PLASMA GLUCOSE, INSULIN AND NONESTERIFIED FATTY ACIDS OF MALE OSBORNE MENDEL RATS FED A HIGH OR LOW FAT DIET

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ABSTRACT

PLASMA GLUCOSE, INSULIN AND NONESTERIFIED FATTY ACIDS OF MALE OSBORNE MENDEL RATS FED A HIGH OR LOW FAT DIET

By

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Male Osborne Mendel rats were fed from weaning either a high fat diet (44% Crisco, w/w) or a high carbohydrate diet (69% cerelose, w/w) up through 20 weeks of age. At that time, all rats were fasted for 18 hours prior to an oral glucose tolerance test. Using the cardiac puncture technique, blood samples were obtained at fasting, 30, 60, 120 and 180 minutes from the time of the ingestion of the glucose solution for analyses of plasma glucose insulin and nonesterified fatty acids.

Rats fed the high fat diet weighed 633 ± 14 gm at the end of 20 weeks, whereas rats fed the high carbohydrate diet weighed 486 ± 12 gm at the same age. These values were significantly different. The results of the glucose tolerance test indicated that rats fed the high fat diet exhibited: (1) a significantly higher plasma glucose level than rats fed the high carbohydrate diet, which was sustained over the three hours following the oral glucose load, (2) a higher, but not significantly so, fasting

insulin level than rats fed the high carbohydrate diet. This value returned to the basal level prior to the fall of plasma glucose, and (3) a lower, but not significantly so, plasma nonesterified fatty acid response in comparison to the rats fed the high carbohydrate diet. This value fell to a nadir level at one hour after the glucose load.

At the end of 20 weeks, the diets were reversed and the animals were fed for an additional five weeks; that is, the rats originally fed the high fat diet now received the high carbohydrate diet and vice versa.

Rats changed from the high fat to the high carbohydrate diet decreased their body weight by a mean weight of 36 gm at the end of the fifth week of the new regimen and showed an improvement in glucose utilization. Their plasma insulin and nonesterified fatty acid responses were improved and resembled that of the rats originally fed the high carbohydrate diet.

Rats changed from the high carbohydrate diet to the high fat diet increased their body weight by a mean weight of 138 gm over the five weeks. This group of rats now exhibited: (1) an impaired glucose utilization during the oral glucose tolerance test, (2) a higher plasma insulin response than the rats which were changed from the high fat diet to the high carbohydrate diet. The

values were statistically nonsignificant, and (3) a lower, but not significantly so, plasma nonesterified fatty acid response than the rats which were changed from the high fat diet to the high carbohydrate diet.

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A THESIS

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To my father

RICHARD S. L. CHANG

in loving memory

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INTRODUCTION

Caloric intake above caloric expenditure over prolonged periods of time is responsible for the development of obesity. Nevertheless, individuals who maintain a constant body weight are able to balance their caloric intakes and expenditures at varying energy levels; others are constantly confronted with the problem of positive energy balance. The latter has led to a great many studies in the area of obesity and its etiology which show that a variety of factors can influence it. Some of these may be of genetic origin; others may be associated with the regulation of appetite, especially when high energy diets are consumed. Psychological factors are also involved.

Nutritional obesity resulting from feeding a high fat diet occurs in many strains of rats (Mickelsen et al., 1955; Schemmel et al., 1969, 1970). This nutritional obesity is characterized by an increased caloric intake and an elevated feed efficiency (Schemmel et al., 1972).

High intakes of dietary fat have been reported to cause several metabolic disturbances in humans (Hales and Randle, 1963a) and rats (Zaragoza-Hermans and Felber, 1972). These include: (1) impaired glucose utilization

in vivo and *in vitro* with resistance to exogenous insulin (Christophe and Mayer, 1959; Blazquez and Quijada, 1968), (2) an increase of body weight and distinct hypertrophy of the pancreatic islets (Katsumata, 1970a, b), (3) a decrease of liver glucokinase activity (Vaughan *et al.*, 1960), (4) an increased oxidation of fatty acids in muscles (Hales and Randle, 1963a; Randle *et al.*, 1963), (5) hypoinsulinemia (Blazauez and Quijada, 1968) and (6) low liver glycogen content (Zaragoza and Felber, 1970).

All of the factors listed above are likely to be adaptations to the feeding of a high fat diet. Additional changes associated with feeding a high fat diet, especially where obesity occurs, are likely to include: (1) an increase in body fat (Schemmel *et al.*, 1969), (2) hypertrophy of the fat cells (Lemonnier, 1969) and (3) an increase in cell number (Salans *et al.*, 1968). These enlarged fat cells are also associated with a decrease in insulin sensitivity (Salans *et al.*, 1968).

In light of current reports and knowledge, obesity is a hazard to health and a shortener of life span (Duncan *et al.*, 1964; Wohl, 1968). Therefore, it seems important to study and further elucidate the conditions and complications involved in nutritional obesity. The primary purpose of the present study is to elucidate the effect of a glucose tolerance test on plasma glucose,

insulin and nonesterified fatty acids, when administered to fasted obese and "normal weight" Osborne Mendel male rats fed either a high or low fat diet.

REVIEW OF LITERATURE

Living organisms are endowed with a limited capacity to store carbohydrate and therefore most of them depend on the storage of fat in the adipose tissues for a major supply of energy. Recent work has shown that adipose tissue is one of the most metabolically active tissues of the body (Jeanrenaud, 1961). A constant turnover of constituent lipid components takes place when new fatty acids are synthesized and esterified into triglycerides, and when the existing stored fat is hydrolyzed and released as albumin-bound fatty acids, capable of providing energy for cells. These two functions, lipogenesis and lipolysis, are controlled by a multiplicity of nervous and complex hormonal factors (Exton et al., 1972; Saggerson and Greenbaum, 1970).

Lipogenesis and Triglyceride Synthesis

The carbon and hydrogen molecules for the synthesis of fatty acids within the fat cells come from glucose, whose entry into the cells is stimulated by the action of insulin (Stetten and Boxer, 1944). The key intermediate is actyl CoA. This reacts with carbon dioxide to form malonyl CoA. Condensation of malonyl CoA with further molecules of actyl CoA occurs, followed by

decarboxylation, reduction, dehydration and further reduction. The whole cycle continues with a two carbon unit being added in each cycle until long chain fatty acids are formed (Lehninger, 1970).

The hydrogen for the reduction comes from reduced $NADPH_2$ which is derived from the hexose monophosphate shunt pathway. The high activity of the two dehydrogenases of the shunt pathway emphasizes the capacity of the tissues for $NADPH_2$ production, which is presumably a contributing factor to the capacity of adipose tissue for the synthesis of fatty acids (Weber et al., 1960).

The adipose tissue cell is not only capable of the synthesizing of fatty acids from acetyl CoA, but also of esterifying them to produce triglycerides. The esterification occurs with α-glycerophosphate derived from cell glycolysis. This biochemical transformation is very efficient and takes place at an accelerated rate with large food intakes; especially when carbohydrate intakes are beyond the capacity for normal utilization and storage (Gordon, 1964).

Although triglycerides are the storage form of fat, this lipid fraction is also found in blood plasma in various lipoprotein forms. Under normal conditions, triglycerides are believed not to enter the circulation from adipose tissue, but rather from (1) liver where

they are being produced from fatty acids and glycerol and, (2) intestine where they are absorbed after the ingestion of a fat-containing meal. Therefore, their plasma concentration is a reflection of the liver and intestine function. Other factors such as the rate of triglyceride uptake and FFA release by the muscles and adipose tissues can also alterate the plasma triglyceride concentration.

Lipolysis and Nonesterified Fatty Acids

Lipolysis, or mobilization of fat, is constantly occurring and to a limited extent occurs concurrently with lipogenesis. It appears to be subjected to many different controls, and one of the most important of these is the availability of fuel food. During fasting or starvation, after the available carbohydrate stores have become depleted, there is a rising efflux of nonesterified fatty acids from the adipose tissues. Conversely, ingestion of food, especially carbohydrate, at any time during the fast is associated with a fall in plasma NEFA concentrations, indicating a suppression of the release of fatty acids from adipose tissues and a facilitation of the uptake and utilization of glucose. This decreased release of fatty acids from adipose tissue and the increased utilization of glucose are aided by the action of insulin. Serum insulin is elevated as a result of

the elevated blood glucose after food absorption (Dole, 1956).

Other controls for lipolysis involve hormonal agents such as ACTH, TSH, vassopressin, melanophore stimulating hormone and luteotrophin which are all active in mobilizing fat from storage (Harper, 1971). Most powerful of all are the catecholamines, epinephrine, norepinephrine and growth hormone (Rudman et al., 1963).

Free fatty acids which are released from the cell are bound to plasma albumin and transported throughout the circulatory system. Since the human adipose tissue cell lacks the enzyme glycerolkinase to restore a high energy phosphate bond, the glycerol portion is not reutilized for triglyceride synthesis. This glycerol portion of the triglyceride is then transported to the liver where it is converted to glucose and glycogen (Steinberg et al., 1961). Glycerolkinase is present in the adipose tissue of some animal species (Treble and Mayer, 1963). Frederickson and Gordon (1958) have written that the FFA content of the blood plasma is derived almost solely from adipose tissue, although a small and insignificant amount may arise from chylomicrons following a fat-containing meal.

Physiological and Metabolical Differences between Normal and Obese Human Individuals

The obese individual is obviously different from the normal individual. He is noted for a configurational change, which is mainly a concern of vanity. Of greater importance is the alteration in the size and number of fat cells, and the functioning and metabolism of these fat cells. Abnormalities in lipid, carbohydrate and hormone metabolism in obese humans are well documented (Dole, 1956; Ogilvie, 1935; Yarlow et al., 1965).

Abnormalities in Lipid Metabolism

In "normal weight" adult humans, the mean early morning plasma FFA concentration is about 450 to 500 μeq per liter, and upon fasting, the plasma FFA level may increase to 1500 μeq per liter (Gordon, 1964). The ingestion of glucose results in a decrease in plasma FFA, which returns to the initial level or, more often, to a higher level after blood glucose becomes normal (Bonnet et al., 1970). In humans who are obese, the nonfasting plasma FFA is significantly elevated when compared to "normal weight" counterparts (Dole, 1956). It has further been observed that prolonging the fasting of obese subjects often does not result in a progressive elevation in plasma FFA as it does in "normal weight" adults (Corvilain et al., 1961; Opie and Walfish, 1963).

Measurement of plasma FFA turnover by Issekutz et al. (1967) showed that the obese subjects had a higher total turnover than did the lean. In addition, both lean and obese subjects oxidized about 33% of the total FFA turnover. This would indicate that the obese individuals do not have a diminished capability to mobilize and utilize their fat stores, but instead have a greater total fat oxidation.

If fat utilization is not diminished in the obese, one may hypothesize that fat synthesis is disproportionately increased. The observed high plasma level of insulin in the obese individuals (Yarlow et al., 1965) seems to support the suggestion, since insulin enhances glucose entry into the fat cell for the synthesis of triglycerides. In contrast, Golderick and Hirsch (1964) have shown the lack of difference in lipogenesis between obese and nonobese adipose tissue biopsy samples. In short, there is a lack of conclusive data on the relative rates of fat formation in obese compared to "normal weight" individuals.

Abnormalities in Glucose Metabolism

Although fasting blood glucose levels are usually normal in obese subjects, their glucose responses are frequently elevated during a glucose tolerance test. Such

intolerances resemble those of diabetic subjects (Morse et al., 1960; Newburgh and Conn, 1939).

A report by Sims and Horton (1968) has shown that impaired glucose metabolism is associated with both the severity and duration of the obesity, and can frequently be corrected with weight reduction. They concluded, therefore, that such defective glucose metabolism was the result of human obesity.

Abnormalities in glucose metabolism have also been observed in humans fed a low carbohydrate diet (5%, w/w) with no restriction on fat or protein intake (Hales and Randle, 1963a). They reported that such a dietary restriction of carbohydrate in humans had led to a reduction of glucose tolerance with increased concentrations of glucose, NEFA and insulin in the plasma. It was suggested that normal people on such a high fat, low carbohydrate diet showed antagonism not only to the hypoglycemic action of insulin but also to the important action of insulin in suppressing the release of NEFA. This antipolytic action of insulin had been confirmed by Dole (1956).

Randle et al. (1963) later hypothesized the "Glucose-Fatty Acid Cycle" to explain such glucose abnormality and its mechanism. They suggested that excessive lipolysis of triglycerides in fat stores leading to an elevated plasma FFA level could impose a direct inhibition

on the membrane transport of glucose and other key glycolytic enzymes, like hexokinase and phosphofructokinase. They also emphasized the reciprocal relationship between the metabolism of glucose and fatty acids in muscles and adipose tissues, indicating that not only did glucose utilization affect the fat metabolism, but that fat utilization could also profoundly assert an effect on glucose and insulin metabolism.

Direct experimental verification of the effect of the plasma FFA on glucose metabolism as proposed by Randle et al. (1963) has been demonstrated by others. In 1969, Waterhous et al. confirmed the fact that glucose could inhibit fatty acid oxidation. Evidence was also brought forth indicating a paradoxical inhibition of fatty acid esterification as induced by glucose feeding. The infusion of noradrenaline by Nestle et al. (1964) to produce a three- to five-fold increment in plasma FFA led to a mean increase of 10% in blood glucose concentration. Similar results showing the effect of high plasma FFA on the abnormality of glucose metabolism were also reached by Schalch and Kipnis (1965).

Pelkonen et al. (1968) could not observe the rise in blood glucose in their test subjects whose plasma FFA level was increased by a fat meal ingested after a nine hour fast, and into whom intravenous heparin had been administered 15 minutes before the start of the

glucose injection. The reduction of plasma FFA level by nicotinic acid in obese human subjects also did not improve the utilization of glucose (Butturini et al., 1966).

Other in vitro studies (Herrera and Cahill, 1965; Kruger et al., 1964) showed a heightened hepatic gluconeogenesis as a result of increased perfusate concentration of FFA. Seyffert and Madison (1967), however, showed that the acute elevation of plasma free fatty acids produced a 37% decrease in the hepatic output of glucose and a 30% inhibition of peripheral glucose utilization. This decrease in hepatic glucose output was the consequence both of a significant increase in the secretion of insulin and a decrease in glucagon secretion. These data suggested that there was a feedback of FFA on the secretion of insulin and glucagon, which played an important role during starvation in providing a safe transition from carbohydrate to fat metabolism without the risk of progressive ketoacidosis. Therefore, they concluded that it was likely that during starvation, FFA could contribute to the control and maintenance of a low but vital concentration of plasma insulin.

Borrebaek and Spydevold (1970) reported a decreased hexokinase activity in the rat epididymal fat pad caused by a high level of free fatty acids. Weber

et al. (1969) observed further that long and short chain fatty acids selectively inhibited the key enzymes of glucose catabolism, such as glucokinase, hexokinase, phosphofructokinase and pyruvate kinase. Others like Bortz and Lynen (1969) indicated that it was not the FFA per se, but rather the fatty acid acyl CoA derivatives that were directly responsible for the changes in enzyme activities, thus resulting in the alteration in glucose-lipid metabolism.

Abnormalities in Insulin Metabolism

Numerous reports attest to the elevated plasma insulin levels both as immunoactive insulin (IRI) and as insulin-like activity (ILA) in obese individuals (Yarlow et al., 1965; Karam et al., 1965). This has been shown to correlate both with the degree and duration of the obesity (Merimee, 1971). The obese individual is more resistant in vivo to the effects of exogenous insulin (Rabinowitz and Zierler, 1962) and his adipose tissue is believed to be more irresponsive to this hormone in an in vitro study (Salans et al., 1968).

Other reported endocrine changes may involve: glucagon, growth hormone, fat mobilizing hormone, thyroid hormone and ACTH.

Metabolic Abnormalities in Rats Fed a High Fat Diet

Rats fed a high fat ration (60% Crisco, w/w) throughout their lives become extremely obese compared to "normal weight" controls fed a grain diet (Mickelsen et al., 1955; Schemmel, et al., 1959, 1970). The high fat fed rats are more efficient in converting feed energy to body fat than are the controls (Schemmel et al., 1972). Both male and female rats fed a high fat ration have larger fat deposits (Schemmel et al., 1970) than rats fed a low fat ration.

Whitney and Roberts (1955) reported that adult male Sprague Dawley rats fed a diet high in fat (85% Mazola corn oil in lactalbumin) for two to three months, showed a decreased incorporation of acetate into fatty acids and liver glycogen when compared to those rats fed a chow type ration. On the other hand, incorporation of acetate into cholesterol was stimulated when rats were previously fed a high fat diet. Blazquez and Quijada (1968) confirmed that the intake of a high fat diet (vegetable oil, 45%; sucrose, 29%; vitamin-free casein, 18%) in male rats of the Wistar strain resulted in alterations in glucose and insulin metabolism. Their in vitro study of muscles and adipose tissue showed a

decreased insulin sensitivity while the plasma glucose remained high.

Zaragoza and Felber (1970) studied the early effect of a high fat diet (35.5%, w/w; 67%, cal/cal) on six to eight week old male Wistar rats. The mean caloric intake, when related to 100 gm of body weight, decreased gradually during the growth of the animals. Serum glucose in both fed and 24 hour fasted rats was about 10% higher in the high fat fed group as compared to the control group. In the fed state, high fat fed rats also exhibited a higher plasma FFA and a lower plasma insulin level. However, after 24 hours of fasting, these rats had a significantly lower plasma FFA level than the control rats which were fed a low fat diet (2%, w/w; 5%, cal/cal). They proposed two processes which might contribute to maintain the high serum glucose concentration: (1) enhanced hepatic production and (2) decreased peripheral utilization of glucose.

Katsumata (1970a, b) studied the long-term metabolic effect of the high fat diet (48%, w/w) on male rats of the Wistar strain. These rats fed the high fat diet for 400 days had greater increases in body weight compared to the lean controls fed a low fat diet (10%, w/w), and thereafter remained obese. Glucose tolerance and plasma insulin sensitivity curves demonstrated significant impairment in carbohydrate metabolism. In vitro

study with adipose tissue showed: (1) insensitivity to exogenous insulin and (2) decreased glucose utilization. There was also an increased gluconeogenesis in the liver of the high fat fed group. The pancreatic insulin level was about the same in both groups of rats, although the high fat fed rats showed distinct hypertrophy in the Langerhan Islets. When feeding the rats with a diet high in protein (62%, w/w), Katsumata was unable to observe any abnormalities in carbohydrate or lipid metabolism.

Malaisse et al. (1969) and Blazquez et al. (1971) reported a decreased insulin release by the pancreas in rats fed high fat diet. To explain the hyperglycemic state in rats fed a high fat diet, Zaragoza-Hermans and Felber (1972) recently have investigated and found that a high fat diet has no effect on the intestinal glucose absorption rate. The rate of disappearance of an orally ingested glucose load was similar in both the high fat fed rats and the lean controls. Therefore, the high level of exogenous glucose in the serum of the high fat fed rats was explained as the result of decreased sensitivity of the body tissues to exogenous insulin.

Further in vitro study by Bringolf et al. (1972), using rats' diaphragms, showed that there were reductions in glycogen synthesis from glucose, diminished glycogen storage under fed state and impaired glycogen utilization

during fasting in fat adapted rats. The concentration of glucose-6-phosphate and glycerides was increased. An inhibition of pyruvate dehydrogenase was also reported. Mayes (1960), using rats' diaphragms, also found a similar decrease in glycogen storage.

Bringolf et al. (1972) proposed that the impairment of glucose of high fat fed rats occurred chiefly in glycolysis and in pyruvate oxidation. This was the result of an increased oxidation of fatty acids rather than total enzymatic adaption, since the incorporation of glucose carbon into CO_2 and fatty acids was severely reduced in fat fed rats. The lactate released was found to be doubled.

In short, glucose, lipid and insulin abnormalities in rats fed a high fat diet resemble closely those of human obesity and diabetes mellitus. Whether the mechanism for such development is similar in all these groups demands much further investigation.

Methodology of Plasma Glucose, NEFA and Insulin Determination

Plasma Glucose Determination

Muller (1928) showed that B-glucose oxidase oxidized glucose to gluconic acid. Later, Keston (1956) employed the simultaneous use of two enzymes--glucose oxidase and peroxidase; this resulted in a great advance toward the determination of glucose. In this system,

glucose oxidase catalyzed the oxidation of glucose, by molecular oxygen, to gluconic acid and hydrogen peroxide. The peroxide formed in the presence of peroxidase and a chromagenic hydrogen donor such as O-dianisidine formed a product that was stoichimetrically related to the amount of original glucose present.

Teller (1956) and McComb et al. (1957) showed that glucose oxidase could not be used for the analysis of D-glucose in the presence of 2-deoxy-d-glucose since this latter sugar was also oxidized by glucose oxidase. Saifer and Gerstenfeld (1958), using glucostat reagent containing glucose oxidase, horseradish peroxidase, phosphate buffer and O-dianisidine, found that this method could not be applied directly to serum or plasma due to the presence of substances inhibitory to the enzyme. The reagent was only usable when the protein portion was first removed by protein precipitation.

"Glucostat," the commercially prepared reagents using this system by Worthington Biochemical Co., Freehold, N.J., contains two vials. One has the hydrolytic enzyme and 0.005 M phosphate buffer, pH 7, and a second vial contains the solid chromogenic O_2 acceptor, O-dianisidine. The reaction is as follows:

$$\text{Glucose} + O_2 + H_2O \xrightarrow[\text{glucose oxidase}]{} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{O-dianisidine} \xrightarrow[\text{peroxidase}]{} \text{Chromogen (amber color)}$$

Since proteins do interfere with the reaction of glucostat reagents, they are precipitated with Somogy zinc and barium reagents prior to the use of glucose oxidase (Glucostat reference).

Plasma NEFA Determination

Dole's (1956) titrimetric method for the analysis of plasma FFA level has been in wide use. Later, this method was modified by Ko and Royer (1967) to improve its specificity and sensitivity.

The modified method by Ko and Royer (1967) changes the water: heptane mixture from 28:36 as used by Dole (1956) to 46:18. This increase of the water portion results in a better recovery of palmitic acid and a reduction of interferences from polar acids.

Further, in the modified method, tetrabutylammonium hydroxide is used. This reagent is more soluble in organic solvents than are the corresponding sodium salts used in Dole's method. Therefore, difficulties due to precipitation are avoided.

Thymolphthalein (0.01%) dissolved in heptane/acetone (10/1, v/v) is the optimal indicator. There are two reasons for this: (1) the end point of the reaction is reached when 97% of the palmitic acid has been titrated, and (2) it turns from colorless to blue at the end point, which is easily detected. In contrast, Dole (1956) used thymol blue as an indicator which turned from yellow to

green and finally to blue at the end point; therefore, it was difficult for one to detect and decide when the end point was reached.

Finally, the decreased volume of heptane in the modified method reduced the blank potentiometric titration value and also sharpened the end point. Positive interferences of lactate, B-hydroxybutyric and pyruvic acids in plasma are only about 0.4 to 0.6% while under Dole's condition, the interference is three to four times higher.

Dole (1956) reported that extending the time period between the titration and extraction stage brought no significant changes in the titratable acidity of the heptane phase, but prolonged evaporation of the solvent before titration could remove most or all of the volatile short chain fatty acids. Also, incubation of plasma at 37°C before extraction could increase the extractable acidity, presumably by enzymatic hydrolysis of esterified fatty acids. Heparin, the most commonly used anticoagulant, did not affect the result of plasma NEFA determination.

Plasma Insulin Determination

The radioimmunoassay method for determining plasma insulin was developed by Yarlow and Berson (1960) and contributes a great advance in assay methods, not only for insulin but for other hormones in plasma as well. The method has brought much needed specificity and sensitivity in the assay of plasma insulin.

Various modifications of the original method are in current use. The Hales and Randle (1963b) modified method employs competition between 125-I labeled insulin and the unlabeled insulin in the unknown sample for binding sites on anti-insulin globulin, usually produced by immunizing guinea pigs against insulin. The antibody bound insulin, and the amount of unlabeled insulin, are determined by comparison with a standard curve which relates the ratio of antibody bound to free insulin to known hormone concentrations.

During the procedure, a fixed amount of labeled insulin is added to a limited amount of antibody. The larger the quantity of unlabeled insulin in the sample to be assayed, the smaller will be the quantity of labeled insulin bound to antibody, which is assumed to have the same affinity for both insulins.

This immunoassay method offers other advantages in addition to its high degree of specificity and sensitivity. It enables the assays of much larger numbers of samples than do the bioassay methods. It only requires 0.1 ml of plasma for each assay, and does not suffer from the complication of dilution effects. Endogenous insulin concentrations decrease proportionately upon dilution of plasma over the range of 1:2 to 1:100 (Rieser, 1967).

"Insulin Kit," the commercially prepared reagents using Hale's system by Amersham/Searle Corporation,

Arlington Heights, Illinois, contains the antibody insulin binding reagent, human insulin standards, insulin I-125 (100 nanogram, specific activity of 50 μCi/μg) and membrane filters.

According to the instructions, the precipitation of the insulin antibody complex is incubated with standard or unknown solutions of unlabeled insulin for six hours prior to the addition of I-125 labeled insulin. This method increases the sensitivity of the assay because alpha globulin in plasma will not be introduced until after the precipitation of insulin antibody complex and consequently cannot interfere by cross-reacting with the precipitated antiserum.

METHOD

Animals and Diets

Sixty-six weaning male littermate Osborne Mendel rats were weighed and assigned to be fed a high fat (44% fat, w/w) or high carbohydrate (3% fat, w/w) ration in a manner which equalized body weights at the start of the experiment. The two rations were designed so that about 30% of the kilocalories were derived from protein in each ration with the remainder of the kilocalories derived primarily from fat (Crisco) or carbohydrate (cerelose). The exact composition of the rations, as well as the energy content, are given in Table 1.

All animals were housed individually in wire screen cages (18 x 18 x 25 cm) in a laboratory with a controlled temperature of 23° ± 1°C throughout the study. Their movement was neither limited nor forced. Once every week, the animals, their feed cups and food spills were weighed, and the feed consumption values for each rat were corrected for the spillage. The rats were fed their specific diets and had free access to water throughout the experimental period of 20 weeks.

Although 33 rats fed the carbohydrate diet were used at the beginning of the study, only 20 were used in the final analysis. The others were eliminated on the

basis of death or illness. Similarly, for rats fed a high fat diet, 18 were used for the final data collection. Half of the animals fed either one of the diets were used to evaluate plasma glucose and insulin, and the other half were used to determine plasma glucose and free fatty acids under similar dietary glucose tolerance tests.

This procedure was necessary in order to provide sufficient blood for sampling. Details of the experimental design are presented in Table 2.

Procedure for Glucose Tolerance Test and Blood Collection

Anesthesia

All animals were fasted overnight for 18 hours preceding the oral glucose tolerance test and were then anesthetized by a subcutaneous injection of nembutal sodium solution (Abbott Co., Chicago, Ill.) to the neck region that had been washed with disinfectant, zephiran chloride 17% (Winthrop Lab., N.Y.). The nembutal sodium solution was injected slowly into the body in order to prevent adverse respiratory and circulatory reactions, since the drug exerts a direct depressant action on the total central nervous system besides providing the sedative hypnotic action typical of all the barbiturates. The usual dosage given was 6.20 mg per 100 gm of body weight.

An initial blood sample was collected by cardiac puncture using a 26 gauge, 1/2" heparinized disposable

syringe (Sargent Co., Detroit, Mich.) at about 40 to 50 min. after the injection of nembutal sodium solution. The small needle was used in order to keep the tissue damage of the heart to a minimum. Blood was drawn very slowly into the syringe to prevent the possible mass hemolysis of the erythrocytes which might introduce an error in plasma glucose, insulin and NEFA level.

Oral Glucose Tolerance Test

A glucose solution containing 250 mg/ml was prepared from anhydrous D-glucose (Mallinckrodt Chem. Co.) and 1/2 ml was given to each rat for every 100 gm of body weight. This meant that rats fed the high fat ration received about 750 mg glucose, and those fed the high carbohydrate ration received around 625 mg glucose. The predetermined glucose solution was delivered to each by means of a syringe connected to a polyethelene tube approximately 15 cm long and 3 mm in outside diameter. While holding the rat in one hand, the tube was inserted quickly back over the tongue and downward until a certain length had been passed which might be expected to reach the stomach. The glucose solution was dispensed and the tube was withdrawn at once to avoid interference with respiration.

The blood samples were delivered to the glass blood tubes containing 4 drops of heparin (Fisher Scientific). These were kept cold in a pan of ice.

More blood samples were obtained from each rat at 1/2, 1, 2, and 3 hours after the administration of glucose solution for analysis of glucose and insulin. Similarly, the blood samples were obtained from one rat at fasting and 1 hour after the glucose administration and from another rat, treated with the same diet, at 1/2 and 3 hours after the glucose load for analysis of glucose and free fatty acids.

A small portion of each blood sample (about 0.1 ml) was used for the determination of hematocrit for each rat. Microcapillary tubes (heparinized) were filled with blood and sealed at one end with plasticine. These were placed in the slots of the revolving plate with the unsealed ends towards the center and centrifuged using the microhematocrit high speed centrifuge (International Micro-Capillary Centrifuge, Model MB) at 11,500 rpm and 13,000 g for 7 min. Hematocrit values were used to detect whether or not internal hemorrhage had occurred as the result of frequent cardiac puncture. Values were determined in duplicate for each of the blood samples and an average of these two readings represented the hematocrit for each rat.

The blood samples in the glass blood tubes were centrifuged using the clinical centrifuge (International Clinical Centrifuge, Model 0843, International Equipment Co., Mass.) for 10 min. Plasma was then divided into

two aliquots--one for glucose determination and the other for insulin or free fatty acid analysis. These were frozen as quickly as possible and kept in the frozen state until analyzed.

After this test, rats were changed from a high fat to a high carbohydrate diet and vice versa for five weeks. Similar test procedures were conducted to evaluate changes. All rats then were sacrificed with an overdose of ether. Immediately thereafter, the carcasses were dissected. Rats with hemorrhage in their chest cavity were not used as part of the test subjects in the experiment.

Plasma Glucose Determination

The amount of circulating glucose was measured by the "Glucostat" glucose-oxidase method (Teller, 1956). A Beckman Model DB spectrophotometer was used. The procedure outlined was applicable without change to the Bausch and Lomb Spectronic 20 and both instruments were used. The reagents included deproteinizing solutions (1.8% barium hydroxide and 2% zinc sulfate solution), standard glucose solutions, 4N hydrochloric acid and glucostat reagents (Worthington Biochemical Co., Freehold, N.J.).

A series of clean 15 ml centrifuge test tubes (Sargent Co., Detroit, Mich.), each containing 1.9 ml distilled water, was set up, with two tubes for each unknown, reagent blank and each glucose standard.

To each appropriate test tube was added 0.1 ml of plasma, glucose standard or distilled water, 1 ml of barium hydroxide solution and 1 ml of zinc sulfate solution, with mixing after each addition (Vortex mixer, Scientific Industries, Inc., Springfield, Mass.) The samples were centrifuged with a Clinical Centrifuge (International Equipment Co., Boston, Mass.) for 20 min.

An amount of 2 ml supernate from each tube was pipetted into the respective tube. At the timed interval, 2.0 ml glucostat reagent was added to each tube, mixed and let stand at room temperature for 10 min. The solutions exhibited a range of amber color relating to the amount of original glucose present. Test tubes containing blank or distilled water were colorless. At the same time intervals, one drop of 4N hydrochloric acid was added and mixed. After the color had been developed, about 5 min., all samples were read for absorbance in the Beckman Spectrophotometer (Beckman Co., Model DB) at 420 mλ using a 1 cm Beckman cuvette against the cuvette with reagent blank. The photometer was allowed to warm up into working capacity about one hour before using and was set to zero absorbance on the scale with reagent blank prior to all other sample reading.

After the absorbance of each standard was plotted against the concentration on linear graph paper, the

concentration of plasma glucose in the unknown samples could be read directly or calculated from the standard curve as follows:

$$\frac{\text{Absorbance of unknown} \times \text{Concentration of known standard}}{\text{Absorbance of known glucose standard}}$$

Whenever the amount of glucose present in the sample mixture exceeded the highest known standard value, the whole assay procedure was repeated with a more diluted sample of plasma.

Details of plasma glucose determination are presented in Appendix I.

Plasma Insulin Determination

The plasma insulin level during the glucose tolerance test was determined by the modified radioimmunoassay method originally described by Hales and Randle (1963b). The "Insulin Kit" containing insulin binding reagent, bovine insulin, I-125 labeled insulin and filter discs, was supplied by the Radiochemical Center/Amersham (Arlington Heights, Ill.). This was kept frozen upon receiving and used within a two month period.

A Packard beta liquid scintillation counter was used. The reagents included deionized water, phosphate buffer A, isotonic buffer B-1, buffer C-1, scintillant, insulin standard and I-125 labeled insulin. (See Appendix III.)

In this procedure, all clean small glass tubes (75 x 10 mm) were first soaked in a silicad solution (one part silicad to 100 parts of deionized water) about 3 min., then rinsed in deionized water and air dried before use. All disposable pipettes and syringes were rinsed immediately before use with buffer A or buffer B as appropriate, and then washed out with solutions to be used.

A series of glass tubes was set up in a pan filled with ice, with at least three tubes for the determination of each standard, unknown, zero (with no unlabeled insulin) and washing blank as controls of the washing procedure.

The insulin binding reagent was reconstituted immediately before use by adding 8 ml deionized water to one bottle of reagent; 0.1 ml of this reagent was dispensed into each tube except those for the washing blank to which 0.1 ml of buffer A and B were added. The same microsyringe was used throughout the experiment. It was rinsed carefully with each new solution before dispensing. The same amount (0.1 ml) of buffer B was dispensed to those tubes labeled zero and 0.1 ml of plasma unknowns or standards was delivered to the respective tubes.

From the preliminary test, plasma insulin was found to exceed that of the highest standard value and a dilution with buffer B was necessary. Thus, unknowns in this experiment were diluted with a known amount of

buffer B and 0.1 ml of the mixture was then taken for the radioimmunoassay of insulin.

The contents of each tube were mixed, corked and refrigerated at 2° to 4°C for six hours. The contents of each tube were again mixed well afterwards and 0.1 ml of working solution of insulin I-125 was added to each tube. At this stage, the total radioactivity added to each tube was measured by dispensing 0.1 ml of insulin I-125 to a scintillation vial containing approximately 10 ml of liquid scintillant. These were returned to the refrigerator for 18 hours.

After this period of incubation, the precipitates in the tubes were collected by micro filtration through a membrane filter disc mounted on a microanalysis filter holder on a vacuum filtration flask. The filter discs were floated one at a time on the surface of a suitable quantity of buffer C contained in a beaker. Within 10 to 20 seconds, all filters were soaked with buffer C; they were kept in the buffer solution until required. The test tubes and buffer C and soaked filters were kept cold throughout the experiment in a pan of crushed ice. They were not allowed to be warmed up to room temperature during this filtration stage.

The filter holder (Millipore Co., Bedford, Mass.) was rinsed with buffer C and then mounted on the vacuum filtration flask with the disc assembled in place. Details of the apparatus set up are shown in Appendix II.

The contents of each tube were mixed as before then delivered onto the filter using a disposable pipette (Sargent Co., Mich.). The filter was washed with two successive portions (approximately 1 ml each) of ice cold buffer C. It was convenient to use one pipette for transferring buffer C to each precipitin tube and another for transferring the assay samples and washing solution from the precipitin tubes to the filter.

When the filtration of a sample was completed, and while the filter was still under vacuum, the glass reservoir was removed from the filter. The filter disc was lifted off with the aid of a scalpel blade and forceps. It was transferred to a respective glass counting vial, and dried for 10 min. in an oven at 120°C. After cooling to room temperature, the filter was freed from the surface of the vial and 10 ml of scintillant were added to each counting vial.

The filter discs for each standard, unknown, zero and washing blank in the immunoassay, the clean filter for background count rate measurement and unfiltered samples of insulin I-125 for the total count rate measurement were counted by Packard beta liquid scintillation counter for 10 min., using the following settings:

	A - B Red Channel	C - D Green Channel	E - F Blue Channel
Gain	8.7	8.7	2.0
Window	50 1,000	120 800	300 1,000

All counts were calculated to counts per minute. The average of three readings represented the count for each assay. The background counts were subtracted from each sample assay and standard. Any count rates which were grossly aberrant from each series of replicates were rejected. Washing procedure was considered as satisfactory when the controls showed not more than 5% of the total count. After the counts per minute of each insulin standard were plotted against the known concentration, and for the "zero," on a semilogarithm graph paper, the concentration of insulin in each of the unknowns, expressed in terms of the particular species of insulin used as a standard, could then be read directly from the graph.

Details of the reagent preparation and the summary of the procedure are presented in Appendix III.

Plasma NEFA Determination

Plasma FFA was estimated by the titrimetric method of Ko and Royer (1967), as modified by Dr. Romsos (personal communication).

A microburet (Micro Metric Instrument Co., Cleveland, Ohio) equipped with a gas tight syringe was used in the present study. Reagents used included indicator solutions, the extraction mixture, TBAH titrant and a palmitic acid standard.

A series of clean test tubes was set up, with at least three tubes for the determination of the sample, standard, and blank (containing no lipids), but additional replicates would be necessary for increased precision.

An automatic dispenser (Schwartz and Mann Co.) was used to deliver 0.5 ml of sample, standard or heptane (for blank) to the respective test tubes containing 3 ml of extraction mixture, followed by 1 ml of heptane and 2.5 ml of distilled water, with vigorous mixing after each addition on a Vortex mixer. The contents in each tube separated rapidly into two phases forming a sharp interface without centrifugation. One ml of the upper phase, mostly heptane and lipids, was transferred to a small glass tube containing 0.1 ml indicator solution, mixed and titrated with TBAH titrant from the microburet syringe.

Nitrogen gas (National Cylinder Gas Co.) was bubbled into the bottom of the tube with a fine glass pipette to expel the carbon dioxide from the sample and keep the two solutions mixed during the titration process. The gas stream was interrupted from time to time

for the examination of the approach of a light blue end point.

Good lighting, given by a fluroescent light above and a white background on the working bench, were helpful in reading the end point.

An average of the three readings represented the amount of titrant used for each sample, standard and blank. Any values which grossly deviated from the series of replicates were rejected. The value for the blank was subtracted from each sample and standard assay. When the titrant used for each standard was plotted against the known concentration on a linear graph paper, the amount of plasma NEFA in the unknown could be read directly or calculated from the standard curve as follows:

$$\frac{\text{Titrant value of unknown} \times \text{Concentration of known standard}}{\text{Titrant units of known palmitic acid standard}} \ \mu\text{eq/litre}$$

As in all chemical analysis procedures, the whole assay was replaced for any sample whose value exceeded that of the highest standard value. Plasma was diluted with a known proportion of distilled water and rechecked for NEFA level.

The details of reagent preparations and a summary of the procedure are given in Appendix IV.

STATISTICAL ANALYSES

Means and standard errors were calculated for weekly food intake (gm and kilocalorie), body weight, and plasma glucose, insulin and NEFA levels during the oral glucose tolerance test. Significant differences between means for the high fat and high carbohydrate fed rats were determined by the student's T test (Sokal and Rohlf, 1969).

RESULTS AND DISCUSSION

Food Intake--Before Diet Change

Immediately after weaning, all of the rats consumed a large quantity of food within the first six weeks, irrespective of their particular diet. Toward the latter eleven weeks, their food intake continued to increase but at a smaller degree (Table 3, Figure 1).

The rats consumed significantly more grams ($P < 0.001$) of the carbohydrate diet than the high fat diet throughout the first part of the study. This difference was clearly associated with the greater weight density of the high carbohydrate diet (Table 1). However, when the data were expressed on an energy basis (Kcal consumed/week; Table 4, Figure 2), significant differences occurred in only 7 of the 17 weeks ($P < 0.1$).

Food intake, as measured in grams or kilocalories, was greatest during the first 6 weeks after weaning and continued to increase gradually during the remaining 11 weeks. These food intake data were in agreement with that reported by Schemmel *et al.* (1970).

Food Intake--After Diet Change

Immediately after changing from a high fat to a high carbohydrate diet or vice versa, all of the rats went

through a period whereby their food intakes were greatly and significantly reduced (Table 3, Figure 1). More specifically, during the first week of the new diet regimen, the 21-week-old male OM rats which were being changed from high fat to high carbohydrate diet ate 8 gm per day. By contrast, the 20-week-old male rats fed the high carbohydrate diet throughout their lives consumed about 22 gm per day. Rats consistently exposed to a calorically dense high fat diet have, no doubt, a smaller stomach capacity and this plus other unknown factors could account for the small intake of the high carbohydrate diet during the first week of diet change. By the end of the fifth week, the feed intake of this group had increased greatly. However, the amount they consumed was still significantly less than that of the 20-week-old rats which were fed this high carbohydrate diet since weaning ($P < 0.01$).

The rats being changed from the high carbohydrate to the high fat diet also had a drop in food intake on the first week of the new diet. They consumed about 15 gm per day. By contrast, rats fed the high fat diet since weaning would have consumed about 19 gm per day at 20 weeks of age ($P < 0.001$). The high calories, the lack of flavor and the greasy texture of the high fat diet might have contributed to this decrease of food intake in this group of rats. Later, toward the end of the

fifth week, their food intake increased to exceed that of the high fat fed rats at 20 weeks of age. This difference was not significant.

Before the diet change, the group fed the high carbohydrate diet consumed significantly ($P < .001$) more food on a weight basis than the rats fed the high fat diet. However, after the diet change, the groups fed the high fat diet consumed slightly more than the other group. This could be accounted for by the fact that rats which were consistently exposed to a calorically light, high carbohydrate diet had, no doubt, a slightly larger stomach capacity. Therefore, this possibly explains the slightly increased intake of the high fat diet during the first week of the diet change in this group of rats.

The kilocalorie intake after the diet change was significantly higher in the group being changed to a high fat diet ($P < 0.001$). This was in contrast to the kilocalorie intake pattern during the period before the diet change whereby the high fat fed rats consumed about the same as the high carbohydrate fed rats (Table 4, Figure 2). The difference could be attributed to the reason mentioned above.

Body Weight--Before Diet Change

The body weight of the weanling rats in both diet groups was exactly the same--54 gm. After the start of the dietary treatment, both groups gained markedly and

very steadily during the first six weeks and continued to increase in body weight at a slower rate as they grew older (Table 5, Figure 3).

The high fat fed rats always were heavier than the high carbohydrate fed rats. This difference in body weight became more and more significant toward the end of 20 weeks ($P < 0.1$ at first two weeks, $P < 0.01$ at next three weeks, $P < 0.001$ from then on). The body weight of the high fat fed rats was 633 ± 14 gm, while the body weight of the high carbohydrate fed rats was 486 ± 12 gm, at 20 weeks of age. Schemmel et al. (1972) has concluded that in these high fat fed animals, the energy of the diet could be readily transferred into body fat tissue with a minimum of energy expended by the animals. Normally the deposition of fat is thought to proceed from the lipogenesis of glucose, an insulin requiring process.

Body Weight--After Diet Change

Rats changed from the high fat to the high carbohydrate diet lost 33 gm of body weight ($P < 0.05$) in the first week and 14 gm in the second week. After that, the change in body weight was slight in either direction. The body weight at the end of the fifth week of the new regimen, however, was significantly lower ($P < 0.05$) than before these rats were changed from the high fat to the low fat diet. The primary factor for such a weight loss

was the reduction of the total calorie intake in these animals during the period of the new regimen.

Rats, being changed from the high carbohydrate to the high fat diet, rapidly gained weight. More specifically, the gain was 29%. By the end of the fifth week of the new regimen, their body weight was significantly heavier ($P < 0.001$). This gain in body weight could be explained by the increase in total kilocalorie intake and the higher feed efficiency of the high fat ration (Schemmel et al., 1972).

The weight data on both groups of rats after the diet change was significantly different ($P < 0.001$) at the beginning of the new regimen. This initial difference in body weight disappeared toward the end of the fifth week. The continued increase in total kilocalorie intake in the group being changed to the high fat diet caused a significant weight gain. At the same time, the total low food and kilocalorie intakes resulted in significant weight loss in the other group of rats which were being changed from the high fat to the high carbohydrate diet. Therefore, these two processes together contributed to the small weight difference between both groups at 25 weeks of age.

Plasma Glucose--Before Diet Change

Both groups showed a sharp rise in plasma glucose after the glucose administration (Table 6, Figure 4).

In the high carbohydrate fed rats, plasma glucose reached a maximum (180 mg %) within 1/2 hour and started to decline at 1, 2 and 3 hours after the glucose load. By contrast, those fed a high fat diet had elevated plasma glucose during the oral glucose tolerance test. This rise was sustained up to 2 hours after the glucose load. The difference in plasma glucose level between the two diet groups was significant at fasting ($P < 0.1$), 1/2 ($P < 0.1$), 1, 2 and 3 hours ($P < 0.05$).

Impaired glucose utilization as found in this study has also been reported by Whitney and Roberts (1955), Samuels et al. (1942), Blazquez and Quijada (1968), Zaragoza and Felber (1970) and Katsumata (1970a, b) in rats fed a high fat diet. Similar findings were also found in humans fed a high fat, low carbohydrate diet by Himsworth (1935), Hales and Randle (1963a) and Anderson (1968). Hales and Randle suggested that normal people on a low carbohydrate diet showed antagonism not only to the hypoglycemic action of insulin but also to the important action of insulin in suppressing the release of nonesterified fatty acids.

Zaragoza-Hermans and Felber (1972) reported that the fat diet had no effect on the intestinal glucose absorption in vivo after an oral C-14 glucose load. If this factor is eliminated, three other possible processes

can maintain a high serum glucose concentration in fat fed animals:

1. Decreased peripheral utilization of glucose involving insulin. This has been reported by Christophe and Mayer (1959) who found a 54% decrease in glucose utilization *in vivo* and resistance to the administered insulin in rats fed a high fat diet for three weeks. Malaisse *et al.* (1969) found a 40% decrease in glucose utilization. Blazquez and Quijada (1968), too, reported that insulin sensitivity *in vitro* of isolated muscle and adipose tissues was decreased. All these reports indicated the existence of a decreased peripheral tissue utilization of glucose which then produces a heightened level of exogenous glucose in the plasma of high fat fed rats during the oral glucose tolerance test, as in the present study.

2. Enhanced hepatic production of glucose. Blazquez *et al.* (1971) found an increase in gluconeogenesis associated with enzymes such as glucose-6-phosphatase in liver, as well as a heightened liver capacity to synthesize glucose from physiological concentrations of alanine. This might contribute partly to the high glucose level in the high fat fed rats.

3. An impairment in the glycolysis cycle. There has been evidence showing a lower activity of the glycolytic enzymes such as hexokinase (Niemeyer *et al.*, 1962),

glucokinase (Vaughan et al., 1960) and pyruvate dehydrogenase (Bringolf et al., 1972). All of these impairments could have occurred in the high fat fed rats in the present study.

Plasma Glucose--After Diet Change

The group being changed from the high fat to the high carbohydrate diet had essentially the same fasting level of glucose as before the diet change (Table 6, Figure 4). However, at 1, 2 and 3 hours after the glucose administration, the plasma glucose levels were now significantly lowered ($P < 0.05$) than that before the diet change. Two things possibly could have led to this. First, the introduction of more carbohydrate to the diet might have enhanced and improved the glucose metabolism. Second, the significant weight loss might have improved glucose metabolism. Zaragoza and Felber (1970) switched young rats from a high fat diet to a high carbohydrate diet and found there was an improvement in glucose utilization.

Brunzell et al. (1970) reported similar findings in their human test subjects whose plasma glucose was decreased after the increase of the carbohydrate portion in their diet. In Hales and Randle's study (1963a), human individuals were changed from a low carbohydrate diet to a high carbohydrate diet and, as a result, their plasma glucose response during oral glucose became normal. Sims and Horton (1968) by placing obese test subjects on

a weight reduction diet were able to produce significant weight loss, as well as improvement in glucose metabolism.

The high carbohydrate fed rats, after being subjected to the high fat regimen for five weeks, showed a significant impairment in glucose metabolism. There was a higher and significant glucose level at fasting ($P < 0.001$), 1/2 hour ($P < 0.01$), 1 and 2 hours ($P < 0.05$) after the glucose load.

The high fat diet, nearly devoid of carbohydrate, had indeed contributed to the impairment of glucose metabolism, as well as to a significant increase in body weight and fat storage. Questions then arose. Did the diet first cause a significant increase in body weight gain which then in turn led to an impaired glucose metabolism? Or, did the diet itself directly cause such an impairment? Further studies in this area should provide the answer.

At the end of the fifth week of the new regimen, the body weight of both groups was not significantly different nor did their plasma glucose response differ significantly during the oral glucose tolerance test.

Plasma Insulin--Before Diet Change

The concentration of plasma insulin, in general, paralleled that of glucose (Table 7, Figure 5). After fasting, the high fat fed rats had a slightly higher plasma insulin level than the high carbohydrate fed rats. This, however, was not significant. After the glucose load,

the plasma insulin level rose to a peak within half an hour and returned to the basal level after three hours. This early drop of plasma insulin which preceded the fall of plasma glucose was possibly related to an altered secretion of insulin by the pancreas.

The present data are consistent with the report by Zaragoza-Hermans and Felber (1972). However, at 1/2 hour they found a slightly higher plasma insulin level in rats fed a high fat diet. This difference between their and our results might be due to the relatively heavier and older rats used in the present study. This condition, plus the fact that our animals were fed a high fat diet for a much longer time prior to the oral glucose tolerance test, means that our animals were much more adapted to a metabolic scheme involving dietary fat. The animals used by them were of the Wistar strain and they were fed a high fat diet (35.5%, w/w; 67%, cal/cal) for only three to five weeks after weaning.

A low plasma insulin level has been reported by Malaisse et al. (1969) who fed rats a high fat diet for six months. All of his animals had a decrease in insulin release by their pancreas. Blazquez and Quijada (1968) also reported a decrease in plasma insulin, but claimed the pancreas insulin level was the same in both the high fat fed and the control animals whether in the fed or fasted state. Katsumata (1970a) found similar insulin levels in

the rats fed a high fat diet for 400 days when compared to the control rats. Using histological studies, he reported the presence of hypertrophy in the Langerhan islets.

These studies all indicate that long-term, high fat feeding would result in the decreased secretion of insulin and consequently a lower plasma insulin response during an oral glucose tolerance test. This, plus the report of the decrease of peripheral utilization of glucose in high fat fed rats, explains the occurrence of higher plasma glucose levels after the glucose load in these animals. It is also possible that in these rats the peripheral tissues, especially fat, might have had a lower requirement for the hormone insulin, despite their reduced insulin sensitivity. The fact that this high fat diet was nearly devoid of carbohydrate could have removed and changed the pattern of insulin secretion by the pancreas.

In the present study, the plasma insulin levels were not significantly different ($P < 0.1$) in both diet groups, although the high fat fed rats showed a significantly ($P < 0.05$) higher plasma glucose. Again, the decrease of peripheral sensitivity to insulin could clarify this difference in plasma glucose levels.

Plasma Insulin--After Diet Change

The plasma insulin response to the glucose load after the diet change is listed in Table 7 and graphed in Figure 5. In both groups, plasma insulin level rose to a peak within half of an hour and returned to the basal level afterwards. The group being changed to a high fat diet had a slightly higher but insignificant plasma insulin level response than before their diet change. This slight increase could possibly be the result of:

1. The increase in body weight, and
2. The development of insulin insensitivity in tissue as seen by the heightened plasma glucose level after the diet had been changed to high fat.

In the other group, the insulin level was also essentially the same as before their diet was changed from high fat to high carbohydrate. The fasting insulin level was also slightly lower, while at 1/2 hour the insulin level was raised. This indicated a possible improvement in

1. The insulin secretion pattern of the pancreas as the result of the increase in carbohydrate in the diet, or
2. The insulin sensitivity of the tissues.

The decrease of plasma glucose level in this group, after being changed to a high carbohydrate diet, could well be the product of these two processes individually or together. More studies are necessary to determine the exact process.

Plasma NEFA--Before Diet Change

In both diet groups, there was a closely correlated reciprocal variation between glucose and fatty acid response (Table 8, Figure 6). Dole (1956) using human subjects reported the similar inverse fatty acid response during the glucose tolerance test.

The explanation for this reverse pattern is: After an overnight fast, the available glucose is greatly decreased and fatty acids are released from adipose tissues and muscles, and are oxidized by body cells for energy. Conversely, the administration of an oral glucose load creates a suppression of the release of fatty acids and a facilitation of the uptake and utilization of glucose. This decreased release of fatty acids and increased glucose utilization are aided by the action of insulin secretion as a result of the heightened blood glucose after intestinal absorption.

The mean plasma NEFA concentration in the high carbohydrate fed rats was higher than in the high fat fed rats, but it was not significant. The mean percentage fall of plasma NEFA after glucose administration was 38% for high carbohydrate fed rats and 19% for the high fat fed rats, respectively, at 1/2 hour. The plasma NEFA in the high fat fed group continued to fall after 1/2 hour, while the high carbohydrate fed group after reaching the nadir level at 1/2 hour began to recover at 1 hour. Even though the high carbohydrate group had

higher plasma NEFA level, the T test showed there was no significant difference between the two groups.

Using rats, starved for 24 hours, Zaragoza and Felber (1970) reported the high fat group had a lower plasma FFA (679 μeq/litre) when compared to the high carbohydrate fed group (1277 μeq/litre). This was in agreement with the data obtained in the present study. The high fat fed rats had a fasting plasma FFA of about 791 μeq/litre, while the high carbohydrate rats had a fasting plasma FFA of about 1104 μeq/litre. Since no other investigator has measured the complete plasma NEFA response during an oral glucose tolerance test in the high fat fed or high carbohydrate fed rats, it is impossible at the present time to compare our data obtained at 1/2, 1 and 3 hours after the glucose load to those of similar types of studies.

The lower but insiginificant plasma NEFA level in the high fat diet group could be the result of three processes:

1. A decreased release of fatty acid from adipose tissue or liver, leading to the excess body fat in these rats,
2. An enhanced fatty acid turnover rate and utilization, and/or
3. An elevated plasma glucose level.

Further *in vivo* or *in vitro* studies will be needed to conclude the exact process. Also, the oxygen consumption of these rats could be helpful in supporting or rejecting the above three possible processes.

Plasma NEFA--After Diet Change

The pattern of plasma NEFA response was essentially the same in both groups after the diet change. Both showed a higher fasting plasma NEFA level which started to fall after the glucose administration and began to recover after two hours. There was no significant difference between the values of plasma NEFA levels in both groups.

Compared to the data before the diet change, the group being changed from the high carbohydrate to the high fat diet now showed a lower NEFA level response during the oral glucose tolerance test. The values were significantly different ($P < 0.01$) except at 1/2 hour. This response resembled the plasma NEFA response of the 20-week-old rats which were fed the high fat diet throughout that whole period. It then indicated that the lipid metabolism might have been changed as the result of the high fat feeding.

In the same manner, the group being changed from the high fat to the high carbohydrate diet now had a higher plasma NEFA response than before their diet change. This elevation was, however, not significant. Their plasma response resembled the plasma NEFA response of the 20-week-old rats which were fed the high carbohydrate diet throughout that whole period of study. The only existing difference between them was the plasma NEFA level at 1 hour after the glucose load. In the 20-week-old rats

fed low fat diets, their plasma NEFA level was already on the course of returning to the fasting level, while the group being changed to the high carbohydrate diet for five weeks was still exhibiting a continuous decline after 1/2 hour and did not return to the fasting level until after 2 hours. This indicates that there was only a partial improvement in lipid metabolism. Maybe with prolonged feeding of a low fat diet, this group would eventually have a total and complete improvement in their lipid metabolism.

SUMMARY AND CONCLUSION

To evaluate the effect of a high fat diet on food intake, body weight, plasma glucose, insulin and NEFA levels, male Osborne Mendel rats were fed either a high fat diet or a high carbohydrate diet for 17 weeks after weaning. After an 18 hour overnight fast, all were subjected to an oral glucose tolerance test. Blood samples were obtained from all for glucose, insulin and NEFA analyses.

The animals fed the high carbohydrate diet responded normally to the oral administration of a glucose solution with an increase in plasma glucose and insulin levels at 1/2 hour after the load, followed by a decrease in both parameters after 1 hour. Plasma NEFA response was practically the opposite. The fasting level of plasma NEFA fell to the lowest level at 1/2 hour and started to increase afterwards.

The response of the high fat fed rats was quite different. There was a continuous increase in plasma glucose up to 2 hours after the glucose load. Plasma insulin level increased at 1/2 hour but dropped to the basal level prior to the decline of the plasma glucose level. In these rats, their plasma NEFA level was low

and continued to fall after 1/2 hour, while the high carbohydrate fed group began to recover.

In summary, rats fed a high fat diet for a prolonged period of time resulted in a greater body weight gain along with a decreased glucose tolerance to oral glucose administration and changes in insulin and lipid metabolism. In order to confirm the above parameters and to observe the possibility of improving such impairment in glucose, insulin and NEFA metabolisms, the diet was reversed between the two groups of rats for five weeks.

Now the rats being changed from a high fat to a high carbohydrate diet showed a significant weight loss and improvement in glucose utilization. Insulin and NEFA metabolism were improved, but not significantly. The opposite occurred in rats being changed from a high carbohydrate to a high fat diet. This group gained significantly in body weight and showed impairment in glucose utilization. Their insulin and NEFA metabolism were also impaired. However, these values were not significant.

Both groups after the diet change showed comparable body weight, yet the glucose metabolism was clearly affected in the group which was changed from the high carbohydrate to the high fat diet. At the same time, the other group showed improvement in glucose utilization after being changed from a high fat to a low fat diet.

Suggestions for Further Studies

Effect of Carbohydrate or Fat in Diets for Patients with Diabetes

A high fat diet fed to rats has produced significant weight gain and impaired glucose tolerance as often seen in adult diabetes mellitus. A reverse to a high carbohydrate diet has improved this abnormality in carbohydrate metabolism. Brunzell et al. (1970) treated some mild diabetics with high carbohydrate feedings and found an improvement in their glucose tolerance. Stone and Connor (1963) also reported a similar result. Therefore, it seems some alteration in the long-held dietary treatment for diabetes mellitus should be required. An increase of carbohydrate, without an increase of total calories beyond that required by the patients, may in fact lead to an improvement in glucose utilization.

However, one of the risky factors associated with increase in dietary carbohydrate is the elevated plasma triglyceride level--a contributing factor to arteriosclerosis. Therefore, it is important to find out what is the optimal portion of carbohydrate and fat which should be recommended for diabetes. Second, what will be the long-range effect of a high carbohydrate diet on diabetes mellitus and its complications.

Further studies in this area may eventually bring an improved treatment of diabetes.

The Role of Chromium in Glucose Metabolism

Schwartz and Mertz (1959) showed that impaired glucose tolerance in experimental rats might be due to the contents of the glucose tolerance factor in the diets. Ashing the preparation failed to destroy the activity of glucose tolerance factor which indicated the presence of an inorganic material was responsible for the biological effects. Upon a series of testing, they detected the activity in trivalent chromium complexes. They also reported the impaired glucose tolerance in chromium deficient animals might be due to their decreased response to their endogenous insulin (Mertz, 1969). Rats fed the high fat diet have been reported to be insensitive to exogenous and endogenous insulin, as well as being distinctly glucose intolerant. An analysis of the composition of this mineral in high fat and control diets used in the present study may explain such difference in carbohydrate metabolism. If the high fat diet used in this study is low in chromium, as found in the original M-16 high fat ration, then will an increase of this mineral overcome all the metabolic abnormalities induced by such a diet? And what will be the optimal level of chromium which one can add to the diet without causing some other adverse effect on body metabolism?

Weight Reduction and Its Effectiveness

The first aim of the treatment for obese patients should always be the provision of a diet that is restricted in calories yet nutritionally balanced and adequate. The success of this measure alone is often reported with the reversal of the glucose intolerance and reduction of prominent risk factors involved in obesity. However, studies also have shown that regularity of food intake appears to have an effect on several biological mechanisms (Leveille, 1970). Therefore, will such a dietary management be successful in correcting the associated metabolic abnormalities when the obese individual is accustomed to consume all his calories in one meal? Conversely, will such metabolic abnormalities seen in the obese be corrected at a higher rate when the food intake is divided into several smaller meals per day?

TABLE 1.--Composition of high carbohydrate and high fat rations.

Ingredients	High Carbohydrate (gm)	High Fat (gm)
Mineral mix[a]	4.00	4.00
Vitamin mix[b]	2.00	2.00
Fiber[c]	2.00	2.00
Aureomycin[d]	0.01	0.01
dl-methionine	0.25	0.25
Casein	20.00	20.00
Corn oil	3.00	3.00
Cerelose[e]	68.74	3.40
Crisco[f]	--	27.20
Total weight	100.00	61.86
Energy Value of Rations		
Digestable energy[g]		
Casein (kilocalories)	85.40	85.40
Corn oil (kilocalories)	26.52	26.52
Glucose (kilocalories)	252.96	12.51
Crisco (kilocalories)	--	240.45
Total digestable energy (kilocalories)	364.90	364.90
Digestable energy (kilocalories per gm)	3.65	5.90
Ratio--Fat and carbohydrate to protein (kilocalories)	280:85	280:85
Ratio--Fat and carbohydrate to protein (kilocalories in per cent)	70:30	70:30

[a]Purchased from General Biochemicals, Inc., Chagrin Falls, Ohio.
[b]Purchased from Nutritional Biochemicals Corp., Cleveland, Ohio.
[c]Purchased from General Biochemicals, Inc., Chagrin Falls, Ohio.
[d]Generously provided by American Cyanamid Co., Princeton, N.J.
[e]Purchased from Corn Industry, Division of CPC International Co., Englewood, N.J.
[f]Purchased from Proctor and Gamble, Cincinnati, Ohio.
[g]Reference from Composition of Foods, Handbook No. 8, U.S. Department of Agriculture, p. 160.

TABLE 2.--Experimental Design.

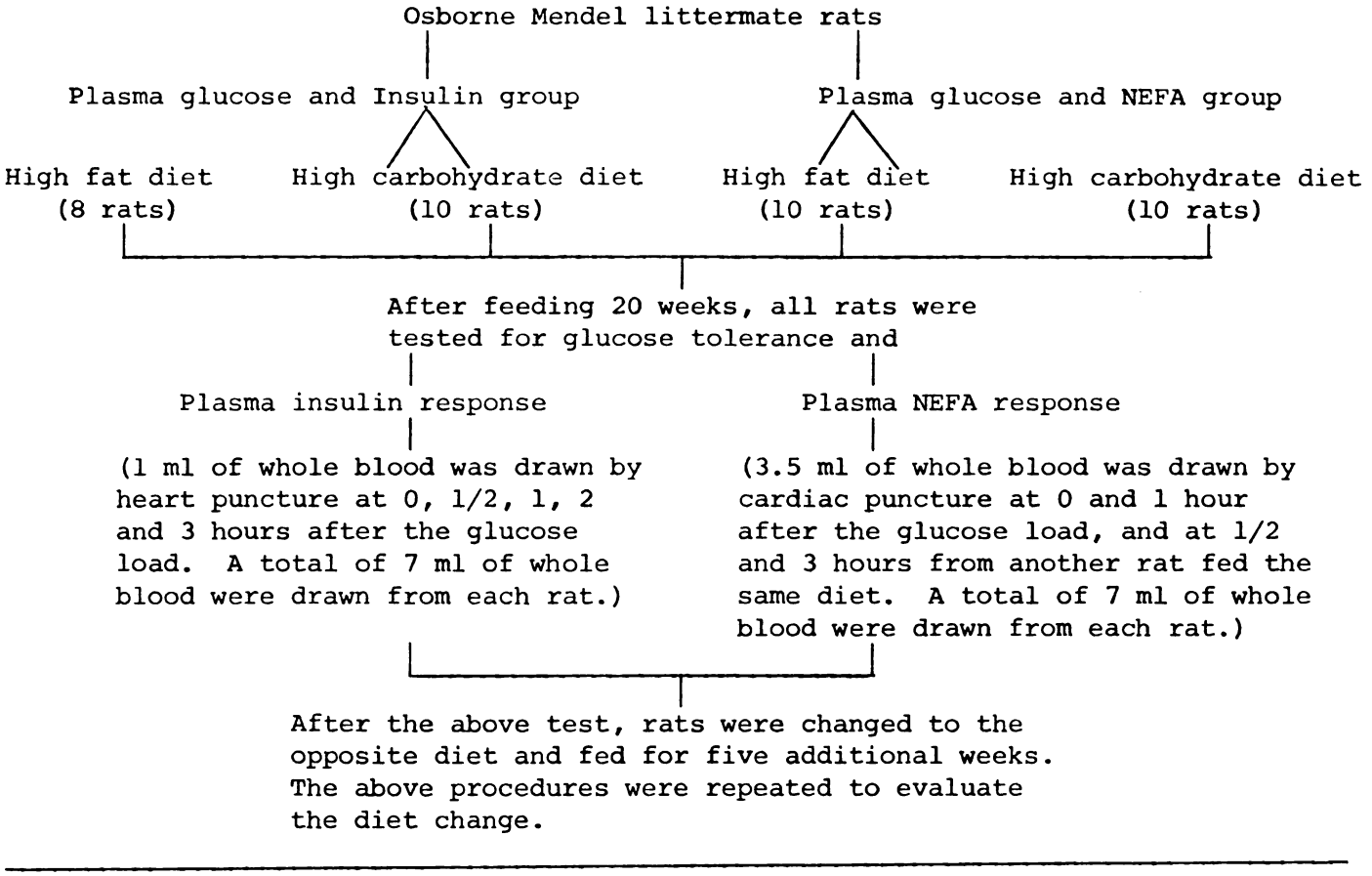


TABLE 3.--Weekly mean food intake of male Osborne Mendel rats fed either a high carbohydrate or high fat diet.

Age (weeks)	Food Intake (gm/week) High Carbohydrate (20 rats)	High Fat (18 rats)	P <
4	66 ± 2[a]	52 ± 2	0.001
5	94 ± 3	62 ± 2	0.001
6	109 ± 3	77 ± 2	0.001
7	126 ± 4	84 ± 3	0.001
8	126 ± 7	89 ± 4	0.001
9	153 ± 6	102 ± 3	0.001
10	164 ± 6	105 ± 4	0.001
11	153 ± 6	106 ± 3	0.001
12	171 ± 6	106 ± 3	0.001
13	176 ± 4	111 ± 4	0.001
14	180 ± 6	120 ± 3	0.001
15	172 ± 4	110 ± 4	0.001
16	181 ± 5	119 ± 5	0.001
17	181 ± 6	120 ± 2	0.001
18	185 ± 4	127 ± 4	0.001
19	189 ± 6	130 ± 4	0.001
20	194 ± 6	131 ± 6	0.001
	High Fat[b] (17 rats)	High Carbohydrate[b] (17 rats)	
21	104 ± 5	86 ± 9	0.1
22	141 ± 5	129 ± 6	NS
23	140 ± 5	157 ± 7	0.1
24	128 ± 6	154 ± 6	0.01
25	145 ± 9	156 ± 8	NS

[a]Mean ± standard error.

[b]Rats were changed to the opposite diet.

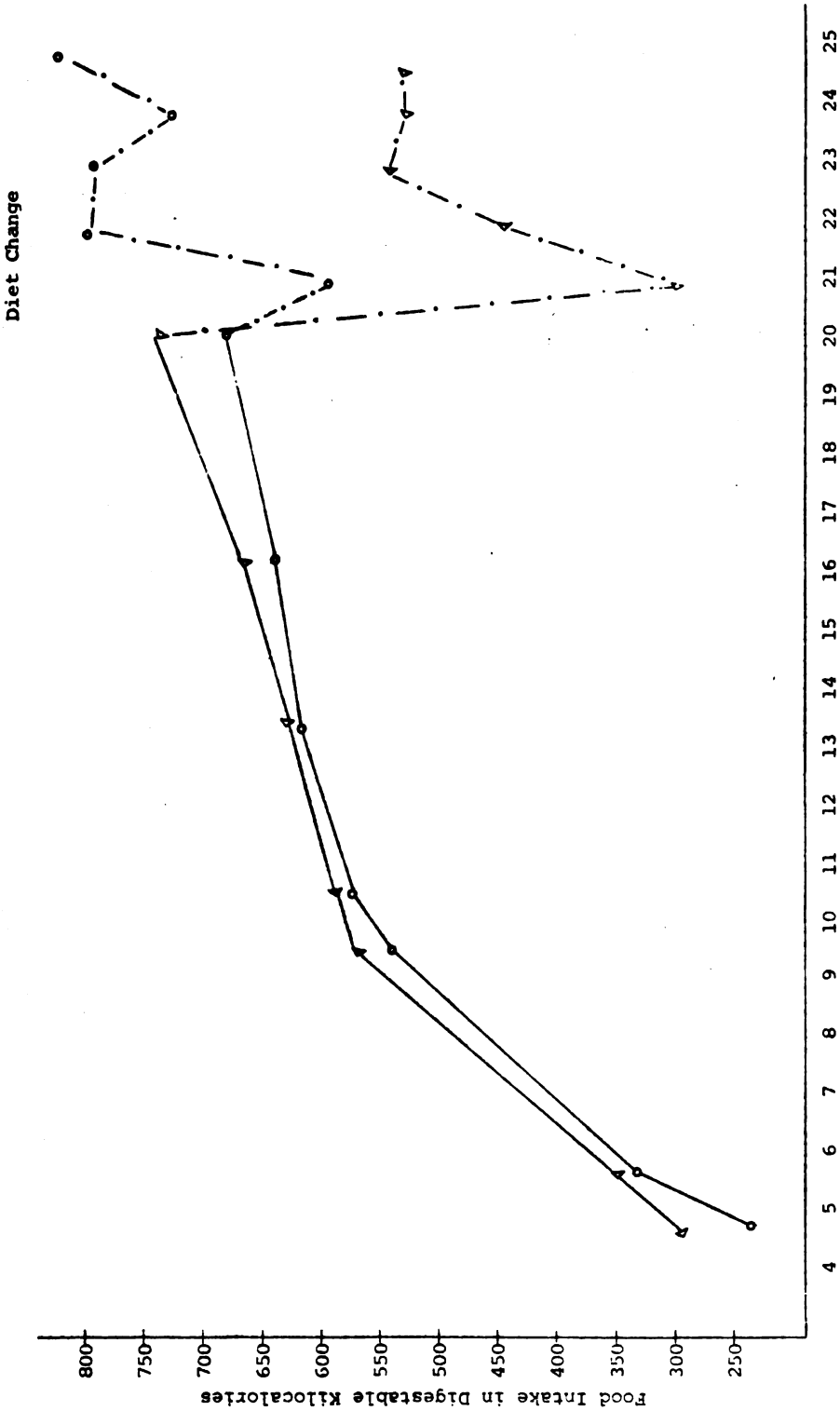


Figure 1.--Weekly mean food intake of male Osborne Mendel rats fed either a high fat or high carbohydrate diet.

Δ——Δ high fat
Δ—·—·—Δ high fat to high carbohydrate
o——o high carbohydrate
o—·—·—o high carbohydrate to high fat

TABLE 4.--Weekly mean kilocalories consumed by male Osborne Mendel rats fed either a high carbohydrate or high fat diet.

Age (weeks)	Food Intake (kilocalorie/wk) High Carbohydrate (20 rats)	High Fat (18 rats)	P <
4	241 ± 8[a]	307 ± 9	0.001
5	343 ± 10	366 ± 14	NS
6	398 ± 10	454 ± 10	0.001
7	460 ± 14	496 ± 15	NS
8	460 ± 20	525 ± 23	NS
9	559 ± 21	602 ± 20	NS
10	599 ± 23	620 ± 23	NS
11	559 ± 22	625 ± 17	0.1
12	624 ± 23	625 ± 20	NS
13	642 ± 13	655 ± 21	NS
14	657 ± 21	708 ± 16	0.1
15	628 ± 15	649 ± 23	NS
16	661 ± 19	702 ± 32	NS
17	661 ± 22	708 ± 12	0.1
18	675 ± 16	749 ± 22	0.01
19	690 ± 22	767 ± 23	0.01
20	708 ± 21	773 ± 33	NS
	High Fat[b] (17 rats)	High Carbohydrate[b] (17 rats)	
21	614 ± 29	314 ± 33	0.001
22	832 ± 31	471 ± 20	0.001
23	826 ± 30	573 ± 26	0.001
24	755 ± 35	562 ± 21	0.001
25	856 ± 51	569 ± 28	0.001

[a]Mean ± standard error.

[b]Rats were changed to the opposite diet.

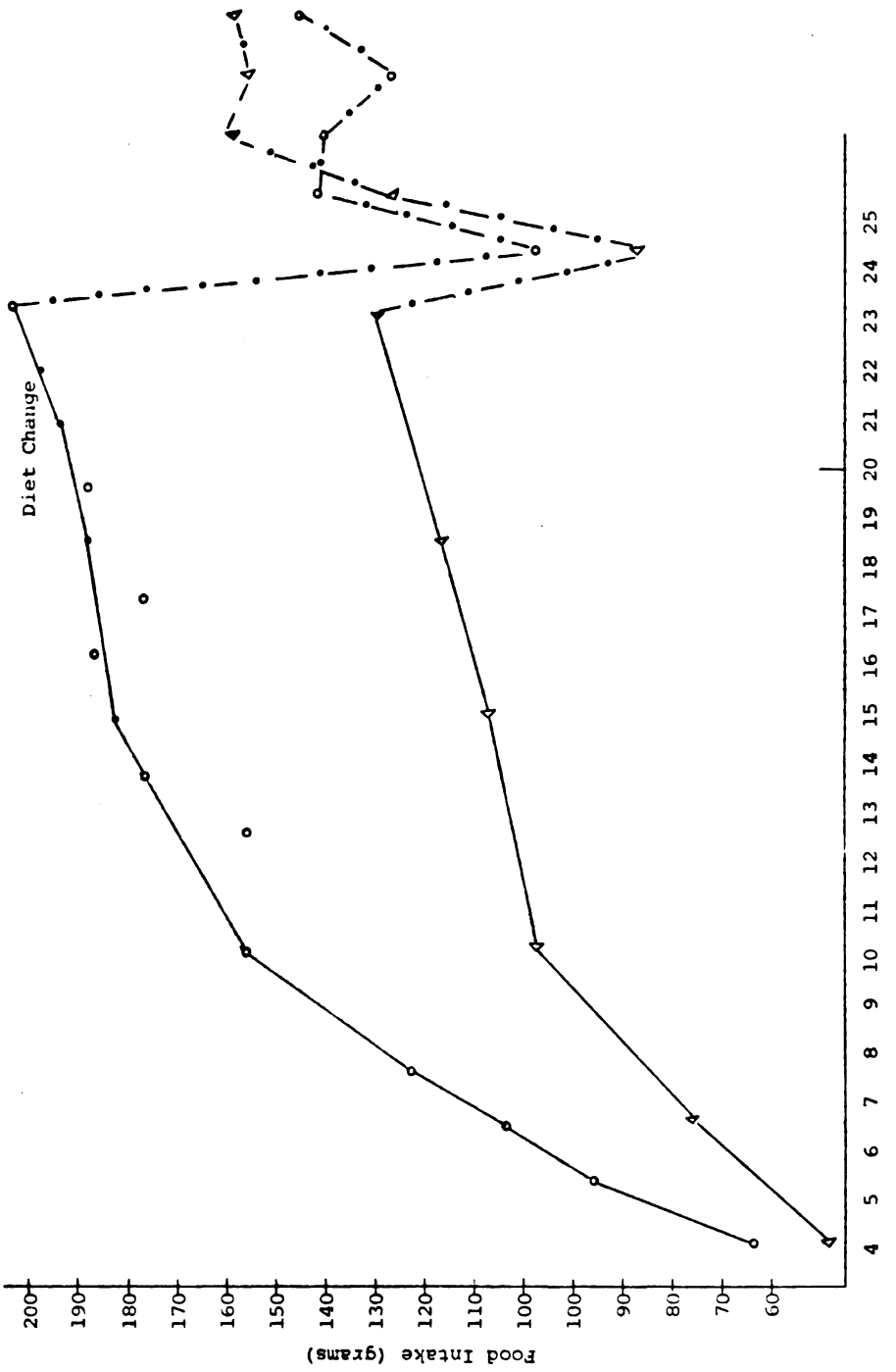


Figure 2.--Weekly mean kilocalories consumed by male Osborne Mendel rats fed either a high fat or high carbohydrate diet.

△——△ high fat
△—·—·—△ high fat to high carbohydrate
o——o high carbohydrate
o—·—·—o high carbohydrate to high fat

TABLE 5.--Weekly mean body weight in grams of male Osborne Mendel rats fed either a high carbohydrate or high fat diet.

Age (weeks)	Mean Body Weight (gm/wk) High Carbohydrate (20 rats)	High Fat (18 rats)	P <
Weaning	54 ± 2[a]	54 ± 1	NS
4	83 ± 3	91 ± 2	0.1
5	122 ± 3	136 ± 6	0.1
6	166 ± 5	173 ± 17	NS
7	208 ± 6	234 ± 6	0.01
8	246 ± 6	282 ± 9	0.01
9	286 ± 8	324 ± 10	0.01
10	320 ± 8	368 ± 10	0.001
11	346 ± 9	405 ± 11	0.001
12	370 ± 10	435 ± 10	0.001
13	394 ± 10	465 ± 11	0.001
14	411 ± 10	499 ± 11	0.001
15	434 ± 10	525 ± 11	0.001
16	446 ± 10	548 ± 11	0.001
17	460 ± 10	567 ± 11	0.001
18	475 ± 10	590 ± 11	0.001
19	486 ± 11	616 ± 12	0.001
20	486 ± 12	633 ± 14	0.001
	High Fat[b] (17 rats)	High Carbohydrate[b] (17 rats)	
21	503 ± 12	600 ± 13	0.001
22	540 ± 14	586 ± 12	0.01
23	573 ± 14	583 ± 11	NS
24	604 ± 16	593 ± 12	NS
25	624 ± 17	597 ± 11	NS

[a]Mean ± standard error.

[b]Rats were changed to the opposite diet.

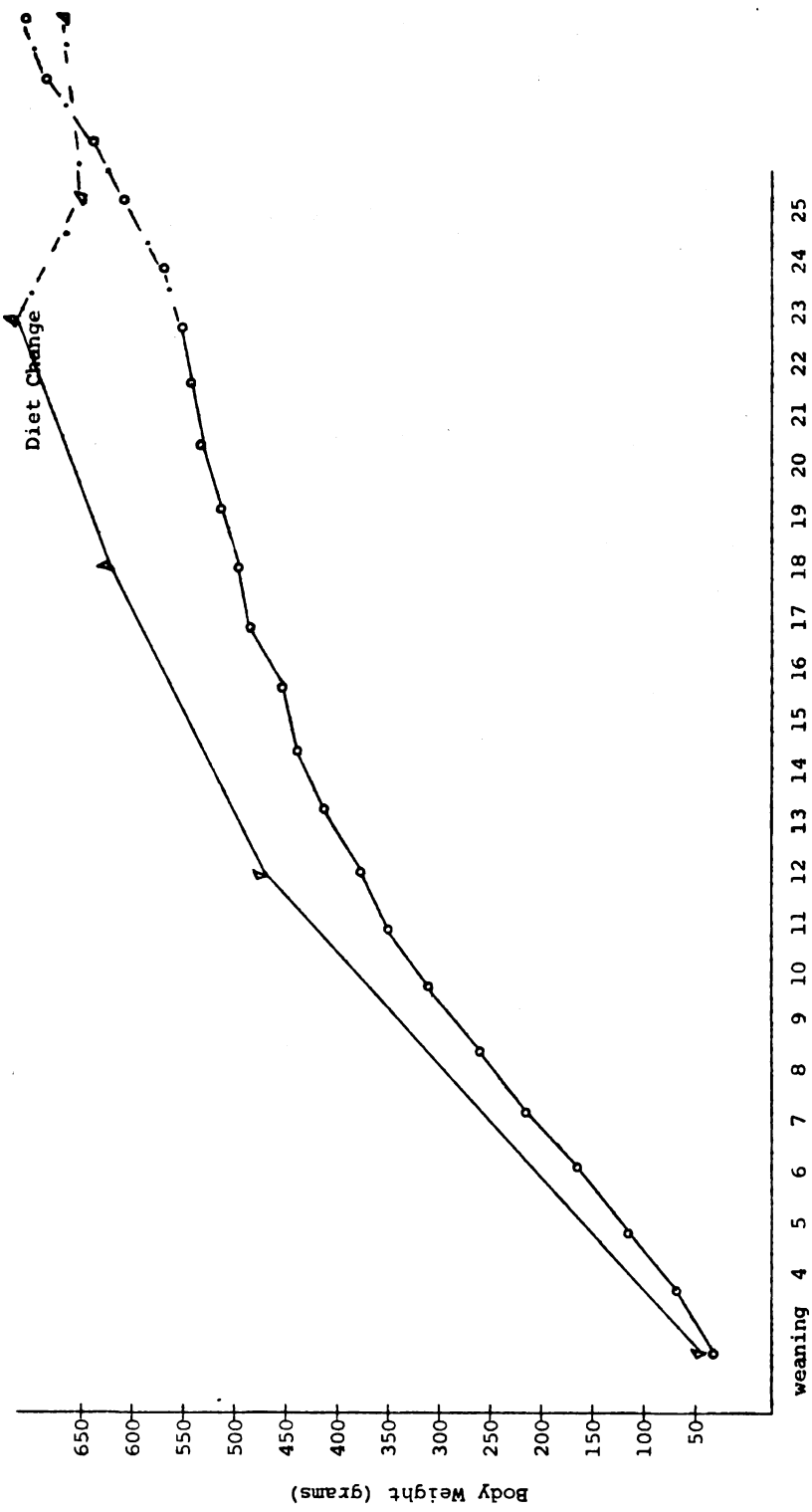


Figure 3.--Weekly mean body weight of male Osborne Mendel rats fed either a high fat or high carbohydrate diet.

TABLE 6.--Glucose tolerance test results of male Osborne Mendel rats fed either a high carbohydrate or high fat diet.[a]

Hours	Plasma Glucose (mg %) High Carbohydrate[b]		High fat[c]		P <
Age--20 weeks					
0	103 ± 8[d]	(14)[e]	131 ± 11	(13)	0.1
1/2	170 ± 13	(15)	216 ± 21	(15)	0.1
1	158 ± 16	(15)	239 ± 30	(12)	0.05
2	151 ± 12	(15)	287 ± 54	(13)	0.05
3	152 ± 19	(15)	258 ± 39	(13)	0.05
	High Fat[f]		High Carbohydrate[g]		
Age--25 weeks[h]					
0	140 ± 4	(12)	133 ± 5	(11)	NS
1/2	199 ± 9	(11)	174 ± 10	(12)	0.1
1	200 ± 9	(12)	178 ± 10	(12)	NS
2	183 ± 7	(12)	162 ± 11	(12)	NS
3	154 ± 9	(12)	151 ± 9	(12)	NS

[a]Rats were fasted overnight for 18 hours before the test.

[b]Mean body weight in gm of 20 rats: 489 ± 19.

[c]Mean body weight in gm of 17 rats: 629 ± 17.

[d]Mean plasma glucose in mg % ± standard error.

[e]Number of rats.

[f]Mean body weight in gm of 17 rats: 628 ± 17.

[g]Mean body weight in gm of 17 rats: 595 ± 12.

[h]Rats were changed to the opposite diet.

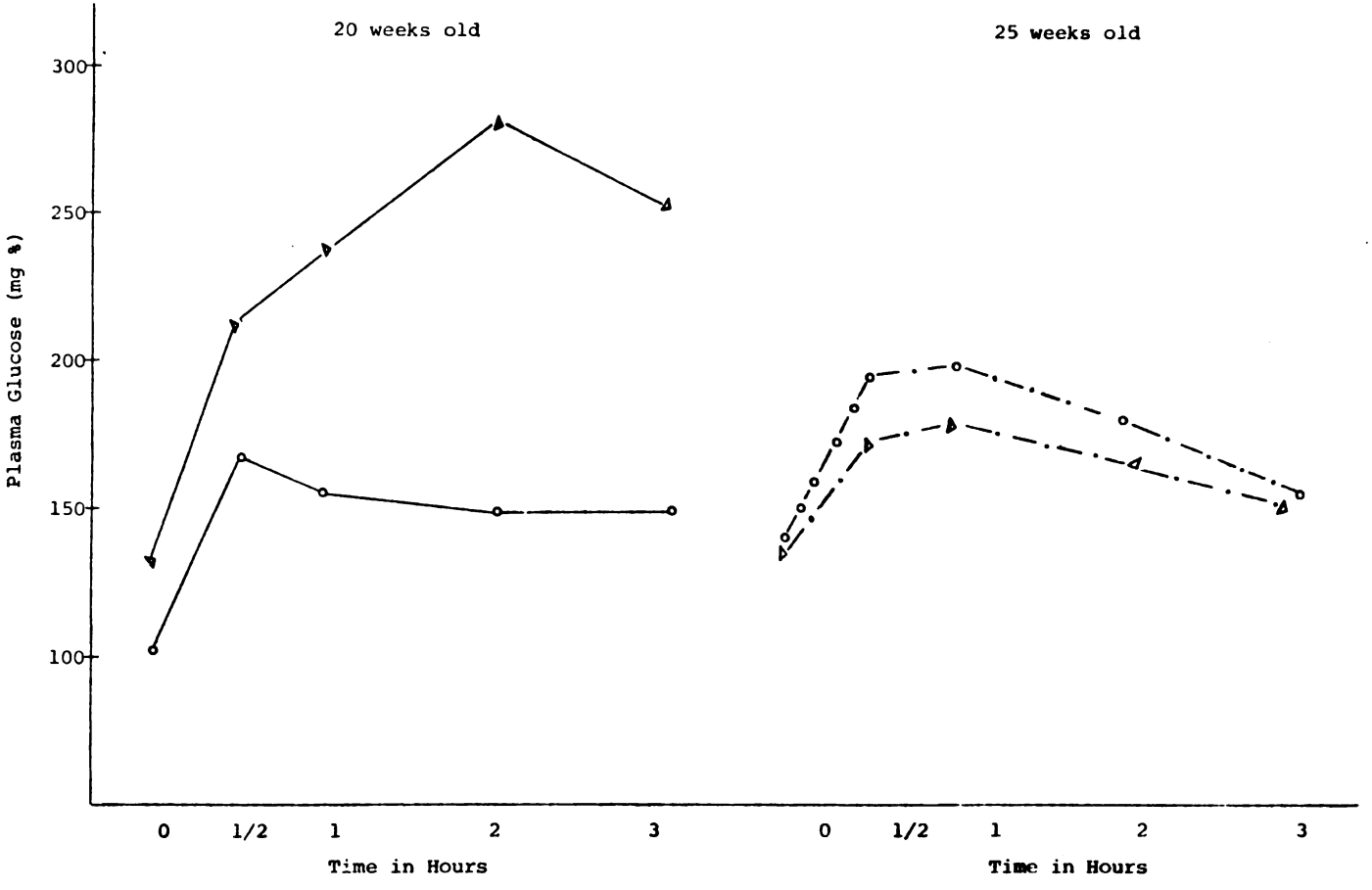


Figure 4.--Response of glucose during an oral glucose tolerance test of male Osborne Mendel rats fed either a high fat or high carbohydrate diet.

Δ———Δ high fat
Δ—•—•—Δ high fat to high carbohydrate
o———o high carbohydrate
o—•—•—o high carbohydrate to high fat

TABLE 7.--Plasma insulin response of male Osborne Mendel rats fed either a high carbohydrate or high fat diet.[a]

Hour	Plasma Insulin (μU/ml)				P <
	High Carbohydrate		High Fat		
	Body Weight (gm) 469 ± 19[b]	(10)[c]	Body Weight (gm) 654 ± 23	(8)	0.001
Age--20 weeks					
0	24 ± 3	(10)	27 ± 5	(8)	NS
1/2	38 ± 5	(10)	36 ± 6	(7)	NS
1	28 ± 2	(9)	32 ± 6	(7)	NS
2	33 ± 4	(10)	27 ± 5	(8)	NS
3	27 ± 3	(10)	27 ± 5	(6)	NS
	High Fat		High Carbohydrate		
	Body Weight (gm) 643 ± 23	(7)	Body Weight (gm) 599 ± 11	(7)	
Age--25 weeks[d]					
0	32 ± 7	(7)	22 ± 3	(6)	NS
1/2	53 ± 15	(7)	54 ± 11	(7)	NS
1	42 ± 8	(7)	31 ± 4	(7)	NS
2	40 ± 10	(7)	25 ± 3	(6)	NS
3	30 ± 15	(6)	31 ± 4	(6)	NS

[a]Rats were fasted for 18 hours before the test.

[b]Mean ± standard error.

[c]Number of animals.

[d]Rats were changed to the opposite diet.

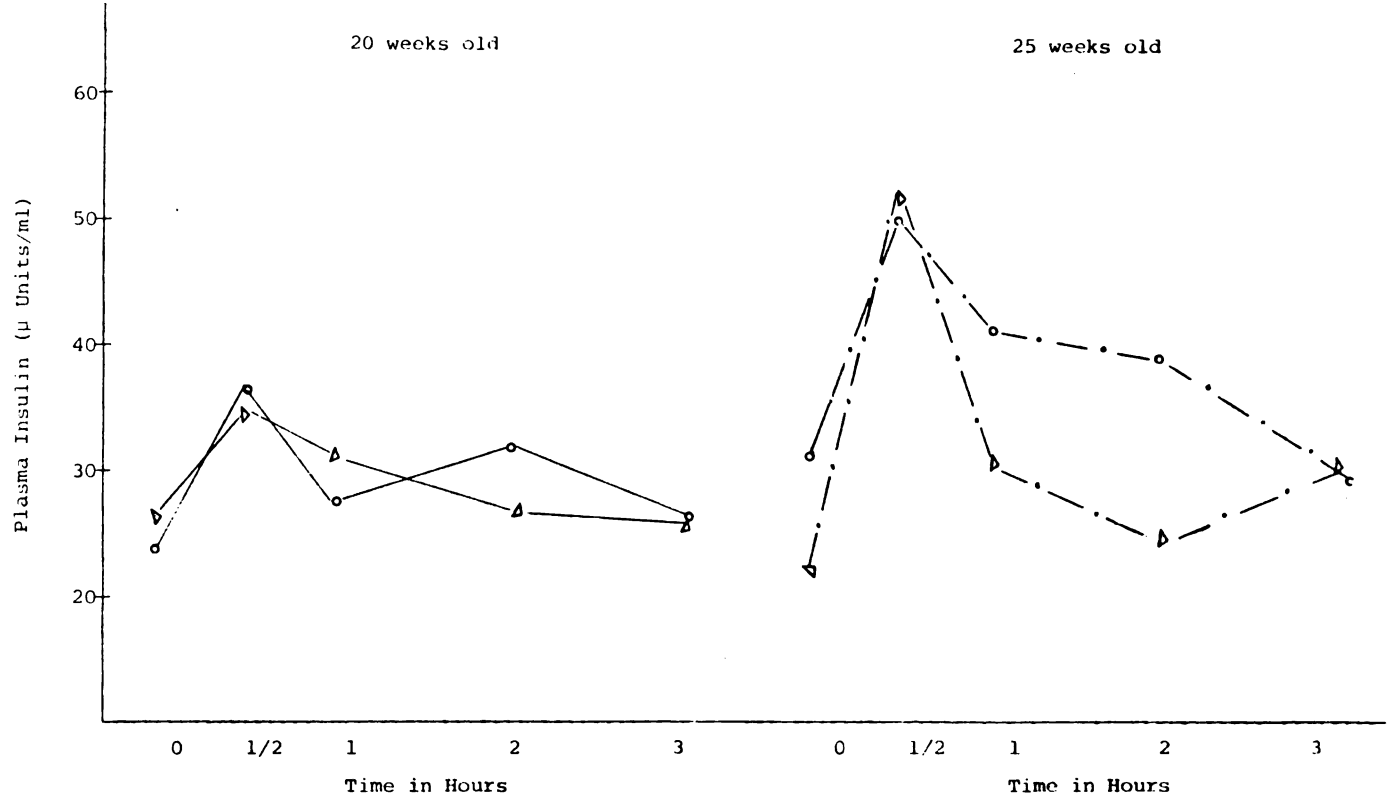


Figure 5.--Plasma insulin response during an oral glucose tolerance test of male Osborne Mendel rats fed either a high fat or high carbohydrate diet.

Δ———Δ high fat
Δ—•—•—Δ high fat to high carbohydrate
o———o high carbohydrate
o—•—•—o high carbohydrate to high fat

TABLE 8.--Plasma NEFA response of male Osborne Mendel rats fed either a high carbohydrate or high fat diet.[a]

Hour	Plasma NEFA (μeq/litre)				P <
	High Carbohydrate		High Fat		
	Body Weight (gm) 481 ± 19[b]	(10)[c]	Body Weight (gm) 617 ± 16	(10)	0.001
Age--20 weeks					
0	1104 ± 179	(5)	791 ± 58	(5)	NS
1/2	683 ± 118	(5)	639 ± 104	(5)	NS
1	789 ± 124	(5)	617 ± 93	(5)	NS
3	1040 ± 84	(5)	1025 ± 45	(5)	NS
	High Fat		High Carbohydrate		
	Body Weight (gm) 591 ± 13	(10)	Body Weight (gm) 612 ± 25	(10)	NS
Age--25 weeks[d]					
0	1015 ± 265	(5)	729 ± 47	(5)	NS
1/2	695 ± 274	(5)	609 ± 252	(5)	NS
1	558 ± 132	(5)	424 ± 74	(5)	NS
3	1280 ± 367	(5)	789 ± 101	(5)	NS

[a]Rats were fasted overnight for 18 hours before the test.

[b]Mean ± standard error.

[c]Number of animals.

[d]Rats were changed to the opposite diet.

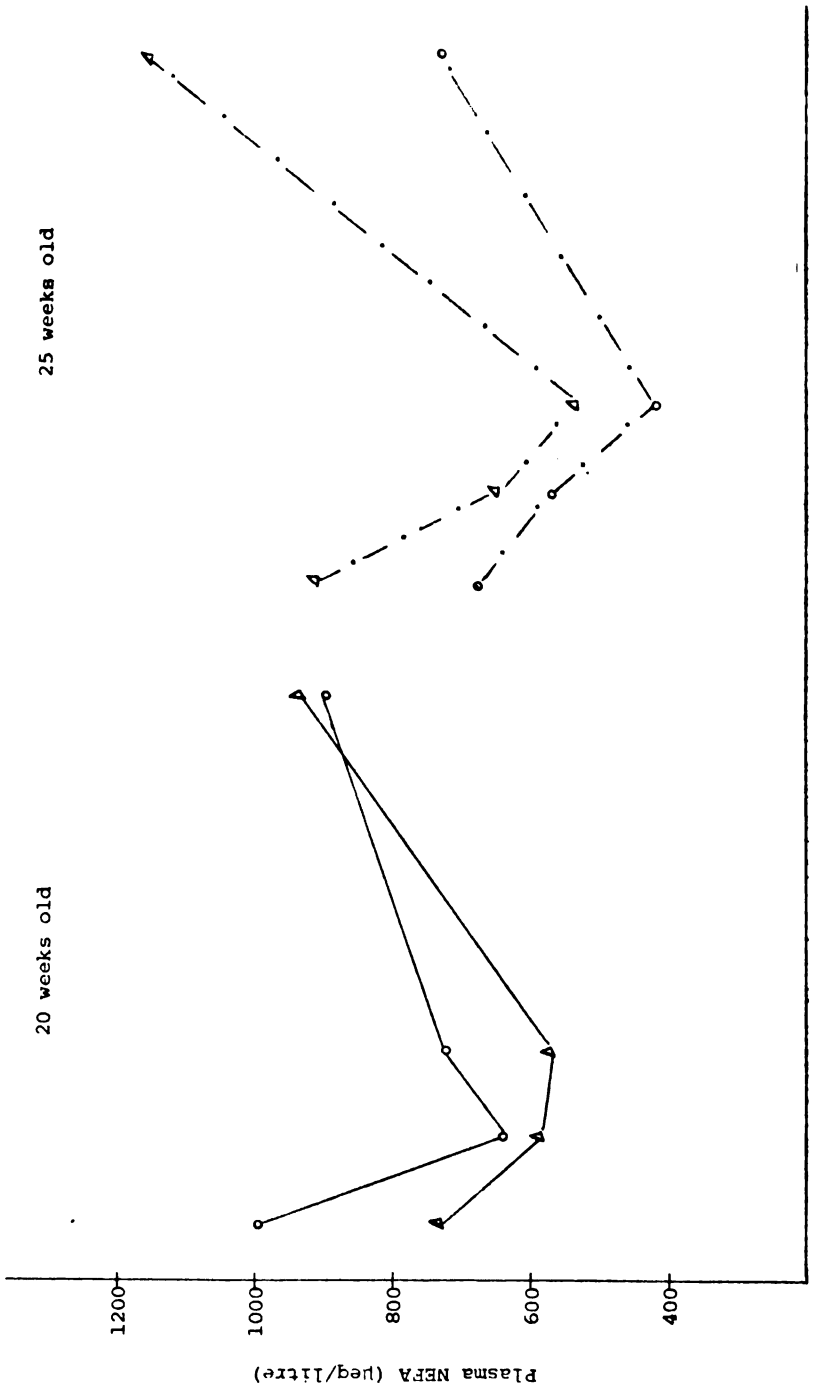


Figure 6.--Plasma NEFA response during an oral glucose tolerance test of male Osborne Mendel rats fed either a high fat or high carbohydrate diet.

Δ——Δ high fat
Δ—·—·—Δ high fat to high carbohydrate
o——o high carbohydrate
o—·—·—o high carbohydrate to high fat

APPENDICES

APPENDIX I

PLASMA GLUCOSE DETERMINATION

APPENDIX I

PLASMA GLUCOSE DETERMINATION

Reagents

Deproteinizing Solutions

A solution of 1.8 gm of barium hydroxide ($Ba(OH)_2 \cdot 8H_2O$, Fisher Scientific Co.) per 100 ml and a solution of 2 gm zinc sulfate septihydrate ($ZnSO_4 \cdot 7H_2O$, Fisher Scientific Co.) per 100 ml were prepared. These Somogyi solutions should neutralize each other. About 10 ml of 2% zinc sulfate solution diluted with 50 ml of distilled water was titrated by 10.05 ml of 1.8% barium hydroxide solution, using two drops of phenolphthalein as indicator, to a faint pink color. When necessary, the stronger solution was diluted and rechecked. The barium hydroxide solution was stored with soda lime in the stopper.

Standard Glucose Solution

A solution of 1 gm d-glucose anhydrous and 0.27 gm of benzoic acid per 1000 ml distilled water was prepared and stored in the refrigerator at 2° to 4°C. This stock solution was stable for months. Aqueous working standards were prepared daily prior to glucose analysis. The stock solution was diluted with distilled water into

concentrations of 450, 400, 350, 300, 250, 200, 150, 100 and 50 mg % in the following manner:

Mg %	50	100	150	200	250	300	350	400	450
1000 mg % stock (ml)	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5
Distilled water (ml)	9.5	9.0	8.5	8.0	7.5	7.0	6.5	6.0	5.5

Hydrochloric Acid 4N

One part of concentrated hydrochloric acid was mixed into two parts of distilled water.

Glucostat Reagents

This commercially prepared reagent by Worthington Biochemical Co. (Freehold, N.Y.) contained two vials. One had the hydrolyzed enzyme glucose oxidase with 0.005 M phosphate buffer, pH 7 and a second vial contained the solid chromogen (O-dianisidine). All reagents were stored in the refrigerator when received and used before the expiration date marked on each package.

In the present experiment, the semi-micro method was followed. The chromogen was dissolved in a small amount of distilled water and added to a graduate cylinder containing 30 ml of distilled water. The content of the glucostat vial was dissolved and added to the graduate. The final volume was adjusted to 50 ml with distilled water.

The reagent, once made up, slowly became colored as it stood. This was a photosensitive reaction. Thus, this reagent had to be prepared a few hours before use and stored in dark bottles to retard the reaction. The reagent was used on the same day for glucose analysis, as excessive color often developed on prolonged storage.

Summary of Procedure

1. Prepare all reagents as described above.

2. Add 0.1 ml of plasma to 1.9 ml of distilled water. Mix. Add 1 ml barium hydroxide and mix. Add 1 ml of zinc sulfate solution; mix and centrifuge to precipitate the protein portion.

3. 0.1 ml of glucose standard containing from 50 to 450 mg % is substituted for plasma in the preparation of standards using the above procedure for the unknown.

4. Pipette 2 ml of each filtrate (unknowns and standards) into the respective tubes. Into reagent blank, pipette 2 ml of distilled water.

5. Add 2 ml glucostat reagent to each tube at timed intervals. Mix and let stand for at least 5 minutes. Color is stable for several hours.

6. Read absorbance of solution in Beckman spectrophotometer at 420 mλ. First set at zero absorbance with reagent blank.

7. Calculate the concentration of plasma glucose in unknowns as follows:

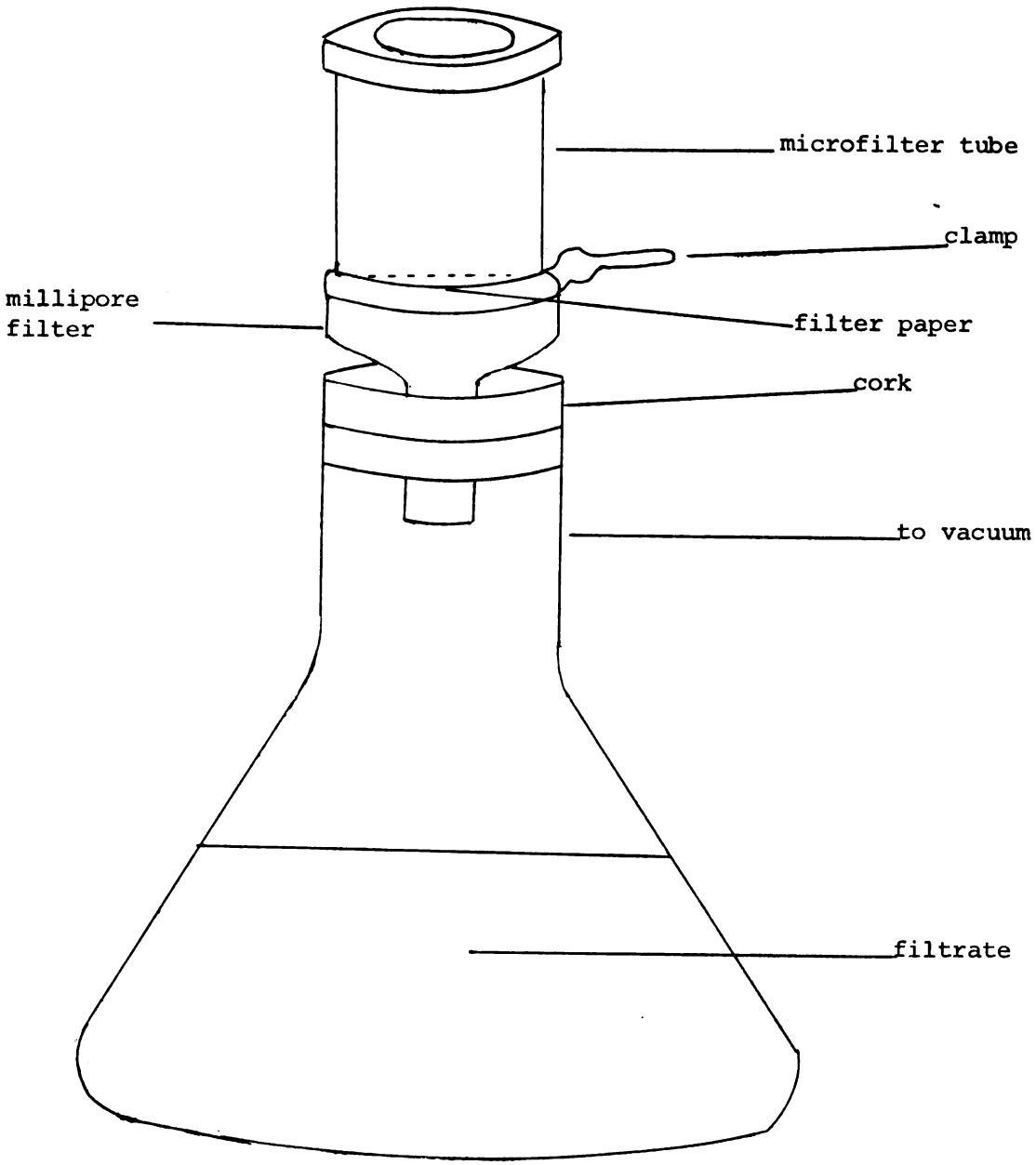
$$\frac{\text{Absorbance of unknown x Concentration of known standards}}{\text{Absorbancy of known glucose standard}}$$

APPENDIX II

MICRO FILTRATION APPARATUS OF IMMUNOASSAY OF INSULIN

APPENDIX II

MICRO FILTRATION APPARATUS OF IMMUNOASSAY OF INSULIN



APPENDIX III

IMMUNOASSAY OF PLASMA INSULIN

APPENDIX III

IMMUNOASSAY OF PLASMA INSULIN

Reagents

Deionized Water

The variations in the quality of ordinary distilled water might give rise to considerable variations in the amount of insulin bound to the antibody precipitate; therefore, distilled water was passed through a mixed bed ion exchanger (Sargent Co., Detroit, Mich.) adjusted to a flow rate of about 5 ml per minute.

Phosphate Buffer A

This buffer solution containing sodium ethyl mercuriothiosalicylate (0.6 millimolar) and bovine plasma albumin (0.5%) was used for the dilution of antisera and iodinated insulin. It was prepared with 6.2 gm sodium phosphate disbasic crystals ($NaH_2PO_4.7H_2O$, Baker Chemical Co., N.J.); 0.25 gm sodium ethyl mercurithiosalicylate (thimerosal, K and K Lab., N.Y.); 5.1 gm bovine albumin (Sigma Chemical Co., Miss.) and made up to 1000 ml with deionized water, and adjusted to pH 7.4 using aqueous 2 N sodium hydroxide solution (Mallinckrodt Chemical, Mo.).

Isotonic Buffer B-1

This buffer was used for dilution of standard insulin and plasma sample, containing 0.9 gm sodium chloride (Mallinckrodt Chemical, Mo.) and made up to 100 ml with phosphate buffer A.

Buffer C-1

This buffer of high protein content, used for washing antibody precipitates, contained 21 gm bovine albumin and 600 ml phosphate buffer A.

All three buffers were stored in the refrigerator at 2° to 4°C.

Scintillant

It was prepared using 150 ml ethanol (spectro-analyzed); 300 ml p-Dioxane (spectroanalyzed); 50 gm naphthalene crystals; 5 gm 2-5 diphenyloxazole (scin-tillanalyzed) and made up to 1000 ml with tolulene (scintillanalyzed). It was stored in a dark bottle and refrigerated. Ethanol, p-dioxane, naphthalene and tolulene were obtained from Fisher Chemical, Detroit, Mich. 2-5 diphenyloxazole was purchased from Amersham/ Searle Co. (Arlington Heights, Ill.).

Insulin Standards

The contents of the standardized human insulin bottle was dissolved in 2 to 3 ml of buffer B-1 and made up to 10 ml with the same buffer. This stock solution

now contained 200 micro units per ml. Further dilutions for standards were made up as follows:

Reconstituted Standard ml	Buffer B-1 ml	Final Insulin Concentration µUnits/ml
2.0	to 5.0	80
1.5	to 5.0	60
2.5	to 10.0	50
1.0	to 5.0	40
1.5	to 10.0	30
0.5	to 5.0	20
0.5	to 10.0	10
0.25	to 10.0	5

Repeated freezing and thawing of solutions of protein nature was not recommended; thus, each standard solution was divided into several aliquots and stored deep frozen at -40°C.

Insulin I-125

This was prepared from specially purified crystalline ox insulin potency 24.3 international units/mg. Unbound iodine had been removed by gel filtration. Each "Insulin Kit" had one vial containing 0.1 µg of insulin I-125 dissolved in 5 ml of buffer A. The working solution was prepared by adding 1 ml of the reagent, with the aid of a micro syringe (Hamilton Co.) to 7 ml of buffer A. A total of five bottles of stock solutions could be prepared and stored in the deep frozen state until use. Once thawed, the working solution was used immediately for insulin analysis. Any unused solutions could be kept in

the refrigerator for not more than a week. Refreezing and thawing of I-125 insulin was not recommended.

Summary of Procedure

1. Set up siliconized tubes for blanks, standards and unknowns. Add 0.1 ml prepared insulin binding reagent to all tubes. Add 0.1 ml buffer B to blanks. Add 0.1 ml sample plasma diluted with buffer B to tubes labeled unknowns. Add 0.1 ml of known insulin solution to tubes labeled standards. Mix the content in each tube; cork tightly and refrigerate at 2° to 4°C for six hours.

2. Add 0.1 ml of prepared iodinated insulin solutions to each tube. Mix and refrigerate again for 18 hours.

3. Collect the precipitate by microfiltration through the membrane filter disc (soaked in buffer C until needed) using a micro analysis filter holder and flask. Remove disc while under vacuum. Place disc in the corresponding counting vial and dry for 10 min. in the oven.

4. After the vials have cooled to room temperature, add 10 ml of scintillant to each.

5. Measure the radioactivity using a liquid beta scintillation counter. Calculate results in counts per minute and average count rates for each series of replicates.

6. Plot the amount of bound iodinated insulin of each standard against the corresponding known concentrations; unknowns can be read off directly from the graph.

APPENDIX IV

PLASMA NEFA DETERMINATION

APPENDIX IV

PLASMA NEFA DETERMINATION

Reagents

Indicator Solution

0.005 gm thymothylein in 50 ml spectroscopic grade heptane/acetone solution (10:1, v/v).

Extraction Mixture

A solution containing 40/10/1 (v/v/v) of spectroscopic grade isopropyl alcohol (Fisher Chemical, Detroit, Mich.), heptane and 1 N sulfuric acid.

Palmitic Acid Standard

A stock solution was made of 10.25 mg palmitic acid dissolved in 50 ml heptane. This solution contained 800 µeq/litre. Working standards were prepared from this stock prior to each fatty acid analysis in the following manner:

µeq/litre	700	600	500	400	300	200	100
800 µeq/L stock (ml)	8.75	7.5	6.25	5.0	3.75	2.5	1.25
Heptane (ml)	1.25	2.5	3.75	5.0	6.25	7.5	8.75

Summary of Procedure

1. Prepare all the reagents as described above.

2. Set up a series of tubes for blanks, standards and unknowns. Add 0.5 ml plasma, standard or heptane to each corresponding test tube containing 3 ml of extraction mixture. Mix and add 1 ml heptane. Mix and add 2.5 ml of distilled water. Mix and remove 1 ml supernate containing NEFA to corresponding tubes which contain 0.1 ml of indicator solution.

3. Mix and titrate with TBAH solution to a light blue end point, with nitrogen gas being bubbled into the solutions during the whole titration process.

4. Record units of titrant used for each known standard, unknown and blank. Minus the value of the blank from the standards and unknown. Plot units of titrant for each standard against known concentration on a linear graph paper. Read directly or calculate the plasma NEFA level from the standard curve as follows:

$$\frac{\text{Units of titrant of unknown} \times \text{Concentration of known standard}}{\text{Units of titrant of known palmitic standard}} \ \mu eq/L$$

LITERATURE CITED

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Anderson, J. W., R. H. Herman and D. Zakim. 1968. Glucose tolerance and insulin response to prolonged high carbohydrate feeding in normal men. Amer. J. Clin. Nutr. 21, 529.

Blazquez, E. and C. Lopez Quijada. 1968. The effect of a high fat diet on glucose, insulin sensitivity and plasma insulin in rats. Endocr. 42, 489.

Blazquez, E., M. Castro and E. Herrera. 1971. Effect of a high fat diet on pancreatic insulin release, glucose tolerance and hepatic gluconeogenesis in male rats. Revista Espanola De Fisologia 27, 297.

Bonnet, F., L. Gosselin, J. Chantraine and J. Senterre. 1970. Free fatty acids pattern in the plasma of normal and obese children during fasting and intravenous glucose tolerance test. Arch. Intern. Phys. Biochim. 78, 495.

Borrebaek, B. and Ø. Spydevold. 1970. Decreased hexokinase activity of the rat epididymal fat pad caused by high level of free fatty acid. A possible factor in the mechanism of dietary adaption. Biochim. Biophys. Acta. 201, 215.

Bortz, W. M. and F. Lynen. 1963. The inhibition of acetyl CoA carboxylase by long chain acyl CoA derivatives. Biochem. Z. 337, 505.

Bringolf, M., N. Zaragoza, D. Rivier and J. P. Felber. 1972. Studies of metabolic effects induced in the rat by a high fat diet. Inhibition of pyruvate metabolism in diaphragm *in vitro* and its relation to the oxidation of fatty acids. Eur. J. Biochem. 26, 360.

Brunzell, J. D., M.D., R. L. Lerner, M.D., W. R. Hazzard, M.D., D. Porte, Jr., M.D. and E. L. Bierman, M.D. 1970. Improved glucose tolerance with high carbohydrate feeding in mild diabetes. New Eng. J. Med. 248, 521.

Butturini, U., A. Gnudi, C. Coscelli, E. Valenti and U. Palami. 1966. Glucose tolerance associated with elevated plasma nonesterified fatty acids. J. Clin. Inves. 44, 2010.

Christophe, J. and J. Mayer. 1959. Influence of diet on utilization of glucose and incorporation of acetate C-14 into liver fatty acid and cholesterol in rats. Amer. J. Phys. 197, 55.

Corvilain, J., M.D., H. Loeb, M.D., A. Champenois, M.D. and M. Abramow, M.D. 1961. Effect of fasting on levels of plasma nonesterified fatty acids in normal children, normal adults and obese adults. Lancet 1, 534.

Dole, V. P. 1956. A relation between nonesterified fatty acids in plasma and the metabolism of glucose. J. Clin. Invest. 35, 150.

Duncan, G. G., M.D., F. C. Cristotori, M.D., J. K. Yue, M.D. and M. S. J. Murphy, M.D. 1964. The control of obesity by intermittent fasts. Med. Clin. of N. Amer. 48, 1359.

Exton, J. H., N. Friedmann, E. H. A. Wong, J. P. Brineaux, J. D. Corbin and C. R. Park. 1972. Interaction of glucocorticoids with glucagon and epinephrine in the control of gluconeogenesis and glycogenolysis in liver and of lipolysis in adipose tissue. J. B. C. 247, 3579.

Frederickson, D. S. and R. S. Gordon, Jr. 1958. Transport of fatty acids. Physiol. Rev. 38, 585.

Golderick, R. S. and J. Hirsch. 1964. Serial studies on the metabolism of human adipose tissue. II. Effects of caloric restriction and refeeding on lipogenesis and the uptake and release of free fatty acids in obese and nonobese individuals. J. Clin. Inves. 43, 1793.

Gordon, E. S. 1964. New concepts of the biochemistry and physiology of obesity. Med. Clin. N. Amer. 48, 1285.

Hales, C. N. and P. J. Randle. 1963a. Effects of low carbohydrate diet and diabetes mellitus on plasma concentrations of glucose, nonesterified fatty acids and insulin during oral glucose tolerance test. Lancet 1, 790.

Hales, C. N. and P. J. Randle. 1963b. Immunoassay of insulin with antibody precipitate. J. Biochem. 88, 137.

Harper, H. A. 1971. Review of physiological chemistry, Lange Medical Publications, Los Altos, California, p. 414.

Herrera, M. G. and G. F. Cahill, Jr. 1965. Control of hepatic gluconeogenesis. Clin. Res. 13, 323.

Himsworth, H. P. 1935. The dietetic factor determining the glucose tolerance and sensitivity to insulin of healthy men. Clin. Sci. 2, 67.

Issekutz, B., Jr., W. M. Bortz, H. I. Miller and P. Paul. 1967. Turnover rate of plasma FFA in humans and in dogs. Metabolism 16, 1001.

Jeanrenaud, B. 1961. Dynamic aspects of adipose tissue metabolism. A review. Metabolism 10, 535.

Karam, J. H., G. M. Grodsky and P. H. Forsham. 1965. The relationship of obesity and growth hormone to serum insulin levels. Ann. N.Y. Acad. Sci. 131, 374.

Katsumata, K. 1970a. Studies on the diabetic state of rats fed a high fat diet for 400 days. Nagoya J. Med. Sci. 32, 261.

Katsumata, K. 1970b. The disturbance of the carbohydrate metabolism and the specific retinal lesions of rats fed with a high fat and high calory diet. Ibid. 407.

Keston, A. S. 1956. Specific colorimetric enzymatic analytical reagents for glucose. Absr. of papers, 129th meeting, A.C.S. Dallas, Texas. p. 31c.

Knittle, J. L. and J. Hirsch. 1968. Effect of early nutrition on the development of rat epididymal fat pads. Cellularity and metabolism. J. Clin. Inves. 47, 2091.

Ko, H. and M. E. Royer. 1967. A submicromolar assay for nonpolar acids in plasma and depot fat. Analy. Biochem. 20, 205.

Kruger, F. A., J. M. Carhart and R. A. Austad. 1964. The glucose fatty acid cycle and the liver. J. Lab. Clin. Med. 64, 876.

Lehninger, A. L. 1970. Biochemistry. The molecular basis of cell structure and function. Worth Publishers, Inc., N.Y. ch. 23, p. 513.

Lemmonier, D. 1969. Experimental obesity in the rat by high fat diet. Physiopathology of adipose tissue. Third International Meeting of Endocrinology. Excerpta Medica Foundation, Vague, J., Amsterdam, p. 197.

Leveille, G. A. 1970. Adipose tissue metabolism: Influence of periodicity of eating and diet composition. Fed. Pro. 29, 1294.

Malaisse, W. J., D. Lemmonier, F. Malaisse-Lagae and I. M. Mandelbaum. 1969. Secretion of and sensitivity to insulin in obese rats fed a high fat diet. Horm. Metab. Res. 1, 9.

Mayes, P. A. 1960. An inverse relation between the liver glycogen and the blood glucose in the rat adapted to a high fat diet. Nature 187, 325.

McComb, R. B. and W. D. Yuslack and D. B. Bratti. 1957. 2-Deoxy-D-Glucose. A new substrate for glucose oxidase. J. Franklin Inst. 263, 161.

Merimee, T. J., M.D. 1971. Obesity and hyperinsulinism. New Eng. J. Med. 285, 856.

Mertz, W. 1969. Chromium occurrence and function in biological systems. Physiol. Review. 49, 163.

Mickelsen, O., S. Takahashi and C. Craig. 1955. Experimental obesity. 1. Production of obesity in rats by feeding a high fat diet. J. Nutr. 57, 541.

Morse, W. I., J. J. Sidorov, J. S. Soeldner and R. C. Dickson. 1960. Observations on carbohydrate metabolism in obesity. Metabolism 9, 666.

Muller, D. 1928. Studien uber ein neues enzym Glykose-oxydase. Biochem. Z. 199, 136.

Nestle, P. J., K. F. Carroll and M. S. Silverstein. 1964. Influence of free fatty acids metabolism on glucose tolerance. Lancet 1, 116.

Newburgh, L. H. and J. W. Conn. 1939. A new interpretation of hyperglycemia in obese middle aged persons. J.A.M.A. 112, 7.

Niemeyer, H., L. Clark-Turri, E. Garces and F. E. Vergara. 1962. Selective response of liver enzymes to the administration of different diets during fasting. Arch. Biochem. Biophy. 98, 77.

Ogilvie, R. F. 1935. Sugar tolerance in obese subjects. A review of 65 cases. Quarterly J. Med. 4, 345.

Opie, L. H. and P. G. Walfish. 1963. Plasma free fatty acid concentrations in obesity. New Eng. J. Med. 268, 757.

Pelkonen, R., M.D., T. A. Miettinen, M.D., M. R. Taskinen, M.D. and E. A. Nikkila, M.D. 1968. Effect of acute elevation of plasma glycerol, triglyceride and FFA levels on glucose utilization and plasma insulin. Diabetes 17, 76.

Rabinowitz, D. and K. L. Zierler. 1962. Forearm metabolism in obesity and its response to intra-arterial insulin. Characterization of insulin resistance and evidence for adaptive hyperinsulinism. J. Clin. Inves. 41, 2173.

Randle, P. J., P. B. Garland, C. N. Hales and E. A. Newsholme. 1963. The glucose fatty acid cycle, its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. Lancet 1, 785.

Resier, P. 1967. Insulin, membrane and metabolism, Williams and Wilkins, Wilkins, Baltimore. p. 25.

Rudman, D., S. J. Brown and M. F. Malkin. 1963. Adipokinetic actions of ACTH, TSH, vasopressin, melonophore stimulating hormone, fraction A, epinephrine and norepinephrine in rabbit, guinea pig, hamster, rat, pig and dog. J. Lipid Res. 4, 527.

Saggerson, E. D. and A. L. Greebaum. 1970. The regulation of triglyceride synthesis and fatty acid synthesis in rat epididymal adipose tissue. Effect of altered dietary and hormonal conditions. Biochem. 119, 221.

Saifer, A. and S. Gerstenfeld. 1958. The photometric microdetermination of blood glucose with glucose oxidase. J. Lab. Clin. Med. 51, 448.

Samuels, L. T., R. M. Reineke and H. A. Ball. 1942. Effect of diet on glucose tolerance and liver and muscle glycogen of hypophysectomized and normal rats. Endoc. 31, 35.

Salans, L. B., J. L. Knittle and J. Hirsch. 1968. The role of adipose cell size and adipose tissue insulin sensitivity in the carbohydrate intolerance in human obesity. J. Clin. Inves. 47, 153.

Schalch, D. S. and D. M. Kipnis. 1965. Abnormalities in carbohydrate tolerance associated with elevated plasma nonesterified fatty acids. J. Clin. Inves. 44, 2010.

Schemmel, R., O. Mickelsen and Z. Tolgay. 1969. Dietary obesity in rats: influence of diet, weight, age and sex on body composition. Amer. J. Physiol. 216, 373.

Schemmel, R., O. Mickelsen and J. L. Gill. 1970. Dietary obesity in rats: body weights and fat accretion in seven strains of rats. J. Nutr. 100, 1041.

Schemmel, R., O. Mickelsen and K. Motawi. 1972. Conversion of dietary to body energy in rats as affected by strain, sex and ration. J. Nutr. 102, 1187.

Schwarz, K. and W. Mertz. 1959. Chromium (III) and glucose tolerance factor. Arch. Biochem. Biophy. 65, 292.

Seyffert, W. A., M.D. and L. L. Madison, M.D. 1967. Physiologic effects of metabolic fuels on carbohydrate metabolism. 1. Acute effect of elevation of plasma free fatty acids on hepatic glucose output, peripheral glucose utilization, serum insulin and plasma glucagon levels. Diabetes 16, 765.

Sims, E. A. H. and E. S. Horton. 1968. Endocrine and metabolic adaptation to obesity and starvation. J. Clin. Nutr. 21, 1455.

Sokal, R. R. and F. J. Rohlf. 1969. Biometry. The principles and practice of statistics in biological research. W. H. Freeman and Company, San Francisco.

Steinberg, D., M. Vaughan and S. Magolis. 1961. Studies of triglyceride biosynthesis in homogenates of adipose tissue. J. B. C. 236, 1631.

Stetten, D., Jr., and G. E. Boxer, 1944. Studies in carbohydrate metabolism. III. Metabolic defects in alloxan diabetes. J. B. C. 156, 271.

Stone, D. B. and W. E. Connor. 1963. The prolonged effect of a low cholesterol, high carbohydrate diet upon the serum lipids in diabetic patients. Diabetes 12, 127.

Teller, J. D. 1956. Direct, quantitative, colorimetric determination of serum or plasma glucose. Abstr. of Papers, 130th meeting. A.C.S. Atlantic City, J.J., p. 69c.

Treble, D. H. and J. Mayer. 1963. Glycerolkinase activity in white adipose tissue of obese hyperglycaemic mice. Nature (London) 200, 363.

Vaughan, D. A., J. P. Hannon and L. N. Vaughan. 1960. Effects of diet on selected glycolytic enzymes of the rat. Amer. J. Physiol. 199, 1041.

Waterhous, C., N. Baker and H. Postami. 1969. Effect of glucose ingestion on metabolism of free fatty acids in human subjects. J. Lipid Res. 10, 487.

Weber, G., M. A. Lea and N. B. Stamm. 1969. Regulation of hepatic carbohydrate metabolism by FFA and acetyl CoA: sequential feedback inhibition. Lipids 4, 388.

Weber, G., G. Banerjee and J. Ashmore. 1960. Activities of enzymes involved in glycolysis, glucogenesis and hexosemonophosphate shunt in rat adipose tissue. Bioch. Biophy. Res. Comm. 3, 182.

Whitney, J. E. and S. Roberts. 1955. Influence of previous diet on hepatic glycogenesis and lipogenesis. Amer. J. Physiol. 181, 446.

Wohl, M. G. 1968. Obesity. Modern nutrition in health and disease, Lea and Febiger. Philadelphia. ch. 33, p. 973.

Yarlow, R. S. and S. A. Berson. 1960. Immunoassay of endogenous plasma insulin in man. J. Clin. Inves. 39, 1157.

Yarlow, R. S., S. M. Glick, J. Roth and S. A. Berson. 1965. Plasma insulin and growth hormone levels in obesity and diabetes. Ann. N.Y. Acad. Sci. 131, 357.

Zaragoza, N. and J. P. Felber. 1970. Studies on the metabolic effects induced in the rat by a high fat diet. 1. Carbohydrate metabolism in vivo. Hormone Metab. Res. 2, 323.

Zaragoza-Hermans, N. and J. P. Felber. 1972. Studies of metabolic effects induced in the rat by a high fat diet. 11. Disposal of orally administered C-14 glucose. Horm. Metab. Res. 4, 25.