washings with 30% methanolic KOH, to 20 ml screw-cap glass vials and stored at -10°C for later analyses of acetate-1-^{14}C incorporation into fatty acids of liver and epididymal adipose tissue.

a. ii. Saponification

Vials containing liver and epididymal adipose tissue in buffer and methanolic KOH were brought to room temperature and contents transferred, together with five one-ml washings with 30% methanolic KOH, to 125 ml Erlenmeyer flasks. Flask contents were refluxed (80 to 85°C) for three hours. Then the amount of heat applied was reduced, condensors removed from the flasks, five ml water added and heating continued until no odor of alcohol was detectable.

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[1]Eberback Corporation, Ann Arbor, Michigan, U.S.A.

a. iii. Extraction of non-saponifiable and saponifiable lipid

Flasks were cooled and the contents transferred, together with two five-ml washings with petroleum ether (boiling point 30 to 60°C), to 25 x 200 mm screw-cap culture tubes. Following thorough mixing with a Vortex Jr. mixer[1], the petroleum ether layer containing the non-saponifiable lipid was removed with a water aspirator and discarded. The aqueous mixture was extracted with another two ml of petroleum ether and the ether layer removed with a Pasteur pipette fitted with two-ml rubber bulb and discarded.

The saponified aqueous mixture was acidified with six normal HCl (change in pH determined with litmus paper). Three five-ml portions of petroleum ether were used to extract the fatty acids from the acidified aqueous mixture. The petroleum ether was transferred with a Pasteur pipette to a culture tube and evaporated to dryness by a stream of warm air from a hair dryer. The residue remaining in the tube was dissolved in five ml petroleum ether and the ether washed with five ml distilled water. The ether layer was transferred to a liquid scintillation vial[2] and the aqueous phase re-extracted with two five-ml portions of petroleum ether which also were transferred to the liquid scintillation

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[1]Scientific Industries Inc., Queens Village, New York, U.S.A.

[2]Packard Instrument Inc., Downers Grove, Illinois. U.S.A.

vial. The petroleum ether then was evaporated to dryness.

a. iv. Radioactivity determination

The residue in the vial was dissolved in ten ml toluene (containing 5.0 g of PPO[1] and 0.3 g POPOP[2] per liter). Total radioactivity in each sample (expressed as dpm/g tissue) was determined using a Liquid Scintillation spectrometer[3]. The efficiency of counting was ascertained by the Channels Ratio method (Wang and Willis, 1965).

b. Total hepatic lipid extraction

Frozen livers (see section V. D.) were thawed at room temperature, and the total lipid extracted following the procedure of Bligh and Dyer (1959). Approximately seven g of liver was homogenized with 95 ml of a monophasic solvent mixture containing chloroform, methanol and water (1:2:0.8 v/v/v) for two minutes at full speed in a Virtus 23 tissue homogenizer[4]. The homogenate was filtered with slight suction through a No. 1 Whatman filter paper and the residue washed with 25 ml of chloroform and 25 ml of water. The filtrate was allowed to separate in a 500 ml separatory funnel. The chloroform layer then was filtered through a

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[1]PPO: 2-5 diphenyl ozazole-Packard Instrument Inc., Downers Grove, Illinois, U.S.A.

[2]POPOP: 2,2-p-phenylene bis (5-phenyl ozazole)-Packard Instrument Inc., Downers Grove, Illinois, U.S.A.

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

[4]Virtus Research Equipment, Gardiner, New York, U.S.A.

No. 2 Whatman filter paper into a 100 ml tarred round-bottom boiling flask and evaporated to dryness under vacuum using a Buchler Flash Evaporator[1] equipped with a vacuum pump and constant temperature water bath. The flasks were flushed with nitrogen and placed over concentrated sulphuric acid in a dessicator to remove any remaining traces of moisture. Total lipid was then determined gravimetrically.

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[1]Model PFE-1BN-Buchler Instruments, Fort Lee, New Jersey, U.S.A.

V. F. Statistical Analyses

Statistical analyses were carried out on the data for G-6-P dehydrogenase and malic enzyme in liver and adipose tissue according to the methods described by Snedecor and Cochrane (1967). Bartlett's Chi-square test for equality of variance between several samples was performed on the data for G-6-P dehydrogenase and malic enzyme activity in both tissues. The calculated Chi-square value was greater than the tabulated Chi-square value at $P < 0.05$ with 28 degrees of freedom for each enzyme in both tissues. Therefore, a log transformation was performed on the original data. This brought the Chi-square values into the acceptable range of values for both enzymes in the liver (Appendix Tables I and II) while the calculated Chi-square value for malic enzyme in adipose tissue was slightly higher than the tabulated value even after log transformation (Appendix Table IV). Nevertheless, the transformed data for both enzymes in the liver and malic enzyme in the adipose tissue was subjected to analysis of variance and the significance determined by the F-test at $P < 0.05$ (Appendix Tables I, II and IV). The analysis of variance on malic enzyme in the adipose tissue, however, must be viewed with some reservation due to the high Chi-square value. The calculated Chi-square value for G-6-P dehydrogenase activity in the adipose tissue was extremely high in comparison with the

tabulated value (56.7 vs. 41.34), and for this reason no statistical analysis will be reported for this enzyme in the discussion of experimental results.

The difference in activities of G-6-P dehydrogenase and malic enzyme in the liver, were calculated for each rat in order to determine the degree of similarity in the response of these two enzymes. The values for the differences between the two enzymes were then subjected to log transformation and differences among treatments determined by analysis of variance (Appendix Table III). The calculated Chi-square value was slightly higher than the tabulated Chi-square value and hence the analysis of variance must be interpreted with some caution. A similar determination was not performed on G-6-P dehydrogenase and malic enzyme activities in the adipose tissue since the calculated Chi-square value for G-6-P dehydrogenase activity in this tissue was much greater than the tabulated Chi-square value.

Statistical analyses were not performed on the data for acetate-1-^{14}C incorporation into fatty acids by liver slices or adipose tissue due to the number of missing values in the data and the extreme variability between duplicate samples from the same animal.

VI. RESULTS AND DISCUSSION

A. Results

Starvation and restriction of diet to 5 g daily resulted in significantly ($P < 0.05$) lower G-6-P dehydrogenase and malic enzyme activities in liver and adipose tissue than those observed in ad libitum-fed controls (controls, Tables II to V; Figs. I to IV). There was no significant ($P < 0.05$) difference however in NADP-linked dehydrogenase activities due to method of inducing weight loss (starvation or diet restriction) or composition of the diet fed during restriction (fat-free or 12% fat). Restriction may have been so severe that any effects due to diet composition were masked.

Similar results were observed for acetate-1-^{14}C incorporation into fatty acids by liver slices and adipose tissue although incorporation into fatty acids by liver slices and adipose tissue was lower in the group restricted on the fat-free diet (controls, Tables VI and VII; Fig. VI). Young et al. (1964) and Pande et al. (1964) also found that fasting depressed G-6-P dehydrogenase and malic enzyme activities in liver and adipose tissue. By contrast, Jomain and Hanson (1969) reported that fasting did not suppress G-6-P dehydrogenase and malic enzyme activities in adipose tissue but did suppress fatty acid synthesis. Allmann et al. (1965) also noted that fasting depressed fatty acid

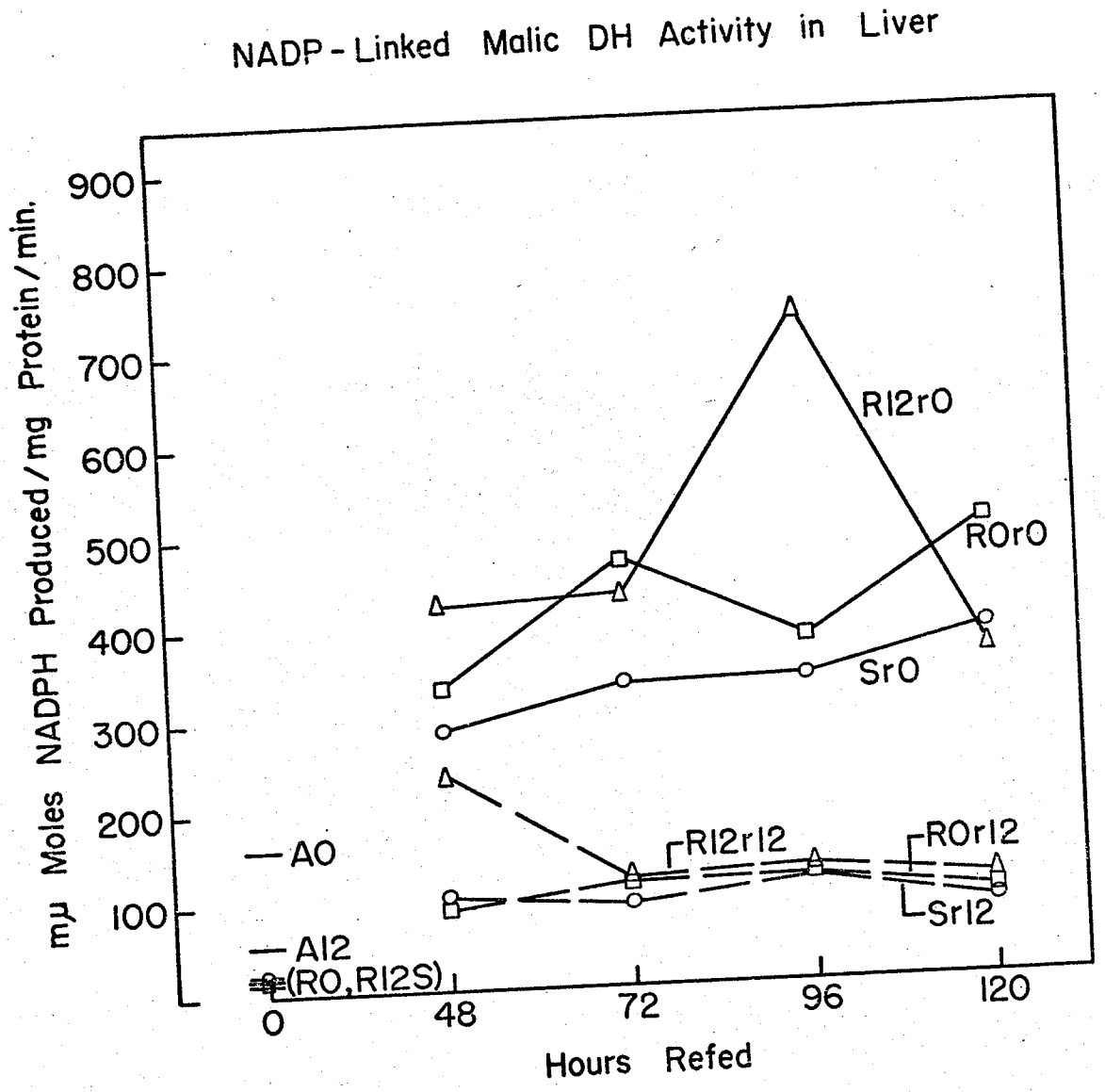

Fig. I. Liver NADP-linked malic dehydrogenase activity during refeeding following starvation or diet restriction. Key: S denotes starvation for 4 days; R-diet restriction for 8 days; A-ad libitum feeding for 10 days; and r-refeeding. The numbers following the letters specify the type of diet fed during the restriction or refeeding periods. O denotes fat-free and 12-12% fat diet.

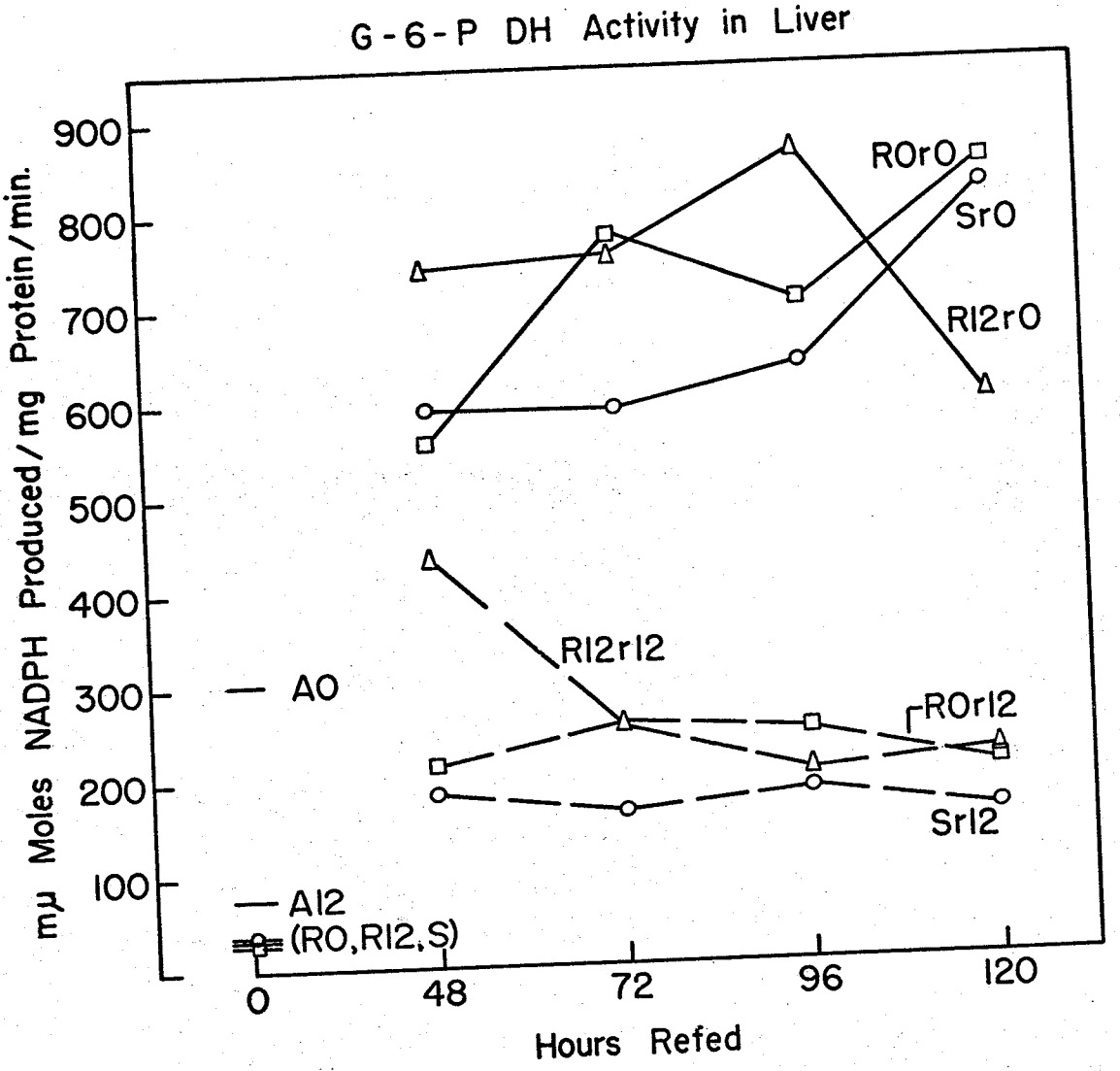

Fig. II. Liver glucose-6-phosphate dehydrogenase activity during refeeding following starvation or diet restriction. Key: S denotes starvation for 4 days; R-diet restriction for 8 days; A-ad libitum feeding for 10 days; and r-refeeding. The numbers following the letters specify the type of diet fed during the restriction or refeeding periods. 0 denotes fat-free diet and 12-12% fat diet.

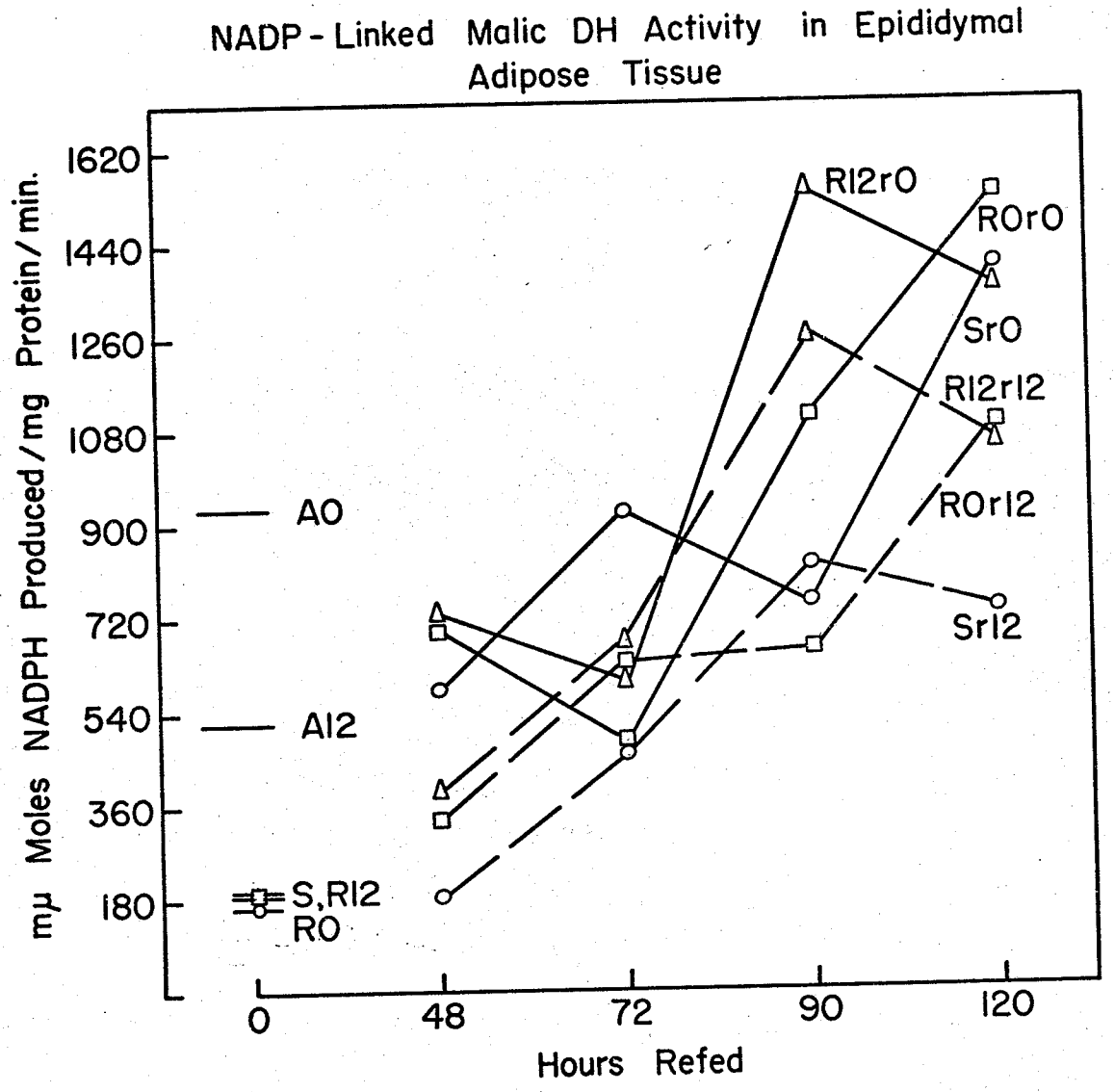

Fig. III. The response of NADP-linked malic dehydrogenase activity in epididymal adipose tissue during refeeding following starvation or diet restriction. Key: S denotes starvation for 4 days; R-diet restriction for 8 days; A-ad libitum feeding for 10 days; and r-refeeding. The numbers following the letters specify the type of diet fed during the restriction or refeeding periods. 0 denotes fat-free diet and 12-12% fat diet.

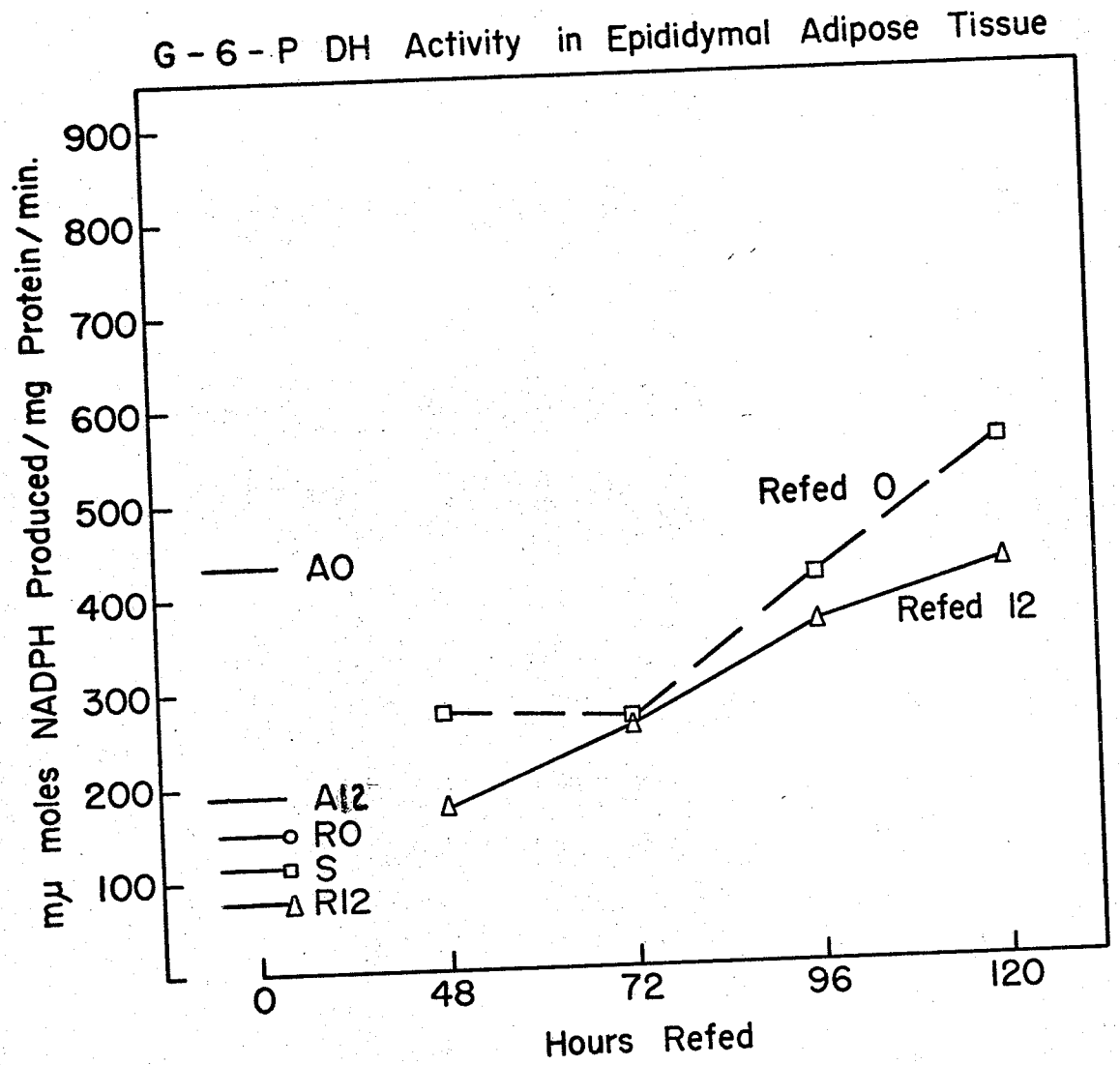

Fig. IV. The response of glucose-6-phosphate dehydrogenase activity in epididymal adipose tissue during refeeding following starvation or diet restriction. Each point is the mean G-6-P DH activity for the three groups of rats refed the fat-free diet and for similar groups refed the 12% fat diet.

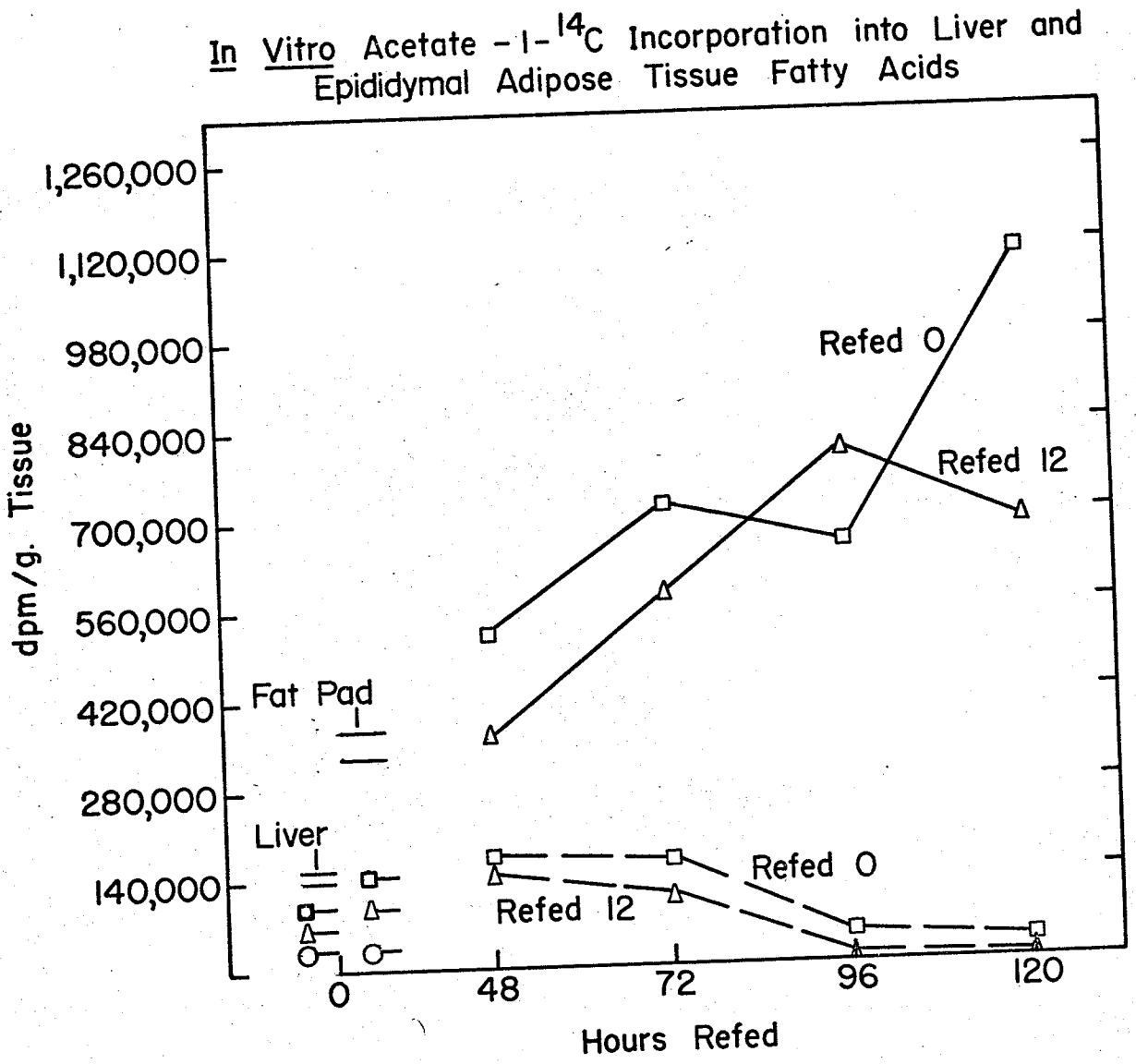

Fig. V. The effect of starvation and diet restriction on *in vitro* acetate-1-^{14}C incorporation into fatty acids by the liver and epididymal adipose tissue during refeeding. Each value is the mean for the three groups of rats refed the fat-free diet and for similar groups refed the 12% fat diet.

TABLE II

Liver malic enzyme activity during refeeding following starvation or diet restriction[1]

Treatment Prior to Refeeding	Hours Refed	Malic Enzyme Activity (n moles NADPH produced/mg protein/min) Diet Refed: Fat-Free	Diet Refed: 12% Fat
Starved-4 days	48	286 ± 25[2]	100 ± 12
	72	333 ± 50	91 ± 11
	96	333 ± 70	112 ± 13
	120	384 ± 108	85 ± 12
Restricted-Fat-Free 5g/rat/day 8 days	48	330 ± 31	92 ± 4
	72	468 ± 22	112 ± 13
	96	379 ± 45	115 ± 13
	120	501 ± 20	99 ± 10
Restricted-12% Fat 5g/rat/day 8 days	48	420 ± 55	237 ± 111
	72	431 ± 31	117 ± 23
	96	732 ± 145	124 ± 6
	120	360 ± 32	111 ± 8
Controls			
Starved, 4 days		17 ± 3[3]	
Restricted, 5g fat-free/day, 8 days		22 ± 3	
Restricted, 5g 12% fat/day, 8 days		18 ± 3	
Ad libitum-fed, fat-free, 10 days		160 ± 5	
Ad libitum-fed, 12% fat, 10 days		54 ± 1	

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

[2] Mean ± S.E. of 3 rats

[3] Mean ± S.E. of 4 rats

TABLE III

Liver glucose-6-PO_4 dehydrogenase activity during refeeding following starvation or diet restriction[1]

Treatment Prior to Refeeding	Hours Refed	Glucose-6-PO_4 DH Activity (n moles NADPH produced/mg protein/min) Diet Refed: Fat-Free	Diet Refed: 12% Fat
Starved-4 days	48	596 ± 45[2]	190 ± 35
	72	591 ± 117	164 ± 40
	96	633 ± 87	187 ± 24
	120	821 ± 216	159 ± 40
Restricted-Fat-Free 5g/rat/day 8 days	48	556 ± 46	212 ± 43
	72	772 ± 8	256 ± 20
	96	701 ± 79	249 ± 27
	120	849 ± 19	207 ± 56
Restricted-12% Fat 5g/rat/day 8 days	48	741 ± 91	434 ± 188
	72	754 ± 53	256 ± 49
	96	861 ± 43	201 ± 28
	120	599 ± 97	215 ± 11

Controls	
Starved, 4 days	37 ± 5[3]
Restricted, 5g fat-free/day, 8 days	39 ± 3
Restricted, 5g 12% fat/day, 8 days	35 ± 3
Ad libitum-fed, fat-free, 10 days	296 ± 34
Ad libitum-fed, 12% fat, 10 days	73 ± 6

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

[2]Mean ± S.E. of 3 rats

[3]Mean ± S.E. of 4 rats

TABLE IV

The response of malic enzyme in epididymal adipose tissue[1] during refeeding following starvation or diet restriction

Treatment Prior to Refeeding	Hours Refed	Malic Enzyme Activity (n moles NADPH produced/mg protein/min) Diet Refed: Fat-Free	Diet Refed: 12% Fat
Starved-4 days	48	586±174[2]	182± 35
	72	933± 89	470±121
	96	753± 91	831± 48
	120	1,405±112	735±293
Restricted-Fat-Free 5g/rat/day 8 days	48	706±109	334± 10
	72	484±181	634± 95
	96	1,119±125	663±164
	120	1,530±295	1,092±153
Restricted-12% Fat 5g/rat/day 8 days	48	723±335	383± 31
	72	617±171	686±142
	96	1,547±220	1,265±196
	120	1,365±202	1,070±131

Controls	
Starved, 4 days	191±113[3]
Restricted, 5g fat-free/day, 8 days	172± 40
Restricted, 5g 12% fat/day, 8 days	185± 47
Ad libitum-fed, fat-free, 10 days	933±419
Ad libitum-fed, 12% fat, 10 days	514±132

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

[2]Mean ± S.E. of 3 rats

[3]Mean ± S.E. of 4 rats

TABLE V

The response of glucose-6-PO_4 dehydrogenase activity in epididymal adipose tissue during refeeding following starvation or diet restriction

Treatment Prior to Refeeding	Hours Refed	Glucose-6-PO_4 DH Activity (n moles NADPH produced/mg protein/min) Diet Refed: Fat-Free	Diet Refed: 12% Fat
Starved-4 days	48	250 ± 22[1]	159 ± 13
	72	347 ± 29	241 ± 32
	96	263 ± 123	368 ± 63
	120	537 ± 19	281 ± 113
Restricted-Fat-Free 5g/rat/day 8 days	48	264 ± 45	192 ± 30
	72	238 ± 121	233 ± 86
	96	427 ± 55	318 ± 25
	120	624 ± 76	407 ± 96
Restricted-12% Fat 5g/rat/day 8 days	48	323 ± 55	191 ± 19
	72	199 ± 80	316 ± 41
	96	553 ± 80	394 ± 82
	120	498 ± 61	586 ± 80
Controls			
Starved, 4 days		116 ± 27[2]	
Restricted, 5g fat-free/day, 8 days		153 ± 3	
Restricted, 5g 12% fat/day, 8 days		89 ± 30	
Ad libitum-fed, fat-free, 10 days		431 ± 373	
Ad libitum-fed, 12% fat, 10 days		175 ± 126	

[1] Mean ± S.E. of 3 rats

[2] Mean ± S.E. of 4 rats

TABLE VI

The effect of starvation and diet restriction on *in vitro* acetate-1-^{14}C incorporation into fatty acids in the liver during refeeding

Treatment Prior to Refeeding	Hours Refed	Acetate-1-^{14}C Incorporation into Fatty Acids (dpm x 10^3/g Liver) Diet Refed: Fat-Free	Diet Refed: 12% Fat
Starved-4 days	48	94±19[1]	78±54
	72	214±83	108±69
	96	49±17	10± 3
	120	17± 3	4± 1
Restricted-Fat-Free 5g/rat/day 8 days	48	95±26	236±62
	72	238±72	171±91
	96	34± 7	6± 1
	120	55±11	13± 2
Restricted-12% Fat 5g/rat/day 8 days	48	337±49	137±66
	72	47±21	89±39
	96	76±27	9± 1
	120	26±12	8± 3
Controls			
Starved, 4 days			56±24[2]
Restricted, 5g fat-free/day, 8 days			6± 1
Restricted, 5g 12% fat/day, 8 days			39±22
Ad libitum-fed, fat-free, 10 days			124±56
Ad libitum-fed, 12% fat, 10 days			109±54

[1] Mean ± S.E. of 3 rats

[2] Mean ± S.E. of 4 rats

TABLE VII

The effect of starvation and diet restriction on *in vitro* acetate-1-^{14}C incorporation into epididymal adipose tissue fatty acids during refeeding

Treatment Prior to Refeeding	Hours Refed	Acetate-1-^{14}C Incorporation into Fatty Acids (dpm x 10^3/g epididymal adipose tissue) Diet Refed: Fat-Free	Diet Refed: 12% Fat
Starved-4 days	48	484±204[1]	196± 87
	72	596±103	484± 74
	96	743±243	851±176
	120	1,119±121	469± 70
Restricted-Fat-Free 5g/rat/day 8 days	48	282±106	265±141
	72	869±193	781±108
	96	746± 25	854±226
	120	1,198± 79	1,012±170
Restricted-12% Fat 5g/rat/day 8 days	48	811±285	543± 87
	72	683±103	469± 67
	96	657± 96	718±105
	120	1,010±127	659± 80

Controls	
Starved, 4 days	129±32[2]
Restricted, 5g fat-free/day, 8 days	23± 5
Restricted, 5g 12% fat/day, 8 days	65±39
Ad libitum-fed, fat-free, 10 days	362±78
Ad libitum-fed, 12% fat, 10 days	379±73

[1] Mean ± S.E. of 3 rats

[2] Mean ± S.E. of 4 rats

synthesis by the soluble supernatant fraction of rat liver.

Dietary fat had a suppressing effect on NADP-linked dehydrogenase activities in ad libitum-fed controls. G-6-P dehydrogenase and malic enzyme activities were significantly lower in liver and adipose tissue from rats fed the corn oil diet ad libitum than from those fed the fat-free diet ad libitum (controls, Tables II to V; Figs. I to IV). These results in liver agree with those by Tepperman and Tepperman (1965). By contrast dietary fat was ineffective in suppressing acetate-1-^{14}C incorporation into fatty acids by liver and adipose tissue from ad libitum-fed controls (controls, Tables VI and VII; Fig. VI). These observations suggest that dietary corn oil is more effective in suppressing NADP-linked dehydrogenase activity than fatty acid synthesis in ad libitum-fed controls, although several authors (Sabine et al., 1969; Bortz et al., 1963; Hill et al., 1958; Leveille, 1970) have shown that dietary corn oil suppressed fatty acid synthesis in non-fasted animals. However, these data are not directly comparable to the present study due to the variable lengths of time that the fat-containing diets were fed.

Neither method of inducing weight loss nor diet composition during the restriction period had any influence on dehydrogenase activities or fatty acid synthesis in liver or adipose tissue during refeeding (Tables II to VII; Figs. I to IV and VI). In general the metabolic response in rats

refed the fat-free diet was the same whether the animals had been starved or restricted to 5 g daily of either the fat-free or 12% fat diet. Similar results were observed for rats refed the 12% fat diet following starvation or severe calorie restriction. The similarity in the refeeding response of starved and restricted rats is contrary to the suggestion by Johnson and Sassoon (1967) that starvation must precede carbohydrate refeeding to obtain an 'overshoot' in dehydrogenase activity. Although the metabolic response during refeeding was not affected by the treatment prior to refeeding it was markedly influenced by the fat content of the diet refed.

The response of the dehydrogenase enzymes to refeeding and to the presence of corn oil in the diet refed was greater in the liver than in the adipose tissue. G-6-P dehydrogenase and malic enzyme activities in liver from rats fed the fat-free diet for 120 hours were 28 to 25 times those of the starved or restricted controls (Tables II and III; Figs. I and II). Inclusion of corn oil in the diet significantly ($P < 0.05$) suppressed the induction to a 5-fold increase over that of controls. In adipose tissue, malic enzyme increased to only 8 times and G-6-P dehydrogenase to 4-5 times that of starved or restricted controls when the fat-free diet was refed for 120 hours (Tables IV and V; Figs. III and IV). Inclusion of corn oil in the diet suppressed the activity of both enzymes to a 3- to 5-fold increase above

the values for control animals at 120 hours of refeeding. These results show that the magnitude of change of malic enzyme in adipose tissue was different from that of G-6-P dehydrogenase when the fat-free diet was refed. On the other hand, the extent of increase was the same for both enzymes in adipose tissue from rats refed on the 12% fat diet. Both enzymes also showed a similar degree of increase in livers of rats refed each diet, although the magnitude of the response varied with the fat content of the diet. Therefore, different mechanisms may exist for the control of enzyme activity in the liver and adipose tissue since the magnitude of suppression and induction of enzyme activity in response to the diet refed was different in the two tissues. Large increases in G-6-P dehydrogenase and malic enzyme activities in liver and adipose tissue have been reported by other authors during refeeding on a high-carbohydrate diet following starvation (Fabry et al., 1969; Young et al., 1964; Oliver, 1967; Arès, 1969). It has also been noted that hepatic G-6-P dehydrogenase and malic enzyme activities were suppressed by feeding a diet containing corn oil or ethyl linoleate (Tepperman and Tepperman, 1965; Oliver, 1967; Arès, 1969). In addition, Wise and Ball (1964) reported that malic enzyme exhibited a more pronounced increase in activity in the liver than in the adipose tissue from fasted rats refed for three days. The highly adaptive nature of the liver to dietary manipulation attests to the central role the liver

plays in the metabolism of the animal.

The type of diet refed appeared to be more important in suppressing the enzymatic response which occurred in the liver during refeeding than the length of time the diet was refed; although activity determination did not commence until 48 hours post-refeeding. The effect of diet on hepatic G-6-P dehydrogenase and malic enzyme activity was statistically significant ($P < 0.05$) whereas the effect of time was not statistically significant (Appendix Tables I and II). By contrast, the effects of time and the type of diet refed on malic enzyme activity in the adipose tissue were statistically significant ($P < 0.05$) (Appendix Table IV). The effect of time is illustrated in Fig. III where malic activity showed a progressive increase from 48 to 120 hours.

There was a striking similarity between the pattern of G-6-P dehydrogenase and malic enzyme response to diet during refeeding in liver as well as in adipose tissue (Fig. I vs. II, Fig. III vs. IV and Fig. V) although the pattern and magnitude of the enzymatic response to diet differed for the two tissues. The similarity in pattern of response was confirmed by the fact that the difference between the activities of the two enzymes in response to the various treatments was not significant (Appendix Table III). In addition, G-6-P dehydrogenase activity was consistently higher than malic enzyme activity in the liver, whereas the reverse

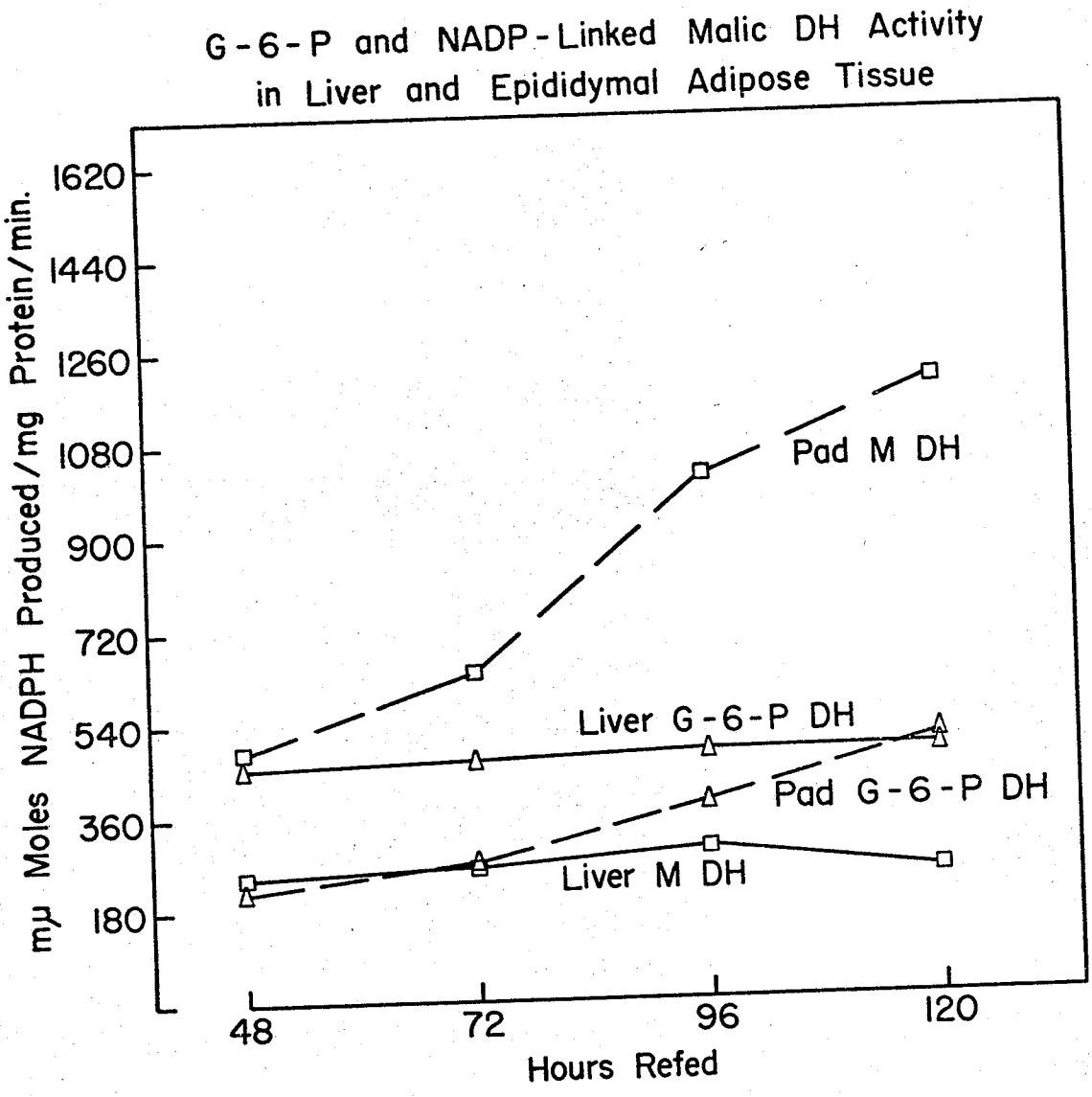

Fig. VI. The pattern of glucose-6-phosphate dehydrogenase and NADP-linked malic dehydrogenase activity in liver and epididymal adipose tissue during refeeding following starvation or diet restriction. Each point is the mean enzyme activity of all rats refed for that period irrespective of diet.

relationship existed in the adipose tissue (Fig. V). A similar relationship between the level of G-6-P dehydrogenase and malic enzyme activity in liver and adipose tissue from starved-refed rats was observed by Young et al. (1964) and in epididymal adipose tissue from normal rats by Pande et al. (1964). The question therefore arises whether the enzymes found in adipose tissue are the same as those present in liver or whether they are different proteins or isoenzymes catalyzing similar reactions. Immunological, electrophoretic and enzyme kinetic data are required in order to answer this question.

The pattern and magnitude of the lipogenic response differed in the two tissues. Fatty acid synthesis was much higher in adipose tissue than in liver (Tables VI and VII; Fig. VI). Acetate-1-^{14}C incorporation into fatty acids in adipose tissue progressively increased above control values throughout the entire refeeding period whether the animals were fed the fat-free or the corn oil diet while acetate-1-^{14}C incorporation by hepatic tissue was elevated at 48 and 72 hours but decreased to one-half that of starved or restricted control values by 120 hours. Hollifield and Parson (1962) also observed smaller changes in in vitro acetate-1-^{14}C incorporation into fatty acids in liver than in adipose tissue from meal-fed rats. In the present study the increase in hepatic fatty acid synthesis up to 72 hours refeeding on the fat-free diet is in agreement with the

increase reported by Allmann et al. (1965). The subsequent decrease found in the present study has not been investigated by other workers. The decrease in hepatic lipogenesis after 72 hours of refeeding may indicate that liver stores have been replenished to the pre-starvation or pre-restriction levels.

Refeeding the corn oil diet failed to appreciably suppress fatty acid synthesis in either tissue although there was a slight tendency toward lower acetate-1-^{14}C incorporation than with the fat-free diet. These observations agree with those of Goodridge (1969) for the chicken but are contrary to those of Oliver (1967), Arès (1969), Allmann and Gibson (1965), and Sabine et al. (1969) who noted that fatty acid synthesis was suppressed in liver from fasted rats and mice that were refed a diet containing linoleic acid. Therefore, factors other than chain length and degree of unsaturation of the fatty acids of the fat in the diet must be involved in the inhibition of fatty acid synthesis in these tissues.

Lipid concentrations in livers of rats refed the fat-free diet were elevated above those refed the corn oil diet, although hepatic fatty acid synthesis was not markedly influenced by the composition of the diet refed. Liver lipid concentration in rats refed the fat-free diet increased to approximately three times that of the ad libitum-fed, fat-free controls at 72 hours of refeeding and

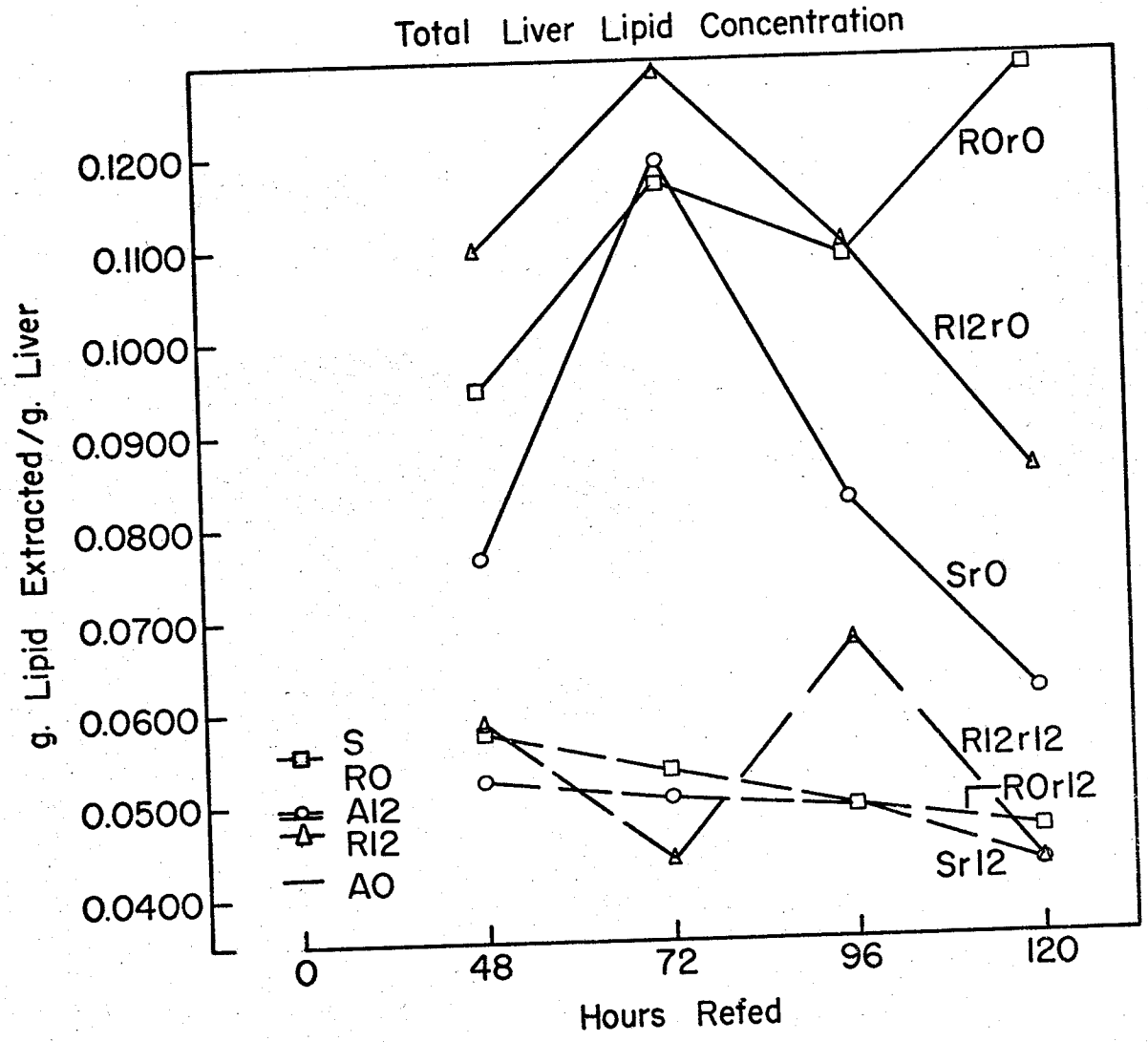

Fig. VII. Changes in liver lipid concentration during refeeding following starvation or diet restriction. Key: S denotes starvation for 4 days; R-diet restriction for 8 days; A-ad libitum feeding for 10 days; and r-refeeding. The numbers following the letters specify the type of diet fed during the restriction or refeeding periods. 0 denotes fat-free diet and 12-12% fat diet.

TABLE VIII

Changes in liver lipid concentration during refeeding following starvation or diet restriction

Treatment Prior to Refeeding	Hours Refed	Total Liver Lipid (mg lipid extracted/g liver) Diet Refed: Fat-Free	Diet Refed: 12% Fat
Starved-4 days	48	77 ± 5[1]	52 ± 3
	72	119 ± 15	50 ± 5
	96	82 ± 18	50 ± 6
	120	61 ± 0	42 ± 0
Restricted-Fat-Free 5g/rat/day 8 days	48	95 ± 8	58 ± 6
	72	117 ± 18	54 ± 5
	96	108 ± 50	49 ± 5
	120	130 ± 14	47 ± 0
Restricted-12% Fat 5g/rat/day 8 days	48	109 ± 21	59 ± 0
	72	130 ± 3	43 ± 3
	96	104 ± 11	68 ± 22
	120	85 ± 0	43 ± 0

Controls	
Starved, 4 days	56 ± 7[2]
Restricted, 5g fat-free/day, 8 days	50 ± 5
Restricted, 5g 12% fat/day, 8 days	48 ± 0
Ad libitum-fed, fat-free, 10 days	42 ± 0
Ad libitum-fed, 12% fat, 10 days	49 ± 5

[1] Mean ± S.E. of 3 rats

[2] Mean ± S.E. of 4 rats

then decreased to 1½ to 2 times the controls except for rats restricted on the fat-free diet where liver lipid concentration tended to plateau after 72 hours. The high liver lipid concentrations probably represent faulty lipid release and transport from the liver because rate of fatty acid synthesis was essentially the same as those refed the 12% fat diet whose lipid concentrations remained at the same level as control animals. These results suggest that dietary linoleate may facilitate lipid release from the liver.

The fact that treatment prior to refeeding had no marked effect on liver lipid levels during refeeding agrees with earlier results with dehydrogenase activities and fatty acid synthesis in liver and adipose tissue. In fact, dehydrogenase activity in liver and adipose tissue and lipid concentration in liver tended to be higher in restricted-refed rats than in starved-refed rats. These results further support the earlier contention that complete starvation is not necessary to induce an 'overshoot' in metabolic response to the feeding of a high-glucose diet as suggested by Johnson and Sassoon (1967). However, these authors compared the response in rats refed a high-glucose diet following starvation with those refed the same diet after ad libitum feeding on a high-fat, carbohydrate-free diet whereas the present study compared starvation with severe diet restriction. Nevertheless these observations, together with those of the present experiment, suggest that body

weight loss is the factor that predisposes to the marked elevation in enzyme activity which occurs during feeding on a high-glucose diet.

VI. B. Integrated Discussion

It is tempting to postulate that a relationship exists between enzyme activity and fatty acid synthesis in adipose tissue but does not exist in liver since the enzymatic response and the lipogenic response to the type of diet refed tended to parallel each other in adipose tissue but not in the liver (Tables II to VII; Figs. I to IV and VI). If enzyme activity and fatty acid synthesis are related, they should respond in a similar manner to dietary alterations. The present study showed that the lipogenic and dehydrogenase response were similar in adipose tissue when the fat-free and 12% fat diets were refed whereas dehydrogenase activity in the liver was considerably higher than fatty acid synthesis in animals fed the fat-free diet. These results agree with those of Jomain and Hanson (1969) who found an association between fatty acid synthesis and dehydrogenase activity in adipose tissue and Tepperman and Tepperman (1965) who found no association in the liver.

If NADPH-generating enzyme activity is not related to rate of fatty acid synthesis in liver, the question then arises as to the function of the elevated enzyme activity which occurs in the liver during refeeding on the fat-free diet. Tepperman and Tepperman (1965) suggested that the elevated G-6-P dehydrogenase and malic enzyme activities which occur when a coconut oil diet is refed probably

represent an increased demand for NADPH for desaturation of saturated fatty acids. Desaturation may represent an attempt by the body to synthesize unsaturated fatty acids that could perhaps perform the functions of linoleic acid. Since the body can not synthesize linoleic acid and since other unsaturated fatty acids can not carry out the functions of linoleic acid, the processes of desaturation and fatty acid synthesis and accumulation continue at accelerated rates. These data coincide with those of Allmann and co-workers (1965) who showed that lipid accumulation, increased monoene synthesis, and low linoleate concentrations occurred in fatty acid deficiency and starvation-refeeding. Furthermore, Arès noted a high correlation between hepatic lipid concentrations and G-6-P dehydrogenase and malic enzyme activities. In general it appears that a decrease in the level of polyunsaturated fatty acids is accompanied by an increase in liver lipid accumulation and an elevation in NADP-linked dehydrogenase activity. Allmann et al. (1965) for example noted an inverse relation between rate of fatty acid synthesis and concentration of linoleic acid. Therefore, linoleate-containing diets should overcome the signs of an apparent essential fatty acid deficiency which occur when a fat-free diet is fed. Supplying linoleate may stimulate lipid transport and inhibit 9-desaturase activity as suggested by Inkpen et al. (1969). The effects of linoleate just mentioned may have been operating in the

present experiment since refeeding the corn oil diet suppressed hepatic lipid concentration and NADPH-generating enzymes from the levels observed when the fat-free diet was refed. Validation of these conclusions would require an analysis of the fatty acid composition of the hepatic lipids and a time course study of the response of desaturase and NADPH-generating enzymes upon refeeding fat-free and linoleate-containing diets following food deprivation.

The present study suggests that NADPH-generating enzyme activity is related to fatty acid synthesis in the adipose tissue. Adipose tissue appeared to be the major site of the hyperlipogenic induction to refeeding following starvation or diet-restriction since the rate of lipogenesis and enzyme activity was much higher in the adipose tissue than in liver. However, the data on _in vitro_ fatty acid synthesis is difficult to interpret in terms of the metabolism of the whole animal. Synthesis _in vivo_ may be limited by the supply of substrates, coenzymes, activators and inhibitors. Nevertheless, Leveille (1967), working with _in vitro_ systems, concluded that adipose tissue plays a major role in lipid synthesis especially under conditions of enhanced lipogenesis such as meal-feeding. Hollifield and Parson (1962) also observed greater changes in _in vitro_ acetate-1-^{14}C incorporation into fatty acids by adipose tissue than by liver from meal-fed rats. Furthermore, the distribution of HMP shunt dehydrogenase enzymes in various

rat tissues has been related to their lipogenic capacities and shown higher in the adipose tissue than in liver (Pande et al., 1964; O'Hea and Leveille, 1969; Hollifield and Parson, 1962; Fritz, 1961). Although Weber (1965) noted that G-6-P dehydrogenase activity was five times more active in adipose tissue than in liver, G-6-P dehydrogenase activity was similar in liver and adipose tissue in the present experiment (Fig. V). Observations from the present experiment also support the suggestion that malic enzyme activity is more closely related to lipogenesis than G-6-P dehydrogenase activity (Young et al., 1964; Pande et al., 1964). Malic enzyme activity in adipose tissue was more active than G-6-P dehydrogenase activity in adipose tissue from either starved or diet-restricted rats that were refed. Adipose tissue malic enzyme was also more active than either enzyme in liver and it tended to adapt in a manner identical to overall lipogenesis.

VII. SUMMARY

The present study compared the metabolic response (ie. physiological stress) due to unregulated food intake following starvation and severe diet restriction. In addition, the study investigated the effect of dietary fat fed during the restriction and refeeding periods on the response accompanying ad libitum refeeding.

Diet refed had a marked effect only on hepatic enzyme activity and hepatic lipid concentrations. In these instances, the 12% fat diet suppressed the induction in response observed when the fat-free diet was refed. Corn oil also suppressed dehydrogenase activity in ad libitum-fed controls. On the other hand, the 12% fat diet had no suppressing effect on dehydrogenase activity in liver or adipose tissue from diet-restricted controls and in adipose tissue from refed rats. Similarly, dietary fat had no suppressing effect on fatty acid synthesis in either the liver or adipose tissue of any of the treatment groups. The failure of dietary corn oil to consistently suppress lipogenesis and enzyme activity further confuses the present understanding of the mechanism which regulates fatty acid synthesis.

Neither method of inducing weight loss nor diet fed during restriction had a profound effect on NADP-linked dehydrogenase activities and fatty acid synthesis in liver

and adipose tissue or lipid concentrations in liver during refeeding on either the fat-free or corn oil diet. The metabolic response during refeeding was the same whether animals had been starved for four days or restricted to five grams of either a fat-free or 12% fat diet daily for eight days. Furthermore, the inclusion of an unsaturated fat in the diet fed during restriction did not appear to have an ameliorating effect on the physiological stress which accompanies refeeding.

The response of NADP-linked enzymes and lipogenesis to diet composition during refeeding followed a similar pattern in adipose tissue suggesting that dehydrogenase activity was dependent on rate of fatty acid synthesis in the adipose tissue but not in the liver.

Refeeding the fat-free diet induced metabolic changes in the liver similar to those observed in essential fatty acid deficiency since refeeding the corn oil diet suppressed enzyme activity and lipid concentrations in the liver. These results suggest that NADP-linked dehydrogenase activity may be associated with fatty acid desaturation in the liver whereas in the adipose tissue activity appeared to be related to rate of lipogenesis.

The adipose tissue appeared to be the major site of fatty acid synthesis since the rate of lipogenesis and enzyme activity were higher in the adipose tissue than in the liver. Moreover, malic enzyme activity was consistently

higher than G-6-P dehydrogenase activity in the adipose tissue, whereas the opposite relationship existed in the liver. The enzymatic response to refeeding appeared to be more responsive in liver than adipose tissue since the suppression and induction of enzyme activity due to diet were more pronounced.

The significance of these observations as they relate to methods of inducing weight loss in humans must await further study.

VIII. APPENDIX TABLES

APPENDIX TABLE I

Analysis of variance for malic enzyme activity in liver following log transformation of the original data

Source of Variation	Degrees of Freedom	Sums of Squares	Mean Square	F Value	Significance $P < 0.05$
Total	91	101.69	1.12		
Within treatment	63	4.41	0.07		
Between treatment					
1. Controls					
Ad libitum-fed vs others	1	1.50	1.50	21.45	s[1]
Ad libitum-fed, fat-free vs ad libitum-fed, 12% fat	1	2.36	2.36	33.69	s
Starved vs diet-restricted	1	0.07	0.07	1.03	ns[2]
Diet-restricted, fat-free vs diet-restricted, 12% fat	1	0.65	0.65	0.93	ns
2. Not refed vs refed	1	60.00	60.00	855.97	s
3. Refed, fat-free vs refed, 12% fat	1	29.64	29.64	422.87	s
4. Pre-refeeding	2	1.42	0.71	10.10	s
Starved and refed vs diet-restricted and refed	1	1.04	1.04	14.86	s
Diet-restricted, fat-free, and refed vs diet-restricted, 12% fat, and refed	1	0.37	0.37	5.32	s
5. Time	3	0.19	0.06	0.90	ns
6. Interactions					
Pre-refeeding x refeeding	2	0.10	0.05	0.68	ns
Pre-refeeding x time	6	0.80	0.13	1.91	ns
Refeeding x time	3	0.41	0.14	1.96	ns
Pre-refeeding x refeeding x time	6	0.69	0.11	1.64	ns
Chi-square value =		40.396			
Chi-square critical (28, $P < 0.05$) =		41.340			

[1] significant

[2] not significant

APPENDIX TABLE II

Analysis of variance for glucose-6-phosphate dehydrogenase activity in liver following log transformation of the original data

Source of Variation	Degrees of Freedom	Sums of Squares	Mean Square	F Value	Significance P< 0.05
Total	91	96.860	1.06		
Within treatment	63	5.180	0.080		
Between treatment					
1. Controls					
Ad libitum-fed vs others	1	2.860	2.860	34.88	s[1]
Ad libitum-fed, fat-free vs ad libitum-fed, 12% fat	1	3.860	3.860	46.98	s
Starved vs diet-restricted	1	0.003	0.003	.04	ns[2]
Diet-restricted, fat-free vs diet-restricted, 12% fat	1	0.018	0.018	.22	ns
2. Not refed vs refed	1	56.850	56.850	692.40	s
3. Refed, fat-free vs refed, 12% fat	1	25.291	25.291	308.06	s
4. Pre-refeeding	2	0.922	0.461	5.61	s
Starved and refed vs diet-restricted and refed	1	0.873	0.873	10.63	s
Diet-restricted, fat-free, and refed vs diet-restricted, 12% fat, and refed	1	0.048	0.048	0.59	ns
5. Time	3	0.036	0.012	0.15	ns
6. Interactions					
Pre-refeeding x refeeding	2	0.215	0.107	1.31	ns
Pre-refeeding x time	6	0.728	0.121	1.48	ns
Refeeding x time	3	0.404	0.135	1.64	ns
Pre-refeeding x refeeding x time	6	0.474	0.079	.96	ns
Chi-square value =		39.13			
Chi-square critical (28, P< 0.05) =		41.34			

[1] significant

[2] not significant

APPENDIX TABLE III

Analysis of variance for the difference between G-6-P dehydrogenase and malic enzyme activities in liver following log transformation of the original data

Source of Variation	Degrees of Freedom	Sums of Squares	Mean Square	F Value	Significance P< 0.05
Total	91	3.99			
Within treatment	63	2.13	0.03		
Between treatment					
1. Controls					
Ad libitum-fed vs others	1	0.22	0.21	6.43	s[1]
Ad libitum-fed, fat free vs ad libitum-fed, 12% fat	1	0.18	0.18	5.42	s
Starved vs diet-restricted	1	0.05	0.05	1.37	ns[2]
Diet-restricted, fat-free vs diet-restricted, 12% fat	1	0.02	0.02	0.43	ns
2. Not refed vs refed	1	0.04	0.04	1.27	ns
3. Refed, fat-free vs refed, 12% fat	1	0.17	0.17	5.13	s
4. Pre-refeeding	2	0.16	0.08	2.40	ns
5. Time	3	0.12	0.04	1.19	ns
6. Interactions					
Pre-refeeding x refeeding	2	0.43	0.22	6.38	s
Starved and refed vs diet-restricted and refed	1	0.42	0.42	12.55	s
Diet-restricted, fat-free, and refed vs diet-restricted, 12% fat, and refed	1	0.01	0.01	.18	ns
Pre-refeeding x time	6	0.38	0.06	1.86	ns
Refeeding x time	3	0.05	0.02	0.48	ns
Pre-refeeding x refeeding x time	6	0.05	0.01	0.27	ns
Chi-square value =		44.797			
Chi-square critical (28, P< 0.05) =		41.340			

[1] significant

[2] not significant

APPENDIX TABLE IV

Analysis of variance for malic enzyme activity in epididymal adipose tissue following log transformation of the original data

Source of Variation	Degrees of Freedom	Sums of Squares	Mean Square	F Value	Significance P < 0.05
Total	91	57.87	0.64		
Within treatment	63	16.01	0.25		
Between treatment					
1. Controls					
Ad libitum-fed vs others	1	0.001	0.001	0.003	ns[2]
Ad libitum-fed, fat free vs ad libitum-fed, 12% fat	1	0.159	0.159	0.628	ns
Starved vs diet-restricted	1	0.003	0.003	0.010	ns
Diet-restricted, fat-free vs diet-restricted, 12% fat	1	0.004	0.004	0.014	ns
2. Not refed vs refed	1	21.656	21.656	85.226	s[1]
3. Refed, fat-free vs refed, 12% fat	1	2.639	2.639	10.385	s
4. Pre-refeeding	2	1.123	0.561	2.209	ns
5. Time	3	11.237	3.746	14.740	s
6. Interactions					
Pre-refeeding x refeeding	2	0.748	0.374	1.473	ns
Pre-refeeding x time	6	1.342	0.224	0.880	ns
Refeeding x time	3	1.227	0.409	1.610	ns
Pre-refeeding x refeeding x time	6	1.451	0.242	0.952	ns
Chi-square value =		43.22			
Chi-square critical (28, P < 0.05) =		41.34			

[1] significant

[2] not significant

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