COMPARATIVE STUDIES ON THE EFFECT OF DIETARY CARBOHYDRATES ON FAT SYNTHESIS

by

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ABSTRACT

Rats and fish were used to study the effect of diets differing only in carbohydrate supplement on the specific activity of the enzyme acetyl coenzyme A carboxylase which catalyzes the first step in the *de novo* biosynthesis of lipids. The carbohydrates fed were glucose, fructose or glycerol. Glucose is metabolized in an insulin-regulated pathway while the other two sugars are not in mammals. It was found that normal rats have about the same acetyl coenzyme A carboxylase activity regardless of diet when fed for a seven day period. The marine catfish, *Arius felis* apparently cannot tolerate a diet containing 17.7% fructose.

The project was divided into two separate but related studies. The first was a study on the effect of high carbohydrate diets on lipid *de novo* biosynthesis in the rat and the second was an attempt to perform a similar study on the marine catfish, *Arius felis*.

It has been reported that in mammals fructose and glucose are metabolized via different pathways. Figure 1 shows the differences between the degradation of the two sugars. The key regulation points, which are marked with asterisks in the diagram, differ for fructose and glucose. The glucose degradation pathway (glycolysis) is regulated at its first step by the constitutive enzyme glucokinase and at the step catalyzed by phosphofructokinase (PFK). Insulin, a hormone produced in the islets of Langerhans of the pancreas, regulates the enzyme

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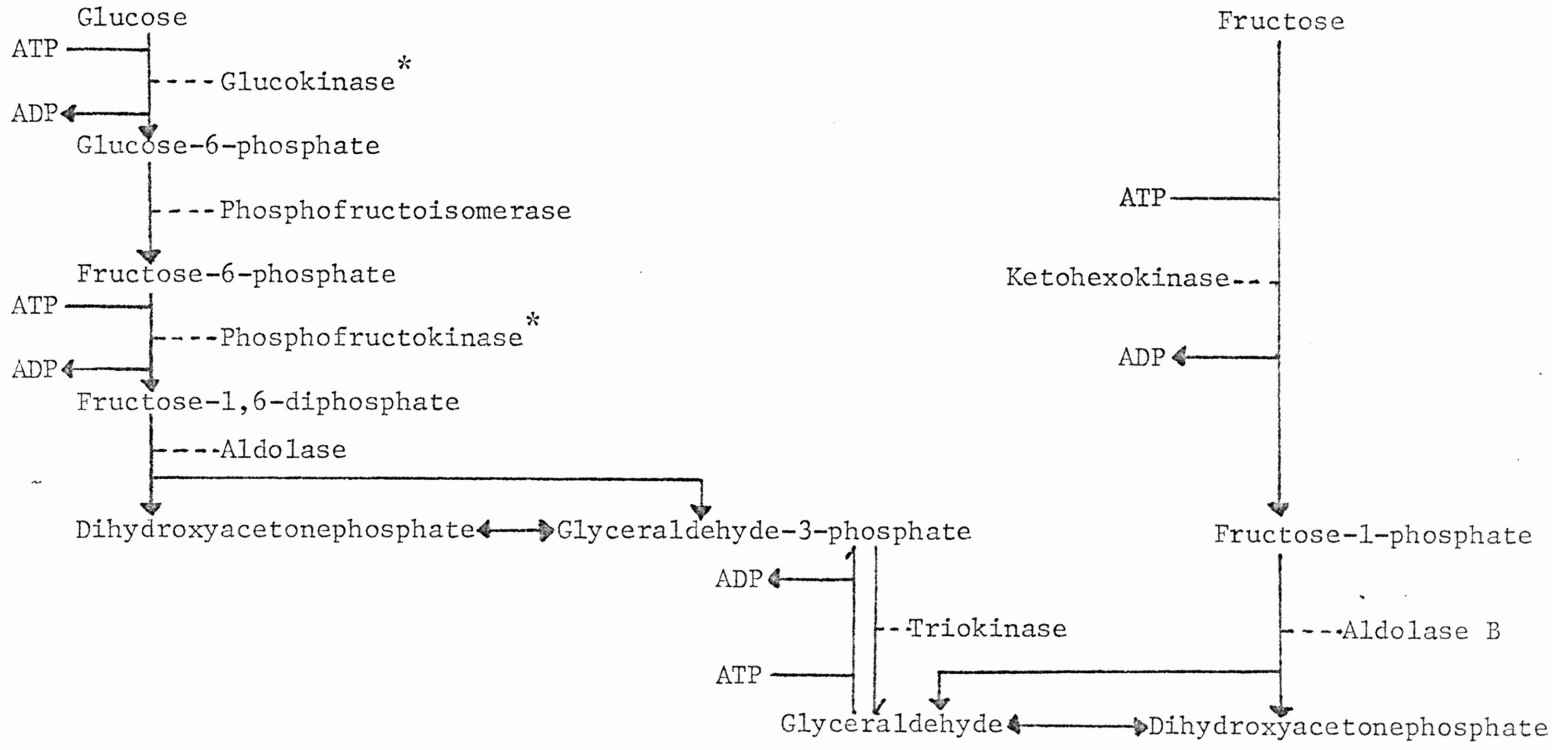

Figure 1. A comparison of glucose and fructose degradation.

glucokinase. Therefore, diabetic animals, which are characterized by having insulin deficiency, are restricted in their utilization of glucose. In contrast, the fructose degradation pathway is not regulated by insulin. The first enzyme in fructose degradation, ketohexokinase, is not constitutive, that is, it is not dependent on substrate concentration for activity. The fructose degradation pathway also bypasses the PFK control step. This could mean that fructose is metabolized faster than glucose. It has been found that diabetic rats, which have difficulty with the glucokinase reaction, could utilize fructose and that the effect of its use was manifested in a higher activity of the enzymes of lipid de novo biosynthesis over those of diabetic rats fed glucose-containing diets.

Many fish are "natural diabetics". Their normal diet contains very little carbohydrate and thus the fish is probably unable to utilize carbohydrate to a great extent. Experiments by Warman (1975) showed that the activities of catfish liver lipogenic enzymes, acetyl coenzyme A carboxylase (ACC) and fatty acid synthetase (FAS), were low and independent of whether the fish were starved, fed a fat-containing or fed a fat-free glucose-rich diet (1). In land animals and birds, eating the latter could raise the lipogenic activity previously lowered by starvation or by eating fat-containing diets. The administration of insulin to land animals and birds raises their lipogenic activity by allowing glucose to be utilized more effectively. However, insulin injections (5 IU/100 g body weight) did not affect catfish liver lipogenic activity (1). It was unknown what effect a fructose-containing, fat-free diet would have on the fish ACC enzyme. If the

fish was a "natural diabetic", perhaps the pathway for fructose degradation which is not regulated by insulin would allow an increase in endproducts for lipid biosynthesis and increased activity of the enzymes ACC and FAS.

A possible flaw was the fact that certain fish tissues are devoid of the ketohexokinase enzyme necessary for fructose degradation to occur (2). However, marine organisms differ so widely in their physiological and metabolic characteristics that it could not be assumed from the observation of the two species studied by Heinz and Weiner that no fish possesses the ketohexokinase enzyme. Heinz and Weiner extended their observation to state that probably no ectotherm possessed it. They however failed to take into account that the amphibia and fish they tested differed in other enzymes of fructose metabolism; the fish they tested did possess triokinase while the amphibia did not. The fish also had the other enzymes used in fructose degradation including the specific aldolase. This fact allowed a reasonable hope that some fish could also possess the ketohexokinase enzyme.

To account for possible absence of the enzyme in the catfish Arius felis, an intermediate in the pathway was sought that would avert the ketohexokinase step in fructose degradation as well as the glucokinase and PFK steps in glycolysis. Glycerol was used due to its point of entrance into the degradation pathway as shown in Figure 2. The enzymes required for glycerol's entrance into the degradation pathway had been found in Heinz and Weiner's fish, which would provide a better chance that the fish Arius felis would have the enzymes also. Another reason for feeding glycerol was based on previous findings in rats, glycerol

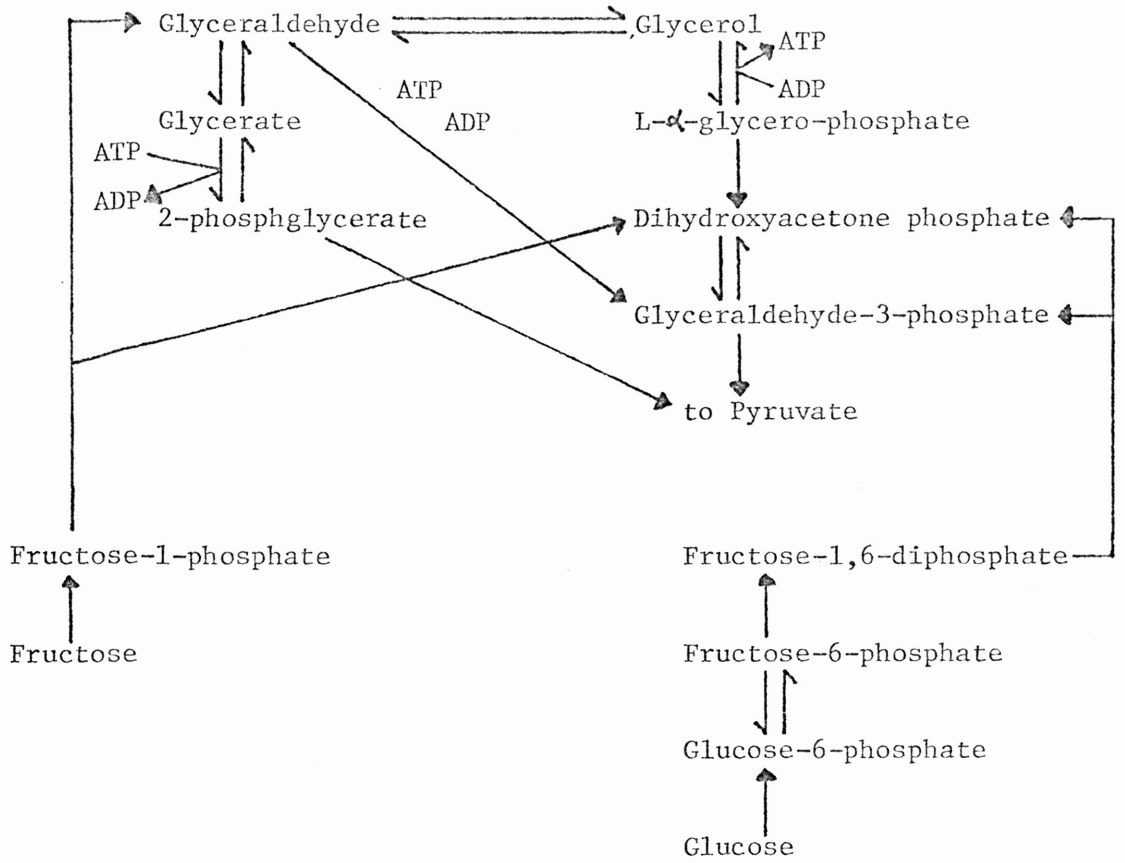

Figure 2. Entrance of glycerol into carbohydrate metabolic pathways.

diets mimicked the effects of fructose diets, including an increase in ACC activity over the activity found after glucose was fed (3). Glycerol can be converted to glycerol-3-phosphate, the triglyceride backbone. Therefore, glycerol can be more readily converted to fat than glucose or fructose. The direct conversion to fat would occur if there were sufficient material for the de novo biosynthesis so that eventual breakdown of glycerol to form acetyl-CoA was not necessary. Glycerol seemed to possess many of the characteristics desired in addition to a more favorable price than other possible choices. Therefore, one group of fish was fed glycerol-containing diets.

The endproduct of the breakdown of fructose or glucose is pyruvate. Since glycolysis occurs in the cytoplasm, the pyruvate must enter the mitochondria to form acetyl-CoA. After entering the mitochondria, pyruvate is converted to acetyl-CoA by pyruvate dehydrogenase. Acetyl-CoA then combines with oxaloacetate to form citrate. A carrier system in the membrane transports the citrate into the cytoplasm. There it is cleaved to oxaloacetate and acetyl-CoA. The acetyl-CoA is then available for lipid biosynthesis. The initial step of de novo fatty acid biosynthesis is the carboxylation of acetyl-CoA to form malonyl-CoA. The enzyme involved in this reaction is ACC with a bound biotin cofactor. ATP is required for the reaction. The malonyl-CoA formed undergoes a series of reactions with the FAS complex of enzymes. The endproduct is a fatty acid of an even number of carbon atoms because one two-carbon residue is added for each elongation of the synthesizing fatty acid. Carbon dioxide (CO_2) is released during the synthesis. It had been found that the activity of ACC parallels that of FAS in nearly all cases.

Many authors have postulated that the formation of malonyl-CoA is the rate limiting step of fatty acid biosynthesis. The assay procedure for ACC involves following the reaction for malonyl-CoA formation by using a radioactive bicarbonate source, $NaH^{14}CO_3$. The reaction is diagrammed in Figure 3.

Characteristics of the ACC enzyme require that it be activated prior to the assay. ACC can exist in two forms: one is an inactive protomer and the other is an active polymer. The protomer is thought to have two or more binding sites - one for acetyl-CoA and the other for an activator such as citrate or isocotrate. The activator causes the promoters of enzyme to aggregate into active, filamentous form. It is thought that a type of covalent modification control may exist also in which an insulin-promoted dephospho-form is active and a phospho-form is inactive (4). It is possible that citrate allows the enzyme to remain in the dephospho-form. The citrate may compete with acyl-CoA which is involved in feedback inhibition of the ACC enzyme (5). To allow for maximum activation of the enzyme, potassium citrate (10 mM) is added to the reaction mixture in a preincubation step. One favorable characteristic of fructose diets in regard to ACC activation is that these diets tend to reduce the concentration of fatty acyl-CoA (6).

In the first part of the project, I worked with rats in order to become familiar with the assay. Rats were fed fat-free diets and the activity of their ACC enzyme was determined. A considerable amount of time was spent to obtain values for the activity of their ACC enzyme consistent with those reported in the literature. A problem existed somewhere in the procedure that several people in the lab who were

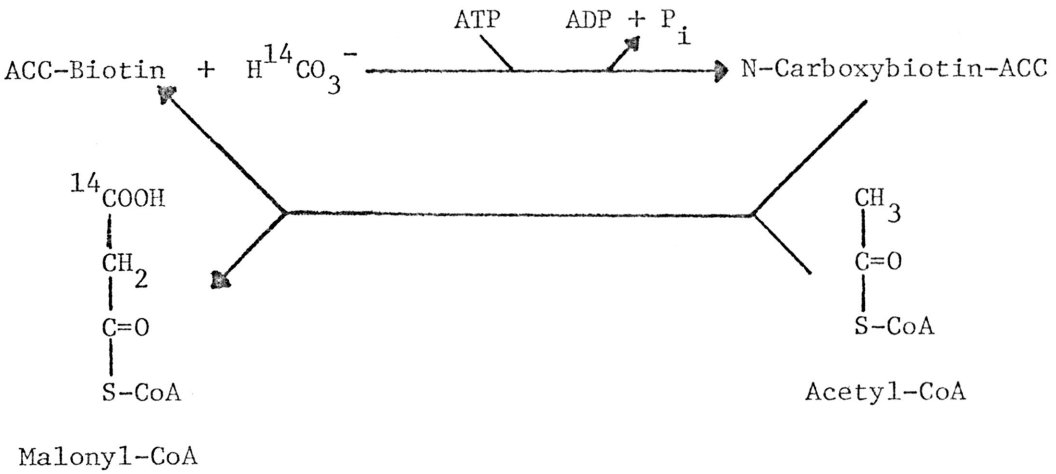

Figure 3. The formation of malonyl-CoA from acetyl-CoA.

working with the enzymes tried to pinpoint. Despite the use of different sets of reagents, different operators and different rats kept in different environments, the values we obtained were low. However, we felt that whatever the factor was that was lowering the values for ACC and FAS activity, perhaps it would be a constant factor in the dietary experiment planned so that I could still observe differences between the groups. Another problem encountered was the unusually cold winter. Because of this, fish were not available until midway through the spring semester. Consequently, it was decided to perform an experiment on rats which would parallel the one to be performed on the fish.

MATERIALS AND METHODS

Dietary Experiment

Experiment A: Rats

The rats were divided into three groups, each containing three rats. They were fed the same fat-free diet with different carbohydrate supplements (7). The diets contained 54.7% fructose, glucose or glycerol. After arrival, the rats were starved for two days, then fed for seven days. They were then killed by decapitation, their livers were immediately removed and placed in a 0.25 M sucrose solution at $4^{o}C$. The liver was then weighed and homogenized in a solution of sucrose (0.25 M) - ethylenediaminetetraacetate disodium salt (EDTA, 3 mM) - 2-mercaptoethanol (5 mM) in a volume equal to two times the weight in a Potter-Elvehjem homogenizer also at $4^{o}C$. The homogenate was centrifuged at 10,000 x g for 30 minutes to remove cellular debris and mitochondria. The resulting supernatant was centrifuged at 100,000 x g for one hour to remove microsomes. The resulting particle-free supernatant (PFS) was immediately placed on ice. The enzyme assay for ACC was carried out immediately.

Experiment B: Fish

Live fish were obtained from Corpus Christi Bay in the Gulf of Mexico. They were transported in plastic bags within styrofoam chests. The bags contained water which had been oxygenated. After exposure to formaldehyde solution (1 part formaldehyde to 15,000 parts water) for one hour to kill surface bacteria, the fish were divided into groups and placed into aquaria which had been set up for two weeks with "Instant Ocean", a product that simulates sea water. The salinity was

measured with a hydrometer and approximated that of the water the fish had been living in (20 parts per thousand). After two days of starvation the fish were fed a fat-free diet (8) containing 17.7% fructose, glucose or glycerol. Most of the fish appeared healthy upon arrival, but it was later found that, except for the fish in one tank, they were contaminated with bacterial pathogens, gill parasites, and/or tapeworms.

Acetyl Coenzyme A Carboxylase

The assay for ACC activity was conducted according to the method of Gregolin et al. (9) modified by including a preincubation with citrate to activate the enzyme and by changing the concentration of the citrate. The reaction followed was the condensation of $H^{14}CO_3^-$ with acetyl-CoA to form ^{14}C malonyl-CoA. The reaction mixture used was, in micromoles: Tris (Cl^-), 60, pH 7.5; ATP, 2; $MgCl_2$, 8; $NaH^{14}CO_3$ (specific activity 2.5×10^5 CPM per μmole), 10; acetyl-CoA, 2; potassium citrate, 10; glutathione, 3; bovine serum albumin (BSA), 0.6 mg; and carboxylase enzyme preparation (PFS diluted in 0.01 to 0.05 M potassium phosphate buffer pH 7 containing 0.1 mM EDTA and 5 mM 2-mercaptoethanol to 0.03 - 0.05 ml). Final volume: 1 ml.

The procedure followed was first a preincubation step in which the enzyme preparation was mixed with the substances not directly involved in the reaction; the ATP, acetyl-CoA and $H^{14}CO_3^-$ were omitted. The mixture was placed in a 37°C water bath for 30 minutes (protein dilutions of 0.1 ml PFS to 9.9 ml water were also prepared at this time). After the preincubation, the tubes were placed in ice while the ATP and acetyl-CoA were added. The $H^{14}CO_3^-$ was added individually and each tube was incubated at 37°C for exactly 10 minutes. The reaction was stopped

with 0.2 ml of 6 N HCl, making the total volume in each tube 1.2 ml. Two counting vials for the liquid scintillation counter were prepared at this time as standards by pipetting 10 µl of the $H^{14}CO_3^-$ into each vial then adding 10 ml of Bray's counting solution [napthalene, 6 g: 2,5-diphenyloxazole (PPO), 4 g ; 1,4-bis-2-[5-phenyloxazolyl]-benzene (POPOP), 0.2 g; absolute ethanol, 100 ml; and p-dioxane to make 1 liter of solution].

The tubes were centrifuged for 10 minutes at 2000 rpm. A 0.6 ml aliquot was placed in a counting vial and evaporated under a hood by placing the vial in a sand bath at 80°C for 45 minutes. After evaporation, 10 ml Bray's counting solution and then 0.1 ml of water were added, mixed and then the vial was placed in the scintillation counter overnight. This was done to allow time for the product to dissolve in the liquid. The samples were counted for 20 minutes in the Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3375.

The results were calculated by correcting the values for quenching by use of a previously calculated quench curve. Quenching is a phenomenon which interferes with the transmission of the beta particles in the sample to the counter, resulting in lower counts than are actually present. The scintillation spectrometer operates by printing a ratio that is a measure of the quenching taking place in the sample. It does this by counting an external standard present in the machine for one half of a minute and then recounting the external standard for the same length of time with the sample lowered in front of it in the counter. The comparison between the two counts is the AES printed ratio. A quenching curve and table was made by pipetting a known volume of a

standard toluene compound, labeled and assessed for radioactivity by New England Nuclear, into a counting vial with Bray's counting solution. This vial was then counted which yielded the efficiency of the machine. It was recounted after addition of a series of aliquots of water, a chemical quencher. The efficiency goes down as the quenching effect increases, as does the ratio printed by the machine. A table of efficiency vs ratio was constructed using the data from this process. Thereafter, the ratio printed could be converted to % efficiency of the counts obtained by using the table.

The protein content of the PFS diluted at the time of the assay was determined by the method of Lowry et al. (10). A standard curve was obtained by plotting % transmittance vs concentration of five dilutions of a standard BSA solution. A statistical evaluation of the standard curve data was done which yielded an equation of the best possible straight line. The values of the % transmittance of the PFS dilutions were substituted into the equation and the concentration obtained.

The specific activity of the ACC enzyme was calculated by using the method shown in Figure 4.

$$\text{Substrate consumed (umoles)} = \frac{10{,}000 \text{ umoles} / 0.1 \text{ ml } H^{14}CO_3^-}{DPM^* \text{ of standard vial} / 0.1 \text{ ml } H^{14}CO_3^-} \times \text{DPM sample} \times \frac{\text{total vol.}}{\text{aliquot vol.}}$$

$$\text{Enzyme Specific Activity} = \frac{\text{substrate consumed / tube}}{\text{time of rxn. (minutes)} \times \text{mg protein / tube}}$$

$$^*\text{DPM} = \text{disintigrations per minute} = \frac{\text{counts per minute (CPM)}}{\text{efficiency}}$$

Figure 4. Calculation of specific activity.

RESULTS AND DISCUSSION

Rat Feeding Experiment

The results of the experiment with rats suggest that no difference exists in specific activities of ACC (Table 1) when normal rats are fed fat-free diets varying in carbohydrate supplement. This would seem to indicate that glucose, fructose and glycerol are metabolized at the same rate in normal rats, allowing the same amount of acetyl-CoA to be produced for use in ACC catalyzed reaction, or that the regulation of the enzyme ACC is equivalent despite the dietary carbohydrate source.

Rat weight differed among dietary groups (Table 2). While the rats in the glucose and fructose groups either gained weight or stayed substantially at the same level, the rats in the glycerol group lost weight. The rats in the glycerol group had a different appearence than the other rats. Their coats appeared wet and sticky and they took in a great deal more water than the other rats. They did eat the diet however, and became more active later in the week when they began to eat more. It is interesting to note that the activity of ACC (Table 1) was about the same as found in the other groups, despite the fact that the glycerol-fed rats were losing weight. Therefore there was more activity per unit body weight. A longer feeding experiment in which the rats could better adapt to the diet would be a better indication of glycerol's effect.

There was also a difference observed in the weight of the livers (Table 2). The rats fed glucose or fructose weighed about the same, but the livers of the fructose group were larger and heavier. It could be possible that the fructose group was metabolizing its respective sugar

Table 1.

Total counts (DPM) and enzyme specific activity

	Total Counts (DPM)	Enzyme Specific Activity (nmoles / min mg protein)	
			Average
Standard $H^{14}CO_3^-$	1.44076×10^6		
Glucose #1	5898	5.3	
Glucose #2	6093	4.7	5.4
Glucose #3	7053	6.3	
Fructose #1	8746	9.6*	
Fructose #2	6645	5.8	5.75
Fructose #3	3657	5.7	
Glycerol #1	7477	5.7	
Glycerol #2	6960	5.9	5.7
Glycerol #3	5782	5.5	

*Eliminated by "t" test ($P < 0.5$).

Table 2.

Weight of rats and livers after seven days feeding (g)*

	Glycerol			Glucose			Fructose	
Number	Rat wt.	Liver wt.	Number	Rat wt.	Liver wt.	Number	Rat wt.	Liver wt.
1	83	4.94	1	100	4.64	1	116	6.35
2	67	4.39	2	100	5.58	2	109	7.34
3	75	4.27	3	97	4.28	3	91	6.61
Average	75	4.53		99	4.83		105	6.77

*Pre-feeding weight, 90-100 g.

at a faster rate than the glucose fed rats and synthesizing fatty acids faster. These fatty acids could have lowered by feedback inhibition the activity of ACC at the time the assay was done. Again, a change in the timing of the experiment - a longer or shorter feeding period might have shown differences in ACC activity from the glucose control group.

It would be interesting to investigate the possibilities that glycerol and fructose might cause differences in ACC activity upon prolonged feeding. If there is indeed no difference among ACC activities with the three different diets, this would suggest that in normal rats, the enzymes that control carbohydrate metabolism leading to acetyl-CoA formation work in a way that regardless of carbohydrate source the ultimate effect on lipid biosynthesis is the same.

The results of the experiment do not agree with Volpe and Vagelos' experiments in which they tested fatty acid synthetase (FAS). If the assumption is made that ACC and FAS activities run in parallel, the ACC activities of the experiment should have shown differences among the dietary groups. In the Volpe-Vagelos experiments, it was found that FAS activity was raised by fructose diets fed to either normal or diabetic rats. They used a longer feeding period (two weeks) which could have been the reason for the differences (11). The glycerol diet seems to have greater potential for causing higher ACC activity if longer feeding periods are used. Although much work has been done on rats in the area of carbohydrate dietary effects, this would have been the next logical step in my research so that I could have used the data on the rats as a control for the assay on fish. If there were no differences in ACC or FAS activities in diabetic rats, I would have had to look for

possible flaws in the assay procedure or reagents that was giving unreliable results.

Fish Dietary Experiment

As stated in the introduction, the fish I received varied in health. Fish are difficult to transport from the Gulf Coast to College Station; great care must be taken to keep them alive. When the proper precautions were taken, the fish survived the trip, although some arrived in a less than ideal state. The fish were exposed to formaldehyde solution to kill surface bacteria before they were distributed into tanks. The fish were to be fed according to their placement in the tanks (Table 3). The fish eating fructose were the only fish who ate the diet more than a two day period. The fish were fed once a day, were observed eating the food, and appeared to be doing well until the fifth day when all fish died. Upon examination of the stomach and intestines of the fish, it was found that food was present. No parasites were found in the intestines of the fish but tapeworms had apparently killed one or more fish in another tank. From this it was thought that the fish on fructose could have died from toxicity of the fructose diet. It was also possible that the fish could not utilize the fructose due to lack of ketohexokinase, but this would probably not cause the toxic effect. Two diseases of fructose intolerance have been recorded in mammals. One of these diseases is fructoseuria, a rare disease that is indicated by the presence of fructose in the urine. It is thought to be caused by a lack of ketohexokinase. The second is fructose intolerance caused by a lack of aldolase B, the special aldolase used in fructose metabolism. It results in a severe hypoglycemia, i.e. deficiency of

Table 3.

Observations on the fish feeding experiment

Dietary Group	Remarks
Fructose	The fish ate the prepared diet for a period of four days after a two day starvation period. The fifth day they were all found dead. Food was found in the stomach and intestines. There was no evidence of parasites.
Glucose	Two of the three fish ate the prepared diet in small quantities the first day of feeding but refused to thereafter. One of the fish was apparently afflicted with some type of parasite. They died after three more days, but the cause could not be determined.
Glycerol	Apparently none of the fish consumed the prepared diet. All were apparently afflicted with external parasites which were visible after three to four days. One had an external red lesion which grew progressively worse and spread. Although glycerol had probably dissolved in the water and thus could have been taken in through the gills, these fish probably died from their various afflicitions. The fish liver for eight days after the feeding was started.

sugar to the blood (12). Perhaps the fishes' death was the result of something similar to these diseases.

The fish fed glucose and glycerol could not be used to draw conclusions due to the presence of at least one fish afflicted with parasites in each tank. It was not known until death occured that the reason was the presence of external and/or internal parasites and in one case a possible bacterial pathogen. Some of the fish in the glucose tank did begin eating the diet but would not eat after they began to become sluggish with the onset of the disease. None of the glycerol-fed fish ever was observed eating - an apparent sign of illness. One thing was interesting about the ill fish, however they were able to live up to a week longer than the fish which had been eating the diet. This adds credence to the possibility that the fish fed fructose died from diet toxicity.

The results of the experiments were not extensive enough to draw any firm conclusions. It would be necessary to repeat the experiments again in order to reaffirm the data. This was impossible to do because of the time factor involved in the present program. The unfortunate events that prevented the schedule of the research from proceeding as planned could not have been averted because they were not foreseen. The work that I have done can serve as a basis for additional curiosity in regard to fish carbohydrate metabolism, but it is obvious that at least a control group fed glucose must survive the experiments as they did in the experiments of Warman (1). Possible additional work could be first repeating the experiment, with diets reduced in carbohydrate content until the fish could survive a fructose-containing diet. The activities

of the ACC enzyme among the various dietary groups could then be compared.

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VITA

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The typist for this paper was Arthur W. Warman.