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The Ohio State University, Ph.D., 1977
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THE SHORT-TERM EFFECTS OF HIGH STARCH OR HIGH SUCROSE DIETS
ON HEPATIC LIPOGENESIS IN THE RAT

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Karla Louise Roehrig, B.S.

The Ohio State University
1977

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Most of all, I would like to express my appreciation to my husband, Dr. Fred K. Roehrig, for his constant encouragement, his enthusiasm for the endeavor and his continuing emotional and moral support. Without his help and cooperation, the task would not have been begun or completed.
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PUBLICATIONS

Abstracts of papers presented orally:
acetyl Co A carboxylase. Federation Proc. 36, 1159 Abs.

Full-length papers:
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INTRODUCTION

In the last few years, there has been increasing concern that the high carbohydrate consumption of much of the Western world may be an important factor in the etiology of cardiovascular disease. Suggestions have been made that diets high in sucrose might have a more deleterious effect than diets containing more complex carbohydrates. Since cardiovascular disease nearly always contains components of altered lipid metabolism, it is supposed that the effects of a high carbohydrate diet on the inception and progress of the disease would be due to its effects on lipogenesis.

This project was commenced to attempt to answer two questions. First, is there a difference in the effect of a high starch as opposed to a high sucrose diet on hepatic lipogenesis? Second, if there is a difference, what is the site and mechanism of action? It has long been known that refeeding a high carbohydrate diet to previously fasted rats is an effective way to stimulate hepatic lipogenesis to levels far above those of control rats fed ad libitum. Accordingly, a model system was designed in which rats fasted for seventy-two hours were refed a high starch or a high sucrose diet for one, two, three or five days in an effort to accentuate any differences in the effect of the two diets on hepatic lipogenesis.

The experiments which will be described show that the two dietary
regimes result in differences in both time and magnitude of maximum effect. Substrate concentrations and enzyme activities at sites likely to be important in the control of lipogenesis have been measured in an attempt to better define the mechanism of the observed differences.
Evidence suggests that even the ancient Egyptians knew that high carbohydrate diets could result in high levels of fat in the liver (1). Studies of the biochemistry of this phenomenon, however, were begun only in the last century when Lawes and Gilbert, two British researchers, in an investigation of the carcass composition of sheep and pigs relative to the composition of various natural diets were able to demonstrate that much of the fat in the animal body comes from carbohydrate in the diet (2). In 1916, Higgins (3) provided the first evidence that not all carbohydrates are equally effective in producing fat. Specifically, sucrose and fructose led to respiratory quotients (RQ) of 1.15 and 1.10, respectively about 20 minutes after oral ingestion which indicates that lipogenesis is occurring while glucose reached a peak RQ of only 0.96 nearly 80 minutes after ingestion. It was not until the late 1950's and early 1960's though that serious attention was given to the possible metabolic consequences, especially for the potential victim of coronary artery disease, of the consumption of diets high in a particular type of carbohydrate. Prior to this time, it was felt that dietary effects on the etiology of atherosclerosis were due predominately to the consumption of diets high in saturated fat and cholesterol and that the amount and type of carbohydrate in the diet was of little consequence.

This opinion began to change when Albrink and Man (4) in a study of
82 male coronary patients and 134 age-matched controls showed that only 18% of the coronary patients had fasting serum cholesterol levels above the normal range while 70% had fasting serum triglyceride values above normal. Fasting serum triglyceride is likely to have been derived mostly from triglyceride produced as the result of the consumption of carbohydrate and would be carried in the serum as a component of very low density lipoproteins (5). Further confirmation of the observation of Albrink and Man was provided by Carlson in 1960 (6) when he showed that young men who had had myocardial infarctions also had elevated serum triglycerides but low serum cholesterol.

That the type of carbohydrate was important to the serum lipid pattern in man was demonstrated by Keys et al. (7) in a study of 28 men confined to a mental hospital. It was found that diets high in starch fed for six weeks resulted in lower serum cholesterol levels than comparable diets in which sucrose was the carbohydrate source.

At approximately the same time, the results of a series of population and epidemiological studies appeared which also supported an apparent role for carbohydrate in the etiology of heart disease. The Samburu tribes of Kenya were found (8) to consume diets adequate in calories and very high in saturated fats from meat and milk and yet they had a very low incidence of cardiovascular disease compared to other aged matched populations. Masai tribesmen (9) eating substantially the same diet as the Samburu also had little heart disease. The population of St. Helena Island, on the other hand, consumed a diet low in fat (predominately unsaturated fat) and high in carbohydrate. They had a high incidence of coronary disease (10). Cohen (11) in a study of Yemenite Jews who
migrated to Israel found a low incidence of cardiovascular disease before and for a few years after migration when their diet consisted mostly of meat products and starch. They had a relatively high incidence of this health problem after 25 years of residence in Israel where they consumed about the same amount of meat products but where nearly 30% of their carbohydrate intake was in the form of sucrose.

Yudkin, who has been a principle proponent for the role of sucrose in cardiovascular disease, has based his belief largely on epidemiological studies concerning the correlation between the consumption of diets high in sucrose and increased cardiovascular disease (12-15). He has pointed out that increased affluence brings an increased consumption of meat but also a simultaneous shift from starch to sucrose as the source of carbohydrate calories. In addition, Yudkin noted that populations which are relatively wealthy become less physically active and that this might contribute to increased heart disease.

In further studies, workers began performing more controlled investigations than some of the early work on the effect of the source of carbohydrate on serum lipid levels (16-25). In general, the experimental diets in these studies were consumed for 3 to 4 weeks in a carefully monitored setting, and the subjects served as their own controls. It was found that dietary sucrose increased serum triglyceride levels far more than starch or glucose but there was usually less effect due to carbohydrate source on the levels of serum cholesterol. Hodges and Krehl (20) suggested that reductions in serum cholesterol in some studies using diets high in vegetable starch could have been due to an effect of increased fiber content of the diet which could enhance the
excretion of cholesterol from the body.

Studies were also performed using human subjects who had carbohydrate-induced hypertriglyceridemia. Kuo et al. (26) found that hyperlipidemia was accentuated by high sucrose diets and that fructose could also produce this effect. Kaufmann and coworkers (27-29) in similar studies also observed greater increases in plasma triglycerides in hypertriglyceridemic patients on a high sucrose versus a high starch or a high glucose diet. In two of their studies (28,29), fructose led to an even greater elevation of plasma triglycerides than did sucrose. Little et al. (30), however, could make a correlation between serum lipids and dietary constituents only in coronary patients but not in normal controls. This led them to suggest that a metabolic lesion renders a coronary patient more sensitive to dietary influences. It was also noted that when high carbohydrate diets were fed instead of polyunsaturated fat diets, there was an increase in serum cholesterol but not when they replaced saturated fat diets (31). The effects on serum triglyceride concentration observed in shifting from a high fat to a high carbohydrate diet (32) were age and sex related, with old men and postmenopausal women being the most responsive to the change in diet.

Investigators next began to look for the reason sucrose diets were more effective than starch diets in elevating serum triglycerides. Cohen (33) found impaired glucose tolerance curves in male volunteers who ate high sucrose diets for five weeks. In a group of 19 apparently healthy men, all experienced a rise in serum triglyceride levels (34). Six of the 19 also experienced a rise in serum immunoreactive insulin (IRI), an increase in weight and an increase in platelet adhesiveness.
Yudkin suggested that the sucrose effect on IRI, rather than its effect on blood lipids per se, might be the basis for an effect of sucrose on heart disease. Studies by Gertler et al. (35) indicated that patients with ischemic heart disease had elevated IRI. In another investigation, however, healthy young women, a group not very prone to heart disease, also showed an elevated IRI in response to high sucrose diets (36). Diabetics (maturity onset (MO)) have a far greater risk of being afflicted with cardiovascular problems (37), and it has been demonstrated (38) that one preclinical lesion in MO diabetes is an elevated IRI level in the serum.

Besides the changes in serum IRI, high sucrose diets led to a rise in the concentration of serum 11-hydroxycorticosteroid, increased platelet adhesiveness and decreased plasma fibrinogen (39). Sucrose diets also altered the fatty acid patterns in lecithins of serum, liver, and bile (40). An increased content of oleic acid in lecithins from the three sources was observed.

The effects of dietary carbohydrate in man have been extensively reviewed (41,42).

The induction of hypertriglyceridemia by high sucrose or by high fructose diets has been confirmed by investigators using the rat as an experimental model (43-47). Allen and Leahy (48) were able to demonstrate the existence of higher serum cholesterol levels in rats fed sucrose or fructose as compared to glucose for 26 weeks, but this was not confirmed by a separate study by Carrella and coworkers (47) who used a 10 day feeding period. Several investigators believe that the rise in plasma triglyceride which appears with sucrose or fructose diets
compared to starch or glucose diets is due to an alteration in the rate of triglyceride clearance from the plasma (49-52). In addition to an elevated plasma triglyceride level, investigators have found higher rates of hepatic triglyceride formation (50, 51) and increased liver fat (48) in rats fed sucrose or fructose relative to glucose-fed controls.

In a study (53) using meal-fed rats trained to eat a high starch diet, it was found that switching them to a high fructose diet for one meal led to elevated plasma triglycerides, lowered serum IRI, no effect on plasma glucose concentration and elevated plasma fructose levels relative to switching them to an equivalent amount of glucose for one meal. The same group of investigators found very little difference in IRI levels of rats fed glucose or fructose for 30 days (46). Cryer et al. (51) has suggested that the lower IRI in plasma which they observed in fructose-fed rats might be responsible for the lower adipose tissue clearing factor activity which they found in these rats. The lowered enzyme activity would then be expected to result in elevated levels of plasma triglycerides.

Investigations have been carried out on the effects of fructose on the perfused liver in the belief that it is the fructose moiety of sucrose which is primarily responsible for the alterations in lipid metabolism observed in both man and rats eating a high sucrose diet. Topping and Mayes (54) in an extensive investigation of the effects of perfusing rat liver with insulin ± fructose concluded that fructose uptake into liver was insulin independent, that both insulin and fructose enhanced the secretion of triglyceride from the liver and depressed the oxidation
of fatty acids. Further, there appeared to be no interaction of insulin and fructose on any of the observed effects. Fructose also elevated perfusate lactate concentrations, an effect which was also noted by Woods and Krebs (55).

Other studies have been carried out to examine the effects of various carbohydrates on specific enzymes in an effort to isolate the locus of action of these diets. There are almost as many opinions as to the most important site of action as there are potential regulatory sites. Since fructose enters the liver and is phosphorylated to fructose-1-phosphate by fructokinase (FK) (56), it has been suggested that fructose provides substrate for lipogenesis faster than glucose does. In one investigation it has been found that IP injection of fructose leads to an elevation in F-1-P concentration (58). Other studies have found little effect on FK activity in the liver (59). Some researchers have been able to show an enhancement of FK activity by fructose feeding (60,61). In a study on the aging response to dietary sugars (62), it was found that fructose or sucrose diets consistently elevated the activities of F-1-P aldolase and F-1,6-diP aldolase but that the adaptation to dietary carbohydrate became increasingly blunted with the advancing age of the rat. Elevation of hepatic aldolase activity by sucrose as compared to starch feeding has also been observed by Yudkin's group (63) and by Fitch and Chaikoff (64) for fructose verses glucose diets. High sucrose or high fructose diets have also been shown to lead to a rise in the gluconeogenic enzyme glucose-6-phosphatase (G-6-Pase) (65-67).
Stifel et al. (67) have found that refeeding rats fructose for three days after a 72 hour fast as compared to refeeding glucose elevates hepatic activities of phosphofructokinase (PFK), pyruvate kinase (PK) and glyceraldehyde-3-phosphate dehydrogenase (G-3-PD). The effect on PK has also been noted by other investigators (63,67).

The hepatic activities of glucose-6-phosphate dehydrogenase (G-6-PD) and malic enzyme (ME), both of which produce reducing equivalents in the form of NADPH which could be used for fatty acid biosynthesis, have been found to be increased by feeding sucrose to rats (69,70). In addition to a rise in the activities of enzymes which can produce NADPH for lipogenesis, a 4 to 6 fold increase in the activity of acetyl CoA carboxylase (AC), generally thought to be the rate-limiting enzyme for fatty acid biosynthesis, has been reported (67) for rats fed sucrose verses chow. This is in contrast to only a 2 to 3 fold rise for rats fed starch verses chow. An increase in the hepatic activity of fatty acid synthetase (FAS) with sucrose or fructose feeding has also been noted (71,72). It has been suggested that the effect on FAS could be due to an increase in α-glycerophosphate concentration (71) or to an increased rate of synthesis of FAS (72). Not only does sucrose and/or fructose appear to have an effect on enzyme activity while the diets are actually being fed, but one group has reported that the type of carbohydrate fed to rats from 21 to 50 days of age has an enduring influence on the metabolic response of those rats regardless of the type of carbohydrate fed after that time (73).

Thus, it appears that there is a general consensus of opinion that the type of carbohydrate does influence the hepatic lipogenic response.
Attempts to determine the site of apparent sucrose/fructose action have so far produced a variety of results and opinions. Indeed, the Teppersmans have concluded (74) that there is no single site of regulation for lipogenesis. Instead, a favorable "lipogenic climate" consisting of factors such as NADPH and cytoplasmic acetyl CoA availability, an appropriate ionic environment and concentrations of metabolic effectors is the result of synchronous control of each of the various factors.

A discussion of work which concerns regulation of lipogenesis and/or specific enzymes involved under dietary conditions other than starch/glucose versus sucrose/fructose will be included in the discussion section.
MATERIALS AND METHODS

Male, Sprague-Dawley rats\(^a\) weighing from 100 to 150 grams were housed singly in galvanized, wire mesh bottom cages and had access to control diet and water ad libitum for at least two weeks before beginning the experiment. The animals were maintained on a 7:00AM to 7:00PM light cycle with the light portion of the cycle during the day.

The animals were divided into ten groups of four each and assigned numbers randomly from one to forty. The numbers were used to assign a position in the cage banks so that any one group would not be subjected preferentially to potential variations in temperature, light, sound or other zeitgebers. After division into groups, a protocol for fasting, refeeding and killing was designed (Table 1). Due to experimental limitations, only six animals could be killed per day, so care was taken to spread the animals of any one group across the entire experimental period. Further, if a starch refeed one day animal (St-1) was killed, the corresponding sucrose refeed one day animal (Suc-1) was also killed the same day. Except for the group of control animals, the rats were fasted for 72 hours before being refeed the appropriate diet. The composition of the control, starch and sucrose diets is shown in Table 2.

\(^a\) Animals were obtained from Laboratory Animal Supply, The Ohio State University, Columbus, Ohio.
<table>
<thead>
<tr>
<th>Group</th>
<th>#</th>
<th>Days</th>
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<tr>
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<tr>
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<td>K</td>
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<td>25</td>
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<tr>
<td>St-1</td>
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<td>F</td>
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<tr>
<td>Suc-1</td>
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### Table 2. Composition of diets.

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<th>Component</th>
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<th>Starch</th>
<th>Sucrose</th>
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<tr>
<td>Glucose</td>
<td>55%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Argo Corn Starch</td>
<td>0%</td>
<td>60%</td>
<td>0%</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0%</td>
<td>0%</td>
<td>60%</td>
</tr>
<tr>
<td>Vitamin-free casein(^a)</td>
<td>21%</td>
<td>21%</td>
<td>21%</td>
</tr>
<tr>
<td>α-cel Nonnutritive fiber(^a)</td>
<td>13%</td>
<td>13%</td>
<td>13%</td>
</tr>
<tr>
<td>Salt Mix USP XIVA(^a)</td>
<td>4%</td>
<td>4%</td>
<td>4%</td>
</tr>
<tr>
<td>Vitamin diet fortification mixture(^a,(^b))</td>
<td>2%</td>
<td>2%</td>
<td>2%</td>
</tr>
<tr>
<td>Mazola Corn Oil</td>
<td>5%</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

\(^a\) Obtained from ICN Life Sciences Group, Cleveland, Ohio
\(^b\) Contains (in gms/kg of mixture): Vit. A concentrate 4.5 (20,000 U/gm); Vit. D 0.25 (400,000 U/gm); α Tocopherol 5.0; Ascorbic Acid 45; Inositol 5.0; Choline chloride 75; Menadione 2.25; p-Aminobenzoic Acid 5.0; Niacin 4.5; Riboflavin 1.0; Thiamine Hydrochloride 1.0; Calcium Pantothenate 3.0; Biotin 0.02, Folic Acid 0.09; Vit. B\(_12\) 0.0135. The vitamin diet fortification mixture is triturated with dextrose.
For experiments where the rats were weighed, the process was completed approximately one hour before the killing time in an attempt to avoid extra trauma to the animals. The rats were killed at 10:30AM ± 15 minutes by a blow to the head and subsequent decapitation. The large lobes of liver which were used to prepare liver slices were rapidly excised and each was placed in 50 ml of ice cold saline. Smaller lobes were excised and homogenized immediately (except as noted) for enzyme assays. In experiments where substrates were determined, a portion of the liver was freeze-clamped in situ with tongs cooled to -195°C in liquid nitrogen. The frozen liver was stored at -195°C until being finely powdered in a stainless steel percussion mortar cooled to -195°C. A weighed portion of the powder was homogenized in 6% perchloric acid. Denatured protein was then centrifuged out and the extract was neutralized in preparation for the substrate assays.

Blood samples were obtained from the hepatic portal vein immediately after decapitation. The blood was placed in heparinized screw cap tubes which were stored on ice for blood glucose determination.

All biochemical reagents were obtained from the Sigma Chemical Co., St. Louis, Mo. except for acetyl CoA which was obtained from P and L Biochemicals, Milwaukee, Wisc. and acetate-1-14C and 3H2O which were purchased from New England Nuclear Corp., Boston, Mass.

Liver Slice Experiments

Slices from each liver were prepared in 0.9% ice cold saline using a Stadie-Riggs microtome. One litre of fresh saline was used for each liver. Incubations were performed in duplicate in siliconized 25 ml
erlenmeyer flasks with 3 ml of Krebs Ringer buffer (75) plus 11 mM glucose and either acetate-1-\( ^{14} \text{C} \) or \( ^{3} \text{H}_2\text{O} \). Each flask contained three slices of liver (approximately 0.5 gm total) which had been blotted on filter paper and weighed. The incubations were performed in a Dubnoff metabolic shaker at 37°C for one hour with gentle shaking (Dubnoff setting 3) under a flow of 95% \( \text{O}_2 \)/5% \( \text{CO}_2 \) (6 liters/min.). At the end of the incubation period, the samples were placed on ice and then homogenized with 20 ml of chloroform:methanol (1/3) in a Potter Elvejhem homogenizer. The homogenates were next heated to 55°C for approximately 5 minutes then centrifuged at 500 x G for 10 minutes in an International Refrigerated Centrifuge. The supernatant was transferred to a 250 ml round bottom flask and the pellet was re-extracted with ethanol:diethyl ether (3/1). After recentrifugation, the supernatant from the second extraction was combined with the first and both were evaporated to dryness in vacuo. The dried contents of the flasks were extracted with a total of 15 ml of chloroform:methanol (4/1) in two portions. The chloroform: methanol extract was washed with 5 ml of water, and the two phases were allowed to separate overnight after which the upper water phase was removed by aspiration. The lower phase was then poured over anhydrous sodium sulfate which was then washed with several small portions of chloroform. The lipid extract was transferred to pre-tared tubes and evaporated to dryness under a stream of nitrogen. Total lipid was determined gravimetrically. Dioxane-counting solution (76) was added to the samples and the \( ^{14} \text{C} \) or \( ^{3} \text{H} \) incorporation into lipid was determined on a Packard Tricarb Liquid Scintillation Counter.
For determination of acetate-1-\(^{14}\)C incorporation into fatty acids and cholesterol of liver slices, aliquots of the lipid samples were saponified in ethanolic KOH after which the fatty acids and cholesterol were extracted and streaked on 250 \(\mu\)m thick Silica Gel H thin layer plates. The plates were developed first in a hexane:diethyl ether (80/20) solvent system, then again in the same direction in hexane:diethyl ether (90/10). Regions corresponding to cholesterol or fatty acid standards were scraped into scintillation vials and counted using dioxane counting solution (76).

**Substrate Determinations**

Blood glucose was determined colorimetrically by the glucose oxidase/peroxidase method using a Sigma kit (77).

Two methods were used for glycogen determinations. The initial experiments were done using a sulfuric acid digestion method with subsequent determination of glucose (78) using the Sigma kit for glucose (77). Later a rapid, enzymatic method for glycogen which had been developed in this laboratory was used (79). This method entailed the digestion of tissue homogenates with amyloglucosidase then the subsequent determination of the glucose liberated. Use of this method yielded a more sensitive assay for glycogen than the previous methods.

Free fatty acid concentrations were determined by microtitration with NaOH (80) using an American Instrument Company Microtitrimeter.

Lactate, \(\alpha\)-glycerol phosphate and glycerol concentrations in the neutralized extract were determined sequentially in the same cuvette by the fluorimetric method of Hohorst et al. (81). After lactate
and diglycerol phosphate had been determined, ATP, MgCl₂ and purified glycerol kinase were added to the cuvette in order to obtain the glycerol concentration. Glucose-6-phosphate and fructose-6-phosphate were also determined by the method of Hohorst (81).

The cytoplasmic and mitochondrial concentrations of CoA and acetyl CoA were determined by first rapidly separating the two fractions by centrifugation. A perchloric acid extract of each fraction was made. Acetyl CoA and CoA were measured in the neutralized extracts by the method of Allred and Guy (82).

Protein concentration of the livers was determined (83) on the various fractions used for enzyme assays.

**Enzyme Activity Determinations**

Glucose-6-phosphate dehydrogenase [E.C.1.1.1.49 D-glucose-6-phosphate:NADP+ oxidoreductase] (G-6-PD), isocitrate dehydrogenase [E.C.1.1.1.42 L-isocitrate:NADP+ oxidoreductase (decarboxylating)] (ICD), malic enzyme [E.C.1.1.1.40 L-malate:NADP+ oxidoreductase (decarboxylating)] (ME), fructokinase [E.C.2.7.1.3 ATP:D-fructose-1-phosphotransferase] (FK) and phosphofructokinase [E.C.2.7.1.11 ATP:D-fructose-6-phosphate-1-phosphotransferase] (PFK) were all assayed in a 700 x G supernatant of a 10% homogenate prepared in ice cold water. G-6-PD, ICD and ME were assayed fluorimetrically by the methods of Taketa and Pogell (84), Plaut (85) and Ochoa (86), respectively. FK was assayed by the system of Adelman et al., while PFK activity was determined by the method of Mansour and Ahlfors (88). Citrate cleavage enzyme (89) [E.C.4.1.3.8 ATP:citrate oxaloacetate-lyase (ATP dephosphorylating)]
(CCE) was determined in a 10% homogenate prepared in water using liver which had been frozen and stored for 24 hours at -20°C. The activity of glucose-6-phosphatase \([\text{E.C. 3.1.3.9 D-glucose-6-phosphate phosphohydrolase}] (\text{G-6-Pase})\) was determined by the method of Nordlie and Arion (90).

Acetyl CoA synthase \([\text{E.C.6.2.1.1 acetate:CoA ligase (AMP)}] \text{(AS)}\) was measured by a modification of the radioactive acetate incorporation method of Reijnierse et al. (91). The assays were performed using fresh liver homogenized with 1.5 volumes of cold water and centrifuged for 15 minutes at 700 x G. The total volume of the reaction mixture was reduced from 0.9 mls to 0.251 mls while the concentrations of the constituents remained the same. In place of column chromatography to separate the labelled acetyl CoA from the other components, aliquots of the boiled reaction mixtures were plated on microcrystalline cellulose thin layer plates\(^{a}\) and developed in a butanol:glacial acetic acid:water system (5/2/3). Acetyl CoA standards were plated simultaneously, and after development, the spots were visualized with a UV light. The spots corresponding to acetyl CoA were scraped off and put into scintillation vials. The cellulose scrapings were moistened with 0.1 mls of water and 0.1 mls of absolute ethanol before dioxane scintillation counting fluid (76) was added.

Acetyl CoA carboxylase \([\text{E.C.6.4.1.2 Acetyl CoA:CO}_2 \text{ ligase (ADP)}] \text{(AC)}\) was assayed in 27,000 x G supernatant prepared from fresh liver homogenized with 1.5 volumes of cold water. The supernatants were

\(^a\) Avicel plates were obtained from Analtech Inc., Newark, Delaware.
filtered through four layers of cheesecloth before the assay procedure. The method, developed earlier in this laboratory (92,93), measures acid stable $^{14}$C fixed from $^{14}$C bicarbonate in a one minute assay. The reaction mixture contained 60 mM tris acetate; pH 7.8; 0.5 mM potassium citrate; 0.3 mg BSA/sample; 3 mM reduced glutathione; 0.99 mM ATP; 25 mM potassium bicarbonate (2 $\mu$C $^{14}$C bicarbonate per sample); 8 mM magnesium acetate; and 0.35 mM acetyl CoA. A premix of the reagents was prepared fresh daily with the components being added in the order given above. After a one minute incubation period of the premix and the enzyme, the reaction was stopped with 0.05 mls of concentrated HCl. In experiments to determine the ability of the enzyme to be heat activated, the homogenates were preincubated at 37°C for 15 or 30 minutes before being assayed. Aliquots of the stopped reaction mixtures were put into scintillation vials and dried overnight at 45°C in a stream of filtered air. After drying, 0.1 mls of water and 0.1 mls of absolute ethanol were added to solubilize the residues before dioxane counting solution (76) was added and the samples were counted.

Glutamic dehydrogenase [E.C.1.4.1.3 L-glutamate:NAD(P) oxidoreductase (deaminating)] (GD) was determined (94) in mitochondria and cytoplasm of 10% homogenates of the livers of dextrose control rats in order to assess the degree of integrity of the mitochondria. The assays were performed immediately after centrifugation (35 minutes after the excision of the liver) and after 30 minute or 60 minute incubations at 0 to 4°C of the homogenates prior to centrifugation.

Since a large number of parameters were studied, not all could be feasibly accommodated in the same experiment. In all cases, however,
acetate-1-\textsuperscript{14}C incorporation into total lipids of liver slices was performed as a part of each experiment in an effort to be certain that any particular experiment was as nearly equivalent to any other experiment as possible.
RESULTS

In the experiments reported here, rats were refed isocaloric diets ad libitum containing either corn starch or sucrose for periods of one, two, three or five days after a 72 hour fasting period. One group of rats which served as the basal control group remained continuously on the pre-experimental diet which contained 55% glucose and 5% corn oil. As shown in Figure 1, the rats lost an average of 48 grams during the 72 hour fasting period. None of the pre- or post-fasting average weights for any one of the ten groups deviated significantly from the values observed as the average for all of the rats combined. There was no difference due to diet in the amount of weight regained, and by the end of the five day refeeding period, both starch and sucrose refed rats had completely regained the weight lost during the fasting period. Although per diem food consumption was not measured, these data support the qualitative observation that starch-refed and sucrose-refed rats consumed the same amount of food.

In Figure 2 the weight of the liver as a percentage of body weight for the fasting and refed groups is shown. It should be noted that refeeding causes a nearly two-fold increase in the liver weight with respect to body weight. On days three and five, the liver is a higher percentage of the body weight for sucrose-refed rats than for starch-refed rats. The livers also differed markedly in appearance during the
Figure 1. Change in body weight from prefast value. The initial average weight for 40 rats was 266.7 ± 4.0 gms (S.E.M.). The value after 72 hours of fasting is at 0 days.
Figure 2. Liver as a per cent of body weight. Values ± S.E.M. For analysis of variance, see Appendix B.
refeeding period. Livers from fasted rats were always very dark, small and quite firm. Livers from rats refed starch were lighter in color and most enlarged after two days of refeeding. The livers from the sucrose-refed rats were invariably the palest in color regardless of the number of days of refeeding. They approached a light beige on the third day of refeeding and were very enlarged and extremely fragile. After five days of refeeding, they began to return to a more normal color, texture and size.

There was also a change in cytoplasmic protein content per gram of liver during the experimental period (Figure 3). The precipitous drop in protein concentration after one day of refeeding with either starch or sucrose may well be due to the storage of large amounts of glycogen during this time period. In Figure 4, the enormous rise in liver glycogen content on the first day of refeeding is shown. At this point, nearly 11.5% of the weight of the liver is glycogen in the case of sucrose-refed rats and nearly 8.5% for St-1 rats. By day two, the glycogen content had dropped to the normal pre-fast values and did not show any differences due to diet.

The amount of total lipid accumulated in the liver over the experimental period is given in Figure 5. By day three, the amount of lipid in the livers of the sucrose-fed rats was higher than in the starch-fed rats. The greater lipid content of the livers of rats fed sucrose may also have played a role in the altered appearance and texture of these livers. By five days of refeeding, the extent of lipid accumulation approached 8% of the weight of the liver for sucrose-refed rats.

In order to ascertain whether the greater lipid accumulation in
Figure 3. Cytoplasmic protein content of liver (mg protein/gm liver). Values ± S.E.M. are for the post mitochondrial supernatant.
Figure 4. Glycogen as a % of liver weight. Values ± S.E.M.
Figure 5. Total lipid content in liver (mg lipid/gm liver).

Values ± S.E.M. For analysis of variance, see Appendix B.
livers from sucrose-refed rats was due, at least in part, to enhanced hepatic lipogenesis, liver slices were incubated in duplicate assays for 1 hour at 37°C in Krebs Ringer buffer containing 11 mM glucose and a tracer amount of acetate-1-^{14}C. The results in Figure 6 clearly demonstrate that there is a substantial difference in incorporation of the labelled acetate into total lipid for the two diets. In addition, starch-refed rats may have reached their peak incorporation slightly earlier than did the sucrose-refed rats. Although this pattern was observed in all of the experiments, it was noted that the magnitude of the difference between the maximum incorporation values for the two diets appeared to be dependent upon the age/weight of the rats. For younger, lighter weight rats, the differences were less pronounced.

Aliquots of these samples of total lipid were saponified in ethanolic KOH. The resultant fatty acid and cholesterol fractions were then extracted. The results are given in Figures 7 and 8. As would be expected, most of the labelled acetate incorporated was found in the fatty acid fraction (about 2.5 orders of magnitude greater incorporation than for cholesterol). The peak of acetate-1-^{14}C incorporation into liver cholesterol is shifted to three days compared to the two days for fatty acids in the sucrose-refed rats. There were no significant differences in the amount of free fatty acids found in the liver during the experimental period (Figure 9).

In several experiments both acetate-1-^{14}C and ^3H_2O incorporation into total lipid (using separate flasks for each isotope) was measured (Table 3). It will be noted that the amount of ^14C incorporation
Figure 6. Incorporation of Acetate-1-\(^{14}\)C into total lipid of liver slices. The incubations were performed at 37°C for 1 hour with an atmosphere of 95%O\(_2\)/5%CO\(_2\). Values ± S.E.M. For analysis of variance, see Appendix B.
Figure 7. Incorporation of acetate-1-$^{14}$C into fatty acids of liver slices. Fatty acids were extracted after saponification of an aliquot of the total lipid fraction. Values ± S.E.M. For analysis of variance, see Appendix B.
Figure 8. Incorporation of acetate-1-$^{14}$C into cholesterol of liver slices. Cholesterol was extracted with petroleum ether after saponification of an aliquot of the total lipid fraction. Values ± S.E.M. For analysis of variance, see Appendix B.
Figure 9. Free fatty acid content of liver (µeq FFA/gm of liver). Values ± S.E.M. were obtained by extraction and microtitration of FFA.
Table 3. Tritium incorporation as compared to acetate-1-\(^{14}\)C incorporation into total lipids from liver slices

<table>
<thead>
<tr>
<th>Dietary Treatment</th>
<th>(^{3}\text{H}) obs</th>
<th>(^{14}\text{C}) exp. obs</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Fasted</td>
<td>26</td>
<td>26</td>
<td>10</td>
</tr>
<tr>
<td>St-1</td>
<td>300</td>
<td>300</td>
<td>504</td>
</tr>
<tr>
<td>St-2</td>
<td>337</td>
<td>337</td>
<td>422</td>
</tr>
<tr>
<td>St-3</td>
<td>219</td>
<td>219</td>
<td>93</td>
</tr>
<tr>
<td>Suc-1</td>
<td>221</td>
<td>221</td>
<td>512</td>
</tr>
<tr>
<td>Suc-2</td>
<td>405</td>
<td>405</td>
<td>342</td>
</tr>
<tr>
<td>Suc-3</td>
<td>70</td>
<td>70</td>
<td>54</td>
</tr>
</tbody>
</table>

\(^{3}\text{H}_{2}\text{O}\) or \(^{14}\text{C}\)-acetate was incubated with liver slices in duplicate flasks as described in the text.
expected based upon what was found with tritium is in some cases at variance with the values actually obtained. These experiments were performed using young rats (about 175 grams each) and, as stated before, a smaller difference in the incorporation of $^{14}$C or $^{3}$H into lipid due to diet was noted than was observed for older rats (about 270 grams each).

Glucose was measured in hepatic portal blood (Figure 10) in an effort to obtain a practical approximation of the hormonal status of the rats. St-2 rats had slightly higher blood glucose levels than Suc-2 rats, but the situation was reversed after three days of refeeding.

In addition to determining blood glucose levels, the concentrations of several hepatic substrates were measured in liver. The concentration of glucose-6-phosphate (G-6-P)(Figure 11), the first metabolite of glucose, dropped sharply during fasting, but either starch or sucrose restored the hepatic G-6-P content in one day. After the first day of refeeding, the G-6-P level of the liver began declining such that Suc-5 animals had markedly lower G-6-P values nearly equal to that found in fasting rats.

Hepatic fructose-6-phosphate (F-6-P) (Figure 12) also declined nearly two-fold during the fasting period. F-6-P levels were higher in Suc-1 than in St-1 rats but lower for Suc-2 and Suc-5 rats as compared to their starch-fed counterparts. In general, the pattern of fluctuations was the same for both G-6-P and F-6-P for the experimental period.

The lactate content of the liver (Figure 13) dropped during fasting and returned to the control level by one day of refeeding. Sucrose-refed rats tended to have slightly higher liver lactate levels than starch
Figure 10. Blood glucose levels (mg %). Values ± S.E.M. for blood obtained from the hepatic portal vein immediately after decapitation.
Figure 11. Glucose-6-phosphate content of liver (mMoles/gm of liver). Values ± S.E.M.
Figure 12. Fructose-6-phosphate content of liver (mumoles/gm of liver). Values ± S.E.M.
Figure 13. Lactate content of liver (μmoles/gm of liver).
Values ± S.E.M.
refed rats until the fifth day of refeeding.

Since it has often been suggested that the availability of α-glycerol phosphate (α-GP) with which to make triglycerides could be a prime regulatory site in lipogenesis, the concentrations of both α-GP (Figure 14) and glycerol (Figure 15) were measured. After the fasting period, there was an initial build-up of α-GP followed by a decline in its concentration on days two and three. By day five, the St-5 rats had an increased concentration of α-GP relative to the St-3 rats while the value for Suc-5 rats remained essentially the same as for the Suc-3 rats. The drop in α-GP content to below the fasting level undoubtedly reflects the increased use of α-GP for esterification with free fatty acids to produce triglycerides. The hepatic glycerol content declined steadily during the experimental period to approximately the same extent for both diets.

The availability of cytoplasmic acetyl CoA might also be expected to be rate-limiting for lipogenesis under certain circumstances. Accordingly, the concentrations of both cytoplasmic and mitochondrial acetyl CoA pools were determined (Figure 16). The rise in mitochondrial acetyl CoA during fasting is undoubtedly due to an increase in the β-oxidation of fatty acids. By day three, the amount of cytoplasmic acetyl CoA in livers of Suc-3 rats was substantially higher than in starch-refed rats. Although the general shape of the cytoplasmic acetyl CoA curve is similar to that for acetate-1-14C incorporation into total liver lipid (Figure 6), the magnitude of the difference in acetyl CoA content between the two diets is not well correlated with the difference between the two diets for 14C incorporation into total liver lipids. In a separate
Figure 14. $\alpha$-glycerol phosphate content of liver (mumoles/gm of liver). Values ± S.E.M.
Figure 15. Glycerol content of liver (\(\mu\)moles/gm of liver).

Values ± S.E.M.
Figure 16. Cytoplasmic and mitochondrial acetyl CoA content of liver (mumoles/gm of liver). Values ± S.E.M. obtained for mitochondria and post-mitochondrial supernatant separated by centrifugation. For analysis of variance, see Appendix B.
experiment, the ratio of acetyl CoA to CoA in the cytoplasm as a function of diet was calculated (Figure 17). This ratio appears to correlate well with acetate-1-\(^{14}\)C incorporation into total liver lipids (Figure 6). In these experiments, studies of the distribution of the activity of glutamic dehydrogenase (GD), a mitochondrial marker enzyme, using the control group of rats were performed (Table 4) in order to make some assessment of mitochondrial integrity. Even though GD would undoubtedly leak out of a slightly damaged mitochondrion to a less extent than would CoA or acetyl CoA, it was felt that this marker enzyme would provide an approximation at least of the feasibility of the approach for the analysis of the compartmentalization of acetyl CoA and CoA.

In addition to the substrates which were measured, the activities of nine enzymes at potentially regulatory sites were also determined. The first of these enzymes is fructokinase (Figure 18). Since fructose entering the liver is believed to be phosphorylated primarily by FK to yield fructose-1-phosphate, it might be expected that FK activity would be higher in livers of sucrose-fed rats. FK activity declined during the experiment and by day five the activity of FK was substantially lower for Suc-5 rats than for the control group and also less than for the St-5 rats.

Phosphofructokinase (PFK) activity was also assayed (Figure 19). Although the enzyme has often been thought to be one of the most important regulatory sites for glycolysis, there seems to be little apparent effect due to diet on its activity in this experiment.

In Figure 20, the activity of glucose-6-phosphatase is indicated.
Figure 17. The ratio of Acetyl CoA to CoA in the cytoplasm of liver. The values were calculated from data for the content of acetyl CoA and CoA obtained in the same experiment.
Table 4. Distribution of Glutamic Dehydrogenase in Cytoplasm and Mitochondria of Control Rats.

<table>
<thead>
<tr>
<th>Time of Incubation (Minutes)</th>
<th>Cytoplasm</th>
<th>Mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.3</td>
<td>91.7</td>
</tr>
<tr>
<td>30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.9</td>
<td>92.1</td>
</tr>
<tr>
<td>60&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.0</td>
<td>92.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Assay performed 35 minutes after excision of the liver from the rat.
<sup>b</sup> Assay performed 55 minutes after excision of the liver from the rat.
<sup>c</sup> Assay performed 85 minutes after excision of the liver from the rat.

The incubation temperature was from 0 to 4°C. The two fractions were separated by centrifugation immediately after the incubation period.
Figure 18. Fructokinase activity in liver. Values ± S.E.M. are for the rate of disappearance of NADH in units/min/mg protein.
Figure 19. Phosphofructokinase activity in liver. Values are ± S.E.M.
Figure 20. Glucose-6-phosphatase activity in liver. Values ± S.E.M. are for the amount of phosphate liberated from glucose-6-phosphate in a 10 minute incubation period.
This enzyme would be expected to exhibit higher levels of activity in a gluconeogenic situation, but in this experiment, fasting did not appear to alter the level of activity of the enzyme as compared to the control rats. There was a steady, though not statistical, decline in G-6-Pase activity for both starch and sucrose-refed rats during the refeeding period.

Lipogenesis could potentially be limited by NADPH availability. Accordingly, three of the enzymes which produce NADPH were assayed: malic enzyme (Figure 21), glucose-6-phosphate dehydrogenase (Figure 22) and isocitric dehydrogenase (NADP-linked) (Figure 23). ME and G-6-PD showed increased activities during the refeeding periods while ICD activity became generally lower. Suc-2 and Suc-5 rats had higher ME than comparable starch-fed rats. ICD activity, however, was lower in Suc-1, Suc-2 and Suc-3 rats than in St-1, St-2 and St-3 rats, respectively.

If one assumes that these assays accurately reflect the in vivo activities of these enzymes (a dubious premise), then by adding the activities of all three enzymes, the potential for NADPH production could be assessed. These calculated values are given in Table 5. Although the capacity for NADPH production rises during the five day refeeding period, there is no difference attributable to the type of carbohydrate in the diet.

In Figure 24, the activity of citrate cleavage enzyme in the liver is shown. This enzyme is believed to be primarily responsible for the presence of acetyl CoA in the cytoplasm as the result of cleavage of citrate (transported from the mitochondria to the cytoplasm) into oxaloacetate and acetyl CoA. Thus, low activity of this enzyme could
Figure 21. Malic enzyme activity in liver. Values ± S.E.M. for the rate of appearance of NADPH in μmoles/min/mg protein. For analysis of variance, see Appendix B.
Figure 22. Glucose-6-phosphate dehydrogenase activity in liver. Values ± S.E.M. for the rate of appearance of NADPH in μmoles/min/mg protein.
Figure 23. Isocitric dehydrogenase (NADP linked) activity in liver. Values ± S.E.M. are for the rate of appearance of NADPH in mumoles/min/mg protein. For analysis of variance, see Appendix B.
Table 5. Potential for NADPH production from malic enzyme, glucose-6-phosphate dehydrogenase and isocitrate dehydrogenase (NADP linked).

<table>
<thead>
<tr>
<th>Diet</th>
<th>Days of Refeeding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>my moles NADPH/min/mg protein</td>
</tr>
<tr>
<td>Starch</td>
<td>51.02</td>
</tr>
<tr>
<td>Sucrose</td>
<td>45.04</td>
</tr>
</tbody>
</table>

The totals for the control and fasting groups are 53.64 and 45.26, respectively.
Figure 24. Citrate cleavage enzyme activity in liver. Values \( \pm \) S.E.M. are for the rate of disappearance of NADH in \( \mu \)moles/min/mg protein.
limit acetyl CoA availability in the cytoplasm for lipogenesis. CCE activity was slightly higher in livers from sucrose-fed than in starch-fed rats. The activity of the enzyme was still well above the control levels after five days of refeeding when incorporation of acetate-1-$^{14}$C had already returned to the control level. Since the assays were performed on livers which had been frozen, this treatment might possibly alter the activity observed. The values obtained, however, correlated very well with the acetyl CoA content of the cytoplasm (Figure 16) which one would expect if the CCE values accurately reflect the in vivo CCE activity and if CCE is indeed the enzyme responsible for providing acetyl CoA to the cytoplasm.

The acetyl CoA pool of both the mitochondria and cytoplasm could also be altered if the activity of acetyl CoA synthase were altered substantially by diet (assuming that free acetate is available in the liver). An alteration in the activity of this enzyme could have considerable effect on the observed incorporation of acetate-1-$^{14}$C into total lipid as a result. The results of the determination of the activity of AS are given in Figure 25. Even though the data suggests that Suc-5 animals were able to provide labelled acetate at a faster rate than St-5 animals, the data in Figure 6 do not indicate a greater incorporation of $^{14}$C into total lipids. Since fasting decreased the activity of the enzyme nearly two-fold, it is conceivable that this could have some influence on the labelled acetate incorporation for the fasted animals. The $^{3}$H$_2$O incorporation study (Table 3), however, indicates that the drop in lipogenesis upon fasting is indeed a real effect and not due merely to a drop in AS activity. This experiment, which also
Figure 25. Acetyl CoA synthase activity in liver. Values ± S.E.M. are cpm x 10^{-3} from acetate-1-^{14}C incorporated into acetyl CoA during a 15 minute incubation period at 37°C.
included the assay of acetyl CoA carboxylase activity, was carried out only to day three in an effort to conserve both on rats and reagents.

Figure 26 indicates the effect of refeeding either starch or sucrose on the activity of acetyl CoA carboxylase. This enzyme is believed to be the rate-limiting enzyme for fatty acid synthesis. Since AC activity has been demonstrated to be increased by incubation at 37°C in other experiments from this laboratory (92,93), the ability of the enzyme to be heat activated under these dietary conditions was also investigated. From the graph it is seen that the fasting animals have the least ability to be heat activated and the lowest zero time activities also. After one day of refeeding, Suc-1 rats have only a slightly lower zero time activity than St-1 rats, but heat incubation led to a substantial difference in their activities at the end of 30 minutes. For the two and three day periods of refeeding, the activities achieved after 30 minutes of incubation are higher, but the two diets did not yield different results.

The implications of the findings reported in this section will be discussed in the following section.
Figure 26. Acetyl CoA carboxylase activity in liver and the effect of heat activation of the enzyme in vitro. Values are cpm x 10^{-3} from $^{14}$C bicarbonate incorporated into an acid stable form during a one minute assay at 37°C. The enzyme was assayed at 0, 15, and 30 minutes of incubation.
DISCUSSION AND CONCLUSIONS

The model system used in these experiments sought to take advantage of the well-known "overshoot" in lipogenesis (95-102) observed when starved animals are refed diets high in carbohydrate. Although the biochemical mechanism of "overshooting" has not been elucidated, it is easy to speculate that this phenomenon would give a distinct evolutionary advantage over a species whose lipogenic systems were restored only to the prefast level of activity. After a period of fasting, an animal who finally obtains food and whose hepatic metabolism allows lipogenic "overshooting" would presumably be able to restore body fat stores at a greater rate. It was felt that this system might provide a good way to accentuate any differences in the effects of isocaloric high starch and high sucrose diets on hepatic lipogenesis. A continuous or a long-term feeding study, on the other hand, might indeed have real differences attributable to the dietary regimes, but due to animal and experimental variation, the differences might not prove to be statistically significant. In addition, there would be no way to magnify any differences due to the type of carbohydrate in such a system. Even in our system, experimental variation has in some cases posed serious problems in demonstrating a statistical significance of the differences due to diet. The trends in differences, however, are clearly observable.

The age and strain of rats used has also been shown by other
investigators to have a bearing on the results obtained in a study of lipogenesis (103,104). In this study, Sprague-Dawley rats were used. Although no systematic study of the effect of age and type of carbohydrate was performed as a part of our experiments, it appeared that the use of younger rats led to higher incorporation of labelled acetate or tritium into total liver lipids but to less difference in incorporation due to diet. Most of the work was done using rats of approximately the same weight (about 250 grams).

Sampling was done on rats after one, two, three, or five days of refeeding in an effort not to miss changes in enzyme activities or substrate concentrations which might not all occur at the same time. Indeed, it would be unlikely that all metabolic systems would reequilibrate at the same rate. Since the rate of lipogenesis apparently returned to control levels by five days, it was felt that a five day refeeding would be sufficient for the enzyme and substrate studies. It is interesting to note that even though lipogenesis had returned to pre-fasting rates by five days of refeeding, many of the enzyme activities measured were still at or near their peak activities.

In confirmation of the work of many other investigators using both humans and rats, the data presented here appears to demonstrate an effect of sucrose on hepatic lipogenesis which is different than that of starch. There is a slight increase in the lipid content of the liver from rats fed a high sucrose diet and a much higher rate of incorporation of acetate-1-\(^{14}\)C into free fatty acids, cholesterol and total lipid. Taking the striking differences in appearance and texture of livers from starch compared to sucrose-refed rats, there is little doubt that these
two carbohydrates differ in their effects upon liver metabolism.

In order to avoid the problems of changes in acetate pool size, in the rate of transformation of acetate-\(^1\)C into \(^{14}\)C-acetyl CoA or in compartmentation of acetyl CoA, measurement of the lipogenic rate by tritium incorporation into total liver lipids would be the method of choice (105-109). In order to achieve sufficient levels of tritium incorporation into total lipid of liver slices to avoid statistical problems in lipid extraction and scintillation counting, the amounts of tritium required for this experimental design (approximately 50uC/flask) would have exceeded the maximum allowable use of tritium per day as established by the Office of Radiological Health and Safety, The Ohio State University. This would have necessitated a change in the design of the experiments, either to study fewer groups or to spread the experiment over a greater number of days. Neither change was deemed desirable. In addition, many of the studies of other investigators were based upon \(^{14}\)C-acetate incorporation into liver lipids. Therefore, for purposes of comparison with the work of others and for radiation safety, acetate-\(^1\)C was selected. When tritium was used, there were some differences in tritium versus acetate incorporation which can undoubtedly be attributed to one or several of the potential problems in use of acetate mentioned above.

There are a variety of possible sites which might be involved in the differential effects of the two carbohydrates. The asterisks in Figure 27 indicate some of the likely metabolic steps at which one might expect to see an effect of diet. The first possibility is a differential entry of substrate into the glycolytic sequence. Starch, of course, consisting solely of glucose, would enter the liver as blood
Figure 27. Potential sites of regulation of hepatic lipogenesis.
glucose from the hepatic portal circulation and be phosphorylated by hexokinase/glucokinase. Sucrose would be cleaved in the gut wall to one molecule each of fructose and glucose which would also enter the portal circulation. The fructose entering the portal circulation would then be efficiently taken up by the liver from the portal blood supply and phosphorylated to fructose-1-phosphate (56) and to a lesser extent to fructose-6-phosphate by hexokinase. In these studies, fructokinase was not increased in activity and actually appeared to decline upon refeeding. There was a concurrent change in cytoplasmic protein/gm liver, but that does not completely explain the decline. The activity of FK was not well-correlated with the lipogenesis profile (Figure 6) and thus the enzyme does not seem to be a likely candidate for the observed effects of diet. Since changes in the concentrations of G-6-P and F-6-P were well-correlated with each other but not with the differences in lipogenesis due to diet, it would seem that this segment of glycolysis was not responsible for the observed effects on lipogenesis.

The observations of others (65-67) concerning the rise in hepatic glucose-6-phosphatase activity with sucrose (or fructose) refeeding were not confirmed in these experiments. Since fructose alone should be a gluconeogenic substrate, one might expect to see a rise in G-6-Pase activity in order to provide glucose to the blood stream. It is possible, however, that the 60% sucrose diet used in these experiments provided sufficient glucose to avoid the necessity for appreciable gluconeogenesis. Even though the blood glucose level (Figure 10) of Suc-2 rats was a little lower than for St-2 rats, sucrose-refed rats did not have a consistently lower blood glucose level.
It has been suggested that α-glycerol phosphate availability might be the rate-limiting step in lipogenesis (110,111). Presumably, lack of α-glycerol phosphate would lead to a build-up of fatty acyl CoA which would then inhibit lipogenesis. Since fructose-1-phosphate is cleaved to dihydroxyacetone phosphate (DHAP) and glycerol, it might provide α-GP more readily than would glucose. In the experiments reported here α-GP levels dropped (Figure 15) as expected during the period of highest lipogenic activity but there were no differences in α-GP concentration due to carbohydrate source until day five. The glycerol pool became progressively depleted during the experiment. The data do not suggest that α-GP and/or glycerol concentrations are the controlling factors in lipogenesis in accord with the beliefs of other investigators (112-114). It has also been suggested that in place of α-GP being the substrate for esterification, DHAP esterification of fatty acids is the main pathway (115). If this pathway is a major route for the esterification of fatty acids, then the DHAP concentration would also become important if the α-GP-DHAP couple is not at equilibrium. However, DHAP was not measured in these experiments.

Another factor which has been considered to be a regulator of lipogenesis under some conditions is NADPH availability (116-119). Two of the enzymes supplying NADPH are in the hexose monophosphate shunt (HMPS). The Teppermans have been able to show elevated HMPS activity after re-feeding a high carbohydrate diet to rats which had been starved for 48 hours (120,121). Glucose-6-phosphate dehydrogenase has been studied under a variety of conditions (122-124). Its activity is elevated in a meal-eating verses a nibbling situation (122) and when a high carbohydrate
diet is substituted for a pelleted lab chow (123). Induction of G-6-PD activity by a high carbohydrate diet can be prevented by injection of cyclic 3',5' adenosine monophosphate (c-AMP) (124). Since hepatic G-6-PD has been shown to exist in at least three different dimers (125), it is not clear whether the carbohydrate effect is due to an effect on one dimer in particular or to all of them. The activities of malic enzyme and isocitrate dehydrogenase have also been reported to parallel changes in the rate of lipogenesis (126-128) and to be increased by sucrose feeding (69,70). It was noted by Lockwood et al. (128) that ME responded to dietary change at weaning faster than did ICD or G-6-PD but that both G-6-PD and ICD had higher activities than did ME. A similar phenomenon was noted in our experiments. Even at its lowest activity, ICD was considerably higher than the activity of ME. The activities and the rates of increase in the activities of ME and G-6-PD were somewhat closer together. There may be several forms of ME (129) so there could be a potential problem in correlating the activity measured by assay with the actual in vivo activity of the enzyme. None of the individual activities of the NADPH forming enzymes correlated well with the lipogenic rate profile nor did the profile of the aggregate of their activities. Thus, in agreement with others (121), it seems unlikely that a limitation in the amount of NADPH available could account for the differences observed between the two dietary regimes.

The availability of acetyl CoA in the cytoplasm must be considered as a potential point of regulation for lipogenesis. An actual measurement of the concentration of cytoplasmic acetyl CoA indicated there were differences in the content of acetyl CoA in the livers of animals
fed sucrose as opposed to those fed starch. Although the shape of the curve was relatively similar to that for lipogenic rate (Figure 6), the magnitude of the difference was not. In addition, the peaks of the cytoplasmic acetyl CoA curves appeared to be displaced slightly from lipogenesis with respect to time. The graph of the cytoplasmic acetyl CoA to CoA ratio (Figure 17) is more similar to the curve for lipogenesis, but again it is displaced by one day. It might easily be argued that CoA is a competitive inhibitor with acetyl CoA for a site on acetyl CoA carboxylase, but how this mechanism could function with a time displacement involved is difficult to explain.

The provision of acetyl CoA to the cytoplasm by citrate cleavage enzyme is well-demonstrated by the excellent correlation between cytoplasmic acetyl CoA concentration and the CCE curves (Figure 16 and Figure 24). CCE has been suggested as a potentially regulatory enzyme for lipogenesis (130-132). It undergoes an increase in activity in the same situations where there is an increase in lipogenesis. It is decreased in the livers of diabetic rats (132) and a diet high in fructose but not high in glucose was shown to be able to elevate CCE activity in diabetic rats. In subsequent work, Foster and Srere (133) reported that in recovery from a fasting state, liver CCE activity increases lag behind those in lipogenesis. Also, addition of purified CCE to a fatty acid synthesizing system from fasted rats did not return the lipogenic rate to the levels observed for normal fed rats. For these reasons, Foster and Srere do not consider CCE to be regulatory for fat synthesis. The citrate transporter (CT) has been reported to be a regulated step (134,135). If so, provision of citrate to CCE might be a control point.
Eventually, however, regulation at the CT step should lead to differences in cytoplasmic acetyl CoA which should correlate well with the lipogenic rate. This was not the case in our experiments.

Acetyl CoA synthase could conceivably contribute some acetyl CoA to the cytoplasm if there was appreciable free acetate available. Murthy and Steiner (136) have found about a two-fold elevation in the concentration of free acetate in fasting animals without a change in deacylase activity. If the rise in acetate was due to lowered activity of AS, then part of the apparent lowered activity during fasting as measured by acetate-1-\(^{14}\)C could be due to an inability to metabolize acetate to acetyl CoA. Murthy and Steiner (137) have suggested that AS may be regulatory for lipogenesis. In our studies tritium incorporation confirmed the acetate incorporation data and there was no difference in AS activity due to the type of carbohydrate until day three. Although AS activity was higher in Suc-3 rats than in St-3 rats, the value was not far above the control level and was certainly not enough different to account for the far greater incorporation of labelled acetate into liver lipids in sucrose-refed rats. Kornacker and Lowenstein (138) were not able to demonstrate much change in AS upon refeeding a high carbohydrate diet to fasted rats either. Since cytoplasmic acetyl CoA concentrations are higher in sucrose-refed rats, it is also unlikely that the apparent increased incorporation of labelled acetate into liver lipid was due to a greatly increased specific activity of acetyl CoA.

The most likely site for an effect on lipogenesis due to the type of carbohydrate in the diet is at the level of acetyl CoA carboxylase
which is considered to be the rate-limiting enzyme for fatty acid synthesis. Zakim, et al. (57) has suggested that a part of the differential effects of high carbohydrate diets is at this site. The various aspects of AC regulation have been recently reviewed (139,140). Bortz and Lynen have reported (141,142) that the enzyme is inhibited by fatty acyl CoA and that a decrease in the \( \alpha \)-GP concentration would lead to an inhibition of AC. Free fatty acids in a physiological concentration perfused into rat liver have also been shown (143) to inhibit hepatic lipogenesis (measured by tritium into total lipid), but in our studies the liver free fatty acid concentration did not vary in response to diet.

In this laboratory, it has been shown (144) that hepatic lipogenesis and AC activity are inhibited to about the same extent by c-AMP. Klein and Weiser have reported (145) that glucagon injection in vivo reduced the synthesis of fatty acids from glucose and that the activity of AC was reduced but not the activities of CCE, ME, G-6-PD, ICD or fatty acid synthetase. Carlson and Kim (146,147) have presented evidence that AC activity is regulated by a phosphorylation-dephosphorylation mechanism. In a study by Numa's group (148), data was presented which supported the view that the rapid rise in hepatic lipogenesis observed when a high carbohydrate diet is refed to starved rats is due to a modulation of the catalytic efficiency of the enzyme. In another study (149), an inactive form of AC having a lower molecular weight was found to be present in homogenates of liver from fasted rats. In other studies from our laboratory, AC activities have been shown to increase upon incubation for a short period (less than 1 hour) at 37°C (92,93). Addition
of glycogen at levels equal to from 2% to 8% of the weight of the liver to 105,000 x G supernatants enhanced heat activation of the enzyme in a manner proportional to the amount of glycogen added. In the studies reported here, enormous amounts of glycogen were found in the liver after just one day of refeeding, an effect also noted by Hers et al. (150). The Teppermans (120,151) have noted a good correlation between hepatic glycogen levels and lipogenesis from 0-6 hours after refeeding a high carbohydrate diet to starved rats. During some phases of regulation of lipogenesis, at least, this glycogen-AC interaction may serve a useful regulatory role. It would tend to insure that hepatic glycogen stores were repleted before available substrate was stored as fat which cannot be used (except for glycerol) for glucose synthesis.

The determination of the activity of AC under the two dietary regimes used in this study, however, indicated very little difference after the first day of refeeding either in the zero time activity or in heat activability of AC in starch or sucrose-refed rats. This may have been due in part to two things. First, the rats used were younger, and there was less difference in effect on lipogenesis between the two diets when young rats are used. The second possibility perhaps is that the assay system does not yield results which reflect the in vivo activity of the enzyme. The third alternative, of course, is that this enzyme is not a regulatory site for lipogenesis.

It has been noted that AC and fatty acid synthetase (FAS) rise and fall in concert under several dietary and hormonal situations (152-155). Insulin is believed to be required for the induction of FAS activity observed when a high carbohydrate diet is refed to fasting rats (156,157).
Several authors believe that the increase in enzyme activity upon refeeding is due to a change in enzyme content rather than a change in catalytic efficiency (155, 158-161). A discussion of the potential problems with this interpretation can be found in Appendix A.

There are some other potential sites for sucrose/fructose action which have not been investigated in the experiments reported here. Two possibilities for a site of action of carbohydrate occur at the level of the gut. It has been reported (162) that the disaccharidases in the gut wall undergo a circadian rhythm. In our experiments care was taken to process the animals at the same time each day. If, however, the activities of enzymes for getting the moieties of starch or sucrose across the gut wall had a differential activity peak with respect to time, then there might be a difference in the time when maximum substrate was available to the liver and potentially a difference in the amount of insulin released in response to feeding. Our data indicate that there was little difference in the portal blood glucose values which may imply that the amount of substrate coming through the gut wall (at the time of sampling) was approximately equivalent for the two diets. The other possibility has been suggested by Bartley et al. (163). They proposed that sucrose/fructose might stimulate a particular type of intestinal cell to produce a gut hormone which would then enter the blood and act at the level of the liver cell. On the basis of the direct effects in liver of fructose perfusion studies (54), one might argue that an effect of sucrose/fructose at the level of the gut is not required to explain the data.

In this laboratory, we have shown (164) that the use of 11 mM
fructose instead of glucose in liver slice incubation media leads to less inhibition of lipogenesis by c-AMP in liver slices from chickens (as measured by tritium incorporation into total liver lipids). These data could perhaps be explained in two ways. One, there is a block to lipogenesis near the level of entry of fructose to glycolysis. Two, fructose (or an immediate metabolite) specifically protects acetyl CoA carboxylase (or some other lipogenic enzyme) from the inhibitory effects of c-AMP, or perhaps it activates the enzyme in some way. It has been suggested by Goodridge (165) that enzymes responsible for fatty acid synthesis in the livers of chick embryos are latent and that additions of fructose to incubation medium induced them to become active. The advantages of a direct effect of fructose or an immediate metabolite on AC activity are obvious for an animal whose diet is high in carbohydrate from fruit and honey. Even though the amount of substrate available for fat synthesis would be expected to be quite high, there would be relatively little insulin (compared to a diet with an equivalent amount of glucose) and thus the lipogenic enzymes would not be induced. A specific effect of fructose, on the other hand, would overcome this impairment and allow excess carbohydrate to be stored as fat.

Also at the level of the liver, one must consider the possibility of an interaction of fructose with other hormones which alter liver metabolism such as the glucocorticoids (166) or somatomedin (167).

In conclusion, these studies appear to demonstrate an ability of a high sucrose diet to stimulate hepatic lipogenesis to a greater extent than does an isocaloric high starch diet in the model system used for these experiments. It seems clear that the effect is not due to the
availability of $\alpha$-GP, NADPH nor acetyl CoA. The Tepperman's suggestion of a generalized change in the "lipogenic climate" of the cell (74) must still be translated into some kind of molecular mechanism in order to work. The most likely site for regulation of lipogenesis is AC since the enzyme appears to be regulated by a wide variety of mechanisms. The difficulty in determining the \textit{in vivo} activity of the enzyme renders a definitive statement about its importance to lipogenic regulation under the conditions of this study difficult.

The regulation of lipogenesis and, indeed, all metabolic systems is finely balanced through a set of hormonal and intracellular control systems. The fine interactions of the body were appreciated and pointed out long ago by the Apostle Paul (168). It seems likely that the effects of a high sucrose diet are not localized to just the rate-limiting enzyme in lipogenesis. Regardless of the mechanism of action of the effects of these diets, a host of enzyme activities and substrate pools must re-equilibrate to accommodate the new metabolic situation.
Several authors believe that the increased activity of the "lipogenic" enzymes observed when a high carbohydrate diet is fed to starved rats is the result of a change in the enzyme content of the cell rather than to a change in the catalytic efficiency of the enzyme (155, 158-161, 169). Most of these studies were based upon labelled leucine incorporation into protein which was then recovered by antibody (Ab) precipitation. This technique is adequate for studying enzymes which exist in one and only one form. Even in this case, care must be taken to have very pure Ab, and the rate of synthesis and degradation of the enzyme must be sufficiently different to avoid severe errors in the overall rate determinations. For the case where the enzyme exists in two or more forms, either or both of which may be active, this technique can lead to an artifact which appears to be consistent with an increase in enzyme synthesis.

Acetyl CoA carboxylase (149), malic enzyme (129) and fatty acid synthetase (170) have all been reported to exist in more than one form. For the cases of AC and FAS, one of the forms appears to be inactive. Figure 28 shows a situation which would be expected to be similar to the case for AC or FAS. Since AC has been reported (146,147) to be interconverted from active to inactive forms by phosphorylation-dephosphorylation, a mechanism which may be common for many enzyme systems,
Figure 23. Interconversion scheme for an enzyme which exists in an active, inactive, and super active forms.
this method of interconversion will be assumed for the hypothetical case shown in Figure 28. There would be at least two pools of enzyme, active and inactive. In addition, if there are multi-subunits which make up the enzyme monomer, there would be pools of these all of which would not necessarily be synthesized at the same rate. Several enzymes, of which AC may be an example, undergo a polymerization of the active form of the enzyme under the influence of metabolic effectors to form a "super active" enzyme.

The usual procedure, of which the AC studies are typical (171), is to isolate and purify the enzyme. If the purification procedure were adequate only the active (and/or "super active") form(s) of the enzyme would be purified. Antibody would then be prepared by injection of the purified enzyme into another species of animal. After a suitable titre of Ab is attained, it is harvested and purified. The antibody would be expected to react well with the enzyme species with which it was produced, but it may react to a lesser extent or not at all with other forms of the enzyme.

In the case of a fasting or a control rat, the majority of a "lipogenic" enzyme might be expected to be in an inactive form. When a fasted or chow-fed rat is switched to a high carbohydrate diet, there would be a large shift of the inactive enzyme to the active form. For this case, assume that the rate of de novo synthesis (and degradation) is constant and not dependent upon diet and that the Ab reacts only with the super active form. If the rate of synthesis were constant, a pulse label of $^{14}$C-leucine will tend to label the inactive enzyme pool in a fasted or control rat. When the basal leucine incorporation is
determined, there will be only low levels of leucine labelled enzyme recovered because the Ab reacts only to the super active form. When the rats are then switched to a high carbohydrate diet, the leucine label which was incorporated into the inactive enzyme as the result of the steady state synthesis and degradation of the enzyme now appears in the active and super active forms as a result of a shift in equilibrium toward those forms. Now when the enzyme is challenged with Ab, there would be a great deal of labelled leucine recovered in the enzyme protein.

In an actual fasting-refeeding situation, there may be a combination of increases in the rate of synthesis (or decreased degradation) of an enzyme and a shift toward a more active form of the enzyme. Szepesi et al. (172) has reported that a second fasting-refeeding cycle led to a greater increase in lipogenic "overshoot" than did the first cycle. It is possible that this dual mechanism could account for their data. In the first cycle, there was not only a shift toward more active enzyme but also an increased rate of synthesis. During the second fasting sequence, the active enzyme (both old and new) could have been returned to the inactive pool. The second refeeding then could cause activation of a larger pool of inactive enzyme.
APPENDIX B

In figures where there appeared to be a potential effect of dietary treatment as well as an effect due to the number of days of refeeding, the data were treated statistically using a two way analysis of variance (ANOVA) (173) on a pre-recorded calculator programa.

The following equations apply to all of the ANOVA tables given below:

$$C_{ij} = \sum_k x_{ijk}$$

$$RS_i = \sum_j \sum_k x_{ijk}$$

$$CS_j = \sum_i \sum_k x_{ijk}$$

where

i = number of rows (r) days of treatment

j = number of columns (c) dietary treatment

k = cell size (n) where n = 4.

The degrees of freedom are:

$$df_1 = r - 1$$

$$df_2 = c - 1$$

$$df_3 = (r - 1)(c - 1)$$

$$df_4 = rc(n - 1)$$

In this mixed model, the rows are the days of treatment from one through five and the columns are the two dietary treatments (starch or sucrose).

The F statistic for each case is obtained as follows:

Row F = RMS/IMS

Column F = CMS/EMS

Interaction F = IMS/EMS

For row effects to be significant at the 0.05 level, the F test statistic must be greater than $F_{.05}(3,3) = 9.28$. The F test statistic for column effects significant at the 0.05 level must be greater than $F_{.05}(1,24) = 4.26$. For interaction effects to be significant at the 0.05 level, the F test statistic must exceed $F_{.05}(3,24) = 3.01$. The ANOVA tables for selected figures are given below.

Table 6. ANOVA for Figure 2. Liver as a per cent of body weight.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>SS</th>
<th>MS</th>
<th>df</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days</td>
<td>.35</td>
<td>.12</td>
<td>3</td>
<td>1.0</td>
<td>N.S.</td>
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<td>Diet</td>
<td>2.37</td>
<td>2.37</td>
<td>1</td>
<td>12.75</td>
<td>.05</td>
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<tr>
<td>Interaction</td>
<td>.36</td>
<td>.12</td>
<td>3</td>
<td>.64</td>
<td>N.S.</td>
</tr>
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<td>Error</td>
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<td>.19</td>
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<td></td>
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<tr>
<td>Total</td>
<td>7.53</td>
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<td></td>
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</table>

Table 7. ANOVA for Figure 5. Total lipid content in liver.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>SS</th>
<th>MS</th>
<th>df</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days</td>
<td>8701.7</td>
<td>2900.6</td>
<td>3</td>
<td>17.15</td>
<td>.05</td>
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<tr>
<td>Diet</td>
<td>1296.7</td>
<td>1296.7</td>
<td>1</td>
<td>6.21</td>
<td>.05</td>
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<tr>
<td>Interaction</td>
<td>507.4</td>
<td>169.1</td>
<td>3</td>
<td>.81</td>
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<td>Error</td>
<td>5008.4</td>
<td>208.7</td>
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<td></td>
</tr>
<tr>
<td>Total</td>
<td>15514.2</td>
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Table 8. ANOVA for Figure 6. Incorporation of acetate-$1^{14}$C into total lipid of liver slices.

<table>
<thead>
<tr>
<th>Source of variation</th>
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<th>MS</th>
<th>df</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days</td>
<td>$2.4 \times 10^{10}$</td>
<td>$8.1 \times 10^9$</td>
<td>3</td>
<td>2.03</td>
<td>N.S.</td>
</tr>
<tr>
<td>Diet</td>
<td>$2.1 \times 10^{10}$</td>
<td>$2.1 \times 10^{10}$</td>
<td>1</td>
<td>16.19</td>
<td>.05</td>
</tr>
<tr>
<td>Interaction</td>
<td>$1.2 \times 10^{10}$</td>
<td>$4.0 \times 10^9$</td>
<td>3</td>
<td>3.14</td>
<td>.05</td>
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<tr>
<td>Error</td>
<td>$3.0 \times 10^{10}$</td>
<td>$1.3 \times 10^9$</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>$8.7 \times 10^{10}$</td>
<td></td>
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</table>

Table 9. ANOVA for Figure 7. Incorporation of acetate-$1^{14}$C into fatty acids of liver slices.

<table>
<thead>
<tr>
<th>Sources of Variation</th>
<th>SS</th>
<th>MS</th>
<th>df</th>
<th>F</th>
<th>Sig.</th>
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</thead>
<tbody>
<tr>
<td>Days</td>
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<td>1.91</td>
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<tr>
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<td>$1.8 \times 10^{10}$</td>
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<td>19.13</td>
<td>.05</td>
</tr>
<tr>
<td>Interaction</td>
<td>$9.4 \times 10^{10}$</td>
<td>$3.1 \times 10^9$</td>
<td>3</td>
<td>3.42</td>
<td>.05</td>
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<tr>
<td>Error</td>
<td>$2.2 \times 10^{10}$</td>
<td>$9.1 \times 10^8$</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>$6.7 \times 10^{10}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

Table 10. ANOVA for Figure 8. Incorporation of acetate-$1^{14}$C into cholesterol of liver slices.

<table>
<thead>
<tr>
<th>Sources of variation</th>
<th>SS</th>
<th>MS</th>
<th>df</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days</td>
<td>189246</td>
<td>63082</td>
<td>3</td>
<td>.55</td>
<td>N.S.</td>
</tr>
<tr>
<td>Diet</td>
<td>66248</td>
<td>66248</td>
<td>1</td>
<td>3.62</td>
<td>N.S.</td>
</tr>
<tr>
<td>Interaction</td>
<td>345528</td>
<td>115176</td>
<td>3</td>
<td>6.29</td>
<td>.05</td>
</tr>
<tr>
<td>Error</td>
<td>439616</td>
<td>18317</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1040637</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Table 11. ANOVA for Figure 16. Cytoplasmic acetyl CoA content of liver.

<table>
<thead>
<tr>
<th>Sources of variation</th>
<th>SS</th>
<th>MS</th>
<th>df</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days</td>
<td>853.2</td>
<td>284.4</td>
<td>3</td>
<td>5.20</td>
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</tr>
<tr>
<td>Diet</td>
<td>87.2</td>
<td>87.2</td>
<td>1</td>
<td>2.99</td>
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<tr>
<td>Interaction</td>
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<td>57.6</td>
<td>3</td>
<td>1.88</td>
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</tr>
<tr>
<td>Error</td>
<td>698.9</td>
<td>29.1</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1803.2</td>
<td></td>
<td></td>
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<td></td>
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</tbody>
</table>
Table 12. ANOVA for Figure 21. Malic enzyme activity in liver.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>SS</th>
<th>MS</th>
<th>df</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days</td>
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<td>123.8</td>
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<td>4.17</td>
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<tr>
<td>Diet</td>
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<td>134.6</td>
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<td>9.47</td>
<td>.05</td>
</tr>
<tr>
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<td>29.7</td>
<td>3</td>
<td>2.09</td>
<td>N.S.</td>
</tr>
<tr>
<td>Error</td>
<td>341.1</td>
<td>14.2</td>
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<td>Total</td>
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</tr>
</tbody>
</table>

Table 13. ANOVA for Figure 23. Isocitric dehydrogenase (NADP linked) activity in liver.

<table>
<thead>
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<th>SS</th>
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<th>df</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days</td>
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<tr>
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<td>333.7</td>
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<tr>
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REFERENCES


