THE EFFECT OF A HIGH-SUCROSE INTAKE DURING GESTATION AND LACTATION UPON THE METABOLIC PATTERNS OF GROWING AND MATURE RATS

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ABSTRACT

THE EFFECT OF A HIGH-SUCROSE INTAKE DURING GESTATION AND LACTATION UPON THE METABOLIC PATTERNS OF GROWING AND MATURE RATS

By

William Davidson Evers

The research described in this thesis was conducted to ascertain whether maternal diet has any long-term effects on development of offspring. Specifically, does feeding a high-sucrose diet during pregnancy and lactation alter certain metabolic processes in the offspring from weaning through maturity, even though the offspring are not fed a high-sucrose diet.

Female rats were fed either a 65% cornstarch or a 65% sucrose diet during pregnancy and lactation. Their offspring were fed the 65% cornstarch diet from weaning.

At approximately 30, 50 and 110 days of age offspring were given a glucose load test and their glucose tolerance was measured. At 30, 60 and 120 days of age, offspring were killed and the following parameters were analyzed: body weight, serum triglycerides and cholesterol, relative liver size, liver glucose-6-phosphate dehydrogenase activity and liver lipid and cholesterol.

No consistent major differences in body weight, serum triglycerides, relative liver size, liver enzyme activity, liver cholesterol or glucose tolerance were noted at any of

the ages. These findings are inconsistent with reports that body weight and glucose tolerance were elevated and serum triglycerides were depressed in the offspring from dams fed a high-sucrose diet.

Serum cholesterol was significantly higher at 120 days of age in the offspring of sucrose-fed dams, but not at 30 or 60 days of age. Liver lipid was higher at 60 days but lower at 120 days in the offspring from mothers fed the high-sucrose diet.

Based upon these results, it was concluded that in the rat there does not seem to be any consistent long-term metabolic differences in offspring of mothers who had been fed a high-sucrose diet during gestation and lactation, if the offspring were fed a cornstarch diet from weaning.

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INTRODUCTION

This study reviews two areas of human nutrition; maternal-fetal nutritional relationships, and the effects of dietary sucrose on metabolic patterns. Then there is an examination of studies that indicate that dietary sucrose is a nutritional factor which influences the maternal-fetal relationship. The question being asked is: Does the feeding of a high-sucrose diet to a pregnant and lactating rat have any effect upon the subsequent growth and development of the offspring, even though the offspring are fed a normal diet? The research was conducted to answer this question.

REVIEW OF LITERATURE

Maternal-Fetal Relationships

Many connections from fetus to mother have been shown to occur. For example, the pregnant rat during the second half of gestation shows large increases in food intake and in insulin secretion, but still loses fat from adipose tissue because the tissues appear less sensitive to insulin. This development is considered to be an adjustment to fetal growth whereby ingested glucose is channelled to the fetus and the mother draws upon stored lipid for energy(1). The fetus may act as a parasite during periods of maternal starvation, with the continuous consumption of glucose, and possibly alanine, despite decreased levels in the blood of the mother. However there are limitations upon this ability to obtain nutriture from the mother. With prolonged fasting it would appear that the fetus, like the mother, must utilize ketones as an alternative source of energy(2). Maternal dietary restriction in rats can lead to growth stunting of offspring(3,4), lending credence to the belief that the fetus is not always able to extract the nutrients needed from the maternal blood. Food restriction may demonstrate another type of interaction. The malnourished fetus may be less able to withstand other stresses when these

also occur during pregnancy(5). Intrauterine growth retardation, caused by maternal undernutrition, placental insufficiency or extrauterine factors; such as disease, maternal smoking or high altitude living; can lead to small-for-gestational-age (SGA) infants. The SGA infant in turn may be more prone to hypoglycemia, have a higher mortality rate and display a greater incidence of mental-motor retardation at a later age(6).

The maternal role of nutrient supplier has led many investigators to explore the effects of maternal diet on fetal development. Deficiencies of specific nutrients in maternal diets have been shown to alter fetal development. For example, zinc deficient diets fed to pregnant rats result in increased reabsorptions, stillborns and brain and skull malformations(7-11). A thiamin-deficient diet fed during pregnancy has been related to reduced number of offspring(10). Increased perinatal mortality and uneven hair development in newborn rats was observed wnen pregnant rats were fed a diet that was made deficient in nickel(12). Numerous studies of restricted caloric and/or protein intake during gestation have shown that growth and possibly brain development are retarded(3-5,13-16).

Sucrose

Sucrose has been intensively studied over the past few decades as the consumption of this form of carbohydrate has risen as a percent of total carbohydrate consumed(17).

Formerly the general belief was that all metabolizable carbohydrates had the same physiological effects. Now the literature is replete with studies showing a relationship (causal or otherwise) between sucrose and the incidence of obesity (17-20), heart disease and atherosclerosis(17,21-23), serum triglycerides(24) and serum cholesterol(25), vascular and neural changes in the eye(17,26) and dental caries(17).

Current research deals with the effects that sucrose produces on pathways of carbohydrate and lipid metabolism. Usually the diet containing sucrose is compared with diets containing starch, high fat, monosaccharides or other disaccharides. Blood and liver lipid concentrations and activities of lipogenic enzymes in liver and adipose tissue are the parameters most often examined. High intakes of sucrose have been shown to cause increases in blood triglycerides in the rat(27-32), primates(33,34) and man(35-38); and increases in blood cholesterol in the rat(27,31,39) and man(35-38). In other investigations a rise in blood cholesterol did not occur(25,30,40).

Possible explanations for these findings have been offered by several groups. Since the most apparent difference between sucrose and starch, other than chain length, is the presence of fructose in the former, much of the research has centered around this monosaccharide. Bar-on and Stein (41) compared the effect of glucose and fructose on lipid metabolism in the rat and found that in the liver more fructose was converted to triglyceride than was glucose, and that fructose did not stimulate lipoprotein lipase activity

in the adipose tissue. Their explanation was that fructose was absorbed into the portal circulation and converted in the liver to α-glycerophosphate. At the same time the outflow of free fatty acids from the adipose continued, leading to more triglyceride formation in the liver and its subsequent release into the blood. Since lipoprotein lipase activity did not increase, there was a buildup of triglyceride levels in the blood.

Chang et al.(42) concluded that dietary carbohydrates affected several enzymes. Depending upon the age and strain of the rat, they found elevated levels of glucose-6-phosphatase, glucose-6-phosphate dehydrogenase and aldolase in rats fed a 50% sucrose diet as compared to rats fed 50% glucose or cornstarch. Other researchers have also reported increased activity of glucose-6-phosphate dehydrogenase(42,45), glucose-6-phosphatase(42,43) and pyruvate kinase(44,45) in animals fed a high-sucrose diet.

Antar et al.(36) proposed that the hyperlipidemic effect of sucrose was dependent upon the percent of total calories as sucrose or starch, the amounts of saturated and unsaturated fat and the amount of cholesterol in the diet. They suggested two possible mechanisms of action: 1) An apparent inability of fructose to stimulate lipoprotein lipase in adipose tissue, resulting in an accumulation of blood triglycerides; or 2) the rapid absorption of simple sugars in comparison to starch thus causing a flooding effect on the liver and a stimulation of the release of lipoprotein into the blood.

Macdonald(33) suggested that the type of fat and protein consumed may also influence the lipid response to a high-sucrose diet. In studying primates, Macdonald suggested that presence of fructose in sucrose may be the important factor. According to Macdonald, fructose does not stimulate insulin output so lipoprotein lipase activity would not be stimulated. Therefore serum lipids would increase. Macdonald (33) and Coltart and Macdonald(34) proposed that the female sex hormone, estrogen, plays a role after fructose or glucose has been metabolized to α-glycerophosphate to give the glycerol moiety of triglycerides.

Naismith and Khan(32), however, reported that a high-sucrose diet induced a large increase in the activity of lipoprotein lipase in the adipose tissue, and concluded from this that in rats the elevated blood triglycerides were not due to an inability to clear the lipid from the blood. Naismith et al.(37) also examined the effect of a high-sucrose diet on humans to determine if the hypertriglyceridemia associated with this diet might be caused by some unrecognized increase in energy intake. Comparing a high energy-high-sucrose diet to a high energy-starch diet in humans, they found that only the high-sucrose diet caused elevated plasma triglycerides, total cholesterol and phospholipids. They concluded that the elevated blood lipid levels were induced by the sucrose and not by a change in energy intake.

In addition, Naismith(29) found that while fructose was a potent stimulator of hepatic lipogenesis, maltose, which has no fructose, stimulated lipogenesis as effectively as did sucrose. Therefore he concluded that fructose could not be used to explain the hyperlipidemic effect of sucrose. Instead, he suggested that the primary stimulus was the flooding of liver cells with large amounts of monosaccharides, which induced the enzymes of fatty acid synthesis.

Naismith and Rana(27,28) proposed further that the hyperlipidemic effect of sucrose was due to the rapid rate of hydrolysis and absorption of sucrose and the increased output of triglycerides from the liver to the adipose tissue. It has been recently demonstrated that there may be a different and more rapid absorption system for sucrose as compared to more complex carbohydrates(46).

Naismith and Khan(39)showed a rise in blood cholesterol in rats fed a high-sucrose diet. This was largely confined to the esterified fraction and there was a concomitant rise in activity of lecithin:cholesterol acyltransferase which catalyses the esterification of free cholesterol in the blood.

Using labelled glucose, Bender and Thadani(43) found that feeding a sucrose diet to rats caused a marked depression of both glucose oxidation and lipogenesis in the liver. While they concluded that these metabolic pathways had been altered, they did not indicate the direction of change of the pathways. They did suggest that the effect of sucrose might be a reduction in quantity of glucose-6-phosphate,

which resulted from the stimulation of glucose-6-phosphatase and by altering the ratio of glucose metabolized through the pentose pathway, which would occur as a result of the stimulation of the formation of glucose-6-phosphate dehydrogenase.

Moser and Berdanier(47) researched the effects of feeding a high-sucrose diet during growth. They developed four experimental groups: 1) one fed a 65% starch diet from weaning; 2) one fed a 65% sucrose diet from weaning; 3) one fed a starch diet from weaning to 50 days of age and then fed a sucrose diet; and 4) one fed a sucrose diet from weaning to 50 days of age and then fed a starch diet. At 142 days the animals fed sucrose up to 50 days of age had higher liver lipid and liver cholesterol than control animals fed starch from weaning. Differences in activity of several liver enzymes were also noted, although the differences were not necessarily attributable to sucrose feeding from weaning to only 50 days of age.

Sucrose and Maternal-Fetal Relationships

Using the BHE strain of rat, which has been bred to develop lipemia and diabetes in response to various types of carbohydrate, Berdanier(48) fed female rats sucrose or starch diets during pregnancy and lactation, switching some of the mothers' diets during lactation. Male offspring were then fed the starch or sucrose diet. This produced six experimental groups:

Gestation:	Starch	Starch	Starch	Sucrose	Sucrose	Sucrose
	↓	↓	↓	↓	↓	↓
Lactation:	Starch	Starch	Sucrose	Starch	Sucrose	Sucrose
	↓	↓	↓	↓	↓	↓
Growth:	Starch	Sucrose	Starch	Starch	Sucrose	Starch
Group no.:	1 (control)	2	3	4	5	6

At 150 days of age the progeny in groups 3,4 and 6 had <u>decreased</u> levels of serum triglycerides as compared to controls. Group 2 had elevated serum triglycerides, similar to data reported by other researchers(27-32). However, group 5 did not differ from controls in this respect. Group 6 also showed elevated liver lipid, while groups 2 and 3 had lower liver lipid, when each was compared to controls. Groups 2 and 5 also showed higher activity of glucose-6-phosphate dehydrogenase activity when compared to control, but no difference was noted in any group in the activity of malic enzyme when compared to controls. From these data, Berdanier suggested that the post-weaning period is of greater importance in regards to the lipogenic response of sucrose feeding than is either gestation or lactation.

Davis et al.(49,50) conducted experiments in mice to examine the effects of feeding sucrose during gestation. They increased the sucrose content of the maternal diet by diluting a regular stock diet with 35% sucrose. At weaning pups were fed the normal stock diet. Male progeny from dams fed sucrose during gestation and lactation were heavier in weight and remained heavier throughout the experiments. Some of these experiments lasted over 80 weeks. They also presented results which they interpreted as showing an

impaired response to a glucose load by offspring from mice fed the sucrose diet during gestation and lactation. In other results they found that the pre-beta lipoprotein fraction in serum of mice at birth from dams fed the sucrose diet was over 50% while the control group was less than 5%. The lipoprotein pattern of the offspring from dams fed the sucrose diet is described as similar to that of Type IV hyperlipoproteinemia. The pre-beta lipoprotein levels were still elevated at 2 months of age, but had decreased significantly by 4 months. They also report higher serum cholesterol and body fat in the mice from dams fed the sucrose diet but do not present any data.

The present study was undertaken to confirm some of the same parameters used by Berdanier and Davis. However, a strain of rat was used which does not show any specific genetic tendencies toward alterations in carbohydrate as does the BHE rat. Instead of diluting the diet with sucrose as was done by Davis and thus reducing the protein, vitamins and minerals as well, semipurified diets similar to Berdanier's were used, where the only difference was the type of carbohydrate.

MATERIALS AND METHODS

Preliminary Study

The following preliminary research was conducted to decide upon possible parameters to study in future experiments. It is only briefly described here. The more detailed description given for the main study will elucidate what was done in this preliminary work.

Eight female Sprague-Dawley rats[1], weighing approximately 250 g, were fed a semipurified diet with either cornstarch (4 rats) or sucrose (4 rats) as the sole source of carbohydrate (Table 1). These rats were mated and kept on the same experimental diet. After parturition, litter size was adjusted to 8 pups per dam.

At weaning, pups were divided into 4 experimental groups based on diets:

Mothers:	Sucrose	Sucrose	Cornstarch	Cornstarch
Weanlings:	Sucrose	Cornstarch	Sucrose	Cornstarch
Group No.:	1	2	3	4 (control)

At approximately 9 weeks of age, 2-4 rats from each group were killed and the following parameters were examined: body weight, serum cholesterol (51,52), serum

[1]Spartan Research Animals, Inc., Haslett, MI 48840.

TABLE 1 Diet composition.

Ingredient	Percent of diet
Sodium caseinate[1]	20.0
Mineral mix[2]	4.0
Vitamin mix[3]	1.0
Corn oil[4]	5.0
Cellulose	4.0
Choline Cl	0.2
D,L methionine	0.3
Cornstarch or sucrose	65.5

[1]Donated by Quaker Oats Co., Barrington, IL. [2]No. 4164 salt mix, Teklad Test Diets, Madison, WI 53713. [3]In mg/kg diet: thiamin HCl, 22; pyridoxine, 22; riboflavin, 22; Ca pantothenate, 60; p-amino benzoic acid, 110; ascorbic acid, 200; niacin, 100; vitamin B_{12}, 0.3; biotin, 0.6; folic acid, 4.0. In IU/kg diet: vitamin A, 20,000; vitamin E, 100; vitamin D_3, 2,200. [4]Mazola.

triglycerides (53), liver weight, liver lipid and liver cholesterol (51,52). At this same age blood samples from 2-4 of the remaining rats in each group were drawn from the posterior orbital region of the eye (54). These samples were analyzed for serum glucose[1] and cholesterol.

At 18 weeks of age, blood samples were again taken. Serum glucose, cholesterol and triglycerides were determined.

At 20 weeks of age, the remaining 2-4 rats from each diet were killed and the same parameters, plus serum glucose, were studied as with the first group at 9 weeks of age.

Main Study

Thirty female Sprague-Dawley rats[2], weighing approximately 225 g, were distributed five to a cage. Three groups were fed the cornstarch diet and three groups the high-sucrose diet (Table 1). Water was available ad libitum. After 3 days on the experimental diets, a male Sprague-Dawley rat[3], weighing approximately 500-700 g, was placed with each group. After 8 days, the males were removed, and the females were separated and weighed every other day. Whenever a rat had a weight gain of from 5-7 g/day over a 4 day period, it was placed in a solid bottom plastic cage which contained wood shavings. Each female remained on the experimental diet that

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[1]Glucostat, Worthington Biochemical Corp., Freehold, NJ.
[2]Spartan Research Animals, Inc., Haslett, MI 48840.
[3]These rats were almost one year old. An attempt had been made to get younger male rats at an age where they were capable of mating. At that time the supplier was unable to provide such animals, and it was decided that the older animals would be used.

was originally fed.[1] Within 24 hours of delivery, pups were sexed and litter size was reduced or increased to 8 pups/dam. If the litter was reduced, the procedure was to maximize the number of male pups in the litter that remained. Litters were increased by taking female pups from other dams who had delivered larger litters within the same 24 hour period. This latter procedure was used twice. Food and water were available to the dams at all times and no attempt was made to prevent the pups from eating from the food jar during the 21 day lactation period.

Pups were weaned at 21 days of age, weighed, placed in hanging wire cages and fed the cornstarch diet. This produced two experimental groups: weanlings from dams fed the high-sucrose diet (the SUC group); and weanlings from dams fed the cornstarch diet (the CS or control group). Water was available ad libitum, the room was lighted from 0700 hours to 2000 hours and room temperature was a constant $22^{\circ}\pm1^{\circ}$. Humidity tended to flucuate with the humidity external to the building. Food intake was measured whenever food cups were filled, usually every 3-4 days, and body weights were recorded weekly.

To develop skill in performing the analytical procedures and to obtain some initial data, intraperitoneal glucose tolerance tests, as described by Romsos and Leveille (55), were conducted on 16 weanlings, 8 from each group (4 males

[1]Since initially only 7 out of the 30 females delivered pups, the procedure was repeated 3 weeks later with 16 of the remaining 23 females, 8 of which subsequently had litters.

and 4 females), at 30 days of age. Blood glucose concentration was analyzed by the glucose oxidase procedure.[1]

At 30-35 days of age, 4 female weanlings from each group were killed by decapitation. Blood was collected for serum triglyceride (53) and serum cholesterol (56) determinations. The liver was rapidly removed and a 1 g sample excised for the determination of glucose-6-phosphate dehydrogenase (EC 1.1.1.49, G6PD) activity (57). Protein content was analyzed using the method of Lowry et al. (58). The rest of the liver was frozen. After thawing the liver, duplicate 1 g samples from different lobes were homogenized in 10 ml of methanol. Total lipids were determined gravimetrically after two extractions in chloroform:methanol. Liver cholesterol was assayed after redissolving the lipid material in 10 ml of chloroform and taking a 3 ml aliquot.

Only male rats were used for the remainder of the experiment. A total of 81 males were weaned, 39 CS weanlings and 42 SUC weanlings. At 53 days of age, glucose tolerance tests were performed on 8 weanlings from each group, and at 113 days of age on 10 weanlings from each group. Offspring from several different dams were used each time. At 60 and 120 days of age, one-half of the CS weanlings and one-half of the SUC weanlings were killed. The same parameters as those studied at 30-35 days of age were analyzed.

Analyses as used in this study, and percent error, are described in detail in the Appendix. Student's t test was used to test the data for significance (59).

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[1]Glucostat, Worthington Biochemical Corp., Freehold, NJ.

RESULTS AND DISCUSSION

Preliminary Study

Results from the preliminary study are summarized in Table 2. Body weight, serum cholesterol and triglycerides, relative liver size (RLS) and liver total lipid and cholesterol are given for the two groups which had the same experimental design as the groups subsequently used in the main study. These groups were: the offspring from dams fed the cornstarch diet during pregnancy and lactation (the CS or control group); and the offspring from dams fed the high-sucrose diet during pregnancy and lactation (the SUC group). All offspring were fed the cornstarch diet after being weaned.

No difference in body weight was observed between the SUC and control groups either at 66 days of age or 139 days of age. This finding was not in accordance with results obtained by Davis et al. (49,50) in mice. Serum cholesterol was the same at 66 days, but was somewhat elevated at 127 and 139 days of age for the SUC group. Serum triglycerides were not altered by the experimental regimen at either 66 or 127 days of age. At 139 days there was a trend toward lower serum triglycerides in the SUC animals. This trend in triglycerides is in agreement with results reported by Berdanier (48).

TABLE 2 Results of preliminary study.[1]

Parameter	Group CS[2] n=3	SUC[2] n=3
Body weight(g)		
66 days	384	365
139 days	539	503
Serum cholesterol(mg/100 ml)		
66 days	99	94
127 days	95	120
139 days	105	124
Serum triglycerides(mg/100 ml)		
66 days	61	60
127 days	76	77
139 days	111	87
RLS[3](g liver/ 100 g body wt.)		
66 days	3.20	3.50
139 days	2.94	2.87
Liver total lipid(mg/g liver)		
66 days	55.2	58.2
139 days	49.0	59.0
Liver cholesterol(mg/g liver)		
66 days	5.0	5.3
139 days	5.2	6.9

[1]No significant differences ($p < 0.05$) were found for any parameter. [2]CS = offspring of dams fed cornstarch diet during pregnancy and lactation; SUC = offspring of dams fed high-sucrose diet during pregnancy and lactation; all offspring fed cornstarch diet after weaning. [3]RLS = relative liver size.

Liver size was not affected by the experimental design either at 66 or 139 days of age. Total lipid in the liver, while not different at 66 days, tended to be higher in the SUC group at 139 days. This apparent elevation at 139 days would agree with the work of Berdanier(48). For liver cholesterol no difference was observed at 66 days of age, but the SUC group results tended to be higher than control at 139 days.

Since some of the results in this preliminary experiment differed from those found by other investigators, a more detailed experiment using larger numbers of animals was undertaken to look at additional parameters related to carbohydrate and lipid metabolism and to see if the present results could be replicated.

Main Study

Litter Size and Weaning Weights

The feeding of sucrose during pregnancy did not have any effect on the size of the litter(Table 3). There were no grossly apparent differences in the physical appearance of the pups, either at parturition or during lactation. Weaning weights of all pups, and specifically of all male pups, were not altered by the diet fed the dams during gestation and lactation(Table 3). The pups in the SUC group consumed some of the high-sucrose diet during the last week of lactation. Apparently this brief period of sucrose intake did not have any immediate effect on body weight and, as will be shown later, did not affect body weight during the rest of the study.

Parameters Studied in Dams

If the parameters being studied were dramatically different in the mothers, this fact might explain any differences noted in their offspring. Whether any differences were due to the experimental design or just random variation among animals is of some importance. Possibilities might exist that offspring were exhibiting traits inherited from the mothers or were exhibiting long-term effects that resulted from the diets fed to the dams during gestation and lactation. When the dams were killed they had been fed the experimental diets continuously for an 8-10 week period. All dams had finished lactation 10-14 days prior to being killed.

Body weights of the dams were similar although the rats fed the sucrose diet had somewhat lower weights (Table 4). Serum cholesterol and triglycerides were not significantly different (Table 4). This result is not in agreement with other studies (27-32) in which sucrose was fed to mature rats and elevations of triglycerides were noted. One possible reason for this difference in results might be the fact that the rats in this study had recently finished lactating.

The relative size of the liver (RLS) was not different between the two groups (Table 4). Nor was glucose-6-phosphate dehydrogenase (G6PD) activity significantly changed by the experimental design (Table 4). However, for both parameters, the dams fed the high-sucrose diet had the higher values. The difference in G6PD activity was rather marked but variation in activity within the groups was such that this

TABLE 3 Litter size and weaning weight.

Dietary group	No. of dams	Litter size	No. of male offspring	Weaning weights(g)
CS[1]	7	12.3±0.5[2]	39	59±1
SUC[1]	8	11.4±1.1	42	60±1

[1]CS = dams fed cornstarch diet during pregnancy and lactation; SUC = dams fed high-sucrose diet during pregnancy and lactation. [2]Mean±SEM.

TABLE 4 Effect of high-sucrose diets in dams.[1]

Parameter	CS group n=4	SUC group n=4
Body weight(g)	313±6[2]	294±7
Serum cholesterol (mg/ 100 ml)	98±7	110±8
Serum triglycerides (mg/100 ml)	180±23	179±36
Relative liver size (g liver/100 g body wt.)	3.74±0.13	4.39±0.35
G6PD activity[3]	41.5±12.8	54.3±10.7
Liver total lipid (mg/g fat-free dry matter)	169±10	222±14*[4,5]
Liver cholesterol (mg/g fat-free dry matter)	6.0±0.3	7.7±1.0*

[1]1-2 weeks after termination of lactation; total length of time on diet was 8-10 weeks. [2]Mean±SEM. [3]Given as nmoles substrate converted/ mg protein/min. [4]Values having an asterisk are significantly different ($p < 0.05$) from CS group. [5]Data for 3 animals.

difference could be attributed to a random selection of animals. Total lipid in the liver was significantly higher in the mothers fed the high-sucrose diet (Table 4). Other studies (29,41) have also demonstrated that increases in liver lipid did occur when a high-sucrose diet was fed. Liver cholesterol was also significantly higher in the sucrose-fed dams (Table 4).

Parameters Studied in Offspring

Body weight

A comparison of body weights revealed no significant differences between the SUC group and controls either at 60 or 120 days of age (Table 5). The results of Davis et al. (49,50) in mice, where offspring from sucrose-fed mothers were found to be heavier then controls throughout the study, may well be due to other alterations in the diet since the diet was diluted with sucrose rather then having the sucrose isocalorically replace different carbohydrates. The species difference also cannot be disregarded as a possible causative factor for the difference in weight gain.

Between approximately 80 and 100 days of age, most animals contracted a respiratory infection of varying degrees of severity. All animals were given an antibiotic[1] in their drinking water for 14 days. While some animals experienced weight loss, by 112 days all were eating amounts equal to or

[1]Cosa-Terramycin (oxytetracycline hydrochloride with glucosamine HCl and vitamins); Dept. of Veterinary Medicine Chas. Pfizer and Co., Inc., New York, NY. Approximately 2 tablespoons per liter of water.

TABLE 5 Body weight, serum triglycerides and cholesterol.

Parameter	CS[1] group	SUC[1] group
Body weight(g)		
60 days	339±4[2] (20)[3]	334±5 (21)
120 days	450±7 (19)	443±7 (21)
Serum triglycerides(mg/100 ml)		
30 days[4]	84±10 (8)	79±11 (8)
60 days	258±28 (20)	219±17 (21)
120 days	135±10 (19)	123±12 (21)
Serum cholesterol(mg/100 ml)		
30 days[4]	107±3 (8)	111±4 (8)
60 days	98±4 (20)	97±4 (21)
120 days	84±4 (19)	97±5*[5] (21)

[1]CS = offspring of dams fed cornstarch during pregnancy and lactation; SUC = offspring of dams fed high-sucrose diet during pregnancy and lactation; all offspring fed cornstarch diet after weaning. [2]Mean±SEM. [3]Number of animals in group. [4]Only female offspring were killed at 30 days of age and only male offspring were used for the remainder of the study. [5]Values with an asterisk are significantly different ($p < 0.05$) from CS group.

greater than before the infection and all were gaining weight. Although an effect upon weight gain and the other parameters studied cannot be conclusively ruled out, the close agreement of body weights within groups at 120 days leads to the supposition that the illness did not have a long-lasting effect and most likely did not alter the results obtained at 120 days of age.

Serum triglycerides and cholesterol

Contrary to the findings of Berdanier(48), no significant differences were noted in serum triglycerides(Table 5). Since a larger number of animals per group were used in this study(21 vs 6 for Berdanier's work), these results indicate that no difference in triglyceride concentration exists. At all ages when triglycerides were analyzed the SUC rats tended to have the lower values. This difference might have been significant if the analyses had been performed on fasted rats (as was done by Berdanier) instead of nonfasted rats. While the large variation that is observed may result from not having fasted the animals, it also should be noted that Berdanier's results displayed similar or even greater variations in the findings.

Berdanier offers a possible explanation for the lower serum triglycerides that she reports. The hypothesis is that the fetus or suckling rat in the SUC group develops a more efficient system for clearing the blood triglycerides as a consequence of an increase in lipoprotein lipase activity and an increase in the receptor sites for lipoproteins. As

long as excess lipids are present in the blood, the increased enzyme activity and more numerous receptor sites would remain. The problem with this hypothesis is that at 60 or 120 days of age(or 142 days or Berdanier's study), the SUC rats would not be synthesizing any excess "sucrose-induced" lipids since they have been fed a cornstarch diet from weaning. Therefore there should not be any overly large amounts of lipid in the blood and the two factors would supposedly have returned to their normal activity or number.

Serum cholesterol was the same in both groups at 30 and 60 days of age (Table 5). At 120 days of age the SUC group had significantly higher serum cholesterol. Why this increase occurred is not clear. That the elevation might be due to the difference in maternal diet appears rather unlikely, for one would question why this was not observed at 60 days of age. And yet, given that the only difference between the two groups was the diets fed to the mothers and given the large n value for each group, it is difficult to ignore the hypothesis that the diet fed to the dams during gestation and lactation might have had some long-term effect on the offspring, at least with regard to serum cholesterol.

Liver total lipid and cholesterol

Differences in total lipid in the liver did not show a consistent pattern (Table 6). At 30 days of age both groups had similar amounts of lipid in the liver. At 60 days of age the SUC animals had significantly higher liver lipid, but at 120 days the CS group had significantly higher lipid.

TABLE 6 Liver total lipid and cholesterol.

Parameter	CS[1] group	SUC[1] group
Liver total lipid (mg/g fat-free dry matter)		
30 days[2]	189±9[3] (8)[4]	180±11 (8)
60 days	213±24 (20)	341±23*[5] (21)
120 days	202±8 (19)	174±8* (21)
Liver cholesterol (mg/g fat-free dry matter)		
30 days[2]	7.3±0.4 (8)	7.2±0.3 (8)
60 days	5.4±0.2 (20)	5.7±1.1 (21)
120 days	5.5±0.1 (19)	5.6±0.3 (21)

[1]See footnote 1, Table 5. [2]See footnote 4, Table 5. [3]Mean±SEM. [4]Number of animals in group. [5]Values having an asterisk are significantly different ($p < 0.05$) from CS group.

Berdanier reported that at 142 days of age the group comparable to the SUC group had significantly higher amounts of lipid in the liver. Since neither study showed any elevation of G6PD activity (see following section), nor did Berdanier's study show any elevation of malic enzyme activity, an increase in the rate of fatty acid synthesis is not likely. Because the results of this present study are not consistent from one age to the next, the reason why liver lipid was different is not clear.

Liver cholesterol values were very similar at all 3 ages (Table 6). From these results, it would seem that the maternal diet had no long-lasting effect on the amount of cholesterol in the liver. That total lipid in the liver should show changes while cholesterol did not would indicate that any difference in lipid was in the triglyceride or phospholipid fraction.

Relative liver size (RLS) and G6PD activity

RLS was lower at 60 and 120 days of age, although the difference was statistically significant only at 60 days (Table 7). Berdanier (48) also found a significantly lower RLS at 142 days of age in the group that is comparable to the SUC group of this study. Changes in liver size often result from changes in total liver lipid. At 60 days in this study and at 142 days in Berdanier's study, the rats in the SUC group had a significantly smaller RLS but significantly more total liver lipid. The greater amount of lipid but smaller RLS might imply a decrease in water, glycogen or

TABLE 7 RLS[1] and G6PD activity.[2]

Parameter	CS[3] group	SUC[3] group
RLS(g liver/100 g body wt.)		
30 days[4]	4.59±0.14[5] (8)[6]	4.58±0.06 (8)
60 days	4.34±0.06 (20)	4.15±0.07*[7] (21)
120 days	3.54±0.06 (19)	3.42±0.03 (21)
G6PD activity[2]		
30 days[4]	21.3±4.6 (7)	20.6±4.0 (7)
60 days	9.5±0.9 (20)	10.4±0.7 (21)
120 days	13.9±1.2 (19)	14.6±1.0 (21)

[1]RLS = relative liver size. [2]Glucose-6-phosphate dehydrogenase activity expressed as nmoles substrate (NADP) converted per mg protein per min. [3]See footnote 1, Table 5. [4]See footnote 4, Table 5. [5]Mean±SEM. [6]Number of animals in group. [7]Values having an asterisk are significantly different ($p < 0.05$) from CS group.

other nonlipid material. Since Berdanier's rats were fasted, glycogen would not be present in the liver in any significant amounts. In this study the fat-free dry matter was not different between the two groups. The possibility of a decrease in water content of the liver cannot be ruled out. A reason for the water content of the liver to be lower is not known.

G6PD activity was similar in both experimental groups at all three ages(Table 7). Since the substrate available was the same for both groups, differences in activity would not be expected. However, if the hypothesis is that the maternal diet has some long range effect on lipogenic activities of the liver, then one would have expected to see some alteration in the activity of this lipogenic enzyme.

Glucose tolerance

The glucose tolerance test is often administered to ascertain if diabetes mellitus is present as a metabolic disease. The concentration of glucose in the blood over time is an indication of the rates of addition and removal of blood glucose(60,61). In the present experiment, at the three ages studied, fasting blood glucose was nearly the same for both groups(Table 8). Compared to their control, Davis et al. consistently found significantly higher fasting values in the mice which were comparable to the SUC group of this study. At 30 days of age and in the animals tested the second time at 120 days of age, the CS group had a significantly higher peak in bloodglucose 15-30 minutes after the

TABLE 8 Glucose tolerance tests.

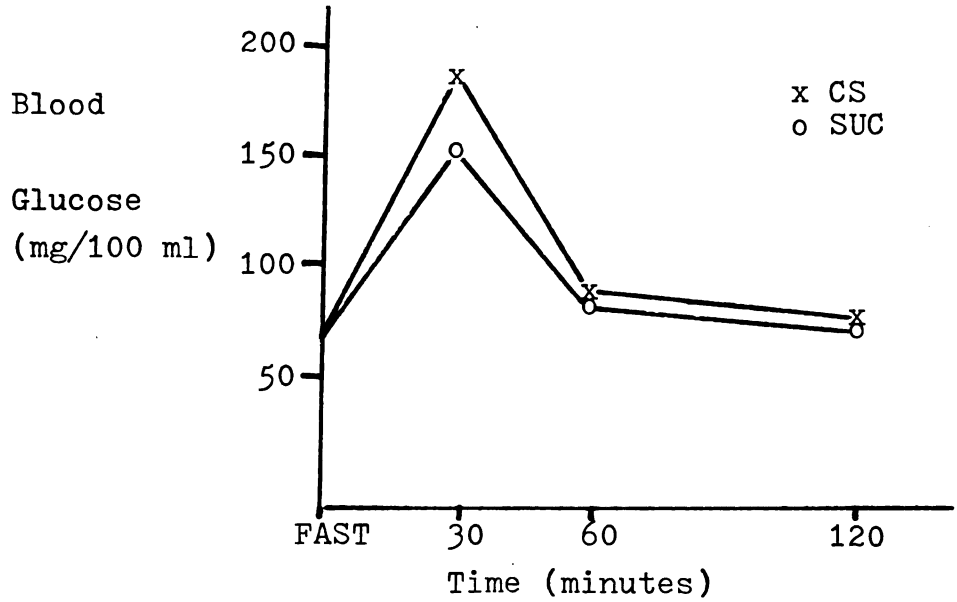
Group[1]	Age[2]	No. rats[3]	Blood glucose (mg/100 ml) Fast	15[4]	30	60	120
CS	30		72±5[5] (4,4)[3]		186±6 (4,3)	89±3 (4,3)	78±2 (1,4)
SUC			68±3 (4,4)		153±14*[6] (4,3)	83±3 (4,4)	74±2 (4,4)
CS	60	8	90±1	231±30	246±32	176±21	128±9
SUC		8	85±2	280±25	284±26	182±15	126±9
CS	120 (1st test)[7]	5	87±3	172±27	172±24	142±15	120±9
SUC		5	87±5	185±15	208±16	192±10*	125±7
CS	120 (2nd test)[7]	5	72±2	218±10	209±7	166±5	126±5
SUC		5	72±4	159±15*	157±10*	141±6*	104±4*

[1]See footnote 1, Table 5. [2]Days. [3]Number of rats varied at 30 days of age; values in parenthese represent number of males and females, respectively. [4]Minutes. [5]Mean±SEM. [6]Values having an asterisk are significantly different ($p < 0.05$) from CS group at time indicated. [7]The first test at 120 days of age was performed on the rats from females that became pregnant initially, and the second test was performed on rats from females that became pregnant 3 weeks later. Two tests were also performed at 60 days of age, but the data from the first test was not valid due to problems with the colorimetric technique that was used.

administration of a glucose load(Table 8, Figure 1 and Figure 2). However at 60 days and in the animals tested the first time at 120 days of age, the SUC animals had the higher peak although it was not significantly higher. The higher peak in glucose concentration in the CS group is in contrast to the results of Davis et al.(49,50) in which the mice from mothers fed the sucrose diet always had the higher mean peak values.

These investigators also reported that the blood glucose remained higher for a longer period of time in these mice; that is, these mice appeared to display a lag time in the clearance of glucose from the blood. A better understanding would have resulted if the rate of clearance of glucose from the blood had been calculated. Amatuzio et al.(60), Scow and and Cornfeld(61) and Chernick and Scow(62) have developed methods for finding the rate of removal of glucose from the blood. The method of Amatuzio et al. requires that a blood sample be taken very soon after a glucose load is administered. This was not done here; therefore the formula presented by Chernick and Scow(62) was utilized. The removal rate(K) is given as: $K = \frac{2.3(\log G_{t_1} - \log G_{t_2})}{t_2 - t_1}$; where G_t is the excess glucose concentration at time t; that is, G_t is the blood glucose concentration at time t minus the fasting blood glucose concentration. (K x 100) then represents the percentage decrease in the excess glucose concentration per minute over time t_1 to t_2. In this study, t_1 was taken as 30 minutes and t_2 as 120 minutes.

a. 30 days of age.[1]



b. 60 days of age.

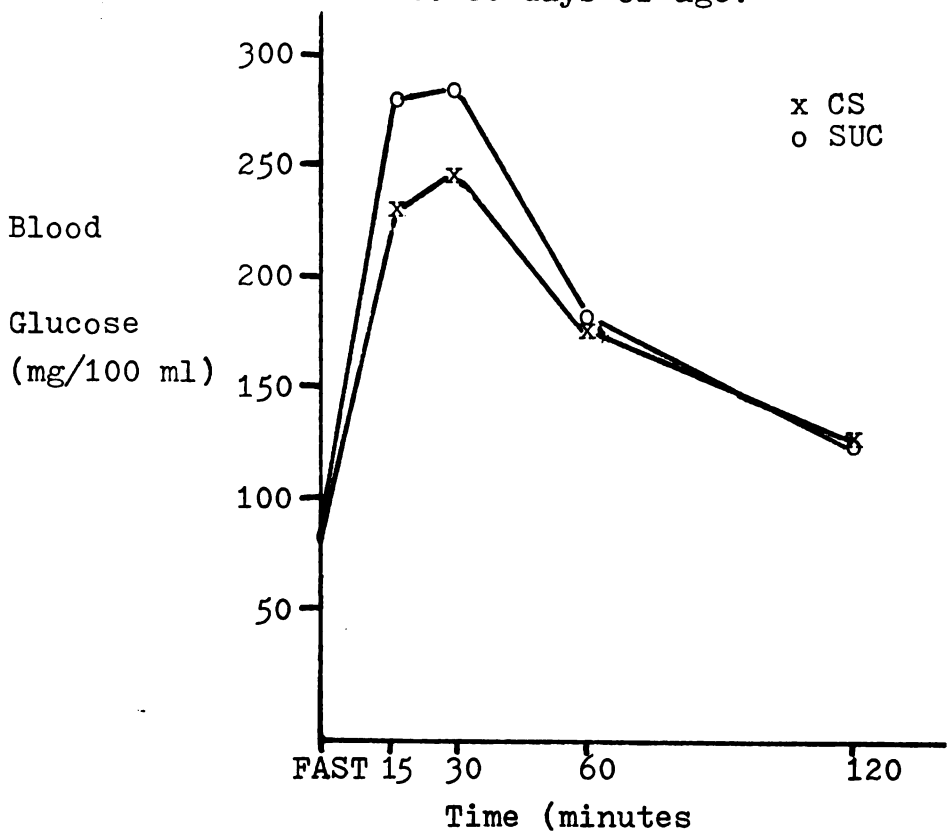
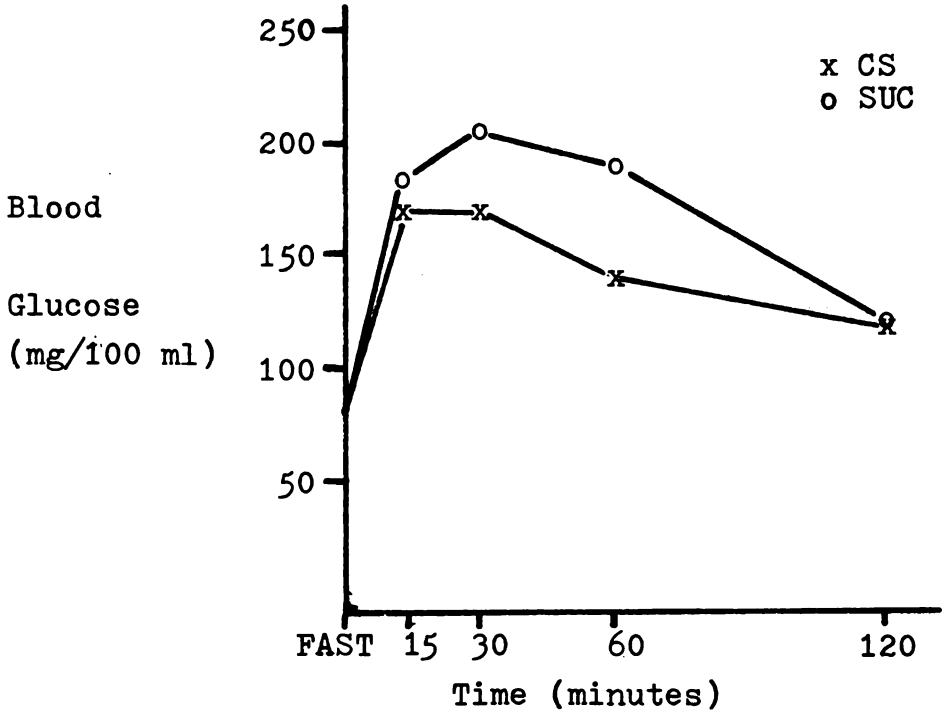


FIGURE 1 Glucose tolerance, 30 and 60 days of age.

[1]Male and female rats, see Table 8.

a. 120 days of age, 1st test.[1]



b. 120 days of age, 2nd test.[1]

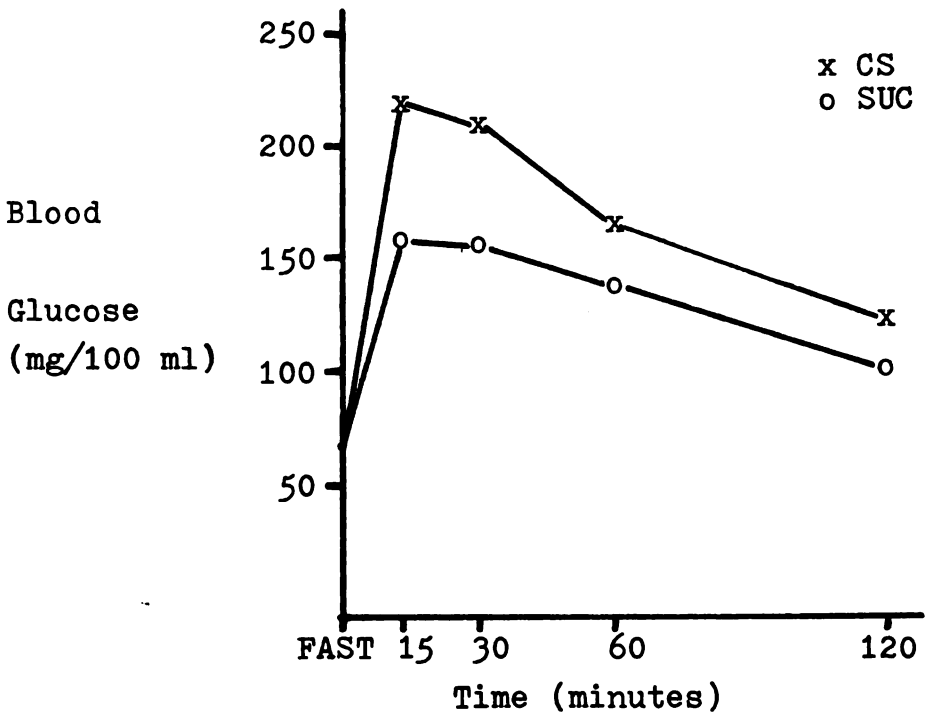


FIGURE 2 Glucose tolerance, 120 days of age (2 tests).

[1]For description of 2 tests, see footnote 7, Table 8.

Table 9 gives the removal rats at the three ages studied. There is no indication that the removal rate differed between the two groups at any age. The glucose load administered at 30 days of age was only one-quarter of the amount given at 60 and 120 days of age. This may account for the more rapid rate of removal.

The results of Davis et al. might indicate that the maternal diet somehow altered the fasting glucose values of the mice. The present study would seem to clearly indicate that this was not true for the rat. Of course the species difference is a factor that cannot be ignored when attempting to compare results.

Since the present study placed greater restrictions on possible dietary variables other than the carbohydrate source then did the study conducted by Davis et al., it appears relatively certain that, at least in the rat, the feeding of a high-sucrose diet during gestation and lactation does not alter the response to a glucose load in growing or mature offspring who have been fed a diet without sucrose.

TABLE 9 Glucose removal rate.

Group[1]	Age[2]	No. rats[3]	Removal rate of excess glucose(%/min.)[4] 30-120 min.
CS	30		4.0±0.8[5] (1,3)[3]
SUC			3.3±0.5 (4,3)
CS	60	8	1.8±0.2
SUC		8	1.8±0.2
CS	120	10	1.0±0.1
SUC		10	1.2±0.2

[1]See footnote 1, Table 5. [2]Days. [3]See footnote 3, Table 8. [4]Kx100, see text for derivation of removal rate, K. [5]Mean±SEM.

SUMMARY AND CONCLUSIONS

This study was undertaken in an attempt to clarify some results of investigations previously reported in the literature (47-50). Various parameters of lipid and carbohydrate metabolism were evaluated in male offspring of rats fed a high-sucrose (SUC group) or cornstarch diet (CS or control group) during pregnancy and lactation. After weaning the offspring from both groups of dams were fed a cornstarch diet. In previous studies the diets were either diluted with sucrose, thus reducing the protein, vitamins and minerals as well, or a strain of rat was used that was susceptible to carbohydrate induced lipemia.

The present study did not show the increase in body weight or the inability to handle a glucose load by the offspring in the SUC group as had been reported in mice by Davis et al. (49,50). Likewise, a depression in serum triglycerides and an elevation of liver lipid was not noted in the SUC group, although these results had been reported by Berdanier (48). Analyses at 30, 60 and 120 days of age of body weight, serum triglycerides and cholesterol, relative liver size, liver G6PD activity, liver lipid and cholesterol, and glucose tolerance revealed that while some other differences were apparent, they did not follow the same patterns that had been

reported previously. Given the large n value of each group, the results of this study lead one to the conclusion that in the rat there does not seem to be any consistent long-term metabolic effects in offspring of mothers that had been fed a high-sucrose diet during gestation and lactation, if the offspring were fed a cornstarch diet from weaning. This conclusion would be true at least for the parameters chosen for this research.

APPENDIX

APPENDIX

METHODS OF ANALYSIS

Glucose-6-Phosphate Dehydrogenase (G6PD) Activity

1g samples of liver were homogenized in 9 ml of the following solution: 0.15 M KCl, 10 mM N-acetyl-l-cysteine and 1 mM $MgCl_2 \cdot 6\ H_2O$, made up in distilled water. This solution was mixed 2 days prior to use and refrigerated. The day before the analysis was to be done, the solution was adjusted to pH 7.6 while still cold. The solution and the subsequent homogenates were kept on ice throughout the procedure.

Homogenates were centrifuged for 15 minutes at 31,000 X g in a Model RC2-B Sorval refrigerated centrifuge[1] (-4° to 0°). The supernatant was removed using disposable pipettes, with care being taken to avoid pipetting the top layer of lipid and the bottom layer of non-cytosolic material. This supernatant was then centrifuged for 1 hour at 100,000 X g in a Model L3-40 Beckman centrifuge.[2] The supernatant was removed, with care being taken not to draw off any lipid from the top or precipitate from the bottom. The supernatant was kept in tubes on ice throughout the assay.

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[1]Ivan Sorval, Inc., Newtown, CT 06470.
[2]Beckman Instruments, Inc., Palo Alto, CA 94304.

The reaction mixture consisted of:

(a) 2.7 ml of a solution prepared as follows, for 100 assays:

Tris buffer[1], 1 M, pH 7.5, 25°	10 ml
NADP, monosodium salt, 3 mM	10 ml
$MgCl_2 \cdot 6\ H_2O$, 200 mM	10 ml
Distilled water	250 ml

(b) 0.1 ml 6-phosphogluconic acid (6PG), trisodium salt, 25 mM.
(c) 0.1 ml glucose-6-phosphate (G6P), monosodium salt, 25 mM.
(d) 0.02 ml sample[2]
(e) 0.08 ml distilled water.

The initial mixture contained (a), (d) and (e). The 6PG(b) was then added, the solution mixed and then run, 4 samples at a time, on an ACTA CIII Beckman spectrophotometer[3] with span set at 0.5 of full range and chart paper speed of 1.2 inches per minute. The temperature was kept constant at 25°. After 4-5 minutes, G6P(c) was added, the solution rapidly mixed and then once again run. The wavelength was set at 430 nm, with a slit width of approximately 0.15 mm. A tungsten lamp was used.

The principle of the assay is based on the reduction of NADP to NADPH by glucose-6-phosphate dehydrogenase(G6PD) in the pentose pathway: G6P $\xrightarrow[NADP \rightarrow NADPH]{G6PD}$ 6-phosphogluconolactone $\xrightarrow[H_2O]{lactonase}$ 6PG $\xrightarrow[NADP \rightarrow NADPH]{6PGD}$ D-ribulose-5-phosphate. Centrifuging at 100,000 X g eliminates the probability that reduction reactions in the mitochondria and microsomes would be present to interfere with the cytosolic readings. A change in absorbance due to this conversion of NADP is read

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[1] Sigma Technical Bulletin No. 106 B, Sigma Chemical Co., St. Louis, MO 63178.

[2] 0.01-0.05 ml samples were used to obtain a good rate and to check for linearity, and 0.02 ml was chosen as the best concentration.

[3] Beckman Instruments, Inc., Scientific Instruments Division, Fullerton, CA 92634.

maximally at 430 nm. By adding 6PG first and then waiting several minutes before adding G6P, a rate can be determined for the conversion of 6PG to D-ribulose-5-phosphate by the enzyme, 6-phosphogluconate dehydrogenase (6PGD). The addition of G6P then will give a rate for both enzymes (G6PD and 6PGD), and the activity of G6PD is found by taking the difference. This activity is expressed in nanomoles of substrate (NADP) converted per milligram of cytosolic protein per minute, and is calculated as follows:

1) Determine the rate of change of squares per minute on the chart paper and the span used. If the span was 1.0 (full range), then each square would be equal to 0.01 OD units on chart paper that went from 0 to 1.00 OD. If the span was 0.5, then each square would be equal to 0.005 OD units and so on.

2) Record the change in OD units per minute (Δ OD/min) by multiplying the number of squares per minute by the number of OD units per square.

3) Find the number of micromoles substrate utilized per minute:

$$\frac{\Delta \text{OD/min}}{1 \text{ cm} \times 6.22 \text{ cm}^2/\mu\text{mole}} \times 3 \text{ ml}$$

This is based on the fact that absorbance (in OD units) is equal to the length of the lightpath (1 cm in this case) times the concentration of the substrate times the molar absorptivity (the extinction coefficient, ε) for the substrate. That is, $A = l \cdot c \cdot \varepsilon$. For a 1 molar solution of NADP with a 1 cm lightpath, the molar absorptivity (and consequently the absorbance since the lightpath(l) is 1 and the concentration(c) is 1) is 6.22×10^3. Therefore a 1 millimolar solution in a 1 cm lightpath would have a molar absorptivity of 6.22, and the units of the solution would be millimoles per liter or micromoles per milliliter. The units $cm^2/\mu mole$ in the above equation come from $\varepsilon = \frac{A}{l \cdot c}$, when c=1 millimolar (1ml = $1cm^3$). The equation at the beginning of 3) is essentially obtained by solving $A = l \cdot c \cdot \varepsilon$ for c. Since the concentration is based upon 1 ml it is then necessary to multiply by 3 ml, as this was the volume of the reaction mixture.

4) Divide the value obtained in 3) by the milligrams of protein in the reaction mixture to obtain the activity.

Lowry Protein Determination

Reagents

A 2% Na_2CO_3 in 0.1 N NaOH, prepared each day

B 0.5% $CuSO_4 \cdot 5\ H_2O$ in 1% sodium tartrate

C 50 ml A + 1 ml B

Procedure

A stock solution containing 1 mg/ml of protein (bovine albumin) was used to make standards containing 100, 300 and 600 µg of protein per 1.2 ml of distilled water. To find a sample dilution that would fall within these standards (preferably between the 100 and 300 µg standards), 1:20, 1:50 and 1:100 dilutions (in distilled water) were tried first. In this study the 1:50 dilution produced the most satisfactory result. 6 ml of reagent C was then added to 1.2 ml of the diluted sample or the standard or blank (distilled water) and mixed. This was allowed to stand for 10 minutes at room temperature. Then 0.3 ml of reagent E was added, while the solution was being mixed. This was allowed to stand at room temperature for 30 minutes. Absorbance was estimated by reading at 500 nm on a Model 6/20A Coleman Jr-IIA spectrophotometer.[1] For this study, the above method was found to have a c.v. = 4.7% based on 10 replications.

Glucose Tolerance Tests and Blood Glucose Determinations

Food was withdrawn from the rats between 2200 and 2300 hours the night before the tests. The tests were initiated

[1]Coleman Instruments, Maywood, IL 60153.

at approximately 0900 hours. Rats were placed in plastic restrainers only while blood was drawn. At 30 days of age the rats were held by hand during blood collections. Between drawings all animals were kept in their cages. Blood was drawn by clipping the tail and drawing two 100 µl samples of blood into calibrated capillary tubes which had been previously heparinized. After the fasting bloods, subsequent samples were drawn without clipping the tail further. Samples were taken for fasting levels, and then at 15, 30, 60 and 120 minutes after injection of the glucose test load. The glucose load for 53 and 113 day old rats consisted of 800 mg of glucose in 2 ml of normal saline (0.9% NaCl). It was administered intraperitoneally using a 25 gauge, 5/8 inch needle and a 2.5 cc syringe. Due to smaller body size, only 0.5 ml of the glucose solution was injected into the 30 day old rats.

The blood samples were put into test tubes containing 1.9 ml of distilled water. The tubes were set in ice prior to the blood drawings. Each tube had received one drop of a NaF solution (10 mg/ml), which was then dried before the distilled water was added. Keeping the tubes cold and adding NaF aids in maintaining glucose stability for approximately 24 hours.[1]

Blood glucose determinations were based on the reactions:

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} H_2O_2 + \text{D-glucono-}\delta\text{-lactone.}$$

$$H_2O_2 + \text{reduced chromogen} \xrightarrow{\text{peroxidase}} \text{oxidized chromogen} + H_2O.$$

[1]From the circular that comes with the Glucostat Reagent Set, Worthington Biochemical Corp., Freehold, NJ 07728.

The color produced by the oxidized chromogen is proportional to the glucose concentration.

It was discovered that the stated concentrations for the $ZnSO_4$ and NaOH deproteinizing steps were not strong enough to insure complete precipitation of the protein from whole blood. Therefore, concentrations of 6% $ZnSO_4$ and .46 N NaOH were finally settled upon as being adequate to completely deproteinize the blood. These were titrated the day before the tests to be certain that they neutralized each other. The rest of the deproteinization was carried out exactly as explained using the semi-micro method.[1] It should be noted that the solutions must be mixed well after the addition of both the NaOH and the $ZnSO_4$ so that complete deproteinization will occur. After deproteinization, the solutions were centrifuged for 20 minutes in a Model U.V. International centrifuge.[2] For the enzymatic reaction, 0.8 ml of supernatant was added to 1.2 ml of distilled water. 2.0 ml of the Glucostat reagent was then added and the solution mixed. The tube was stoppered and placed in a water bath at 37° for 30 minutes. This allows the reaction to go to completion and therefore it was necessary to dilute the supernatant (as described above) before the Glucostat reagent was added. After removal from the water bath the reaction was stopped by adding one drop of 4 N HCl and mixing. Each sample was read at approximately the same time after the addition of the HCl (usually 1 hour) at 420 nm on the Model

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[1] Glucostat, Worthington Biochemical, Freehold, NJ 07728.
[2] International Equipment Co., Needham, MA.

6/20A Coleman Junior IIA spectrophotometer. Based upon 9 replications this author found a coefficient of variation for this method of 4.7%.

Liver Lipid Extraction

The extraction technique is based on the solubility of lipid in a chloroform:methanol mixture. Protein, as well as any carbohydrate, will not dissolve in this mixture and therefore the lipid may be quantitatively extracted, after water is removed by filtering through sodium sulfate.

After thawing, two 1g samples of liver were excised. Each was completely homogenized in 10 ml of methanol, using a Model S63C Tri-R-Stir-R homogenizer.[1] This solution was then transferred to a 125-200 ml Erlenmeyer flask. The homogenizing pestle and tube were washed in 15 ml and then 5 ml of methanol. The washings were added to the flask. Then 50 ml of chloroform was added and mixed and the flask was placed in a water bath for 30 minutes at 45°-50°. After cooling, the solution was filtered into 250-300 ml Erlenmeyer flasks. The 125-200 ml flask was rinsed 3 times with chloroform. This was then filtered into the larger flask after each rinsing. The filter paper was then rinsed twice and removed and the funnel rinsed once with chloroform. The filter had been previously weighed, and after filtering the solution and rinsing the paper, the paper was dried and placed in a desiccator until a constant weight was obtained (approximately 48 hours). This fat-free dry matter was used

[1]Tri-R Instruments Inc., Rockville Centre, NY.

as a constant for expressing the amount of lipid (and liver cholesterol) as mg/g fat-free dry matter. The sample and filter paper were evaporated to dryness in a constant temperature drying cabinet[1] at 50°.

50 ml Erlenmeyer flasks were weighed to the nearest 0.1 mg. Funnels with filter paper were set up and a spatula of anhydrous sodium sulfate added to remove any moisture in the extract. Approximately 10 ml of chloroform and some sodium sulfate were then added to the flasks containing the lipid material. After mixing well this solution was poured through the filter paper into the 50 ml flasks. The first flask was rinsed 3 times, the filter paper rinsed twice and the funnel once with chloroform. This solution was evaporated overnight in the drying oven. The flasks were placed in a desiccator until a constant weight to the nearest 0.1 mg was obtained. Total lipid was determined by finding the difference between the weight of the flask and the weight of the flask plus the lipid material.

Serum Triglyceride Determination

The method used was exactly the same as described by Fletcher(53). Glycerides (after removal of phospholipids) were saponified to free glycerol. The glycerol was then converted to formaldehyde by oxidation. The formaldehyde was reacted with diacetylacetone and ammonia in a condensation reaction which yields a fluorescent product (3,5-diacetyl-1,

[1]Blue M Electric Co., Blue Island, IL.

4-dihydrolutidine) absorbing at 405 nm. This procedure was found to have a coefficient of variation of 5.8% based on 13 replications.

Serum and Liver Cholesterol Determinations

Gas-liquid chromatography was used for cholesterol determinations. For serum, the procedure was to make a standard by adding 0.5 mg cholesterol and 0.2 mg 5-α cholestane (as the internal standard) to a tube. The solvent was then evaporated, and the tubes were covered and stored in a desiccator. When ready to use, 0.1 ml of trimethylsilyl reagent (TMS) was added. The TMS solution was prepared by adding, under a hood, 7.7 ml trimethylchlorosilane and 23.1 ml hexamethyldisilazane to 69.2 ml pyridine (1:3:9).[1] Anhydrous sodium sulfate was placed in the bottom of the jar to absorb moisture. The purpose of the TMS is to make silyl ether derivatives of the cholesterol, which makes the cholesterol more volatile and stable for analysis by gas-liquid chromatography. For the serum samples, 0.2 mg 5-α cholestane(as the internal standard) was added to a screw cap tube and the solvent evaporated. Then 0.5 ml of serum was added, followed by 5 ml of .33 N ethanolic (90% ethanol) NaOH. The tube was covered, mixed and the solution was saponified at 37° for 3 hours. After cooling, 1 ml of heptane was added and the solution was shaken vigorously by hand. Then 5 ml of distilled water was added and again the solution was shaken

[1]Applied Science Catalog 18 (1975), p. 101.

vigorously by hand. The top layer of heptane containing the extracted cholesterol and cholestane was removed and the heptane evaporated in the drying oven at 50°. After drying, the samples were stored in a desiccator until a large number were ready to be injected into the gas chromatograph. At that time, exactly 0.1 ml of the TMS solution was added. After addition of TMS the samples or standard were allowed to sit between 30 and 40 minutes at room temperature before injection into the gas chromatograph.

The gas chromatograph used was a model No. 900 Perkin Elmer.[1] The column was a 1.8 meter, 2 mm i.d., stainless steel column packed with 3%-5% SP2401. The chromatographic conditions were: Column temperature, 230°; injector port temperature, 285°; detector temperature, 285°; helium carrier gas flow rate, 30 ml/min. and range, X 100. Attenuation was usually X 16 and 2 µl injections were normally made. The standard was injected initially and after every 8 samples each day.

For the determination of liver cholesterol the procedure was the same except that the liver lipid was dissolved in 10 ml of choloroform and a 3 ml aliquot was taken. This was added to a screw cap tube, along with 0.1 mg or 0.2 mg cholestane, and the solvents evaporated before the saponification procedure.

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[1]Perkin Elmer, Norwalk, CT.

The amount of cholesterol was determined as follows:

1. Take the peak height or the area under the curve as the proportion for comparison.
2. Find the amount of cholestane in sample injection;

$$\frac{\text{area(peak) of sample cholestane}}{\text{area(peak) of standard cholestane}} \times \frac{\text{theoretical amt. of cholestane in sample injection}}{}$$

3. Determine the factor for cholesterol conversion;

$$\frac{\text{theoretical amt. of cholestane in WHOLE sample}}{\text{amount of cholestane in sample injection(2.)}}$$

4. Repeat 2. for cholesterol to get amount of cholesterol in sample injection.
5. 3. X 4. = amount of cholesterol in initial serum sample(0.5 ml in this study) or liver sample (3 ml aliquot of 10 ml/g liver in this study).
6. Convert serum value to mg% cholesterol and liver value to mg cholesterol/mg fat-free dry matter.

On 10 replications of serum, this method was found to have a coefficient of variation of 3.8%. Figure 3 is a typical example of a chromatogram of a standard and duplicate sample injections.

FIGURE 3 Example of a chromatogram.

A and B, duplicate liver samples; C, standard.
I, 5-α cholestane; II, cholesterol; III, unknowns.
Chart speed = 1 in./4 min.

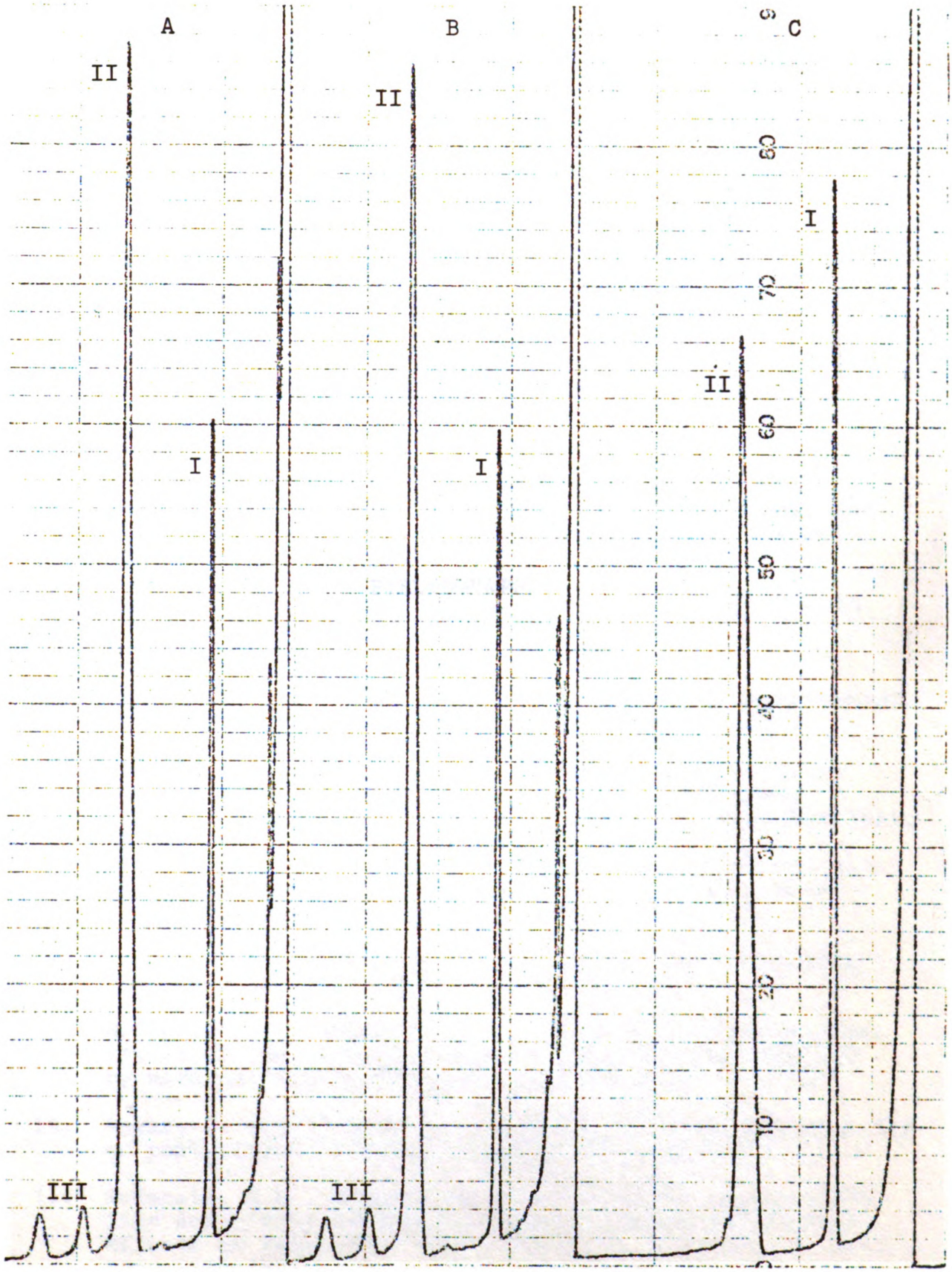

FIGURE 3 Example of a chromatogram.

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